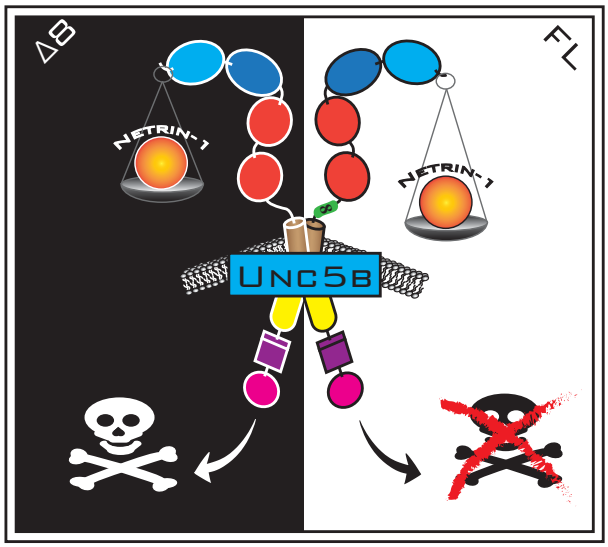
UNIVERSITA' DEGLI STUDI DI PAVIA

Dipartimento di Biologia e Biotecnologie "*L. Spallanzani*"

Nova2 alternative splicing regulation of the netrin receptor Unc5b during angiogenesis



Davide Pradella

Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
Ciclo XXX – A.A. 2014-2017

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Abstract

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Angiogenesis, the growth of new blood vessels from pre-existing vasculature, is essential to sustain tumor growth and metastasis formation. Although targeting angiogenesis is a promising anti-cancer therapy, all attempted strategies so far have shown modest therapeutic effects. This indicates that tumor angiogenesis is a more complex phenomenon than previously anticipated. Hence, a better understanding of the molecular mechanisms supporting growth of tumor vessels will be crucial to identify novel and more specific anti-angiogenic therapies.

Alternative splicing (AS) is the post-transcriptional molecular process that generates, from a single gene, multiple mRNAs leading to the production of protein isoforms with different structural and functional properties. Notably, increasing evidence supports a causative role of AS regulation in cancer. In particular, as many as 15'000 cancer-specific AS events have been identified suggesting that AS could serve as a powerful source of new diagnostic, prognostic and therapeutic tools for human cancer. Despite its well-established function(s) in tumor progression, the role of AS during angiogenesis has so far been largely ignored. Indeed, the molecular pathways involved in angiogenesis have been traditionally suggested to act primarily through regulation of transcription.

For the first time, our group demonstrated an important role for the regulation of AS during angiogenesis. We found that the organization of the vascular lumen during angiogenesis is controlled at post-transcriptional level by the AS factor Nova2, previously considered neural cell-specific. Consequently, Nova2 ablation in zebrafish embryos causes vascular lumen formation defects, reminiscent of aberrant morphology of tumor vasculature. By RNA-seq and CLIP-seq, we recently identified novel Nova2-mediated AS variants of several receptors implicated in vascular patterning. Among these is *Unc5b*, encoding for a Netrin-1 receptor involved in both axon guidance and vessel morphogenesis. During my Ph.D., I found that Nova2 directly promotes the production of a novel Unc5b isoform deleted of 11 amino acids in the extracellular portion of the receptor (Unc5b-Δ8). I found that Unc5b-Δ8 is increased in ovarian cancer and I showed that the Nova2-dependent production of Unc5b-Δ8 plays a fundamental role in the formation of specific vessels during vascular development *in vivo*. Mechanistically, I found that Unc5b-Δ8 is not able to respond to the canonical Netrin-1 pro-survival signal. Collectively, my data suggest that Nova2 regulation of *Unc5b* AS plays a key role in angiogenesis thus identifying a novel pathway that could be relevant for cancer progression.

Acknowledgments

Acknowledgments

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A special thank goes to my long-time collegues Anna and Elisa, and also to Daniele, Chiara e Cristina for the criticisms, suggestions and especially for the support during my work.

Abbreviations

DNA	deoxy ribonucleic acid
RNA	ribonucleic acid
RNA pol II	RNA polymerase II
mRNA	messenger RNA
5' ss	5' splice site
3' ss	3' splice site
BPS	branch point sequence
PTT	polypirimidine tract
RNP complex	ribonucleoprotein complex
snRNP	small nuclear RNP
AS	alternative splicing
UTR	untranslated region
ESE	exonic splicing enhancers
ESS	exonic splicing silencers
ISE	intronic splicing enhancers
ISS	intronic splicing silencers
SR proteins	serine-arginine proteins
hnRNPs	heterogeneous nuclear ribonucleoproteins
NMD	non-sense mediated decay
POMA	paraneoplastic opsoclonus-myoclonus ataxia
RBD	RNA-binding domain
CNS	central nervous system
SELEX	systematic evolution of ligands by exponential enrichment
CLIP	cross-linking and immunoprecipitation
EC	endiothelial cell
EMT	epithelial-to-mesenchymal transition
ASO	antisense oligonucleotides
ECM	extra cellular matrix
MMP	matrix metalloproteinases
AD	Alzheimer's disease
PD	Parkinson’s disease
ALS	amyotrophic lateral sclerosis
TKR	tyrosine kinase receptor
GPI	glycosylphosphatidylinisotol
DR	dependence receptor
RT-PCR	reverse transcription – polymerase chain reaction
qPCR	quantitative polymerase chain reaction
PAV	parachordal vessel
DA	dorsal aorta
ISV	inter-segemental vessel

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Hardcover picture: Schematic representation of the netrin receptor Unc5b alternative splicing variants as two arms of a balance able to differently weight the same ligand, Netrin-1. Unc5-Δ8, distinguishable by the absence of a short alternative exon close to the transmembrane domain, is incapable to respond to the canonical pro-survival signal induced by netrin-1, thus inducing apoptosis. On the contrary the full-length variant, under the force of its ligand, is able to prevent cell death and promote survival.

Introduction and literature review

An introduction to the "pre-mRNA processing field"

In eukaryotes, gene expression is a finely tuned process that occurs in different compartments of the cell (Orphanides & Reinberg, 2002). Information stored in the nucleus flows to produce functional gene products in the correct cell at the correct time. When RNAs and proteins are required, transcription from a template of deoxyribonucleic acid (DNA) takes place by RNA polymerases (Maniatis & Reed, 2002). Independently of the nature of the functional gene product, primary pre-mRNA molecules transcribed by RNA polymerase II (RNA pol II) undergo extensive modifications after their synthesis (Proudfoot et al., 2002). This complex process of maturation includes a series of modifications that are required for the proper localization, stability and translation of the mature transcript (mRNA) in the cytoplasm.
Attachment of a 7-methylguanosine cap to the 5' end (capping), intron removal (splicing), cleavage and addition of a poly(A) tail (polyadenylation) at 3' and chemical modification of RNA (editing), in the majority of the cases, occur co-transcriptionally thus generating an additional layer of regulation of gene expression beyond the transcriptional control (Bentley, 2014).

The "splicing code"

The majority of eukaryotic genes contain non-coding regions (introns). On average introns sizes varies from thousands to 50-100 nucleotides, whereas the size of exons span typically from 50 to 250 nucleotides (Lee & Rio, 2015). Independently of the size, all introns must be removed during the splicing reaction. Splicing is a fundamental step in the maturation of eukaryotic protein-coding genes and is directed by the recognition of three *cis*-acting consensus sequences in the pre-mRNA (Figure 1):
- a 5' splice site (5' ss), or "donor site", is located in the exon/intron junction at the 5' of the intron and, in mammals, it is characterized by a short consensus sequence of eight nucleotides in length: AG|GURAGU (R for purine, | for splice site);
- a 3' splice site (3' ss), also called "acceptor site", of four conserved nucleotides (YAG|G, Y for pymiridines) in the intron/exon junction at the 3' of the intron;
- a third element, approximately located 18-40 nucleotides upstream of the acceptor site, is the branch point sequence (BPS). Instead of the highly conservation found in 5' and 3' ss the consensus sequence of the BPS is YNYURAY (N for any nucleotide), where the adenosine is the only conserved nucleotide in metazoans (Mueller & Hertel, 2012).
In higher eukaryotes, in addition to the tetramer of the 3' ss, a tract of polypyrimidine (PPT) of variable length (5-30 nucleotides) may be found upstream

the intron-exon junction (Will & Lurhmann, 2011). These four *cis*-acting elements represent the "docking sites" for the ribonucleoprotein (RNP) machinery deputed to remove the non-coding part of the pre-mRNA, the spliceosome.

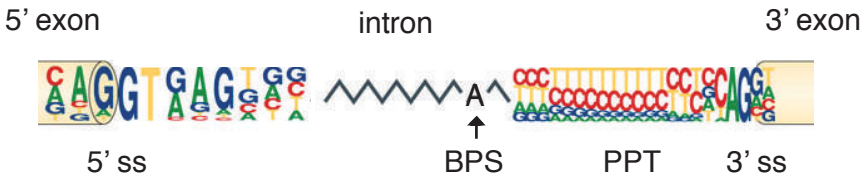


Figure 1. *Cis*-acting elements required for pre-mRNA splicing. Consensus *cis*-acting sequences present at the exon-intron boundaries are recognized by the spliceosome, thus allowing the correct definition of exons in the mature mRNA. The 5' splice site (5' ss or "donor site": AG|GURAGU), the 3' splice site (3' ss or "acceptor site": YAG|G) with the polypyrimidine tract (PPT) and the branch point sequence (BPS) are shown (Y = pyrimidine and R= purine) (modified from Matera & Wang 2014).

The spliceosome is a large and dynamic ribonucleoprotein complex that consists of proteins (~ 300) and RNAs. Key spliceosomal components are the small nuclear RNPs (U1, U2, U4/6 and U5 uridine-rich snRNPs), which are assembled in the cytoplasm and re-imported in the nucleus (Kiss, 2004). SnRNPs are composed by RNA, known as small nuclear RNA (snRNA), and complex-specific proteins. snRNAs are transcribed in the nucleus by RNA pol II, capped at their 5' end and exported in the cytoplasm, with the exception of U6 snRNA that is transcribed by RNA polymerase III and never reach the cytoplasm. SnRNP biogenesis occurs in the cytoplasm where several Sm proteins, snRNPs and additional non-snRNP proteins are properly assembled before translocation into the nucleus (Matera & Wang, 2014).

Definition of exon/intron boundaries in the pre-mRNA is ensured by the recognition of the three consensus sequences by the RNA molecules of snRNPs. These RNA-RNA contacts are further stabilized by RNA-protein and protein-protein interaction networks within the spliceosome in order to properly locate the reactive sites of the pre-mRNA for the splicing reaction (Lee & Rio, 2015). The importance of the precise recognition of 5' ss, 3' ss and the BPS is highlighted by the fact that mutations in these sequences may cause aberrant intron retention or alteration of the splicing pattern leading to a wide range of inherited and acquired human genetic disorders (Scotti & Swanson, 2016; Bentley et al., 2014, Pistoni et al., 2010).

The spliceosome machinery

The spliceosomal complex is directly assembled on the pre-mRNA, thus allowing the highly fidelity of the process at nucleotide level. From a biochemical point of view, introns are removed from the pre-mRNA through two trans-esterification reactions. First, the 2-hydroxyl group of the adenosine of the BPS carried out a nucleophilic attack on the phosphodiester bond at the 5' ss. This result in the formation of an intron lariat-3' exon and a free 5' exon. Second, the 3' hydroxyl group of the 5' exon attacks the phosphodiester bond at the 3' ss, leading to the ligation of the 5' and 3' exons and excision of the lariat intron (Will & Lurhmann, 2011, Fica et al., 2013) (Figure 2).

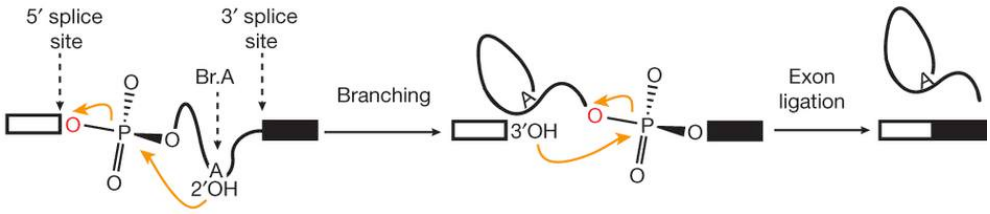


Figure 2. The splicing reaction. White and black boxes represent respectively the 5' exon and the 3' exon, the black line is the intron. The two transesterification steps are indicated by the orange arrows. The first catalyzes the formation of a free 5' exon and an intron lariat-3' exon. The second performs the exon ligation and the release of the lariat intron (modified from Fica et al., 2013).

The spliceosomal assembly is a highly dynamic process in which multiple conformational and compositional changes occur in a step-wise assembly of snRNPs and non-snRNP proteins on the pre-mRNA (Matera & Wang, 2014).
The first step involves the recognition of *cis*-acting elements, 5' and 3' ss, by U1 and U2 snRNPs, respectively. Recognition of the 5' ss via base pairing of the U1 snRNA is facilitated by the interaction with the C-terminal domain of RNA pol II, thus generating the early complex (Complex E). Next, the U2 snRNP recognizes the opposite intron/exon junction together with additional non-snRNP factors, such as SF1 and the U2 auxiliary factor (U2AF). The U2 snRNP is also able to contact the BPS sequence and to interact with the U1 snRNP at the 5' ss in an ATP-dependent process. The pre-spliceosomal complex (or Complex A) is formed, and the remaining particles of the core (U4, U5 and U6 snRNPs) are recruited in order

to form the pre-catalytic spliceosome, also known as Complex B. The resulting Complex B undergoes a series of conformational and compositional changes. In particular, U1 and U4 leave the complex to allow to the first catalytic reaction to be completed (catalytic spliceosome or Complex C). ATP-dependent rearrangements of the remaining snRNPs and recruitment of different auxiliary factors are required for the second catalytic reaction, leading to the release of the intron in the form of a lariat structure. Several RNA helicases drive the disassembly of the post-catalytic spliceosome, in which U2, U5 and U6 are released and used for additional new rounds of splicing (Matera & Wang, 2014).

The entire cycle, summarized in Figure 3, starts with the *de novo* assembly of the spliceosome on the pre-mRNA molecules when, in average, 5 kb have been elongated by RNA pol II. Nevertheless, splicing of introns can also take place post-transcriptionally, thus preventing pre-mRNA export outside the nucleus and its translation in the cytoplasm (Jacob & Smith, 2017).

The entire "spliceosome cycle" is a tightly regulated and the high metabolic energy cost is necessary to ensure the fidelity of pre-mRNA processing, thus avoiding the production of imprecisely spliced mRNAs (Bertram et al., 2017; Agafonov et al., 2016). For this reason, ATP-dependent helicases, such as Prp5, Prp2 and Prp22, are required to drive different conformational changes in the RNA structure of the snRNP particles thus favoring base-pair recognition of the splice sites and the BPS in the early steps of the reaction and during the formation of the catalytic core. In addition, a number of accessory or auxiliary proteins (omitted for simplicity in this introduction) are also important for the recognition of exon/intron boundaries, since in many cases the interactions between splicing factors and snRNPs are very weak and instable.

Finally, it is important to note that a non-canonical splicing reaction, which involve less than 1% of human introns (Younis et al., 2013), is carried out by an equivalent class of snRNP (U11, U12, U4atac and U6atac) that constitute the so called minor spliceosome (opposite to the major spliceosome composed by U1, U2, U4/6) (Turunen et al., 2012).

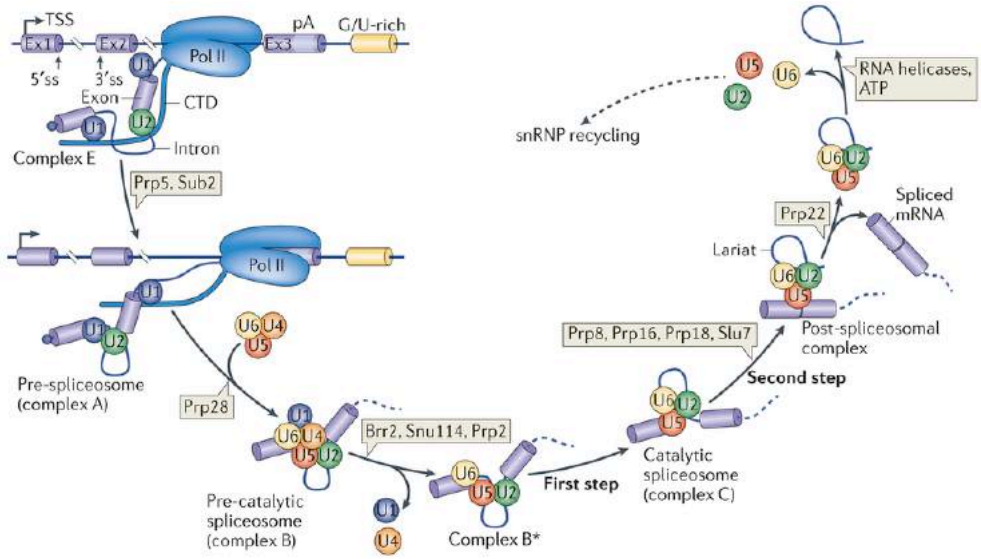


Figure 3. The spliceosome cycle. The spliceosome assembly is a multi-steps process in which the various snRNPs and accessory proteins bind sequentially to the pre-mRNA. The different complexes formed by snRNPs, pre-mRNA and proteins are shown. The two transesterification steps occur between the formation of Complex B* and Complex C (first transesterification) and after Complex C assembly (second transesterification), respectively (modified from Matera & Wang, 2014).

Alternative splicing

The amount of the coding part of the genome does not correlate with the organism complexity (Mattick, 2001). For instance, *Drosophila melanogaster* has fewer protein-coding genes than the less evolved nematode *Caenorhabditis elegans* (~14,000 versus ~19,000). Meanwhile, mammals have similar number of protein-coding genes as *Arabidopsis thaliana* (~24,000–27,000) and only four times the number found in the budding yeast *Saccharomyces cerevisiae* (~6,000) (Lin et al. 2007; Hillier 2005; Swarbreck et al. 2007; Lin et al. 2013). Moreover, the number of protein-coding genes is not able to explain the complexity of many organs or cell types in the same individual. For example, neurons generate an intricate network of connection among them (Lau, 2016). The establishment of these interactions require the combined action of pre- and post-synaptic signaling molecules combined to generate more than trillions of chemically differentiated synapses. Even if considering all protein-coding genes presents in the human genome their combination is not sufficient to explain such synaptic diversity.

Alternative splicing (AS) plays a major role in the generation of proteomic diversity in eukaryotes and it accounts for primates-species specific characteristics (Calarco et al., 2007). Protein-coding genes in humans are ~20,000–25,000, the same number of mouse. However, the number of human transcripts is 200,000 compared to less than 100,000 in mice (Lee & Rio, 2015). Indeed, it is estimated that approximately 95% of human genes undergo AS (Pan et al., 2008; Wang et al., 2008). Thus, it represents the primary source to expand the proteome encoded by the relatively small number of genes of our genome.

Mechanistically, AS is the process by which a pre-mRNA molecule, derived from a single gene, can generate multiple and distinct mature mRNAs leading to production of a number of protein isoforms that differ for structural and functional properties or have a diverse sub-cellular localization (Ghigna et al. 2008; Kelemen et al. 2013). To emphasize the role of AS, the *Drosophila Dscam1* (Down Syndrome cell adhesion molecule) gene is able to generate nearly 38,000 distinct isoforms through AS, more than the number of *Drosophila* genes (Hattori et al., 2009). Moreover, in human brain, cell-adhesion molecules (such as neurexin) play important roles during synaptic transmission (Aoto et al., 2013). Considering only a single region of the brain in a particular developmental stage, more than 2,500 AS transcripts of *neurexin* have been identified to date (Treutlein et al., 2014).

Coordination of specific AS events plays essential roles to establish fundamental properties during developmental processes, such as spermatogenesis (Liu et al., 2017; Hannigan et al., 2017), angiogenesis (Giampietro et al., 2015), erythropoiesis (Yamamoto et al., 2009), neurogenesis (Baralle & Giudice, 2017), immune system maturation (Martinez & Lynch, 2013) and in response to extra-cellular stimuli (Shin & Manley, 2004). Different AS modalities are summarized in Figure 4 and include:

- exon skipping, or cassette exon; the most common type of AS in higher eukaryote (Nagasaki et al. 2005). In this event one exon is sometimes included or sometimes excluded (skipped) from the mature mRNA.
- alternative 5’ or 3' splice site; the alternative selection of these splice sites gives rise to a mRNA molecule with an exon of different length due to addition or depletion of nucleotides in its final or initial part, respectively.
- intron retention; more common in lower eukaryotes, such as fungi, due to the short length of introns compared to higher eukaryotes (Kornblihtt et al. 2013).
- mutually exclusive exons; two or more adjacent exons are spliced in a mutually incompatible way, when one is present the other is not and *vice versa*;

Other less frequent AS events, which involve the untranslated regions (UTR) of the pre-mRNA, are: alternative promoter usage and alternative polyadenylation.

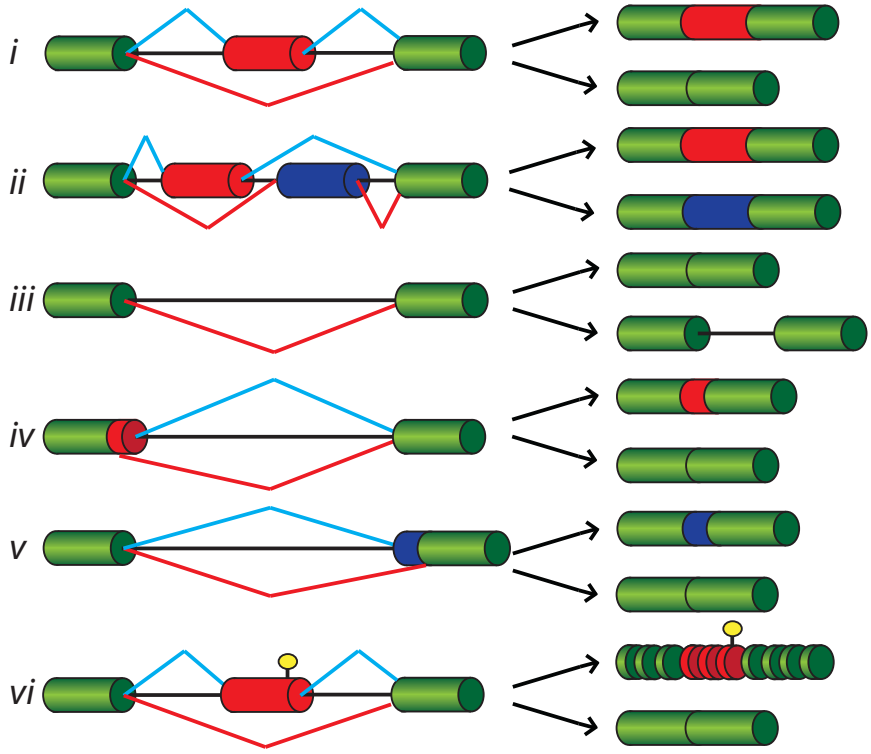


Figure 4. Scheme of the different types of AS modalities. The use of alternative splice sites at the 5' and 3' of exons gives rise to the formation of different mature mRNAs from a single gene. The different events of AS are reported: (*i*) cassette exons; (*ii*) mutually exclusive exons; (*iii*) intron retention; (*iv*) alternative 5' splice sites; (*v*) alternative 3′ splice sites; (*vi*) inclusion of a poison exon containing a premature stop-codon (yellow) leading to mRNA degradation through NMD. Precursor transcripts and final spliced products are shown (modified from Pradella et al., 2017)

Alternative splicing regulation and mechanism of action

Similarly to transcriptional control, AS events are regulated by the combined action of *trans*-acting factors and *cis*-acting sequences, which act in coordinated manner in order to determine when and where a particular AS transcript is produced.

An important characteristic of AS exons is the intrinsic weakness of their splice sites (5' and 3'ss) that are short and degenerated (Faustino & Cooper, 2003). These sequences are not sufficient to trigger exon definition since they have a reduced affinity for spliceosomal components. Thus, the recognition of AS exons relies on

the presence of additional *cis*-elements (located within or near the alternative exons) and the expression of *trans*-acting factor in a specific cellular context (Fu & Ares, 2014).

In addition to the combined action of *cis*-acting sequences and *trans*-acting factors (described in more details in the following paragraphs) AS decisions can be also modulated by other factors, such as transcription rate, chromatin architecture and epigenetic modifications (Luco et al., 2011). For example, histones and DNA methylation, as well as, nucleosome positioning and G/C content of the transcribed gene can influence the RNA pol II elongation rates thus affecting the AS profile of the nascent transcript (Kornblihtt et al., 2013). In particular, high elongation rates of RNA pol II is able to impair the recognition of weak splice sites, leading to exclusion of a specific AS exon. On the contrary, slow RNA pol II allows the recruitment of either positive or negative *trans*-acting factors on the nascent pre-mRNA with an opposite effect on the splicing outcome (Figure 5)

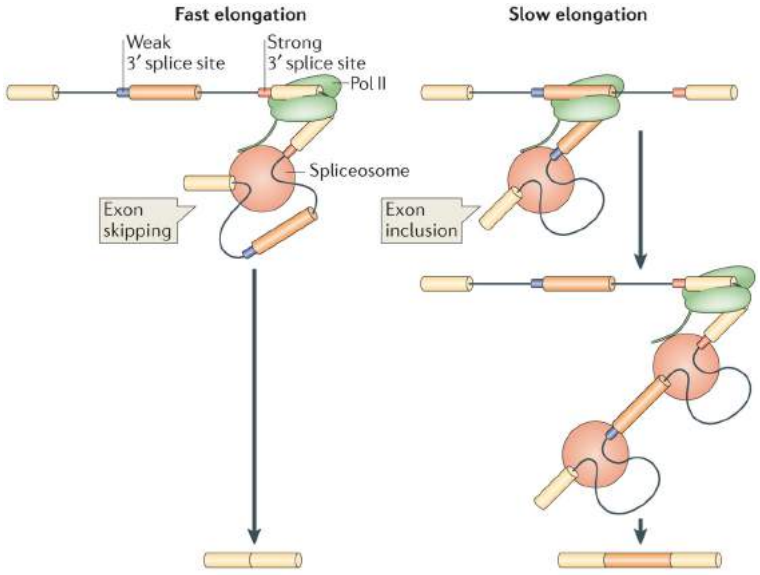


Figure 5. Kinetic models of splicing regulation mediated by the processivity of RNA pol II. RNA pol II elongation rate is able to influence AS events. A fast elongation rate (left) does not allow the recognition of weak 3' ss but facilitates the use of downstream strong 3' ss, causing exon skipping. The recognition of weak splice sites by the spliceosomal machinery is promoted when elongation occurs slowly (right) (modified from Kornblihtt et al., 2013).

Splicing-sensitive microarrays and RNA-sequencing have shown differences in the AS patterns in various cellular context and different biological processes, including cell division and cell cycle progression (Moore et al., 2010), apoptosis (Schwerk & Schulze-Osthoff, 2005), epithelial-to-mesenchymal transition (EMT) (Pradella et al., 2017) or upon genotoxic stress (Chandler et al., 2006). Notably, extensive AS changes have been annotated in different stages of tissue and organs development. For instance, different AS regulators change their expression levels during neurogenesis, including polypyrimidine tract-binding protein 1/2 (PTBP1/2) (Licatalosi et al., 2012; Li et al., 2014), SRRM4 (Quesnel-Vallières et al., 2015), RBFOX1 (Fogel et al., 2012) and Nova proteins (Jensen et al., 2000; Yano et al., 2010). Consequently, alterations in the AS profile of their pre-mRNA targets occur during neural development (Baralle & Giudice, 2017).
Similarly, numerous muscle-specific AS events take place during skeletal muscle development (Castle et al., 2008) and these required the combined and coordinated action of different families of AS factors, such as PTBP1/2, RBFOX1/2, Quacking (QK), CELF and muscleblind-1 (MBNL1) (Pistoni et al., 2010). Interestingly, during hearth development these factors change their expression levels thus leading to the transition from fetal- to adult-specific AS isoforms (Wang et al., 2016).
In the pancreas Nova1, through the regulation of the AS of genes involved in exocytosis, apoptosis, insulin signaling and transcription, is able to orchestrate some important aspects of the pancreatic beta cells biology (Villate et al., 2014).
Finally, two Ser-Arginine rich splicing factors (SRSF3 and SRSF10) and the Epithelial Splicing regulator protein 2 (ESRP2) have been shown to support postnatal liver maturation (Bhate et al., 2015).

Cis-acting splicing sequences

In addition to the BPS, 5' a 3' splice sites, numerous *cis*-acting sequences are present in the pre-mRNA and serve to enhance or inhibit the use (or "definition") of specific AS exons (Lee & Rio, 2015). These sequences, by recruiting different *trans*-acting factors, are able to promote or inhibit the assembly of the spliceosome in exons characterized by the presence of weak splice sites.
Depending on their position and function these *cis*-acting elements are classified as:

- exonic splicing enhancer (ESE);
- exonic splicing silencer (ESS);
- intronic splicing enhancer (ISE);
- intronic splicing silencer (ISS);

Exonic splicing enhancers and silencers are short, highly degenerated sequences of about 6-8 nucleotides located inside exons, generally within 200-300 base pairs to

the splice sites. Notably, it has been reported that approximately 22-25% of point mutations linked to human genetic disease generate splicing defects. This percentage increase up to 50% in genes, such as *Duschenne muscular dystrophy* (*DMD*), *PAX3* and *BRAF*, which are more susceptible to aberrant pre-mRNA splicing (Sterne-Weiler & Sanford, 2014). Many of these mutations do not occur at the splice junctions, but affect exonic splicing elements (Blencowe, 2000). For example, a mutation in exon 10 of the *microtubule-associated protein tau* (*MAPT*) gene impairs the activity of an ESS element located within this exon and increases exon 10 inclusion in the *MAPT* mRNA. Consequently, aberrant AS splicing of the *MAPT* gene affects microtubule assembly thus leading to the development of the frototemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) disease (Iovino et al., 2014).
Intronic splicing elements are less characterized than exonic elements. However, the importance of *cis*-acting elements located in intronic region for the proper recognition of weak splice sites is highlighted by the fact that intronic region adjacent to exon/intron boundaries of AS exons are more conserved at nucleotide level than regions flanking constitutive exons (Sorek and Ast, 2003). Examples of intronic enhancers are: G triplet (GGG) or G run (Gn; n >= 3) located adjacent to exon/intron boundaries and CA repeats, which provide the binding site of the *AS* regulator factor hnRNP L (Hung et al., 2007) that are able to repress exon inclusion.

Trans-acting factors

Trans-acting splicing factors, are non-snRNP proteins able to interact with the pre-mRNA and regulate the assembly of the spliceosomal machinery on the 3' and 5' splicing sites. Serine-arginine (SR) factors and heterogeneous ribonucleoproteins (hnRNPs) are the two major classes of ubiquitously expressed *trans*-acting factors (Black, 2003). On the contrary other splicing factors, such as the aforementioned Nova1/2, ESRP1/2, PTB2, Fox1/2, MBNL1/2/3, CELF1/2, TIA and STAR families, display a cell-type-restricted pattern of expression (Braunschweig et al., 2013).
SR proteins, by binding to splicing enhancers sequences (ESE or ISE), generally promote exon recognition, whereas hnRNPs repress splicing through the recognition of silencer *cis*-elements (Figure 6) (Singh & Varcarcel, 2005). The mechanism of action of tissue-specific splicing factors is more peculiar and depends also on the position of the recognized binding sites on the pre-mRNA target (see below).

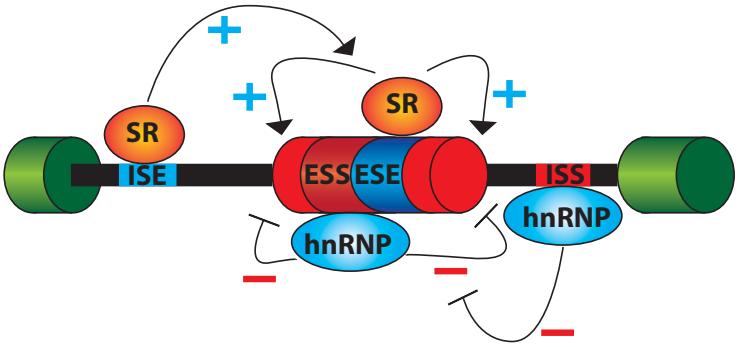


Figure 6. *Cis*-acting sequences. Intronic and exonic splicing enhancers (ISE and ESE) promote the inclusion (+) of the AS exon (red) by providing the binding sites for activators (orange circles), whereas intronic and exonic splicing silencers (ISS and ESS) are bound by repressors (blue circles) and promote exon skipping (-). Generally, ESE-bound SR factors stimulate the assembly of the splicesome on the variant exon or counteract the inhibitory activity of hnRNPs bound to ESS elements. On the contrary, hnRNPs interfere with the assembly of spliceosome to the variant exon leading to exon skipping. In addition, hnRNPs by binding ISSs located in the introns flanking a variant exon cause its looping out and skipping, whereas when bound to ESSs they may polymerize along the exon and displace the ESE-bound SR proteins (not shown) (modified from Pradella et al., 2017).

Activity of splicing factors is controlled and regulated by different mechanisms in eukaryotic cells (Heyd and Lynch, 2011). The first level of regulation is the transcriptional control. Importantly, in contrast to the mechanism of action of several transcription factors, even relatively modest changes in the expression of a specific *trans*-acting factor are able to dramatically alter splicing pattern profiles of several target pre-mRNAs (Heyd and Lynch, 2011). Expression levels of splicing factors dynamically changes in many biological processes (Braunschweig et al., 2013). However, in many cases the molecular mechanisms at the base of these alterations remains unknown. An interesting example in which a splicing factor increases its expression level upon transcriptional activation comes from CELF2 during T-cell activation. In the immune system CELF2 expression is required for the proper response during antigen-induced T-cell activation (Mallory et al., 2011). CELF2 regulates around 70 specific AS events, which are altered upon stimulation of cultured T-cell. Remarkable, *CELF2* transcription is controlled by the activation of NF-kB that binds to highly conserved regions upstream of the *CELF2* transcription start site (Mallory et al., 2015).
Post-transcriptional and post-translational modifications are also able to modulate

the expression of splicing factors by affecting their stability at both protein and mRNA levels. Interestingly, in several genes for SR factors and hnRNP proteins there are alternatively spliced cassette exons carrying premature stop-codon able to induce mRNA degradation through non-sense mediated decay (NMD) pathway (Lareau & Brenner, 2015). This process is known as alternative splicing activated NMD (AS-NMD) (Isken & Maquat, 2008). Interestingly, for several splicing regulators AS-NMD cassettes overlap highly conserved or ultraconserved elements (UCEs) longer than 200 bp, with 100% identity among rat, mouse, and human genomes (Lareau et al., 2007; Ni et al., 2007). A number of SR proteins, such as SRSF1, SRSF3, SRSF4, can control their own mRNA level by regulating AS-NMD in a feedback loop mechanism that act to maintain the protein homeostatic level in the cell (Laureau et al., 2007; Valacca et al., 2010; Guo et al., 2015; Änkö et al., 2010). Otherwise the recognition of the stop-coding containing exon is mediated by other SR proteins, as described for SRSF3 that recognizes *SRSF5* pre-mRNA (Änkö et al., 2012).
Finally, post-translational modifications, including methylation, acetylation and phosphorylation of specific residues, can affect the recruitment of the splicing factors on the pre-mRNA or alter their import in the nucleus, where the splicing reaction takes place (Zhou & Fu, 2013). A prototypical example is given by SRSF1. In addition to be regulated at transcriptional (Das et al., 2012) and post-transcriptional levels (Valacca et al., 2010), SRSF1 is also regulated through different post-translational mechanisms. For instance, both methylation of arginine residues of its RNA-binding domains and phosphorylation in its RS region promote SRSF1 import in the nucleus, an essential step for SRSF1 activity as a splicing factor (Sinha et al., 2010; Serrano-Gomez et al., 2016).

SR proteins

SR proteins are a highly conserved family of *trans*-acting factors involved in both constitutive and AS regulation (Shepard & Hertel, 2009). Generally, SR proteins by binding to splicing enhancer sequences, through one or two RNA-recognition motifs (RRM) in their N-terminal region, promote exon inclusion (Shepard & Hertel, 2009). In addition to the RRM motifs, SR proteins are characterized by the presence, at the *C-terminus,* of a domain of variable length rich in serine – arginine dipeptides (RS domain) (Shepard & Hertel, 2009) (from which the name of SR protein). SR proteins are more commonly found in the nucleus (in the nuclear speckles), but several SR proteins are known to shuttle between the nucleus and the cytoplasm (Cáceres et al., 1998). Nuclear localization of the SR proteins is favored upon phosphorylation of the RS domain, whereas unphosphorylated (or partial dephosphorylated) SR proteins are found in the cytosol (Zhou & Fu, 2013).

Biochemically, RRM domains determine the RNA-binding specificity for the pre-mRNA target, whereas the RS domain is deputed to protein-protein interactions required during spliceosome assembly (Tacke & Manley, 1999). Upon binding to ESE elements, SR factors favor the formation of the early spliceosomal complex by promoting the formation of U1-dependent complexes at 5' ss of the pre-mRNA (Eperon et al., 1993). Moreover, SR proteins are important for the binding of U2 snRNP to the BPS and during late steps of splicing reaction, such as the recruitment of U4/U6 snRNP and U5 snRNP (Blencowe, 2010).
As reported above, SR proteins are well-known for their role in promoting exon inclusion during AS reactions. Mechanistically, exon recognition is promoted by either stabilization of spliceosomal components or preventing the recruitment of splicing repressor, such as an hnRNP proteins. However, increasing evidence suggest also a role of SR proteins in favoring exon skipping events (Han et al., 2011; Ghigna et al., 2005). SR proteins by binding to intronic sequences adjacent to splice sites generate steric hindrances that prevent the correct assembly of the spliceosome (Ibrahim et al., 2005), thus blocking exon recognition. A different mechanism by which SR proteins promote exon skipping is due to strong binding of these factors to adjacent constitutive exons (Ghigna et al., 2005). In this case, SR binding forces the communication between two constitutive exons, thereby suppressing the inclusion of the internal alternative exon (Han et al., 2011).
Finally, in addition to splicing regulation, SR proteins have also been associated to different steps of the gene expression, including mRNA export and translation, mRNA degradation, pri-miRNA processing, (Twyffels et al., 2011; Sanford et al., 2004) and genome stabilization (Zhong et al., 2009)

hnRNPs

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are the most abundant proteins present in the nucleus (Chaudhury et al., 2010). Similarly to SR proteins, hnRNPs have a modular structure characterized by the presence of RNA-binding domains flanked by auxiliary domains with different functions and properties (Chaudhury et al., 2010). hnRNPs are able to bind to ESS and ISS, thus repressing both constitutive and AS decisions (Geuens et al., 2016).
Interaction with the pre-mRNA is mediated by different type of RNA binding domain (RBD). The four major types of RBD are RRM, structurally related to the RBD of SR proteins (Görlach et al., 1992); the quasi-RRM domain; the K-homology (KH) domain found in hnRNP E and HnRNP K (Valverde et al., 2008) and the RGG (Arginine - Glycine - Glycine) box. Auxiliary domains, including glycine-rich, proline-rich and acidic domains, are required for protein-protein interactions (Jean-Philippe et al., 2013).

The ability of hnRNPs to function as splicing repressor can be explained through different molecular mechanisms (Busch & Hertel, 2011):
- "direct competition"; by binding to ESS adjacent ESE, hnRNPs can sterically block the recruitment of SR proteins, thus preventing spliceosomal assembly.
- "looping out" model; dimerization of hnRNPs bound on flanking regions of an alternative exon is sufficient to mask the internal exon from the spliceosome.
-"multimerization and cooperative binding"; hnRNPs, through their auxiliary domains, are able to multimerize along the pre-mRNA. Polymerization process usually starts from a strong exonic or intronic splicing silencers and is able to displace splicing activators bound at distant sites. Different hnRNPs may cooperate to this process.
Importantly, hnRNPs are also able to promote exon inclusion when bound to ISE (Chou et al., 1999). Examples of this situation is represented by the hnRNP G, which favor the inclusion of exon 7 of *SM* gene (Moursy et al., 2014), and hnRNP E that affect *CD44* AS regulation (Meng et al., 2007).

Tissue-specific splicing factors

While SR and hnRNPs factors are ubiquitously expressed proteins, a number of tissue-specific splicing factors have also been described. Importantly, the tissue-restricted expression of these splicing factors is fundamental to establish the proper spatio-temporal generation of multiple splice variants in many cellular- and/or developmental-specific processes.
Many tissue-specific AS regulators are enriched in central nervous system (CNS) tissues. Compared to other tissues, mammalian CNS is characterized by the presence of several specialized cell types and need to plays complex and particularly plastic functions that are involved in the growth of neuronal dendrites, synapse development, as well as the development of the neuromuscular junction. In line with this, CNS shows high levels of tissue-specific AS regulation and AS isoforms expressed in the brain are more conserved compared to other tissues (Lipscombe, 2005). In the brain the establishment of an intricate network of connection is achieved by the generation of thousand of AS isoforms. Nova1/2, nPTB, Fox1/2 are among the best-studied brain-specific splicing factors involved in different biological processes. In particular Nova and Fox proteins regulate synapse development and activity (Ule et al., 2003; Lee et al., 2016a), whereas nPTB inhibits splicing of alternative exons in genes encoding for proteins associated with proliferation, cytoskeleton organization and cell fate (Licatalosi et al., 2012).
Skeletal muscle is another tissue that shows an elevated degree of AS. In this regard, MBNL, CELF, RBM20 and RBM24 play fundamental roles in the

production of muscle-specific splicing isoforms required during myogenesis (Kino et al., 2009; Yang et al., 2014). Recently, through gain- and loss-of-function experiments, it has also been reported that Rbfox1 and Rbfox2 splicing factors cooperate in regulating splicing of *Mef2D* gene that encodes a transcription factor essential durig muscle cell differentiation (Runfola et al., 2015).
In testis, expression of testis-specific AS variants involved two *trans*-acting factors, such as RBMY (RNA-binding motif encoded by the Y chromosome) and T-STAR (testis-signal transduction and RNA processing) (Elliott & Grellscheid, 2006; Yeo et al., 2004).
Finally, two epithelial-restricted splicing regulators, ESRP1 and ESRP2, have been also described (Warzecha et al., 2009). These are key regulators of epithelial identity that plays important functions during epithelial-to-mesenchymal transition (EMT), a process associated with metastasis formation as well as with generation and maintenance of cancer stem cells (Pradella et al., 2017).
The importance of splicing factors for neurogenesis, muscle development and other developmental processes is underscored by the fact that splicing defects play a causative role in several neurodegenerative diseases (Licatalosi & Darnell, 2006; Tazi et al., 2009), myotonic dystrophies (Kino et al., 2009) and cardiomyopathies (Guo et al., 2012).

Nova1 and Nova2

The Nova (neuro-oncological ventral antigen) family consists of two RNA-binding proteins, Nova1 and Nova2, initially discovered as auto-antigens in paraneoplastic opsoclonus-myoclonus ataxia (POMA) (Buckanovich et al., 1996; Yang et al., 1998). POMA is an autoimmune disorder in which patients with breast, ovarian or small cell lung cancer develop involuntary movements of eyes, limbs or face and in some cases ataxia or dementia. The syndrome is thought to be induced by the solid tumor through the expression of proteins, which are normally present in the CNS (Buckanovich et al., 1993). Importantly, the presence of onconeural antigens in paraneoplastic syndromes has provided a means, for instance anti-sera, to identify neuro-specific proteins, such as Nova factors extensively studied by the group of Prof. Robert B. Darnell (Darnell, 1996).
Nova1 displays restricted expression in the CNS (specifically in the hypothalamus, ventral midbrain, hindbrain and spinal cord) while Nova2 is expressed with a reciprocal pattern of expression to Nova1 since it is enriched in the neocortex and in the hippocampus (Buckanovich et al., 1993; Racca et al., 2010).
Structurally, Nova proteins are characterized by the presence of three K-homology domains - RNA-binding elements first identified in the human hnRNP K and involved in RNA stabilization and translational (Burd & Dreyfuss, 1994) -

interposed by flexible linkers (Buckanovich et al., 1993; Lewis et al., 1999). Nova proteins are highly conserved in their amino acid sequences. In particular, the overall 75% identity increases to 87-93% at the level of KH domains (Lewis et al., 1999). KH domains are RNA-binding motifs present in single or multiple copies in a wide variety of regulatory proteins, from prokaryotes to eukaryotes (Valverde et al., 2008). Crystallographic and NMR structure resolutions of Nova KH-3 domain have shown the molecular organization of this element: a specific pattern of hydrophobic residues, an invariant Gly-X-X-Gly segment and a variable loop that allow both RNA and protein interactions (Lewis et al., 1999; Musco et al., 1996). Similarly, KH-1 and KH-2 domains are able to interact with specific RNA sequences and form hydrophobic intramolecular dimer (Teplova et al., 2011).

Several approaches, such as SELEX (*systematic evolution of ligands by exponential enrichment*) and CLIP (*ultraviolet cross-linking and immunoprecipitation*) have been used to identify the consensus binding site recognized by Nova proteins on the pre-mRNA target (Lewis et al., 2000; Ule et al., 2003). A cluster of YCAY (at least three YCAY tetramers), interspersed in a various number of nucleotides, are required for RNA:Nova high-affinity binding (Ule et al., 2006). Following these studies, a number of pre-mRNAs were subsequently identified based on the presence of these YCAY elements (Buckanovich & Darnell 1997; Lewis et al. 2000; Dredge & Darnell, 2003).

These studies have also shown that, depending on the position of YCAY clusters in the pre-mRNA targets, Nova proteins are able to promote or repress the inclusion of specific AS exons. In particular, Nova proteins by binding to upstream intronic or exonic YCAY repeat elements support exon skipping, while they stimulate exon inclusion when bound to downstream intronic region (Ule et al., 2006). It is demonstrated that Nova proteins, by binding ISS immediately upstream of AS exon, block the recognition of the 3' ss by the U2 snRNP component of the spliceosome (Ule et al., 2006). Similarly, ESS recognized by Nova prevents the recruitment of U1 snRNP to the 5' ss (Figure 7) (Teplova et al., 2011).

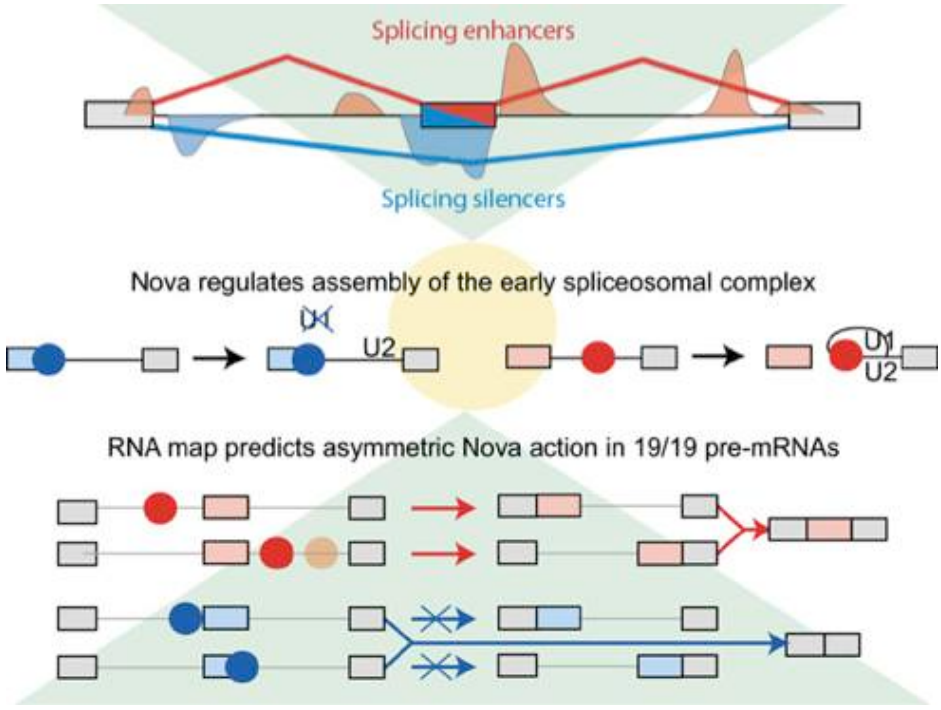


Figure 7. Nova-RNA binding map. Depending on the location of Nova clusters on the pre-mRNA target, different mature mRNAs can be generated. Clusters (YCAY, Y= pyrimidine) located down-stream of the alternative exon promote the proper assembly of the spliceosome (in red). On the contrary, clusters positioned up-stream and inside the alternative exon impair U1 snRNP association with the 5' ss, resulting in a exon skipping event (in blue) (modified from Ule et al. 2006).

Nova1 and Nova2 genetically null mice have been extensively used to assess Nova functions in the CNS. Nova1 knockout (KO) mice die between 7 to 10 days after birth for motor deficiencies as a consequence of apoptosis of brainstem and spinal cord neurons (Jensen et al., 2000). Interestingly, these mice display some phenotypes similar to that observed in POMA patients (Jensen et al., 2000). Histopathological analysis shown an increase of pyknotic cells in the spinal cord, in the deeper cerebellar but not in regions of the brain that do not express Nova1. Remarkable, Nova1 null mice shown aberrant AS profiles of neuron-specific pre-mRNAs containing YCAY cluster elements (Jensen et al., 2000). These results demonstrated for the first time the role of Nova1 in neuronal development, thus

proposing Nova1 as the first tissue-specific AS regulator (Jensen et al., 2000).
Similarly to Nova1, Nova2 KO mice born indistinguishable from littermates, but exhibit progressive motor dysfunction and died between 14-18 days after birth (Saito et al., 2016). Notably, some of the phenotypes of these mice (such as agenesis of the corpus callosum, axonal outgrowth defects specific to ventral motoneuron axons and efferent innervation of the cochlea), were specific to Nova2 deficiency (Saito et al., 2016). At the molecular levels these defects were associated to aberrant AS of pre-mRNAs encoding for key components in cortical, brainstem and spinal axon guidance/outgrowth pathways during neural differentiation (Saito et al., 2016).
Nova double knockout mice (Nova1 Nova2 DKO) have been also generated in order to unmasking compensatory effects in neuronal compartments in which both proteins are expressed (Ruggiu et al., 2009). Nova DKO mice born alive but are completely paralyzed since they fail to clusterize the acethylcoline receptor at the neuromuscular junction (Ruggiu et al., 2009).
The generation of Nova1, Nova2, and Nova DKO mice in combination with the advances in biochemical, computational and bioinformatics tools helped to reveal the complex network of Nova-regulated genes during neural development. In particular, it has been reported that Nova2 regulates a significant fraction (approximately 7%) of all brain-specific AS events (Ule et al., 2006). These target genes encoding for regulators of synapse development and transmission, neuronal migration and axon guidance, cell-cell signalling and dynamic organization of the cytoskeleton (Ule et al., 2003; Ule et al., 2005; Zhang et al., 2010a). In addition, many of these targets interact with each other, suggesting that Nova2-mediated AS plays an important role in affecting physical interactions between these factors in protein complexes.
Specific examples of Nova targets in the brain include the neuronal ionotropic receptor *GABAA Rγ2*, the glycine receptor α2 gene (*GlyRα2*) and the cell adhesion molecule involved in synaptic transmission, *Neurexin1* (Zhang et al., 2010). Importantly, it has been found that Nova2 functions in neuronal migration, axon outgrowth and axon guidance (Leggere et al., 2016). In line with this, Nova2 uniquely regulates AS splicing of different axon guidance genes, including *Robo2 (Roundabout guidance receptor 2)*, *Slit2 (Slit Homolog 2)*, *Epha5 (Ephrin type-A receptor 5)*, the Netrin-receptors *DCC (Deleted in Colorectal Cancer)* and *Neogenin* (Saito et al., 2016).
Recent works have also been shown that the expression and functions of Nova proteins are not restricted to the CNS. Outside the CNS, Nova proteins expression has been found in endothelial cells of blood vessels (only Nova2) (Giampietro et al., 2015), pancreatic β cells (only Nova1) (Villate et al., 2014) and in white adipocytes (both Nova1 and Nova2) (Vernia et al., 2016). In these tissues, Nova

proteins control different biological processes by regulating AS decision of genes harboring YCAY clusters. Our group recently demonstrated that Nova2 plays an important role in vascular development (Giampietro et al., 2015). In particular, Nova2 regulates endothelial cells polarity and vascular lumen formation, which are fundamental steps occurring during invasion and growth of the incipient vascular sprouts through activation of angiogenesis process (Iruela-Aripse & Davis, 2009). Notably, these events are associated to Nova2-dependent AS regulation of target genes encoding for components of the Par polarity complex and its regulators (Giampietro et al., 2015). In pancreatic islets, Nova1 regulates a large number of genes implicated in beta cell functions, including exocytosis, insulin receptor signaling and apoptosis (Villate et al., 2014). Also, Nova proteins have been demonstrated to regulate thermogenesis in adipocytes by regulating AS of genes involved in JNK signaling (Vernia et al., 2016). Finally, in addition to their well-characterized role as splicing regulators, several recent works have revealed that Nova proteins play also important roles in mRNA localization at the synapse (Racca et al., 2010), mRNA stability through activation of AS-NMD program (Eom et al., 2013) and neuronal miRNA function (Storchel et al., 2015).

Alternative Splicing and Cancer

Cancer is a heterogeneus and complex disease that involve a series of genetic, cellular and environmental interactions (Knox, 2010). However, different tumor types are characterized by a common overall phenotype: uncontrollable proliferation and growth (Futreal et al., 2004). Indeed, neoplastic cells gain the ability to divide indefinitely through mechanisms that dramatically change their gene expression profiles (Hanahan & Weinberg, 2000, Biamonti et al., 2014). During each step of this progression, cancer cells acquire mutations that activate oncogenes and disrupt tumor suppressor genes, thus eventually allowing unrestrained cell growth, escape from apoptosis and other important hallmarks of cancer (Lee & Muller, 2010). Moreover, cancer cells are associated with many different stromal cells, including fibroblasts, macrophages and lymphocytes, but also with endothelial cells and pericytes recruited to the tumor vasculature (Balkwill et al., 2012). Thus, a global reprogramming of gene expression takes place in both cancer cells and cells of the tumor microenvironment (Gamazon & Stranger, 2014; Bainer et al., 2016).
Importantly, increasing evidences of a causative role of aberrant AS in cancer have been provided (Ghigna et al., 2008, Bonomi et al., 2013b). The identification of cancer-related splicing variants has supported the notion that splicing fidelity is lost during cancer progression (Oltean & Bates, 2013; Ghigna et al., 2008; Biamonti et al., 2014) and splicing factors can act as *bona-fide* oncoproteins (Dvige et al.,

2016). In particular, AS is used by the cancer cell to quickly change their proteome and generate novel proteins involved in different cancer hallmarks, including angiogenesis (Vorlová et al., 2008), cell proliferation (Yanagisawa et al., 2008), invasion and metastasis (Oltean & Bates, 2013), evasion of apoptosis and senescence (Karni et al., 2007). Moreover, it has been estimated that more than 15.000 AS isoforms are cancer-specific (He et al., 2009) suggesting that these isoforms not only elucidated many fundamental hallmarks of cancer, but also offer innovative chances to improve the efficacy of anti-cancer therapies (Bonomi et al., 2013; Lee & Abdel-Wahab, 2016).

Two different classes of mutations are able to affect the fidelity of the splicing reaction in cancer cells: i) mutations that occurs in *cis*-elements or splice sites in the pre-mRNA; ii) mutations affecting the expression and/or activity of *trans*-acting factors (Climente-Gonzàlez et al., 2017).

i) Splicing mutations of the first group can be divided into two subclasses. Subclass I, associated with severe diseases, comprises splicing mutations occurring in splice sites and that block exon definition. Subclass II, associated with a mild phenotype, comprises mutations in not conserved sequence (for example the poly-P tract) or that create novel donor or acceptor splice sites. The best characterized examples are provided by *BRCA1* and *BRCA2* two genes that confer an increased risk for early onset breast and ovarian cancer (Yurgelun et al., 2017). BRCA1 and BRCA2 are part of complexes involved in maintenance of genome stability upon different stresses, such as double-strand breaks or defective replication forks (Nielsen et al., 2016). In the recent years, many efforts have been done to assess the pathogenicity of several mutations in the *BRCA1* that potentially alter the outcome of the splicing reaction (Ahlborn et al., 2015; Esposito et al., 2016; Wappenschmidt et al., 2012). Interesting examples are reported below:

- *mutations in exonic splicing enhancers*: a G>C transversion in *BRCA1* exon 18 has been reported to impair SRSF1 binding on the pre-mRNA, resulting in inappropriate skipping of the constitutive exon 18 and production of an inactive BRCA1 protein (Mazoyer et al., 1998; Liu et al., 2001);
- *creation of criptyc splice sites*: a mutation in *BRCA2* intron 12 creates the perfect consensus sequence for a new splice site, thus promoting the inclusion in the final mRNA of additional nucleotide sequences (Anczukov et al., 2012);
- *disruption of natural splice site*: a A>G mutation, identified in four unrelated Spanish families (Vega et al., 2002), affects the use of the donor splice site in intron 5 and causes a deletion of 22 nucleotides in exon 5 and the formation of a premature stop codon.

ii) The second group includes mutations affecting the activity, localization and/or expression of *trans*-acting splicing factors. Somatic mutations of spliceosomal components have been frequently detected in several types of hematological

malignancies and in solid tumors (Dvinge et al., 2016). These mutations, generally in heterozygosis, affect splicing regulators engaged in the initial steps of spliceosome assembly, such as U2AF1, SRSF2 (or SC35), SF3B1 and ZRSR2 (Yoshida & Ogawa, 2014). Specifically, *U2AF1, SRSF2* and *SF3B1* are found mutated in different hematopoietic malignancies, from 50% in chronic myelomonocytic leukaemia (CMML) (Chesnais et al., 2012) to approximately 20% in chronic lymphocytic leukaemia (CLL) (Oscier et al., 2012). Interestingly, among the different components of the spliceosome, the vast majority of mutations have been found in only these four genes, suggesting that specific mutations can confer a gain-of-function that drives tumorigenesis. Importantly, *U2AF1*, *SRSF2* and *SF3B1* may be classified as *bona-fide* proto-oncogenes (Dvinge et al., 2016). SF3B1 is required for the recognition of the 3' ss during splicing reaction and mutations that affect the HEAT domains of this protein were associated with an increased ability of SF3B1 to bind criptyc 3' ss (normally not recognized by the spliceosome) (DeBoever et al., 2015). For instance, a K700E substitution in SF3B1 protein was reported to alter splicing of the *ABCB7* (iron transporter ATP-binding cassette subfamily B member 7) pre-mRNA leading to recognition of a cryptic 3' ss and to introduction of a premature stop codon in *ABCB7* mRNA that, as a consequence, is degradated by NMD pathway (Dolatshad et al., 2016). Down-regulation of ABCB7 protein ultimately causes accumulation of mitochondrial iron that potentially enhances reactive oxygen species (ROS) production and genome instability, important processes to sustain tumor progression (Torti & Torti, 2013). Similar findings were also reported for U2AF1, involved in the recognition of the 3' ss during splicing reaction (Shao et al., 2014). Mutations in U2AF1 zinc finger domains are able to change the RNA binding capability of this splicing factor, thus altering at the same time the AS profiles of different target genes. A typical example of abnormal splicing caused by mutation in U2AF1 zinc finger domain involves the anti-apoptotic gene *BIRC6*. In which retention of intron 59-60 causes the production of a loss-of-function BIRC6 isoform, which partially explain the enhanced apoptosis found in mutant U2AF1-transduced cells (Yoshida et al., 2011).

Expression of several SR and hnRNP proteins have been frequently found altered in tumors and this observation was linked to aberrant production of AS variants with predictable effects on tumor cell behavior (Bonomi et al., 2013a; Ghigna et al., 1998; Ghigan et al., 2008). Salient examples that show how changes in the level of splicing regulators in cancer cells may severely impact gene expression programs involved in tumor progression are reported below.

Resistance to cell death. Apoptosis is a process of programmed cell death used by multicellular organisms to maintain tissue homeostasis during development or in response to stress and pathogens (Taylor et al., 2008). Several genes involved in the propagation of the apoptotic signals are regulated through AS process. A well-characterized example involves the production of the pro- or anti-apoptotic isoforms of Bcl-X, a member of the Bcl-2 family that control apoptosis by governing mitochondrial membrane breakdown (Youle & Strasser, 2008). Different Bcl-X isoforms result from the selection of two alternative 5' ss in exon 2 of *Bcl-X* pre-mRNA: Bcl-xL, the longest anti-apoptotic form that promotes cell survival and Bcl-xS, which instead induces cell death (Bielli et al., 2011). Importantly, Bcl-xL/Bcl-xS ratio is altered in several cancers (Zhu et al., 2005) and has significant roles in determining the sensitivity of tumor cell to apoptotic agents, such as chemotherapeutic drugs (Zhu et al., 2005). Several splicing factors are involved in the regulation of these two alternative 5' ss in exon 2 of *Bcl-X* pre-mRNA, including Sam68, hnRNP A1, SRSF1, SRSF9, hnRNP F/H, RBM25, RBM11 and RBM4 (Ghigna et al., 2008). In particular, RBM4 promotes the formation of the longest variants, whereas SRSF1 has an opposite effect (Wang et al., 2014). In line with the promotion of the pro-apoptotic isoform, RBM4 has been found down-regulated in many cancer types, whereas SRSF1 is often up-regulated during tumor progression (Yong et al., 2016; Karni et al., 2007; Gout et al., 2012; Wang et al., 2014).

The opposite functions of Bcl-X isoforms and their altered expression in cancers (compared to normal tissues) (Zhu et al., 2005) suggested that *Bcl-X* pre-mRNA splicing could be used to develop new therapeutical interventions based on highly selective splicing correction approaches. Several groups, by using antisense oligonucleotides (ASO), have redirected *Bcl-X* splicing from the production of the anti-apoptotic Bcl-xL isoform toward the production of the pro-apoptotic Bcl-xS isoform in various cancer types, including prostate, breast and melanoma (Mercatante et al., 2002; Bauman et al., 2010).

Other genes involved in apoptosis program, such as Caspase-9, BIM and bridging integrator 1 (BIN1), are regulated by AS and altered expression of SRSF1 was associated to the production of the anti-apoptotic splicing variants of these genes (Shultz et al. 2011; Anczuków et al., 2012).

Evading growth suppressors. *BIN1* encodes for a tumor suppressor able to inhibit tumor growth by binding to the transcription factor MYC, a proto-oncogene implicated in the pathogenesis of most types of human cancers (Sakamuro et al., 1996; Gabay et al., 2014). SRSF1, in addition to promote the production of anti-apoptotic splicing isoforms in cancer cells, stimulates the inclusion of exon 12a in BIN1 pre-mRNA, thus generating a protein variant lacking of tumor suppressor activity (Karni et al., 2007). Also RBM4 regulates *BIN1* exon 12a splicing in an

opposite fashion respect to SRSF1, since it promotes the production of an anti-oncogenic splice variant (Wang et al., 2014).

Invasion and metastasis formation. During tumor progression of epithelial tumors, cancer cells acquire an invasive and motile phenotype in order to invade adjacent tissues and disseminate toward distant organs. EMT (Epithelial-to-mesenchymal transition) has been proposed as mechanism by which epithelial cancer cells lose their cohesive cell-cell junctions and acquire a motile and invasive phenotype (Thiery et al., 2009). EMT requires a robust reprogramming of gene expression through the involvement of different transcription factors (Thiery et al., 2009). However, AS has recently emerged as an important player that cancer cells use to quickly modify their proteome during activation of the EMT program (Pradella et al., 2017).

In addition to the two epithelial-restricted splicing proteins ESRP1 and ESRP2, other splicing factors are required to generate AS signatures of EMT, including RBFOX2, MBNL1, hnRNP A2/B1, SRSF1 and SRSF3 (Pradella et al., 2017). Aberrant expression of all these factors during EMT regulates AS of a large plethora of pre-mRNAs encoding for proteins involved in different fundamental steps of this program, such as migration (FGFR2, RON and CD44), polarity and cytoskeleton organization (NUMB, RAC, p120) and transcriptional regulation (TCFL2) (Pradella et al., 2017).

In the past our group provided the first example of a gene with specific AS event associated to activation of EMT (Ghigna et al., 2005). The RON proto-oncogene encodes a tyrosine kinase receptor that activates a signaling cascade leading to cell dissociation, migration and matrix invasion (Collesi et al., 1996). Through skipping of exon 11, AS process generates a constitutively active isoform (called ΔRON isoform) able to confer increased motility to the expressing cells (Collesi et al., 1996). ΔRON is frequently over-expressed in epithelial cancers (Ghigna et al., 2005; Zhou et al., 2003) and its production is stimulated by SRSF1 overexpression, which in turn activates the EMT program (Ghigna et al., 2005). In addition, skipping of exon 11 is also stimulated by hnRNP A2/B1, another splicing regulator involved in EMT with altered expression in cancers, whereas it is inhibited by hnRNP A1 that its able to antagonize SRSF1 binding (Bonomi et al., 2013). Notably, inhibition of SRSF1 binding (through bifunctional ASO) or SRSF1 activity (by using small molecule inhibitors) was found sufficient to prevent ΔRON production. Interestingly, inhibitors of SRSF1 activity were also able to reverse the invasive phenotype of transformed cell (Ghigna et al., 2010) suggesting that these treatments may have therapeutic applications for anti-cancer purposes.

Finally, matrix metalloproteinases (MMPs), important components of the tumor microenvironment, are upregulated in almost all types of human cancer and

associated with poor survival (Egeblad & Werb, 2002). Notably, over-expression of MMPs also triggers a cascade of events that determine activation of EMT process (Radisky et al., 2005). Interestingly, treatment of tumor epithelial cells with MMP3 promotes the production of a constitutively active alternatively spliced isoform of Rac1, a small GTPase of the Rho family implicated in the organization of the actin cytoskeleton, cell growth, cell-cell adhesion and migration (Bosco et al., 2009). This isoform, called Rac1b, is down-regulated by hnRNP A1 splicing factor (Pelish et al., 2012) and an inverse correlation between expression levels of hnRNP A1 and Rac1b in breast cancer has also been reported (Pelish et al., 2012). Additional examples regarding the contribution of AS to EMT in cancer cells have been summarized in a review published recently in the journal "*Molecular Cancer*" in which I am first author (Pradella et al., 2017).

Sustaining proliferative signaling. Deregulated cell proliferation in cancer cells occurs through altered expression or activity of cell cycle proteins or by constitutive activation of components of signal transduction pathways (Feitelson et al., 2015). There are several examples of genes sustaining proliferative signaling, which are regulated by AS process and characterized by AS isoforms frequently altered in cancer cells (Sveen et al., 2016). Among them, it is important to mention *NUMB*, *BRAF*, *FGFR2*, *HRAS*, *KRAS* and *PTEN* (Sveen et al., 2016). For instance *NUMB* gene, encoding an endocytic protein that antagonizes Notch signaling (Santolini et al., 2000), is characterized by AS isoforms that sustain cellular proliferation (Bechara et al., 2013). Importantly, skipping of *NUMB* exon 9 AS, an event frequently altered in lung adenocarcinomas (Bechara et al., 2013), generates a NUMB splicing variant (NUMB-PPR^{S}) able to repress Notch signaling and, thus, cell proliferation (Cieply & Cartens, 2015). On the contrary, inclusion of exon 9 drives the production of NUMB-PRR^{L} able that reduces NUMB expression (Cieply & Cartens, 2015), thus enhancing transcription of Notch target genes. Different *trans*-acting splicing factors are involved in *NUMB* exon 9 regulation (Bechara et al., 2013; Zong et al., 2014). Among these, RBM10 or QK1 can directly interact with different intronic *cis*-elements upstream exon 9 and prevent the recognition of the alternative exon by the spliceosomal machinery, thus promoting the production of the anti-proliferative NUMB-PPR^{S} isoform (Bechara et al., 2013; Zong et al., 2014). Interestingly, the observation that mutations in RBM10 are frequent in lung cancer (Bechara et al., 2013) and that QK1 is often down-regulated in the same tumor (Zong et al., 2014) suggests the importance of NUMB-PPR$^{S/L}$ splicing switch for cancer progression (Cieply & Cartens, 2015).

Avoiding immune destruction. Tumor escape from immunological surveillance is well represented by the death receptor Fas (or CD95). Fas, a cell surface proteins belonging to the tumor necrosis factor receptor family, is able to mediate cell death upon binding with its ligand, CD95L (Peter et al., 2015). Expression of Fas and its ligand by cancer cells confer resistance to Fas-induced apoptosis and in the same way allows tumor cells to "counterattack" immune system (Igney et al., 2000). *Fas* pre-mRNA is alternatively spliced and this process has been extensively investigated. The RNA-binding protein TIA-1, by binding a sequence downstream exon 6 in *Fas* pre-mRNA, favors the recruitment of U1 snRNP, thus promoting inclusion of exon 6. Interestingly, several additional *trans*-acting splicing factors have been reported to inhibit exon 6 recognition (exon skipping event), including PTB (Izquierdo et al., 2005), RBM5 (Bonnal et al., 2008) and HuR (Izquierdo, 2008). Functionally, skipping of exon 6 removes the transmembrane domain of the receptor, generating a soluble isoform (sFas) able to prevent the interaction between CD95L and Fas receptor. Notably, elevated expression of sFas has been detected in several tumors and shown to correlate with tumor grade (Konno et al., 2000; Kondera-Anasz et al., 2005).

Deregulating cellular energetics. Tumorigenesis process relies on the reprogramming of cellular metabolism by cancer cell in order to satisfy the biosynthetic demands associated with an increased proliferation (Pavlova & Thompson, 2016). Aerobic glycolysis, rather than TCA cycle and oxidative phosphorylation, generates a number of reducing intermediates and precursor molecules that can divert into different metabolic pathways. Indeed, is not surprisingly that glycolytic enzymes are often overexpressed in cancer (Wang et al., 2012; Ying et al., 2012), including lactate dehydrogenase, the enzyme that catalyze the final step of aerobic glycolysis, which is the production of lactate from pyruvate (Zhao et al., 2009).
AS of the pyruvate kinase (PKM), encoding the enzyme that generates pyruvate, is also essential to allow cancer cells to use aerobic glycolysis. Two different splicing isoforms result from mutually exclusive use of exon 9 and 10 in the *PKM* pre-mRNA. Inclusion of exon 9 leads to formation of the adult PKM1 isoform, whereas mRNA harboring exon 10 encodes the embryonic PKM2 isoform. In cancer, re-expression of PKM2 promotes aerobic glycolysis since it allows tumor proliferating cells to convert glucose to pyruvate and lactate (Christofk et al., 2008b). Notably, PKM2 is expressed at higher levels in several cancer types (Christofk et al., 2008a; David & Manley., 2010). Even if the role of PKM2 in tumor progression is not fully elucidated, it has been reported that its production is controlled through the binding of hnRNPA1, hnRNPA2 and PTBP1 to sequences flanking exon 9 (David & Manley, 2010; Calabretta et al., 2016). Notably, the

oncogenic transcription factor c-Myc, which is overexpressed in several tumors (Miller et al., 2012), promotes transcriptional upregulation of hnRNPA1, hnRNPA2 and PTBP1 in this way ensuring a high PKM2/PKM1 ratio in cancer cells.

Tumor-promoting inflammation. Cells of the immune system are recruited to tumor site and constitute a fundamental part of tumor microenvironment (Gajewski et al., 2013). Cytokines and chemokines profoundly affect tumor microenvironment by promoting cell growth, survival, inflammation and differentiation (Grivennikov & Karin, 2011). Interestingly, interleukine genes are extensively regulated by AS. Inteleukine-7 (IL-7) expression is associated with tumor development and progression in different human cancers, including colorectal, renal and breast (Maeurer et al., 1997; Vudattu et al., 2009; Pan et al., 2012). *IL-7* undergoes an AS reaction that remove exon 5 from the mature mRNA thus generating the short IL-7δ5 variant, which promotes cell proliferation and cell cycle progression through AKT activation in breast cancer cell line (Pan et al., 2012).

Enabling replicative immortality. Activation of telomerase is another key step occurring during malignant transformation (Akincilar et al., 2016). Throughout the course of an organism's lifetime TTAGGG repeats of telomeres are progressively lost due to incomplete replication of chromosome ends. Once telomeres become critically short, cells enter in a state of irreversible growth arrest, the cellular senescence (Campisi, 2013). Elongation and maintenance of telomere is made possible, during embryonic development and in a restricted subset of stem-like progenitors in the adult organism, by the action of ribonucleoprotein complexes known as telomerase (or hTERT in human). Importantly, cancer cells are able to reactivate telomerase and this event represents a challenging target for anti-cancer therapies (Jäger & Walter, 2016). Interestingly, telomerase activity does not correlate with its expression (Ito et al., 1998). Several post-transcriptional and post-translational modifications impact the ability of hTERT to amplify telomeric repeats. Depending on the tissue, hTERT is alternative spliced in different protein variants: full-length, α-deleted variant, β-deleted variant and α/β-deleted variant. Among these variants, only the full-length isoform retains enzymatic activity, whereas the α-deleted variant represents a dominant negative inhibitor and the β-deleted is subjected to AS-NMD control (Wong et al., 2013). Notably, in tumor cells AS redirected *hTERT* splicing toward the production of the full-length isoform indicating that this mechanism plays a relevant role to generate active telomerase enzyme needed to sustain cancer cell growth (Jeung et al., 2017).

Angiogenesis. The requirement of a high demand of nutrients and oxygen by proliferating tumor cells is ensured through the formation of a proper tumor vasculature (or angiogenesis process; see next sections for details). Modifications of the tumor microenvironment, such as hypoxia, are recognized as a selective pressures that promote angiogenesis. Tumor cells respond to hypoxia by activating transcription factor HIF-1 (hypoxia-inducible factor-1) (Semenza, 2003). One important target of HIF-1 is VEGFA (Vascular Endothelial Growth Factor A), a cytokine that stimulates blood vessel growth and angiogenesis (Hoeben et al., 2004). *VEGF* pre-mRNA is widely regulated by AS process of two mutually exclusive terminal exons 8a and 8b. This process generates diverse VEGF protein isoforms with different C-terminal domains and distinct affinity for their receptors. These isoforms are classified in two families: $VEGF_{xxx}$ have pro-angiogenic activity and $VEGF_{xxx}b$ with anti-angiogenic effects (xxx indicates the position of the amino acid residue in the final protein). Use of the proximal 5' splice site in exon 8a generates $VEGF_{xxx}$, while the distal 5' ss in exon 8b promotes $VEGF_{xxx}b$ isoforms that are preferentially downregulated in cancer cells (Qiu et al., 2009). AS of *VEGF* pre-mRNA is controlled by the splicing regulators SRSF1 and SRSF6 that, respectively, promotes pro-angiogenic or anti-angiogenic VEGF isoforms (Nowak et al., 2008). Since expression of SRSF1 and SRSF6 is frequently altered in many tumors, it is tempting to speculate that AS could affect tumor progression by ensuring the appropriate balance of pro- and anti-angiogenic isoforms in cancer cells.

Collectively, these examples shown how aberrant expression of specific splicing factors can affect at the same time the processing of several genes. These genes encode for proteins involved in all major aspects of cancer cell biology, including cell cycle control, proliferation, signal transduction pathways, cell death, angiogenesis, invasion, motility and metastasis (Ghigna et al., 2008). Based on their preferential expression in tumor, cancer-associated AS variants have a clear diagnostic/prognostic value that can provide potential therapeutic targets for innovative approaches.

Vasculature development and angiogenesis

The vasculature system consists in an extensively branched network of blood vessels that allow an efficient circulation of oxygen and nutrients to all tissues of the body and clean out, at the same time, waste and toxic compounds (Schuermann et al., 2014). Blood vessels formation, or neovascularization, can occurs either by vasculogenesis or angiogenesis (Patan, 2004). Vasculogenesis is the process by which new blood vessels are generated *de novo* from mesodermal precursors, known as angioblasts (Goldie et al., 2008). Briefly, upon stimulation angioblasts start to migrate, proliferate and form lumens, thus creating primitive vascular structures (Goldie et al., 2008). Vasculogenesis predominantly occurs during early development, whereas angiogenesis, the formation of new vessels by sprouting from pre-existing vasculature, is subsequential (Ribatti & Crivellato, 2012). In particular, after first vessels are formed by vasculogenesis, angiogenesis is responsible for the expansion of the intricate network of blood vessels required to supply tissues with oxygen and nutrients (Carmeliet & Jain, 2011b).

Angiogenesis and vasculogenesis are fundamental during embryogenesis. However, in adulthood, most vessels remain quiescent and formation of new vessels occur only in restricted circumstances. For instance, in physiological growth of endometrium during menstrual cycle or in response to exercise training in skeletal muscles (Demir et al., 2010; Gustafsson & Kraus, 2001), but also in pathological conditions, such as inflammation, ischemia, infective disorders and to support cancer development and progression (Carmeliet, 2005).

Angiogenesis is a tightly regulated multi-steps process, during which endothelial cells (ECs) sprout, connect one to each other, and subsequently remodel into a functional network (Potente & Mäkinen, 2017). Quiescent ECs, in response to different angiogenic stimuli, are able to convert their resting phenotype to cells with a high mitotic index and increase capacity of migration and proteolytic capabilities (Ucuzian et al., 2010). Acquisition of specialized phenotypes and functions by a restricted number of ECs is strictly required during angiogenesis. For example, angiogenic stimuli are read by specialized cells (called tip cells) that are able to extend numerous filopodia to sense the surrounding environment. Upon activation, tip cells acquire a migratory phenotype and, at the same time, inhibit the acquisition of the same morphology by neighboring cells. Tip cell migration starts with the destruction of tight junctions, adherences junctions and gap junctions between neighboring ECs and mural cells (Patel-Hett & D'Amore, 2011). Next, secretion of metalloproteases (MPPs) allows the invasion into the basement membrane and in the surrounding (Extra Cellular Matrix) ECM (van Hinsbergh & Koolwijk, 2008), a process also referred as sprouting. Subsequently, additional

chemotactic cues promote sprouting extension with tip cells supported in their migration by a second type of specialized ECs, the stalk cells. Differently from tip cells, stalk cells are highly proliferative and allow the elongation and stabilization of the neo-formed primitive vessel. Stalk cells are also responsible for the establishment of the vascular lumen, a hallmark of angiogenesis (Ucuzian et al., 2010). Lumenogenesis and the subsequent tube formation are genetically programmed processes of ECs, which allow the creation of luminal compartments within multicellular chains through different mechanisms (Charpentier & Conlon, 2014). Upon lumenization and initial blood flow endothelial sprouts fuse and become connected (anastomosis) to establish a continuous lume, the resulting blood perfusion stabilizes and promotes the maturation and remodeling of the new formed vessel (Jain, 2003). Finally, complete maturation of the nascent vessel requires the spatio-temporal recruitment of different specialized cells, such as pericytes and mural cells, which are essential for structural support and regulation of vessel function (Fantin et al., 2010; Rymo et al., 2011) (Figure 8).

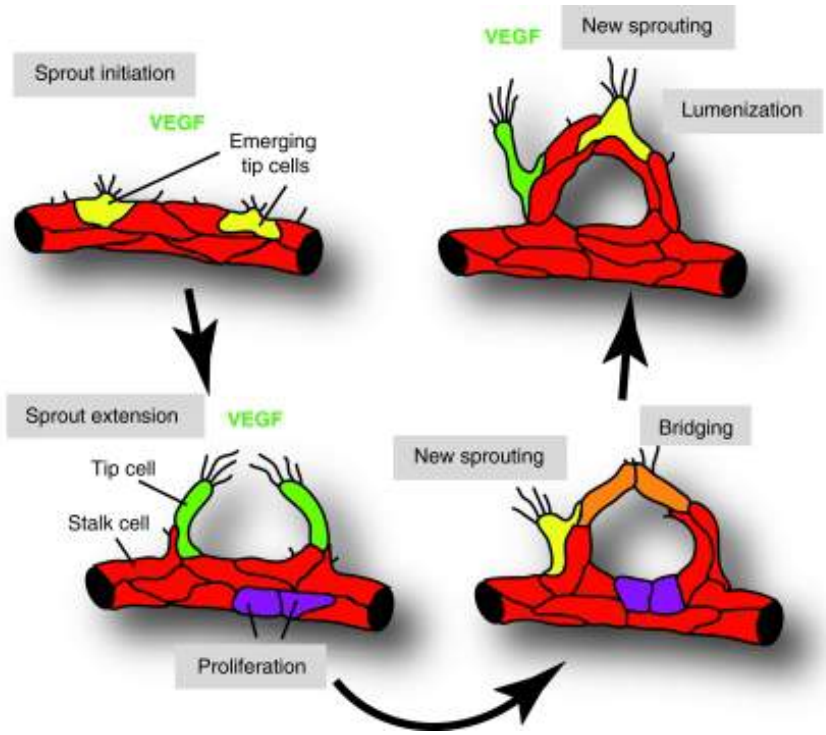


Figure 8. Angiogenic sprouting and new blood vessel formation. Angiogenesis starts with the sprouting of tip cells in response to external signalling cues, such as that mediated from the Vascular Endothelial Growth Factor (VEGF). Then tip cells migrate followed by

proliferative cells, named stalk cells, to form the primitive vessel. Migration finishes when other tip cells are encountered. resulting in the establishment of a new connection (anastomosis). Subsequently, lumen formation takes place to allow the flow of blood carrying nutrients and oxygen (modified from Adams & Eichmann, 2010).

Cell death during angiogenesis

During angiogenesis a programmed cell death process (apoptosis) is required in different fundamental steps (Mallat & Tedgui, 2000). In particular, the balance between pro-survival and pro-apoptotic signals is critical to maintain blood vessel integrity and maturation. Thus, impairment of apoptosis results in formation of abnormal vessels networks (Pollman et al., 1999). Furthermore, apoptosis is required for proper lumen formation in specific vascular beds (Fierlbeck et al., 2003). Accordingly, inhibition of programmed cell death has been shown to impair vascular-like structure formation *in vitro* and capillary formation *in vivo* (Segura et al., 2002).

Importantly, the central role of apoptosis in ECs during angiogenesis is highlighted by the fact that cell death occurs before capillary formation, but not in quiescent vessels (Segura et al., 2002). At the same time, increased ECs apoptosis, triggered by different stress conditions, promotes development of pathological conditions including atherosclerosis (Norata et al., 2002), diabetic retinopathy (Mizutani et al., 1996) and several vasculopathies (Dong et al., 1996; Panizo-Santos et al., 2000). These findings suggest the importance of a correct balance between apoptosis and survival signals both during vascular development and pathological angiogenesis (Murakami et al., 2011; Giampietro et al., 2012; Korn & Augustin, 2015).

Eukaryotic cell death machinery is redundant (Elmore, 2007), with many different pathways regulating the protease activity of caspases, which ultimately lead to a rapid cell death (Taylor et al., 2008; Strasser et al., 2011). Activation of both *intrinsic* (or mitochondrial) and *extrinsic* (or death receptor) pathways occurs in ECs during vascular development and pathological conditions (Stupack & Cheresh, 2003). For instance, the mitochondrial apoptotic pathway, which is achieved through interactions between different Bcl-2 proteins, is activated in lung ECs upon lipopolysaccharide stimulation during gram-negative sepsis (Wang et al., 2007). The same pathway, which also involves the mitochondrial release of cytochrome c, is essential for physiological EC death in stress conditions such as growth factors deprivation (Koenig et al., 2014), a process necessary to adapt the vascular arborization to organism demands. Notably, withdrawal of survival factors has been suggested as one of the main contributor of vessel pruning and regression by stimulating EC apoptosis (Baffert et al., 2006).

The extrinsic mechanism is activated in ECs by different death receptors during development and in response to pathological conditions. Examples of death receptors involved in angiogenesis include the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor family DR4 and DR5, which specifically mediate oligomeric Aβ induction of extrinsic apoptotic pathways in human microvascular cerebral ECs of Alzheimer's patients (Fossati et al., 2012). Another member of the TNF family required for the proper control of angiogenesis and vessel density is Fas (and its ligand CD95L), which exert a pivotal role during mouse retina vascularization (Kaplan et al., 1999).
Finally, in addition to ECs cell-independent intrinsic and extrinsic apoptotic pathways, pericytes and tissue-specific macrophages influence EC viability by promoting or suppressing apoptosis, in different cellular context (Franco et al., 2011; Bouillet et al., 1999).
Once again, these examples highlight the importance of a correct balance between pro-apoptotic and pro-survival signals in ECs not only during physiological conditions but also in several vascular pathologies (Stefanec, 2000; Mallat & Tedgui, 2000).

Molecular mediators of angiogenesis

Along with the aforementioned control of apoptosis, angiogenenesis and vascular development require the coordinated action of different processes, including cell proliferation, differentiation, migration and cell-cell signaling (Adams & Alitalo, 2007). VEGF-A represents the most important molecule that control vascular development (Coultas et al., 2005). Additional angiogenic growth factors and cytokines are: the fibroblast growth factors (FGF), the tumor necrosis-factor α (TNF-α), the angiopoietins family, the transforming growth factors beta (TGFβ-1 and TGFβ-2) and bone morphogenetic factors family (BMPs) (Chauveut et al., 2013). Importantly, these growth factors are not only produced by ECs but are also secreted by additional cells including fibroblast, smooth muscle cells, platelets, pericyte and, during tumor angiogenesis, by cancer cells (Papetti & Herman, 2002).

Vascular Endothelial Growth Factor (VEGF). The VEGF family comprises six different members: VEGF-A/B/C/D/E and PIGF (or placenta growth factor) (Holmes & Zachary, 2005). The biological effects of VEGFs are mediated via three specific transmembrane receptors, VEGFR-1, VEGFR-2 and VEGFR-3 (Shibuya, 2011). In addition to canonical VEGF receptors, several members of VEGF family also bind to non-tyrosine kinase receptors of neuropilin (NRP) family, Nrp1 and Nrp2 (Koch, 2012). VEGF signaling exerts a pivotal role in vascular biology. Through, activation of different pathways (such as MEK–MAPK,

the PI3K–Akt pathway, and the Src–eNOS), VEGF and its VEGFR receptors regulate different processes fundamental for vascular development, including proliferation, migration, survival and permeability (Olsson et al., 2006). For example, in the early phases of vasculogenesis, expression of VEGFR2 by angioblast precursor is a pre-requisite for differentiation from mesodermal cells in response to VEGF-A. Later VEGFR2 and its co-receptor Nrp1 in a positive feedback loop have a critical role in arterial differentiation, which occurs before circulation starts (Mukouyama et al., 2005). Moreover, VEGF gradient are required for vascular development along the mammalian axis by promoting vessel sprouting from the dorsal aorta and the cardinal vein (Coultas et al., 2005).

Given these roles during development, it is not surprising that VEGF downstream signaling is tightly regulated in adults (Kowanetz & Ferrara, 2006). High levels of VEGF are present during embryo development, whereas after birth VEGF decreases and it is up-regulated in tissues undergoing active angiogenesis (Hoeben et al., 2004). Notably, cancer cells and stromal cells of the tumor microenvironment are able to produce and secrete VEGF molecules in response to hypoxia condition and, furthermore, VEGF expression levels increase with tumor progression (Dvorak, 2002).

Once secreted VEGF stimulates ECs, which express VEGFRs, commitment to angiogenesis. Activated ECs begin to produce MMPs that degrade the ECM (Eble & Niland, 2009). Proteolytic degradation of ECM has a double function: first it permits vessel enlargement, second sustain angiogenic process by liberating pro-angiogenic factors (such as TGFβ), which are normally trapped in the matrix (Arroyo & Iruela-Aripse, 2010). This first phase, known as ECs activation (Zhang et al., 2010), is followed by specification of ECs into tip and stalks cells (Potente et al., 2011). Tip and stalk selection is mediated by the coordinated action of VEGF and Dll4/Notch signaling (Gerhardt et al., 2003; Bray, 2006). VEGF induces filopodia formation in the tip cells and it is able to activate a specific gene expression program (Hellstrom et al., 2007). However only a subset of ECs exposed to the pro-angiogenic VEGF gradient undergoes this morphological and metabolic specification.

Interestingly, VEGF:VEGFR2 interaction promotes the expression of different receptors and ligands, including PDGFB, Unc5b and Dll4. Dll4 is one of the canonical transmembrane ligand of Notch (Maihlos et al., 2011). Expression of Dll4 by committed tip cell, inhibit adjacent ECs, which express Notch receptors, by down-regulating VEGFR2 (Jakobsson et al., 2010). Additionally, Jagged-1, a Notch ligand highly expressed in stalk cells (Benedito et al., 2009), is able to antagonize Dll4 regulation of endothelial branching and also promotes vascular maturation (Pedrosa et al., 2015).

Importantly, Notch/Dll4 and VEGF pathways are involved in tumor angiogenesis (Liu et al., 2014) (see Angiogenesis and Cancer section). Briefly, both VEGF and Dll4 are up-regulated during tumor angiogenesis (Liu et al., 2014). In particular, Dll4 has been found expressed in tumor ECs of both murine and human tumors (Li et al., 2007). Moreover, different VEGF isoforms are produced and secreted by hypoxic tumor cells or by stromal cells of the tumor microenvironment, leading to a dis-regulated vessel growth (Morfoisse et al., 2015).

Fibroblast Growth Factor (FGF). FGFs are potent angiogenic inducers. The importance of FGFs relies on their ability to promote different biological processes, including cell proliferation, differentiation, survival and as well as angiogenesis (Lieu et al., 2011). Notably, FGF receptors (FGFRs) are ubiquitously expressed tyrosine kinase receptors that are alternative spliced; notably, different FGFRs splicing isoforms display different ligand affinities (Chellaiah et al., 1994). ECs of different tissues express FGFRs and are sensitive to FGF signal (Presta et al., 2005). In particular activation of FGFR-1, the predominant FGF receptor in endothelium (Presta et al., 2005), increases ECs migration (Zou et al., 2012) and proliferation (Larsson et al., 1999). Moreover, FGF-2 cross-talks with VEGF signaling by enhancing its production and stimulating ECM proteolysis, an essential step necessary for endothelial sprouting in the early invasive phase of angiogenesis (Ucuzian et al., 2010). Nevertheless, FGFR expression is not only restricted to ECs; indeed, mural cells and smooth muscle cells are also sensitive to FGF signaling. In particular, FGFR-2 promotes pericytes recruitment during vessel maturation (Bergers and Song, 2005).

Angiopoietins. Angiopoientins are secreted factors that bind to the EC-specific Tie receptors (Hansen et al., 2010). Two different angiopoietins, Ang-1 and Ang-2, have been extensively characterized for their role in ECs (Fagiani & Christofori, 2013). Both Ang-1 and Ang-2 bind the same receptor, Tie-2; whereas Tie-1 remains an orphan receptor that mediate its function by heterodimerizing with Tie-2 (Savant et al., 2015). Despite the binding with the same receptor the two secreted angiopoietins elicit very different response. Ang-1, which is produced by mesenchymal cells associated with the endothelium (Klagsbrun & Moses, 1999), promotes EC survival and induces vessel maturation (Suri et al., 1996). On the contrary, Ang-2 induces sprouting angiogenesis by cooperating with other angiogenic factors, including VEGF-A (Felcht et al., 2012). Ang-2 is secreted by ECs after exposure to angiogenic stimuli, such as hypoxia, and VEGF-A, or TNF gradient (Ray et al., 2000). After its release, Ang-2 antagonizes Ang-1 signaling by preventing Ang-1/Tie-2 interaction and thus promotes neovascularization. Importantly, increased Ang-2 expression has been found in several pathological

conditions, including cancer (Thurston & Daly, 2012), where it plays a pivotal role in stimulating tumor angiogenesis (Saharinen et al., 2011).

Tumor necrosis factor-alpha (TNF-α). TNF-α is a major cytokine that mediate inflammation. Mainly produced by activate macrophages during inflammation (Fajaro et al., 1992), it is able to promote angiogenesis in different ways: i) directly, by promoting ECs differentiation o ii) indirectly, by stimulating the production of angiogenic factors from other cells (Baluk et al., 2009). Interestingly, TNF-α is able to promote the inducing a "tip cell" phenotype through the upregulation of *PDGF*, *VEGFR2* and *Jagged-1* genes (Sainson et al., 2008).

Transforming growth factors beta. Cytokines belonging to the Transforming growth factors beta (TGFβ) family are secreted molecules with pleiotropic effects in different tissues and biological processes (Chaudhury & Howe, 2009). In the vasculature system, TGFβ via its binding to the TGFβ receptor complex (formed by type I and type II receptors) is indispensable in both vasculogenesis and angiogenesis (Chaudhury & Howe, 2009).
TGFβ type I receptors, also known as activin receptor-like kinase (ALK), are a family of seven transmembrane receptors (de Caestecker, 2004). ALK5, which is broadly expressed in different tissues, and the EC-specific ALK1 are implicated in TGF-β mediated regulation of angiogenesis (Cuhna & Pietras, 2011). ALK1 is particularly expressed during embryogenesis in regions undergoing angiogenesis (Roelen et al., 1997). Here, TGF-β/ALK1 signaling induces Smad1/5 activation, thus promoting EC migration, proliferation and tube formation (Goumans et al., 2003). On the contrary, TGF-β/ALK5 signaling through activation of Smad2/3 induces vessel stabilization and exerts a key role in maintaining quiescent ECs. However, the role of TGFβ in vascular system is cellular-context dependent, since many different ligands compete with various receptors on the same ECs (van Meeteren et al., 2011).
TGF-β1, one of TGF-β isoforms, is also involved in the control of apoptosis in ECs (Pollman et al., 1999). Intriguingly, TGF-β1 induces vessel formation *in vitro* and *in vivo* (Yang & Moses, 1990), but it inhibits EC proliferation and migration *in vitro* (Pollman et al., 1999). Notably, inhibition of apoptosis prevents the ability of TGF-β1 to activate angiogenesis *in vitro* (Choi & Ballermann, 1995; Ferrari et al., 2009). Mechanistically, TGF-β1 is able to convert the pro-survival pathway of VEGF via p38 MAPK in a pro-apoptotic signal by shifting the expression of different p38 isoforms (Ferrari et al., 2012).
Finally, TGF-β, in synergy with the Platelet-derived growth factor (PDGF), is also involved in recruitment of pericytes and vascular smooth-muscle cells, allowing the stabilization of the capillary sprouting (Bergers & Sons, 2005).

Bone morphogenetic protein (BMP). BMP factors (including BMP-2, 4, 6, 7 and GDF5) typically promote angiogenesis by increasing EC motility, migration and proliferation (Wiley & Jin, 2012). Similarly to TGF-β, BMP recognize different serine/threonine kinase receptors and activate Smad-mediated signaling in ECs (Dyer et al., 2014). For instance, BMP-2 is able to activate Smad 1/5/8 and ERK-1/2 pathways, thus promoting migration, proliferation and tubule formation of ECs (Langenfeld & Langenfeld, 2004; Finkenzeller et al., 2012). Particularly interesting is the fact that BMP-2 is overexpressed in different cancers, including pancreatic and hepatic carcinomas (Hatakeyama et al., 1997; Zuo et al., 2016). Finally, another member of BMP family, BMP-9, inhibits EC proliferation counteracting the effect of VEGF (Scharpfenecker et al., 2007).

Angiogenesis and cancer

Tumor cells require a high amount of nutrients and oxigen to sustain their growth and high proliferative rate (Eales et al., 2016). Indeed, 1-2 mm in diameter is the maximun size of an avascular tumors (Nishida et al., 2006) and subsequent growth is made possible only after the formation of the tumor vasculature through a process known as angiogenesis (Carmeliet 2003). Importantly, angiogenesis does not initiate malignancy but it promotes tumor progression and it also provides tumor cells with the metastatic route to colonize distant organs (Bielenberg & Zetter, 2015).

The "angiogenic switch" of the tumor is promoted by the secretion of different chemokines and growth factors by proliferating cancer cells and stromal cells of the hypoxic tumor microenviroment (Papetti & Herman, 2002). As previously described, these molecules are able to stimulate EC proliferation, migration, sprouting, and maturation (or stabilization) of new tumor vessels (Jain, 2003). Importantly, tumor vasculature is fundamentally different from that of normal vasculature. In tumors, the excessive production of pro-angiogenic factors, driven by the highly hypoxic environment, leads to the development of disorganized blood vessel networks Indeed, blood vessels have a chaotic organization (which varies from wide, irregular and tortuos shape to thin vessels with small lumens), are highly fragile and hemoraggic and are characterized by the presence of tumor ECs with multiple fenestration and trans-endothelial channels (Carmeliet & Jain, 2011a; Jain, 2005). However, tumor ECs possess peculiar characteristic interesting for a number of reason: i) they are easily accessible for drugs via the blood circulation; ii) differently from cancer cells, tumor ECs are genetically stable and are supposed to respond in a predictable way to the therapeutic drugs; iii) inhibition or regression of tumor vessels leads, in most of the cases, to cancer cell death (Figure 9) (Carmeliet, 2005; Neri & Bicknell, 2005; Ferrara & Kerbel, 2005).

These observations suggested that targeting tumor angiogenesis is a promising approach to develop innovative anti-cancer therapies. In this line, several efforts have been focused on the development of anti-angiogenic ("vessel pruning") strategies focused on preventing new vessel growth (Goel et al., 2011). Many of them are able to specifically block the VEGF signaling (Meadows & Hurwitz, 2012). Interestingly, monoclonal antibodies (for example Bevacizumab), or tyrosine kinase inhibitor (TKI) against VEGF receptors (including Sunitinib and Sorafenib), have been approved in 2003 for the treatment of different cancer types, including colorectal, non-small lung cancer, renal and ovarian cancers (Ferrara et al., 2005; Ichihara et al., 2011). However, while the use of anti-VEGF as mono-therapy has shown only modest results (Potente et al., 2011; McIntyre & Harris, 2015) anti-angiogenic therapies in combination of chemotherapy have achieved successful outcomes in different clinical studies (Ma & Waxman, 2008; Welti et al., 2013). Several issues have been proposed to explain the failure of anti-VEGF therapies. Indeed, it has been proposed that inhibition of a single pro-angiogenic target leads to up-regulation of other pro-angiogenic factors. In particular, upon VEGF inhibition tumor microenvironment become more hypoxic - an adaptive resistance mediated by the metabolic reprogramming through HIF signaling - that ultimately lead to up-regulation of additional pro-angiogenic factors released by tumor or stromal cells (Bergers & Hanahan, 2008). Another problem is related to the observation that anti-VEGF drugs are able to inhibit growth of new vessels, but they do not affect pre-existing tumor vessels, suggesting that therapy, to have successful, should be not only anti-angiogenic but also anti-vascular. Indeed, combination of anti-angiogenic inhibitors and anti-vascularagents have been tested in patients with advanced cancer (Nathan et al., 2012). In addition, it is important to note that some patients are refractory to anti-VEGF therapy, thus indicating the presence of VEGF-independent pro-angiogenic mechanism(s) that drives the development of tumor vasculature (Carmeliet & Jain, 2011). An example is the vessel co-option exploited by tumors that develop in densely vascularized organs, such as the brain (Leenders et al., 2002).

Angiogenesis is essential for metastasis formation (Bielenberg & Zetter, 2015), thus allowing cancer cells to reach and colonize distant organs (Zetter, 1998; Carmeliet & Jain, 2011). Even if inhibition of angiogenesis has shown reduction of metastatization in specific tumors (Drevs et al., 2000), in other cases antiangiogenic therapies were able to stimulate tumor cells local invasion and metastatization at distant sites (Ebos et al., 2009; Paez-Ribes et al., 2009).

It has been proposed that an alternative strategy is to "normalize" the tumor vasculature by restoring the balance of pro- and anti-angiogenic factors (Jain, 2005). This goal could be achieved by the adjustment of anti-angiogenic treatments in dose and duration, as suggested by the appearance of a "normalization window"

within 1-2 days of starting treatment and lost after prolonged exposure to anti-angiogenics factors (Kamoun et al., 2009). Importantly, restoration of a normal vasculature has been shown to enhance drug delivery and produce a favorable tumor microenvironment, highlighting once again the important concept of the combination of traditional chemoterapeutic drugs with novel and more precise anti-angiogenic therapies (Goel et al., 2011). However, the analysis presented in this paragraph indicates that a better understanding of the molecular mechanisms sustaining growth of tumor vessels could help to overcome anti-angiogenic limitations and to develop novel and more efficient anti-angiogenic therapies for cancer treatment.

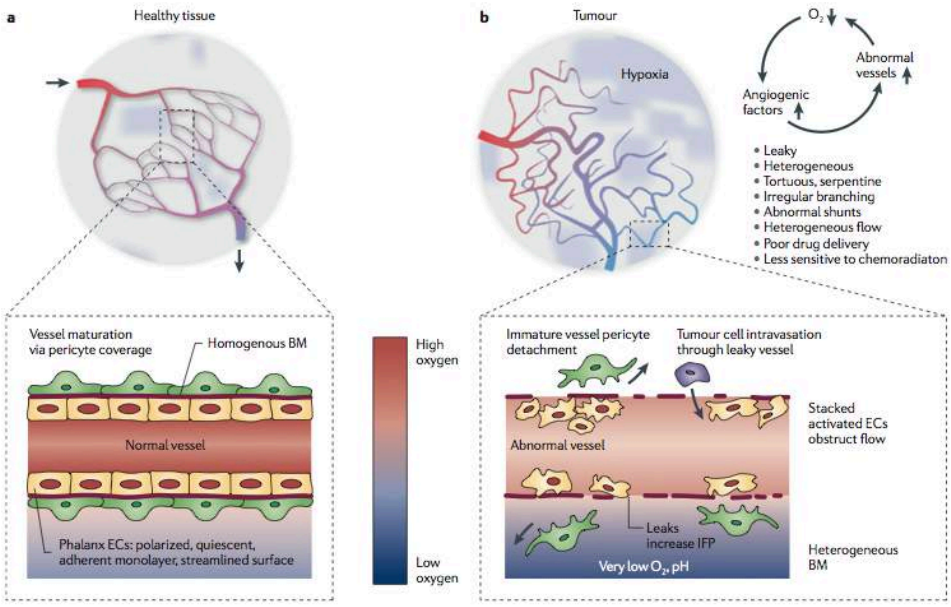


Figure 9. Comparison between healthy and tumoral vascular architectures. On the left is represented a vascular network in a healthy tissue. Vessels are homogeneous and are able to provide oxygen to the entire tissue. Furthermore, the vascular structure, composed by ECs, mural cells and pericytes, is stable and compact. On the right is shown the tumoral vasculature, characterized by tortuous shape, and irregular lumen. Moreover, ECs are not correctly polarized in the lumen and their junctions are fragile. These lead to: *(i)* inefficient delivery of oxygen, *(ii)* increase of hypoxia in the tissue and *(iii)* causing a positive feed-back mechanisms that drive the production of additional pro-angiogenetic factors by the tumor microenvironment (modified from Carmeliet & Jain 2011).

Anatomic, structural and molecular similarities between the nervous and vascular system: the neurovascular link

In vertebrates, vascular and nervous systems have been developed as route of communications that go through the entire body to supply oxygen and nutrients or transmit electrical signals, respectively (Ruiz de Almodovar et al., 2009). Both systems consist of an intricate network of efferent and afferent branches able to reach the different organs of the body. In addition to the similarities in the arborization of the two systems, already documented by the Belgian anatomist Andreas Vesalius, other structural and molecular similarities have been highlighted only recently (Figure 10) (Quaegebeur et al., 2011).

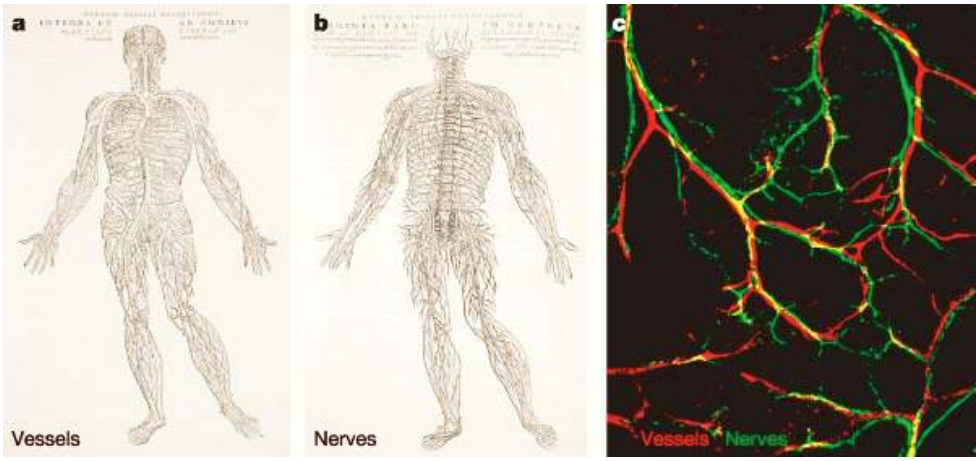


Figure 10. Anatomical parallelisms between vessels and nerves. **a** and **b**: drawing by the Belgian anatomist Andreas Vesalius, illustrating the similarities in the arborization of the vascular and nervous system. **C**: close association between vessels (red) and nerves (green) (modified from Carmeliet & Tessier-Lavigne 2005).

Similar specialized structures (tip cells at the forefront of endothelial sprouts and axonal growth cones) and common repulsive and attractive signals direct the specification, differentiation and patterning of these two systems during development (Adams & Eichmann, 2010). Accordingly, molecules belonging to the Netrin/DCC/UNC, Slit/Robo, semaphorin/Nrp/plexin and Eph/ephrin families, that were originally described as axon guidance cues, have also been shown to act also on ECs (Zacchigna et al., 2008). Molecules, affecting both neuronal and vascular functions, have been termed "angioneurins" (Zacchigna et al., 2008).

In addition to share similar mechanisms and molecular cues responsible for their development, nerves and vessels cross-talk and are functionally interdependent, a concept referred as "neurovascular link" (Quaegebeur et al., 2011). Importantly, the neurovascular link is bidirectional with molecules produced by the nervous system that act on vessel development and vice versa since ECs are able to secrete factors required for neural maintenance (Quaegebeur et al., 2011). For instance, neural cells stimulate vessels growth by releasing angiogenic factors, such as VEGF, whereas ECs secrete signals that sustain neuronal precursor proliferation and differentiation in neuronal stem cells (NSC) niches, including the brain-derived neurotrophic factor (BDNF) (Shen et al., 2004; Louissaint et al., 2002). Notably, defects in these cross-talk have been implicated in the pathogenesis of several neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) (Zlokovic, 2005; Zhong et al., 2008). An interesting example is provided by the VEGF, the most important angiogenic factor, which is up-regulated in the brain during stroke and ischemic episodes (Kovacs et al., 1996). VEGF expression is fundamental not only during angiogenesis, but also promotes neuronal survival and neuroprotection through the activation of VEGFR2 and MEK1/2 pathways in neurons (Beazley-Long et al., 2013). Hence, VEGF has been implicated in various neuronal disorders, such as AD, PD, Huntington disease, diabetic neuropathy, and ALS (Storkebaum et al., 2004), where alterations in VEGF expression directly contribute to ALS development in humans (Lambrechts et al, 2003). Due to its role in neuroprotection and impairment in ALS patients, VEGF has been proposed as therapeutic target for neurodegeneration. Indeed, delivery of VEGF in ALS mouse models reduces motor neuron death and prolongs neuronal survival (Ruiz de Almodovar et al., 2009). Hopefully, these findings suggested that in the next years other angioneurin members could be exploited for novel therapeutic opportunities for several vascular and neuronal disorders.

Angioneurins: repulsive and attractive signals involve in axon guidance and vascular patterning

As mentioned before, co-patterning of vascular and nervous system is possible by the action of molecules able to affect both systems (angioneurins) (Zacchigna et al., 2008). Interestingly, avascular organisms (such as *Drosophila melanogaster*) express VEGF orthologs to regulate neuronal development, suggesting that vessels might have co-opted some of the neural mechanisms and molecules for their development (Ruiz de Almodovar et al., 2009).

The discovery of axon guidance factors involved in vascular patterning and morphogenesis was one of the first cues indicating the existence of a close

neurovascular link between vessels and nerves (Carmeliet & Tesser-Lavigne, 2005). Examples of classical axon guidance molecules affecting vascular guidance include: Semaphorins, Netrins, Slit Ephrins and their receptors neuropilin/plexins, Robo, Eph, and DCC/Unc5 receptors, respectively (Adams & Eichmann, 2010) (Figure 11).

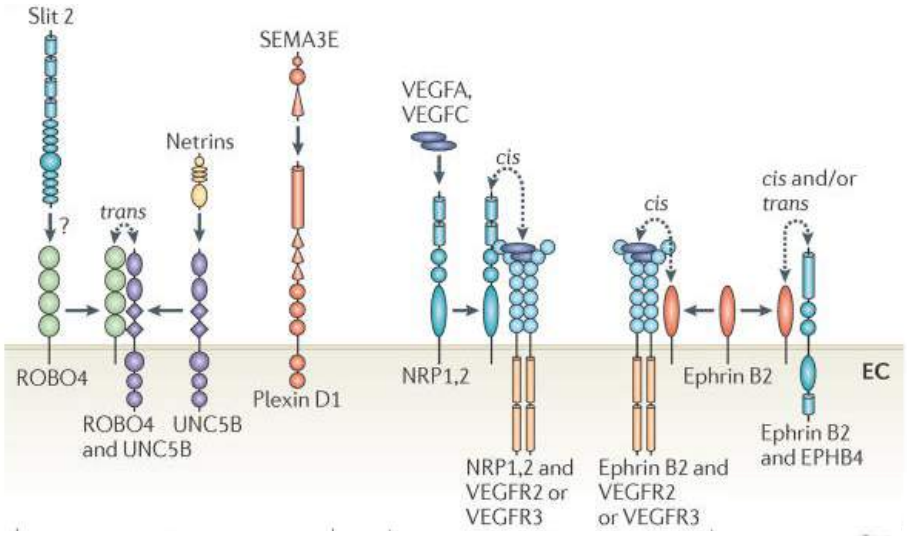


Figure 11. Axon guidance receptors that control angiogenesis. Axon guidance molecules expressed in ECs. Receptors (Robo4, Unc5b, PlexinD1, Nrp1 and 2, EPHB4) and their respective ligands are represented (modified from Herbert & Stainier, 2011).

Semaphorins and their receptors, neuropilin and plexin. Semaphorines are both membrane bound and secrete proteins characterized by the presence of a cysteine-rich semaphorin protein domains (Yazdani & Terman, 2006). In mammalian 20 different semaphorines are present, which are classified in five classes depending on their structural properties: class 3 semaphorins (SEM3) that are secreted molecules, classes 4 to 6 (SEM4-6) characterized by a transmembrane domain and class 7 (SEM7), which is anchored to the cytoplasmic membrane via a glycophosphatidylinositol (GPI) residue (Worzfeld & Offermanns, 2014). All different classes of semaphorines are able to intact with two different receptor families: Neuropilin (Nrp1 and Nrp2) and Plexins (PlexinA1-4, PlexinB1-3, PlexinC1, PlexinD1) (Sakurai et al., 2012).

Semaphorines are involved in axon guidance and neuronal cell migration, by functions as repulsive signal (Chen et al., 2000). For instance, SEM3A, by specifically binding to Nrp1, is able to properly direct axonal pathfinding (He et al., 1997; Telley et al., 2016). Notably, SEM3A is also released from ECs in the vasculature system, where it reduces EC migration and vessel remodeling, all functions depending on its ability to antagonize integrins signaling (Serini et al., 2003). In accord with its anti-angiogenic effect, SEM3A is down-regulated in tumor ECs (Maione et al., 2009). Similarly, other members of class 3 semaphorines (such as SEMA3B, SEMA3D and SEMA3F) exert anti-angiogenic properties (Kigel et al., 2008; Sabag et al., 2012) through the binding with neuropilin receptors (Nrp1 and/or Nrp2). Biochemically, Nrp1 and Nrp2 do not signal themselves but act as co-receptors for Plexins signaling (Sakurai et al., 2012), but also for other tyrosine kinase receptors (TKRs), including the kinase insert domain-containing receptor (KDR) involved in VEGF signaling (Herzog et al., 2011).
An exception of the class 3 of semaphorins is SEM3E, which directly binds to PlexinD1 and acts as a potent chemorepellent (Bagri et al., 2009). Notably, PlexinD1 is predominantly expressed in the vascular endothelium (van der Zwagg et al., 2002) and particularly in tip cells at the forefront of endothelial sprouting, suggesting that PlexinD1-SEM3E axis could act as a negative regulator of vascular patterning (Gu et al., 2005).
The pro-angiogenic role of semaphorines in the vasculature system is instead exerted by SEM4D, SEM5A and SEM6A through the direct action on Plexin receptors. These molecules play important roles in regulating tip cell extension and VEGF-dependent vessel sprouts (Adams & Eichmann, 2010; Sakurai et al., 2012). In particular, SEM4D-Plexin B1 interaction induces migration of ECs and angiogenesis *in vivo* (Zacchigna et al., 2008; Yukawa et al., 2010).
In addition, some semaphorins display dual activity depending on the cellular context in which they were investigated (Neufeld et al., 2016). A possible explanation is due to the ability of Nrp1 and Nrp2 to interact with other ligands or membrane bound receptors to induce even opposite signals. For instance, SEM3AC competes with $VEGF_{164}$ in the vascular system to mediate pro-angiogenic signal, whereas in the CNS, which do not express $VEGF_{164}$, promotes a chemorepulsive signal upon binding with Nrp1 (Fantin et al., 2009).

Slits and their Robo receptors. In vertebrates, three Slit ligands (Slit1, Slit2, Slit3) and four single-pass transmembrane roundabout receptors (Robo1, Robo2, Robo3, Robo4) are present (Grieshammer et al., 2004). Robo 1-3 are expressed in the CNS, whereas Robo4 shows a selective expression in ECs (Huminiecki et al., 2002). In the nervous system Slit molecules by binding through their LRR2 domain to the first Ig domain (Ig) of Robo extracellular portion acts as chemorepulsive

signals and regulate dendritic as well as axonal branching (Ma & Tessier-Lavigne, 2007).
Slit-Robo signaling in the vascular system is mainly mediated by Robo4, which has been reported to control angiogenesis and blood vessel permeability (Jones et al., 2008). However, only about 40% of Robo4 Ig domain is conserved between Robo4 and Robo1-3, arguing the possibility that Robo4 cannot bind Slit ligands, a concept that is not fully supported by experimental data (Adams & Eichmann, 2010). Indeed, the role of Robo4 in ECs has been demonstrated to be partially Slit-independent (Kaur et al., 2008). For instance, Robo4 is able to interact with the netrin receptor Unc5b (expressed by neighbor ECs) to maintain vessel integrity and inhibit angiogenesis by counteracting VEGF signaling (Koch et al., 2011). Moreover, due to the fact that Robo1-2 are expressed in ECs and the ability of Robo1 to heterodimerize with Robo4, it is possible that Slit acts on ECs without the direct binding with Robo4 in regulating ECs migration and motility, but require the activation of other Robo receptors (Sheldon et al., 2009). Indeed, Slit2-activation of Robo1-2 signaling can cooperate with VEGF signaling via VEGFR2 in the control of cell polarity during sprouting angiogenesis (Dubrac et al., 2016).

Ephrins and their Eph receptors. Eph receptors are TKRs that regulate morphogenesis in different tissues (Lisabeth et al., 2013). Signaling cascade is activated by Ephrins, cell surface proteins divided in two different subclasses: ephrin-A subclass, characterized by glycosylphosphatidylinisotol (GPI) anchor and ephrin-B subclass, retained in the membrane by the presence of a transmembrane domain (Poliakov et al., 2004). Similarly, depending on binding preference and sequence homology, Eph receptors are subdivided in two groups, EphA and EphB.
Blood vessels express several Eph receptors and ephrins family members (Mosch et al., 2010). For instance, EphB4 and ephrinB2 interaction are important role during vascular development (Adams et al., 1999; Gerety et al., 1999). In particular, ephrinB2/EphB4 signaling is involved in arterial-venous specification, vascular remodelling (Kuijper et al., 2007), but also has a function in determining artery and vein size during angiogenesis (Kim et al., 2008). Interestingly, the membrane-bound ephrinB2 ligand is also able to induce a reverse signaling fundamental for vessel morphogenesis by promoting endothelial and mural cells assembly into vascular structure (Adams et al., 2001; Salvucci et al., 2009).
Finally, class A of Eph and ephrins, mainly EphA2 and ephrinA1, seem to be important in the vasculature system. EphrinA1 is expressed during vascular development in regions of active angiogenesis (McBride & Ruiz, 1998), where it enhances new vessel formation (McBride & Ruiz, 1998); EphA2 signaling has been demonstrated to be crucial for EC migration and capillary assembly in response to ephrinA1 (Chen et al., 2006).

Netrins and their receptors, DCC and Unc5. Netrins are a small family of highly conserved ligands initially discovered as chemotropic guidance cues in the CNS (their name derives from the Sanskrit word *netr* which means "one who guides"). In particular, these molecules were initially characterize for their role in axon migration and elongation during the development of the nervous system (Moore et al., 2007). However, in mammals Netrin-1, -3, -4 and -5 are also expressed in different organs, including the vascular system, lung and pancreas (Beamish et al., 2017).

Netrins are lamin-related secreted extracellular proteins, approximately of 600 aminoacids characterized by lamin-related domain VI and V and a carboxy-terminal domain (called domain C or netrin-like). These ligands bind to two canonical receptor families, both belonging to the Ig superfamily of cell adhesion molecule (CAM-like) type 1 transmembrane proteins: the deleted in colorectal cancer (DCC), including also the DCC paralog Neogenin, and the uncoordinated 5 (Unc5) family.

Several works reported that Netrins are able to function as attractive or repulsive cues during axon guidance (Colamarino & Tesser-Lavigne, 1995). In particular, attractive signal is mediated by the association of Netrin with DCC (Chan et al., 1996), whereas repulsion requires the presence of the Unc5 receptors (Leonardo et al., 1997). Similarly, netrins and their receptor have been reported to exhibit both pro- and anti-angiogenic activity in the developing of the vascular system (Castets & Mehlen, 2010). Interestingly, Netrin-1 and Netrin-4 were deeply investigated for their role in angiogenesis. In particular, Netrin-4 exerts an anti-angiogenic function by modulating EC response to VEGF gradient, through the interaction with Neogenin and Unc5b receptors (Lejmi et al., 2008). Moreover, Netrin-4 stimulates vascular smooth muscle cell (VSMC) activity increasing their coverage on EC tube, an important process involved in blood vessel maturation (Lejmi et al., 2014). On the contrary, Netrin-3 and Netrin-5 were predominantly found expressed in axons of motor, sensory and sympathetic neurons (Wang et al., 1999; Seaman et al., 2001), and in neurogenic regions of the brain (Yamagishi et al., 2015), respectively.

Recently, a high affinity Netrin-1 receptor, CD146, has been identified in ECs, where it plays an important role in positively regulating angiogenesis (Tu et al., 2015). Finally, among Unc5 family receptors only Unc5b is predominantly expressed in vascular endothelium and characterized for its ability to selectively binds to Netrin-1 but not Netrin-4 (Lejmi et al., 2008). Due to relevance for the results of this thesis, the different roles of Netrin-1 and the Netrin-receptor-Unc5b signaling are discussed in separated sections.

Netrin-1

Netrin-1 was initially discovered in the CNS and characterized for its ability to establish a gradient for axon guidance (Ishii et al., 1992). Several studies then shown that Netrin-1 is not restricted to the nervous system. In particular, it has been reported that Netrin-1 is important also in different non-neural tissues, including the mammary gland, lung and vascular system (Cirulli & Yebra, 2007).
Through the binding of a large assay of receptors, Netrin-1 is able to activate different pathways, such as MAPK/ERK (Tang & Kalil, 2005), PI3K (Ming et al., 1999) or Fak/Src/CD151 signaling (Yang et al., 2017). In the vascular endothelium, Netrin-1 inhibiths sprouting angiogenesis (Larrivèe et al., 2007) and mediates short-range repulsion during growth and pathfinding of blood vessels (Lu et al. 2004). In line with this, depletion of Netrin-1 with antisense oligonucleotides in zebrafish lead to abnormal vessel branching during the development of the vascular system (Wilson et al. 2006).
Netrin-1 gradients are established in the ECM through the binding of its C-terminal Netrin domain to negatively charged heparan sulphate proteoglycans (Serafini et al., 1994; Kappler et al., 2000). Notably, release of the lamin-related domains VI and V of Netrin-1 by the action of MMPs in pathological conditions, such as diabetic retinopathies, has been shown to facilitate Netrin-1 diffusion, thus exacerbating its anti-angiogenetic effects on the vasculature (Miloudi et al., 2016). Nevertheless, it has been reported that Netrin-1 can also promotes proliferation and migration of ECs (pro-angiogenetic effects) (Park et al., 2004). Two different mechanisms have been hypothesized to explain this paradoxical phenomenon. The first relies on the ability of Netrin-1 to regulate dependence receptor (DR) mediated cell death by binding with different DRs, including Unc5b, DCC and Neogenin (Mehlen & Guenebeaud, 2010). In this case, the pro-angiogenic effect of Netrin-1 is due to the increased survival of ECs that express DR receptors. Accordingly, treatment of Netrin-1 depleted zebrafish embryos with apoptotic inhibitors, partially restore vascular abnormalities (Castets et al., 2009). Another possibility is the presence of additional Netrin-1 receptors that are able to mediate pro-angiogenic signal. The recently identified Netrin receptor CD146 is in fact able to activate ECs and promote angiogenesis. In particular, CD146:Netrin interactions, by acting in synergy with VEGF signaling, increase ECs proliferation, whereas by stimulating ERK and p38 pathways, promote EC migration (Tu et al., 2015). Notably, CD146 has a greater affinity for Netrin-1 compared to others DRs such as Unc5b (Tu et al., 2015). Indeed, it has been demonstrated that low concentration of Netrin-1 promotes proliferation, migration and tube formation *in vitro* via CD146 signaling, whereas at high concentration Netrin-1 binds Unc5b thus generating inhibitory effects (Tu et al. 2015).

The restricted specific expression of additional known Netrin receptor could also explain its different functions in specific vascular beds. An example is the expression of DCC in arterial, where after Netrin-1 stimulation, it increases the level of nitric oxide (NO) leading to an increased EC proliferation and migration (Nguyen & Cai, 2006). Similarly, ECs of the subventricular zone of adult brain express another Netrin-1 receptor, such as Neogenin receptor (Cayre et al., 2013). In this context, injection of Netrin-1 has been demonstrated to contribute to vascular remodeling and endogenous stem/progenitor cell mobilization, processes fundamental for spontaneous brain repair after demyelination (Cayre et al., 2013).
Due to its role in regulating apoptosis, proliferation and migration not only in the nervous and vascular systems but also in epithelial cells, Netrin-1 expression has also been investigated in cancer progression (Kefeli et al., 2017). Indeed, Netrin-1 was found up-regulated in several cancer types, including colorectal, breast, pancreatic, lung, hepatic cancers and in brain tumors, such as glioblastoma, medulloblastoma and neuroblastoma (Ylivinkka et al., 2016). In particular, Netrin-1 expression has been shown to confer a selective advantage for tumor cell survival and progression in metastatic breast cancer and neuroblastoma (Fitamant et al., 2008; Delloye-Bourgeois et al., 2009). In colorectal cancer Netrin-1 promotes migration and invasion (Ko et al., 2014). Pro-invasive activity of premalignant colon adenoma cells and colon adenocarcinoma cells is mediated by activation of Rho-associated protein kinase (ROCK) (Rodrigues et al., 2007). The same pathway is also exploited by glioblastoma cells to increase their invasiveness (Shimizu et al., 2012). Moreover, Netrin-1 has been shown to promote cell proliferation and invasion through activation of PI3K/AKT signaling in gastric cancer and hepatocellular carcinoma (Yin et al., 2017; Han et al., 2015). Interestingly, loss of the expression of Netrin-1 receptors is associated with a poor prognosis in patients with breast, colorectal and glioblastoma tumors (Austrup et al., 2000). Whereas, ectopic expression of the Netrin-receptor DCC, by promoting pro-apoptotic pathway, was found able to suppress the pro-invasive phenotype observed in cultured colon adenocarcinoma cells (Rodrigues et al., 2007).

The netrin receptor Unc5b

Unc5b (Uncoordinated-5 Homolog B, also known as Unc5H2 or p53RDL1) is a transmembrane receptor involved in both axon guidance process and vascular patterning during angiogenesis (Adams & Eichmann, 2010).
The vertebrate Unc5 family of netrin receptors was discovered in the CNS and, beside Unc5b, it includes Unc5a, Unc5c and Unc5d (Ackermann et al., 1997). Unc5 receptors were identified as repulsive Netrin receptors involved in neuronal cell migration in different organisms, including *Caenorhabditis elegans*, *Drosophila* and mouse (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; Leonardo et al., 1997). Interestingly, in addition to their role in axon guidance, Unc5b has been linked to other developmental processes such as vascular development (Klagsbrun & Eichmann, 2005) and immune response (Ly et al., 2005; Mediero et al., 2016).
Unc5b is more expressed in the vascular system compared to CNS (Lu et al., 2004). However, Unc5b expression in ECs is not uniform in the vascular system; indeed Unc5b is upregulated in vessels undergoing active angiogenesis and it becomes down-regulated in the quiescent adult vasculature (Larivée et al., 2007). Notably, in adult organisms, Unc5b is re-expressed in pathological conditions, such as tumor angiogenesis (Larivée et al., 2007).
Biochemically, Unc5b is a single-pass transmembrane protein characterized by the presence in the extra-cellular portion of two Ig domains and two thrombospondin type I (TSP) domains. Its cytoplasmic tail includes a ZU5 domain (initially discovered in *Z*O-1 and *U*NC5), a DCC-binding domain (DB or UPA, due to its conservation in *U*NC5, *P*IDD, and *A*nkyrins) and a death domain (DD) (Hong et al., 1999). The precise structure of the cytoplasmic portion of Unc5b receptor has been recently solved (Wang et al., 2009). In particular, the three cytoplasmic domains adopt an L-shaped architecture in which the ZU5 domain interacts with both the DB and DD domains, simultaneously (Wang et al., 2009). Importantly, core aminoacids of each residue are well conserved among Unc5 family members and in different species (Wang et al., 2009). A partial resolution of the extra-cellular part has been only obtained for Unc5d in complex with other two cell guidance receptors, FLRT2 and Lphn3 (Jackson et al., 2016).
Unc5b function as receptor for secreted axonal guidance molecules and specifically mediates the repulsive responses of Netrins (Hedgecock et al., 1990). In particular, Unc5b:Netrin-1 interaction, which occurs between Netrin-1 laminin-related V-2 subdomain and Unc5b Ig1/Ig2 domains (Grandin et al., 2016), is able to mediate both short-range and long-range signalings (Keleman & Dickson, 2001). In addition, a number of Unc5b co-receptors, such as Neogenin, have been described in the CNS. Neogenin is a member of the DCC receptor family (Meyerhardt et al.,

1997) and mediates repulsion through interaction with guidance cues such as RGMa (Rajagopalan et al., 2004). Neogenin also binds Netrin-1, however its ability to form a co-receptor complex with Unc5b is Netrin-independent (Hata et al., 2009). Unc5b:Neogenin interaction mediates growth cone collapse by transduce the signal from RGMa to the small GTPase RhoA. Importantly, RhoA activation occurs through the leukemia-associated guanine nucleotide exchange factor (LARG), which constitutely binds to the DB domain of Unc5b (Hata et al., 2009). Different Unc5b partners were recently suggested to mediate its vascular role. In particular, it has been reported that, by binding to the EC-specific guidance receptor Robo4, Unc5b is able to maintain vascular integrity by counteracting VEGF signaling (Koch et al., 2011).

Recently, Unc5b has been described as a "dependence receptor" (Tanikawa et al., 2003). Unc5b is able to induce p53-dependent apoptosis in the absence of its ligand Netrin-1, whereas ligand binding triggers survival signals (Arakawa, 2004). Since the absence of a death-inducing receptor should increase cell survival, this can explain the increased number of blood vessels and hyper-vascularization seen in *Unc5b*$^{-/-}$ mice (Lu et al., 2004). On the contrary, loss of *netrin-1* was associated to increase of pro-apoptotic signaling that in turn prevented blood vessel formation (Wilson et al., 2006; Castets et al., 2009). All these findings reconcile the initial apparently contradictory conclusions that Netrin-1 is either a pro- or an antiangiogenic factor.

Due to their ability to induce cell death in the absence of its ligands, Unc5 has been proposed to act as tumor suppressor (Thiebault et al., 2003). Accordingly, Unc5 genes were down-regulated in many cancers, including colorectal, breast, ovary, uterus, stomach, lung and kidney cancers (Thiebault et al., 2003). Unc5b is a direct transcription target of p53 (Tanikawa et al., 2003). Indeed, upon genotoxic stress Unc5b expression is induced in p53-dependent manner only in cancer cells wild type p53 (Arakawa, 2004). In ECs, Unc5b expression has been demonstrated to be induced by hypoxia and requires HIF-1a in both physiological and pathological conditions (Dakouane-Giudicelli et al., 2011; Ramkhelawon et al., 2013). Notably, also BMP9 and Dll4 additively induced Unc5b expression in tip ECs through Smad1/5 pathway (Larivée et al., 2011; Morikawa et al., 2011).

The molecular mechanism by which Unc5b induces cell death has been partially depicted through structural and functional studies. Importantly, Unc5b can induce apoptosis via two different mechanisms: i) cleavage of its cytoplasmic tail by caspases (Llambli et al., 2001; Tanikawa et al., 2003) and ii) through recruitment and activation of Death-Associated-Protein kinase (DAPk) (Llambli et al., 2005) or NRAGE (Williams et al., 2003). In the first case, caspases are able to recognize a classic caspase sites DXXD (Thornberry et al., 1997) localized in the intracellular portion of the receptor and amplified caspase signaling, resulting in activation of

apoptotic pathway (Llambli et al., 2001). Notably, mutations of Unc5b DXXD site (D412N) have been shown to strongly inhibit Unc5b-induced cell death (Llambli et al., 2001). Unc5b-induced apoptosis during angiogenesis is mediated by recruitment (via death domain) and activation (by dephosphorylation) of Death-Associated-Protein kinase (DAPk), which ultimately lead to Caspase-3 activation and cell death (Guenebeaud et al., 2010). The importance of the conformation state of the cytoplasmic tail of Unc5b has been highlighted by its structure resolution through X-ray crystallography and structure-based functional analysis (Wang et al., 2009). In particular, the cytoplasmic domains ZU5, DB and DD can adopt two different conformation: an *open conformation* that allow the recruitment of the apoptotic machinery required for cell death induction and a *close conformation* in which the DD interact with the ZU5 domain and prevent DAPk activation (Wang et al., 2009). Notably, Netrin-1 treatment induces Unc5b dimerization and stabilizes the *close conformation*, whereas post-translational modifications, such as phosphorylation in ZU5 and DD domains weaken their interaction (Wang et al., 2009; Li et al., 2006). Moreover, mutations in the interface between DD and ZU5 (V619Q) or complete deletion of ZU5 domain (ΔZU5) shown an enhancement of cell death in transfected HEK293 cells, probably due to the full release of DD from the *close conformation* (Wang et al., 2009). Accordingly, Netrin-1 treatment of HEK293 overexpressing Unc5b mutants, V619Q or ΔZU5, was found to be not more able to stabilize the *close conformation* and to prevent apoptosis activation (Wang et al., 2009).

DAPk is a stress-regulated Ser/Thr protein kinase that mediates a range of processes, including signal-induced cell death and autophagic signaling (Lin et al., 2010). Notably, DAPk is frequently down-regulated through promoter methylation in different cancers type (Toyooka et al., 2003; Grandin et al., 2016). Unc5b has been shown to recruit DAPk via binding of their respective death domains and to activate kinase activity of DAPk through dephosphorylation of the Ser308 (Llambli et al., 2005).

DAPk dephosphorylation is mediated by PP2A holoenzyme, an ubiquitously expressed Ser/Thr phosphatase composed by a catalytic subunit (PP2Ac) and a PR65 scaffold protein. Notably, also PP2A activity is frequently inactivated in cancer, mainly through the up-regulation of the PP2A-inhibitor CIP2A (Junttila et al., 2007) or mutations in the scaffold subunit PR65 (Eichhorn et al., 2009). Unc5b, PR65, PP2Ac and DAPK form a protein complex in the absence of Netrin-1, whereas in the presence of Netrin-1, Unc5b, in its *close conformation,* is unable to bind the phosphatase PP2A holoenzyme and activate Ser308 DAPk dephosphorylation (Guenebeaud et al., 2010). Also, Unc5b and DAPk interactions require a series of post-translational modifications, including palmitoylation, which are required for Unc5b localization in lipid rafts (Maisse et al., 2008).

Differently from Unc5b, other members of Unc5 family activate a cell death program through diverse mechanisms. For instance, in the absence of Netrin-1, the intracellular domain of Unc5d is cleaved by Caspase-3 (Zhu et al., 2013) generating a cytoplasmic fragment able to translocate into the nucleus where activates the transcription factor E2F1, which ultimately stimulates transcription of apoptotic genes in neuronal cells (Zhu et al., 2013). Unc5a is able to interact with NRAGE (neurotrophin receptor-interacting MAGE homolog) via its ZU5 domain (Williams et al., 2003), thereby promoting apoptosis through degradation of the caspase inhibitor XIAP or activating the pro-apoptotic JNK pathway (Williams et al., 2003).
Collectively, these examples illustrate how different mechanisms are exploited by dependence receptors of the Unc5 family to induce cell death/survival in different tissues and organs depending on the expression of different intracellular effectors.

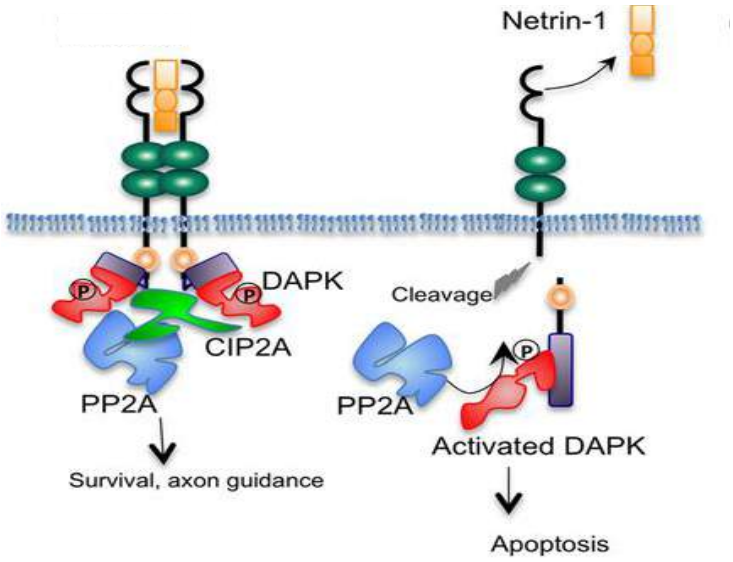
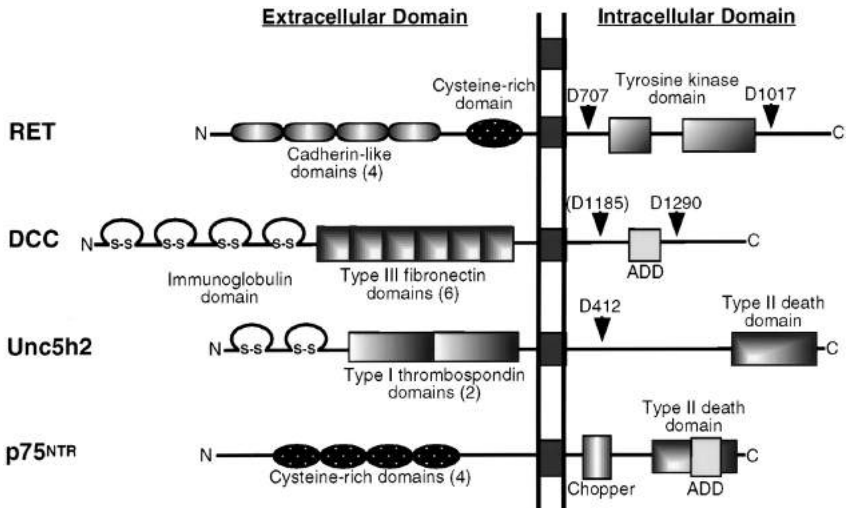


Figure 12. Unc5b as Dependence receptor. Unc5b transmembrane receptor (in black with green thrombospondin type 1 domain) acts as dependence receptor able to to induce apoptosis in the absence of its ligand Netrin-1, whereas ligand binding triggers survival signaling. In the monomeric state, Un5b cytoplasmic tail adopts an *open conformation* that allows the cleavege of Caspase-3 and the PP2A-mediated DAPk dephosphorylation (at Ser308), which ultimately lead to cell death. Oppositely, Netrin-1 binding promotes receptor dimerization and survival signal (modified from Mehlen & Tauszig-Delamasure, 2014)

Dependence receptors

Dependence receptors (DR) family includes membrane receptors that differs from their structural organization but share similar functional traits (Goldscheider & Mehlen, 2010). DRs are able to induce cell death in the absence of their ligands, whereas ligand binding activates classic signaling pathways implicated in cell survival, migration and differentiation (Goldscheider & Mehlen, 2010).
DR family includes more than 20 transmembrane receptors. Initially introduced with $p75^{NTR}$ (p75 neurotrophin receptor) and its ligand NGF (Rabizadeh et al., 1993), the concept of dependence receptor has been applied to many other receptors, including Unc5a-d (Llambi et al., 2001), DCC (Mehlen et al., 1998), Neogenin (Matsunaga et al., 2004), Plexin D1 (Luchino et al., 2013), RET (rearranged during transfection) (Bordeaux et al., 2000), TrkC (tyrosine kinase receptor C) (Tauszig-Delamasure et al., 2007), Ptc (patched) (Thibert et al., 2003), EphA4 (ephrin type A receptor 4) (Furne et al., 2009), ALK (anaplastic lymphoma kinase) (Mourali et al., 2006), MET (Tulasne et al., 2004) and some integrins (Stupack et al., 2001). An exception of the DR family is represented by the androgen receptor (AR) (Ellerby et al., 1999). AR is, in fact, a nuclear receptor that localizes in the cytoplasm and translocate into the nucleus upon its activation (Figure 13).



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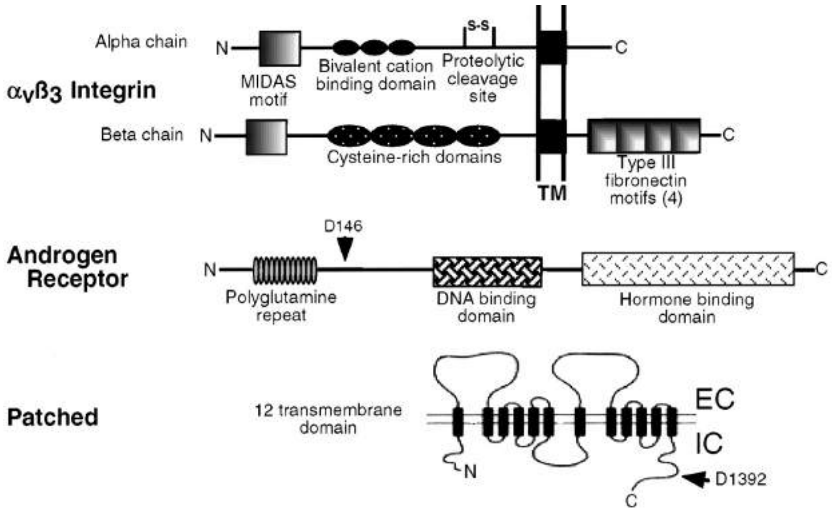

Figure 13. Dependence receptor (DR) family. Schematic representation of DRs with their functional domains. Notably, DRs differs from their structural organization but share the ability to induce cell death in the absence of their ligands, whereas ligand binding activates promotes cell survival, migration and differentiation. Intracellular cleavage sites are indicated by black arrowheads. Unc5b is also known as Unc5h2 (modified from Bredesen et al, 2004).

Nevertheless, AR shows all the peculiar features of a DR summarized as: i) induction of apoptosis in the absence of the ligand; ii) inhibition of cell-death upon ligand binding; iii) presence of intracellular cleavage sites (Goldscheider & Mehlen, 2010).

Cleavage by caspase, in the absence of the ligand, is one of the most common characteristics of DR (Goldscheider & Mehlen, 2010). The position of caspase cleavage sites varies among receptors, from the proximity to the plasma membrane in Unc5b (Llambi et al., 2001) to the middle of the intracellular portion in DCC and Neogenin (Mehlen et al., 1998; Matsunaga et al., 2004), whereas RET, TrkC and MET have instead two different cleavage sites (Bordeaux et al., 2000; Tauszig-Delamasure et al., 2007).

Interestingly, caspase cleavage sites are well conserved in mammals and variably conserved in other vertebrates, suggesting that the appearance of a "dependence state" may be arisen late in the evolution (Goldscheider & Mehlen, 2010). For instance, DCC caspase cleavage site (LSVD) is not perfectly conserved in *Xenopus laevis* and absent in zebrafish (*Danio rerio*), whereas Unc5b (DITD) and Neogenin

(CCTC) are well conserved from human to zebrafish, with only slightly variations (Goldscheider & Mehlen, 2010).

Recognition and cleavage of the caspase site is a requisite for DR-mediated apoptosis. Notably, ectopic overexpression of the truncated receptor is sufficient to induce cell death also in the presence of the ligand (Mehlen et al., 1998). Inhibition of apoptosis induction by ligand binding is thought to be mediated by the prevention of caspase cleavage and different mechanisms have been proposed to explain protection of caspase sites. For instance, Unc5b dimerization plays a key role in apoptosis abrogation (Mille et al., 2009). Similarly, receptor multimerization has been demonstrated to block $p75^{NTR}$ induced cell-death (Wang et al., 2000). However, some DRs do not multimerize in the presence of their ligands and examples include Ptc and αvβs integrin (Goldscheider & Mehlen, 2010).

A pivotal role in the activation and amplification of caspase signaling involved the addiction/dependence domain (ADD). It has been reported that ADD release or exposition activate and amplify apoptosis through different mechanisms, one of these well characterized for DCC. Indeed, in the absence of Netrin-1, DCC recruits and activates caspase-9. Notably, caspase-9 does not directly interact with DCC but require the presence of adaptor proteins (Forcet et al., 2001), such as APPL1 (adaptor protein containing PH domain, PTB domain and leucine zipper motif 1). In particular APPL1 is able to interact with DCC ADD and, as a consequence, induces cell death through direct or indirect recruitment of caspase-9 (Liu et al., 2002).

DRs has been hypothesized to be important not only during embryogenesis and development but also in tumor progression. Indeed, DRs expression (or activity) is lost in many aggressive cancers (Goldscheider & Mehlen, 2010). For instance, DCC was found to be deleted in more than 70% of colorectal cancers (from this observation derives that name of DCC, *deleted in colorectal cancer*) as well as in many other tumors, including gastric, prostate, endometrial, ovarian, esophageal, breast and testicular cancer (Mehlen and Fearon, 2004). Importantly, loss of DCC was associated with a poor prognosis and metastasis formation (Shibata et al., 1996; Saito et al., 1999).

Similar to DCC, Unc5 receptor family members are down-regulated in human cancers, including colorectal, breast, ovary, uterus, stomach, lung or kidney cancers (Thiebault et al., 2003). Moreover, other DRs have been found repressed in a wide fraction of cancers; this includes EphA4 in breast (Fox & Kandpal, 2004), liver and kidney cancers (Hafner et al., 2004) and in metastatic melanomas (Easty et al., 1997) and Ptc in basal cell carcinoma and medulloblastoma (Wicking & McGlinn, 2001) or $p75^{NTR}$ in prostate cancers (Pflug & Djakiew, 1998). Interestingly, it has been reported that cancer cells have develop the

possibility to overcome DRs induced cell death signaling. Indeed, high levels of Netrin-1 were detected in a large panel of human cancers, including breast (Fitamant et al., 2008) and lung tumors (Delloye-Bourgeois et al., 2009). Moreover, decreased expression of downstream proteins involved in the apoptotic pathway can be used by tumor cells to escape the pro-apoptotic activity of DRs (Goldscheider & Mehlen, 2010). In addition to the already described down-regulation of DAPk expression through promoter methylation, two other DRs effectors, DRAL and caspase 8, have been shown to be functional inactivated in human cancers. For example, DRAL, the effector of Ptc-mediated apoptosis, is down-regulated in rhabdomyosarcoma, lymphoblastic leukemia, promyelocytic leukemia and Burkitt's lymphoma cells (Genini et al., 1997; Johannessen et al., 2006; Desmond et al., 2007), whereas Caspase-8 expression is selectively lost in neuroblastoma, thus preventing unbound integrin-mediated cell death (Stupack et al., 2006). Collectively, these findings suggested that targeting DRs signaling represent a promising approach for anti-cancer treatments. Indeed, silencing of Netrin-1 by using small interfering RNA or decoy recombinant DCC ectodomain protein (DCC-5Fbn) were found able to inhibit growth of lung tumor in xenografted mice (Delloye-Bourgeois et al., 2009). It is possible to speculate that these approaches will be more efficient in combination with conventional chemotherapies since these drugs are known to induce Netrin-1 upregulation (Paradisi et al., 2013). Recently the use of Netrin-1 monoclonal antibody was shown to inhibit tumor growth in mice model of colorectal adenocarcinoma *in vitro* (Grandin et al., 2016). Notably, this monoclonal antibody, in combination with epidrugs that revert the down-regulation of both DRs and their downstream effectors, is also able to decrease tumor growth in mice *in vivo* (Grandin et al., 2016).

Aims of the research

Angiogenesis is a hallmark of cancer that allows oxygen and nutrients to reach proliferating cancer cells and provides tumor cells with the metastatic route to colonize distant organs (Potente et al., 2011). Traditionally, the molecular pathways regulating angiogenesis have been suggested to act primarily through regulation of transcription. For the first time our group demonstrated that alternative splicing (AS) plays a key role in regulating endothelial cells (ECs) biology (Giampietro et al., 2015).

In particular, we found that the AS factor Nova2, previously considered brain-specific (Darnell, 1996), is also expressed in vascular endothelium (Giampietro et al., 2015). Notably, we showed that Nova2, by controlling AS of genes encoding for members of the Par polarity complex and its regulators, is required for the establishment of EC polarity and vascular lumen formation, fundamental steps occurring during angiogenesis (Iruela-Arispe & Davis, 2009).

Since molecules affecting both nerves and vessels function were recently termed "angioneurins" (Zacchigna et al., 2008), on the basis of our results we propose that Nova2 is a novel member of this family. Interestingly, Nova2 is the only angioneurin that acts as post-transcriptional regulator.

Given the observation that AS and transcription predominantly regulate different group of genes (Le et al., 2004; Pan et al., 2004), the main goal of our research is to characterize the contribution of the AS regulation during angiogenesis. In addition, our findings highlighted the importance to use highthroughput approaches to identify novel Nova2-mediated AS isoforms that are responsible for this process. Interestingly, by using RNA-seq (high-throughput sequencing of RNA) of Nova2 gain- and loss- of-function ECs, we discovered new Nova2 targets encoding for regulators of angiogenesis and/or vascular development. Among these, are axon guidance receptors recently linked to vascular development, such as *Unc5b*, *Neogenin*, *Slit2* and *Nrp1* (Adam & Eichmann, 2010). Notably, the Netrin receptor Unc5b is predominantly expressed in ECs where it functions in repulsive guidance during vessel patterning (Lu et al., 2004; Larrivée et al., 2007). We identified *Unc5b* exon 8 as novel Nova2-regulated AS event in ECs. In particular, Nova2 promotes skipping of this exon thus generating a novel protein isoform deleted of 11 amino acids in the extracellular domain of the Unc5b.

Due to the importance of Unc5b for vascular development and angiogenesis (Lu et al., 2004; Larrivée et al., 2007), we decided to validate this novel AS event in ECs, as well as characterize the function/activity of the novel Unc5b AS variant by using *in vitro* and *in vivo* models of angiogenesis.

Briefly, the specific aims of my PhD project can be summarized as follows:

Chapter I. Validation of Nova2-mediated AS regulation of *Unc5b* in ECs;

Chapter II. Characterization of the functional role(s) of the novel AS variant of Unc5b in *in vitro* angiogenesis;

Chapter III. Definition of the contribution of *Unc5b* AS during vascular development *in vivo*.

Materials and methods

Cell Culture

VE100 Mouse ECs were isolated, immortalized and extensively characterized by our collaborators (Taddei et al., 2006; Lampugnani et al., 2006). Culture medium of VE100 mouse ECs was DMEM (w/ glucose 4.5 g/l w/o L-Glutammine; Lonza, #LOBE12614F) supplemented with 10% fetal bovine serum (FBS; Euroclone, #ECS0180L), L-Glutamine (2 mM, Euroclone, #ECB3000D), penicillin/streptomycin (100 U l^{-1}, Euroclone, #ECB3001D), sodium pyruvate (1 mM, Euroclone, #ECM0542D), heparin (100 mg ml^{-1}, from porcine intestinal mucosa; Sigma-Aldrich #H3149) and EC growth supplement (5 mg ml^{-1}, Sigma-Aldrich, E2759). The same medium, supplemented with puromycin (3 µg ml^{-1}; InvivoGen, #ant-pr-1) or Hygromycin (50 ng ml^{-1}; Santa Cruz Biotechnology, #sc-29067), was used for stable VE100 mouse ECs cells knockdown or overexpressing *Nova2* (Giampietro et al., 2015) or carrying inducible *Unc5b-FL-GFP*, *Unc5b-Δ8-GFP* or *GFP* pSLICK constructs. VE100 Mouse ECs were grown as sparse or confluent by placing 500,000 cells in 100 mm and 35 mm Petri dishes, respectively.
Human Umbilical cells (HUVEC) were obtained from the Yale University Vascular Biology and Therapeutics Core Facility and maintained in EGM™2-BulletKit™ medium (Lonza, #CC-3156 and #CC-4176). The cells were verified by CD31 and VE-cadherin staining for endothelial cell identify.
Culture medium of human cervix carcinoma (HeLa) cells (ATCC, CCL-2) and human embryonic kidney (HEK) 293T cells (ATCC, CRL-1573) was DMEM glucose (w/ 4.5 g l^{-1} w/o L-Glutammine; Lonza, #LOBE12614F) with 10% fetal bovine serum (FBS; Euroclone, #ECS0180L), L-Glutamine (4 mM, Euroclone, #ECB3000D), penicillin/streptomycin (100 U l^{-1}, Euroclone, #ECB3001D). All cells were free of mycoplasma contamination. Cells were maintained in a humidified, 5% CO_2 atmosphere at 37 °C.

Plasmids

Mouse Unc5b-FL was amplified with primers *Unc5b-For-HindIII* and *Unc5b-rev-EcoRI-GFP* from 100VE mouse ECs cDNA and it was cloned in pEGFP-N1 vector (Clonentech) with standard procedure in order to add an in-frame C-terminal GFP-tag. Mouse Unc5b-Δ8 was generated by PCR-mediated mutagenesis of Unc5b-FL by using primers *Unc5b-deletion_ex8-for* and *Unc5b-deletion_ex8-rev*. The same approach (Primers *Unc5b-For-HindIII* and *Unc5b-rev-EcoRI-HA*) was used to generate Unc5b-FL and Unc5b-Δ8 with a C-terminal HA tag in a pcDNA 3.1(-) backbone (Invitrogen; # V79520).

Unc5b AS variants lentiviral vectors of the Tet-on inducible system were generated by Gateway technology (Gateway LR clonase II, Invitrogen; #11791) from pEN_Tmcs vectors, previously subcloned with Unc5bFL-GFP and Unc5bΔ8-GFP or GFP cDNA, in a pSLICK-Hygro backbone (Addgene, #25737).
To obtain *Unc5b* AS variants adenoviral constructs Unc5bFL-GFP and Unc5bΔ8-GFP cDNAs were subcloned into pENTR11 vector (Invitrogen; #A10467) and then transferred into pAd/CMV/V5/DEST using the Gateway Cloning System (Invitrogen; #11791).
Plasmids for *in vivo unc5b* mRNA injection, *unc5b* minigene splicing assay were described in the apropiate sections. All constructs were verified by sequencing.

Lentivirus and adenovirus production and transduction

Lentivirus production and transduction was performed in collaboration with Dr. Costanza Giampietro at IFOM Vascular Biology Unit. HEK293T cells were seeded in DMEM supplemented with 10% FBS without antibiotics in 60-mm Petri dishes (one Petri per infection). The day after, cells at 60–70% confluence were transfected (calcium phosphate transfection method) with: 5 µg of packaging plasmid, 5 µg of envelope plasmid and 20 µg of Unc5b lentiviral vectors. After 18 h, the medium was replaced with DMEM supplemented with 20% FBS and 1% penicillin/streptomycin. Cells were incubated for 24 h and the medium containing the lentiviral particles was harvested, filtered using a 0.45-µm filter unit and used to infect the cells. For viral transduction, VE100 mouse EC cells were seeded in 100-mm Petri dishes and were infected at 70% of confluence. Cells were incubated overnight with the viral supernatant supplemented with 0.2 mM proline and polybrene (final concentration 8 µg ml^{-1}; Sigma-Aldrich). After 48 h, hygromycin selection (50 µg ml^{-1}) was started and it was continued until all non-infected control cells died.
Adenovirus production was performed into HEK293 cells. HEK293A cells were seeded in 100-mm Petri dishes in DMEM medium without antibiotics to have 50-70% confluency next day. HEK293A at the correct confluency were transfected (Lipofectamin 2000; Invitrogen; #11668027) with *Unc5b-GFP* AS constructs linearized with PacI digestion, overnight at 37 °C. At day 3 after seeding medium was replaced, at 12 days cells and supernatant media were collected and freeze at -80 °C. Upon three cycles of freeze and thaw the medium containing the adenoviral particles was harvested, filtered using a 0.45-µm filter unit and used to infect HUVEC cells. Adenoviral infection was carried out by incubating cells with the viral supernatant for 24 hours.

Plasmid transfection

HeLa cells or VE100 ECs transfection was carried out by using Lipofectamine 3000 (Invitrogen; #L3000001) as indicated by the supplier. Briefly, cells were seeded in a 6-well or 24-well plate to reach 80% of confluence the next day. Cells were then transfected and assayed after the indicated time.

RNA interference with siRNA oligonucleotides

RNA interference was carried out on HUVECs using Lipofectamine RNAiMAX (Invitrogen; #13778030) following supplier's protocol. Briefly, HUVECs were transfected at 50-60% confluence with the indicated siRNA oligonucleotides (25 nmol). The following oligonucleotides were used: ON-TARGETplus human *Unc5b* siRNA (pool of four siRNA) and ON-TARGETplus non-targeting pool (as a negative control) (Dharmacon).

Immunofluorescence

Transfected HeLa cells were seeded in 35 mm Petri HeLa were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich). Nuclei were stained with 0.1 g ml^{-1} DAPI (Sigma-Aldrich). The same protocol was used for mouse 100VE ECs and human HUVECs, which were seeded in 35 mm Petri dishes coated with Gelatin (Difco) 0.1%.
For imaging, epifluorescence microscope (Optical Microscope Olympus IX71) equipped with 60X objective was used. Photomicrographs were taken with digital camera Cool SNAPES (Photometrics). Data acquisition was done using the MetaMorph 7.7.5 software (Universal Imaging Corporation). Images were exported to Photoshop (Adobe). No manipulations were performed other than adjustments in brightness and contrast.
High-resolution pictures were acquired using a Leica SP5 confocal microscope with a Leica spectral detection system (Leica 15 SP detector) and the Leica application suite advanced fluorescence software or Zeiss LSM800 confocal microscope and Zeiss Zen 2.3 Software.

Co-immunoprecipitation

Co-immunoprecipitations were carried out in HeLa co-transfected cells which the indicated plasmids and treated with Netrin-1 (150 ng ml-1) or PBS/0.2% BSA for 30 minutes. Adherent cells were washed with ice-cold PBS and collected in an IP Buffer (50 mM HEPES pH 7.6, 150 mM NaCl, 5 mM EDTA, and 0.1% NP-40

with the addition of protease and phosphatase inhibitors). GFP-tagged Unc5b AS variants were immunoprecipitated using GFP-trap magnetic beads (Chromotek; #gtma) for1 hour at 4 °C. Magnetic beads where washed three time with a Wash Buffer (50 mM HEPES pH 7.6, 150 mM NaCl, 5 mM EDTA) and immunoprecipitated proteins were eluted in a 2 x SDS-sample buffer (120 mM Tris/Cl pH 6.8; 20% glycerol; 4% SDS, 0.04% bromophenol blue; 10% β-mercaptoethanol) by heating at 95 °C for 10 minutes.
Half of the eluted proteins were loaded in a SDS-PAGE gel and immunoblotting with the indicated antibodies was performed. As control GFP-tagged Unc5b FL were immunoprecipitated with aspecific magnetic beads).

Vital exclusion dye assay

Transfected HeLa cells were serum-starved (Complete DMEM with 0.2% FBS) 24 hours upon transfection. Mouse recombinant Netrin-1 (150 ng l-1 in PBS/0.2% BSA, Bovin Serum Albumin; R&D) or the equivalent amount of PBS/0.2% BSA were added during starvation. Collected cells were then incubated with a vital exclusion dye (Erythrosin B) that is impermeable to biological membrane (Kim et al., 2016) and counted for positive and negative staining. Percentage of cell death was calculated as the number of Erythrosin B-positive cells with respect to the total. Percentage cell death index is the ratio of the percentage of cell death of each sample to the control (GFP untreated).
For mouse VE100 ECs, plated cells at 70% of confluency were induced with Doxycycline in a serum-starved medium (Complete DMEM with 0.2% FBS) for 6 hours or 24 hours as indicated. The same procedure described for HeLa cells was then utilized for the assay.

Caspase-3 Activity assay

Caspase-3 activity was determined using the “Caspase Glo 3/7” luminescence assay (Promega; #G8090) in a Synergytm HT Microplate reader (BioTeK Instruments). Briefly, 10,000 HUVEC cells were seeded in a 96-well plate and transduced with the indicated adenovirus. Next day, cells were serum-starved (EBM2 medium with 0.2% FBS) for 8 hours and assayed for Caspase- 3 following manufacturing instructions. Mouse recombinant Netrin-1 (150 ng l-1) or the equivalent amount of PBS/0.2% BSA were added during the 8 hours of starvation.

In vitro wound healing assay

HUVECs were transfected with the indicated siRNA, the following day they were transduced with the specified adenovirus particle and plated into 6-well plates. Transduced HUVECs were cultured until confluence (tipicaly 1 day) and starved overnight in 0.2% FBS/EBM-2. Scratch wounds were created with a 200-µl pipette tip. The wounded cell monolayer was cultured in 0.2% FBS/EBM-2 supplemented with mouse recombinant Netrin-1 (500 ng ml^{-1}; R&D) or 6 nM VEGF-A as indicated. Cell-free areas were photographed at 0 h and 12 h post wounding under an inverted light microscope connected with a digital camera. Wound closure was calculated by measuring cell free areas at 0h ($Area_{0h}$) and 12h ($Area_{12h}$) by using NIH Image J software (version 1.48v). Percentage of wound closure is calculated as: $[(Area_{0h})-(Area_{12h})]/ (Area_{0h})$ x 100.

Co-culture experiments of human primary ECs

HUVEC cells transduced with Robo4ΔCD-mCherry adenovirus and Unc5bFL-GFP or Unc5bΔ8-GFP were plated into 6-well gelatin-coated (Difco, 0.1% in PBS) plates at 1:1 ratio. After 24 h, cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) and images were acquired by Leica SP5 confocal microscope (see immunofluorescence section).

Immunoblot analysis

Cells were lysed in Laemmli buffer, supplemented with protease and phosphatase inhibitors (cOmplete™ and EDTA-free Protease Inhibitor cocktail; Roche). Proteins were separated using SDS–PAGE and analyzed by western blotting by standard procedures. After protein transfer, the nitrocellulose membranes (0.45 µm; Whatman PROTRAN) were blocked by incubation with 5% non-fat dry milk. The following primary antibodies were used: anti-Nova2 (1:200; Santa Cruz Biotechnology, #C-16), anti-α-tubulin (1:100,000 Sigma-Aldrich), anti-haemagglutinin (HA; 1:1,000 Roche), anti-GAPDH (1:5,000 Abcam), anti-GFP (1:3000 Millipore); anti-Cleaved Caspase-3 (1:1000 Cell Signalling). The following secondary antibodies linked to horseradish peroxidase (Jackson ImmunoResearch) were used: anti-Mouse (1:5,000), anti-Goat (1:5,000) and anti-Rabbit (1:10,000). Immunostained bands were detected using the chemiluminescent method (Euroclone, LiteAblot Plus/Extended, #EMP011005/#EMP013001).

RNA extraction, RT-PCR and qRT-PCR

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, #74106) according to the manufacturer's instructions. After DNase I (Ambion, #AM2222) treatment, 0.5–1.5 μg of purified RNA was retro-transcribed with a mix of oligos d(T)18 and random hexamers by using SuperScript III First-strand System (Invitrogen, #18080051). Resulting cDNA (1/20 v/v) was then PCR-amplified with the GoTaq® G2 Flexi DNA Polymerase (Promega, #M7805) according to the manufacturer's instructions. For RT-qPCR, an aliquot of the retrotranscribed RNA was analyzed with QuantiTect SYBR Green PCR (QIAGEN, #204145) by using LyghtCycler 480 (Roche). Target transcript levels were normalized to those of a reference gene (Rplp0). The expression of each gene was measured in at least three independent experiments. Primers used for RT-PCR and RT-qPCR are listed in Table 4 and Table 5. All PCR products were sequenced and the bands intensity was quantified with NIH Image J software (version 1.48v). Percentage of alternative exon inclusion, calculated as (Intesity of Inclusion band) / (Intesity of Inclusion + Skipping bands) x 100, is indicated. All PCR products were verified by sequencing. * indicates aspecific bands.

Minigene Splicing assay

Unc5b genomic DNA encompassing exons 7 and 9 (5.6 kb total) was PCR amplified from mouse fibroblast and cloned into the pcDNA3.1(+) vector containing a Human cytomegalovirus immediate-early (CMV) promoter for transient expression in mammalian hosts (Thermo Fisher, #V790-20). *Unc5b* minigene was transfected into HeLa cells or mouse VE100 ECs together with different splicing factors or an empty vector at 1:1 ratio. Cells were cultured for 24 h and total RNA and protein lysate were collected. Nova2-HA and hnRNPA1-T7 expression vectors were already generated in our laboratory (Giampietro et al., 2015; Bonomi et al., 2013).

Zebrafish strains and maintenance.

Zebrafish (*Danio rerio*) experiments were carried out in the FIRC Institute of Molecular Oncology Institutional (IFOM) Zebrafish facility in collaboration with Dr. De Florian. Zebrafish from wild-type AB and transgenic Tg(fli1a:EGFP)y1 (Lawson & Weinstein, 2002) or Tg(Kdrl:GFP) (Cross et al., 2003) strains were maintained and bred according with standard procedures and national guidelines (Italian decree "4th March 2014, n.26"). All experimental procedures were approved by the IFOM Institutional Animal Care and Use Committee.

RT-PCR of zebrafish embryos.

For RT-PCR experiments of *Unc5b* exon 8 and *Neogenin* exon 26 splicing characterization, total RNA from wild-type AB Tg(fli1a:EGFP)y1, Nova2 morpholino-mediated knockdown Tg(fli1a:EGFP)y1 embryos and Tg(Kdrl:GFP) *nova2* mutants was extracted with TRIzol reagent (Invitrogen) purified with the RNeasy Mini Kit (QIAGEN, #74106) and retro-transcribed with $d(T)_{18}$ oligo Superscript III RT (Invitrogen, #18080051). *Nova2* morphants and *nova2* mutants were already generated by the IFOM zebrafish facility as described in Giampietro et al., 2015.

Unc5b Morpholino and capped mRNA injections of zebrafish embryo.

Zebrafish embryos at one- to two-cell stage were injected with an *Unc5b* morpholino antisense oligonucleotide (MO-unc5b) design to block the splicing of *unc5b* Intron 1 as already performed by Lu and colleagues (Lu et al., 2004). Morpholino efficiency was evaluated by RT-PCR in RNA from 72hpf embryos with primers annealing in unc5b exon 1 and intron 1. Increased amplification results from the prevention of intron 1 removal from the final mRNA.
To rescue morphological and vascular defects due to the knockdown of *unc5b*, we amplified, with primers *Unc5b_Zebra-for/Unc5b_Zebra-rev* by using RT generated from Tg(Kdrl:GFP) 72 hpf embryos, *Unc5b-FL* and *Unc5b-Δ8* cDNAs. We cloned these cDNAs in a modified pCS2plus vector carriyng an in-frame C-terminal mCherry tag and we transcribed *in vitro* the capped mRNAs using SP6 mMessage mMachine kit (Ambion; #AM1340). Co-injection in one- to two-cell stage embryos MO-unc5b unc5b AS variants mRNAs was performed.
Evaluation of vascular defects was performed by immunofluorescence staining of zebrafish embryos. Zebrafish embryos from the Tg(Kdrl:GFP) strain at 72 hpf were dechorionated and fixed in 2% PFA in PBS, overnight at 4 °C. Embryos were then washed four times for 5 min in PBST (PBS, 0.1% Tween20). Permeabilization in PSBT with 0.5% Triton X-100 was performed for 30 min at room temperature. Embryos were then blocked in a solution of PBST with 0.5% Triton X-100, 10% normal goat serum and 1% BSA for 2 h at room temperature. Embryos were then incubated with primary antibodies in blocking solution overnight at 4 °C. Successively, embryos were washed six times in PBST over 4 h at room temperature and then incubated with secondary antibodies in blocking solution, overnight at 4 °C. Embryos were washed finally six times in PBST over 4 h at room temperature and equilibrated in glycerol 85% in PBS. The following

antibodies were used: anti-GFP (1:2,000 Millipore); anti-mouse Alexa-488-conjugated IgG (1:400). For the microscope analysis, we mounted on slides the trunk and tail regions dissected from five to six embryos of each samples. Images were taken with a Leica TCS SP2 confocal microscope, using oil-immersion objective.

Capped mRNA injections of *nova2* mutant zebrafish embryo.

To rescue vascular defects of *nova2* mutants one- to two-cell stage embryos from heterozygous *nova2* mutants were injected with *Unc5b-FL* and *Unc5b-Δ8* capped mRNA as previously described. At 72 hpf, zebrafish were analyzed by immunofluorescence staining as previously described. At least 20 embryos of each sample were dissected to mount on slide trunk and tail regions.

Results

CHAPTER I
Validation of Nova2-mediated alternative splicing regulation of *Unc5b* in ECs

Nova2 regulates alternative splicing of *Unc5b* exon 8

Our previous work demonstrated an important biological role for the alternative splicing (AS) factor Nova2 in regulating vascular functions (Giampietro et al., 2015). In order to identify novel AS transcripts regulated by Nova2 in the endothelium we analyzed Nova2 gain- and loss-of-function endothelial cells (ECs) by RNA-seq. Briefly, stable Nova2 knock-down and Nova2 over-expressing immortalized mouse 100VE ECs were generated in collaboration with Prof. Elisabetta Dejana (IFOM, Milan) by using lentiviral vectors carrying shRNAs (knock-down) or the cDNA (over-expression) specific for Nova2, respectively. Nova2-regulated AS events were identify and quantify in collaboration with Dr. Manuel Irimia (EMBL/CRG, Barcelona) and Prof. Benjamin J. Blencowe (University of Toronto, Canada) by using *vast-tools* (Vertebrate Alternative Splicing and Transcription Tools) program. A part of these analysis was included in our recent article (Giampietro et al., 2015).
By using Gene Ontology (GO) enrichment analysis for AS changes in Nova2 over-expression or knock-down ECs, we identified many genes encoding for regulators of angiogenesis and/or vascular development. Among these are axon guidance receptors involved in vascular patterning, such as *Unc5b*, *Slit2*, *Neogenin* and *Nrp1*. In particular, the Netrin receptor Unc5b is predominantly expressed in ECs and it functions as a repulsive guidance receptor during vessel patterning and morphogenesis (Adams & Eichmann, 2010). Depletion of Unc5b in mouse and zebrafish causes aberrant extension of endothelial tip cell filopodia and excessive vessel branching, whereas inactivation of *netrin-1a* is associated with vessel loss during zebrafish development (Lu et al., 2004; Wilson et al., 2006). Recently, the EC-specific axon guidance receptor Robo4 was described as novel Unc5b interaction partner by our collaborator Prof. Eichmann (Yale University, USA) (Koch et al., 2011). Notably, through Robo4 binding, Unc5b maintains vascular integrity by counteracting VEGF signaling (Koch et al., 2011). Due to the importance of Unc5b for vascular development and angiogenesis, I decided to validate this novel Nova2 target in ECs. RNA-seq indicates that Nova2 promotes skipping of *Unc5b* exon 8. This in frame deletion leads to production of a novel (uncharacterized) AS protein variant of Unc5b that I called Unc5b-Δ8 (Figure 1; Table 1 and 2 in Appendix).

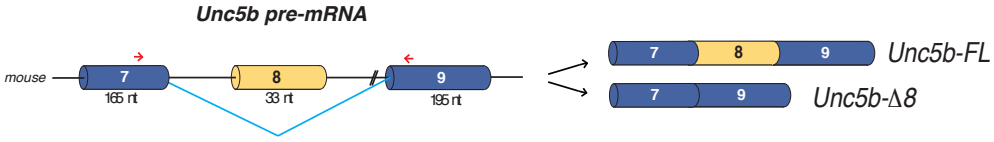

Figure 1. Schematic representation of *Unc5b* AS event. The mouse *Unc5b* genomic region with the AS exon 8 (33 nt in yellow) is indicated. Two different AS transcripts result from the inclusion or skipping of exon 8. Our RNA-seq indicated that Nova2 promotes skipping of *Unc5b* exon 8. Blue bars, Nova-silenced exon inclusion. Red arrows indicate primers used for RT-PCR analysis.

In order to confirm RNA-seq results, I analyzed AS of *Unc5b* exon 8 by using RT-PCR with RNA extracted from Nova2 overexpressing or knockdown ECs. In according to RNA-seq data, I found that high Nova2 expression level increases skipping of exon 8, whereas the opposite effect was observed in Nova2 depleted ECs (Figure 2).

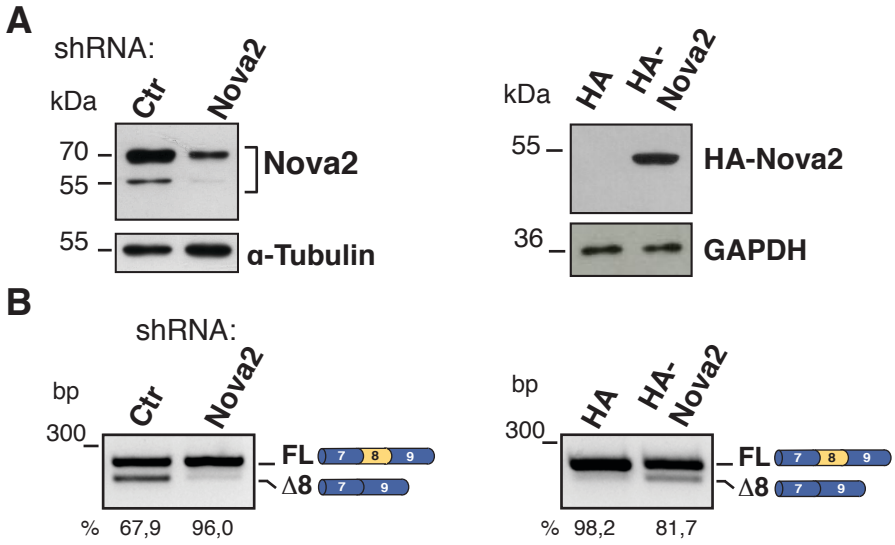

Figure 2. Nova2 controls AS of *Unc5b* exon 8 in ECs. (**A**) Nova2 protein levels in stable knockdown mouse ECs and in stable mouse ECs overexpressing HA-tagged Nova2. α-Tubulin and GAPDH as loading controls. The anti-Nova2-specific antibody recognized two

immunoreactive bands at 50–55 and 70–80 kDa, as previously reported (Yang et al., 1998). (**B**) AS analysis by RT-PCR of *Unc5b* exon 8 in Nova2 knockdown ECs (left) and in ECs overexpressing HA-Nova2 (right). Since Nova2 expression is regulated by EC density (Giampietro et al., 2015 and Figure 3), Nova2 depleted ECs were grown as confluent, whereas Nova2 overexpressing ECs were grown as sparse. FL, transcripts containing exon 8; Δ8, transcripts without exon 8. The percentage of exon inclusion (the ratio between the FL transcripts and the total) was calculated through quantification of the agarose gel bands with the ImageJ software (see Materials and methods).

We have recently shown that Nova2 is up-regulated in confluent versus sparse ECs and that its expression increases during endothelial differentiation of mouse embryonic stem (ES) cells or in adult ECs as compared with embryonic or fetal ECs (Giampietro et al., 2015). By using these models, I found that high Nova2 expression correlated with high *Unc5b-Δ8* production in (Figure 3):

(i) sparse versus confluent ECs;
(ii) during endothelial differentiation of ES cells;
(iii) ECs of different origin.

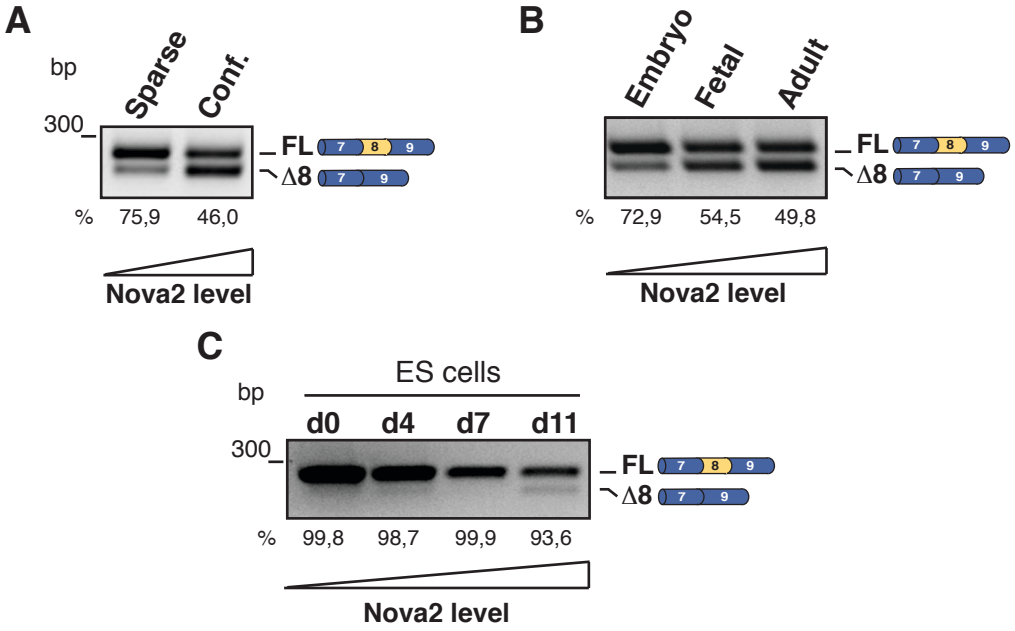

Figure 3. Figure legend next page.

Figure 3. AS of *Unc5b* correlates with Nova2 expression in ECs. RT-PCR analysis of AS of *Unc5b* in ECs grown at different densities (sparse and confluent) (**A**), during endothelial differentiation of mouse ES cells (d= days) (**B**) and in mouse EC lines derived from whole embryo, fetal heart and adult lung (**C**). Nova2 expression levels (as described in Giampietro et al., 2015) and the percentage of exon inclusion is also reported.

In addition to these *in vitro* systems, to confirm *Unc5b-Δ8* expression in vascular endothelium *in vivo,* I analyzed by RT-PCR total RNA extracted from ECs purified from mouse lung and from total testis. Interestingly, I found that the higher expression of Nova2 in freshly purified ECs from mouse lung (as compared with testis) was accompanied to the skipping of *Unc5b* exon 8 (Figure 4).

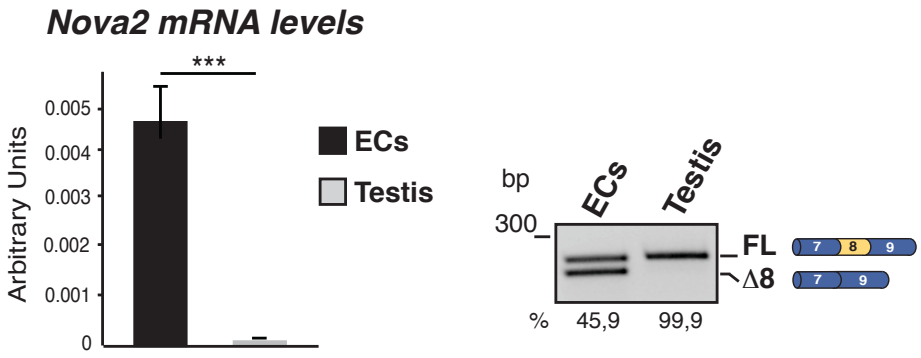

Figure 4. AS of *Unc5b* correlates with Nova2 expression in vascular endothelium *in vivo*. *Nova2* mRNA expression level (left) and RT-PCR analysis of *Unc5b* AS (right) in fresh purified mouse lung ECs and testis. The percentage of exon inclusion is also shown. Error bars indicate ± S.D.; *** P value < 0,001; two-tailed student *t*-test.

As shown in Figure 5, in accord with Nova2 expression in the CNS and its increase during brain development (Saito et al., 2016), I also found that *Unc5b-Δ8* isoform was present in E15.5 mouse whole brain but not in E9.5 that was negative for Nova2 expression (Figure 5).

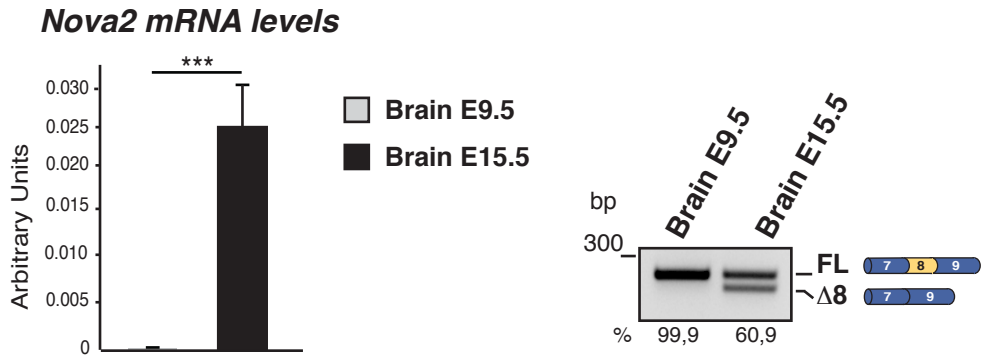

Figure 5. AS of *Unc5b* correlates with Nova2 expression levels in CNS. *Nova2* mRNA expression level and RT-PCR analysis of *Unc5b* AS in E9.5 and E15.5 mouse whole brain. The percentage of exon inclusion is also shown. Error bars indicate ± S.D.; *** P value < 0,001; two-tailed student *t*-test.

Finally, I also validated human *Unc5b* AS in HUVECs (Human Umbilical Veins Endothelial Cells) in which Nova2 was silenced by using shRNA for Nova2 or control shRNA, a sytem that we have used to investigate the role of Nova2 in the vascular endothelium in our recent work (Giampietro et al., 2015). As showed in figure 6, depletion of Nova2 reduces the production of *Unc5b-Δ8* isoform also in human derived ECs (Figure 6).

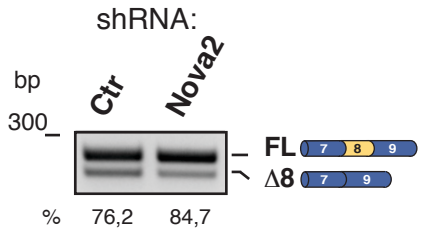

Figure 6. Nova2 controls AS of *Unc5b* exon 8 in HUVEC. RT-PCR analysis of *Unc5b* AS in primary human ECs (HUVEC) knockdown for Nova2 (as described in Giampietro et al., 2015). The percentage of exon inclusion is also indicated.

Collectively, these data demonstrate that Nova2 promotes in ECs the production of a novel transcript (*Unc5b-Δ8)* of the netrin receptor Unc5b.

Nova2 directly binds to *Unc5b* pre-mRNA

Nova2 specifically recognizes clusters of YCAY (Y=C/U) sequences (defined as >3 YCAY sites within 45 nucleotides) on its pre-mRNA targets (Ule et al., 2006). In order to identify putative Nova2 binding sites, I analyzed human and mouse *Unc5b* genes by using RBPmap bioinformatic tool (http://rbpmap.technion.ac.il) (Paz et al., 2014). RBPmap enables accurate prediction and mapping of binding sites of more than 90 different RNA binding proteins (RBPs), by combining the clustering propensity of the binding sites and the over-all tendency of regulatory regions to be conserved (Paz et al., 2014). Of 33 predicted YCAY sites, the top 7 for their scores (human: Z-score ≥ 3.84, P-value < 10^{-4}; mouse: Z-score ≥ 3.84, P-value < 10^{-4}) were localized in the intronic region upstream *Unc5b* exon 8 (Figure 6A; Table Y in Appendix). Interestingly, these YCAY repeats was clustered in 31 nucleotides and, most importantly, located within 200 nucleotides from intron-exon 8 junction, a distance by which Nova2 functions to inhibit exon inclusion (Jelen et al., 2007). Thus, this analysis indicated that the direction of the observed AS changes in *Unc5b* pre-mRNA (Figure 2A) was consistent with the position of Nova2-binding sites (Ule et al., 2006). Moreover, it suggested that the YCAY intronic cluster upstream *Unc5b* exon 8 represent a *bona fide* Nova2 binding sites.

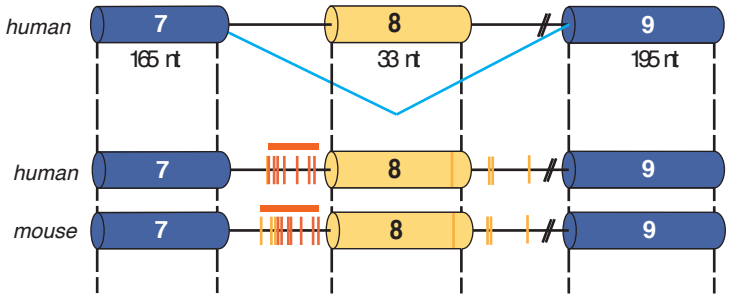

Figure 6. Analysis of Nova2 binding sites in *Unc5b* pre-mRNA. YCAY sites, identified with RBPmap tool and indicated as vertical orange (Z-score ≥ 3.84) or pale orange (Z-score ≥ 2.75) bars, are conserved between human and mouse *Unc5b* genes. The position of YCAY clusters suggested that Nova2 promotes skipping of *Unc5b* exon 8 (blue bars).

To determine if Nova2 directly binds to *Unc5b* pre-mRNA *in vivo*, in collaboration with the laboratory of Prof. Maria Paola Paronetto (University of Rome "Foro Italico"), we carried out individual-nucleotide resolution UV crosslinking and immunoprecipitation (iCLIP), a method that allow identification of direct protein-

RNA interactions (Huppertz et al., 2014). iCLIP relies on the principle that the precise mapping of binding sites is achieved by preserving the *in vivo* protein-RNA interactions through the crosslinking of living cells or tissue. This unperturbed *in vivo* condition allows the maintenance of proper intermolecular interactions, salt and ion concentrations required for an efficient RNA binding.
RNA of *in vivo* cross-linked ECs was immunoprecipitated by using anti-Nova2 or control antibodies. RNAs bound by Nova2 were then analyzed by RT-qPCR with primers spanning the YCAY cluster upstream *Unc5b* exon 8 (primers A) or primers mapping in intron 8 (primers B) as negative control. As shown in Figure 7, Nova2 directly bound to the endogenous *Unc5b* transcript at the level of YCAY cluster upstream of exon 8, but not to *Unc5b* intron 8-9.

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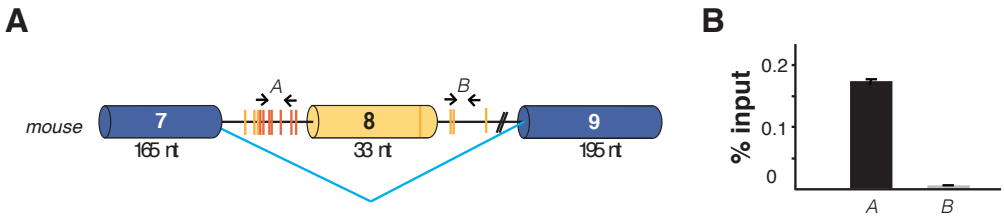

Figure 7. Nova2 directly binds to *Unc5b* pre-mRNA. (**A**) As described in Figure 6**,** single YCAY sites identified with RBPmap tool are represented as vertical orange or pale orange bars. (**B**) The immunoprecipitated RNAs were analyzed by RT-qPCR with primers A (annealing to the YCAY cluster in intron 7, upstream exon 8) and B (annealing in intron 8-9 as negative control). Error bars indicate ± S.D. calculated from two independent experiments.

To further investigate the role of Nova2 in regulating AS of *Unc5b* pre-mRNA, I performed a splicing assay in HeLa cells by using an *Unc5b* minigene encompassing exons 7, 8, and 9 of mouse *Unc5b* gene along with the flanking intron sequences (Figure 8). The minigene was co-transfected in HeLa cells, which express low levels of Nova2 (Giampietro et al., 2015), with plasmids encoding either Nova2 or hnRNP A1 (an unrelated and ubiquitously expressed SRF, (Bonomi et al., 2013), or with the empty vector. As shown in Figure 8, skipping of *Unc5b* exon 8 was drastically enhanced by overexpression of Nova2. Notably, this effect was specific of Nova2 since overexpression of hnRNPA1 was unable to increase *Unc5b-Δ8* transcript.

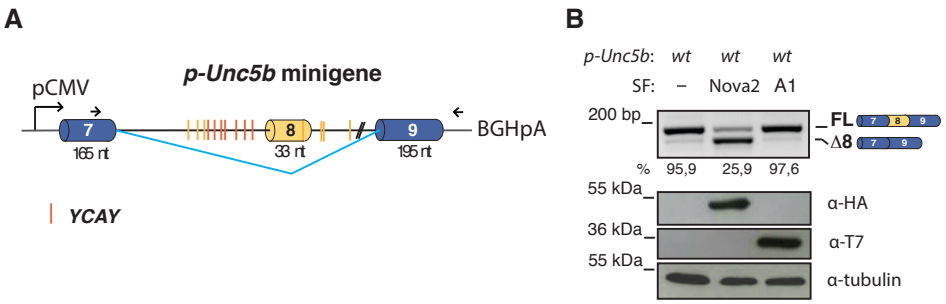

Figure 8. *In vivo* splicing assay with *Unc5b* wild-type minigene. (**A**) The mouse *Unc5b* genomic region encompassing exons 7, 8 and 9, cloned into the pcDNA3.1+ vector to generate the *p-Unc5b wild-type* minigene. Arrows indicate primers used for RT-PCR analysis of pUnc5b splicing. Boxes = exons; thin lines = introns; pCMV= promoter; BGHpA= polyadenylation sequence. (**B**) *p-Unc5b* minigene was co-transfected in HeLa cells (which express low levels of Nova2) with either Nova2, T7-hnRNP A1 (A1), or the empty vector (SF: splicing factor). Ectopic expression of Nova2 and A1 was confirmed by western blotting with anti-HA and anti-T7 antibodies, respectively (α-tubulin as loading control). Quantification of exon inclusion is also shown.

In addition, I generated a mutated *Unc5b* minigene (p-*Unc5b Mut*) in which all YCAY motifs were replaced with YAAY, a sequence not recognized by Nova2 as previously reported (Buckanovich & Darnell, 1997) (Figure 9). As shown in Figure 9, mutations in YCAY repeats led to reduction of Unc5b-Δ8 when Nova2 was co-expressed.

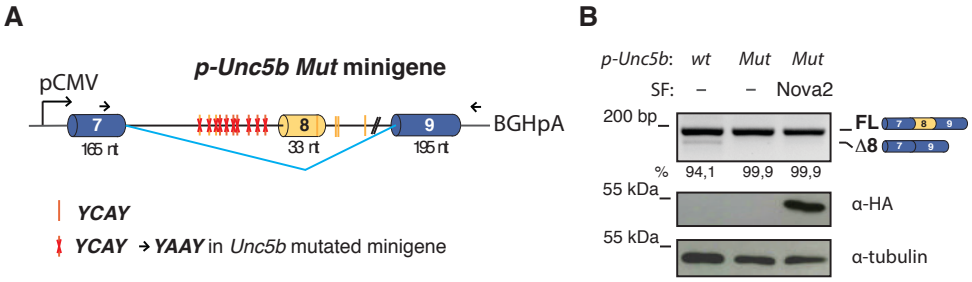

Figure 9. *In vivo* splicing assay with *Unc5b* mutated minigene. (**A**) Scheme of the mouse *pUnc5b mutated* minigene (*Mut*) generated by introducing mutations in all Nova2 binding sites (YCAY repeats were replaced with ACAY). Arrows indicate primers used for RT-PCR. (**B**) RT-PCR analysis of *Unc5b Mut* splicing in HeLa cells co-transfected with the

indicated splicing factor (SF) expression vectors (upper panel). Western blotting with anti-HA was used to confirm Nova2 overexpression (lower panels); α-tubulin as loading control. Quantification of exon inclusion is also shown.

Similar results were also obtained with transient transfection of ECs with *p-Unc5b* minigenes (wild-type and mutated) (Figure 10). In accord with the expression of Nova2 in ECs, *Unc5b-Δ8* transcripts was produced at higher levels in ECs (as compared with HeLa cells) transfected with *p-Unc5b* wild-type minigene. Notably, overexpression of Nova2 in ECs completely abolished the inclusion of *Unc5b* exon 8, leading to production of only *Unc5b-Δ8* isoform. Nevertheless, mutations in YCAY repeats led to a decrease in exon 8 skipping when Nova2 was co-expressed (Figure 10).

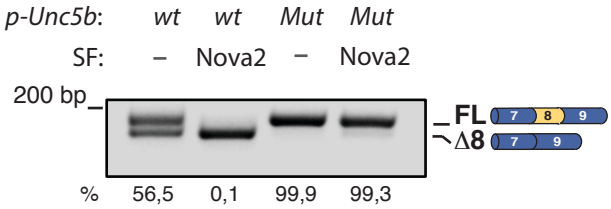

Figure 10. *In vivo* splicing assay with *Unc5b* minigenes in ECs. RT-PCR analysis of pUnc5b (wild-type and mutated) minigenes co-transfected in ECs with the indicated expression vectors (upper panel). The percentage of exon inclusion is also shown.

Collectively, my results demonstrate that Nova2 promotes skipping of *Unc5b* exon 8 by directly binding to the YCAY cluster located upstream this exon.

CHAPTER II
Characterization of the functional role(s) of the novel AS variant of Unc5b in *in vitro* angiogenesis.

Unc5b is a single pass transmembrane receptor able to interact with a plethora of ligands (including Netrin-1, Neogenin and Robo4) through two trombospondin-like domains (TSP1) and two Ig-like domains in its extracellular region, whereas its cytoplasmic tail includes a ZU5 domain, a DCC-binding domain (DB) and a death domain (DD) (Figure 11) (Larrieu-Lahargue et al., 2012; Zuo et al., 2014; Wang et al., 2009). Depending on the type of ligand and the bound or unbound state of the receptor, Unc5b activates different signaling pathways involved in various biological processes such as survival and apoptosis, cell adhesion, migration and angiogenesis (Tang et al., 2008). Importantly, activation of downstream signaling pathways requires the recruitment of different effectors through direct interaction with Unc5b cytoplasmic tail (Hong et al., 1999).

Unc5b exon 8 might encodes for an intrinsically disordered region

Unc5b exon 8 is a cassette of 33 nucleotides encoding for 11 amino acids in the extracellular region of the receptor located in close proximity to the transmembrane domain (TM) (Figure 11). By using different bioinformatic tools, I found that the amino acids encoded by *Unc5b* exon 8 do not overlap with any known functional protein domain (Figure 11). Despite amino acids of this region are poorly conserved among different species, the position of 5 charged residues is strongly conserved among human, mouse and zebrafish and with other members of this family (such as Unc5d) (Figure 11). Interestingly, it has been reported that sequences enriched of charged and polar amino acids and depleted in bulky hydrophobic residues do not mediate co-operative folding (van der Lee et al., 2014). These sequences lack a unique three-dimensional structure either entirely or in parts in their native state (Babu, 2016). For this reason, these sequences are defined as intrinsically disordered regions (IDRs).
To investigate the possible presence of IDRs in Unc5b protein sequence I used GlobPlot software (http://globplot.embl.de), which is able to predict protein structure tendency within the query protein for ordered/globularity and disordered regions (Linding et al., 2003). As shown Figure 11, *Unc5b* exon 8 (in green) was found to overlap with a predicted IDR placed between the hydrophobic TM and the second TSP1 domain. A similar prediction was also confirmed by using another software, PONDR (Predictor of Natural Disordered Regions; (http://www.pondr.com) (Garner et al., 1998).

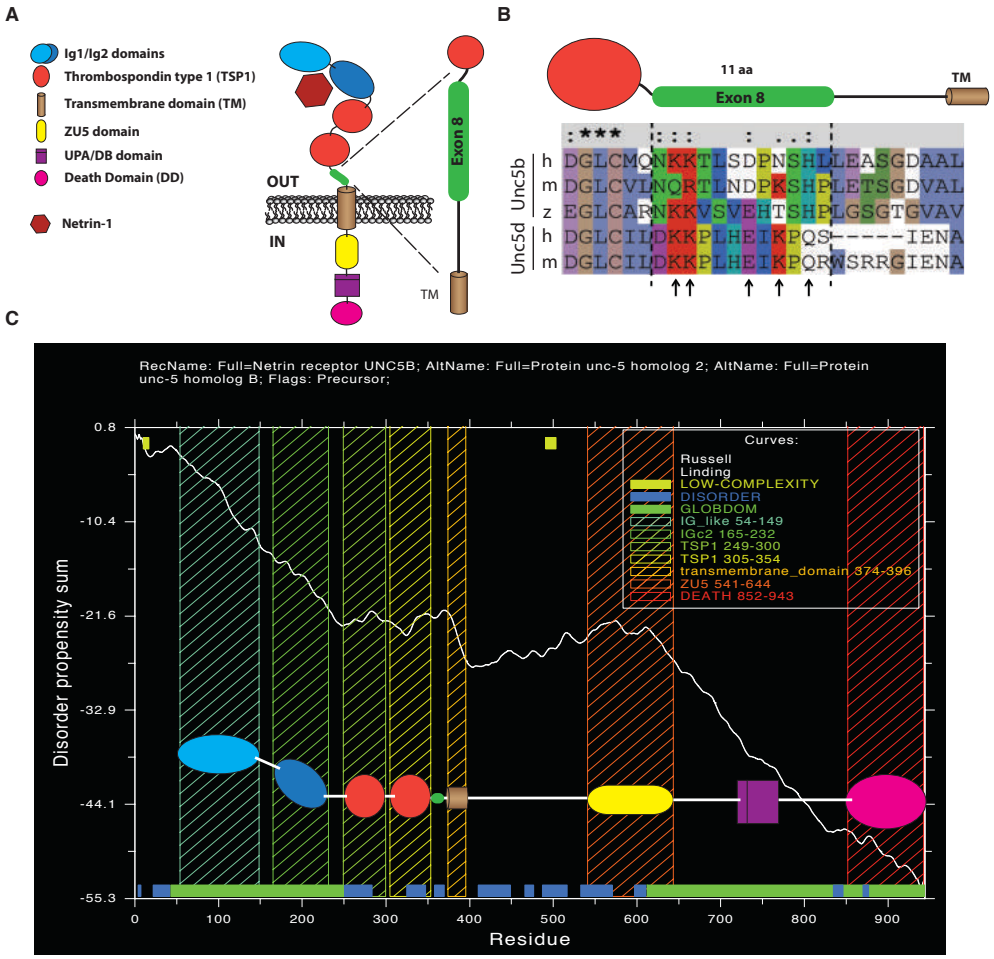

Figure 11. Unc5b protein structure. (**A**) Schematic representation of the Unc5b protein structure. Blue ovals: Ig-like domains; red ovals: trombospondin-like domains (TSP1); beige cylinder: transmembrane domain (TM); yellow dot: ZU5 domain; violet square: UPA/DCC-binding domain (DB); pink dot death domain (DD). *Unc5b* exon 8 (green) encodes 11 amino acids proximal to the transmembrane domain. (**B**) Human (h), mouse (m) and zebrafish (z) Unc5b and Unc5d (h and m) protein sequences analyzed by using ClustalX 2.1 program. Black arrows indicate conserved charge residues in Unc5b exon 8. * fully conserved residue; : "strong" conserved group; . "weaker" conserved group. (**C**) GlobPlot prediction of mouse Unc5b. Low-complexity (yellow), disordered (blue) and globular (green) regions are shown on the x-axis, in which Unc5b residues are indicated (1-943). White line, Russell-Linding propensity of disorder.

Interestingly, tissue-specific alternatively spliced exons frequently encode for IDR (Romero et al., 2006; Buljan et al., 2013). Splicing of these IDR encoding exons can affect a number of functions (or properties) of a protein (Buljan et al., 2013). For instance, IDR often contain binding sites for other proteins, nucleic acids and small molecule ligands, in this case expanding the interactome of the IDR's harboring protein (Buljan et al., 2013). In addition, the flexibility of IDR can favor conformational heterogeneity between structured domains or can harbor sites for post-translational modification (PTM), which ultimately alter downstream signaling through recruitment of different effectors (Buljan et al., 2013).
Hence, my bioinformatic analyses suggest that *Unc5b* exon 8 could encode for a IDR modulating Unc5b activity.

Unc5b-Δ8 is able to dimerize upon Netrin-1 treatment but it remains unresponsive to pro-survival signals

Growing lines of evidence describe the importance of cell death regulation in ECs since the balance between survival and apoptosis is critical to maintain blood vessel integrity both during vascular development and pathological angiogenesis (Murakami et al., 2011; Giampietro et al., 2012; Korn & Augustin, 2015).
Unc5b has been described as a "dependence receptor" (Tanikawa et al., 2003). In particular, Unc5b contains a cytoplasmic death domain able to induce apoptosis in the absence of its ligand Netrin-1, whereas ligand binding induces Unc5b dimerization thus triggering survival signaling (Arakawa, 2004). Importantly, Netrin-1 mediated dimerization of Unc5b has been proposed as a mechanism by which ligand binding is able to prevent cell death activation (Mille et al., 2009). To test if skipping of *Unc5b* exon 8 affects the ability of Unc5b AS variants to dimerize, I cloned both Unc5b AS variants (Unc5b-Δ8 and Unc5b-FL) fused to a C-terminal GFP-tag or a C-terminal HA tag, as previously reported (Larrivée et al., 2007). These vectors were transfected in HeLa cells that are easier to transfect than ECs (Tanner et al., 1997). I found that both GFP-tagged or HA-tagged (Figure 12) Unc5b AS variants were able to reach cell membrane in transient transfected HeLa cells (Figure 12). Next, I performed a co-immunoprecipitation of Unc5b GFP-tagged AS variants co-transfected in HeLa cells together with the corresponding AS variants tagged with an C-terminal HA tag (i.e. Unc5b-FL-GFP + Unc5b-FL-HA or Unc5b-Δ8-GFP + Unc5b-Δ8-HA). GFP-tagged proteins were immunoprecipitated by using GFP-trap magnetic beads (or aspecific magnetic beads as control). Receptor dimerization was evaluated by immunoblotting with an anti-HA antibody as described in *Miller et al., 2009* (Mille et al., 2009). As shown in Figure 12, I found that both Unc5b AS variants increased their dimerization upon Netrin-1 treatment, thus indicating that Unc5b-Δ8 is able to properly dimerize

with others Unc5b-Δ8 receptors, as it has been reported to occur for the Unc5b-FL isoform.

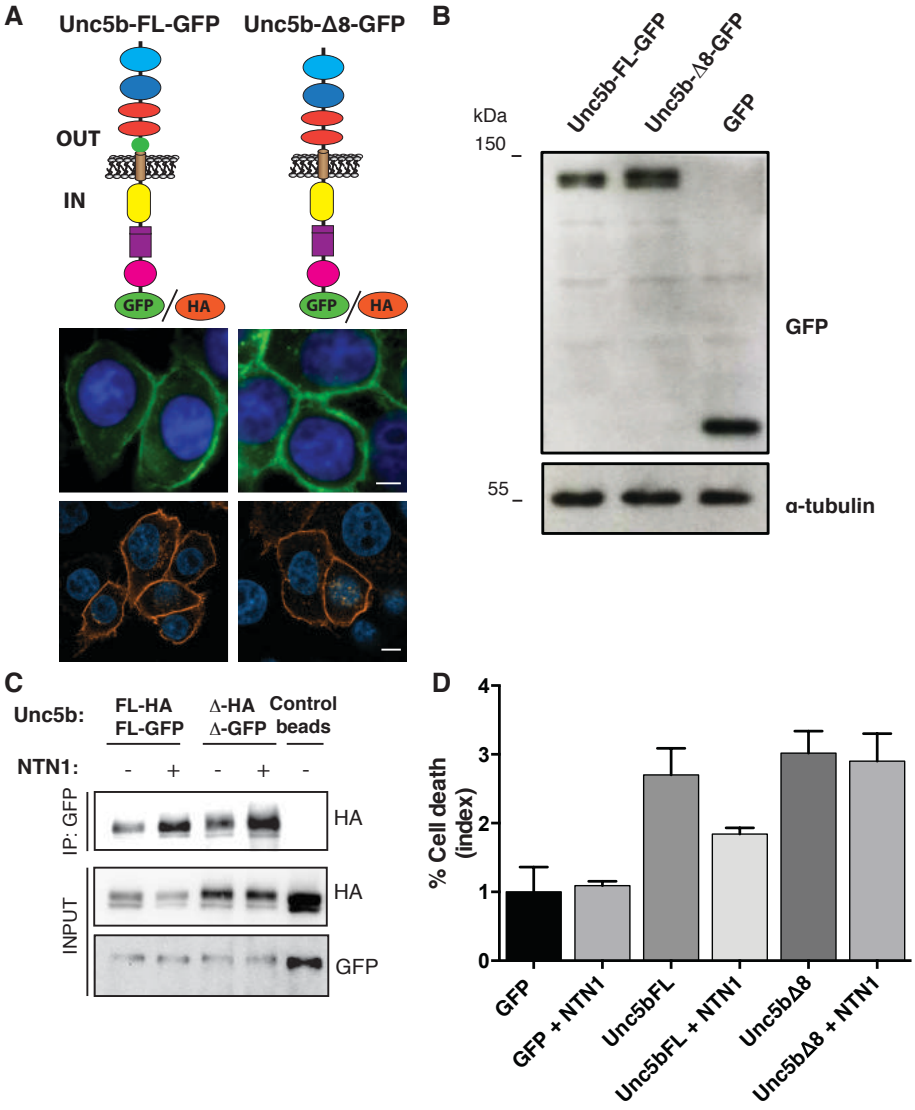

Figure 12. Netrin-1 treatment of HeLa cells overexpressing Unc5b AS isoforms. (**A**) Schematic representation of the Unc5b protein structure as in Figure 11 and cellular localization of GFP and HA-tagged Unc5b-FL or Unc5b-Δ8 in transiently transfected HeLa cells. Both protein isofors are able to reach the cell membrane. Scale bar: 5 μm. (**B**) The ectopic expression of Unc5b AS isoforms was confirmed by western blotting with anti-GFP

antibody in transiently transfected HeLa cells (a-Tubulin as loading control). (**C**) HeLa cells were transiently transfected with the indicated vector expressing (HA or GFP tagged) Unc5b AS variants. Cell lysates were immunoprecipitated with anti-GFP specific magnetic beads as described in Mille et al., 2009. Co-immunoprecipitated Unc5b-GFP proteins were detected by immunoblotting with HA antibody. Control beads: immunoprecipitation with aspecific magnetic (5^{th} lane). Netrin-1 (NTN1) treatment was performed for 30 minutes at 150 ng/ml. (**D**) Vital exclusion dye assay was performed in HeLa cells 24 h after transfection. Cells were serum starved to induce cell stress and treated with Netrin-1 (NTN1; 150 ng/ml) or BSA (-) as control. Error bars indicate ± S.D.; ** P value < 0.01; *** P value < 0.001 two-tailed *t*- test.

I then investigated whether Netrin-1 behaved as a survival factor for HeLa cell overexpressing Unc5b GFP-tagged AS variants. As previously reported (Llambi et al., 2005), I found that addition of Netrin-1 blocked Unc5b-FL induced cell death (Figure 12). On the contrary, Unc5b-Δ8 overexpressing cells were largely irresponsive to Netrin-1 treatment (Figure 12).
To confirm these results, I generated stable inducible mouse 100VE ECs (extensively characterized by our collaborator Prof. E. Dejana, IFOM, Milan-Italy (Taddei et al., 2008)) that express Unc5b-FL or Unc5b-Δ8 under the control of the tetracycline-response element (TRE). To do this, 100VE were transduced with lentiviral vectors pSLICK-Hygro (Shin et al., 2006) in which I cloned Unc5b-FL or Unc5b-Δ8 GFP-tagged cDNA. Induction of transgene expression was achieved by treatment of ECs with doxycycline (Dox), a derivative of tetracycline that binds with high affinity to the rtTA (reverse tetracycline-controlled trans-activator) constitutively expressed by pSLICK-Hygro. Treatment with Dox allows rtTA to recognize the TRE element and promote cDNA expression. By using this system, I confirmed that induction of Unc5b-FL or Unc5b-Δ8 expression are both able to induce cell death (Figure 13). Importantly, compared to Unc5b-FL, Unc5b-Δ8 overexpressing ECs were irresponsive to the pro-survival signal induced by Netrin-1 treatment (Figure 13).

Figure 13 (next page). Unc5b-Δ8 irresponsiveness to Netrin-1 pro-survival signal in mouse ECs. (**A**) Schematic rapresentation of Unc5b Tet-on inducible system. pSLICK-Hygro constitutively express rtTA (yellow circular sector) under control of the Ubi-c promoter (Orange section). In the presence of Doxycycline (Dox; red circle), Dox allows rtTA to recognize the TRE element (green section) and promote *unc5b* cDNA expression of the indicated variant (blue section). For control vector GFP cDNA was inserted instead of *unc5b* cDNA (**B**) Cellular localization of GFP-tagged Unc5b-FL or Unc5b-Δ8 in mouse VE100 ECs induced with Doxycycline (1 ng/ml) for 24h. Scale bar: 10 μm. (**C**) Apoptosis was evaluated by western blotting with an antibody against the cleaved (activated) form of

Caspase-3 (a-Tubulin as loading control) in mouse VE100 ECs induced with Doxycycline (1 ng/ml) for 6 h or 24h. ECs were serum starved to induce cell stress and treated with Netrin-1 (NTN1; 150 ng/ml) or BSA (-) as control. (**D**) Cell death was evaluated in same ECs by using vital exclusion dye assay. Error bars indicate ± S.D.; ** P value < 0.01; *** P value < 0.001 two-tailed *t*- test.

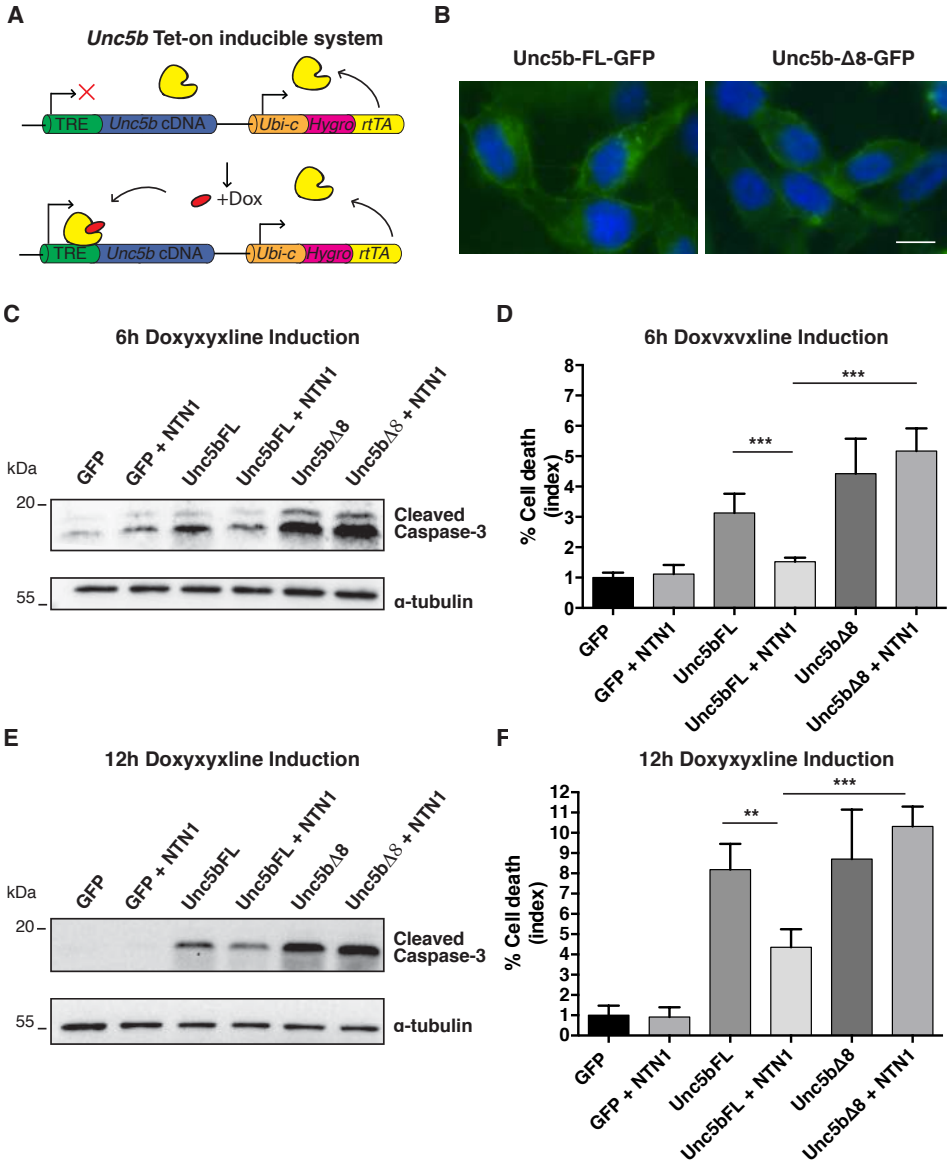

Figure 13. Unc5b-Δ8 irresponsiveness to Netrin-1 pro-survival signal in mouse ECs.

Similar results were obtained in primary human umbilical vein endothelial cells (HUVECs). HUVEC cells were transduced with adenoviral vectors expressing Unc5b-FL-GFP or Unc5b-Δ8-GFP cDNA. Apoptosis was monitored by a luminogenic caspase-3/7 substrate (Promega, see Methods for details), which contains the tetrapeptide sequence DEVD recognized by the activated cleaved caspase 3/7 as previously done (Castets et al., 2009; Llambli et al., 2005; Llambli et al., 2001). Also in HUVECs, I found that Unc5b-FL was able to induce cell death, which was inhibited after addition of Netrin-1 (Figure 14). Notably, compared to Unc5b-FL, Unc5b-Δ8 overexpressing ECs were irresponsive to Netrin-1 treatment (Figure 14).

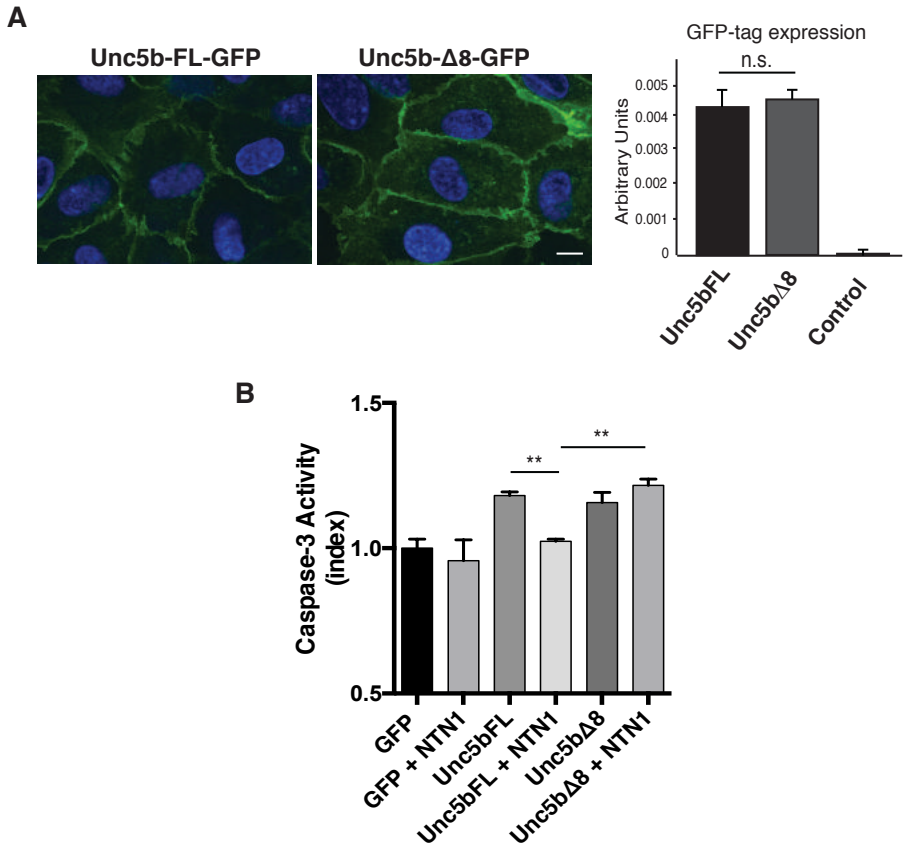

Figure 14. Unc5b-Δ8 irresponsiveness to Netrin-1 pro-survival signal in primary HUVECs. (**A**) HUVEC cells were transduced with adenoviral vectors expressing Unc5b-FL-GFP or Unc5b-Δ8-GFP cDNA. Both protein isoforms are able to reach the cell membrane. Scale bar: 10 µm. Ectopic expression of Unc5b-FL-GFP and Unc5b-Δ8-GFP

upon adenoviral infection was quantified by RT-qPCR with primers mapping to the GFP tag (histogram on the right). (**C**) Apoptosis was evaluated by Caspase-3 activation in HUVECs transduced with adenoviral vectors expressing Unc5b-FL-GFP or Unc5b-Δ8-GFP, with or without Netrin-1 treatment (NTN1; 150 ng/ml). Cells were serum starved to induce cell stress for 6h before evaluation of Caspase-3 activity. Error bars indicate ± S.D.; ** P value < 0.01; two-tailed *t*- test.

Since Nova2 knockdown ECs showed a decreased Unc5b-Δ8 expression whereas the opposite is observed in Nova2 overexpressing ECs (Figure 2), I determined ECs apoptosis levels in these conditions. As shown in Figure 15, I found that Nova2 knockdown led to reduce ECs apoptosis. On the contrary, Nova2 overexpression stimulated ECs apoptosis (Figure 15).

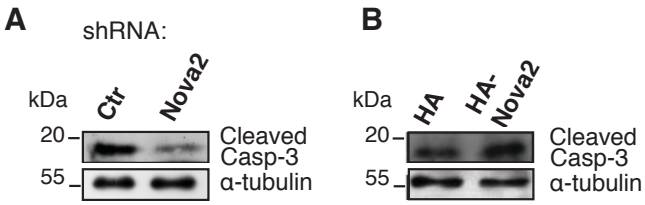

Figure 15. Unc5b-Δ8 expression correlates with increased apoptosis in ECs. (**A-B**) Apoptosis by western blotting with an antibody against the cleaved (activated) form of Caspase-3 in stable *Nova2* knockdown and HA-Nova2 overexpressing mouse ECs (α-tubulin as loading controls).

Collectively, these data strongly suggest that Nova2 regulates, at least in part, ECs survival through *Unc5b* AS. In particular, skipping of *Unc5b* exon 8 profoundly influences the activity of Unc5b protein to act as a dependence receptor. Based on my results, it is tempting to speculate that the absence of the IDR encoded by exon 8 alters the conformation of Unc5b cytoplasmic tail preventing downstream pro-survival signaling.

Other functions of Unc5b AS variants in ECs

Thanks to the mobility program of the University of Pavia, I had the opportunity to join the laboratory of our collaborator Prof. Anne Eichmann (Yale University, USA), a leader in studying common molecular cues that direct growth of blood vessels and nerves. In particular, during my visit in Eichmann's lab I investigated the functional role(s) of Unc5b splicing isoforms (GFP-tag) during different steps of *in vitro* angiogenesis.

It has been reported that, in addition to Netrin-1, Unc5b is able to bind other ligands. In particular, Robo4 acts as an *in trans* membrane-bound Unc5b ligand and Unc5b, on neighboring ECs, acts as a signaling receptor that maintains vessel integrity by counteracting VEGF signaling (Koch et al., 2011). To test if skipping of *Unc5b* exon 8 influences Robo4-Unc5b signaling, I used a recombinant version of Robo4, previously generated in Eichmann's Lab, in which the cytoplasmic tail of the receptor was replaced with the mCherry tag (Robo4ΔCD) (Zhang et al., 2016). To reproduce the Robo4-Unc5b signaling, I performed co-culture experiments with HUVECs over-expressing Unc5b GFP-tagged AS variants (Unc5b-FL and Unc5b-Δ8) and HUVECs over-expressing the mCherry-tagged Robo4ΔCD. As shown in Figure 16, I found no difference between Unc5b-FL and Unc5b-Δ8 over-expressing ECs in terms of repulsion, cell-cell contacts or receptor internalization when these ECs were co-cultured with ECs over-expressing the mCherry-tagged Robo4ΔCD (Figure 16).

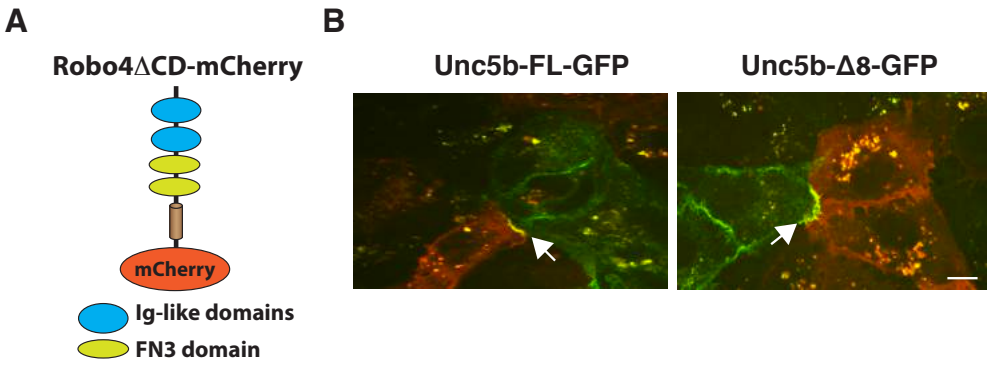

Figure 16. Interaction of Robo4 with Unc5b AS variants. (A) Protein structure of recombinant Robo4ΔCD protein in which the cytoplasmic tail of the receptor was replaced with the mCherry tag (Koch et al., 2011). Blue oval: Ig-like domain; Pale lime oval: FN3 domain; beige cylinder: transmembrane domain; orange oval: mCherry tag. **(B)** Unc5b-FL or Unc5b-Δ8 overexpressing HUVECs (green) were co-cultured with HUVECs overexpressing Robo4ΔCD (red). White arrows indicate similar localization at the cell membrane of the Unc5b AS isoforms. Scale bar: 10 μm.

In addition to apoptosis, Unc5b has been also linked to activation of cell migration (Hong et al., 1999; Tanikawa et al., 2003; Leung-Hagesteijn et al., 1992; Ly et al., 2005; Lv et al., 2015). As recently reported (Tu et al., 2015), I confirmed that *Unc5b* knockdown increased the migration properties of HUVEC cells upon Netrin-1 treatment as measured by wound healing closure (Figure 17). In addition, I also found that overexpression of Unc5b AS variants was able to abolish the increased migration of *Unc5b* knockdown ECs, with the Unc5b-Δ8 isoform slightly but significantly more active in the inhibition of migration (Figure 17).

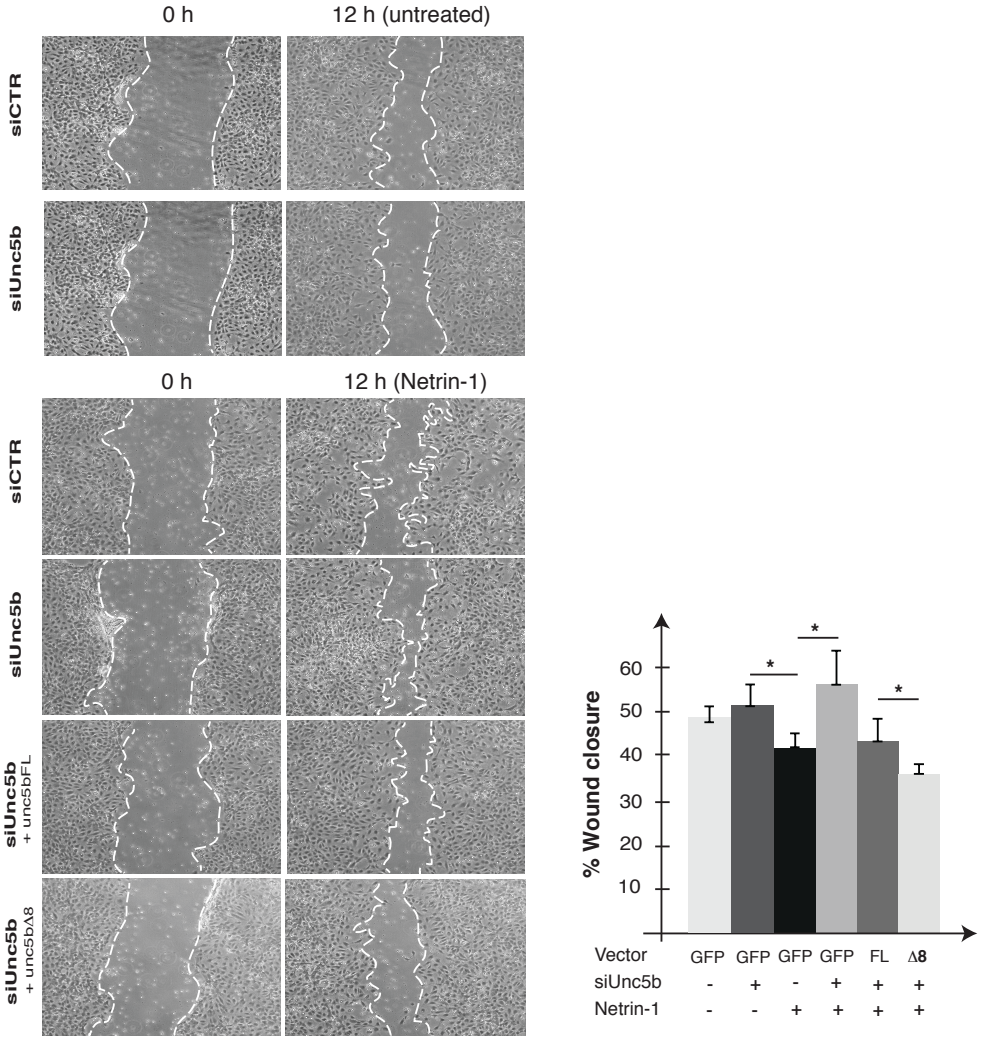

Figure 17. (Figure legend next page)

Figure 17. Unc5b AS variants and EC migration. Representative images after 12 h of wound healing assay in control HUVECs (siCTR), in HUVECs knockdown for *Unc5b* (siUnc5b) or HUVECs depleted of *Unc5b* and overexpressing the indicated Unc5 AS variants or GFP (as control). Quantification of wounds closure were calculated as the ratio between wounds size at 0h and after 12h. Netrin-1 was used at 500 ng/ml. Error bars indicate ± S.D.; * P value < 0.05; two-tailed *t*-test; n >= 3).

However, the observed reduced migration of ECs overexpressing Unc5b-Δ8 could be the consequence of an indirect effect of the increased cell death induced by Unc5b-Δ8 also in the presence of Netrin-1. Indeed, ECs migration upon VEGF treatment, which leads to increased EC migration (Olsson et al., 2006), is not affected by *Unc5b* knockdown or reconstituted expression of the two different Unc5b AS isoforms (Unc5b-FL and Unc5b-Δ8) (Figure 18).

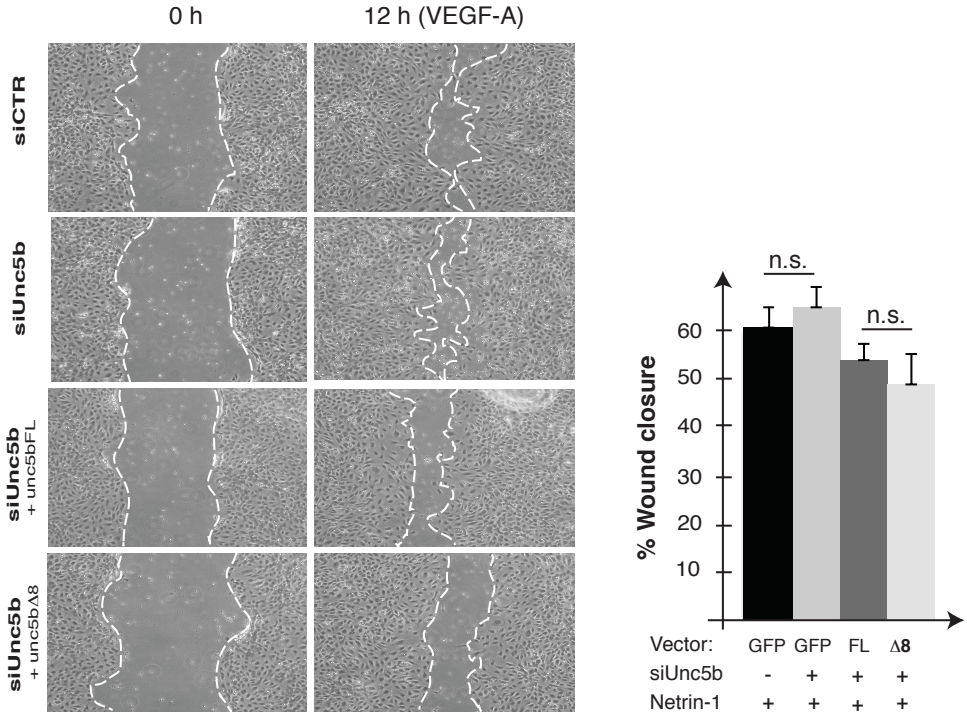

Figure 18. Unc5b AS variants do not affect VEGF-dependent EC migration. Representative images after 12 h of wound healing assay in control HUVECs (siCTR), in HUVECs knockdown for *Unc5b* (siUnc5b) or HUVECs depleted of *Unc5b* and overexpressing the indicated Unc5 AS variants or GFP (as a control). Quantification of wounds closure was calculated as the ratio between wounds size at 0 h and after 12 h. All conditions were treated with VEGF (6 nM). Error bars indicate ± S.D.; n.s.: not significative; two-tailed *t*-test; n >= 3).

Nova2-dependent regulation of dependence receptor signaling

In addition to Unc5b, other axon guidance molecules, such as Neogenin and DCC, act as dependence receptors (Saito et al., 2016). Notably, our RNA-seq identified exon 26 of the *Neogenin* as a novel Nova2 target in ECs. Interestingly, the same exon of *Neogenin* and exon 17 of *DCC* were previously described as Nova2 targets in CNS (Saito et al., 2016; Ule et al., 2003). While skipping of exon 17 in *DCC* pre-mRNA was found to influence the binding of DCC to Netrin-1 (Saito et al., 2016), by using Globplot I found that *Neogenin* exon 26 encodes for 54 amino acids in the cytoplasmic C-terminal tail that overlap with an IDR (Figure 19),
Differently from DCC, which was expressed at low levels in our mouse ECs (not shown), Neogenin was detectable and regulated through AS in ECs. In particular, I found that skipping of *Neogeni*n exon 26 was increased in ECs overexpressing Nova2, thus generating a shorter transcript that I called *Neogenin-Δ26* (Figure 19). On the contrary, *Neogenin-Δ26* was reduced in Nova2 knockdown ECs (Figure 19). Moreover, I have also validated the Nova2-dependent regulation of *Neogenin* exon 26 in zebrafish by using RNA extracted from nova2-morphant and *nova2* mutant embryos.
In analogy to Unc5b, these data suggested that Nova2-dependent skipping of *Neogenin* exon 26 could affect the flexibility of the cytoplasmic region of the receptor and probably the activation of downstream signaling (Lee et al., 2016; Wilson & Key, 2006).

Figure 19 (next page). AS of *Neogenin* exon 26. (**A**) The mouse *Neogenin* genomic region with the AS exon 26 (red) is indicated. Blue bars, Nova-silenced exon inclusion. YCAY motifs (putative Nova2 binding sites) identified with RBPmap tool are represented as vertical pale orange bars. Arrows indicate primers used for RT-PCR analysis. (**B**) AS analysis by RT-PCR of *Neogenin* exon 26 in mouse Nova2 knockdown ECs, in (**C**) ECs overexpressing HA-Nova2 and in (**D**) HUVECs knockdown for *Nova2*. (**E**) RT-PCR analysis with RNA extracted from zebrafish embryos (28 hpf) injected with a control morpholino oligo (ctr) or a morpholino against *nova2*. Altered AS of *neogenin* exon 26 in *nova2* morphants was partially corrected by the co-injection of *nova2* mRNA (MO-nova2+nova2) (**F**) AS of *neogenin* exon 26 in n*ova2* mutant zebrafish embryos showed AS changes similar to that observed in *nova2* morphants (dpf, days post fertilization). The percentage of exon inclusion is also shown. (**G**) Schematic representation of the Neogenin protein structure: Blue ovals: Ig-like domains; violet parallelepiped: FN domains (1-6); yellow dot: cytoplasmic P domains. Exon 26 (red) encodes 54 amino acids in the cytoplasmic tail of the receptor. (**H**) GlobPlot prediction of mouse Neogenin. Low-complexity (yellow), disordered (blue) and globular (green) regions are shown on the x-axis; Neogenin residues (1-1493) are also indicated. Neogenin protein domains as in G, exon 26 in red. White line, Russell-Linding propensity of disorder.

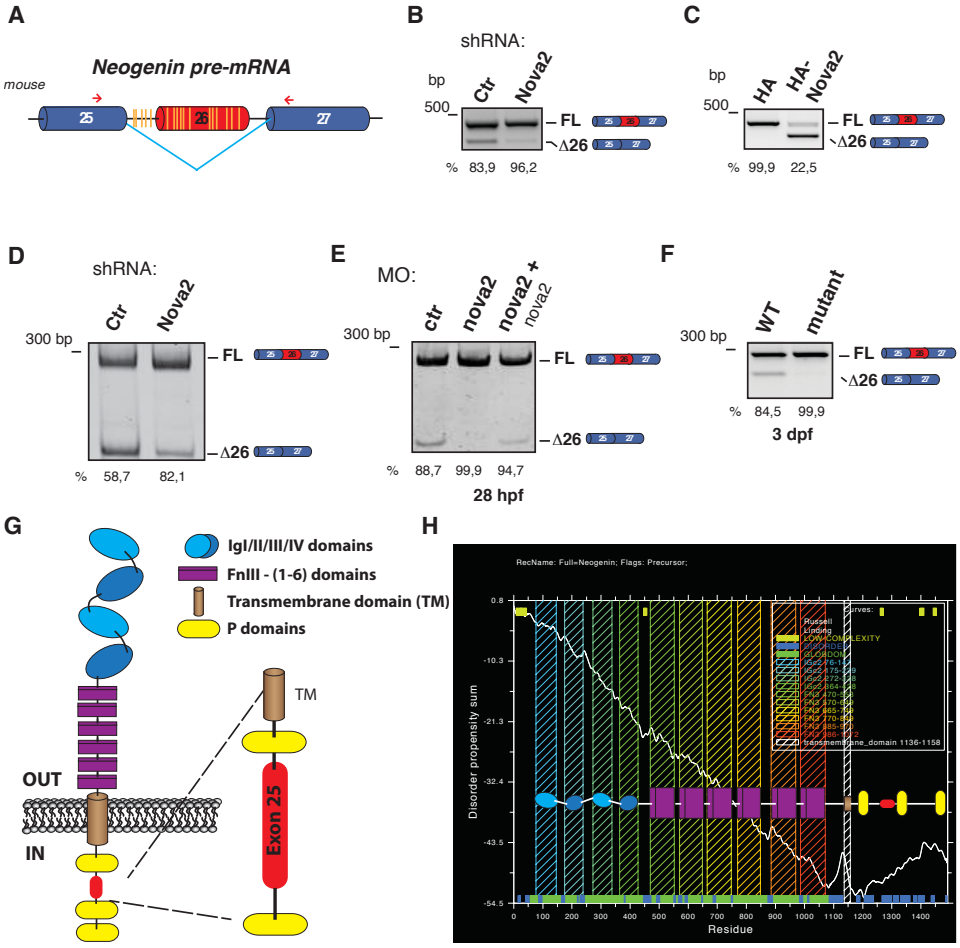

Figure 19. AS of *Neogenin* exon 26.

CHAPTER III
Definition of the contribution of *Unc5b* AS during vascular development *in vivo*

Nova2 expression and *Unc5b* splicing correlates during zebrafish development

In vertebrates, the establishment of a complete network of vessels is essential for embryonic survival (Weinstein, 1999). In line with this, alterations of this fine-tuned process cause, in the majority of the cases, lethality in the first stage of the development (Chàvez et al., 2016).
The formation and patterning of the vasculature could be easily monitored in zebrafish (*Danio rerio*) (Besis & Stainier, 2006). The primary vasculogenic and angiogenic vessels in mammals are also present in Zebrafish and the Zebrafish embryo constitutes a unique and powerful model to study vertebrate cardiovascular development and physiology. Due to its small size, oxygen freely moves through passive diffusion in all surrounding tissues allowing zebrafish embryos to survive and continue to develop normally also without a complete and functional vascular system (Isogai et al., 2001). In addition, zebrafish embryos and larvae provide a large series of additional advantages, including optical transparency, external development, high number of offspring and a large plethora of strategies for forward and reverse genetic manipulation (morpholino oligos, TALENs, CRISPR/Cas) (Bedell et al., 2012; Hwang et al., 2013; Varshney et al., 2015). Importantly, genetic studies have also revealed a strong conservation, between zebrafish, mouse and human, of the molecular pathways involved in vascular development (Chàvez et al., 2016).
The Nova2 RNA-binding domain is 94% identical between zebrafish and human orthologues (Jelen et al., 2007). Importantly, in the past we found that a zebrafish Nova2 orthologous gene (*nova2*) is expressed in the vasculature during development in addition to CNS. Furthermore, approximately 50% of Nova-regulated AS events and most of their YCAY clusters are conserved from mouse to zebrafish (Jelen et al., 2007). Accordingly, we found that Nova2 controls the development of the vascular system in zebrafish by modulating AS of genes involved in EC polarity and lumen formation (Giampietro et al., 2015).
The netrin receptor Unc5b, as well, is conserved among human, mouse and zebrafish (Chisholm & Tessier-Lavigne, 1999). Notably, zebrafish Unc5b orthologous (*unc5b*) shares 65% of amino acid identity to human protein (Kuar et al., 2007), with the highest percentage identity (71%) in the netrin-binding Ig domains (Yang et al., 2013). In the past our group has demonstrated that Nova2

expression increased in adult ECs as compared with embryo or fetal ECs (Giampietro et al., 2015). In accord with this observation, I found that zebrafish *nova2* expression increased from the maternal stage to 48 hours post fertilization (hpf) (Figure 20). Interestingly, RT-PCR analysis with RNA extracted from zebrafish embryos at different developmental stages demonstrated that skipping of *unc5b* exon 8 was also conserved in zebrafish and, more importantly, this AS event was correlated with *nova2* expression levels during zebrafish development (Figure 20).

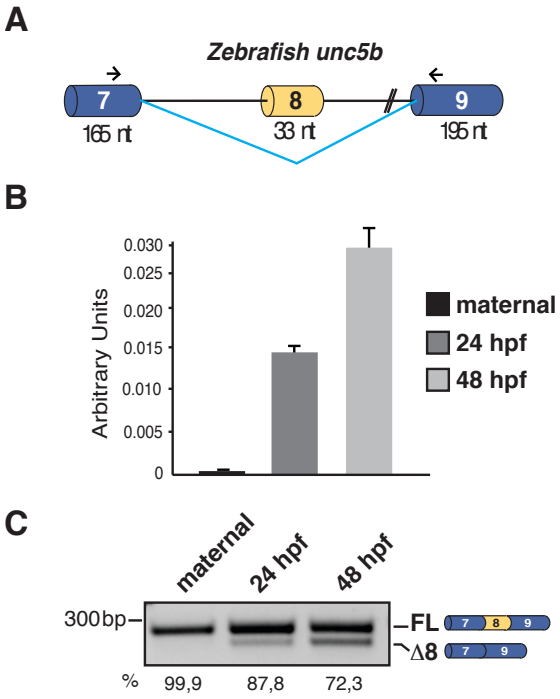

Figure 20. **Nova2 expression and *Unc5b* splicing during zebrafish development.** **(A)** Schematic representation of the *unc5b* zebrafish genomic region containing the AS exon 8 (AS exon 8 in yellow; constitutive exons 7 and 9 in blue). **(B)** *nova2* mRNA levels during zebrafish development stages: maternal, 24 hours post-fertilization (hpf) and 48 hpf. Error bars indicate ± S.D. **(C)** RT-PCR analysis of AS of *unc5b* during zebrafish development as in B. The percentage of exon inclusion is also shown.

Nova2 regulates AS of *unc5b* exon 8 in zebrafish embryos

By performing morpholino oligonucleotide mediated knockdown of *nova2* in zebrafish embryos, which express the EGFP under the control of the endothelial-specific promoter *fli1a* [Tg(fli1a:EGFP)y1] (Lawson & Weinstein, 2002), our group has previously found that Nova2 plays an important role in controlling the development of the vascular system *in vivo* (Giampietro et al., 2015). Notably, these results were independently validated by generating a genetic *nova2* mutant fish with the CRISPR/Cas9 technology (Giampietro et al., 2015).
By bioinformatics analysis, I found that Nova2 binding sites (YCAY clusters) upstream *Unc5b* exon 8 are conserved between human and zebrafish genes. This observation prompted me to investigate whether Nova2 plays also a role in regulating *unc5b* splicing in zebrafish. Interestingly, I found that morpholino-mediated knockdown of *nova2* altered the levels of *unc5b* exon 8 inclusion in the direction predicted by the position of the YCAY motifs (Figure 21).

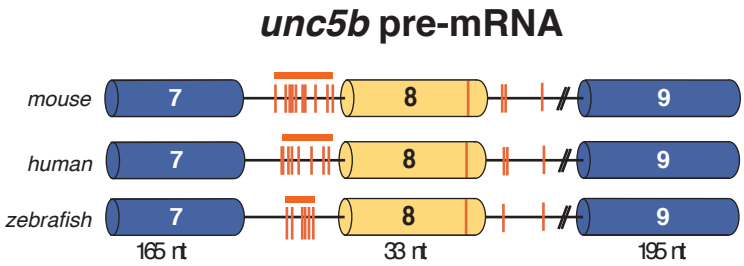

Figure 21. Nova2 binding sites in zebrafish *unc5b* pre-mRNA. The conserved YCAY cluster predicted to function as Nova2 splicing silencer element (horizontal orange rectangle) is shown for the human, mouse and zebrafish *unc5b* genes. Single YCAY sites are indicated as vertical orange.

Importantly, altered AS of *unc5b* in *nova2* morphants was partially rescued by co-injection of morpholino-resistant zebrafish *nova2* mRNA (Figure 22). To independently validate my findings, I used also RNA extracted from *nova2* mutant fish (Giampietro et al., 2015). This allowed me to confirm the conclusion that Nova2 – Unc5b circuitry is conserved in zebrafish embryos.

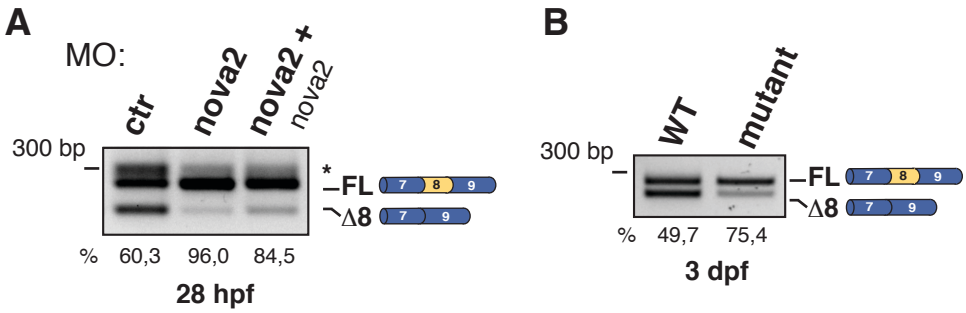

Figure 22. Nova2-dependent AS regulation of *unc5b* exon 8 in zebrafish embryos. (**A)** RT-PCR analysis with RNA extracted from zebrafish embryos (28 hpf) injected with a control morpholino (ctr) or a morpholino against *nova2*. Altered AS of *unc5b* exon 8 in *nova2* morphants was partially corrected by the co-injection of *nova2* mRNA (MO-nova2+nova2) (**B)** Compared to wild-type (WT) organism, n*ova2* mutants zebrafish showed *unc5b* AS changes comparable to that observed in *nova2* morphants (dpf, days post fertilization). Quantification of exon inclusion is also shown. Asterisk indicates a non-specific PCR product.

Vascular phenotype of zebrafish embryos depleted of *Unc5b*

Vascular-specific, as well as complete, *Unc5b* depletion in mice showed incomplete mid-gestational lethality (Lu et al., 2004; Navankasattusas et al., 2008). Homozygous *Unc5b* mutant (*unc5b* KO) mice embryos have a normal vascular plexus, which correctly remodels into arteries and veins (Lu et al., 2004). Nevertheless, *unc5b* KO mice embryos showed increased vessels branching of internal carotid artery, inter-somitic vessels and of vessels in the nervous system (Lu et al., 2004). However, the only vascular phenotype observe in *unc5b* vascular-depleted mice embryos was a reduction in the number of fetal-derived arterioles Navankasattusas et al., 2008), which could explain the lethal phenotype at early stages observed in both animal models.

Unc5b depletion in zebrafish has been shown to cause severe vascular abnormalities (Lu et al., 2004; Navankasattusas et al., 2008). Two different groups (the laboratories directed, respectively, by Prof. Eichmann and Prof. Li) have independently characterized the role of *unc5b* in zebrafish development by using morpholino-mediated knock-down (Lu et al., 2004; Navankasattusas et al., 2008). Formation and perfusion of the axial trunk vessels, comprising of the dorsal aorta (DA) and the posterior cardinal vein (PCV), appeared normal (Lu et al., 2004;

Navankasattusas et al., 2008). Similarly, sprouting of intersegmental vessels (ISV) from the DA initially occurred normally in *unc5b* morphants. However, at 48 hpf, ECs of ISV exhibited aberrant branching and filopodial extension. Notably, these defects depended by the dose of the injected morpholino (Lu et al., 2004). By carefully titrating the amount of injected morpholino, the group of Prof. Li confirmed increased filopodial extensions and aberrant vessel branching of ISVs only at high doses, thus hypothesizing potential off-target effects (Navankasattusas et al., 2008). Importantly, inhibition of parachordal vessel (PAV), formed by secondary sprouts emerging from the PCV (Isogai et al., 2003), was highly penetrant also at low doses of morpholinos. PAV defects in *unc5b* morphants were also confirmed by others groups (Wang et al., 2009; Epting et al., 2010), strongly suggesting that the lack of PAVs is specifically due to loss of *unc5b* function, rather than an off-target effect.

By using transgenic fish expressing the EGFP under the control of the endothelial-specific promoter *kdrl* [Tg(kdrl:EGFP)] (Cross et al., 2003) and the same morpholino oligo against *unc5b* used by our collaborator Prof. Eichmann, aided by the Zebrafish Facility of IFOM, I was able to reproduce PAV defects in *unc5b* morphants, as previously reported (Lu et al., 2004; Wilson et al., 2006) (Figure 23). Therefore, in order to establish the biological relevance of Nova2-regulated *unc5b* AS during zebrafish development, I have co-injected morpholino-resistant zebrafish *unc5b-FL* or *Unc5b-Δ8* mRNAs (fused to the mCherry tag) with morpholino against *unc5b*. By using the PAV formation as readout of unc5b function *in vivo*, I was able to demonstrate that co-injection of *unc5b-Δ8* mRNA in *unc5b* morphants was more efficient to restore vascular abnormalities, such as PAV formation, compared to *unc5b-FL* mRNA (Figure 23). These data are in line with the recent observation that an unc5b mutated protein deleted of the cytoplasmic ZU5 domain (a domain that maintains the receptor in a close *closed conformation* thus inhibiting apoptosis) was found able to increase apoptosis and was more potent than the wild-type receptor in restoring PAV formation of *unc5b* morphants (Wang et al., 2009).

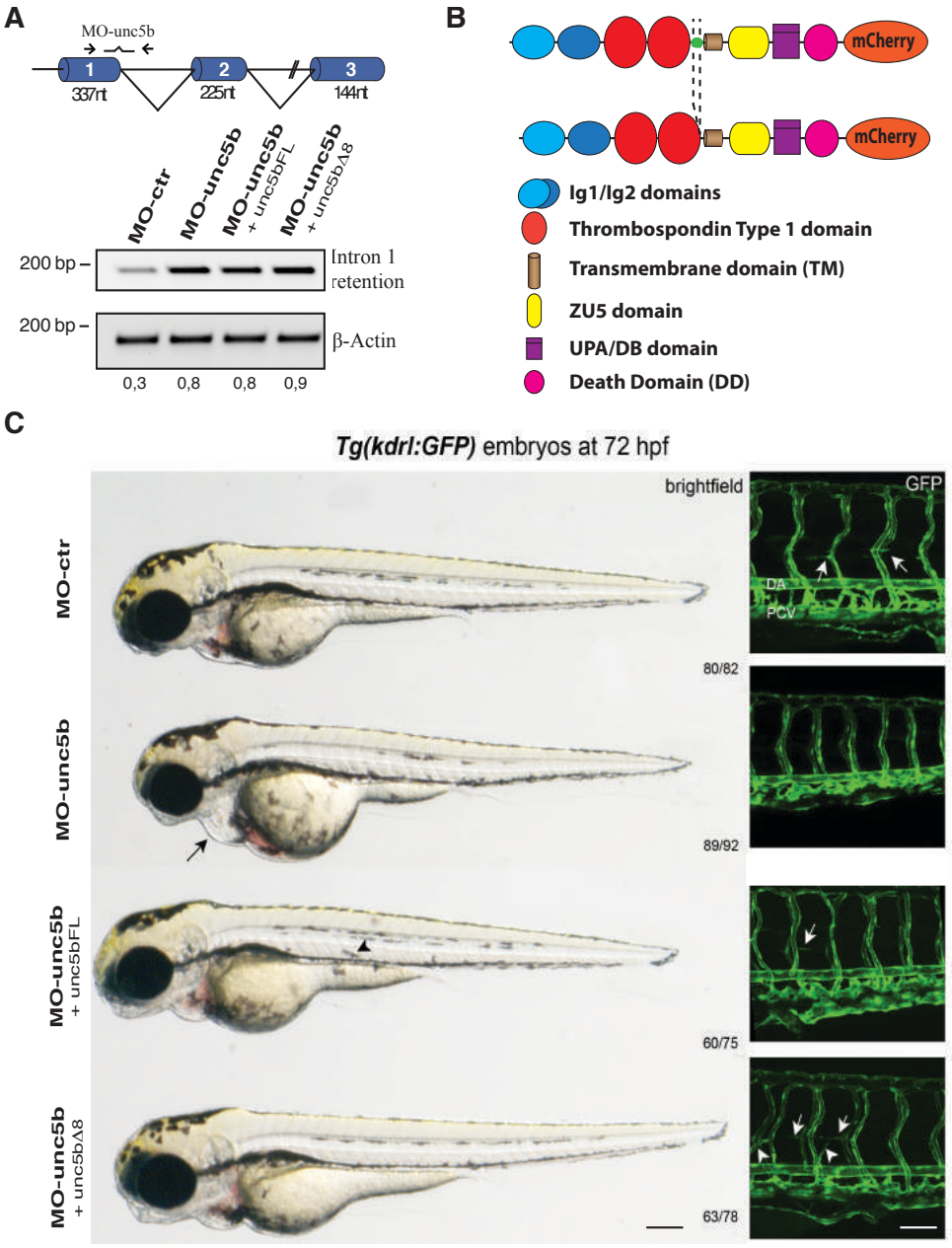

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Figure 23. Depletion of *unc5b* prevents PAV formation in zebrafish. (**A)** Left: schematic representation of the morpholino oligo targeting the Exon1/Intron1 junction of the *unc5b* pre-mRNA (MO-unc5b, splice blocking) used to knockdown *unc5b* expression. Black arrows indicate primers used to detect the event of intron 1 retention. Right: structure of zebrafish unc5b AS variants (FL and Δ8) fused to a C-terminal mCherry tag. (**B)** RT-PCR analysis of the *unc5b* splicing (retention of intron 1) in zebrafish injected with a control morpholino (ctr) or a morpholino against *unc5b* (MO-unc5b); *unc5b* morphants were also co-injected with mRNAs for Unc5b isoforms fused to mCherry tag. β-Actin as loading control. Quantification of intron retention standardized with β-Actin is also shown. (**C)** Lateral views (brightfield, left, and fluorescence, right) of 72 hpf *Tg(kdrl:EGFP)* zebrafish embryos expressing the EGFP under the control of the endothelial-specific promoter *kdrl* injected with a control morpholino (MO-ctr) or with MO-unc5b; *unc5b* morphants were also co-injected with the indicated mRNA encoding for Unc5b proteins. Black arrow = pericardial edema; black arrowhead = melanocyte patterning*;* white arrows = parachordal vessel (PAV) formation; white arrowheads mark vessels extra-branching in MO-*unc5b* + *Δ8-mCherry* co-injected embryos. Scale bar in brightfield 250μm, in fluorescence 50 μm.

Unc5b-Δ8 is required for PAV formation in zebrafish

Our group reported that *nova2* mutants and *nova2* morpholino-mediated knockdown zebrafish embryos displayed many vascular defects (Giampietro et al., 2015). Among them were reduced lumen size of the PCV, enlargement of lateral DA and cephalic vessels and extra-branching of the ISVs (Giampietro et al., 2015). In accord with the literature (Lu et al., 2004; Wilson et al., 2006; Navankasattusas et al., 2008), I confirmed that inhibited PAV formation occurred in *unc5b* morphants zebrafish embryos (Figure 23). Interestingly, in addition to vascular defects that we have described in our initial characterization of *nova2* mutant zebrafish embryos (Giampietro et al., 2015), I found that inhibited growth of the PAV was also observed in these mutants (Figure 24).

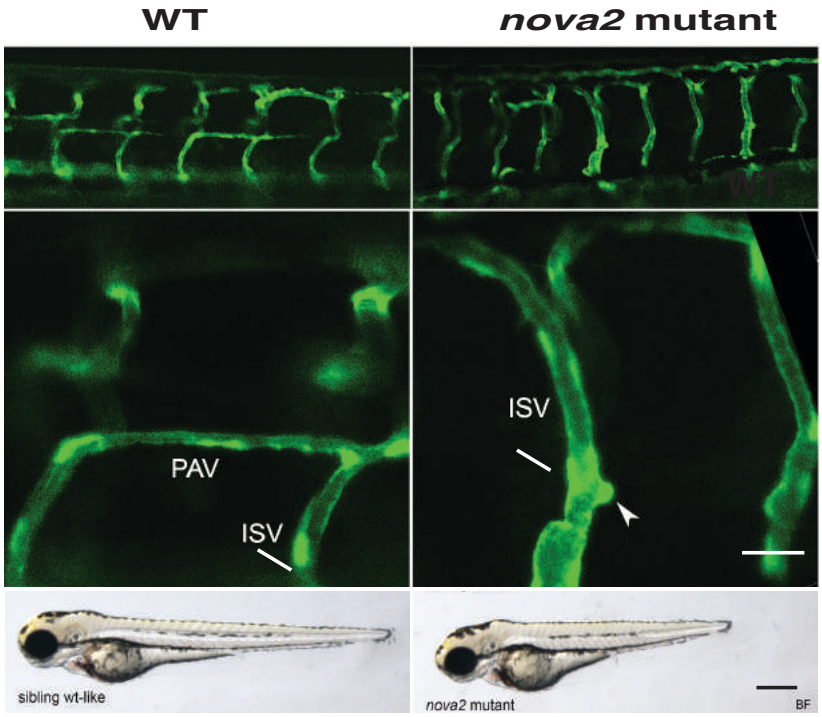

Figure 24. *nova2* mutants show defective PAV formation. **(A)** Lateral views (brightfield, bottom, and fluorescence, top) of trunk of 6 dpf WT and *nova2* mutants zebrafish expressing the EGFP under the control of the endothelial-specific promoter *fli1a*. *nova2* mutants showed inhibition of parachordal vessel (PAV). ISV, intersomitic vessel; white arrowhead indicates absence of PAV sprouting. Bar in brightfield 250μm, in fluorescence 25 μm.

Notably, injection of *nova2* mutants with morpholino-resistant zebrafish *Unc5b-Δ8* mRNA rescued, in 70% of the embryos, the formation of the PAV, whereas *Unc5b-FL* mRNA had no effect (Figure 25). Interestingly, other vascular defects reported in our *nova2* mutants, such as enlargement of the DA (Giampietro et al., 2015), were not rescued by *Unc5b-Δ8* mRNA injection.

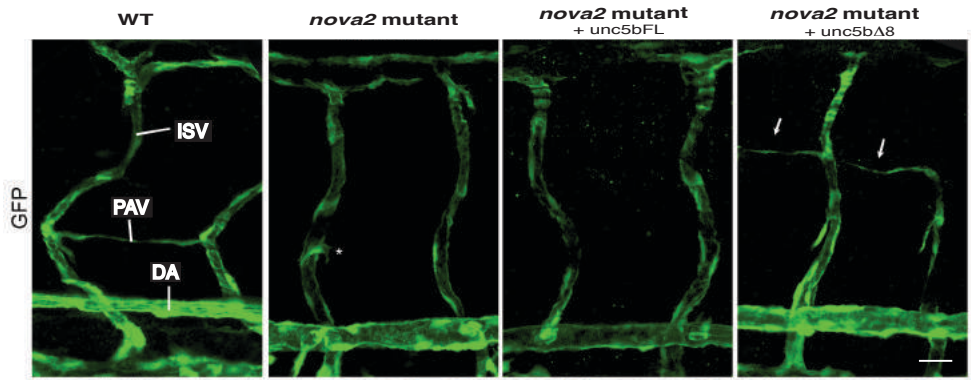

Figure 25. Unc5b-Δ8 is required for PAV formation. (**A)** Lateral views (fluorescence) of 72 hpf *Tg(fli1a:EGFP)y1*) WT and nova2 mutant zebrafish embryos (expressing the EGFP under the control of the endothelial-specific promoter *fli1a*) injected with morpholino-resistant zebrafish mRNAs encoding for Unc5b isoforms (Unc5b-Δ8 and Unc5b-FL) fused to the mCherry tag. Parachordal vessel (PAV), intersegmental vessels (ISV) and dorsal aorta (da) are also indicated. Asterisk indicates absence of PAV sprouting; white arrows: PAV. Scale bar, 25 μm.

Collectively, these data demonstrate that the Nova2-dependent production of Unc5b-Δ8 isoform could have a fundamental role in specific vascular beds for example during the formation of the PAV.

Unc5b splicing in tumors

An appropriate balance between cell death and survival is fundamental for blood vessel maintenance/development during cancer progression (Labi & Erlacher, 2015). Interestingly, very recent findings showed that activation of apoptosis contributes to angiogenesis as well as to chemotherapy resistance (Fianco et al., 2017). Unc5b expression is down-regulated in numerous human cancers Okazaki et al., 2012; Thiebault et al., 2003), whereas it is up-regulated during tumor angiogenesis (Larrivee et al 2007). In addition, consistent with its anti-apoptotic role, Netrin-1 is up-regulated in various types of tumors (Dumatin et al., 2010).
In order to investigate the role of *Unc5b* AS in the tumor vasculature, in collaboration with Dr. Cavallaro (IEO, Milan-Italy), we have performed immunohistochemistry (IHC) analysis of Nova2 expression in ovarian cancers

(OC). Interestingly, our preliminary results revealed that Nova2 was increased specifically in the ECs of the ovarian cancer vasculature compared to non-pathological ovaries (manuscript in preparation). Importantly, by using RT-PCR to examine the AS of *Unc5b* in tumor tissues, I was able to correlate, in a significant fraction of OC samples, splicing of *Unc5b* exon 8 with high levels of Nova2 in the vasculature (Figure 26).

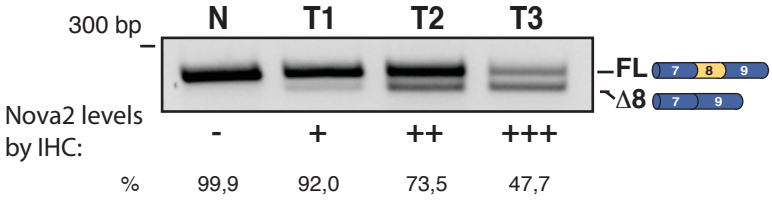

Figure 26. AS of *Unc5b* in in ovarian cancers. AS of *Unc5b* exon 8 in normal (N) and ovarian cancer samples (T1-3). Nova2 expression levels detected by IHC are also indicated (-; +; ++; +++). Bottom: percentage of exon 8 inclusion.

Discussion and conclusions

Alternative splicing (AS) is a post-transcriptional process that plays a pivotal role in the generation of proteome diversity in the highest eukaryotes (Kelemen et al., 2012). By regulating approximately 95% of human genes, AS represent the primary source to expand the human proteome encoded by the relative small number of genes (Pan et al., 2208; Wang et al., 2008).
Notably, increasing evidence of a causative role of aberrant AS regulation in cancers have been provided (Ghigna et al., 2008; Bonomi et al., 2013). In particular, the identification of cancer-specific AS variants has supported the notion that splicing fidelity is lost during cancer progression (Biamonti et al., 2014) and splicing factors can act as *bona fide* oncoproteins (Dvinge et al., 2016). Remarkable, the great plasticity offered by AS to expand tumor proteome will provide precious tools to develop novel diagnostic, prognostic and therapeutic approaches for human cancers.
The main interest of our group is to investigate the role of AS in cancer progression. Recently, we have started to study the relevance of AS during angiogenesis. Angiogenesis is the process by which new blood vessels are generate from pre-existing vasculature in order to supply all tissues of the body with oxygen and nutrients (Schuermann et al., 2014). Angiogenesis is fundamental for embryo development, but in adulthood most vessels remain quiescent and formation of new vessels occurs only in restricted circumstances (Demir et al., 2010). Notably, during tumor progression, angiogenesis allows oxygen and nutrients to reach proliferating cancer cells and provides tumor cells with the metastatic route to colonize distant organs (Potente et al., 2011). Although targeting angiogenesis is a promising anti-cancer therapy, all attempted strategies so far have shown modest therapeutic effects (Potente et al., 2011). This indicates that tumor angiogenesis is a more complex phenomenon than previously anticipated. Hence, a better understanding of the molecular mechanisms sustaining growth of tumor vessels will be crucial to identify novel and more specific anti-angiogenic therapies
For the first time, we demonstrated that the AS factor Nova2, previously considered brain-specific, is also expressed in the vascular endothelium and is important during vascular development (Giampietro et al., 2015). Due to the fact that Nova2 affects both neuronal and vascular processes, we proposed Nova2 as a novel member of the "angioneurins" family, but it is the only member that acts as post-transcriptional regulator.
Our work has highlighted the importance to use high-throughput approaches to identify novel Nova2 pre-mRNA targets in endothelial cells (ECs). Interestingly, by using RNA-seq (high-throughput sequencing of RNA) of Nova2 gain- and loss-of-function ECs, we discovered new Nova2 targets encoding for regulators of angiogenesis and/or vascular development. Among these, are axon guidance receptors recently linked to vascular development and morphogenesis, such as

Unc5b, *Neogenin*, *Slit2* and *Nrp1* (Adam & Eichmann, 2010).
Unc5b contains a cytoplasmic death domain able to induce apoptosis in the absence of its ligand Netrin-1, whereas ligand binding triggers survival signaling (Tanikawa et al., 2003). The observation that Netrin-1 acts as a survival factor for ECs, by blocking the pro-apoptotic effect of the "dependence receptor" Unc5b, reconciles the initial apparently contradictory conclusions that Netrin-1 is either a pro- or an antiangiogenic factor (Castets et al., 2009). Due to the importance of the Netrin-1 receptor Unc5b during angiogenesis and vascular patterning (Larivée et al., 2007), during my PhD I have characterized the Nova2-dependend AS regulation of *Unc5b* in ECs and the functional role of the novel Unc5b AS isoform during vascular development *in vivo*.
I demonstrated that *Unc5b* is a novel and direct Nova2 pre-mRNA target in ECs. In particular, Nova2 is able to bind to an evolutionary conserved YCAY cluster (canonical Nova2 binding site) located upstream of *Unc5b* exon 8, as determined by *in vivo* iCLIP experiment (Figure 7). By using RNA extracted from Nova2 gain- and loss-of-function ECs, I found that Nova2 promotes skipping of *Unc5b* exon 8, thus generating a novel AS variants that I called Unc5b-Δ8 (Figure 2). Interestingly, Nova2-mediated production of Unc5b-Δ8 is in line with the proposed mechanism of action of Nova2 (Ule et al., 2006), which is able to induce exon skipping when bound to intronic upstream YCAY clusters, whereas it induces exon inclusion by binding to exonic or downstream intronic YCAY motifs. In addition to these in vitro systems, I also confirmed Unc5b-Δ8 expression in vascular endothelium *in vivo* by using freshly purified ECs from mouse lung (Figure 4). Remarkable, the YCAY cluster, located upstream of *Unc5b* exon 8, is conserved between mouse and human genes (Figure 6) and, in line with this, I found that skipping of *Unc5b* exon 8 is regulated by Nova2 levels also in human ECs (such as HUVECs).
In cultured ECs, I was able to confirm that the full-length Unc5b isoform, generated by inclusion of exon 8 behaved as a dependence receptor since it induced cell death in the absence of its ligand, whereas it promoted survival in the presence of its ligand Netrin-1. Interestingly, I demonstrated that Unc5b-Δ8 was insensitive to the canonical pro-survival signals induced by Netrin-1 treatment (Figure 12, 13, 14). By using HeLa cells, which are easier to transfect compared to ECs, I found that Unc5b-Δ8 isoform was able to dimerize (o multimerize) in response to Netrin-1 treatment (Figure 12) as already demonstrated by Mille and colleagues for the full-length isoform (Mille et al., 2009). Thus, inhibited Unc5b-Δ8 dimerization is not the mechanism by which this protein activated apoptosis even in the presence of its ligands. However, my bioinformatic analyses indicated that *Unc5b* exon 8 could encode for a IDR (Figure 11) thus suggesting that the absence of this domain could alter the conformation of Unc5b-Δ8 cytoplasmic tail preventing downstream

pro-survival signaling. Moreover, I cannot rule out the possibility that, in the presence of Netrin-1, Unc5b-Δ8 is associated with other co-receptors expressed by ECs and that this interaction is useful to inhibit pro-survival signals. Thus, further studies are necessary to clarify the molecular mechanism by which Unc5b-Δ8 activates apoptosis in ECs

By performing morpholino (MO) mediated knockdown of *nova2* in zebrafish embryos expressing the EGFP under the control of the endothelial-specific promoter fli1a [Tg(fli1a:EGFP)y1], in the past my group found that Nova2 controls the development of the vascular system *in vivo* (Giampietro et al., 2015). Interestingly, I showed that AS of *unc5b* exon 8 is conserved in zebrafish and is co-regulated with Nova2 during development (Figure 20). Importantly, *nova2* knockdown alters exon inclusion levels in the direction predicted by the position of YCAY motifs (Figure 21). Also, altered AS of *unc5b* in nova2 morphants was rescued by coinjection with *nova2* mRNA (Figure 22). To independently support my findings, I used RNA extracted by *nova2* mutant fish generated with the CRISPRs technology (Giampietro et al., 2015). Notably, *nova2* mutants displayed AS changes of *unc5b* exon 8 comparable to those observed in *nova2* morphants (Figure 22). Interestingly, vascular defects, such as increased filopodial extensions, aberrant vessel branching of intersegmental vessels (ISVs) and inhibited growth of the parachordal vessel (PAV) reported in zebrafish embryos knockdown for *unc5b* (Lu et al., 2004; Navankasattusas et al., 2008), were consistently observed also in both *nova2* morphants and *nova2* mutants (Figure 24). In order to establish the biological relevance of Nova2-regulated *unc5b* AS *in vivo*, I have co-injected morpholino-resistant zebrafish *unc5b-FL* and *Unc5b-Δ8* mRNAs (fused to mCherry) with morpholino against *unc5b*. By using the PAV formation as readout of Unc5b function *in vivo*, I confirmed altered PAV formation in *unc5b* morphants, as reported (Lu et al., 2004; Navankasattusas et al., 2008) (Figure 23). Notably, co-injection of *unc5b-Δ8* mRNA in *unc5b* morphants was more efficient to rescue PAV formation compared to *unc5b-FL* mRNA (Figure 23). Notably, injection of *unc5b-Δ8* mRNA in *nova2* mutants is able to restore PAV formation, whereas *unc5b-FL* mRNA has no effect (Figure 25).

Growing lines of evidence describe the importance of cell death regulation in ECs since the balance between survival and apoptosis is critical to maintain blood vessel integrity during vascular development (Murakami et al., 2011; Giampietro et al., 2012; Korn & Augustin, 2015). In particular, Netrin-1 has been demonstrated to control survival of ECs and promote angiogenesis, at least in part by blocking Unc5b-induced cell death (Castets et al., 2009). In line with this, *Unc5b* knockdown reduces apoptosis in ECs by preventing the cleavage and activation of Caspase-3 (Castets et al., 2009). Nevertheless, ECs apoptosis is fundamental for vessel re-modelling and pruning (Pollman et al., 1999) and is required for proper

lumen formation in specific vascular beds (Fierlbeck et al., 2003). Moreover, the importance of apoptosis in ECs during angiogenesis is also highlighted by the fact that cell death occurs before capillary formation, but not in quiescent vessels (Segura et al., 2002). In my thesis, I have shown that Unc5b-Δ8 was insensitive to the Netrin-1 pro-survival signal *in vitro* and co-injection of *unc5b-Δ8* mRNA in *unc5b* morphants was more efficient to rescue PAV formation compared to *unc5b-FL* mRNA (Figure 12, 13, 14 and 23, 25). Interestingly, a similar result was obtained using Unc5b mutated protein deleted of different functional domain (Wang et al., 2009). In particular, Wang and colleagues (Wang et al., 2009), by comparing the ability of Unc5b mutated proteins to rescue PAV defects in zebrafish with the ability of these variants to induce apoptosis *in vitro*, found that the cytoplasmic deletion of ZU5 domain was able to increase apoptosis and was more potent than the wild-type receptor in restoring PAV formation of *unc5b* morphants. These results suggest the importance of EC apoptosis in the formation of specific vascular beds (such as the PAV) during vascular development. Remarkable, the deletion of ZU5 domain did not affect the Netrin-1 binding site, whereas impaired the stabilization of the *closed conformation* adopted by the cytoplasmic tail of Unc5b in response to Netrin-1. Similarly, the absence of an IDR encoded by exon 8 could prevent the functional stabilization of the *closed conformation* of the Unc5b-Δ8 receptor in favor of an *open conformation* able to recruit pro-apoptotic effectors, such as the Protein phosphatase 2 as previously reported for the full-length Unc5b isoform (Guenebeaud et al., 2010).

Accumulating evidence indicates that activation of apoptosis contributes to tumor angiogenesis as well as to chemotherapy resistance (Fianco et al., 2017). Importantly, the function of dependence receptors and their ligands is not limited to developmental programmed cell death but also implicated in cancer progression. In line with the observation that loss of apoptosis confers a selective advantage for tumor development, Unc5b receptor is downregulated in numerous human cancers (Okazaki et al., 2012; Thiebault et al., 2003), whereas Netrin-1 is upregulated in various kinds of tumors (Dumartin et al., 2010). In addition, Netrin-1 confers apoptosis resistance to tumor and ECs suggesting that it functions as an oncogene (Dumartin et al., 2010). The observation that Nova2 is up-regulated in ECs of the ovarian cancer vasculature and this increase was correlated with an upregulation of Unc5b-Δ8 suggests the intriguingly possibility the Nova2 – Unc5b circuitry, in addition to vascular development, could play important role also during tumor progression. Since few samples were analyzed, in collaboration with Dr. Cavallaro (IEO, Milan-Italy) I will extend my preliminary IHC analysis of Nova2 expression in ovarian cancer (versus normal counterpart) as well as I will evaluate by RT-PCR the AS profile of *Unc5b* in these samples.

Finally, it is important to note that, in addition to Unc5, other Netrin-1 dependence

receptors, such as Neogenin and DCC (Figure 19) (Saito et al., 2017), are regulated through AS process by Nova2. While in the case of *DCC* Nova2 promotes the production of a novel variant enable to bind Netrin-1 (Saito et al., 2016), I found that Nova2 generated a short Neogenin isoform (Neogenin-*Δ*26) deleted of a IDR in the cytoplasmic tail of the receptor. This AS event does not affect the interaction between Neogenin and Netrins (Xu et al., 2014). However, it is possible that alterations of the cytoplasmic conformation of the Neogenin-*Δ*26, as postulated for Unc5b-Δ8, could be sufficient to prevent downstream signaling even in the presence of its ligand. These preliminary observations tempt to speculate that Nova-mediated AS regulation of the dependence receptors family could play a relevant role in neovascularization processes in both physiological and pathological condition. Since I found that Nova2 knockdown led to reduce ECs apoptosis, while Nova2 overexpression stimulated ECs apoptosis (Figure 15), these phenotypic changes could be likely the integrated effects of AS modifications in several dependence receptors (Unc5b and Neogenin) that may act in a coordinated manner to regulate survival or pro-apoptotic signals. Collectively, it is tempted to speculate that Nova-mediated AS regulation of the dependence receptors could play a relevant role in neovascularization processes in both physiological and pathological conditions.

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Appendix

Table 1. *Unc5b* (MmuEX0050632) RNA-seq results for *Nova2* knockdown ECs (deposited as PRJNA293346).

Gene	Genomic coordinate	Length	PSI_Cont	PSI_KD	dPSI
Unc5b	chr10:60239933-60239965	33	60,86	97,03	36,17

Table 2. *Unc5b* (MmuEX0050632) RNA-seq results for Nova2 over-expressing ECs (unpublished).

Gene	Genomic coordinate	Length	PSI_Cont	PSI_Over	dPSI
Unc5b	chr10:60239933-60239965	33	93,29	72,84	-20,45

Table 3. RBPmap analysis of human *Unc5b* gene (NM_170744.4) encompassing exon 7, 8 and 9.

Genomic coordinate	Motif	Occurrence	Z-score	P-value
chr10:60777321	ycay	uagggccucugucuggcccccacgcucgcccuucaucucauc	3.380	3.62e-04
chr10:60777308	ycay	uggcccccacgcucgcccuucaucucauccaugccaugcccc	3.920	4.43e-05
chr10:60777303	ycay	cccacgcucgcccuucaucucauccaugccaugcccccauca	3.920	4.43e-05
chr10:60777299	ycay	cgcucgcccuucaucucauccaugccaugcccccaucaccuc	3.920	4.43e-05
chr10:60777294	ycay	gcccuucaucucauccaugccaugcccccaucaccuccucgg	3.920	4.43e-05
chr10:60777286	ycay	ucucauccaugccaugcccccaucaccuccucggccacccuu	3.920	4.43e-05
chr10:60777283	ycay	cauccaugccaugcccccaucaccuccucggccacccuuucu	3.920	4.43e-05
chr10:60777271	ycay	gcccccaucaccuccucggccaccuuucucugucaccugag	3.840	6.15e-05
chr10:60777257	ycay	cucggccacccuuucucugucaccugagucaucaucuuuccc	3.380	3.62e-04
chr10:60777248	ycay	ccuuucucugucaccugagucaucaucuuucccuuuauuucc	3.380	3.62e-04
chr10:60777245	ycay	uucucugucaccugagucaucaucuuucccuuuauuucccuu	2.750	2.98e-03
chr10:60777189	ycay	cucuaaacgacccuaaaagccaccguaagucccauuucaugc	2.750	2.98e-03
chr10:60777177	ycay	cuaaaagccaccguaagucccauuucaugccucucuucuuuc	2.750	2.98e-03
chr10:60777172	ycay	agccaccguaagucccauuucaugccucucuucuuucuucug	2.750	2.98e-03
chr10:60777045	ycay	ucuauaggcucccuguaccucaugcaugcugccacgcuucug	2.750	2.98e-03
chr10:60777033	ycay	cuguaccucaugcaugcugccacgcuucugucauagagaggc	2.750	2.98e-03
chr10:60777022	ycay	gcaugcugccacgcuucugucauagagaggcagugagacucu	2.750	2.98e-03
chr10:60775885	ycay	ccuggguggcuuuuaaaauucauauuucacuucauuaaaacu	2.750	2.98e-03
chr10:60775878	ycay	ggcuuuuaaaauucauauuucacuucauuaaaacuaaauuca	3.380	3.62e-04
chr10:60775873	ycay	uuaaaauucauauuucacucucauuaaaacuaaauucauuagg	3.380	3.62e-04
chr10:60775858	ycay	cacuucauuaaaacuaaauucauuaggaaacauagcucccca	2.750	2.98e-03
chr10:60775644	ycay	gcugugggcucccuggguuccaucuuucuuucccaucccucu	3.380	3.62e-04
chr10:60775631	ycay	uggguuccaucuuucuuuccccaucccucucaccaccucucca	3.840	6.15e-05
chr10:60775622	ycay	ucuuucuuucccaucccucucacaccucuccauccauugcu	3.840	6.15e-05
chr10:60775619	ycay	uucuuucccaucccucucaccaccucuccauccauugcuccg	3.840	6.15e-05
chr10:60775611	ycay	caucccucucaccaccucuccauccauugcuccgccucucac	3.920	4.43e-05
chr10:60775607	ycay	ccucucaccaccucuccauccauugcuccgccucucacccca	3.920	4.43e-05
chr10:60775592	ycay	ccauccauugcuccgccucucacccacgugcuucccucccu	3.690	1.12e-04
chr10:60775587	ycay	cauugcuccgccucucacccccacgugcuucccucccuccacu	3.690	1.12e-04
chr10:60775569	ycay	cccacgugcuucccucccucccacugcaccucccauccaaugc	3.380	3.62e-04
chr10:60775010	ycay	accgacuccucugcggcccucacugguggcuuccacccuguc	2.750	2.98e-03

Table 3. RBPmap analysis of mouse *Unc5b* gene (NM_029770.2) encompassing exon 7, 8 and 9.

Genomic coordinate	Motif	Occurrence	Z-score	P-value
chr10:71288858	ycay	ccucagggccucugucccc**ccac**cacauccaucaucucaucu	3.840	6.15e-05
chr10:71288861	ycay	cagggccucugucccccca**ccac**auccaucaucucaucucau	3.840	6.15e-05
chr10:71288867	ycay	cucugucccccaccacau**ccau**caucucaucucauccuucc	3.920	4.43e-05
chr10:71288870	ycay	ugucccccaccacaucca**ucau**cucaucucauccuucccau	3.920	4.43e-05
chr10:71288875	ycay	ccccaccacauccaucauc**ucau**cucauccuucccauugccu	3.920	4.43e-05
chr10:71288880	ycay	ccacauccaucaucucauc**ucau**ccuucccauugccuucucu	3.960	3.75e-05
chr10:71288889	ycay	ucaucucaucucauccuuc**ccau**ugccuucucugccacccuu	3.840	6.15e-05
chr10:71288904	ycay	cuucccauugccuucucug**ccac**ccuuucccugucaccuaug	3.690	1.12e-04
chr10:71288918	ycay	cucugccacccuuucccug**ucac**cuaugucauugucuuuccc	2.750	2.98e-03
chr10:71288927	ycay	ccuuucccugucaccuaug**ucau**ugucuuucccuuuauuucc	2.750	2.98e-03
chr10:71288986	ycay	cucuaagcgaccccaacag**ccac**cguaagucccauuucaugg	2.750	2.98e-03
chr10:71288998	ycay	ccaacagccaccguaaguc**ccau**uucauggcuguccucuuuc	2.750	2.98e-03
chr10:71289003	ycay	agccaccguaagucccauu**ucau**ggcuguccucuuuccucug	2.750	2.98e-03
chr10:71289100	ycay	gagagcugaaggugccuac**ccac**cccccacgggaucaucuac	2.750	2.98e-03
chr10:71289107	ycay	gaaggugccuacccacccc**ccac**gggaucaucuacaccugac	2.750	2.98e-03
chr10:71289115	ycay	cuacccacccccacggga**ucau**cuacaccugacacugcaug	2.750	2.98e-03
chr10:71289384	ycay	cugggccaaggccgggaca**ucac**cacuccaguccccccucau	2.750	2.98e-03
chr10:71289387	ycay	ggccaaggccgggacauca**ccac**uccaguccccccucaugcc	2.750	2.98e-03
chr10:71289403	ycay	ucaccacuccaguccccccc**ucau**gccccuugcuguaccucuc	2.750	2.98e-03
chr10:71289522	ycay	uccagccccagaggcugcg**ccac**cacugcaaucauaucagag	3.380	3.62e-04
chr10:71289525	ycay	agccccagaggcugcgcca**ccac**ugcaaucauaucagagcag	2.750	2.98e-03
chr10:71289534	ycay	ggcugcgccaccacugcaa**ucau**aucagagcagcaaagccac	3.380	3.62e-04
chr10:71289553	ycay	ucauaucagagcagcaaag**ccac**uugagcagguuggcccaug	2.750	2.98e-03
chr10:71289571	ycay	gccacuugagcagguuggc**ccau**gguaauuaaaccacaauuu	2.750	2.98e-03
chr10:71289585	ycay	uuggcccaugguaauuaaa**ccac**aauuuccaguuaguucugg	2.750	2.98e-03
chr10:71290216	ycay	gcagccauaggauuuucua**ccac**uguagaaagcuccaccaga	2.750	2.98e-03
chr10:71290338	ycay	guggguugccucccugggc**ccau**ccuucuucccacaugcuca	2.750	2.98e-03
chr10:71290350	ycay	ccugggcccauccuucuuc**ccac**augcucaccucuucuccac	3.380	3.62e-04
chr10:71290358	ycay	cauccuucuucccacaugc**ucac**cucuucuccaccgcauugu	3.380	3.62e-04
chr10:71290369	ycay	ccacaugcucaccucuucu**ccac**cgcauuguugugcaucuca	3.690	1.12e-04
chr10:71290389	ycay	caccgcauuguugugcauc**ucau**uccacuucuucagcucccc	2.750	2.98e-03
chr10:71290394	ycay	cauuguugugcaucucauu**ccac**uucuucagcucccguccc	3.380	3.62e-04
chr10:71290418	ycay	ucuucagcucccgucccu**ccac**uucccacccucagagcaug	2.750	2.98e-03

Primer list.

All primer sequences are indicated in 5'-to-3' direction.

Table 4. Primers for RT-PCR.

Primer name	Sequence (5'->3')
Mouse-Unc5b-for	CCAAGAACTGCACTGATGGG
Mouse-Unc5b-rev	GAAGTTGACAGGGTGGAAGC
Human-Unc5b-for	GACGGAGTGGAGCAAGTGGT
Human-Unc5b-rev	ACGAAGATGGCCACCACGAG
Zebrafish-Unc5b-for	TCGTGAATGTCAGGCTCCAC
Zebrafish-Unc5b-rev	CGGCGATAGACCAGAATCCC
Mouse-Neo1-for	GATGCCCTTTGACTCTCAGC
Mouse-Neo1-rev	CTTGGCAGTGCAGGATCATA
Zebrafish-Neo1-for	TGATGCCCTTTGATGCACAAC
Zebrafish-Neo1-rev	GAGCTGTGAAAGCTAATGTCCGTC
BGH-rev	CTAGAAGGCACAGTCGAGGCTGATCACGG

Table 5. Primers for RT-qPCR.

Primer name	Sequence (5'->3')
Unc5b_i7-for	AGCTGTAGGGCCTCTGTCTG
Unc5b_i7-rev	GTGACAGAGAAAGGGTGGCC
Unc5b_i8-for	GTGAGTGTGGAGCTGAGCTT
Unc5b_i8-rev	AAGCCAGGAGGTGCTAGAGA
Rplp0-for	ATGCCCAGGGAAGACAGGGCG
Rplp0-rev	CGAAGGGACATGCGGATCTGCTGC
GFP-for	ATGGCCGACAAGCAGAAGAA
GFP-rev	CTCAGGTAGTGGTTGTCGGG

Table 6. Primers for cloning.

Primer name	**Sequence (5'->3')**
Unc5b-For-HindIII	CCCAAGCTTACCATGAGGGCCCGGAGCGGGGTGC
Unc5b-rev-EcoRI- HA	CCGGAATTCTCAAGCGTAATCTGGAACATCGTATGGGTACATTCCGCAATCGCCATCTGTGGCCATG
Unc5b-rev-EcoRI-GFP	CCGGAATTCGTCCGCAATCGCCATCTGTGGCCATG
Unc5b-deletion_ex8-for	TGATGGGCTGTGCGTGCTGACCCTGGAGACATCGGGAGAT
Unc5b-deletion_ex8-rev	ATCTCCCGATGTCTCCAGGGTCAGCACGCACAGCCCATCA
Unc5b_Zebra-for	GCCGGATCCGGTACCACGCGTGACCGGGTGAAGATGCT
Unc5b_Zebra-rev	TTGGGATCCTCCAGTAAAACC

Table 7. *unc5b* morpholino antisense oligonucleotide.

Morpholino name	**Sequence (5'->3')**
unc5b-morpholino	CATTTAACCGGCTCGTACCTGCATG

Table 8. RBPmap analysis of mouse *Neogenin* gene (NM_008684.2) encompassing exon 25, 26 and 27.

Genomic coordinate	Motif	Occurrence	Z-score	P-value
chr9:58887581	ycay	gcaugggguuuaaaacauu**ucac**cauugacccaugcuccauu	3.380	3.62e-04
chr9:58887578	ycay	ugggguuuaaaacauuuca**ccau**ugacccaugcuccauuuug	3.380	3.62e-04
chr9:58887570	ycay	aaaacauuucaccauugac**ccau**gcuccauuuugucaagcau	3.380	3.62e-04
chr9:58887563	ycay	uucaccauugacccaugcu**ccau**uuugucaagcauauacguu	3.380	3.62e-04
chr9:58887418	ycay	gaagacccaaguucaguuc**ccac**cacucauuucagggagcuc	3.380	3.62e-04
chr9:58887415	ycay	gacccaaguucaguuccca**ccac**ucauuucagggagcucaca	3.380	3.62e-04
chr9:58887411	ycay	caaguucaguucccaccac**ucau**uucagggagcucacaggca	3.380	3.62e-04
chr9:58887397	ycay	accacucauuucagggagc**ucac**aggcaccuguaauuccaac	3.380	3.62e-04
chr9:58887338	ycay	ccucuggccucuaugaaca**ucac**acucauaugcacaaacaua	2.750	2.98e-03
chr9:58886889	ycay	gcaugcacaugcgucuucc**ucac**ucacacacgccucacagug	2.750	2.98e-03
chr9:58886885	ycay	gcacaugcgucuuccucac**ucac**acacgccucacagugcggu	2.750	2.98e-03
chr9:58886874	ycay	uuccucacucacacacgcc**ucac**agugcggucagggaagaua	2.750	2.98e-03
chr9:58885969	ycay	agaucagauccagcuaacu**ucac**ucuacaccaaaacauucac	2.750	2.98e-03
chr9:58885876	ycay	agcccauucugguuuucuc**ucac**cccagaaugcuuucaguuu	2.750	2.98e-03
chr9:58885334	ycay	aagcuucauuaaagaggcu**ucau**aggaaguuauaugugggau	2.750	2.98e-03
chr9:58884877	ycay	aagaaaaagacucccuuaa**ucau**gacugccauuccccaaaau	2.750	2.98e-03
chr9:58884868	ycay	acucccuuaaucaugacug**ccau**uccccaaaauuaccacugu	2.750	2.98e-03
chr9:58884852	ycay	cugccauuccccaaaauua**ccac**uguugauggaauauauacc	2.750	2.98e-03
chr9:58884638	ycay	gcaucucucugucuuguguu**ucau**ccacagcugugauuagugc	2.750	2.98e-03
chr9:58884634	ycay	cucucugucuuguguucau**ccac**agcugugauuagugcccau	3.380	3.62e-04
chr9:58884615	ycay	ccacagcugugauuagugc**ccau**cccauccauuccccucgaua	3.690	1.12e-04
chr9:58884610	ycay	gcugugauuagugcccauc**ccau**ccauuccccucgauaacccu	3.840	6.15e-05
chr9:58884606	ycay	ugauuagugcccaucccau**ccau**ucccucgauaacccucacc	3.840	6.15e-05
chr9:58884588	ycay	uccauucccucgauaaccc**ucac**caucauuuccacuccagca	3.840	6.15e-05
chr9:58884585	ycay	auucccucgauaacccuca**ccau**cauuuccacuccagcagcc	3.840	6.15e-05
chr9:58884582	ycay	cccucgauaacccucacca**ucau**uuccacuccagcagccucg	3.690	1.12e-04
chr9:58884576	ycay	auaacccucaccaucauuu**ccac**uccagcagccucgcuucuc	3.380	3.62e-04
chr9:58884543	ycay	ucgcuucuccagcccgcag**ucau**cucuaccacccaagcagcc	2.750	2.98e-03
chr9:58884534	ycay	cagcccgcagucaucucua**ccac**ccaagcagcccauggccca	3.380	3.62e-04
chr9:58884521	ycay	ucucuaccacccaagcagc**ccau**ggcccauuggcacauccau	3.690	1.12e-04

Genomic coordinate	Motif	Occurrence	Z-score	P-value
chr9:58884514	ycay	cacccaagcagcccauggc**ccau**uggcacauccaugucccuu	3.380	3.62e-04
chr9:58884502	ycay	ccauggcccauuggcacau**ccau**gucccuuucagacagggcc	2.750	2.98e-03
chr9:58883926	ycay	uugccagacaaacaugucc**ucau**uuggaaaucauggcuucau	3.380	3.62e-04
chr9:58883915	ycay	acauguccucauuuggaaa**ucau**ggcuucaucauacaguuuu	3.380	3.62e-04
chr9:58883907	ycay	ucauuuggaaaucauggcu**ucau**cauacaguuuuaaggagaa	3.380	3.62e-04
chr9:58883904	ycay	uuuggaaaucauggcuuca**ucau**acaguuuuaaggagaaaag	3.380	3.62e-04
chr9:58883322	ycay	uuguguccccgccacacuua**ccau**guugaagacuaagggaucc	2.750	2.98e-03
chr9:58883301	ycay	auguugaagacuaagggau**ccau**cccacacaauccucacaua	3.380	3.62e-04
chr9:58883296	ycay	gaagacuaagggauccauc**ccac**acaauccucacauaagguc	2.750	2.98e-03
chr9:58883285	ycay	gauccaucccacacaaucc**ucac**auaaggucgugugugagug	2.750	2.98e-03
chr9:58881350	ycay	acuaugugaggcuuuauug**ucau**gaggucaaagccauucaca	2.750	2.98e-03
chr9:58881336	ycay	uauugucaugaggucaaag**ccau**ucacauuggugauggccau	3.380	3.62e-04
chr9:58881332	ycay	gucaugaggucaaagccau**ucac**auuggugauggccaucuag	3.380	3.62e-04
chr9:58881317	ycay	ccauucacauuggugaugg**ccau**cuaguaccugagcauuggc	3.380	3.62e-04
chr9:58880711	ycay	gacucaggccagagucuuc**ccac**agcccauguccgcccuucc	2.750	2.98e-03
chr9:58880704	ycay	gccagagucuucccacagc**ccau**guccgcccuucccacccuc	2.750	2.98e-03
chr9:58880689	ycay	cagcccauguccgcccuuc**ccac**ccucugaagagcuucgcug	2.750	2.98e-03

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Review Article

SAM68: Signal Transduction and RNA Metabolism in Human Cancer

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Alterations in expression and/or activity of splicing factors as well as mutations in *cis*-acting splicing regulatory sequences contribute to cancer phenotypes. Genome-wide studies have revealed more than 15,000 tumor-associated splice variants derived from genes involved in almost every aspect of cancer cell biology, including proliferation, differentiation, cell cycle control, metabolism, apoptosis, motility, invasion, and angiogenesis. In the past decades, several RNA binding proteins (RBPs) have been implicated in tumorigenesis. SAM68 (SRC associated in mitosis of 68 kDa) belongs to the STAR (signal transduction and activation of RNA metabolism) family of RBPs. SAM68 is involved in several steps of mRNA metabolism, from transcription to alternative splicing and then to nuclear export. Moreover, SAM68 participates in signaling pathways associated with cell response to stimuli, cell cycle transitions, and viral infections. Recent evidence has linked this RBP to the onset and progression of different tumors, highlighting misregulation of SAM68-regulated splicing events as a key step in neoplastic transformation and tumor progression. Here we review recent studies on the role of SAM68 in splicing regulation and we discuss its contribution to aberrant pre-mRNA processing in cancer.

1. Introduction

SAM68 (SRC associated in mitosis of 68 kDa) was originally identified as a protein physically associated with and phosphorylated by the tyrosine kinase c-SRC during mitosis [1, 2], opening the interesting possibility of a signaling circuitry driven by c-SRC and affecting RNA processing and trafficking in a cell-cycle-dependent manner.

SAM68 belongs to the STAR (signal transduction and activation of RNA metabolism) family of RNA binding proteins (RBPs) that link signaling pathways to RNA processing [3, 4]. STAR proteins include *Artemia salina* GRP33 [5], *C. elegans* GLD-1 [6], mammalian QKI [7], SAM68 [8, 9], SLM-1 and SLM-2 [10, 11], *Drosophila* HOW [12], KEP1 and Sam50 [13], and the evolutionary conserved splicing factor SF1 [14]. All STAR proteins, from worms to mammals, share common architecture (Figure 1). They contain a GRP33/SAM68/GLD-1 (GSG) domain for RNA binding and homodimerization, flanked by regulatory regions harboring motifs for protein-protein interactions (Figure 1), often mediated by conserved amino acid residues targeted by posttranslational modifications [15]. SAM68 contains six proline-rich sequences and a tyrosine-rich region at the C-terminus, which form docking sites for signaling proteins containing SRC homology 3 (SH3) and 2 (SH2) domains (Figure 1) [1, 2, 9, 16]. Notably, tyrosine phosphorylation by SRC-related kinases impairs SAM68 homodimerization [17] as well as its affinity for RNA both *in vitro* [16, 18] and *in vivo* [19]. Additional posttranslational modifications were also reported to affect the functions of this RBP. SAM68 binds to and is methylated by the arginine methyltransferase PRMT1 [20], thus affecting SAM68 interaction with SH3 domains [21] and its nuclear localization [20]. SAM68 acetylation, described in tumorigenic breast cancer cell lines [22], by

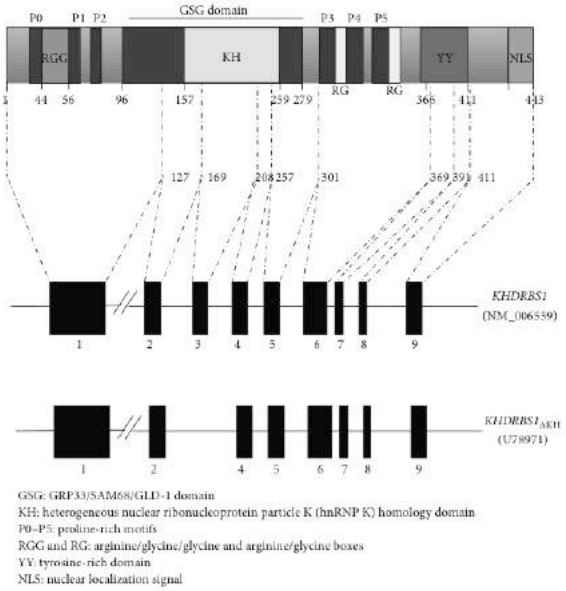

FIGURE 1: Schematic representation of SAM68 domains. In the upper part, schematic model representing the structural/functional domains of SAM68 protein as a prototype of a STAR protein. SAM68 protein is composed of the GRP33/SAM68/GLD-1 (GSG) domain, formed by a single heterogeneous nuclear ribonucleoprotein particle K (hnRNP K) homology domain (KH) embedded in two flanking regions, six consensus proline-rich motifs (P0–P5), arginine/glycine/glycine (RGG) and arginine/glycine (RG) boxes, C-terminal tyrosine-rich domain (YY), and a nuclear localization signal (NLS). In the lower part, the two protein coding mRNA isoforms of human *KHDRBS1* are represented. Black boxes indicate exons (numbered from 1 to 9). The sizes of exons and the protein domains encoded by each exon are indicated.

the acetyltransferase CBP increases SAM68 binding to RNA *in vitro*. Furthermore, SAM68 can be SUMOylated by the SUMO E3 ligase PIAS1, which enhances its transcriptional repression activity [23]. Thus, posttranslational modifications greatly influence the biochemical properties of SAM68 and finely tune its subcellular localization, interaction with signaling proteins, and RNA binding affinity.

Despite the growing interest in STAR proteins, their physiological role has not been completely elucidated yet. Nevertheless, recent mouse models of genetic ablation of STAR proteins are now greatly helping in pursuing this goal. In this review, we discuss the functional properties of SAM68 in signaling and RNA metabolism, with particular emphasis on malignant transformation. In particular, we highlight recent advances and new insights into SAM68-based signaling that have been made in the last two decades, which expand our understanding of STAR-mediated signaling in cancer cells.

2. SAM68 Biological Role(s): Lessons from Mouse Models

The first indication of the involvement of STAR proteins in tumorigenesis came from studies in *C. elegans*. Critical missense mutations in the *gld-1* gene caused germ-line tumors, thus suggesting an important role for *GLD-1* as a tumor suppressor [24]. These null mutations in hermaphrodites caused female germ cells to exit from the meiotic prophase and to start proliferating, thus leading to the formation of a germ-line tumor [3]. In this regard, it is important to notice that the function and localization of GLD-1 appear quite different from the SAM68 subfamily of STAR proteins. Indeed, GLD-1 is localized exclusively in the cytoplasm of germ cells and it does not contain the protein domains flanking the GSG of SAM68, which are involved in cell signaling [3]. Nevertheless, an initial observation seemed to suggest a similar tumor suppressor role also for SAM68.

A random homozygous knockout (RHKO) screen in NIH3T3 murine fibroblasts indicated that functional inactivation of the *Sam68* gene induces tumorigenesis and allows NIH3T3 cells to form metastatic tumors in nude mice [25]. These studies suggested that SAM68 negatively affects neoplastic transformation, like its *C. elegans* ortholog *GLD-1*. However, in contrast to this proposed function, disruption of the *Sam68* gene in chicken DT40 cells showed reduced growth rate, indicating that SAM68 plays a positive role in cell proliferation [26]. Moreover, a natural alternative isoform of *SAM68* with deletion of the KH (RNA binding) domain ($SAM68_{\Delta KH}$) was specifically expressed during growth arrest in normal cells, but absent in SRC-transformed cells (Figure 1) [27]. Importantly, transfection of the $SAM68_{\Delta KH}$ isoform inhibited serum-induced DNA synthesis and Cyclin D1 expression, thus highlighting for the first time the involvement of SAM68 RNA binding activity in cell proliferation [27]. Thus, despite the initial putative role as a tumor suppressor gene, subsequent studies appeared to suggest a positive role of SAM68 in tumorigenesis. These findings were also supported by investigation of the *Sam68* knockout mouse model, which has recently unveiled the physiological processes in which SAM68 is involved.

Sam68-deficient mice displayed high lethality soon after birth [28]. Nevertheless, mice that survived beyond weaning showed a normal lifespan. Importantly, surviving *Sam68−/−* mice lived to old age (~2 years) and were not prone to tumor formation, clearly indicating that SAM68 is not a tumor suppressor *in vivo* [28]. Moreover, haploinsufficiency of SAM68 delayed mammary tumor onset and reduced metastasis [29]. Although the authors reported higher activation of SRC and FAK in the mammary gland of *Sam68* heterozygote females, indicating altered regulation of the SRC signal transduction pathway [29], whether or not this effect was related to the lower tumorigenicity of *Sam68* haploinsufficient cells was not investigated.

Additional phenotypes of the *Sam68−/−* mice revealed the important role played by this RBP in a number of physiological processes. Adult knockout females displayed defects in bone metabolism [28] and delayed development of sexual organs [29]. *Sam68−/−* mice were protected against age-induced osteoporosis and were characterized by preserved bone density. This phenotype was linked to the preferential differentiation of knockout mesenchymal stem cells toward osteoblasts instead of adipocytes [28]. Furthermore, *Sam68−/−* females displayed a reduction in the number of developing ovarian follicles, alteration of estrous cycles, and impaired fertility [30]. Similarly, spermatogenesis and fertility were impaired in *Sam68−/−* males, due to the involvement of both nuclear RNA processing events [31] and translational regulation of a subset of mRNAs during spermiogenesis [32]. Although almost exclusively nuclear in the majority of normal cells, SAM68 localized in the cytoplasm of secondary spermatocytes and associated with polysomes, thus playing a role in translational regulation of target mRNAs [32, 33]. Notably, this function in male germ cells closely resembles that of its orthologue in *C. elegans* GLD-1.

Aberrant regulation of splicing events also contributes to the phenotypes of *Sam68−/−* mice. For instance, stimulation of *Sam68−/−* cerebellar neurons was dramatically attenuated due to the impaired regulation of *Nrxn-1* alternative splicing [34]. *Nrxn-1* encodes a synaptic cell surface receptor that contributes to the assembly of functional presynaptic terminals, and a severe perturbation of *Nrxn-1* splice variants was observed in *Sam68−/−* brains [34]. Moreover, *Sam68−/−* mice exhibited a lean phenotype due to a dramatic reduction in adiposity. The decreased commitment to early adipocyte progenitors and defects in adipogenic differentiation were attributed to aberrant splicing of *mTOR* described in *Sam68−/−* mice [35].

Collectively, the defects documented in *Sam68* knockout mice reflect the multiple roles played by SAM68 in signal transduction and RNA processing and emphasize how aberrant regulation of SAM68 function(s) might contribute to oncogenic transformation [28, 29, 36]. Nevertheless, to what extent SAM68 RNA binding activity contributes to the mouse defects and to neoplastic transformation has not been unraveled yet, and, in this context, knock-in or transgenic mouse models displaying *Sam68* gene with mutations in the RNA binding domain would really help to answer this question.

3. SAM68 Signaling in Human Cancer

SAM68 acts as a scaffold protein in response to different signal transduction pathways [36, 41]. Through its proline-rich motifs, SAM68 interacts with the SH3 domains of different SRC kinases [1, 2], like BRK [42], FYN [18], and Itk/Tec/BTK [43], all involved in different aspects of cell transformation. Importantly, the interaction of SAM68 with the SRC SH3 domain enables SRC kinases to phosphorylate their substrates [9].

The interaction of SAM68 with FYN induces the assembly of a protein complex containing also PLCγ1 (phospholipase C gamma) [18], triggering its phosphorylation and activation [18, 44]. Interestingly, a truncated form of the tyrosine kinase receptor c-KIT, named tr-KIT, stimulates the formation of this complex [18]. Tr-KIT is aberrantly expressed in a subgroup of prostate cancer (PCa) patients and its expression correlates with enhanced activation of SRC and elevated expression and high tyrosine phosphorylation of SAM68 [45]. Moreover, SAM68 is frequently upregulated in PCa patients and promotes PCa cell proliferation and survival to chemotherapeutic agents [46], suggesting a role for this pathway in prostate cancer biology.

The breast tumor kinase BRK, a nonreceptor tyrosine kinase, is also responsible for the tyrosine phosphorylation of SAM68 in cancer cells, which has been associated with SAM68 increased nuclear localization and cell cycle promotion [47, 48]. Importantly, both SAM68 and BRK are upregulated in breast cancer cells and breast tumors [39, 48, 49]. In addition, in the transformed HT29 adenocarcinoma cell line, endogenous BRK colocalized in SAM68

nuclear bodies (SNBs), and BRK-mediated phosphorylation of SAM68 impaired its ability to bind RNA molecules [50]. Consistent with these results, nuclear BRK was also detected in differentiated androgen-responsive LNCaP human PCa cell line, while it was mainly cytoplasmic in the undifferentiated and more aggressive androgen-unresponsive PC3 prostate cancer cell line [50]. Thus, relocalization of the BRK kinase during PCa development and progression may indicate disruption of a signaling pathway important for maintaining the normal phenotype of prostate epithelial cells.

Proteomic analyses revealed that SAM68 is able to form two (large and small) protein complexes, interacting with several RBPs and with regulators of cytoskeletal organization and signal transduction pathways [51, 52]. In accordance with this, *SAM68*-deficient fibroblasts displayed defects in cell migration [53] and an increase in SRC kinase activity [53]. These observations suggest that SAM68 is required for a negative feedback inhibition of SRC and that deregulated SRC activity could be responsible for the defects in actin cytoskeleton and cell migration observed in *SAM68*-deficient fibroblasts. Interestingly, epidermal growth factor (EGF) treatment induced a change in the size of the SAM68-containing complexes, from the large to the smaller one, the latter containing splicing activity [51]. Since EGF receptor (EGFR) stimulation triggers signaling cascades controlling cellular proliferation, migration, differentiation, and survival, and EGFR overexpression has been associated with poor prognosis in several types of epithelial cancers, such as lung, head and neck, colorectal, and breast cancer [54], EGFR-SAM68 signaling could be targeted to attenuate the oncogenic features of cancer cells.

In addition to PCa [46, 52], aberrant expression of SAM68 was detected in several other tumors. In particular, SAM68 was shown to be upregulated in colorectal cancer [55] and in patients with non-small cell lung cancer [56]. Moreover, in patients with renal cell carcinoma high SAM68 expression was inversely associated with overall survival while SAM68 cytoplasmic localization significantly correlated with pathologic grade and outcome of this tumor [57]. Furthermore, in breast cancer patients expression and cytoplasmic localization of SAM68 significantly correlated with clinical characteristics of patients, including clinical stage, tumour-nodule-metastasis classification, histological grade, and ER expression [39]. In line with an oncogenic role played by SAM68 in this tumor type, silencing of SAM68 inhibited proliferation and tumourigenicity of breast cancer cells [39]. Finally, SAM68 was shown to be significantly upregulated in cervical cancer at both mRNA and protein levels [58]. SAM68 upregulation and its cytoplasmic localization were significantly associated with risk factors and correlated with lymph node metastasis and poor prognosis in patients with early-stage cervical cancer [58]. Consistently, downregulation of SAM68 in cervical cancer cells inhibited cellular motility and invasion by the inhibition of the AKT/GSK-3 β/Snail pathway [58].

Collectively, these reports strongly suggest that high SAM68 expression and its cytoplasmic localization are associated with poor overall survival in different types of tumors. Moreover, the deregulation of SRC and AKT pathways could be involved in the oncogenic function of SAM68 in the cytoplasm.

4. SAM68 and Transcriptional Regulation in Human Cancer

The first evidence of the involvement of SAM68 in transcriptional regulation came out in 2002 when Hong and colleagues documented the repressive effect of SAM68 on different mammalian and viral promoter constructs [37]. Direct recruitment of SAM68 to a promoter region resulted in strong transcriptional repression and mutation of the SAM68 RNA binding domain had no influence on this effect, thus suggesting that SAM68 transcriptional activity occurs in a RNA-independent fashion [37]. Mechanistically, the authors described the functional association of SAM68 with the acetyl-transferase CBP, which caused modulation of CBP transcriptional activity (Figure 2(a)) [37].

Other reports confirmed the role of SAM68 as a transcriptional repressor. SAM68 was shown to interact with hnRNP K, leading to inhibition of the *trans*-activating effects of hnRNP K on c-myc target genes [59]. Moreover, overexpression of SAM68 in mouse fibroblasts inhibited accumulation of *Cyclin D1* and *E* transcripts [60], whereas SAM68 SUMOylation by PIAS1 further enhanced repression of Cyclin D1 expression (Figure 2(b)) [23].

In PCa cells, SAM68 was proposed to function as a transcriptional coregulator and to promote the transcriptional activity of the androgen receptor (Figure 2(c)) [38]. Furthermore, in hematopoietic stem cells SAM68 was shown to form an oncogenic transcriptional complex with mixed lineage leukaemia (MLL) and PRMT1 [61]. Chimeric fusion of MLL with PRMT1 or SAM68 enhanced self-renewal of primary hematopoietic cells; conversely, specific knockdown of PRMT1 or SAM68 suppressed MLL-mediated oncogenic transformation [61]. Similarly, SAM68 depletion in breast cancer cells impaired cell proliferation and their tumorigenic features through the upregulation of cyclin-dependent kinase inhibitors p21 (Cip1) and p27 (Kip1). Thus, in this context SAM68 depletion might lead to suppression of AKT phosphorylation and subsequent activation of FOXO factors, which in turn promote the upregulation of p21 (Cip1) and p27 (Kip1) (Figure 2(d)) [39].

In normal and transformed human T cells SAM68 was shown to bind the *CD25* promoter and facilitate p65 recruitment, thus suggesting a novel role for SAM68 in NF-κB regulation of gene expression in human T cell signaling (Figure 2(e)) [40]. In this context, *CD25* expression and aberrant NF-κB signaling led to increased proliferation, expression of antiapoptotic proteins, and drug resistance, while *SAM68* knockdown markedly impaired CD25 upregulation. Remarkably, elevated expression of CD25 has been detected in a large variety of hematopoietic malignancies and solid tumors [62]; thus the p65-SAM68 association might be strategically used to target CD25 expression in those particular tumors that depend on CD25 for survival [40].

Transcription and RNA processing machineries are tightly coupled. Temporal coupling not only provides efficient

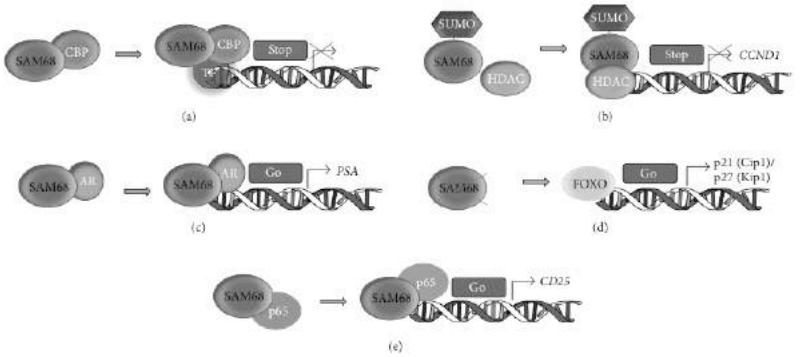

FIGURE 2: Transcriptional regulation by SAM68. (a) SAM68 forms a complex with CBP and transcriptional repressor factors (TF), thus negatively regulating CBP targets transcription [37]. (b) The PIAS1 complex SUMOylates SAM68, which interacts with a histone deacetylase (HDAC) and represses *CCDN1* transcription [23]. (c) SAM68 directly interacts with the androgen receptor (AR) and binds to androgen-responsive elements (AREs) leading to AR targets activation (i.e., *PSA* gene) [38]. (d) SAM68 depletion in breast cancer cells leads to activation of FOXO factors thus inhibiting cell proliferation and tumourigenicity through the upregulation of cyclin-dependent kinase inhibitors p21 (Cip1) and p27 (Kip1) [39]. (e) SAM68 binds the *CD25* promoter and facilitates p65 recruitment, thus contributing to NF-κB regulation of gene expression [40].

gene expression to accomplish rapid growth and proliferation, but also allows rapid response to diverse signaling events [63]. Many splicing regulators are recruited to nascent pre-mRNAs by their interaction with the phosphorylated carboxyl-terminal domain (CTD) of RNAPII thus affecting splicing decisions [64]. Interestingly, SAM68 was shown to interact directly with RNA polymerase II (RNAPII) in meiotic spermatocytes [31] and with the RNAPII associated Brahma (Brm) subunit of the SWI/SNF chromatin-remodeling complex [65]. These observations strongly suggest the involvement of SAM68 in cotranscriptional splicing. Thus, on one hand, SAM68 binding to transcription factors and to the RNAPII itself can affect transcriptional regulation of gene expression; on the other hand, through the cooperation with chromatin remodelers, SAM68 can impact cotranscriptional splicing events. In this regard, interaction of the protooncogenic transcription factor FBI-1 with SAM68 in PCa cells was shown to inhibit SAM68 recruitment on the *BCL-X* pre-mRNA, thus affecting apoptosis [66]. By contrast, binding of SAM68 to the transcriptional coactivator SND1 was required for the efficient association of SAM68 with RNAPII and for the recruitment of SAM68 on the *CD44* pre-mRNA [67]. Remarkably, *CD44* alternative splicing isoforms are associated with tumor progression and metastasis [68]. Thus, the SND1/SAM68 complex might be an important determinant of PCa progression and the concomitant upregulation of these proteins might provide an advantage for cancer cells to invade other tissues, consequently favoring the spreading of metastatic cells [67].

Hence, depending on the cellular partner, SAM68 displays different effects on target genes, modulating in this way different or even antagonistic functions within the cell.

In summary, growing evidence documents the involvement of SAM68 in the transcriptional regulation of gene expression of cancer related genes, both by direct binding to the chromatin and by recruitment of specific transcription factors, which in turn affect its splicing activity.

5. SAM68-Regulated Alternative Splicing Events in Cancer

SAM68 preferentially binds A/U-rich sequences in RNA [16]. SELEX experiments identified the UAAA consensus motif bound with Kd ~12–60 nM. Importantly, a single A to C mutation within this motif abolished SAM68 binding [69], indicating that this motif is involved in high affinity direct binding or in a specific RNA structure. Indeed, SAM68 was then shown to bind cellular RNAs enriched in such U/A-rich sequences [70] and to directly modulate alternative splicing events in target genes [71]. Interestingly, the UAAA motif matches with the last four bases of the mammalian polyadenylation signal AAUAAA, thus opening the hypothesis of SAM68 involvement in RNA stability.

During tumor progression, a variety of oncogenic signaling pathways induce modifications of the downstream effectors of key biological functions [76]. Notably, SAM68 was the first identified "hub factor" able to translate extracellular stimuli to pre-mRNA processing of specific target

genes in the nucleus [71]. As mentioned above, several posttranslational modifications regulate the function and/or localization of SAM68. In particular, serine-threonine and tyrosine phosphorylation of SAM68, which often occurs in cancer cells, are important for SAM68 homodimerization and RNA affinity (Figure 3(a)) [2, 72, 73].

The *CD44* gene represents an interesting example of SAM68-mediated coupling between signal transduction cascades and alternative splicing. *CD44* pre-mRNA is affected by complex alternative splicing events occurring in 10 adjacent exons (v1–v10) to produce multifunctional transmembrane glycoprotein isoforms implicated in cell-cell and cell-matrix adhesion, migration, and invasion [77] and with crucial roles in cancer progression and metastasis [78]. By binding to A/U-rich enhancer element located within exon *v5*, SAM68 promotes the production of the oncogenic *CD44v5* variant (Figure 3(b), (A)) [71], which is upregulated in several cancers [78, 79] and bears prognostic value in gastric and renal carcinoma [80–82].

Several molecular mechanisms (not mutually exclusive) have been proposed to explain the ability of SAM68 to stimulate *CD44* exon *v5* inclusion: (i) SAM68 competes or displaces the antagonistic splicing repressor hnRNP A1 that binds a specific splicing silencer element located within exon *v5* [83]; (ii) SAM68 affects the dynamic recruitment of spliceosomal components, including U2AF65, an auxiliary factor involved in the recognition of the 3′ splice site during the splicing reaction [84]: upon SAM68 phosphorylation this interaction is disrupted and U2AF65 dissociates from pre-mRNA allowing the subsequent spliceosome remodeling and exon *v5* inclusion [85]; (iii) SAM68 interacts with the splicing coactivator SRm160 and they functionally cooperate to simulate *CD44* exon *v5* inclusion [86].

Aberrant regulation of alternative splicing is emerging as a key step in oncogenesis [87]. Recent data demonstrated that genotoxic stress widely modulates alternative splicing events in cancer cells [88, 89]. This regulation is exerted in part through reduced transcription elongation rates as a consequence of RNA polymerase II (RNAPII) phosphorylation [90] and in part through direct involvement of specific RBPs in the repair process or by specific regulation of DNA damage response gene expression [91], also accomplished by RBP relocalization [92]. *CD44* exon *v5* splicing is also influenced by genotoxic stress induced by chemotherapeutic drugs, such as the topoisomerase II inhibitor mitoxantrone (MTX) [93]. Specifically, MTX causes relocalization of SAM68 from nucleoplasm to transcriptionally active nuclear granules and this correlates with changes in alternative splicing of *CD44* exon *v5*. This effect is independent of signal transduction pathways activated by DNA damage [93]. Nevertheless, it appears to be functionally relevant for the cells, as SAM68 was found overexpressed in prostate carcinoma where it promotes resistance and survival to chemotherapeutic treatments [46].

In addition to *CD44*, changes in alternative splicing of other transcripts, including *Caspase 2* (*CASP2*) [94], *BCL-2* [90], the p53 negative modulators *MDM2* and *MDM4* [95], and *Cyclin D1* (*CCND1*), have been observed in cancer cells after treatment with chemotherapy drugs [96, 97]. Notably, *CCND1* pre-mRNA was also identified as a novel alternative splicing target of SAM68 [74]. *CCND1* is a protooncogene that is frequently deregulated in several human cancers through different mechanisms, such as chromosomal translocations, amplification of the *CCND1* locus, and intragenic mutations [97–99]. Alternative splicing also plays an important role in aberrant Cyclin D1 expression. The *CCND1* gene encodes two alternatively spliced transcripts: the canonical *Cyclin D1a* and the alternative *Cyclin D1b*, which results from the retention of intron 4 and premature termination of the transcript [100]. These isoforms display different biological properties and cellular localization [96]. In particular, Cyclin D1b is exclusively nuclear and displays stronger oncogenic potential than Cyclin D1a [74, 100, 101] and its upregulation correlates with poor prognosis in several tumor types [96]. At the molecular level, SAM68 was observed to bind to the proximal region of intron 4 and to interfere with the recruitment of the U1 snRNP, in this way promoting intron 4 retention (Figure 3(b), (B)) [74]. Signal transduction pathways affecting SAM68 phosphorylation status, such as those conveyed by ERK1/2 and SRC kinases, regulate alternative splicing of *CCND1* pre-mRNA by modulating SAM68 affinity for this target [74]. Notably, SAM68 expression positively correlates with levels of Cyclin D1b, but not D1a, in human PCa cells [97], suggesting that increased levels of SAM68 in human PCa contribute to tumorigenesis by elevating the expression of Cyclin D1b in this tumor type.

Recent studies have demonstrated an important contribution of alternative splicing regulation in the cascade of events characterizing the morphological conversion of tumor cells during epithelial-to-mesenchymal transition (EMT) [102], one of the major routes through which cancer cells acquire migratory and invasive potentials [103, 104]. SAM68 phosphorylation by ERK1/2 plays an important role during neoplastic progression of epithelial cells through activation of EMT. This is illustrated by the ability of SAM68 to repress alternative splicing-activated nonsense-mediated mRNA decay (AS-NMD) [105] of a splicing factor of the serine arginine (SR) family, SRSF1 [75]. AS-NMD of *SRSF1* pre-mRNA, which involves a cryptic intron in the 3′ UTR region of the gene, decreases *SRSF1* mRNA stability and protein levels (Figure 3(b), (C)) and, notably, this event is altered in colon cancer [75]. In mesenchymal cells, phosphorylation of SAM68 is controlled by soluble factors expressed by epithelial cells that act through the activation of ERK1/2 kinase [75]. SRSF1, an oncogenic splicing factor upregulated in many human cancers [106], severely impacts on cell physiology. For instance, its overexpression stimulates skipping of exon 11 of the *RON* protooncogene increasing the production of the constitutively active Δ*RON* isoform, which in turn promotes the acquisition of an invasive cellular phenotype [107]. Interestingly, inhibition of ERK activity by small molecules or by using conditioned medium from epithelial cells reverts SAM68 phosphorylation, decreases *SRSF1* mRNA and protein levels, promotes inclusion of *RON* exon 11, and induces the reversal program named mesenchymal-to-epithelial transition (MET) [75]. MET occurs at the final metastatic sites where redifferentiation of mesenchymal cells to an epithelial state is required for the colonization of distant organs [103, 104].

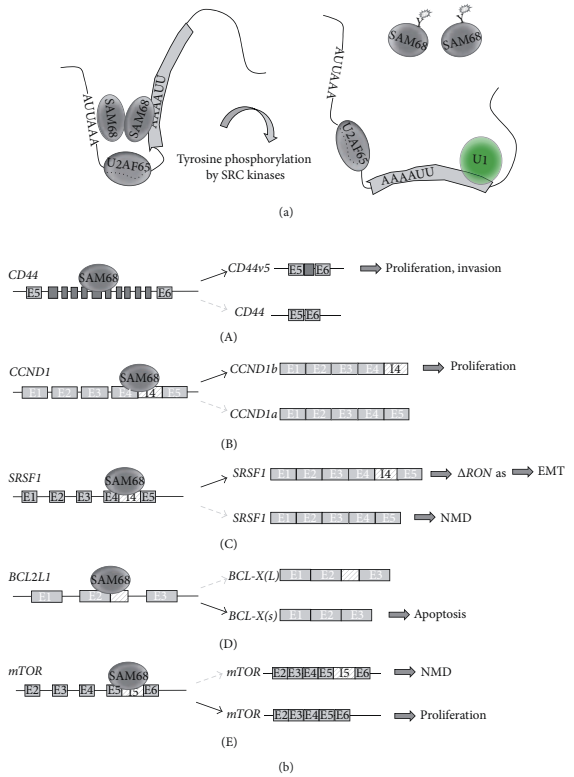

Figure 3: Model of SAM68 interaction with pre-mRNAs and splicing regulation. (a) SAM68 recognition of A/U-rich bipartite sequence in the pre-mRNA homodimerization allows simultaneous binding to the pre-mRNA and to U2AF65 [69–73]. Tyrosine phosphorylation of SAM68 reduces the RNA binding affinity and thus releases SAM68 from the pre-mRNA. (b) Model of alternative splicing events regulated by SAM68 in cancer cells. (A) SAM68 promotes inclusion of *CD44* variable exon *v5*. Inclusion of variable exons in the *CD44* pre-mRNA is specific to cancer cells and correlates with cancer progression and invasiveness [71]. (B) SAM68 promotes splicing events that regulate cell proliferation. Binding of SAM68 to *CCND1* intron 4 interferes with the correct recruitment of U1 snRNP at the exon 4 5′ splice sites, thus enhancing retention of intron 4 and generating the *Cyclin D1b* isoform. In prostate cancer, the expression of Cyclin D1b interrupts a negative feedback in the regulation of androgen receptor (AR) transcriptional activity, thereby promoting cell proliferation [74]. (C) As for *CCND1*, SAM68 promotes retention of *SRSF1* intron 4, thus stabilizing *SRSF1* pre-mRNA and inhibiting its degradation by nonsense-mediated decay (NMD) [75]. Accumulation of SRSF1 in turn favors the splicing of Δ*RON*, an oncogenic variant of *RON* that triggers epithelial-mesenchymal transition (EMT). (D) SAM68 regulates the alternative splicing of *BCL2L1* leading to the short (*BCL-X(s)*) proapoptotic isoform [19]. This activity can be reverted by tyrosine phosphorylation of SAM68 from SRC family kinases, thereby switching the role of SAM68 from being proapoptotic to being antiapoptotic and allowing cells to differentially react to external cues. (E) SAM68 regulates *mTOR* alternative splicing thus leading to the correct mRNA isoform and avoiding retention of intron 5 that generates a premature termination codon and the consequent reduction of mTOR protein levels [35]. Notably, mTOR is a critical effector in cell-signalling pathways commonly deregulated in human cancers and overexpression of the components involved in the PI3K/AKT/mTOR pathway has been shown to induce malignant transformation.

A paradigmatic example of the central role of SAM68 in apoptosis is represented by the regulation of *BCL-X* (*BCL2L1*), a member of the *BCL-2* gene family. *BCL-X* pre-mRNA is alternatively spliced to generate two isoforms with opposite functions in promoting apoptosis. Selection of the proximal 5′ splice site (5′ SS) in exon 2 causes the production of the antiapoptotic long *BCL-X(L)* variant, while the proapoptotic short *BCL-X(s)* variant is produced by the use of the distal alternative 5′ SS [108]. In several cancer types, the *BCL-X(L)* isoform is upregulated thus increasing resistance to chemotherapeutic agents [109, 110]. Targeting this mechanism and switching the splicing of *BCL-X* gene toward the production of the proapoptotic variant thereby offer the opportunity to revert cancer cells resistance to chemotherapeutic drugs and to promote tumor cell death [111, 112]. Due to its relevance in cancer, *BCL-X* alternative splicing has been extensively investigated in the past years and several RBPs were shown to regulate this specific splicing event [19, 113–119]. Among these, SAM68 exerts a proapoptotic function, leading to production of *BCL-X(s)* variant [19]. In particular, SAM68-mediated splicing regulation of *BCL-X* depends on its specific binding to *BCL-X* pre-mRNA and on its ability to interact with the splicing repressor hnRNP A1, thus antagonizing SRSF1, a positive regulator of *BCL-X(L)* splicing (Figure 3(b), (D)) [19, 110]. However, in PCa cells, high levels of SAM68 do not correlate with high levels of *BCL-X(s)* [38, 46, 110]. This apparently contradictory observation can be explained by the fact that tyrosine phosphorylation of SAM68 by the SRC-related kinase FYN counteracts its splicing activity, promoting the antiapoptotic *BCL-X(L)* isoform [19, 120]. In tumors, SRC activity is often increased [121] and it correlates with SAM68 phosphorylation in different cancer types, including prostate cancer [45, 47, 122]. Recently, an additional layer of complexity to the regulation of SAM68-mediated *BCL-X* splicing in cancer has been revealed. This mechanism involves the direct interaction of the transcriptional factor FBI-1 with SAM68, reducing its binding to *BCL-X* pre-mRNA and therefore promoting the production of the antiapoptotic *BCL-X(L)* variant and cell survival [66]. Fascinatingly, FBI-1 function in *BCL-X* splicing regulation is dependent on the activity of histone deacetylases [66], suggesting an important link between this alternative splicing event and dynamic organization of chromatin structure.

The biological consequences and the possible contribution to tumor progression associated with the aberrant splicing in other relevant SAM68-regulated genes have also been recently described. For example, SAM68 is able to promote the production of the oncoprotein E6 of the human papilloma virus (HPV) type 16 [123], which is a known etiological agent for human cervical cancer [124]. *E6* alternative splicing is controlled by EGF through activation of ERK1/2-kinase that promotes SAM68 phosphorylation, suggesting a possible implication of SAM68 in HPV *E6* splicing during differentiation and the viral life cycle processes of cervical cancer.

More recently, SAM68 has been linked to regulation of alternative splicing of the mammalian target of rapamycin (mTOR) [35], which regulates cell size and cell proliferation in response to nutrients and various growth factors [125, 126]. SAM68-depleted cells display intron 5 retention in the *mTOR* mRNA, which generates a premature termination codon and the consequent reduction of mTOR protein levels (Figure 3(b), (E)) [35]. Notably, mTOR is a critical effector in cell-signaling pathways commonly deregulated in human cancers and overexpression of the components involved in the PI3K/AKT/mTOR pathway has been shown to induce malignant transformation [127]. Interestingly, loss of SAM68 reduces breast and PCa incidence [29, 46], suggesting that in cancer cells SAM68 activation may also regulate the expression of PI3K downstream kinases, such as mTOR.

Collectively, these findings indicate that an evaluation of SAM68-associated splicing signatures in diverse sets of tumors can be of medical relevance.

6. SAM68 and Noncoding RNAs

Recent reports have revealed the involvement of SAM68 in noncoding RNAs (ncRNAs) metabolism. ncRNAs are classified into small (18–200 nt) and long ncRNAs (lncRNAs; 200 nt to >100 kb) [128, 129] and play a role in a wide variety of biological processes, including almost all levels of gene expression regulation, from epigenetic to transcriptional and posttranscriptional control [130]. Coimmunoprecipitation studies documented the interaction between SAM68 and key proteins involved in microRNA (miRNA) biogenesis [131]. miRNA genes are transcribed by either RNA polymerase II or RNA polymerase III into long primary miRNA transcripts (pri-miRNAs) [132]. The cleavage of the pri-miRNAs into stem-loop precursors of ~70 nucleotides (pre-miRNAs) is mediated by DROSHA [133], whereas the cytoplasmic processing of pre-miRNAs into mature miRNAs is mediated by DICER [134]. Coimmunoprecipitation experiments performed in male germ cells indicated that SAM68 interacts with both DICER and DROSHA and that the knockout of *Sam68* leads to changes in expression of specific miRNAs in germ cells [131]. Remarkably, a similar functional interaction with components of the miRNA machinery was shown for Quaking (QKI), another member of the STAR family. In the U343 glioblastoma cell line and in primary rat oligodendrocytes QKI interacts with AGO2, a component of the RISC complex involved in miRNA-dependent translational repression, within stress granules [135]. Collectively, these findings suggest a general role for STAR proteins in the regulation of miRNAs.

Interaction between SAM68 and noncoding RNAs might also affect the splicing activity of this RBP. Recently, a long noncoding RNA (named *INXS*) has been described as a novel mediator of SAM68-dependent regulation of *BCL-X* splicing. *INXS* is transcribed from the antisense genomic strand of *BCL-X* gene and is downregulated in various tumor cell lines and in kidney tumor tissues, whereas its expression is induced by treatments that trigger apoptosis [136]. *INXS* interacts with SAM68 and favors its splicing activity, thus increasing the levels of *BCL-X(s)* isoform and enhancing apoptosis [136]. Notably, in favor of a possible role of *INXS* in anticancer therapy, *INXS* overexpression in a mouse xenograft model was sufficient to induce tumor regression and increase *BCL-X(s)* isoform [136].

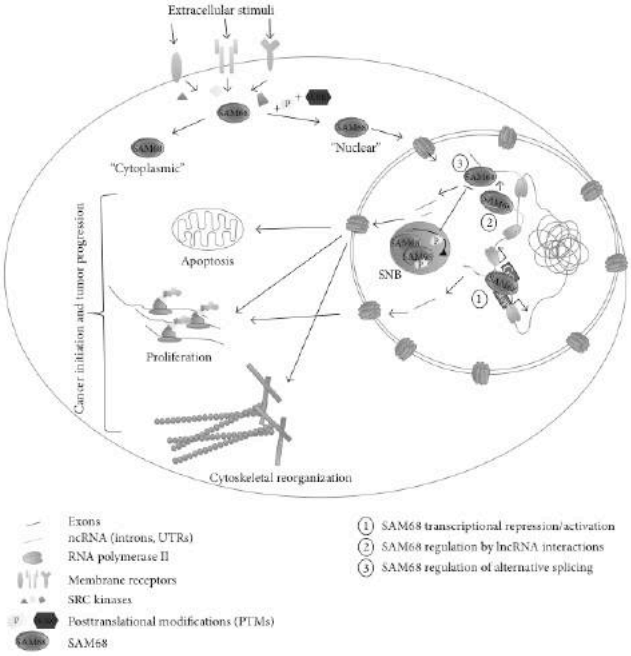

FIGURE 4: Role(s) of SAM68 in transcriptional and posttranscriptional regulation of gene expression in cancer cells. SAM68 and its regulatory networks contribute to important process involved in cancer initiation and progression, such as apoptosis, proliferation, and cytoskeletal reorganization, through different mechanisms. After posttranslational modifications (PTMs) induced by extracellular stimuli and mediated by SRC family kinases, SAM68 is committed to the nucleus where it is able to (1) promote or repress transcription of different targets (see Figure 2 for more details) and (2-3) regulate alternative splicing events through several molecular mechanisms, some of them mediated by lncRNAs (see Figure 3 for more details). In the nucleus, SAM68 can localize in specific bodies (SNB) and associate with other proteins (i.e., BRK kinase) that modify its phosphorylation status, thus affecting its RNA binding activity.

Thus, the complex regulatory network of proteins and ncRNAs orchestrated by SAM68 greatly contributes to the cellular signature in higher eukaryotes and plays a pivotal role in the regulation of gene expression in normal conditions and in oncogenic transformation.

7. Concluding Remarks

Misregulation of cancer-associated alternative splicing events is often correlated with unbalanced expression of splicing factors. SAM68 is a clear example of this concept, as it is upregulated in different types of tumors and it directly affects cancer initiation and progression. Transcriptional and posttranscriptional regulation of gene expression mastered by SAM68 chiefly contributes to changes in gene expression occurring in cancer cells. Moreover, SAM68 orchestrates transcript fate and function (Figure 4). Thus, depicting SAM68 signatures in normal and cancer cells would greatly help in understanding how SAM68 and its regulatory networks contribute to key features of tumor initiation and progression. Although the functional significance of SAM68-regulated alternative splicing events in human cancer has been clearly established, future studies unraveling the positional effect of SAM68 binding to pre-mRNAs would be instrumental for the development of new therapeutic approaches to target SAM68 activities in cancer.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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The alternative splicing factor Nova2 regulates vascular development and lumen formation

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Vascular lumen formation is a fundamental step during angiogenesis; yet, the molecular mechanisms underlying this process are poorly understood. Recent studies have shown that neural and vascular systems share common anatomical, functional and molecular similarities. Here we show that the organization of endothelial lumen is controlled at the post-transcriptional level by the alternative splicing (AS) regulator Nova2, which was previously considered to be neural cell-specific. Nova2 is expressed during angiogenesis and its depletion disrupts vascular lumen formation *in vivo*. Similarly, Nova2 depletion in cultured endothelial cells (ECs) impairs the apical distribution and the downstream signalling of the Par polarity complex, resulting in altered EC polarity, a process required for vascular lumen formation. These defects are linked to AS changes of Nova2 target exons affecting the Par complex and its regulators. Collectively, our results reveal that Nova2 functions as an AS regulator in angiogenesis and is a novel member of the 'angioneurins' family.

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In adulthood most blood vessels remain quiescent; however, in conditions of active physiological tissue growth, such as during embryogenesis or tissue repair, endothelial cells (ECs) migrate and proliferate to form new vessels. This process, known as angiogenesis, is also critical for the pathogenesis of several disorders and to support cancer development and progression[1]. Although angiogenesis does not initiate malignancy, it promotes tumour growth by allowing oxygen and nutrients to reach proliferating cancer cells. Targeting angiogenesis represents a particularly promising anticancer therapeutic approach, and several strategies have been attempted so far[2].

Recent studies have highlighted significant anatomic, structural and molecular similarities between the vascular and the nervous systems[3]. Both systems possess specialized structures—tip cells at the forefront of endothelial sprouts and axonal growth cones—that, through filopodial extensions, probe the environment for guidance cues. Molecules regulating these processes have been termed 'angioneurins'[3]. The prototypic angioneurin is the vascular endothelial growth factor (VEGF), which was originally discovered as a key angiogenic factor, but subsequently shown to be important also in the development of the nervous system[4]. Since blood vessels and nerves are functionally interdependent, the malfunctioning of this 'neurovascular link' can lead to several vascular and neuronal disorders[3].

Until now, the many molecular pathways regulating vascular development and angiogenesis have been suggested to act primarily through the regulation of transcription. However, recent studies indicate that post-transcriptional and epigenetic programmes cooperate to confer tissue-specific vascular properties.

Alternative splicing (AS) is a molecular process that generates multiple, distinct mature mRNAs from a primary transcript (pre-mRNA), leading to the production of protein isoforms with different structural and functional properties. Since more than 90% of human multiexonic genes undergo AS[5,6], this process represents a major mechanism underlying the expansion of the proteome from a limited repertoire of genes[7,8]. AS and transcription predominantly regulate different subsets of genes to generate the molecular and cellular complexity of different cell and tissue types[8–11]. AS thus provides a versatile, additional layer of regulation to both establish and maintain fundamental properties of different cell and tissue types. Despite the importance of AS, the functional roles of the vast majority of AS events is not well understood.

While there are several examples of splicing variants with a role in angiogenesis[12–15], the molecular mechanisms responsible for their production are still unknown. Here we describe a novel role for Nova2, previously described as neural cell-specific[16], as a key AS regulator of angiogenesis. Both its expression and the levels of AS of its target exons are regulated during this process. Through gain- and loss-of-function approaches in ECs, we show that Nova2 regulates AS of factors belonging to the Par polarity complex and its regulators. Consequently, vascular lumen formation defects are developed in zebrafish on *nova2* morpholino-mediated knockdown or clustered regularly interspaced short palindromic repeat (CRISPR)-induced genetic mutation. Collectively, our results provide evidence that Nova2 is a new member of the 'angioneurins' family, and further highlight an important biological role for post-transcriptional regulation of exon networks that contribute to both vascular and neuronal functions.

Results

Nova2 expression and function are regulated in ECs. To identify splicing regulatory factors (SRFs) involved in endothelial growth and quiescence, we studied ECs under sparse and confluent conditions. By mining previously published Affymetrix gene expression data[17] comparing mouse ECs grown at different densities, we identified *Nova2* as an SRF that is significantly upregulated in confluent versus sparse ECs (fold change = 2.3; P value < 0.05, Dunnett test). This result was surprising since Nova2 was considered previously to be neural cell-specific[16].

Nova2 and its paralogue Nova1 are among the best-studied mammalian tissue-specific SRFs. Both proteins bind RNA through KH domains that recognize clusters of YCAY repeats within the pre-mRNA targets[16]. These factors, with indistinguishable biochemical properties but mutually exclusive expression within the central nervous system (CNS)[18], regulate AS programmes involved in neuronal development and synapse activity[16].

Validating the microarray results, we confirmed Nova2 upregulation in confluent versus sparse ECs using reverse transcription–quantitative PCR (RT–qPCR; Fig. 1a). By comparing *VE-cadherin*-null ECs (VEC-null) with the same cells reconstituted with *VE-cadherin* (VEC-positive)[17] we also found that *Nova2* upregulation in confluent versus sparse ECs does not require VE-cadherin expression (Supplementary Fig. 1A). On the contrary, we found that *Nova1* is expressed to a negligible level in ECs, while both factors are expressed in E15.5 mouse whole brain that we used as positive control (Fig. 1a), consistent with recently published results[19,20]. Moreover, expression of the Muscleblind family of tissue-specific AS regulators (*Mbnl1*, *Mbnl2* and *Mbnl3*) was not modified by confluence in ECs (Fig. 1a). We further confirmed the upregulation of Nova2 in confluent ECs at the protein level by immunoblotting (Fig. 1b). Comparable results were obtained by using another EC line (adult ECs from the mouse lung) under sparse and confluent conditions (Supplementary Fig. 1B). Notably, as in the case of mouse cortex (Fig. 1b) and in human neuroblastoma SH-SY5Y cells (Supplementary Fig. 1C), in ECs the anti-Nova2-specific antibody recognized two immunoreactive bands at 50–55 and 70–80 kDa, as previously reported[18]. In agreement with available RNA sequencing (RNAseq) data (Supplementary Fig. 1D), we found that *Nova2* is also expressed in primary human umbilical vein endothelial cells (HUVECs) and that its levels decreased when HUVECs were grown as sparse (Fig. 1c). Moreover, *Nova2* expression increased during endothelial differentiation of mouse embryonic stem (ES) cells (Fig. 1d), or in adult ECs as compared with embryo or fetal ECs (Fig. 1e). Importantly, Nova2 expression correlated with AS changes of its known target *Ank3*: (i) in sparse versus confluent ECs, (ii) during endothelial differentiation of ES cells and (iii) in ECs of different origin (Fig. 1f) and AS of this target parallels that observed in brain of *Nova2*-null mice[21]. Taken together, these data suggest that Nova2 expression and function may play a role in vascular maturation.

To confirm the vascular expression of Nova2 in a more physiological context, we analysed the postnatal mouse retina, which develops a stereotypical vascular pattern following a well-defined sequence of events[22]. In the retina, we found that Nova2-positive nuclei were reduced but still present in the ECs at the sprouting front as compared with the central part of the retina where the majority of ECs of the mature vessels (arteries and veins) and capillaries were Nova2-positive (Fig. 2a,b and Supplementary Fig. 2A). In addition, we found the specific nuclear expression of Nova2 in ECs present in the vessels of different tissues, such as normal human thyroid, skin, bladder, colon and prostate (Fig. 2c and Supplementary Fig. 2B).

Collectively, these data indicate that Nova2 is not exclusively expressed in cells of the nervous system, but it is also present in ECs of different types of vessels.

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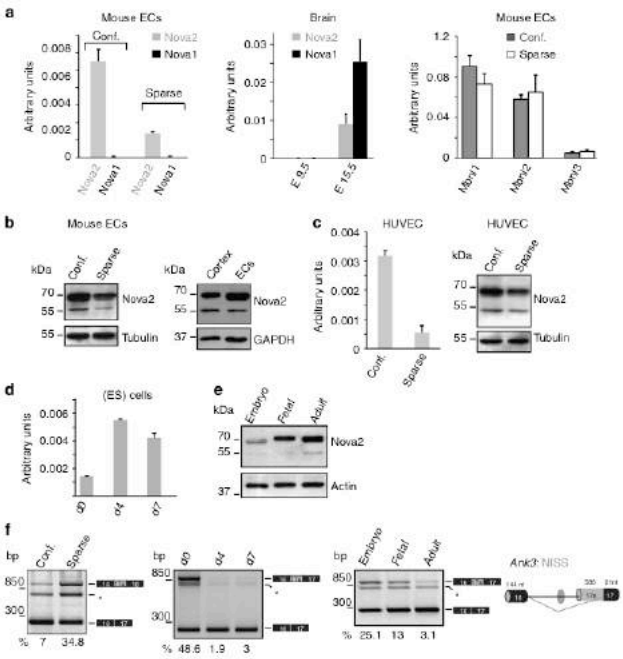

Figure 1 | Nova2 expression levels and AS of its target are regulated in ECs. (**a**) RT–qPCR analysis of *Nova2* and *Nova1* mRNA expression levels in mouse ECs grown as confluent or sparse (left), in E9.5 and E15.5 mouse whole brain (centre), and RT–qPCR analysis of Muscleblind family members (*Mbnl1*, *Mbnl2* and *Mbnl3*) in mouse ECs grown at different densities (right). (**b**) Immunoblotting analysis of Nova2 levels in mouse confluent and sparse ECs (left panel; Tubulin as the loading control) and in confluent ECs and the mouse brain cortex (right panel; GAPDH as loading control). (**c**) Nova2 mRNA and protein expression levels in HUVECs grown at different densities. (**d**) RT–qPCR analysis of *Nova2* during endothelial differentiation of mouse ES cells at the indicated times. (**e**) Immunoblotting analysis of Nova2 in mouse EC lines derived from whole embryo, fetal heart and adult lung; Actin as the loading control. In all histograms, error bars indicate ±s.d. calculated from three independent experiments ($n=3$). (**f**) RT–PCR analysis of AS of a known Nova2 target (*Ankyrin3*/*Ank3*) in mouse ECs (confluent and sparse; left), during endothelial differentiation of mouse ES cells (centre) and in mouse ECs of different origins (right). The schematic representation of the mouse gene structure (AS exon in grey; constitutive exons in black), the YCAY cluster predicted to function as Nova2 silencer (blue dot) and the Nova2-regulated exon-skipping event (blue bars) are also shown. NISS, Nova intronic splicing silencer. The percentage of exon inclusion is shown. Asterisk indicates a nonspecific PCR product.

Nova2 regulates the endothelial apical–basal polarity. An important functional similarity in the development of the vascular and nervous systems is the establishment of the apical–basal polarity, as this is a crucial event for the organization of the vascular lumen and for axon guidance[23,24] respectively. Notably, the partitioning-defective (Par) polarity complex is a key determinant of cell polarity in both systems[23,24]. The specific localization and activity of the Par polarity complex involve the association of four key components: Par3, Par6, the small GTPase Cdc42 and the atypical protein kinase C ($PKC\zeta$). In addition, the small GTPases Rac1 and Rap1 are important regulators of the Par complex during organization of the vascular lumen[23,25].

To investigate the role of Nova2 in the endothelium as a possible regulator of vascular development, we generated stable *Nova2* knockdown ECs (Fig. 3a). Intriguingly, we found that depletion of *Nova2* expression impairs EC polarity. As shown in Fig. 3b, in two-dimensional (2D) cultures, *Nova2* knockdown altered the subcellular localization of the apical surface marker podocalyxin (Podxl) that was distributed all over the cell membrane and also to the basal surface. These findings prompted us to examine whether silencing of *Nova2* alters the junctional staining and/or activity of components of the Par polarity complex. *Nova2*-depleted ECs displayed impaired junctional distribution of Par3, a multiscaffold protein that promotes the assembly of the Par complex (Fig. 3c). Interestingly, *Nova2* knockdown in ECs caused reduced levels of active (GTP-bound) Cdc42 (Fig. 3d), whose association with the Par complex is induced during EC lumen formation[26]. Accordingly, we

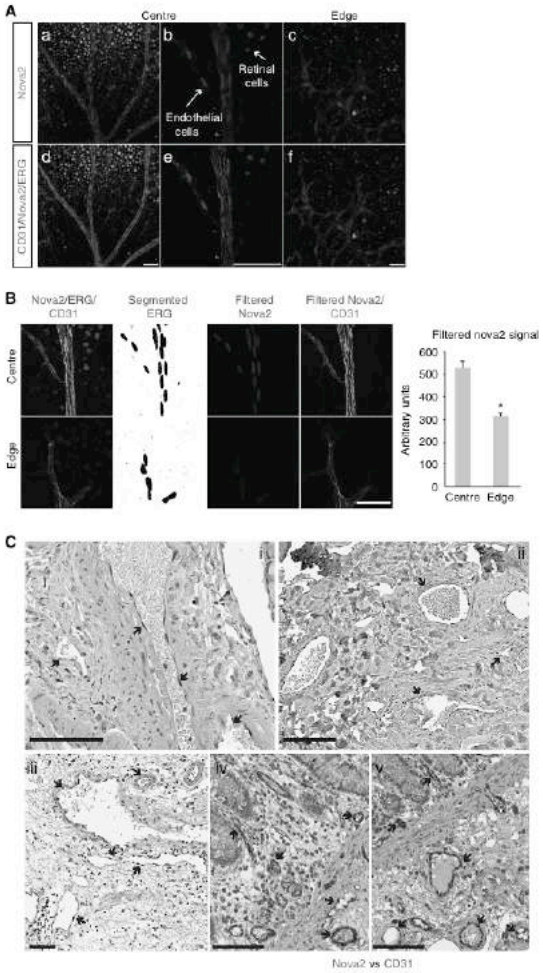

Figure 2 | Nova2 is expressed in the vascular endothelium *in vivo*. (**a**) Immunofluorescence analysis of Nova2 (green), the endothelial markers CD31 (red) and ERG (blue, transcription factor) in whole-mounted postnatal (P6) mouse retina. Optical sections captured using confocal microscopy display large vessels in the central retina region (a,b,d,e) and sprouting ECs in the leading edge of the growing vasculature (c,f). Arrows indicate neural cells of the retina and ECs of vessels expressing Nova2 (scale bar, 50 μm). (**b**) Quantification of Nova2 signal. ERG staining has been segmented with threshold 350–4,096, and segmented images have been filtered to remove speckles and outliers (radius 25). Segmentation results have been used to filter Nova2 staining to isolate EC nuclear staining (scale bar, 50 μm). Chart shows average signals (error bars indicate mean ± s.d.; asterisks *P* value<0.05, two-tailed *t*-test assuming unequal variances; $n = 2$). (**c**) IHC of Nova2 in normal human thyroid (i), skin (ii) and bladder (iii) and IHC of Nova2 and the endothelial marker CD31 in normal human colon (iv, v). Arrows indicate Nova2 nuclear staining of ECs in the blood vessels (scale bar, 100 μm).

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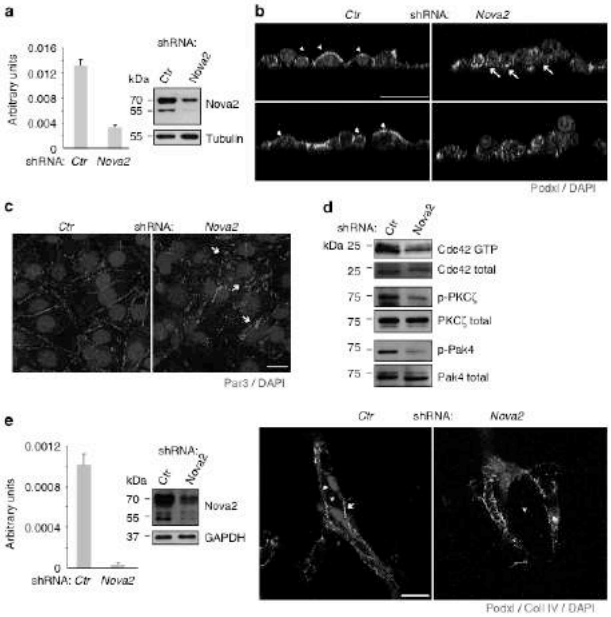

Figure 3 | Nova2 is required for the EC polarization. (**a**) *Nova2* mRNA levels in knockdown mouse ECs (grown as confluent). The Nova2 protein level was analysed by immunoblotting using an anti-Nova2 antibody (Tubulin as loading control). (**b**) Immunofluorescence (IF) analysis of Podocalyxin (Podxl, green) and DAPI (blue) in 2D culture of control (*Ctr*) and *Nova2*-depleted ECs. Podxl is often distributed to the basal (arrows) instead of the apical surface (arrowheads) in *Nova2*-silenced ECs (confocal sections, *z* axis; scale bar, 25 μm). (**c**) IF analysis of Par3 (red) and DAPI (blue) in *Ctr* or *Nova2* knockdown ECs. Arrows indicate altered and fragmented junctional staining of Par3 (bar 20 μm). (**d**) *In vitro* pull down of GTP-bound Cdc42 in *Ctr* and *Nova2*-silenced ECs. Immunoblotting for the phosphorylation status of PKCζ and Pak4 is also shown. (**e**) Left: *Nova2* mRNA levels in knockdown HUVECs. The Nova2 protein level was analysed by immunoblotting using an anti-Nova2 antibody (GAPDH as the loading control). Right: in 3D collagen gel, control HUVECs form vascular structures with a central lumen (asterisk) and apical Podxl and basal collagen IV (Coll IV) proper localization (arrowheads and arrows, respectively), whereas *Nova2*-silenced HUVECs are not correctly polarized (scale bar, 20 μm). Error bars indicate mean ± s.d. calculated from three independent experiments ($n = 3$).

determined that phosphorylation of PKCζ, a Cdc42-GTP-activated protein[27], is also reduced in *Nova2*-depleted ECs (Fig. 3d). Moreover, *Nova2* knockdown ECs were characterized by reduced levels of phosphorylated Pak4 (p21-activated kinase; Fig. 3d), one of the main Cdc42 downstream effectors[28].

EC polarization correlates with lumen formation[25,29]. Thus, to test whether Nova2 is required for the formation of the endothelial lumen, we have carried out its knockdown in primary HUVECs that, when are cultured in three-dimensional (3D) collagen gel, rapidly organize into a network of hollow structures[30]. As shown in Fig. 3e, control HUVEC cells forming vascular-like structures were correctly polarized with Podxl and collagen IV localized to the apical and basal surfaces, respectively. On the contrary, knockdown of *Nova2* resulted in the formation of capillary-like tubular networks with irregular lumen and a non-polarized distribution of endothelial Podxl and collagen IV. These results strongly suggest that Nova2 splicing factor is crucial for EC morphogenesis.

Recently, the Par polarity complex has been shown to regulate endothelial cell-cell contacts and affect migratory behaviour[25,31]. In agreement with these observations we found that silencing of *Nova2* affected junctional clustering of VEC and β-catenin since the architecture of cell–cell boundaries was partially disorganized in *Nova2* knockdown ECs (Supplementary Fig. 3A). Cell polarity is also established during directional cell migration, where cytoskeletal, adhesive and signalling molecules are distributed asymmetrically. We found that *Nova2* knockdown affected the collective behaviour of migrating ECs. During wound closure, migrating ECs depleted of *Nova2* have in part lost their contacts with neighbouring cells positioned at the back (Supplementary Fig. 3B). The leading edge was more tortuous compared with control ECs (Supplementary Fig. 3B). Finally, given the junctional alterations induced by Nova2 knockdown, we tested whether endothelial permeability was also modified. We found that the permeability of confluent endothelial monolayers was indeed increased upon *Nova2* depletion (Supplementary Fig. 3C).

To comprehensively identify AS events regulated by Nova2 in the endothelium, we performed high-throughput RNAseq of two biological replicates of *Nova2* knockdown and control ECs. We used *vast-tools*[32] to identify and quantify all major types of AS events. *vast-tools* maps RNAseq reads to comprehensive sets of annotated and novel splice junctions to derive confident estimates of the percentage of alternative sequence inclusion in a given sample. We identified 365 AS events affected by Nova2 depletion, including 188 (51.5%) cassette exons (Supplementary Fig. 4, Supplementary Table 1 and see Supplementary Methods for details). Gene Ontology (GO) analyses of AS events predicted to generate alternative protein isoforms (41% of all AS events and 64% of cassette exons; Supplementary Fig. 5) showed a significant enrichment for genes involved in cytoskeleton and cell adhesion (including tight and adherens junctions, and integrin binding), consistent with the phenotypes described above (Supplementary Table 2). In addition, the strongest enriched functional terms corresponded to chromatin remodelling and regulators, suggesting a multilayered impact of Nova2 regulation on endothelial formation. Finally, we also observed multiple GO terms related to neuronal differentiation and function (for example, neurogenesis, synapsis, axon part and calcium transport), similar to those reported for Nova-regulated genes in the brain[16]. Indeed, comparison of differentially included cassette exons in genes expressed in both neurons and ECs (see Supplementary Methods) revealed a highly significant overlap between alternative exons predicted to be regulated by Nova proteins in the brain[33] and those showing changes in inclusion levels upon *Nova2* knockdown in the endothelium ($P = 1.93e^{-11}$, hypergeometric test; Supplementary Fig. 6; Supplementary Table 3), despite the very different approaches used in the two studies (see Supplementary Materials for details).

These newly identified Nova2 targets expanded the list of previously known targets involved in apical–basal polarity, actin polymerization dynamics and cytoskeletal remodelling, important processes associated with cell polarity, cell shape, motility and adhesion (Supplementary Tables 4 and 5). Thus, to confirm a possible molecular link between these AS events and the phenotypes described above, we analysed AS changes in selected targets using RNA extracted from *Nova2* knockdown ECs (Fig. 4a). Reduced Nova2 expression in ECs resulted in altered AS of transcripts encoding *Par3*, and regulators of Par activity or localization, including *Magi1*, which recruits Rap1 at junctions[34], *Rap1GAP* (Rap1 inhibitor)[35], *Pix-α*, *Dock6*, *Dock9* and *DBS* (Cdc42 activators)[36–38] (Fig. 4a and Supplementary Fig. 7). Importantly, these AS changes parallel to those observed in the brain of *Nova2*-null mice[16,33], or in two stages of brain development (Supplementary Fig. 8A) characterized by different Nova2 expression levels (Fig. 1a). We confirmed the Nova2-dependent AS of target transcripts in RNA samples extracted from *Nova2*-overexpressing ECs (Fig. 4a, Supplementary Fig. 7 and Supplementary Fig. 8B). Notably, the direction of the observed AS changes is consistent with the position of Nova2-binding sites (YCAY; Fig. 4a and Supplementary Fig. 7), as previously reported[16].

Our data suggest that in cultured ECs Nova2 establishes EC polarity by controlling Par3 localization and by regulating the activity of Cdc42. To begin to address the functional relevance of the AS events regulated by Nova2 in ECs, we focused on the Cdc42 activator Pix-α (ref. 36). Our results indicate that Nova2 promotes the production of a specific Pix-α AS isoform lacking exon 17 (Pix-α-Δ17; Fig. 4a). Intriguingly, we found that Pix-α-Δ17 is more efficient than the Pix-α isoform containing exon 17 (Pix-α-FL) in rescuing the defect of Cdc42 activity caused by *Nova2* knockdown (Fig. 4b).

Collectively, these results provide evidence that Nova2 is required for EC polarity and that it acts by inducing AS of a set of key effectors of cell polarity.

Nova2 promotes vascular lumen formation *in vivo*. Since EC polarity regulates vascular lumen formation, we tested whether vascular development was affected in the absence of Nova2. To this end, we investigated the role of Nova2 in the embryos and larvae of zebrafish, which constitutes a unique and powerful model to study vertebrate vascular development[39]. Importantly, the Nova2 RNA-binding domain is 94% identical between zebrafish and human[40], and we found that a zebrafish *Nova2* orthologous gene (*nova2*) is expressed in the vasculature during development in addition to CNS (Fig. 5a). To assess the role of *nova2* in zebrafish, we performed a morpholino-mediated knockdown of its expression. To specifically visualize the developing blood vessels, we injected morpholino oligos into transgenic embryos expressing the enhanced green fluorescent protein (*EGFP*) gene under the control of the endothelial-specific promoter *fli1a* (*Tg(fli1a:EGFP)y1*)[41]. We used a morpholino targeting the start codon of zebrafish *nova2* (MO-nova2) to block translation of both maternal and zygotic *nova2* mRNAs. While embryos injected with a control morpholino displayed a normal morphology, more than 90% of *nova2* morphant embryos showed defects at the level of the forming blood vessels. Moreover, during early embryogenesis the pattern of some intersomitic vessels (ISVs) displayed extra-branching formation and a delay in the connection with the dorsal longitudinal anastomotic vessel (Fig. 5b). Confocal microscopy analysis confirmed that *nova2* knockdown resulted in altered lumen size of both cephalic vessels and of main trunk blood vessels (Fig. 5b,c). To visualize and further characterize the phenotype at the level of the dorsal aorta and posterior cardinal vein, we performed a morphological analysis using transversal paraffin sections stained with haematoxylin–eosin of embryos at different developmental stages (Fig. 5d). The analysis showed that in most of *nova2* morphants the lumen of the dorsal aorta had a larger diameter if compared with controls, through all developmental stages analysed. The lumen of the posterior cardinal vein appeared irregular along the length of the trunk, with areas of enlargement but also few restrictions (Fig. 5d). Furthermore, starting from 2 days of development, even ISVs display an enlarged lumen (Supplementary Fig. 9A). To better characterize the phenotype of *nova2* morphants at the level of ISVs during the angiogenic process, we performed an *in vivo* time lapse imaging assay. As shown in Supplementary Movies 1–3 in *nova2* morphant embryos the apical cells of some ISVs develop many more filopodia and could barely reach the dorsal longitudinal anastomotic vessel. Importantly, co-injection of *nova2* mutants with a morpholino-resistant zebrafish *nova2* mRNA rescued, in more than 60% of the injected embryos, the morphological phenotype of the vessels, confirming the specificity of the effects (Fig. 5b–d and Supplementary Movies 1–3). The abnormal phenotype of blood vessels observed in *nova2* morphants was not due to haemodynamic problems, since at 48 h post fertilization (hpf) the heart appeared normal for size, shape and beat (Supplementary Movies 4 and 5). In addition, EC proliferation and apoptosis were not significantly modified in the morphants (Supplementary Fig. 9B,C). Collectively, these data indicate that *in vivo* nova2 is required for proper vascular morphogenesis and for the formation of a correct vascular lumen.

Since Par complex members and regulators identified as Nova2 targets have putative orthologues in zebrafish, we analysed their AS using RNA extracted from *ctr* and *nova2* morphants. Of the investigated pre-mRNAs (Fig. 4a), four (*Rap1GAP*, *Pix-α*, *DBS*

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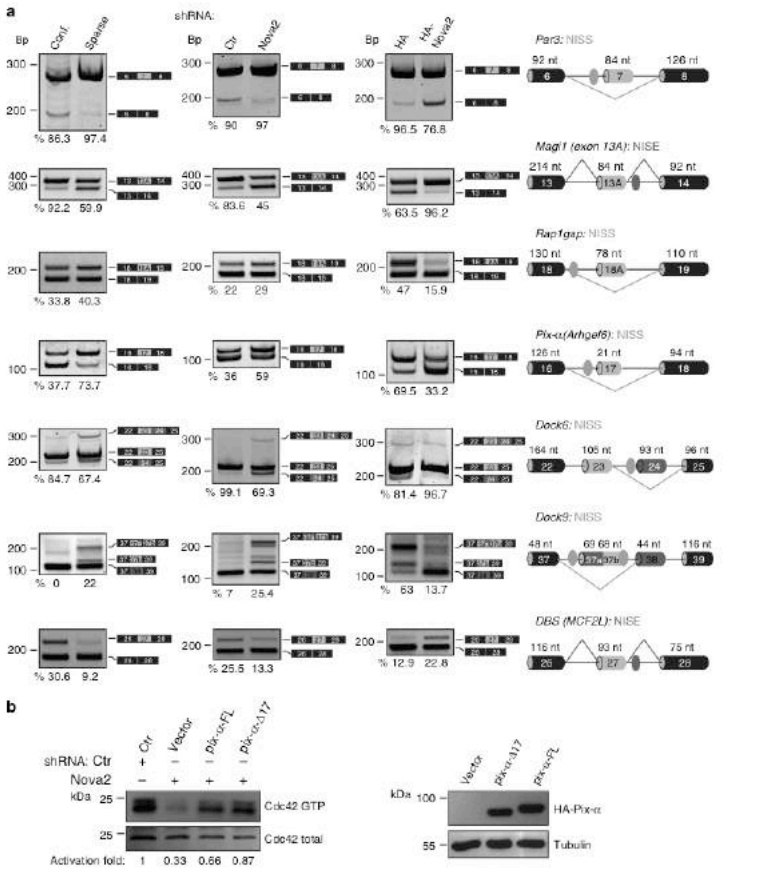
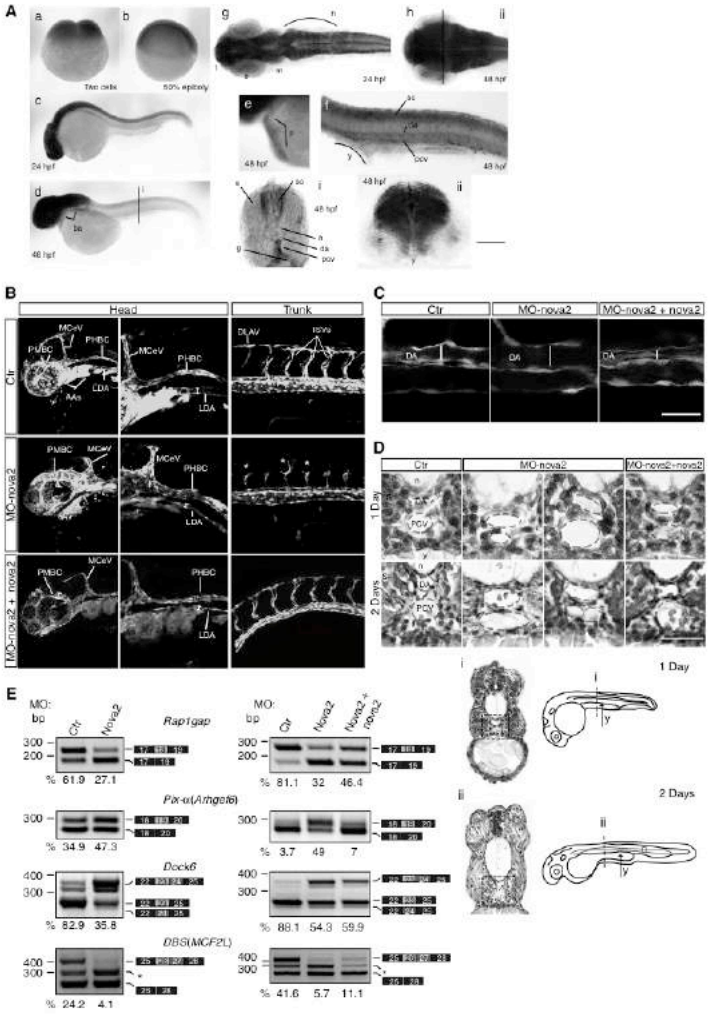


Figure 4 | AS changes in Nova2 overexpression and knockdown ECs. (**a**) AS of the indicated Nova2 targets as determined using RT–PCR in confluent and sparse mouse ECs (left), in confluent *Nova2* knockdown ECs (middle) and in sparse ECs overexpressing HA-tagged Nova2 (right). For each gene, the schematic representations of the genomic region containing the AS exon, the transcripts generated from skipping or inclusion of the AS exon and the calculated percentage of exon inclusion are indicated. For *Dock6*, the percentage indicates the ratio between the isoform containing exon 23 (skipping exon 24) and total, whereas for *Dock9* the percentage is the ratio between the isoform containing exon 37a plus 37b and total. Grey boxes, AS exons; black boxes, constitutive exons; blue/red dots indicate YCAY clusters predicted to function as Nova silencer/enhancer. Blue/red bars indicate Nova-silenced/enhanced exon inclusion. NISS, Nova intronic splicing silencer; NISE, Nova intronic splicing enhancer. (**b**) *In vitro* pull down of GTP-bound Cdc42 in *Nova2*-silenced ECs transfected with the indicated vectors driving the expression of HA-tagged Pix-α deleted of exon 17 (Pix-α-Δ17), Pix-α containing exon 17 (Pix-α-FL) or transfected with the empty vector (vector). The Cdc42 activation fold, calculated as the ratio between GTP-bound Cdc42 and total, is shown (*Ctr* sample used as a reference value). Pix-α isoform expression was analysed by using anti-HA and Tubulin antibodies.

and *Dock6*) are alternatively spliced in zebrafish (Fig. 5e). Interestingly, in all cases nova2 knockdown alters exon inclusion levels (Fig. 5e) in the direction predicted by the position of putative Nova-binding motifs[21] (Supplementary Fig. 10). Importantly, aberrant AS events of *nova2* morphants were rescued by co-injection of *nova2* mRNA (Fig. 5e). Of note, we

found that some nova2 targets (see, for instance, *Rap1gap* and *Pix-α*, Supplementary Fig. 9C) are not expressed at the same level in the different types of vessels suggesting that the effects of the knockdown of *nova2* may have different morphological and functional consequences along the vascular tree.

To assess the apical–basal cell polarity in *nova2* morphant embryos, we analysed the localization of Podocalyxin (Podxl2). As shown in Fig. 6a, in *ctr* embryos Podxl2 is localized at the apical region of the ECs forming ISVs, while in *nova2* morphants Podxl2 is mislocalized in ECs of the ISVs. Moreover, this defect



persisted at late developmental stage (Supplementary Fig. 9A). Likewise, altered Podxl2 localization was also observed in dorsal aorta of *nova2* morphants (Fig. 6a), suggesting an altered establishment of apical–basal polarity. Again, co-injection of *nova2* mRNA rescued Podxl2 localization in ECs (Fig. 6a).

Since Nova2 is expressed in neurons as well as in ECs, and these cells influence their reciprocal differentiation and development[42], it was important to address whether the vascular phenotype of *nova2* morphants was due to alterations in the nervous or vascular systems. To do this, we used a morpholino-resistant zebrafish *nova2* cDNA, fused with the red fluorescent protein mCherry, under the control of the vasculature-specific *fli1a* promoter (Fig. 6b). The vascular defects in *nova2* morphants were restored by the mosaic transient expression in the vascular endothelium of morpholino-resistant *nova2* cDNA (Fig. 6b–d). Moreover, by generating a zebrafish transgenic line, which stably expresses morpholino-resistant *nova2-mCherry* in the vascular endothelium, we found that injection of MO-nova2 did not significantly alter blood vasculature morphology (Fig. 6e), indicating that the vascular phenotype of *nova2* morphants is cell autonomous.

To independently validate our findings, we used CRISPRs genome engineering[43] to generate genetic *nova2* mutant fish (Supplementary Fig. 11A and see Methods for details). The characterization of *nova2* mutants strongly supported our results and conclusions obtained using MO-mediated *nova2* knockdown (Supplementary Fig. 11). In particular, *nova2* homozygous mutants displayed overlapping defects with *nova2* morphants, such as shortening of the anteroposterior body axis, reduced head size, curved trunk and slight pericardial oedema (Supplementary Fig. 11B). Moreover, we observed that *nova2* mutants do not develop the swim bladder and display a strong haemorrhagic phenotype visible both at the level of the head and trunk (Supplementary Fig. 11C,D). Notably, similar to Nova knockout mice[44,45], zebrafish *nova2* mutant embryos displayed paralysis. In particular, they have severe difficulty in swimming (probably because of motor–neuronal dysfunction) and died 7–10 days post fertilization, whereas there was no abnormal phenotype in *nova2* heterozygote mutant embryos. Confocal microscopic analyses performed on the *nova2* mutants transgenic for *kdrl:EGFP* showed that many blood vessels had an enlarged lumen size, both in the head and in the trunk (Supplementary Fig. 11E), as observed in *nova2* morphant embryos. Similar findings were obtained by histological analysis (Supplementary Fig. 11F). Finally, *nova2* mutants displayed AS changes (Supplementary Fig. 11G) that are comparable to those in *nova2* morphants.

Collectively, our results show that Nova2 controls the development of the vascular system *in vivo* by modulating endothelial polarity and lumen formation.

Discussion

Here we report that AS regulation orchestrates some important aspects of EC biology. In particular, our data demonstrate that the AS factor Nova2, known to be neural cell-specific[16], is also expressed in the vascular endothelium and plays a relevant role in vascular morphogenesis.

In spite of its importance, our current understanding of the mechanisms underlying vascular tubulogenesis is only beginning to be unravelled[23,25,29]. We have shown that Nova2 acts as a post-transcriptional regulator of the molecular mechanisms involved in the organization of the vascular lumen. In zebrafish, depletion and genetic knockout of *nova2* prevents proper formation of the lumen of blood vessels and also results in defects in EC polarization. Interestingly, in avascular organisms, such as *Drosophila melanogaster*, the Nova homologue (Pasilla, *ps*), is not expressed in the brain, but instead is expressed at high levels in salivary glands and several other non-neuronal tissues[46,47]. Notably, *ps* mutants have altered apical secretion and are characterized by developmental defects of the salivary gland including regions of lumen alteration[48]. This type of morphology, with a severely altered lumen, is somehow reminiscent of the morphology of the zebrafish vasculature observed on *nova2* depletion. Recently, several regulators of salivary gland lumen formation (such as Cdc42, Pak proteins and cadherins) have been identified in *Drosophila*[48]. Intriguingly, they are also implicated in vascular lumenogenesis[23] and, more importantly, regulators of their activity and localization are known Nova2 AS targets[33].

Our data show that, in cultured ECs, Nova2 establishes EC polarity by controlling Par3 localization, the activity of Cdc42 and the phosphorylated state of PKCζ. Notably, signalling downstream of Cdc42 is impaired in the absence of Nova2, as the active form of PKCζ and Pak4 are reduced in Nova2-depleted ECs. Interestingly, Pak4 phosphorylation correlates with EC lumen formation and RNA interference-mediated suppression of *Pak4* strongly inhibits these processes[26]. Similarly, depletion of *Nova2* impairs the establishment of EC polarity and the organization of the vascular lumen. These defects are associated with aberrant AS of pre-mRNAs encoding factors belonging to the Par polarity complex and its regulators.

Par complex and downstream effectors play important roles in regulating cell–cell adhesions[25], in controlling the organization of the microtubule cytoskeleton[49], and in promoting directional, collective cell migration[31]. Strikingly, we found that downregulation of *Nova2* affected the architecture of cell–cell boundaries and the behaviour of migrating ECs. In particular, in wound closure *Nova2*-depleted ECs failed to move in a cohesive manner with the lack of coordination in the direction of cell movement suggesting that, in addition to its role on lumen

Figure 5 | Nova2 is required for vascular lumen formation in zebrafish. (**a**) *In situ* hybridization of different zebrafish developmental stages showed *Nova2* expression in the pericardium (p in e), in the trunk vessels (f,i) of 48 hpf embryos and in the CNS of 24 (g) and 48 hpf embryos (h,i); scale bars, 250 μm in a,b; 125 μm in c,d,g,h); 50 μm in e,f; 25 μm for vibratome sections (i,ii). (**b**) Lateral views of the head and trunk of 28 hpf *Tg(fli1a:EGFP)y1* embryos, expressing the EGFP under the endothelial-specific promoter *fli1a*, injected with control (*ctr*) or morpholino against *nova2* (MO-nova2). *Nova2* knockdown results in lumen defects of lateral dorsal aorta (LDA), middle cerebral vein (MCeV) and primordial hindbrain channels (PHBC; compare red and/or white bars length between *ctr* and *nova2* morphants). The ISVs display extra-branching formation (*) and a delay in the connection with the dorsal longitudinal anastomotic vessel (DLAV). Co-injection of a morpholino-resistant zebrafish *nova2* RNA (MO-nova2 + nova2) rescues the vascular defects. (**c**) Confocal analysis of the blood vessels in the trunk region of 28-hpf embryos (lateral views); *nova2* morphants display enlarged lumen (white bars) of the dorsal aorta (scale bar, 50 μm). (**d**) High magnifications of paraffin 10 μm transversal sections, stained with haematoxylin–eosin, of the trunk region of 1- and 2-day embryos (dashed black squares in i,ii) highlighted alterations of the lumen size of the dorsal aorta and PCV in more than 90% of *nova2* morphants. Abnormal phenotype was rescued by co-injection of *nova2* mRNA (scale bar, 50 μm). e, eye; g, gut; h, hindbrain; m, midbrain; n, notochord; s, somite; sc, spinal cord; t, telencephalon; y, yolk; AAs, mandibular arches; PMBC, primordial midbrain channel. (**e**) *Nova2* knockdown alters the AS of its targets (24 hpf) that is corrected by the co-injection of *nova2* mRNA (28 hpf). The percentage of exon inclusion is indicated (grey, AS exon); *Dock6* as in Fig. 4, *DBS* is the ratio 26 + 27/total. Asterisk, novel *DBS* AS variant containing 75 nt (exon 27b: ACGCAGGTCCTCACATCACTCTCACCCGAGTGAGATGGCTGAGCACTTC TAGTCTGTTGCAGACTAAACGCAGAG) of intron 27.

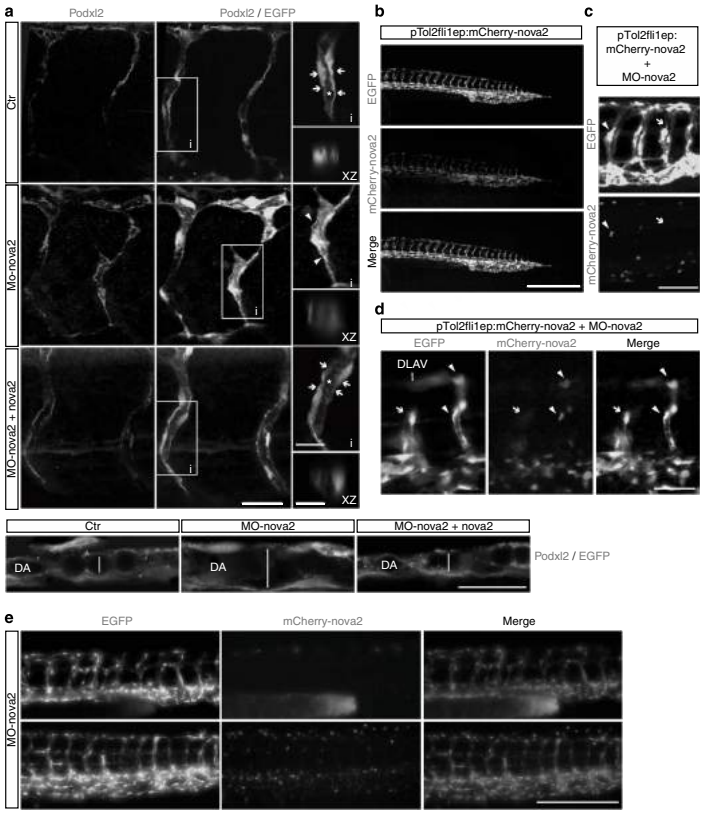


Figure 6 | Endothelial Nova2 is essential for EC polarization. (**a**) *Tg(fli1a:EGFP)y1* zebrafish embryos were treated with control (*ctr*) or *nova2* morpholino (MO-nova2) oligos and analysed with anti-Podocalyxin antibody (Podxl2, red) in the ISVs (upper panel). Co-injection of a morpholino-resistant zebrafish *nova2* mRNA was able to rescue the apical-membrane staining (arrow) of Podxl2 in *nova2* morphant (MO-nova2 + nova2; scale bar, 20 μm). Areas (i) are magnified on the right (scale bar, 10 μm). This is also visualized in the optical transverse sections (XZ, scale bar, 5 μm). Asterisk, lumen; arrowheads indicate Podxl2 mislocalization. Lower panel: Podxl2 localization in the dorsal aorta (scale bar, 50 μm). (**b**) Lateral view of the *Tg(fli1a:EGFP)y1* embryo with EGFP under the control of the endothelial-specific promoter *fli1a*. The same transgenic zebrafish embryos were co-injected at one-cell stage with a vector (pTol2fli1ep:mCherry-nova2) driving the expression of a morpholino-resistant zebrafish *nova2* mRNA fused to mCherry fluorescent protein under the *fli1a* promoter. In this case individual ECs were mosaically labelled with mCherry-nova2 expression (nova2, red) in the nucleus of some cells of the trunk blood vessels (scale bar, 250 μm). (**c**) Endothelial-autonomous rescue of *nova2* morphants. PTol2fli1ep:mCherry-nova2 plasmid was co-injected with the *nova2* morpholino oligo (MO-nova2) in one-cell stage *Tg(fli1a:EGFP)y1* embryos (bar 40 μm; arrowheads and arrows indicate vessels positive and negative for nova2, respectively). (**d**) ISV of the trunk expressing nova2 (arrowheads) showed a normal pattern and developed a correct lumen, whereas an adjacent ISV (arrows), negative for nova2 expression, was not formed properly, so that it failed to reach DLAV (scale bar, 30 μm). (**e**) Lateral view of 28-hpf transgenic embryos injected with the MO-nova2. Embryo showed in the upper row expresses in the vessel endothelium only GFP, whereas the embryo showed in the lower row expresses also a morpholino-resistant *nova2* cDNA fused with mCherry. The presence of *nova2* in the vessel endothelium is sufficient to preserve vessel morphology of *nova2* morphant embryos (scale bar, 50 μm).

formation, Nova2 expression might also control adhesion signals and the directional migration of ECs.

Our current lack of knowledge of the functional implications of most AS events makes it difficult to interpret the global impact of the AS changes we have identified. Moreover, it is plausible that additional AS events are regulated by Nova2 in ECs. Nevertheless, we found that the alteration in Cdc42 activity in *Nova2* knockdown ECs is preferentially reverted by overexpression of a specific AS isoform of Pix-α (Pix-α-Δ17) regulated by Nova2, indicating that AS of this gene plays an important role in Cdc42 activation.

We found Nova2-dependent AS regulation of zebrafish orthologous genes encoding polarity regulators. Among these, there are well-characterized activators (*Pix-α*, *Dock6* and *DBS*) of Cdc42, which—in turn—plays an essential role in controlling lumen formation *in vitro* and *in vivo*[26,48,50–52].

Remarkably, Nova2-regulated pre-mRNA targets encode proteins that interact with each other[16], suggesting that Nova2 regulates a network of apical–basal polarity regulators and that AS plays an important role in affecting physical interactions between these factors during the organization of the vascular lumen. Hence, the phenotypic changes that we observed on *Nova2* knockdown are likely the integrated effects of several AS changes that may act in a coordinated and non-redundant manner.

On the basis of the fact that Nova2 affects both neural and vascular cell processes, we suggest that Nova2 is a novel member of the 'angioneurins' family[5,6]. Interestingly, Nova2 is the only member of the angioneurin family defined so far that functions as post-transcriptional regulator. Malfunctioning or imbalance in angioneurin signalling contributes to various neurological disorders, indicating that non-neuronal defects contribute to these diseases[3]. In line with this, in Alzheimer's disease patients' vascular dysfunction can be observed before the onset of the disease, suggesting that vascular alterations might causally contribute to disease initiation or progression[53]. Notably, significant AS changes associated with decreased Nova activity were reported in Alzheimer's patients[54]. Since Nova2 is an autoimmune target in a severe neurodegenerative disorder paraneoplastic opsoclonus myoclonus ataxia (POMA)[55], an obvious question is whether POMA patients, in addition to displaying neurological symptoms, also develop vascular abnormalities.

Methods

Cell culture. ECs were isolated by dissection and dissociation with collagenase type I (Roche, 1.5 mg ml^{-1}), DNase (Roche, 25 μg ml^{-1}) at 37 °C for 1 h and by passing through a 40-μm cell strainer. ECs were immortalized by infecting them with a retrovirus expressing the polyoma middle-sized T antigen. VEC-null and VEC-positive were grown as sparse or confluent by placing 500,000 cells in 100- and 35-mm Petri dishes, respectively.

Culture medium of mouse ECs, VEC-null and VEC-positive ECs was DMEM (GIBCO) with 20% fetal bovine serum (FBS; HyClone), glutamine (2 mM, Sigma-Aldrich), penicillin/streptomycin (100 U l^{-1}, Sigma-Aldrich), sodium pyruvate (1 mM, Sigma-Aldrich), heparin (100 μg ml^{-1}, from porcine intestinal mucosa; Sigma-Aldrich) and EC growth supplement (5 μg ml^{-1}, made from calf brain; complete culture medium). HUVECs were isolated from umbilical vein by treatment with Collagenase (Roche, 0.1%, for 30 min at 37 °C) and were cultured in MCDB 131 with EC supplements.

Mouse ES cell culture. To obtain endothelial differentiation of mouse ES cells, cells were mildly trypsinized and suspended in Iscove's modified Dulbecco medium with 15% FBS, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 450 μM monothioglycerol, 10 μg ml^{-1} insulin, 50 ng ml^{-1} human recombinant VEGF-A165 (Peprotech Inc.), 2 U ml^{-1} human recombinant erythropoietin (Cilag AG) and 100 ng ml^{-1} human basic fibroblast growth factor (bFGF) (Genzyme). Cells were seeded in Petri dishes and cultured for 4 or 7 days at 37 °C with 5% CO_2 and 95% relative humidity.

Lentivirus production and transduction. GIPZ Lentiviral Nova2 short-hairpin RNAs (shRNAs) were obtained from Open Biosystems, while pLenti-GIII-CMV-humanNova2-HA from THP Medical Products. HEK293T (American Type Culture Collection, CRL-1573) cells were seeded in DMEM-HIGH supplemented with 10% FBS without antibiotics in 60-mm Petri dishes (one Petri per infection). The day after, cells at 60–70% confluence were transfected (calcium phosphate transfection method) using these quantities of DNA: 5 μg of packaging plasmid, 5 μg of envelope plasmid and 20 μg of Nova2 vectors. After 18 h, the medium was replaced with growth medium DMEM supplemented with 20% FBS and 1% penicillin/streptomycin. Cells were incubated for 24 h and the medium containing the lentiviral particles was harvested, filtered using a 0.45-μm filter unit and used to infect the cells.

For viral transduction, mouse EC cells were seeded in 100-mm Petri dishes and were infected at 70% of confluence. Cells were incubated overnight with the viral supernatant supplemented with 0.2 mM proline and polybrene (final concentration 8 μg ml^{-1}; Sigma). After 48 h puromycin selection (3 μg ml^{-1}) was started and it was continued until all non-infected control cells died (typically, 5 days).

2D culture. Mouse ECs transduced with lentiviral vectors carrying shRNA for Nova2 were seeded in 35-mm Petri dishes coated with Gelatin (Difco) 0.1% and cultured for 72 h. The splitting ratio was such that confluence was reached overnight after seeding. For immunofluorescence, ECs were fixed with 4% paraformaldehyde (PFA) and then permeabilized with 0.5% Triton X-100 for 10 min. Blocking (1 h), primary (overnight) and secondary (1 h) antibodies were diluted in PBS with 2% BSA. The following primary antibodies were used: anti-Par3 (1:100 Millipore), anti-Podocalyxin (1:100 R&D), anti-VE-cadherin (1:100 C-19, sc-6458, goat, Santa Cruz Biotechnology) and anti-β-catenin (1:50 BD Transduction Laboratories). Secondary antibodies for immunofluorescence were donkey anti-bodies to the appropriate species conjugated with Alexa Fluor 488, 555 or 647 (dilution 1:200 or 1:400).

For imaging, charge-coupled device camera on epifluorescence microscope (Leica) or Leica TCS SP2 confocal microscopy were used. ImageJ (NIH) was employed for data analysis. Figures were assembled using Adobe Photoshop and Adobe Illustrator. Only adjustments of brightness and contrast were used in the preparation of the figures. For comparison purposes, different sample images of the same antigen were acquired under constant acquisition settings.

Immunoblot analysis. Cells were lysed in Laemmli buffer and proteins were separated using SDS–PAGE and analysed with western blotting. The following primary antibodies were used: anti-Nova2 (1:200 Santa Cruz Biotechnology, C-16), anti-GAPDH (1:5,000 Abcam; 1:50,000 AbFrontier), anti-Tubulin (1:100,000 Sigma-Aldrich), anti-total-Pak4 (1:1,000 Cell Signaling), anti-phospho-Pak4 (1:1,000 Cell Signaling), anti-haemagglutinin (HA; 1:1,000 Covance), anti-total-PKCζ (1:1,000 Abcam), anti-phospho-PKCζ (1:1,000 Cell Signaling) and anti-Actin (1:500 Santa Cruz Biotechnology). The following secondary antibodies linked to horseradish peroxidase (Jackson ImmunoResearch) were used: anti-Mouse (1:10,000), anti-Goat (1:5,000) and anti-Rabbit (1:10,000). Immunostained bands were detected using the chemiluminescent method (Pierce).

Retinal immunohistochemistry. All animal work using mice was conducted in accordance with the Swedish Animal Welfare Board at the Karolinska Institutet, Stockholm, Sweden. Eyes retrieved from pups at P6 were fixed in cold 100% MeOH and stored at −20 °C before dissection. After dissection, retinas were incubated in 5% donkey sera, 1% BSA and 0.5% Triton X-100 in PBS overnight and then incubated with antibodies towards Nova2 (Santa Cruz Biotechnology, C-16), PECAM (BD Bioscience, MEC13.3) and ERG (Abcam, ab92513) overnight. For secondary detection, retinas were incubated with fluorescently conjugated antibodies (Jackson ImmunoResearch) and mounted flat with ProLong Gold (Invitrogen).

Immunohistochemistry. All procedures involving human samples were approved by the Istituto Europeo di Oncologia (IEO) Ethical Committee. When possible, a written informed consent for research use of biological samples was obtained from all patients, and the research project was approved by the Institutional IEO Ethical Committee. Immunohistochemistry (IHC) was performed using 3-μm sections from formalin-fixed and paraffin-embedded tissue samples. Samples were rehydrated through xylene and graded alcohols. Antigen retrieval was accomplished using 10 mM sodium citrate, 0.05% Tween20, pH 6.0. Samples were then incubated with 3% H_2O_2 for 5 min, followed by 30 min of blocking in 2% BSA and 0.05% Tween20, and then by the incubation with goat anti-Nova2 (1:100 Santa Cruz Biotechnology, C-16) overnight at 4 °C in 2% BSA and 0.02% Tween20. Immunocomplexes were visualized with LSAB+ System-HRP, DAKO (K0690) and acquired with the Aperio ScanScope system. Slides were counterstained with haematoxylin for histological evaluation. Double IHC was performed as follows: antigen retrieval was accomplished using 1 mM EDTA, 0.05% Tween. Samples were then incubated with 3% H_2O_2 for 5 min, followed by 30 min of blocking in 2% BSA and 0.02% Tween20, and then were incubated with a mix of goat anti-Nova2 (1:200) and mouse anti-CD31 (1:60 DAKO, Clone JC70A) in 2% BSA and 0.02% Tween20 for 2 h at room temperature. Immunocomplexes were visualized with an

anti-goat horseradish peroxidase (HRP) detection system (Goat-on-Rodent HRP-Polymer, Biocare Medical), for 30 min at RT, followed by incubation with a Goat Anti-Mouse AP Polymer detection system (MACH 2 Mouse AP-Polymer Biocare Medical) for 30 min at RT. CD31 was visualized in red using the Vulcan Fast Red Chromogen (Biocare Medical) and Nova2 was visualized in green using the Vina Green Chromogen Kit (Biocare Medical) according to the manufacturer's protocol.

Migration assay. To analyse cell migration, the wound-healing technique was used. Briefly, confluent EC monolayers on a tissue culture dish were wounded by manually scratching with a pipette tip after an overnight starving, washed with starving medium and incubated at 37 °C for 8 h in complete media containing Mitomycin C (4 μg ml^{-1}). The wound-induced cell migration was followed by staining with fluorescent phalloidin (10 μM, Sigma).

Paracellular flux. Mouse ECs were seeded on 0.4-μm pore size Transwell Permeable Supports (Corning) cultured in complete culture medium before assaying permeability. Next, fluorescein isothiocyanate dextran (70 kDa; Sigma) was added to the medium of the transwell apical compartment. At different times of incubation, a 50-μl aliquot of the medium was collected from the basal compartment, and the paracellular tracer flux was measured as the amount of fluorescein isothiocyanate dextran in the medium using a fluorometer.

3D culture in collagen gels. HUVECs were transduced with GIPZ lentiviral vectors (Open Biosystems) carrying shRNA for Nova2 or control shRNA. Control and *Nova2* knockdown HUVECs were cultured in 3D collagen gel. The final cell density in collagen (3.5 mg ml^{-1} final concentration collagen type1 from rat tail, High Concentration, BD Biosciences) was 5×10^5 cells ml^{-1}. Culture medium was 199 with 1% FCS, Insulin-Transferrin-Selenium supplement (Life Technologies), 50 ng ml^{-1} phorbol myristate acetate, 50 μg ml^{-1} ascorbic acid, 40 ng ml^{-1} VEGF and 40 ng ml^{-1} bFGF. For confocal microscopy, 190 μl cell suspension in collagen was used for each microwell (μ-slide 80826, Ibidi, Germany). 3D cultures were fixed with 3% PAF for 35 min, quenched with 75 mM NH_4Cl and 20 mM glycine in PBS, pH 8, for 10 min and blocked with 0.7% FSG and 0.3% Triton X-100 PBS (blocking buffer) for 30 min. Primary and secondary antibodies were incubated overnight at 4 °C. Primary antibody contained 5% donkey serum. Washes in blocking buffer were performed over the course of a day at room temperature. For immunofluorescence, the following primary antibodies were used: anti-Podocalyxin (R&D, 1:400) and anti-Coll IV (AbD Serotech, 1:200). Secondary antibodies for immunofluorescence were donkey antibodies to the appropriate species conjugated with Alexa Fluor 488, 555 or 647 (dilution 1:200 or 1:400).

Plasmids. Mouse Pix-α-FL fused to HA-tag was amplified with primers SG57-F and SG56-R (Supplementary Table 9) and was cloned in pcDNA3.1(+) vector (Invitrogen), whereas Pix-α-Δ17 was generated by PCR-mediated mutagenesis of Pix-α-FL (using primers SG55-F/-R and SG56-F/-R). All constructs were verified by sequencing.

RNA extraction and RT-PCR. Total RNA was isolated using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. After treatment with DNase (Ambion), 2–3 μg of RNA was retro-transcribed with mix of $d(T)_{18}$ oligos and random hexamers or gene-specific primers (Supplementary Table 10) and Superscript III RT (Invitrogen). An aliquot (1/20th) of RT was then PCR-amplified. For qPCR, an aliquot of the RT reaction was analysed with QuantiTect SYBR Green PCR (QIAGEN) by using LyghtCycler 480 (Roche). Target transcript levels were normalized to those of reference gene. The expression of each gene was measured in at least three independent experiments. All primers are listed in Supplementary Tables 6 and 7. AS bands were quantified using densitometric analysis.

Pull down of GTP-bound Cdc42. Nova2-depleted and control mouse ECs cultured in 100-mm Petri dishes were analysed for Cdc42 activity by using the Cdc42 Activation Assay Kit (Abcam) according to the manufacturer's instructions. For experiments with *Pix-α* minigenes, cells were transfected with Lipofectamine 3000 (Invitrogen) and grown to reach confluence before the beginning of the pull down.

Zebrafish strains and maintenance. Zebrafish (*Danio rerio*) from wild-type *AB* and transgenic *Tg(fli1a:EGFP)y1* (ref. 41) strains were maintained and bred according to the national guidelines (Italian decree '4 march 2014, n.26'). All experimental procedures were approved by the FIRC Institute of Molecular Oncology Institutional Animal Care and Use Committee.

***In situ* hybridization on zebrafish embryos and sections.** Zebrafish *nova2* cDNA was amplified using PCR (primers CG13F and CG13R in Supplementary Table 9) and cloned into pCRII-TOPO vector (Invitrogen). The antisense RNA probe was generated using T7 RNA Polymerase and digoxigenin-labelled UTPs (kit from Roche) and was purified with the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Whole embryos of different developmental stages were fixed overnight in 4% PFA, washed in PBS and pre-incubated for 2 h at 63 °C in hybridization buffer. Next, the *nova2* probe was added to the mix and incubated overnight at 63 °C. Embryos were then washed in SSC buffer and pre-incubated for 2 h in blocking medium at room temperature. A ratio of 1:2,000 anti-DIG antibody conjugated with alkaline phosphatase (Roche) was added and incubated overnight at 4 °C. After several PBS washes, embryos were incubated in NBT/BCIP staining buffer. Stained embeyos were then equilibrated in glycerol 85% in PBS overnight at 4 °C and were observed with a stereomicroscope equipped with optic fibres. To prepare 50-μm transversal sections of 48-hpf embryos, previously stained by *in situ* hybridization, we cut them in PBS with a vibratome after inclusion in 5% low-melting agarose. Sections obtained were equilibrated and mounted in glycerol 85% in PBS on glass slides and observed under a Nikon Upright microscope. All images were acquired with high-resolution digital cameras (Nikon).

To detect the mRNA expression of *Rap1gap* and *Pix-α/Arhgef6* genes and GFP, we use 5-μm paraffin sections of the head of 48-hpf *Tg(fli1a:EGFP)y1* embryos fixed overnight with 4% PFA in PBS. To orientate embryos in the proper way before including in paraffin and cutting microtome sections, we have pre-included them in 1% low-melting agarose (in PBS) under a dissecting microscope with optic fibers, using plastic base moulds of 7 mm of diameter. Paraffin was removed with xylene and sections were rehydrated through graded EtOH washes, permeabilized with Proteinase-K and HCl 2 N and were incubated overnight at 64 °C in hybridization mix with DIG-labelled LNA probes for *Rap1gap* (5′-TTCACCTCTT CACAGACAAGCT-3′) and *Pix-α/Arhgef6* (5′-TAGACGTAGAGGTGTTGG ACT-3′) (designed and produced by Exiqon). Sections were then treated overnight at 4 °C with an anti-DIG antibody (1:2,000 Roche) conjugated with AP and stained with NBT/BCIP. After several washes in PBS, embryo sections were incubated overnight at 4 °C with mouse anti-GFP antibody (Upstate, 1:200) and labelled using the *Vina Green Chromogen kit* (Biocare Medical).

Zebrafish haematoxylin–eosin staining and immunofluorescence. Agarose was used to embed 24- and 48-h zebrafish embryos before including in paraffin and cutting microtome 10-μm sections. Sections were stained with haematoxylin–eosin to assess the histological features. For immunofluorescence, paraffin was removed with xylene and the sections were rehydrated in graded alcohol. Antigen retrieval was carried out using preheated target retrieval solution (pH 6.0) for 45 min. Tissue sections were blocked with FBS serum in PBS for 90 min and incubated overnight with the following primary antibodies: GFP (1:100 Millipore), Phosphohistone H3 (PHH3, 1:100 Millipore), Caspase3 (1:100 Cell Signaling) and Podocalyxin (Podxl2 1:200, kindly provided by Heinz-Georg Belting). Alexa Fluor-conjugated antibodies were used for the immunodetection, and all sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and visualized using a Confocal microscope (Leica SP2).

Morpholino injections and RT-PCR of zebrafish embryo. Zebrafish embryos at one- to two-cell stage were injected with 6.2 ng of an ATG-morpholino antisense oligonucleotide (Supplementary Table 8), designed to block translation of the zebrafish *nova2* gene (ENSDARG00000017673). To analyse the kinetics and the pattern of formation of the ISVs, we mounted in 1.2% low-melting agarose in E3 water *Tg(fli1a:EGFP)y1*-dechorionated embeyos at 22 hpf, previously anaesthetized using 15 mg l^{-1} of tricaine (Sigma). Image acquisition was performed overnight every 10 min with a × 40 water immersion objective on a Leica TCS SP2 confocal microscope. Confocal stacks from each time point were processed for maximum intensity projections and, subsequently, movies were generated with the Leica LCS software. To observe and evaluate the shape and function of the heart, we mounted under a stereomicroscope 48-hpf dechorionated embryos in 3% methyl-cellulose in E3 water, previously anaesthetized using 15 mg l^{-1} of tricaine (Sigma). Using high-resolution digital camera (Nikon), we acquired 13-s movies from both control and *nova2* morphant embeyos. Total RNA was extracted from 24-hpf pooled embryos with TRIzol reagent (Invitrogen), purified with the RNeasy Mini Kit (QIAGEN) and retro-transcribed with $d(T)_{18}$ oligo or gene-specific primer and Superscript III RT (Invitrogen) and then analysed in PCR for AS modification of *nova2* targets. To rescue morphological and vascular defects due to the knockdown of *nova2*, we amplified with primers CG30F/CG13R (Supplementary Table 9) by using RT generated from *Tg(fli1a:EGFP)y1* embeyos, a morpholino-resistant zebrafish *nova2* cDNA, with six mismatches in the pairing region with the morpholino, which, however, do not alter the amino-acid sequence of the translated protein. We cloned this cDNA in pCRII-TOPO vector (Invitrogen) and we transcribed *in vitro* the capped mRNA using SP6 mMessage mMachine kit (Ambion). Co-injection in one- to two-cell stage embryos of 6.7 ng of MO-nova2 and 160 pg of each *nova2* mRNAs was performed. The molecular and morphological changes of *nova2* morphants were scored at 28 hpf.

Generation of new fish line for endothelial-specific rescue. To drive Nova2 expression selectively in the zebrafish vascular endothelium, we cloned the morpholino-resistant zebrafish *nova2* cDNA into the pTolfil1epCherryDest vector[36] in frame with mCherry at the N terminus under the endothelial *fli1a* enhancer/promoter flanked by Tol2 transposable elements (*pTolfil1:nova2-CherryDest*). Fertilized eggs from *casper* x *Tg(fli1a:EGFP)y1* mutant/transgenic line were injected

with 2 nl of a mixture containing 25 ng µl^{-1} of the circular plasmid *pTolfi1:nova2-CherryDest* and 25 ng µl^{-1} of T2 transposase mRNA. Injected embryos were selected for simultaneous expression of GFP and mCherry in the vessels, raised to adulthood and crossed with *casper* × *Tg(fli1a:EGFP)y1* fish. F1 embryos were observed under a fluorescent dissecting microscope from days 1 to 5 after fertilization to select carrier fish of the new mutant/double transgenic line *casper* × *Tg(fli1a:EGFP)y1* + *Tg(fli1:nova2-mCherry)*.

To assay the capability of *nova2* mRNA to rescue *nova2* morphant phenotype specifically in the vessel endothelium, we performed two experiments. Co-injection of 6.7 ng of MO-nova2, 50 pg of the rescuing DNA construct *pTolfi1:nova2-CherryDest* and 120 pg of Tol2 transposase mRNA was performed directly into the cytoplasm of one-cell stage *casper* × *Tg(fli1a:EGFP)y1* embryos. The phenotype of the vessels of the resulting 'mosaic' embryos was scored at 28 hpf. Alternatively, injection of 6.7 ng of MO-nova2 was performed into one-cell stage *casper* × *Tg(fli1a:EGFP)y1* + *Tg(fli1:nova2-mCherry)* embryos. The phenotype of all the vessels of the resulting embryos was scored at 28 hpf.

Immunofluorescence staining of zebrafish embryos. Zebrafish embryos from the *Tg(fli1a:EGFP)y1* strain at 48 hpf were dechorionated and fixed in 2% PFA in PBS, overnight at 4 °C. Embryos were then washed four times for 5 min in PBST (PBS + 0.1% Tween20). Permeabilization in PSBT + 0.5% Triton X-100 was performed for 30 min at room temperature. Embryos were then blocked in a solution of PBST + 0.5% Triton X-100, 10% normal goat serum and 1% BSA for 2 h at room temperature. Embryos were incubated with primary antibodies in blocking solution overnight at 4 °C. Successively, embryos were washed six times in PBST over 4 h at room temperature and then incubated with secondary antibodies in blocking solution, overnight at 4 °C. Embryos were washed finally six times in PBST over 4 h at room temperature and equilibrated in glycerol 85% in PBS. The following antibodies were used: mouse anti-GFP (1:2,000 Millipore); rabbit anti-podocalyxin (1:150 kindly provided by Heinz-Georg Belting); rabbit anti-mouse Alexa-488-conjugated IgG (1:400); and goat anti-rabbit Alexa 546-conjugated IgG (1:400). For the microscope analysis, we mounted on slides the trunk and tail regions dissected from five to six embryos of each samples. Images were taken with a Leica TCS SP2 confocal microscope, using oil-immersion objective × 40.

Generation of nova2 zebrafish mutant by CRISPR/Cas9. To identify the best target site and to design the single guide RNA (gRNA), we submitted to the ZiFiT targeter website (http://zifit.partners.org/ZiFiT) the sequence of the first exon of the zebrafish *nova2* gene. Oligonucleotides corresponding to the target region (see Supplementary Fig. 11A and Supplementary Table 11) were annealed and cloned into the pDR274 gRNA expression vector (Addgene, 42250) immediately upstream of the crRNA:trasRNA backbone. The *nova2* genomic target sequence starts with two GG nucleotides at the 5′ end for efficient transcription from the T7 promoter and ends with the protospacer-adjacent motif. The correct position of the target sequence into the pDR274 plasmid was verified through sequencing, using M13-Rev primer (Supplementary Table 11). The *nova2* gRNA was *in vitro* transcribed and purified from DraI-digested plasmid as template, using the Maxiscript T7 Kit (Life Technologies). Capped and polyadenylated *Cas9* mRNA was *in vitro* transcribed and purified from 1 µg of PmeI-digested pMLM3613 *Cas9* expression vector (Addgene, 42251) as a template, using the mMessage mMachine T7 ULTRA kit (Life Technologies).

A volume of 2 nl of a solution containing ~13 ng µl^{-1} of *nova2* gRNA and ~300 ng µl^{-1} of *Cas9* mRNA was co-injected in one-cell stage zebrafish embryos. On the next day, only embryos with a normal morphological phenotype were allowed to grow. To evaluate the efficiency of Cas9 nuclease activity, genomic DNA was extracted from 48-hpf single injected embryos and the *nova2*-targeted genomic locus was amplified from genomic DNA of each embryo (primers n2-locus-F and n2-locus-R in Supplementary Table 11). PCR products were then processed for the T7 Endonuclease I (T7EI) assay. PCR products were denaturated at 95 °C and rapidly re-annealed using a PCR thermocycler. PCR products were then incubated for 20 min with T7EI enzyme at room temperature, and the digestion products were visualized on 2% agarose gels. We found that 95% of the injected embryos had mutations at the level of the *nova2* target site.

To analyse the kinds of mutations obtained at the level of the *nova2* locus, we sequenced PCR products, cloned into pCRII-TOPO plasmid using the TOPO TA cloning kit (Life Technologies), amplified from genomic DNA extracted from fin-fragments of 2-month fishes. Among these fishes, we isolated those carrying the mutation also in the germline and subsequently we selected from their progeny fishes with nonsense or missense mutations in the *nova2* locus. A male carrying a nonsense mutation was crossed with a female from the *Tg(kdrl:EGFP)* line to generate a double *EGFP* transgenic *nova2* mutant line expressing the EGFP reporter in vascular ECs and allowing to characterize the vascular phenotype of *nova2* mutants.

o-dianisidine staining. For histochemical staining of haemoglobin, 72 hpf live embryos were incubated with o-dianisidine staining solution (40% ethanol, 0.01 M sodium acetate, 0.65% H_2O_2, 0.6 mg ml^{-1} o-dianisidine (Sigma, D-9143) for 15 min. Embryos were then washed with PBS, post-fixed in 4% PFA in PBS overnight at 4 °C and stored in 85% glycerol for microscope analysis.

RNAseq and splicing analysis. RNAseq was conducted on two control and two *Nova2*-depleted ECs. Samples were sequenced on Illumina HiSeq2500 (average of ~93 million, 100-nucleotide (nt) paired-end reads for each run). We employed *vast-tools*[32] to identify and quantify all major types of AS events, including single and multiple cassette exons and microexons, alternative 5′ and 3′ splice sites and alternatively retained introns, from each RNAseq sample. *vast-tools* map reads to comprehensive sets of exon–exon junctions (EEJs) and exon–intron junctions (EIJs) to derive alternative sequence inclusion levels (PSIs, 'Percent Spliced In', for exons; PIR, 'Percent Intron Retention', for introns)[32]. We then compared the two replicates of *Nova2* knockdown ECs versus control ECs. Differentially regulated AS events were defined as those showing an absolute ΔPSI ≥ 15 between knockdown and control means and a ΔPSI ≥ 5 between the ranges of the two groups. Only AS events with a minimum read coverage in all four samples were compared, which was defined as:

- For cassette exons (except for those quantified using the microexon pipeline, see Irimia *et al.*[32] for details): (i) ≥ 10 reads mapping to the sum of exclusion EEJs or (ii) ≥ 10 reads mapping to one of the two inclusion EEJs and ≥ 5 to the other inclusion EEJ.
- For microexons: (i) ≥ 10 reads mapping to the sum of exclusion EEJs or (ii) ≥ 10 reads mapping to the sum of inclusion EEJs.
- For intron retention: (i) ≥ 10 reads mapping to the sum of skipping EEIs or (ii) ≥ 10 reads mapping to one of the two inclusion EIJs and ≥ 5 to the other inclusion EIJ.
- For alternative 3′ and 5′ splice sites: ≥ 10 reads mapping to the sum of all EEJs involved in the specific event.

Additional filtering was used to remove intron retention events with a binomial *P* value score above 0.05 in any of the four samples[57].

This resulted in 365 differentially regulated AS events (Supplementary Fig. 4 and Supplementary Table 1), which were subdivided according to the predicted impact of *Nova2* knockdown on the coding sequence (Supplementary Fig. 5A): (i) AS events predicted to generate protein isoforms both when Nova2 is present or depleted (150, 41.1%); (ii) AS events predicted to trigger nonsense-mediated decay (NMD) or create a truncated protein when Nova2 is present (135, 37.0%); (iii) AS events predicted to trigger NMD or to create a truncated protein when Nova2 is absent (33, 9.0%); (iv) AS events in noncoding regions (40, 11.0%); and (v) AS events not able to be categorized into previous categories (7, 2%).

GO analysis was performed for each of the subdivided groups using ClueGO[58]. The background reference was based on multiexonic genes with the same minimum read coverage in the endothelium used above. Two categories had significant enrichment (*P* value < 0.05), those generating protein isoforms ((i) 49 terms enriched in 150 genes; Supplementary Fig. 5B) and those predicted to trigger NMD/disrupted proteins when Nova2 is included ((ii) 15 terms enriched in 135 genes; Supplementary Fig. 5C). Detail of *P* values (as calculated by ClueGO) are found in Supplementary Table 2.

To compare AS events that show differential inclusion on *Nova2* knockdown in ECs to those regulated by Nova proteins in neural cells, we used the 325 cassette exons previously predicted to be Nova targets in the brain[33] (Supplementary Table 3). Nearly all (319/325) exons could be matched to *vast-tools* AS event IDs based on the exact splice site coordinates and official gene symbols. Of these, 195/319 (61%) had enough read coverage to confidently derive inclusion estimates (PSIs) in all four EC RNAseq samples. From these, 28/195 (14.4%) exons showed a ΔPSI ≥ 10 on *Nova2* knockdown in ECs in the same direction as previously predicted for Nova proteins in the brain, compared with only 2/195 (1%) in the opposite direction (Supplementary Fig. 6). This overlap between Nova-regulated exons in endothelial and neural cells is highly significant ($P = 1.93e^{-11}$, hypergeometric test; background event set corresponded to 14,970 cassette exons with a minimal read coverage in the endothelium as described above and in genes with a cRPKM > 2 in neurons[19]). Moreover, several methodological differences suggest that the actual overlapping of Nova2 regulation between endothelial and neural cells may be even higher. First, predictions of Zhang *et al.*[33] include Nova1 and Nova2 targets, which may not be fully redundant. Second, exons described in Zhang *et al.* are predicted to be Nova protein targets based on Nova binding and presence of binding sites, among others. However, only half of the full set of Nova targets shows measurable differences in PSI in Nova knockout mice[33]. Finally, Nova2 proteins may use different cofactors that may be differentially expressed between endothelial and neural cells.

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Author contributions

C.G.[1,2] and S.G. designed and performed *in vitro* experiments; G.D., V.Q., E.B. and E.P. performed *in vivo* experiments in zebrafish embryos; D.N. performed analysis in mouse retina; S.C. and G.B.[6] performed the IHC analysis and discussed the results with PPDF; S.R., A.D.M., G.B.[3], D.P., E.F.[3], C.T., F.O. and E.F.[7] performed the experiments and discussed the results; C.D.R.W., M.L. and B.J.B. performed bioinformatic analysis and discussed the results; C.G.[1] and E.D. conceived and designed the study and wrote the manuscript.

Additional information

Accession codes. RNASeq reads are available in the NCBI Sequence Read Archive, under Bioproject code PRJNA283346.

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EMT and stemness: flexible processes tuned by alternative splicing in development and cancer progression

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Abstract

Epithelial-to-mesenchymal transition (EMT) is associated with metastasis formation as well as with generation and maintenance of cancer stem cells. In this way, EMT contributes to tumor invasion, heterogeneity and chemoresistance. Morphological and functional changes involved in these processes require robust reprogramming of gene expression, which is only partially accomplished at the transcriptional level. Alternative splicing is another essential layer of gene expression regulation that expands the cell proteome. This step in post-transcriptional regulation of gene expression tightly controls cell identity between epithelial and mesenchymal states and during stem cell differentiation. Importantly, dysregulation of splicing factor function and cancer-specific splicing isoform expression frequently occurs in human tumors, suggesting the importance of alternative splicing regulation for cancer biology.

In this review, we briefly discuss the role of EMT programs in development, stem cell differentiation and cancer progression. Next, we focus on selected examples of key factors involved in EMT and stem cell differentiation that are regulated post-transcriptionally through alternative splicing mechanisms. Lastly, we describe relevant oncogenic splice-variants that directly orchestrate cancer stem cell biology and tumor EMT, which may be envisioned as novel targets for therapeutic intervention.

Keywords: Alternative splicing, EMT, Stem cell differentiation, Cancer stem cells, Tumor progression, RNA binding proteins

Background

Epithelial cells are typically immobile cells, characterized by an apical-basal polarity with cohesive cell-cell junctions connecting adjacent cells in a continuous monolayer [1]. On the contrary, mesenchymal cells exhibit a motile and invasive phenotype by adopting an elongated shape with a front-back polarity [2]. Epithelial-to-mesenchymal transition (EMT) is a developmental program underlying the acquisition of mesenchymal properties by epithelial cells [3]. This process is fundamental during embryogenesis, when regulated migration of restricted population of cells is required for organogenesis [4]. In adult mammals, activation of EMT is mainly exploited in wound healing. However, this process is also reactivated by cancer cells to invade adjacent tissues and to disseminate toward distant organs, representing an essential step during progression of epithelial cancers to more aggressive stages [4]. Furthermore, EMT has also been involved in generation of cancer stem cells (CSCs) [5], the subpopulation of cells identified within leukemias and solid tumors as having self-renewal and expanding capability, thus contributing to tumor growth, metastasis and resistance to conventional therapies [6].

EMT relies on profound changes in gene expression that require multiple layers of regulation, from transcription, to post-transcriptional RNA processing, to translational and post-translational modifications. Although transcriptional regulation by EMT-inducing transcription factors (EMT-TFs), like members of the ZEB, SNAIL and TWIST families, is generally considered the master step in this process, mounting evidence indicates that post-transcriptional events

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strongly contribute to the fine-tuning of EMT [7]. Notably, post-transcriptional mechanisms of gene expression regulation have recently emerged as important tools exploited by cancer cells to acquire unique features that confer advantages over surrounding cells and sustain tumor malignancy [8]. In this regard, splicing of precursor messenger RNAs (pre-mRNAs) appears particularly suited to fine-tune regulation of gene expression because of its extreme flexibility.

It is clear that alternative splicing (AS) of pre-mRNAs plays an essential role in generating proteome diversity in cancer cells, through the production of splice-variants involved in key oncogenic pathways and resistance to chemotherapeutic drugs [9–11]. The advent of next generation sequencing and the development of highly specific bioinformatics tools have offered the possibility to study AS regulation with increasing detail. Through these approaches, a number of cancer-specific AS isoforms have been identified [12], paving the ground for their application in cancer diagnosis and as targets for selective anti-cancer treatments.

AS regulation modulates several molecular and morphological processes involved in EMT [13, 14]. Since AS is a versatile and powerful mechanism to both establish and maintain fundamental properties of different cell and tissue types [15, 16], it is not surprising that it contributes to promote the plasticity required for the EMT process and for establishing the stem-like properties that typify the more aggressive nature of neoplastic cells.

In this review, we offer a brief overview of EMT programs in development, stem cell biology and cancer progression. Subsequently, we assess the contribution of AS in EMT, describing interesting examples of both splicing factors and target genes, and presenting AS profiles that contribute to the dynamic transitional states between the epithelial and mesenchymal phenotypes in cancer. We also focus on the impact of AS regulation in cellular features that are directly related to the oncogenic potential of CSCs and provide examples of AS variants involved in acquisition and maintenance of stem cell-like features.

Main text

Epithelial-mesenchymal transition: a flexible tool for cell plasticity during embryogenesis

EMT was first characterized during embryonic development when a restricted population of epithelial cells differentiate into motile mesenchymal cells in order to form new tissues at specific sites, leading to the three-dimensional organization of developing organs [3, 4].

Differentiation of three embryonic layers during gastrulation of avian and mammalian embryos is the proto-typical example of a developmental program relying on EMT. Epiblastic cells of the primitive streak undergo EMT to move internally and generate the two inner layers of mesoderm and endoderm, while differentiation of the remaining epiblast generates the ectoderm [17]. EMT also promotes migration of neural crest cells from the epithelium near the dorsal midline of the neural tube towards prescribed embryonic regions where they differentiate to give rise to ganglia of the peripheral nervous system and other neural-derived cell types [18]. Interestingly, once their final target destination is reached neural crest cells re-aggregate through a reversible process of mesenchymal-to-epithelial transition (MET), which interrupts cell migration inducing these cells to form novel epithelial tissues [17, 19]. Notably, gastrulation and neural crest migration represent just two of the many examples of EMT/MET processes occurring during embryogenesis, since several rounds of reversible EMT and MET are necessary for proper embryo development [20].

Signals from multiple cues orchestrate the proper execution of EMT/MET cycles during embryogenesis. An example of signaling molecule involved in these programs is provided by WNT, whose signaling pathway promotes EMT and ingression of epiblastic cells from the primitive streak during gastrulation [21]. Furthermore, WNT acts synergically with other regulatory molecules, such as BMP4, for the induction of EMT in the migratory neural crest cells during their delamination from the neural tube [22]. These signal transduction pathways ultimately induce the expression of EMT-TFs [7]. Indeed, both gastrulation and neural crest cell migration require increased expression of SNAIL1 and SNAIL2 (also known as SLUG) [23, 24], which mediate repression of the epithelial adhesion protein E-cadherin, leading to the disruption of adherens junctions (AJ) and acquisition of a mesenchymal migratory morphology. Downregulation of E-cadherin is not sufficient to induce EMT phenotypic changes, and regulation of other adhesion molecules is often required. For example, repression of both E-cadherin and CAD6b coupled with upregulation of less adhesive type II cadherins, such as cadherin 7 and 11, is required during neural crest cell migration [25, 26]. Likewise, another EMT-TF, ZEB1, regulates the E- to N-cadherin switch occurring during the transition from the pre-migratory to the migratory state of the neural crest cells [27], an event necessary for activation of directional migration [28]. Another key step in EMT is the digestion of the extracellular matrix (ECM) of the basal membrane. This process allows the complete detachment of the cells from the original epithelial layer and their migration towards the novel site of destination. Degradation of the ECM is mainly mediated by membrane-bound and/or secreted forms of matrix metalloproteases (MMPs) [29], such as the MMP-2, which also contribute to EMT-driven events during embryogenesis [30].

Molecular processes involved in EMT

Epithelial integrity is ensured by specialized cell-cell junctions organized through the assembly of cell surface

protein complexes: adherens junctions (AJ), tight junctions (TJ) and desmosomes (DS) [31]. TJ are mainly responsible for the sealing of the epithelial layer and acquisition of apico-basal polarity [32]. Transmembrane proteins, such as MARVEL-domain proteins, occludins, claudins and junctional adhesion molecules (JAMs) mediate cell-cell adhesion, whereas cytosolic proteins (mainly zona occludens family members, ZO1/2/3) stabilize the junction by binding cytoskeleton components and providing the docking sites for polarity proteins (PAR3, PAR6, PALS1 and PATJ), signaling components (aPKC, CDC42, RAC and RHOA) and their regulators (RHOGEFs and RHOGAPs) [33].

AJ, similarly to DS, display cadherin clusters as core components [34]. Cadherins are transmembrane proteins that allow cell-cell adhesion among adjacent cells [35]. Both TJ and AJ are able to interact with the actomyosin machinery and this association plays critical functions for cytoskeleton organization and cell-shape remodelling [36]. Mechanistically, the link between the junction and actin or microtubule filaments is provided by catenins (β-catenin, p120 and α-catenin) [37]. Cadherin-catenin clusters facilitate the recruitment of cytoskeletal regulators and polarity proteins to the junctional complex [34, 38].

The prevailing models for EMT regulation propose that a sequential series of events are required for an epithelial cell to acquire mesenchymal features [7] (Fig. 1a, b). During the first step, TJ are disassembled by complete abrogation of occluding and claudin expression [39]. Together with the loss of the transmembrane backbone of the junction, the cytoplasmatic components (ZO1/2/3) diffuse away from cell-cell contacts [40]. In addition, loss of E-cadherin is another fundamental event in EMT [41]. Specifically, E-cadherin is degraded by proteolytic cleavage or through endocytosis from the plasma membrane [42, 43], whereas its expression is repressed (directly or indirectly) by EMT-TFs [44]. As result of E-cadherin disappearance from the cell membrane, catenins are free to move in the nucleus where they act as transcriptional regulators of specific mesenchymal genes [45].

Disappearance of apical-basal polarity is another strictly coordinated event in EMT, which involves both transcriptional repression [46] and re-localization of key cytoskeletal components to the leading edge of the cell. For instance, regulation of Par (PAR3/PAR6/aPKC) and Scribble (Scribble/LGL/DLG) complexes, which specify apical membrane identity, as well as of the Crumbs (PALS1/PATJ/Crumbs) complex, which specifies basal membrane identity, promotes a shift toward a front-rear polarity [47]. Simultaneously, lamellipodia, filopodia and invadopodia are formed by actin cytoskeleton remodeling mediated by the CDC42 and RAC signaling pathways [48]. Globally, these changes shift cell morphology toward a motile and invasive phenotype. Finally, expression of MMPs [29], which degrade the ECM, together with the appearance of mesenchymal markers (N-cadherin, Vimentin, Fibronectin, α5-Integrin) complete the transition to a motile cell that is able to colonize distant tissues [45] (Fig. 1a, b).

The acquisition of mesenchymal properties during EMT occurs progressively along an axis, wherein fully epithelial and mesenchymal cells represent the extreme edges [7]. This plastic and dynamic process comprises several intermediate states, including hybrid phenotypes in which cells concomitantly express epithelial and mesenchymal features [1, 49]. Importantly, cells carrying such hybrid epithelial/mesenchymal phenotype (referred as hybrid E/M) not only exert fundamental roles in embryogenesis, but also during cancer progression [50, 51].

Role of EMT in cancer

During malignant progression of epithelial cancers, tumor cells acquire an invasive and motile phenotype in order to invade adjacent tissues and disseminate toward distant organs. This metastasis formation process is responsible for approximately 90% of cancer mortality [52]. Notably, metastasis is a highly inefficient process. Indeed, it has been estimated that, from 10,000 tumor cells that enter the circulation, only one is able to develop a macroscopic metastasis [53]. Since tumor epithelial cells have cohesive cell-cell junctions that inhibit their movements, the transition toward a mesenchymal phenotype through activation of EMT has been proposed as a key step for tumor dissemination and cancer progression [3]. Although it was initially believed to occur in advance stages of cancer progression, supported by the positive correlation between tumor size and metastatic potential [54], it is now recognized that tumor dissemination and micrometastases can be found in early stages of the disease [55]. Accordingly, epithelial cells undergoing EMT have been found in pre-neoplastic lesions of pancreatic tissues [56]. As in the course of embryonic development, tumor EMT is a reversible process, and regain of epithelial features through MET can also occur at the final metastatic site [57].

Various cues in the tumor microenvironment are implicated in establishing an intricate network of interactions that activate the EMT/MET programs [58]. Cancer cells are associated with a large array of stromal cells, including fibroblasts, myoblasts, macrophages and lymphocytes, but also with endothelial cells and pericytes recruited to the tumor vasculature [59]. Paracrine and juxtacrine signals in such microenvironment include growth factors and cytokines [60]. In addition, oxidative stress, hypoxia and morphogenic (NOTCH and WNT) signaling pathways increase expression of EMT-TFs. The

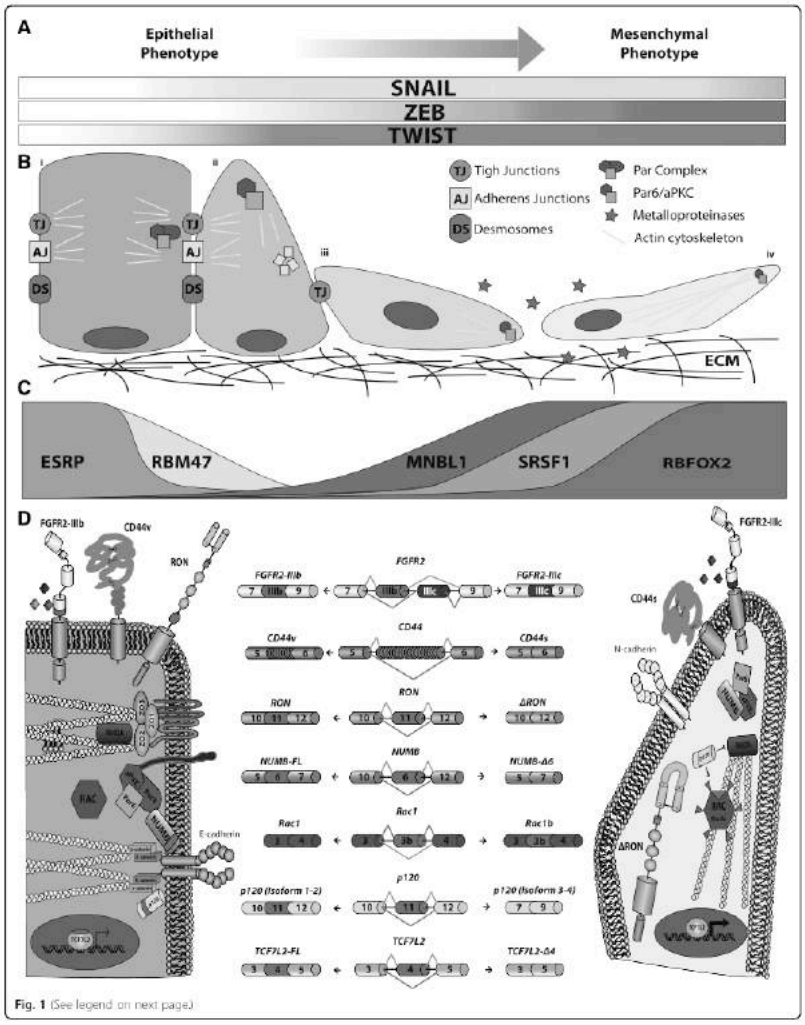

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Fig. 1 Significant alternative splicing changes occurring during EMT. **a** Key transcription factors upregulated during EMT; gradient color represents their expression increase from epithelial to mesenchymal phenotype. **b** Schematic representation of EMT progression. From left to right: (*i*) polarized epithelial cell with strong cell-cell junctions. Par complex and actin filaments localize to the junctions; (*ii*) epithelial cell with residual junctions starts to re-organize its cytoskeleton and change its morphology. E-cadherin disappears from cell membrane (*small yellow square*). The Par complex is disassembled and PAR6/aPKC move to the apical cell surface; (*iii*) the epithelial cell loses its epithelial features and begins to acquire an elongated and spindle-like morphology, while PAR6/aPKC, with other polarity complexes (not shown), allow the establishment of a front-rear polarity. Metalloproteases are secreted in order to degrade the ECM; (*iv*) a motile mesenchymal cell is able to invade the surrounding tissues. **c** Expression gradients of key splicing factors regulated during EMT. **d** *Center*. AS of genes involved in different EMT programs, including migration and invasion (*FGFR2*, *RON* and *CD44*), polarity and cytoskeleton organization (*NUMB*, *RAC* and *p120*) and transcription regulation (*TCFL2*). Alternative exons are represented in red, mutually exclusive exon in blue. *Left*. Scheme of epithelial-specific AS variants. Alternative exons and the encoded amino acids are indicated in red. *Right*. Mesenchymal-specific isoforms are also shown. Differences in functional properties of epithelial versus mesenchymal isoforms are highlighted: *FGFR2* exons IIIb and IIIc confer different ligand binding specificity; ΔRON and Rac1b are constitutively active cytoplasmic isoforms; inclusion of exon 6 in *NUMB* allows it to interact with Par complex and E-cadherin; p120 isoforms 1-2 localize to AJ, whereas p120 isoforms 3-4 localize with the activate RAC and repress RHOA signaling thus promoting re-organization of the actin cytoskeleton; skipping of exon 4 in *TCFL2* generates the more active transcriptional factor TCFL2-Δ4

combined action of these signals, together with the nature of the ECM components, induces cancer cells to adopt molecular and morphological features of either epithelial or mesenchymal identity [61]. EMT in cancer progression follows the same pattern described for physiological EMT programs, with disruption of cell-cell adhesion, loss of polarity and cytoskeleton reorganization, release of mesenchymal-specific MMPs (MMP-1, MMP-2, MMP-9, MMP-12 and MMP-13) and degradation of the ECM that allows invasion of the original tissue and dissemination [62–64]. Notably, high levels of MMPs in the tumor microenvironment affect both stromal and cancer cells. Stromal cells are induced to produce additional MMPs (MMP-7 and MMP-14), thus increasing the degradation of the ECM and promoting tumor invasion [65]. Moreover, MMPs can mediate the proteolytic cleavage of E-cadherin, generating extra-cellular E-cadherin fragments that increase motility [66]. Importantly, expression of different types of MMPs is associated with worse prognosis in several cancers, including ovarian [67], breast [68], gastric [69] and colorectal cancers [70].

EMT has also been linked to other aspects of cancer biology such as inhibition of cellular senescence [71] and chemoresistance [72, 73]. An interesting example is provided by ZEB1/2. These EMT-TFs are induced by TGF-β and repress the cyclin kinase inhibitors $p15^{INK4B}$, $p16^{INK4A}$ and p21, thus abolishing EGFR-dependent senescence in esophageal squamous cell carcinoma [74]. Similarly, TWIST cooperates with Ras signaling to prevent oncogene-induced cellular senescence through abrogation of p53- and Rb-dependent pathways [75]. Finally, reduced susceptibility to apoptosis during EMT is conferred by the action of EMT-TFs on survival pathways, mainly MEK/ERK and PI3K/AKT [76], and pro-apoptotic and anti-apoptotic genes, such as the Bcl2 family members [77].

Activation of EMT has been associated with chemoresistance in different tumor types. Enrichment of cells expressing mesenchymal markers has been detected in breast, colorectal and non-small lung cancers upon chemotherapeutic treatments [78–80]. In line with these observations, inhibition of EMT-TFs and post-transcriptional regulators of EMT was found to abrogate EMT-induced chemoresistance in breast and pancreatic cancer models [72, 73]. Chemoresistance might result from the combined activation of the many cellular processes involved in EMT and may be related to acquisition of stem-like features by cancer cells. High expression of the EMT-TFs ZEB1 [81], SNAIL1 and SNAIL2 [82] in cancer cells triggers the expression of stemness factors SOX2 [81], BMI1 and OCT4 [6, 81, 82]. Notably, mesenchymal and stemness traits are known to characterize the CSC subpopulation within the tumoral mass, which is responsible for tumor metastasis and resistance to conventional therapy [6]. Thus, EMT might revert the phenotype of terminally differentiated epithelial cells to a more plastic, mesenchymal phenotype that mirrors some properties of pluripotent embryonic cells during organogenesis.

EMT has been shown to be a transient process occurring only in a subset of cells at the invasive front of the primary carcinoma, usually associated with stromal components [83]. Nevertheless, hybrid E/M cells have been found in different tumors, including breast, ovarian and lung cancers [84–86] and in some tumor mouse models [56, 87]. Accordingly, circulating tumor cells (CTCs) with a fully mesenchymal state display lower metastatic potential compared to hybrid E/M cells that underwent a partial EMT [88]. A more heterogeneous expression of mesenchymal and epithelial markers is detected in CTC clusters, which are aggregates of 2-50 tumor cells held together through intercellular adhesions and recruitment of platelets [88]. CTC clusters are also characterized by a high metastatic potential taking advantage of both mesenchymal properties, which sustain cell motility and invasion [51], and epithelial features involved in extravasation and colonization propensity [89]. Notably, it was recently reported that also breast CSCs showing an hybrid E/M state, characterized as CD24− CD44+ ALDH+,

displayed the highest invasive ability [90]. These observations strongly suggest that maintenance of a transient epithelial-mesenchymal phenotype reflects an increased cellular plasticity, which allows acquisition and preservation of stemness traits by cancer cells. This hypothesis is also supported by several recent studies showing that, in addition to EMT, the MET pathway can also induce stem-like properties and increase metastatic potential in cancer cells. For instance, downregulation of EMT-TFs in prostate and bladder cancer cells was reported to promote expression of stemness factors and to enhance their growth as spheroids [91], the typical pattern of stem cell growth [92]. Similarly, silencing of PRRX1, a transcription factor that induces EMT, promotes the acquisition of stem cell properties by breast cancer cells, enhancing their self-renewal ability and growth in mammospheres [93].

Mechanisms of regulation of EMT: the emerging role of alternative splicing

EMT requires a robust reprogramming of gene expression [3]. Several EMT-TFs are activated early during EMT to either repress epithelial-specific genes or induce specific mesenchymal features. Epithelial-specific genes, such as *E-cadherin*, *claudins* and *occludins*, are repressed by SNAIL proteins (SNAIL1 and SNAIL2) [41, 94]. SNAIL1/2 bind to epithelial-specific promoters, recruit several epigenetic regulators such as PRC2 [95], HDAC1/2 [96], LSD1 [97], G9a [98] and SUV39H1 [99], and promote chromatin condensation [100]. Similarly, bHLH transcription factors (TWIST1 and TWIST2) and ZEB proteins (ZEB1 and ZEB2) are able to both repress epithelial genes and stimulate expression of mesenchymal-specific genes, such as *N-cadherin*, *fibronectin* and *matrix metalloproteases* [101–103].

However, the proteomes of mesenchymal compared to epithelial cells show significant differences [104] only partially explainable by functions of EMT-TFs. Mounting evidence suggests that post-transcriptional events, and in particular AS, significantly contribute to this diversity. Splicing occurs during transcription, and in some instances post-transcriptionally, when intronic regions are removed by direct interactions of the splicing machinery (the spliceosome) with short, poorly conserved, *cis*-acting sequence elements at exon–intron boundaries (donor or 5′ and acceptor or 3′ splice sites). This poor level of conservation allows high flexibility in splice site recognition, with frequent competition between sites showing variable strength [105, 106]. By using different combinations of donor and acceptor sites, more than 90% of human genes are able to generate different mRNAs through AS of selected exons and introns (Fig. 2a), thus yielding an estimated number of at least 100,000 different proteins [107]. Regulation of AS is modulated by the action of *cis*-acting elements (non-splice site RNA sequence elements) and *trans*-acting factors on the pre-mRNA. *Cis*-acting elements promote (splicing enhancers) or inhibit (splicing silencers) the usage (or definition) of variable exons by providing binding sites for *trans*-acting splicing regulators. *Cis*-elements can be found alone or clustered in introns (ISE/ISS, intronic splicing enhancer/silencer) as well as inside exons (ESE/ESS, exonic splicing enhancer/seilencer) (Fig. 2b) [105]. Serine-arginine (SR) factors and heterogeneous ribonucleoproteins (hnRNPs) are the two major classes of ubiquitously expressed *trans*-acting splicing factors [105]. SR proteins are highly conserved splicing regulators characterized by the presence of a C-terminus serine rich domain (the RS domain) implicated in protein-protein interactions [108] (Fig. 2c). By binding to splicing enhancers, typically purine rich motifs [109] through their RNA-recognition motifs (RRM), SR proteins usually promote exon recognition stabilizing spliceosomal components at exon–intron boundaries or antagonizing splicing repressor (Fig. 2b). However, SR proteins are also able to stimulate exon skipping suggesting that their activity is influenced by a complex network of interactions with the others RNA binding proteins (RBPs) expressed in specific cell types and/or development stages [110, 111]. Similar to SR proteins, hnRNPs have a modular structure with RNA-binding domains flanked by auxiliary domains with different functions and properties (Fig. 2c). Generally, hnRNPs bind to splicing silencers preventing the association of SR proteins or spliceosome components to alternative exons [112], thus leading to exon skipping (Fig. 2b). While SR proteins and hnRNPs are widely expressed across different tissues and cell types, other splicing factors display a cell-type-specific pattern of expression. To date, the best characterized mammalian tissue-specific AS regulators are NOVA1/2, PTBP2 (also known as nPTB or brPTB), SRRM4 (nSR100) and members of the RBFOX, MBNL, CELF, TIA, ESRP and STAR families (Fig. 2c). For some of these factors, the mode of action during the AS reaction is very peculiar since it depends on the position of their binding sites on pre-mRNA targets. For instance, NOVA1/2 proteins are able to promote exon inclusion when they bind to *cis*-acting elements (YCAY clusters) located in exons or near the 3′ splice site of the intron, while they promote exon skipping if their binding sites are located near the 5′ splice site [113]. The tissue-specific expression pattern of these splicing factors help establish the appropriate spatio-temporal generation of splice variants in many cellular and developmental processes [114, 115]. Since some excellent reviews have recently illustrated the general mechanisms of AS regulation, the reader is referred to them for additional insight [106, 116].

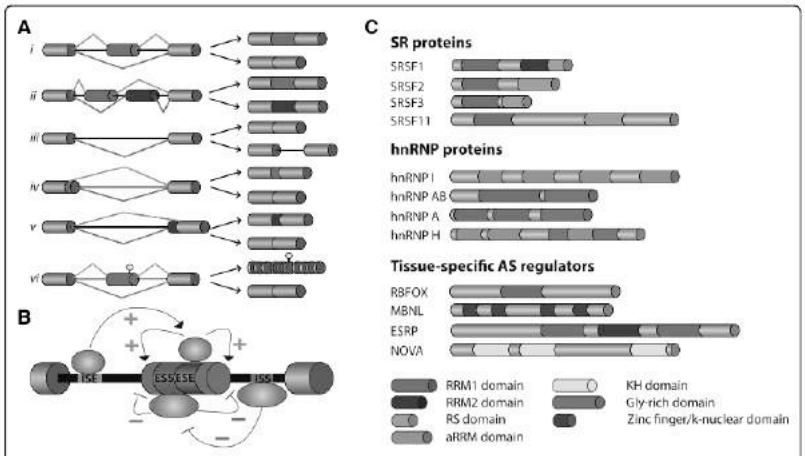

Fig. 2 Alternative Splicing regulation. **a** Scheme of the different AS modalities: (*i*) cassette exons; (*ii*) mutually exclusive exons; (*iii*) intron retention; (*iv*) alternative 5' splice sites; (*v*) alternative 3' splice sites; (*vi*) inclusion of a poison exon containing a premature stop-codon (*yellow*) leading to mRNA degradation through NMD. Precursor transcripts and final spliced products are shown. **b** AS regulation by combined action of *trans*- and *cis*-acting elements. Intronic and exonic splicing enhancers (ISE and ESE) promote the inclusion (+) of the AS exon (*red*) by providing the binding sites for activators (*orange circles*), whereas intronic and exonic splicing silencers (ISS and ESS) are bound by repressors (*yellow circles*) and promote exon skipping (-). Generally, ESE-bound SR factors stimulate the assembly of the spliceosome on the variant exon or counteract the inhibitory activity of hnRNPs bound to ESS elements. On the contrary, hnRNPs interfere with the assembly of spliceosome to the variant exon leading to exon skipping. In addition, hnRNPs by binding ISSs located in the introns flanking a variant exon cause its looping out and skipping, whereas when bound to ESSs they may polymerize along the exon and displace the ESE-bound SR proteins (not shown). **c** Some members of the SR and hnRNP families mentioned in the text are shown with their characteristic domains. SR proteins have a modular structure with one or two RNA recognition motifs (RRM) in the N-terminus able to interact with the pre-mRNA, whereas at C-terminus all members of this family present a domain of variable length rich in serine-arginine dipeptides (RS domain) involved in protein-protein interactions with spliceosomal components. HnRNPs possess one or more RNA-binding domains associated with different "auxiliary" domains that are diverse in sequence and involved in sub-cellular localization or protein-protein interactions. Tissue-specific AS regulators (RBFOX, MBNL, ESRP and NOVA families) are indicated with their own RNA-binding domains

Transcription and AS coordinately control different subsets of genes to generate the molecular and cellular complexity of cell and tissue types [15, 16, 106, 117]. Thus, it is not unexpected that AS also contributes to the dynamic (molecular and morphological) cellular conversion during EMT [118]. In line with this notion, expression of several splicing factors has been reported to be modulated during EMT [119]. Since each of them is able to regulate hundreds of pre-mRNA targets, it is likely that perturbation of their expression levels can simultaneously affect different aspects of EMT progression [7].

The ESRP splicing factors: key regulators of epithelial identity

A salient example of how EMT can be modulated by expression of specific splicing factors is provided by ESRP1 and ESRP2, two epithelial-restricted splicing regulators [120–122]. ESRP gain- and loss-of-function cells and genome-wide based approaches were used to characterize the ESRP-dependent epithelial splicing signature and its contribution to EMT [122]. These high-throughput approaches uncovered an important role of the ESRP-mediated RNA network in affecting exons of genes involved in RNA splicing, vesicle-mediated transport system, cell polarity, cell junction organization, motility and migration, regulation of small GTPase-mediated signal transduction and actin cytoskeleton [14, 121–123]. In addition, this analysis decoded the RNA map by which ESRP1/2 regulate AS. Indeed, similar to NOVA1/2 and RBFOX2 [113, 124], ESRP proteins display a positional effect and promote or repress exon inclusion depending on the locations of their binding sites (UGG-rich motifs) in RNA targets [14].

One of the best characterized ESRP targets is the *Fibroblast growth factor receptor 2* (*FGFR2*) pre-mRNA. ESRPs control mutually-exclusive regulation of two exons (IIIb and IIIc) encoding a protein domain with

critical roles in ligand binding specificity [120]. Splicing of these exons ensures the appropriate expression of FGFR2 isoforms and, as a consequence, the correct FGF/FGFR2 signaling during development. Moreover, altered splicing of exons IIIb and IIIc in *FGFR2* pre-mRNAs was found in primary tumors and metastases and it was associated with tumor plasticity [125]. ESRPs repress exon IIIc and increase inclusion of exon IIIb, leading to production of the epithelial-specific FGFR2-IIIb isoform. On the contrary, downregulation of ESRP proteins promote the inclusion of the mesenchymal-specific exon IIIc and, at the same time, induce molecular and morphological changes associated with EMT progression [120, 122]. In order to properly regulate AS of *FGFR2* pre-mRNAs, ESRPs cooperate with other widely expressed RBPs, including PTBP1 (hnRNP I), hnRNP A1, M, F and H [126–129]. Thus, the net outcome of *FGFR2* AS in any given cell depends on the specific repertoire of splicing factors expressed. These observations suggest that multiple cues could modulate this EMT-related splicing event by affecting expression or post-translational modifications of splicing factors involved in this regulation.

An interesting observation is that, in several cases, ESRP-regulated splice variants exhibit distinct and even opposing functions during EMT. The *p120* pre-mRNA splicing event that generates two variants (p120 isoforms 3 and 4) is able to promote cell-cell adhesion in epithelial cells by increasing p120 binding to E-cadherin in AJ [130]. In contrast, the mesenchymal-specific p120 isoform 1 induces cell migration and invasiveness by inhibiting RHOA-ROCK signaling pathway and stimulating RAC1 activity [131]. Another example of ESRPs target is *NUMB* pre-mRNA, which encodes for a factor involved in maintenance of cell polarity and cell-cell adhesion by binding to Par polarity complex and E-cadherin, respectively [132]. Through its N-terminal phosphotyrosine binding domain (PTB) domain, NUMB binds a conserved NVYY motif in the cytoplasmic portion of E-cadherin. Tyrosine phosphorylation of this motif abolishes NUMB/E-cadherin association, allowing NUMB to interact directly with the Par complex members PAR6 and aPKC [133]. Interestingly, 11 amino acid residues of the PTB domain are encoded by an epithelial-specific exon whose inclusion is controlled by ESRP proteins [122]. Skipping of this exon has been proposed to affect NUMB cellular membrane localization as well as its interaction with E-cadherin, resulting in loss of cell-cell adhesion [122].

ESRPs also regulate cell polarity through AS regulation of *SCRIB* transcripts [121]. SCRIB is a scaffolding protein required for epithelial cell identity and prevents EMT progression by blocking loss of E-cadherin and ZO1 from AJ [134]. In contrast with these roles, SCRIB knockdown has been associated with impaired cell migration and downregulation of mesenchymal markers [135]. The apparent antithetical functions of SCRIB in cell migration and EMT could be partially explained by the ESRP-dependent splicing of *SCRIB* pre-mRNAs, where SCRIB epithelial-isoform is required for AJ stability, whereas the mesenchymal-specific variant is involved in cell motility [136]. Splicing changes of ESRP target exons also affect actin cytoskeleton organization and its regulators. The *ENAH* gene generates an epithelial-specific splice variant, derived from inclusion of a small exon (exon 11A) encoding 21 amino acids in the C-terminal Eva/Vasp homology (EVH2) domain [137]. Downregulation of this variant was linked to tumor invasiveness in vivo [138], whereas a mesenchymal specific isoform lacking exon 6 (ENAH-Δ6) was associated with invasiveness in mesenchymal-like breast tumors [139]. In addition, ENAH interacts with ABI1, another ESRP target gene involved in actin cytoskeleton remodelling and cell-cell adhesion [140]. Remarkably, ESRP-mediated AS of *ABI1* pre-mRNA influences the sequence of the proline region domain important to mediate ABI1 association with several partners, including ENAH [140].

Other ESRP targets include cell membrane proteins such as integrins and receptors (KITLG, MPZL1, ITGA6, CD46, CD44) that are able to sense environmental signals, but also components of signaling pathways involved in EMT (MAP3K7, SOS1 and FYN) [122]. Moreover, ESRPs could act indirectly on expression levels of epithelial transcripts, as they stimulate inclusion of exon 4 of the *TCF7L2* transcription factor, thus promoting an isoform with reduced ability to activate β-catenin-target genes in epithelial cells [141]. Additionally, ESRP-mediated AS of *ITGA6*, *CD46* and *MAP3K7* variant exons causes introduction of premature stop-codons able to induce mRNA degradation through non-sense mediated decay (NMD) [122], a process known as alternative splicing activated NMD (AS-NMD) [142].

Many ESRP-regulated pre-mRNA targets encode proteins that interact with each other (Fig. 1c, d). This observation suggests that ESRPs control a network of epithelial regulators and that AS plays an important role in affecting physical interactions between these factors during activation of EMT programs. Hence, the phenotypic changes reported upon ESRPs knockdown are likely the integrated effects of several AS changes that may act in a coordinated manner. Considering the essential role of ESRPs in coordinating epithelial cell-type-specific AS programs, several groups have investigated how their expression level are regulated. Collectively, it was proposed that downregulation of ESRPs can be induced by transforming growth factor (TGF)-β-induced EMT [143, 144], epigenetic mechanisms [145] and gene mutations [146]. Notably, ESRP1 is among the most downregulated genes in different EMT experimental

models [119, 144, 147–149], indicating that its presence may represent an obstacle to acquisition of mesenchymal features. In line with this hypothesis, the EMT-TF ZEB1, which is upregulated in several human cancers [150, 151], directly inhibits ESRP1 expression, thus causing AS changes in the *CD44* gene [120]. *CD44* encodes a cell surface glycoprotein that binds different components of the extra-cellular matrix [152]. Repression of ESRP1 by ZEB1 promotes expression of a mesenchymal CD44 splice variant (*CD44s*) [153]. Importantly, switch from epithelial isoforms (CD44v) to CD44s was proposed to play a role in EMT [154]. Notably, ZEB1 downregulation was associated to a more invasive phenotype in lung cancer [153], suggesting that ZEB1-induced EMT and ESRP1-mediated splicing of *CD44* could contribute to initial transitions of the metastatic progression. On the other hand, increased expression of ESRPs is linked to better survival in colorectal cancer [155], whereas ESRP1 upregulation is proposed as a favorable prognostic factor in pancreatic ductal adenocarcinoma [156]. In addition, during squamous cell carcinogenesis expression levels of ESRPs seem to be very dynamic with their downregulation observed at the invasive front of the tumor and re-expression in lymph node metastases [157]. All these finding suggest that changes in expression levels of ESRP proteins and, as a consequence, the dynamic regulation of AS of their targets, could contribute to EMT plasticity during malignant transformation.

Other splicing factors involved in EMT/MET processes

In addition to ESRPs, other splicing factors contribute to EMT-associated AS changes [14] (Fig. 1c, d). RBM47, an RBP involved in pre-mRNA splicing, mRNA stability and RNA editing [158, 159], is downregulated during EMT. Notably, RBM47 regulates many splicing cassette exons in the same direction of ESRPs, suggesting a functional combinatorial co-regulation between these splicing factors to promote epithelial splicing patterns [14]. However, some AS events seem to be regulated with opposing effects by ESRPs and RBM47, thus indicating a more complicated pattern of interactions between these proteins during EMT.

If ESRP proteins are fundamental to establish an epithelial-specific splicing program, RBFOX2 and MBNL1 are important contributors of the mesenchymal splicing signatures [160] (Fig. 1c, d). In particular, expression of RBFOX2 is induced during EMT [119], whereas *Rbfox2* transcripts were found more abundant in normal mesenchymal tissues compared to epithelial ones [161]. Notably, RBFOX2 downregulation causes a partial reversion in cell morphology and motility towards an epithelial phenotype [119, 149] and these defects correlate with AS changes in an organizer of actin cytoskeleton (*Cortactin*), a polarity protein (*PAR3*) and a component of the vesicle-mediated transport system (*Dynamin 2*) [149]. Interestingly, some of the RBFOX2 targets (*NUMB* and *MAP3K7*), for which RBFOX2 promotes the production of the mesenchymal-specific isoform, are also regulated by ESRPs in epithelial cells in order to generate their epithelial-specific protein [122]. However, in other cases RBFOX2 was also found to promote epithelial splicing [149]. This scenario is further complicated by the recent observation that RBFOX2 cooperates with Quaking (QKI), an RBP of the STAR (signal transduction and activation of RNA) family [162], in the splicing regulation of common pre-mRNA targets [14].

MNBL1 is another RBP that regulates mesenchymal-specific AS profiles [119]. For instance, MNBL1 cooperates with RBFOX2 in generating mesenchymal isoforms of *ADD3* and *LRRFIP2* genes, whereas a competition between MNBL1 and PTBP1 is present in the case of *PLOD2* and *INF2* pre-mRNAs [161]. Interestingly, MNBL1 is also implicated in other aspects of RNA metabolism [163, 164]. It has been recently reported that MBNL1 promotes the mRNA stability of two genes involved in metastasis suppression (*DBNL* and *TACC1*) and this effect was linked to breast cancer metastatic colonization, a cancer type where MBNL1 expression was associated to metastasis-free survival [165].

Members of the SR family, such as SRSF1 and SRSF3, are also regulated during EMT and play a role in its progression [166, 167] (Fig. 1c, d). SRSF1 (historically known as SF2/ASF) is upregulated in many human tumors and its over-expression increases cell proliferation, delays apoptosis and is sufficient to transform human and mouse mammary epithelial cells in vivo and in vitro [168, 169]. Upregulation of SRSF1 occurs through different mechanisms acting at the transcriptional [170], post-transcriptional [171, 172] and post-translational levels [168, 173]. Notably, in the past our group has contributed to demonstrate that SRSF1 expression levels are dynamically controlled in epithelial and mesenchymal cells through AS-NMD of an intron in the 3′UTR of the *SRSF1* gene. In particular, AS-NMD of *SRSF1* transcripts, which is altered in colon cancer, is controlled through the STAR protein SAM68 [171], a RBP linked to neoplastic transformation and tumor progression [174, 175]. At post-translational level, SRSF1 activity is instead regulated through phosphorylation by kinases that are often upregulated in human cancers [176], such as SRPK1 [166] and NEK2 [177]. Upon phosphorylation, SRSF1 localizes to the nucleus [178] where it modulates AS of several genes involved in motility and invasiveness [10]. Among SRSF1 pre-mRNA targets, splicing of the proto-oncogene *RON* was the first example of an AS event linked to EMT activation [110]. RON is a tyrosine kinase receptor that activates a signaling cascade leading to cell dissociation, migration and matrix invasion [179]. Interestingly, the constitutively active ΔRON isoform,

generated through AS of a cassette exon of 147 nucleotides, is able to confer increased motility to the cell [180] and it is frequently over-expressed during tumor progression of epithelial cancers [110, 181]. SRSF1 stimulates skipping of exon 11 and promotes the production of ΔRON, which in turn activates the EMT program [110]. Importantly, ΔRON production is also promoted by hnRNP A2/B1, another hnRNP proteins involved in EMT and altered in several cancers [182, 183], whereas it is inhibited by hnRNP A1, which in this way activates the reversal MET program [184]. In parallel, the cancer associated ΔRON splice variant was analyzed as a potential target for the development of new anti-cancer therapeutic strategies. Bifunctional antisense oligonucleotides or small-molecule inhibitors of SRSF1 activity showed a positive effect in correcting *ΔRON* splicing toward an increase exon 11 inclusion [185]. Notably, in addition to preventing the production of the *ΔRON* isoform, inhibitors of SRSF1 activity were also able to affect the invasive phenotype of the cells [185]. Several additional splicing targets of SRSF1 have now been identified by RNA-seq in breast cancer cells [186]. Among them, SRSF1 stimulates the production of the constitutive active variant of the *Rac1* gene (called Rac1b), which is generated from the inclusion of a highly conserved cassette exon [187] and is characterized by an increased Rac GDP/GTP exchange activity [188]. Rac1b, expressed in several tumors [189], affects the EMT process in different ways: by increasing reactive oxygen species (ROS) and subsequently inducing the EMT-TF SNAIL [190]; by upregulating of the mesenchymal marker Vimentin [190]; or bypassing oncogenic induced senescence in lung and colorectal cancer [191, 192]. Interestingly, ESRPs contribute to repression Rac1b expression splicing in epithelial cells emphasizing, once again, the integrated effects of several AS factors to determine the epithelial or mesenchymal identity.

AS in stem cell differentiation

EMT represents a typical example of cellular plasticity, which promotes differentiation from one phenotype to another during developmental or pathological programs. The cell types displaying the highest extent of plasticity in our body are the stem cells. Thus, it is not surprising that these cells exploit molecular processes that amplify the flexibility and plasticity of their genome, like AS. Indeed, recent evidence has linked AS regulation to stem cell biology and some remarkable examples are reported below.

Stem cells are undifferentiated pluripotent cells, which are distinguished from other cells because of their ability to asymmetrically divide, to either self-renew themselves or to generate cells committed to differentiation towards a specific cellular lineage [193]. AS of specific genes can modulate the balance between self-renewal and differentiation in response to developmental or environmental cues, thus influencing the developmental potential of tissues and organs [194].

In the last decade, several studies based on high-throughput sequencing have uncovered genome-wide AS programs regulated during differentiation of pluripotent embryonic stem cells (ESCs) into different cellular lineages [195–197]. Moreover, widespread splicing variations have been also observed during differentiation of multi- and unipotent stem cells, as occurring during neurogenesis [198], hematopoiesis [199] and myogenesis [200, 201]. Notably, global changes in AS patterns also occur during the in vitro derivation of ESCs from the inner cell mass of blastocysts [202], suggesting that widespread AS reprogramming are not only required during the differentiation of stem cells, but also for the acquisition of their stemness features. This notion is also supported by high-throughput analyses of transcriptome changes during the cell reprogramming [203–205]. These analyses revealed that reprogramming of somatic cells to induced pluripotent stem cells (iPSCs) is accompanied by a progressive reversion of their splicing profile toward one that closely resemble that of pluripotent ESCs [203]. Intriguingly, orthologous genes display evidence of high conservation in the AS patterns activated during ESCs differentiation and iPSCs induction [160, 197, 206], further supporting an important evolutionary role of AS regulation in the biology of stem cells. Splicing changes occurring during iPSCs induction do not just reflect the phenotypic transition taking place, but they play an active role in reprogramming, as demonstrated by the ability of iPSCs specific splice-variants of the *Ccne1* and *Grlh1* to enhance acquisition of stemness by somatic cells [204, 207]. Importantly, the splicing program activated during iPSCs reprogramming is reversible, as iPCSs redifferentiation to somatic cells leads to re-establishment of the original somatic splicing profile [160]. Overall, these observations highlight the pivotal role of AS in the flexible and reversible regulation of gene expression operated by stem cells upon their switch between self-renewal and differentiation.

One of the major mechanisms by which AS regulates stem cells biology is the generation of splice-variants of key factors controlling the balance between pluripotency and differentiation (Fig. 3). In this regard, an interesting example is represented by the transcription factor FOXP1. Pluripotent ESCs and iPSCs exclusively express a specific FOXP1 splicing isoform (FOXP1-ES), which includes exon 18b and encodes for a protein isoform having different DNA binding properties with respect to the canonical factor expressed in differentiated somatic cells [197]. Differently from the somatic isoform, FOXP1-ES activates the expression of pluripotency genes, such as *Oct4* and *Nanog*, and its expression is

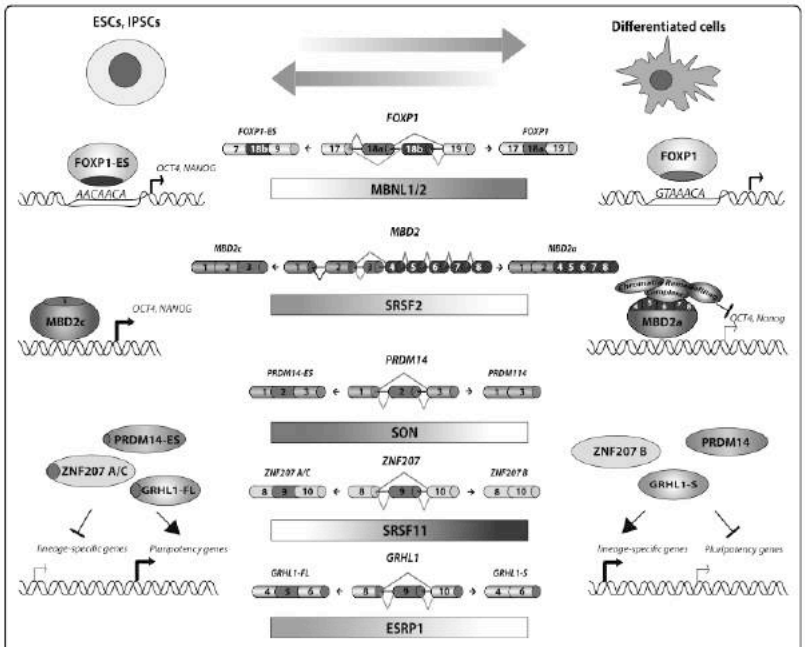

Fig. 3 Significant alternative splicing changes occurring during stem cell differentiation. *Center*. Splicing factors and AS of genes involved in somatic cell reprogramming; gradient color represents splicing factor expression increase/decrease from ESCs or iPSCs to differentiated cells. *Left*. Scheme of ESCs or iPSCs-specific AS variants. Alternative exons and the encoded amino acids are indicated in red. *Right*. Differentiated cells-specific isoforms are also shown. Differences in functional properties of pluripotent versus differentiated isoforms are highlighted. *FOXP1* mutually exclusive exons confer different DNA binding properties; *MBD2* AS variants *c* and *a* are both enriched at the promoters of Oct4 and Nanog, but only MBD2a is able to recruit chromatin remodeling complexes to repress pluripotency factors transcription; PRDM14-ES, ZNF207 A/C and GRHL1-FL enhance somatic cells reprogramming, whereas their AS isoforms, lacking the alternative exon, counteract reprogramming

critical for self-renewal and pluripotency of ESCs, as well as for efficient iPSC reprogramming [197] (Fig. 3). Likewise, pluripotent stem cells preferentially express MBD2c, an AS variant of the methyl-CpG binding protein MBD2a that is mainly expressed by differentiated cells [208]. While both proteins are enriched at the promoters of *Oct4* and *Nanog*, only MBD2a is able to interact with repressive chromatin remodelling complexes (Fig. 3). Accordingly, MBD2a overexpression negatively regulates transcription of core pluripotency factors in iPSCs, whereas MBD2c enhances somatic cells reprogramming [208]. Splice variants with different pluripotency capacity have been described also in other key transcriptional regulators of pluripotency, such as OCT4 [209] and NANOG [210], thus further highlighting the importance of AS in expanding the coding capability of transcriptomes in regulating stem cells biology.

AS may also affect proliferation and differentiation of stem cells by regulating steady-state expression levels of specific mRNAs. Indeed, recent advances in next-generation sequencing technologies have revealed that pervasive intron retention coupled to NMD and other nuclear RNA surveillance mechanisms control developmentally-regulated expression of selected gene subsets during differentiation of multi- and uni-potent stem cells, including neurogenic [211] and hematopoietic [212] lineages. For instance, during early

phases of neurogenesis, the splicing factor PTBP1 promotes intron retention of 3′-terminal introns within genes encoding for presynaptic proteins. Intron retention targets these transcripts to nuclear degradation and prevents their precocious expression during neuronal development. Then, the progressive decrease of PTBP1 expression during neuronal differentiation allows splicing of regulated introns, thus ensuring the appropriate developmentally controlled expression of target mRNAs [211]. Consistently with the great impact exerted by AS regulation in the control of the balance between pluripotency and differentiation of stem cells, different genome-wide RNA interference (RNAi) screenings identified several RBPs and RNA processing factors as key regulators of self-renewal properties of stem cells [203, 213–215]. In particular, search for critical genes required for the reprogramming of mitotic cells in iPSCs identified splicing factors SON [214] and SRSF11 [215]. Notably, these splicing factors behave as crucial players with opposite functions in the acquisition and maintenance of stemness. While SON enhances somatic cell reprogramming and positively regulates maintenance of stemness, SRSF11 acts as a repressor and negatively regulates the acquisition of the stemness phenotype. Both studies also revealed putative splicing targets involved in somatic cells reprogramming. SON regulates splicing of a critical pluripotency transcriptional regulator PRDM14, thus promoting a long isoform containing exon 2 that enhances reprogramming [214]. SRSF11 promotes exon 9 skipping in ZNF207, leading to a shorter isoform that counteracts reprogramming [215] (Fig. 3).

Bioinformatics analyses of potential regulators of the AS changes observed in stem cells and differentiated cells revealed additional splicing factors as critical regulators of the balance between self-renewal and differentiation [160, 205, 206]. Search for binding motifs enriched within AS events regulated between stem and somatic cells discovered MBNL1 and MBNL2 proteins as possible major regulators [206]. Accordingly, MBNL1 and MBNL2 are scarcely expressed in ES cells and actively repress stem-specific AS variants in differentiated cells [206]. In particular, MBNL1 and MBNL2 inhibit FOXP1-ES splicing [206] and their overexpression impairs iPSCs induction [160, 206]. Similarly to MBNL1/2, also RBFOX2 negatively regulates production of stem cell-specific splice variants and its overexpression inhibits somatic cells reprogramming [160] (Fig. 3). On the other hand, ESRP1 enhances reprogramming of somatic cells into pluripotent cells. Putative binding sites for this protein were found upstream of exons undergoing skipping during somatic cells reprogramming. As an example, ESRP1 promotes splicing of the longest isoform of *Grlh1*, which enhances cell reprogramming [205].

The importance of RBPs and splicing factors for the regulation of stem cell fate is also supported by knockout mouse models. For example, genetic ablation of *Ptpb1* causes embryonic lethality shortly after implantation [216, 217], while depletion of its paralog *Ptpb2* impairs the embryonic splicing program required for neuronal maturation [218]. Establishment and maintenance of the AS programs accompanying stem cell fate requires the controlled expression of the splicing factors that regulate these programs. Transcriptome changes occurring during ES differentiation or iPSCs generation revealed regulation in the expression of several RNA processing factors, including RBM47, Zcch4 beside the previously mentioned MBNL1/2, ESRP1 and RBFOX2 [160, 202, 206]. Notably, key transcription factors regulating pluripotency also control the expression of splicing factors with a role in stem cell biology. For instance, SRSF2, which enhances self-renewal of ESCs by promoting MBD2c splicing (Fig. 3) and OCT4 and NANOG expression, is in turn positively regulated by OCT4 [208]. The cross-regulation between SRSF2 and OCT4 suggests the existence of a positive feedback loop between splicing regulators and transcription factors that reinforces stemness features. Importantly, this regulatory loop involves both transcriptional and post-transcriptional regulatory mechanisms, as OCT4 not only binds the SRSF2 promoter, but it also negatively regulates the expression of miRNAs targeting its 3′UTR, such as miR-301b and miR-130b [208]. Moreover, OCT4 promotes the expression of miR-302 family members [208, 219], which specifically target the somatic-specific MBD2a isoform, but not the stem-specific MBD2c variant [208], thus further reinforcing a stemness positive feedback-loop. Additional evidence highlighting the existence of a crosstalk between transcriptional and splicing regulators in stem cell biology arises from a recent study showing that early epigenetic reprogramming occurring during iPSCs induction is functional to control the expression of several splicing regulators leading to activation of an AS program that is crucial for reprogramming [220]. The histone acetyltransferase GCN5 functions as an early mediator of the global epigenetic changes occurring during early phases of iPSCs induction. GCN5 cooperates with the reprogramming factor c-MYC in the regulation of its target genes, including several splicing regulators such as such U2AF1, TRA2B and SNRP70. Depletion of GCN5/c-MYC impacts on the AS program activated during the early phases of somatic cell reprogramming, particularly affecting genes involved in the regulation of cell adhesion and migration [220]. Notably, AS itself may also contribute to regulate the expression of splicing factors controlling stem cells proliferation and differentiation. For example, RBFOX2, which promotes ESC differentiation, directly regulates the steady-state expression levels of several other splicing regulators by AS-NMD mechanisms [124, 221].

Collectively, AS regulation represents an optimal tool to maintain stem cell plasticity and redefine developmental fate according to differentiation signals.

Alternative splicing regulation in CSCs

Acquisition of stem-like features in more aggressive cancer cells has been frequently correlated to the expression of oncogenic splice-variants produced as a consequence of aberrant AS regulation. For instance, widespread alteration in the splicing programs of leukemia stem cells (LSCs) compared to normal stem and progenitor cells were revealed by high-throughput screenings in both chronic myeloid leukemia (CML) [222] and acute myeloid leukemia (AML) [223]. Both studies also identified a global dysregulation in the expression of genes encoding for spliceosomal proteins and RNA processing factors, further suggesting that aberrant AS regulation may contribute to LSCs generation [222, 223] and that this may occur independently from oncogenic mutations in splicing regulatory genes that are frequently observed in different types of leukemia [223, 224]. Interestingly, it has been recently suggested that downregulation of the splicing regulator MBNL3 in LSCs enhances splicing of the CD44 v3 isoform, which positively regulates their self-renewal capacity [225]. As previously described, MBNL3 belong to a family of splicing regulators that promote ESC differentiation [206]. Thus, aberrant splicing events observed in CSCs may be correlated to re-activation of embryonic splicing programs [225], similarly to what described for the activation of the EMT pathway [226]. This hypothesis is consistent with the expression of other oncogenic/embryonic AS variants in cancer cells. For instance, PKM2 is the embryonic splice-variant of the *PKM* gene that promotes aerobic glycolysis and sustains cancer cells proliferation and metabolism [227]. Interestingly, one of the four transcription factors necessary for iPSCs induction, c-MYC [228], induces the expression of oncogenic splicing factors (PTBP1 and hnRNP A1/A2) in cancer cells, which in turn promote PKM2 splicing [229]. Notably, promotion of PKM2 splicing was recently shown to confer chemotherapeutic resistance in pancreatic cancer [230]. Conversely, the tumor suppressor RBM4 [231] promotes neuronal differentiation of human mesenchymal stem cells by enhancing PKM1 splicing [232], thus further suggesting that modulation of the embryonic splicing program might regulate acquisition and maintenance of stemness features.

Splicing events supporting stemness and proliferation of CSCs have been described for genes involved in different cellular functions, such as apoptosis, signal transduction and cell-adhesion. For example, LSCs were shown to express high levels of the anti-apopoptic splice variants of the *BCL-2*, *MCL1*, *BCLXL*, and *BFL1* genes [233], as well as an AS variant of the *GSK3-β* gene that increases LSCs self-renewal [234]. High expression levels of the splicing regulator PTBP1 in brain tumor cells lead to skipping of exon 6 in the *ANXA7* transcripts, generating a shorter isoform of this membrane protein, named isoform 2, which enhances EGFR signaling and promotes cell tumorigenicity [235]. A common splicing event in CSCs of different tumor types is the inclusion of the variable exons of the *CD44* gene. Expression of the CD44v variants is displayed in both LSCs [225] and CSCs of solid tumors, such as colon [236] and gastric [237] cancers, with each tumor type expressing one or more specific variable exons: v3 in LSCs, v6 in colon cancer and v8-10 in gastric cancers. Moreover, splicing of the variable exons of v8-10 has been shown to promote CSC-like features in prostate cancer cells [238] and to increase the invasive and tumorigenic potential of bladder cancer cells [239]. Several splicing factors have been shown to enhance splicing of the CD44 variable exons in cancer, such as SAM68 [240], RBM3 [238] and ESRP1 [120], suggesting that regulation of their expression or activity may underlie CD44 splicing control in CSCs. Intriguingly, CD44v splice variants represent a marker of CSCs even though they are considered epithelial isoforms. Indeed, as aforementioned, the switch from a CD44v toward a CD44s splicing pattern under the control of ESRP1 has been correlated with the EMT of both mammary [154] and bronchial epithelial cells [153]. However, expression of epithelial markers by stem cells is not completely surprising, as a MET phase occurs also during reprogramming of somatic cells into iPSCs [241]. It is thus conceivable that expression of CD44v in CSCs is functional to the re-establishment of an epithelial phenotype, which allows engraftment of cancer cells in the site of secondary lesions during metastasis. Moreover, considering the high heterogeneity in CD44 isoforms expressed by CSCs, which has been documented in breast cancer [242], it is also plausible that regulation of *CD44* splicing may allow CSCs to maintain the hybrid E/M state that has been correlated with higher stemness and tumorigenicity [51, 243]. Regulation of *CD44* splicing clearly demonstrates the great impact that this post-transcriptional regulatory mechanism exerts on CSCs biology, paving the way for further studies aimed at identifying new splice variants and splicing regulators that may represent valuable targets for new approaches interfering with CSCs phenotypic plasticity.

Conclusions

Epithelial and mesenchymal cells, as well as pluripotent and differentiated cells, represent extreme edges of tightly regulated processes: EMT and stem cell differentiation, respectively. In cancers, EMT is linked to metastasis formation as well as CSC generation and maintenance. Tumor populations are highly heterogeneous. Indeed, not all cancer cells are able to undergo EMT at the same time and not all cells

that have activated an EMT program become competent to form metastasis. Tumor heterogeneity is further increased by the existence of epithelial/mesenchymal hybrids in highly metastatic CTCs and CSCs. Together these findings strongly suggest the importance of cellular plasticity for the acquisition of both invasive capabilities and stemness traits.

High-throughput approaches have recently documented remarkable changes in AS profiles of specific genes during activation of EMT programs and CSC generation. Frequently, such alterations are caused by changes in the expression levels of *trans*-acting factors. These analyses point out that AS provides an additional and extremely flexible layer of regulation to rapidly control temporal and spatial expression of protein isoforms, thus shaping cell- and tissue-identity. Importantly, AS variants orchestrate several important aspects of the EMT process, including cell-cell contacts, polarity and cytoskeleton organization, and CSC self-renewal and differentiation. Moreover, the pivotal role of AS regulation in tumor plasticity is underscored by the observation that this mechanism rapidly shifts the expression of protein isoforms with opposite functions. Finally, the recent optimization of antisense oligonucleotides-based approaches to selectively control splicing switches [244–246] suggests that AS variants specifically expressed during tumor EMT and in CSCs could represent valuable diagnostic or therapeutic options for anti-cancer purposes in the near future. However, although an enormous work in the field has already been done, the examples that we have discussed likely represent just the tip of the iceberg, and much more remains to be uncovered in order to draw a more realistic picture. Thus, future studies are warranted to fully elucidate the real contribution of AS regulation to cancer progression.

Abbreviations

AJ: Adherens junctions; AML: Acute myeloid leukeia; AS: Alternative splicing; AS-NMD: Alternative splicing - non-sense mediate decay; CML: Chronic myeloid leukemia; CSCs: Cancer stem cells; CTCs: Circulating tumor cells; DS: Desmosomes; ECM: Extra-cellular matrix; EMT: Epithelial-to-mesenchymal transition; EMT-TF: EMT-transcription factor; ESCs: Embryonic stem cells; iPSCs: Induced Pluripotent Stem Cells; LSCs: Leukemia stem cells; MET: Mesenchymal-epithelial transition; MMPs: Metalloproteases; RBPs: RNA binding proteins; RNAi: RNA interference; TJ: Tight junctions

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Competing interests

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