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Alternative splicing factor or splicing factor-2 plays a key role in intron retention of the endoglin gene during endothelial senescence

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Summary

Alternative splicing involving intron retention plays a key role in the regulation of gene expression. We previously reported that the alternatively spliced short isoform of endoglin (S-endoglin) is induced during the aging or senescence of endothelial cells by a mechanism of intron retention. In this work, we demonstrate that the alternative splicing factor or splicing factor-2 (ASF/SF2) is involved in the synthesis of endoglin. Overexpression of ASF/SF2 in endothelial cells switched the balance between the two endoglin isoforms, favoring the synthesis of S-endoglin. Using a minigene reporter vector and RNA immunoprecipitation experiments, it was shown that ASF/SF2 interacts with the nucleotide sequence of the endoglin minigene, suggesting the direct involvement of ASF/SF2. Accordingly, the sequence recognized by ASF/SF2 in the endoglin gene was identified inside the retained intron near the consensus branch point. Finally, the ASF/SF2 subcellular localization during endothelial senescence showed a preferential scattered distribution throughout the cytoplasm, where it interferes with the activity of the minor spliceosome, leading to an increased expression of S-endoglin mRNA. In summary, we report for the first time the molecular mechanisms by which ASF/SF2 regulates the alternative splicing of endoglin in senescent endothelial cells, as well as the involvement of ASF/SF2 in the minor spliceosome.

Key words: alternative splicing; senescence; endothelial cells; intron retention; minor spliceosome; alternative splicing factor or splicing factor-2.

Introduction

The majority of the genes in high eukaryotes undergo alternative splicing on their premature messenger RNA (pre-mRNA) sequences. This process occurs with amazing fidelity, plays a key role in the regulation of gene expression, and considerably enlarges the complexity of the proteome (Graveley, 2001; Kwan *et al.*, 2007). Briefly, the spliceosome assembly is driven by a set of small nuclear ribonucleoproteins (snRNPs) that recognize the 5′ and 3′ splice sites, as well as the branch point element in between them (Burge *et al.*, 1999). There are two distinct spliceosome complexes, the major spliceosome and the minor spliceosome. The major spliceosome is involved in the vast majority of the splicing events and comprises five snRNPs named U1, U2, U4, U5, and U6 and a multitude of non-snRNP splicing factors (Zhou *et al.*, 2002; Jurica & Moore, 2003). In addition, a structurally and functionally analogous minor spliceosome that mediates the splicing of the so-called non-canonical introns has been reported (Patel & Steitz, 2003; Will & Luhrmann, 2005). The minor spliceosome comprises four unique snRNPs, U11, U12, U4atac, and U6atac, as well as the U5 snRNP, a component that is shared with the major spliceosome (Hall & Padgett, 1996; Tarn & Steitz, 1996). An interesting difference between the major spliceosome and minor spliceosome is their spatial segregation. While the major spliceosome is in the nucleus, the minor spliceosome is located in the cytosol (Konig *et al.*, 2007). Since the discovery of the minor splicing pathway, there is a growing interest in defining its physiological significance and identifying its target genes.

There are three main types of alternative splicing: exon skipping, alternative selection of splice sites, and intron retention (IR). Among these, the least studied is IR because this normally involves defects in mRNA transport to the cytoplasm and a rapid degradation by nonsense-mediated decay (Nott *et al.*, 2003; Lareau *et al.*, 2004). However, in several cases, the retention of an intron in the mature mRNA leads to a biologically active isoform, as reported for the cardiac *ANKRD1* gene (Torrado *et al.*, 2009), the chemokine *CCL27* gene (Ledee *et al.*, 2004), the *FLT1* gene, which can give rise to the soluble-form sFlt-1, (Thomas *et al.*, 2007), and the endoglin (*ENG;* GeneID: 2022) gene (Bellon *et al.*, 1993; Blanco *et al.*, 2008).

The regulation of IR, in particular, and alternative splicing, in general, is a complex but accurate process. In many cases, a single splicing event is under the control of several exonic and/or intronic *cis*-elements, which in turn can act as enhancers and/or silencers. Typically, these *cis*-elements are defined as recognition sites for *trans*-acting splicing factors that stimulate or repress splicing (Black, 2003; Moore & Silver, 2008). Interestingly, genes that give rise to IR-dependent splice variants share some features, including their high expression level, the short length of the intron, and the ratio between the relative isoform frequencies (RIF, the number of cDNAs with retained introns/number of cDNAs defining the introns) (Sakabe & de Souza, 2007).

One of the best characterized splicing factors is alternative splicing factor or splicing factor-2 (ASF/SF2), a member of the family of serine/arginine-rich (SR) proteins which are involved in both constitutive and alternative splicing (Graveley, 2000). The ASF/SF2 splicing factor is primarily found in nuclear speckles, but there is a subset that continuously shuttles between the nucleus and the cytoplasm (Lamond & Spector, 2003). This different localization depends on the phosphorylation state of the RS domain in serine residues, which influences the functions of ASF/SF2 (Sanford *et al.*, 2009). Furthermore, it has recently been reported that there are three arginine residues susceptible to methylation in the glycine-rich linker between the RMM motifs, which also condition the cytoplasmic accumulation of ASF/SF2 (Ong *et al.*, 2004; Sinha *et al.*, 2010). However, the physiological stimuli that regulate the activity and subcellular localization of ASF/SF2 remain largely unknown. The ASF/SF2 presents a modular structure comprising two RNA recognition motifs (RRM) and a C-terminal domain rich in alternating serine and arginine

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residues, known as the arginine/serine-rich (RS) domain. The RRM motifs determine RNA binding specificity to somewhat degenerate sequences of 5–7 nucleotides, whereas the RS domain functions as a protein–protein interaction module (Liu *et al.*, 1998; Wang *et al.*, 2005; Sanford *et al.*, 2008). These *cis*-elements, or binding motifs, are spatially distributed in spliced exons and introns, suggesting differential roles in splice selection (Caceres & Kornblihtt, 2002; Ibrahim *et al.*, 2005; Wu *et al.*, 2005; Sakabe & de Souza, 2007). Indeed, it has been reported that ASF/SF2 can interact with the branch point (Shen *et al.*, 2004).

Vascular physiology progressively declines with age owing to multiple factors such as an increase in oxidative stress, DNA damage, and advanced cellular replication involving telomere attrition, which all contribute to the senescence of endothelial cells (Brandes *et al.*, 2005; Erusalimsky, 2009). At this stage, the expression of many specific genes is modulated, regarding not only their expression levels but also the post-translational modifications and alternative processing of their premature mRNA molecules, which give rise to protein variants (Meshorer & Soreq, 2002). The alternative splicing is an important event in endothelial aging that has been poorly studied so far. For example, the splicing factor SNEV is involved in extending the replicative lifespan of endothelial cells upon overexpression (Voglauer *et al.*, 2006), supporting the view that splicing is of underestimated importance in cellular aging. We recently demonstrated that in contrast to the highly abundant, predominantly expressed L-endoglin isoform, expression of the low-abundance isoform S-endoglin was increased during senescence of human endothelial cells *in vitro*, as well as during aging of mice in vascularized tissues (Blanco *et al.*, 2008). Thus, transcript levels of S-endoglin were significantly increased in lungs from 32- and 60-week-old mice as compared with young animals (4 weeks old). In addition, transgenic mice overexpressing S-endoglin in endothelial cells showed hypertension, decreased hypertensive response to NO inhibition, decreased vasodilatory response to TGF-β1 administration, and decreased endothelial nitric oxide synthase expression in lungs and kidneys, supporting the involvement of S-endoglin in the age-dependent vascular pathology (Blanco *et al.*, 2008). Endoglin is an integral membrane glycoprotein that plays a major role in angiogenesis, vascular remodeling, and homeostasis (ten Dijke *et al.*, 2008; Lebrin & Mummery, 2008; Bernabeu *et al.*, 2009; Lopez-Novoa & Bernabeu, 2010). S-endoglin shares almost all the structural features of L-endoglin, such as a homodimeric conformation and the large extracellular and transmembrane regions, with the only difference being in the composition of their cytoplasmic tails (Bellon *et al.*, 1993; Blanco *et al.*, 2008). These two endoglin isoforms are auxiliary components of the transforming growth factor-β (TGF-β) receptor complex and exert opposite roles to each other. S-endoglin is able to modulate the TGF-β pathway through its preferential interaction with ALK5, a TGF-β receptor type I (Blanco *et al.*, 2005; Perez-Gomez *et al.*, 2007; Velasco *et al.*, 2008), and it shows antiangiogenic activity (Perez-Gomez *et al.*, 2005) and endothelial senescence markers (Blanco *et al.*, 2008). Mutations in the human *ENG* gene are responsible for hereditary hemorrhagic telangiectasia (HHT) type 1, a disease characterized by telangiectases in skin and mucosa, as well as vascular malformations in lung, liver, and brain (McAllister *et al.*, 1994; Shovlin, 2010; Faughnan *et al.* 2011). Hemorrhagic telangiectasia is not apparent at birth but evolves with age. For example, telangiectases of the skin and buccal mucosa typically present from about the third decade of life. Overall, the symptoms of HHT increase and become worse with age (Plauchu *et al.*, 1989; Faughnan *et al.* 2011).

The *ENG* gene consists of 15 exons numbered from 1 to 14 (exon 9 is split into 9a and 9b). Thus, during endothelial senescence, the last intron between exons #13 and #14 remains in the mature mRNA, by means of an IR process, introducing a premature stop codon in the reading frame that gives rise to S-endoglin (Bellon *et al.*, 1993; Perez-Gomez *et al.*, 2005). However, the mechanisms that regulate this process of IR remain to be elucidated. Here, we report the regulation of ASF/SF2 during endothelial senescence and its involvement in the intron retention mechanism that gives rise to S-endoglin.

Results

The structure of the intron responsible for S-endoglin expression is conserved among species

An in silico analysis was performed in order to dissect the genomic sequences of endoglin from different species. The sequences corresponding to the last intron and the flanking exons of the endoglin genes available in the databases were aligned using the ClustalW2 program (Fig. 1). The splicing donor (GU) and acceptor (AG) sites were well defined according to the consensus (score >90%) and so was the polypyrimidine (C/U-rich) zone downstream of the branch point (cugAc) (Gao *et al.*, 2008). Moreover, these motifs were flanked by highly conserved areas among species. As expected, the highest homology level among aligned sequences was found throughout the exonic regions, in contrast to the overall low conservation degree among introns.

Next, because S-endoglin has only been described and characterized in humans and mice, we analyzed in silico the effect of intron retention on the different open reading frames in order to predict theoretical short endoglin isoforms in different species. Although the zone flanking the

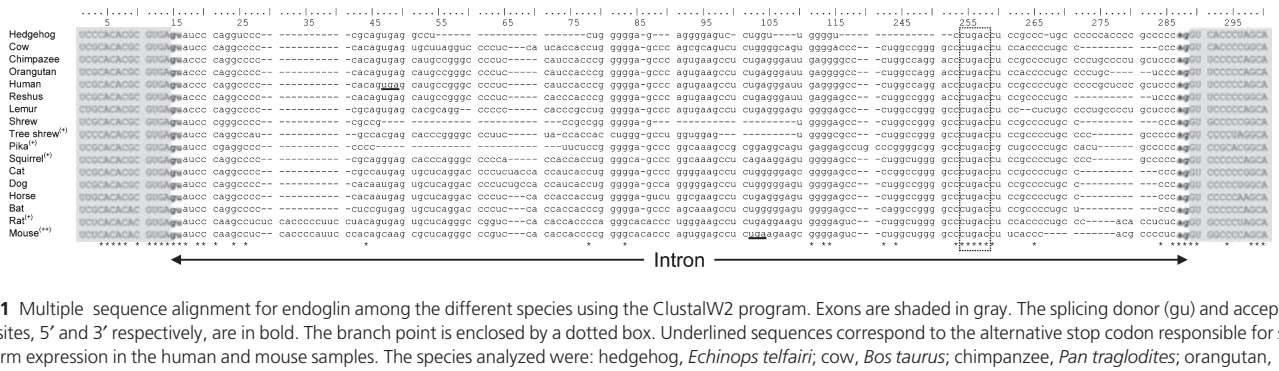

Fig. 1 Multiple sequence alignment for endoglin among the different species using the ClustalW2 program. Exons are shaded in gray. The splicing donor (gu) and acceptor (ag) sites, 5′ and 3′ respectively, are in bold. The branch point is enclosed by a dotted box. Underlined sequences correspond to the alternative stop codon responsible for short isoform expression in the human and mouse samples. The species analyzed were: hedgehog, *Echinops telfairi*; cow, *Bos taurus*; chimpanzee, *Pan traglodites*; orangutan, *Pongo albelii*; human, *Homo sapiens*; Rhesus macaque, *Macaca mulatta*; lemur, *Microcebus murinus*; shrew, *Sorex araneus*; tree shrew, *Tupaia belangeri*; pika, *Ochotona princeps*; squirrel, *Spermophilus tridecemlineatus*; cat, *Felis catus*; dog, *Canis familiaris*; horse, *Equus caballus*; bat, *Myotis lucifugus*; rat, *Rattus norvegicus*; mouse, *Mus musculus*. The species marked with (+) do not contain alternative stop codons in their introns. (++) The short isoform is longer than the one reported for human S-endoglin.

premature stop codon responsible for S-endoglin expression is very similar in almost all species, the reading frame of the intron varies considerably. Interestingly, the generation of a short isoform was not possible in five of the species analyzed (Fig. 1, marked with +) owing to the lack of a premature termination codon in the corresponding reading frame.

In order to find splicing factors that could be involved in endoglin splicing, the genomic sequence of the human gene (*ENG*) corresponding to exon #13–intron–exon #14 was compared using the SpliceAid database. Several putative binding sites were recognized by this bioinformatic tool. However, this result must be taken with caution because binding specificity *in vivo* depends on each factor rather than just sequence recognition of these low-complexity motifs (Long & Caceres, 2009). Thus, we focused on ASF/SF2 and SC35 because of their relatively high prediction scores (5/10 and 4/10, respectively) as possible *trans*-elements that would act as intronic splicing silencers (ISS). It is noteworthy that both factors are members of the SR family of proteins that are used antagonistically on particular target genes.

ASF/SF2 is involved in the IR of endoglin

Based on the SpliceAid prediction, we examined whether ASF/SF2 and SC35 were able to affect endoglin splicing. Thus, the ASF/SF2 or SC35 expression vectors were transfected into the human endothelial cell line HMEC-1, and the endoglin transcripts were detected by RT-PCR in the same reaction (Fig. 2A). The independent overexpression of each splicing factor was able to induce the short isoform of endogenous endoglin, but the effect of ASF/SF2 was markedly higher than that obtained with SC35. Unfortunately, the differential expression of S-endoglin versus L-endoglin at the protein level could not be assessed owing to their similar molecular weight and the lack of antibodies specific for the S-endoglin isoform (data not shown; Blanco *et al.*, 2008). Next, we focused on the intron that shelters the splicing of the endoglin mRNA. Thus, a minigene containing the sequence from the 5′-end of exon #13 to the stop codon codified in exon #14 (MinigEND) was designed. Upon transfection, this construct can be expressed at the cell surface because the vector encodes

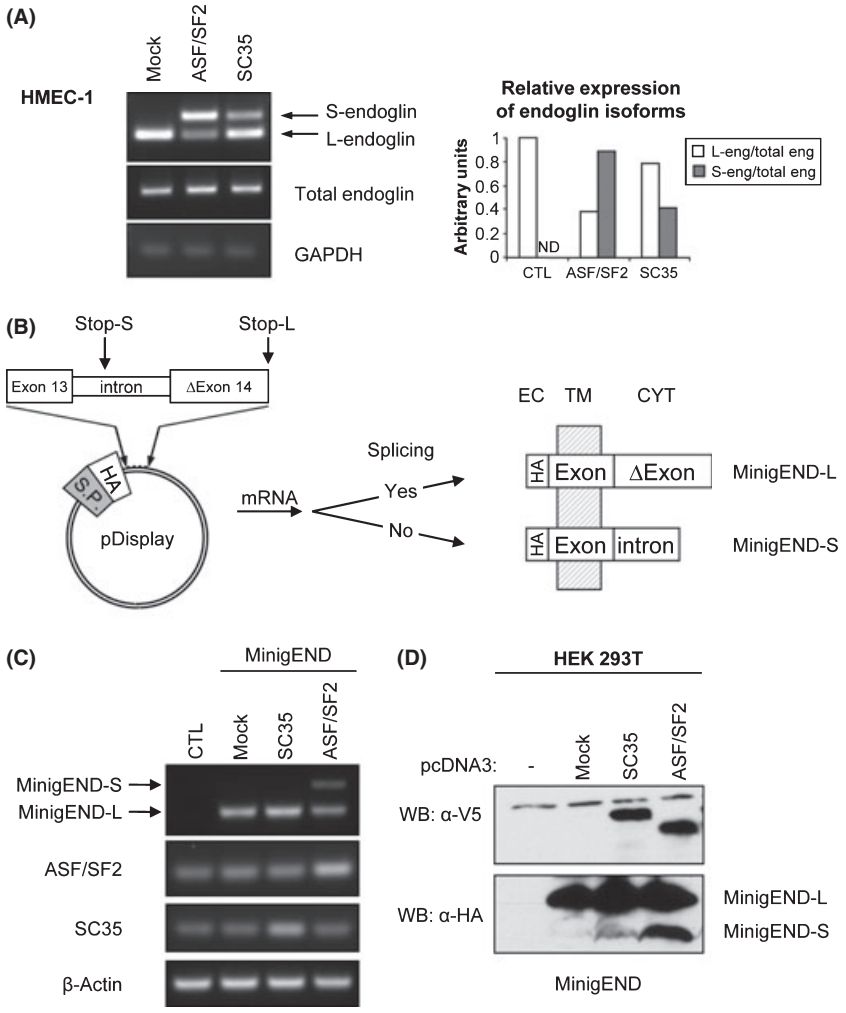

Fig. 2 Intron retention of endoglin. (A) Splicing factors alternative splicing factor or splicing factor-2 (ASF/SF2) or SC35 were overexpressed in the human endothelial cell line HMEC-1. The effect on the endogenous mRNA of endoglin was monitored by RT-PCR. On the right is the densitometry of the bands to quantify the relative expression of endoglin isoforms. The ratio between S and total endoglin was null under mock conditions (ND, not detected). (B) Scheme of the minigene as a splicing reporter vector for endoglin. S.P., signal peptide; HA, hemagglutinin epitope; EC, extracellular; TM, transmembrane; CYT, cytoplasmic. C and D. The human endoglin minigene (MinigEND) was transfected into HEK293T in presence of the splicing factors SC35 or ASF/SF2. The mRNA corresponding to the long and the short variants of the minigenes was detected by RT-PCR (C) or Western blot (D).

a signal peptide and the endoglin exon #13 carries the transmembrane region (Fig. 2B). Thus, the MinigEND was co-transfected with either SC35 or ASF/SF2 in the endoglin-free cell line HEK293T, and the recombinant products were detected by RT-PCR (Fig. 2C). The MinigEND was mainly expressed as its long variant, once the intron was removed, but the intron was only retained in the presence of ASF/SF2. This result was confirmed in parallel experiments by Western blot, showing that ASF/SF2, but not SC35, is able to regulate intron retention of the MinigEND (Fig. 2D).

ASF/SF2 binds to the mRNA of MinigEND

Next, we assessed whether the predicted ASF/SF2 *cis*-elements in the intron between exon #13 and #14 of endoglin were indeed functional. Thus, RNA immunoprecipitation (RIP) was performed on HEK293T cells co-transfected with MinigEND and either ASF/SF2 or SC35. Because both splicing factors were tagged with the V5 epitope in their expression vectors, RIP was performed using a monoclonal antibody against V5, thus reducing the risk of disruption to the RNA/splicing factor interaction. We chose the MinigEND instead of the endogenous endoglin gene to avoid the possibility of immunoprecipitation by means of splicing factors bound to other upstream *cis*-elements. Figure 3 shows that anti-V5 samples allowed the detection of specific PCR fragments from cells transfected with ASF/SF2 or SC35. In contrast, G-Sepharose, either alone or carrying an irrelevant IgG, was unable to yield any PCR products after immunoprecipitation. The length of the PCR fragments was compatible with the mRNA derived from the spliced MinigEND, demonstrating its direct association with ASF/SF2 or SC35. In addition, a higher PCR product was observed in the ASF/SF2 lane, corresponding to the MinigEND mRNA that retained the intron, suggesting that the interaction between ASF/SF2 and mRNA takes place in the intronic sequence. A second PCR was performed using specific primers that hybridize inside the intron. In this experiment, the intron retained was clearly detected when ASF/SF2 was overexpressed (Fig. 3, lower panel), confirming an interaction between ASF/SF2 and the endoglin intron. The possibility that this band corresponded to the intron lariat cannot be ruled out because ASF/SF2 overexpression did not completely achieve the intron retention of MinigEND, as occurs with the endogenous endoglin gene.

ASF/SF2 recognizes a *cis*-element in the endoglin intron near the branch point

Based on the analysis using the SpliceAid database, two putative ASF/SF2 binding sites within the last intron around positions IVS13 + 23G (CACAGUG) and IVS13-36C (CCCUGAC) in the human *ENG* sequence were identified. In order to analyze the contribution of the two theoretical ASF/SF2 *cis*-elements to the intron retention, these sites were destroyed by site-directed mutagenesis on the MinigEND construct. These mutations, referred to as positions +23 and −36, respectively, do not have any effect on the reading frame or the branch point consensus (Fig. 4A). Single or double mutants of the MinigEND were co-transfected with the ASF/SF2 expression vector. As expected, in the absence of exogenous ASF/SF2, all these constructs gave rise to the long variant of MinigEND, confirming the correct expression of the splicing reporter vector under normal conditions (Fig. 4B,C). However, upon ASF/SF2 transfection, intron retention was induced in the wild-type MinigEND, whereas no intron retention was induced in either the single-mutant MinigEND-(−36) or the double-mutant MinigEND-(+23/−36), confirming the critical role of position −36 in the splicing of endoglin.

ASF/SF2 translocates to the cytosol during senescence of endothelial cells

Because S-endoglin is induced during the senescence of endothelial cells, we assessed whether the expression level of ASF/SF2 was also affected in this physiological context. First, we performed an RT-PCR assay to compare young versus senescent HUVECs, but no differences were found at

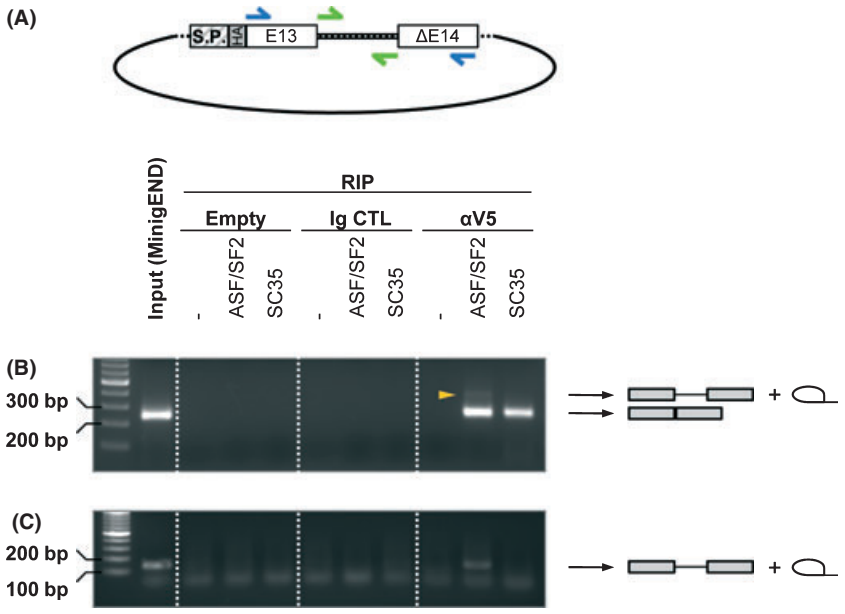

Fig. 3 RNA immunoprecipitation. The HEK293T cells were co-transfected with the human endoglin minigene (MinigEND) and one of the V5-tagged splicing factors alternative splicing factor or splicing factor-2 (ASF/SF2) or SC35. Samples were immunoprecipitated with protein G-sepharose alone, coupled to an IgG isotype control or anti-V5 antibodies, retrotranscripted, and amplified by PCR. (A) The scheme shows the primer pair used in the PCR. The detected PCR products with primer pairs in blue (B) or green (C) are outlined on the right.

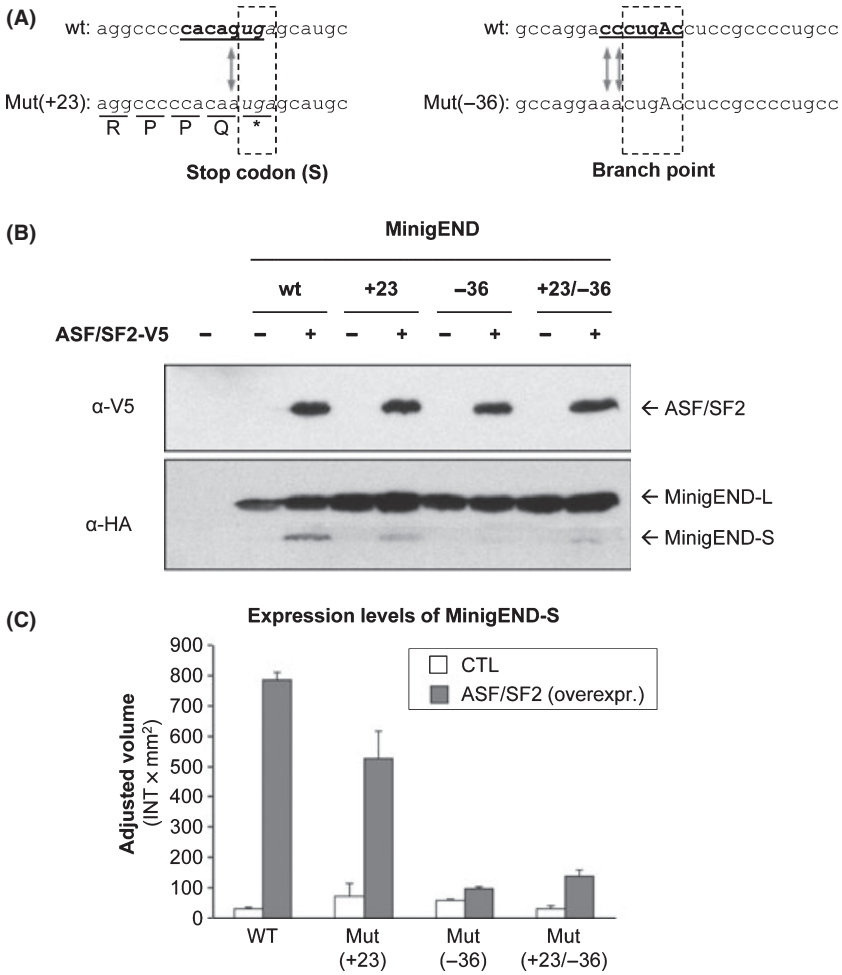

Fig. 4 Site-directed mutagenesis on MinigEND. (A) Schematic representation of the MinigEND mutants. (B and C) HEK293T cells were co-transfected with alternative splicing factor or splicing factor-2 and each MinigEND version: wild-type (wt), and mutants in positions (+23), (–36) or the double (+23/–36). The long and short variants of MinigEND were detected by Western blot using anti-HA antibodies (B). The lower panel shows the densitometry of three different experiments (C).

the ASF/SF2 mRNA level (Fig. 5A,B). Nevertheless, it is well known that some splicing factors, including ASF/SF2, are proteins that continuously shuttle between the cytoplasm and the nucleus depending on their serine phosphorylation or arginine methylation state (Sanford *et al.*, 2005; Sinha *et al.*, 2010). Therefore, we decided to analyze the subcellular distribution of ASF/SF2 by comparing young versus senescent HUVECs using confocal microscopy. While ASF/SF2 was preferentially located in the nucleus during early passages, it was markedly scattered throughout the cytoplasm during senescence (Fig. 5C). The differential localization pattern of ASF/SF2 was confirmed by Western blot analysis using cytoplasmic and nuclear extracts from young and senescent HUVECs (Fig. 5D). Further experimental support for the ASF/SF2 redistribution was obtained by inducing a senescence-like stage in endothelial cells subjected to oxidative stress (Chen, 2000). The HUVECs treated with hydrogen peroxide (H_2O_2) under two different conditions (20 μM H_2O_2 for 24 h or 150 μM for 1 h) showed growth arrest and positivity for SA-β-Gal staining (data not shown), two hallmarks of cellular senescence (Haendeler *et al.*, 2004; Erusalimsky, 2009). As evidenced by qRT-PCR, both treatments with H_2O_2 markedly induced retention of the last intron, whereas total endoglin transcript levels were not affected (Fig. 6A). Moreover, confocal microscopy analysis demonstrated that H_2O_2-induced senescence alters the subcellular distribution of ASF/SF2, shifting its localization from the nucleus into the cytosol (Fig. 6B), these differences being much more evident when HUVECs were treated with 20 μM H_2O_2 for 24 h. Taken together, these results indicate that both replicative and oxidative stress-induced senescence are associated with a redistribution of ASF/SF2 from the nucleus to the cytosol.

The splicing of S-Endoglin takes place in the cytoplasm and involves the minor spliceosome

To assess the role of ASF/SF2 in the cytoplasmic alternative splicing, phosphorylation- and methylation-deficient versions of this factor that target the cytosol were used (Cazalla *et al.*, 2002; Sinha *et al.*, 2010). Thus, ASF/SF2-KS and ASF/SF2-$A_1A_2A_3$ cytoplasmic mutants were co-transfected with the MinigEND construction in HEK293T cells (Fig. S1), and products were detected by RT-PCR and Western blot (Fig. 7A, left and right panels, respectively). Interestingly, the ASF/SF2 cytoplasmic mutants promoted the splicing of the MinigEND to its short variant, similarly as the wild-type version. To corroborate this finding on the *ENG* gene, the cytoplasmic ASF/SF2 mutants were expressed in endothelial cells, and the endogenous endoglin mRNA was detected by RT-PCR. As shown in

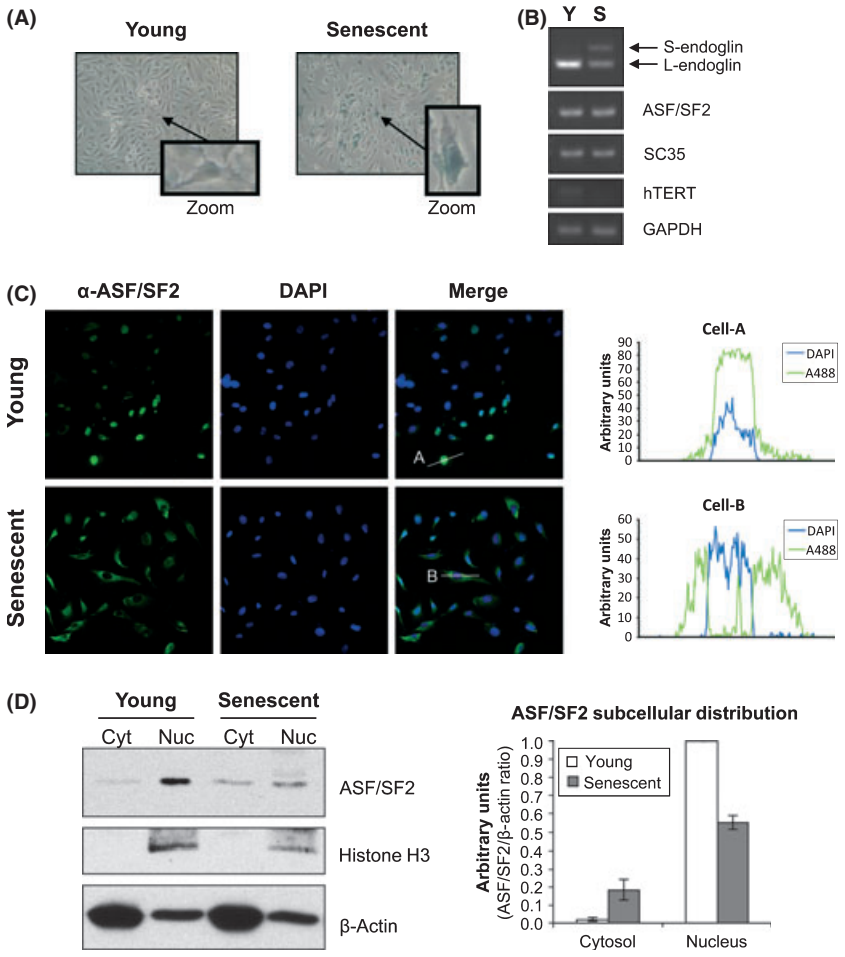

Fig. 5 Subcellular distribution of alternative splicing factor or splicing factor-2 (ASF/SF2) during cellular senescence. (A) Primary cultures of HUVECs were maintained along passages until they reached replicative senescence, as demonstrated by SA-β-Gal staining. Cultures of young and senescent cells were visualized by light microscopy. Inserts in the lower right of each photograph are representative examples of their morphology; senescent cells were larger and with a more rounded appearance. (B) ASF/SF2 and SC35 levels were analyzed by RT-PCR from young (Y) and senescent (S) HUVECs. The induction of S-endoglin and the downregulation of telomerase (hTERT) were used as senescent markers. (C) The subcellular distribution of ASF/SF2 was analyzed by confocal microscopy comparing its staining pattern between young and senescent HUVECs. The fluorescence profiles of representative cells, indicated by a white line, are shown on the right. (D) Western blot of cytosolic (Cyt) or nuclear (Nuc) lysates showing the differential distribution of ASF/SF2 along passages. Immunodetection of nuclear histone H3 and β-actin, both cytosolic and cortical, associated with the nuclear inner membrane (Holaska *et al.*, 2004), were used as loading controls to normalize ASF/SF2 expression.

Fig. 7B, the expression of ASF/SF2 cytoplasmic mutants induced the upregulation of S-endoglin in this endothelial model, supporting the notion that the function of ASF/SF2 is exerted at the cytosolic level.

Taking into account the cytosolic localization of ASF/SF2 during senescence, the cytosolic activity of ASF/SF2, and the existence of a cytosolic spliceosome, we decided to explore the role of this minor splicing complex in the S-endoglin expression. Thus, a knockdown assay was designed to interfere with the small RNA U6atac, an essential component of the minor spliceosome, in order to abolish the minor spliceosome function. The HMEC-1 cells were transfected with three different siRNAs to U6atac (*RNU6ATAC* gene), and the endoglin mRNA was detected by RT-PCR. The U6atac expression was interfered about 50%, and this was associated with an upregulation of S-endoglin levels (Fig. 8). In the same experiment, a scramble and FAM-labeled siRNA was used as a negative control and also to monitor the transfection efficiency. Similar results were obtained when U6atac knockdown was performed in HUVECs, although in this case the transfection efficiency was lower (Fig. S2). Taken together, these data suggest that the minor spliceosome is involved in the S-endoglin alternative splicing.

Discussion

The alternative splicing of premature mRNA molecules is an accurate process that usually changes the biological functions of their codified proteins. This is the case of the short endoglin isoform that exerts an opposite role, in the context of the TGF-β system, with respect to the long variant. S-endoglin is induced during endothelial senescence and contributes to vascular pathology (Blanco *et al.*, 2008), but the underlying mechanisms that regulate its expression are basically unknown. Here, we show that the sequence of the endoglin intron involved in the retention is poorly conserved among different species. Also, we observed that, when this intron is retained, an early stop codon appears in the reading frame of almost all the species analyzed. In this context, the lack of a putative short isoform in the rat was surprising despite its high homology with the mouse sequence (Fig. 1).

In the present study, we report for the first time the engineering of a molecular tool based on a minigene approach, the MinigEND, as a reporter vector for studying the alternative splicing of endoglin. The in silico analysis of the MinigEND sequence showed theoretical *cis*-elements with

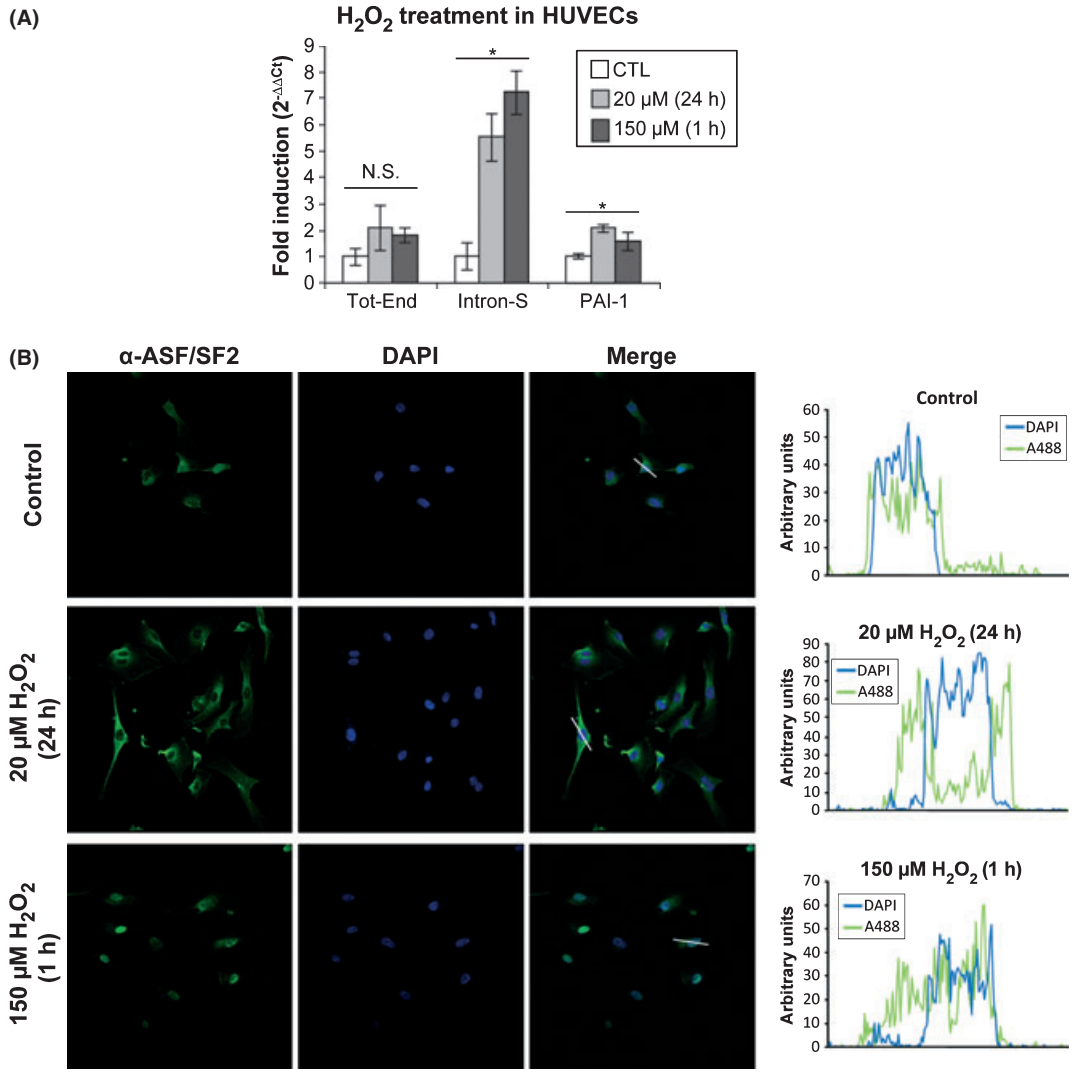

Fig. 6 Effect of oxidative stress-induced senescence on the subcellular distribution of alternative splicing factor or splicing factor-2 (ASF/SF2). HUVECs were treated with H_2O_2 as indicated to achieve cellular senescence. (A) Quantitative RT-PCR analysis of total endoglin, the intron responsible for S-endoglin and PAI-1, an endothelial senescence marker (Comi *et al.*, 1995; Erusalimsky, 2009). (NS, no significant differences; $*P < 0.05$) (B) Subcellular distribution of ASF/SF2 in H_2O_2-treated HUVEC cells. The fluorescence profiles of representative cells, indicated by a white line, are shown on the right.

relatively high scores for SC35 and ASF/SF2, both of which are members of the SR protein family. Hence, we were able to monitor the endoglin IR in the presence or absence of these target splicing factors. We found that ASF/SF2 was able to modulate the IR of the MinigEND, promoting the expression of the short variant, while SC35 had no effect on IR (Fig. 2). Similarly, ASF/SF2 overexpression in HMEC-1 cells caused a co-expression of both endoglin isoforms (Fig. 2). These results are compatible with the antagonistic role widely described for both SR protein family members on the regulation of alternative splicing in different genes (Tacke & Manley, 1995), such as the growth hormone gene (Solis *et al.*, 2008) and the glutamate receptor subunit 2 gene (GluR2) (Crovato & Egebjerg, 2005).

Trans-elements, or splicing factors, act by means of binding to short degenerate sequences of low complexity, and these protein/RNA interactions can be studied by RNA immunoprecipitation (RIP) (Zielinski *et al.*, 2006). Thus, we found that both SC35 and ASF/SF2 co-immunoprecipitate with the mRNA corresponding to the long variant of the MinigEND. Furthermore, when analyzing the interaction between ASF/SF2 and mRNA in detail, we demonstrated that this recognition takes place in the intronic sequence (Fig. 3). Because we also detected the long variant of MinigEND in the ASF/SF2 immunoprecipitate, the possibility that ASF/SF2 recognizes another exonic *cis*-element cannot be excluded. In fact, the SpliceAid tool predicted two exonic ASF/SF2 binding sites centered in positions c.1903T and c.1928G (50 and 75 pb from the start of exon #14, respectively). Nevertheless, site-directed mutagenesis performed on the intronic ASF/SF2 binding sites in the MinigEND confirmed that position −36 is the key *cis*-element for IR (Fig. 4). This result is supported by the following arguments: (i) the ASF/SF2 site centered in the nucleotide IVS13-36G overlaps the consensus for the branch point and (ii) it has been reported that ASF/SF2 can recognize and bind to the consensus branch point (Shen *et al.*, 2004). Altogether, these data suggest that the interaction of ASF/SF2 with the *cis*-element in position −36 would interfere with the correct elimination of the intron.

An increased subcellular localization in the cytoplasm of ASF/SF2 during replicative and oxidative stress-induced senescence was

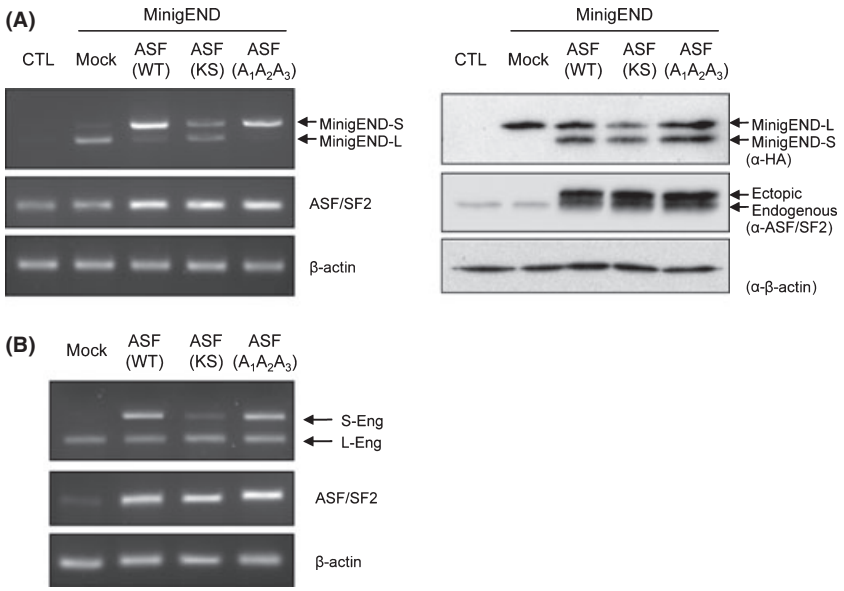

Fig. 7 Intron retention of *ENG* is a cytoplasmic event. (A) Effect of the cytoplasmic alternative splicing factor or splicing factor-2 (ASF/SF2) mutants on the MinigEND vector. HEK293T cells co-transfected with the MinigEND construct and one of the ASF/SF2 versions, as indicated. The expression of endoglin isoforms was analyzed by RT-PCR (left panel) or Western blot (right panel). (B) Effect of the cytoplasmic ASF/SF2 mutants on the endogenous *ENG* gene. The human endothelial cell line HMEC-1 was transfected with the ASF/SF2 constructs, as indicated, and the expression of endoglin isoforms was analyzed by RT-PCR. WT, wild-type; KS, phosphorylation deficient mutant in the RS domain; $A_1A_2A_3$, methylation defective mutant in Arg residues 93, 97, and 109.

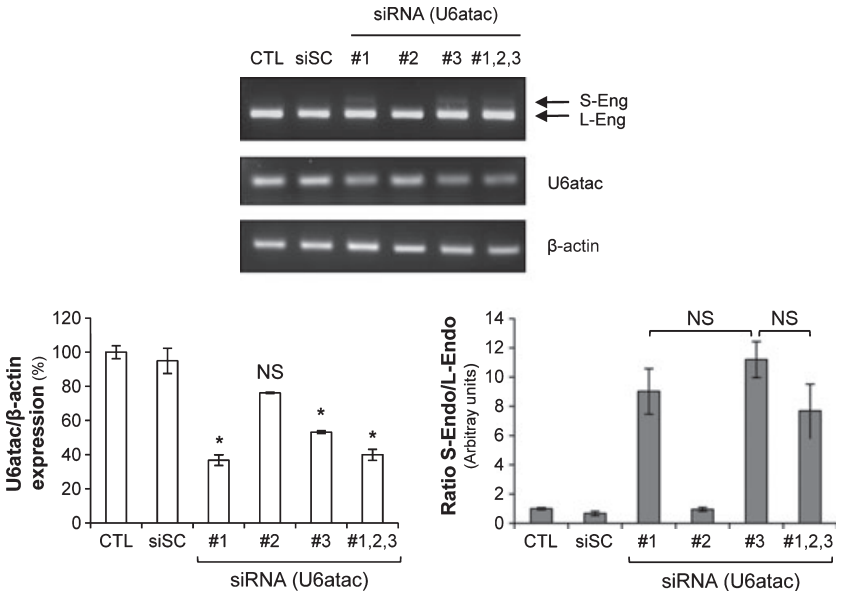

Fig. 8 Effect of the minor spliceosome on the IR of endoglin. Different combinations of three siRNAs targeting the *RNU6ATAC* gene were transfected in HMEC-1 cells to abolish the function of the minor spliceosome. After 48 h, the expression of U6atac and endoglin isoforms was measured by RT-PCR as shown in the histograms. A significant up-regulation of S-endoglin was observed upon silencing the U6atac snRNA. (NS, no significant differences; $*P < 0.05$).

demonstrated in endothelial cells (Figs 5 and 6), suggesting that the splicing factor ASF/SF2 is a new target of senescence. This is in line with the reported role of ASF/SF2 as modulator of the senescence induced by oncogene overexpression in mouse embryonic fibroblasts (Verduci *et al.*, 2010) as well as with the upregulation of ASF/SF2 in various human tumors and with the oncoprotein function of ASF/SF2 itself (Karni *et al.*, 2007). Moreover, a direct correlation was established between the preferential cytoplasmic localization of ASF/SF2 and S-endoglin expression at the senescent stage of endothelial cells. Further investigations are necessary to elucidate the specific post-translational modification/s that take place on ASF/SF2 during endothelial senescence. Because the functional ASF/SF2 mutants assayed in this work localize in the cytoplasm, where the minor spliceosome is preferentially located (Caceres & Misteli, 2007), these data suggested the involvement of ASF/SF2 in the cytoplasmic

spliceosome during endothelial senescence. Supporting this view, specific siRNA-mediated knockdown of the U6atac subunit of the minor spliceosome induced an increase of the S-endoglin mRNA (Fig. 8). Based on these data, a hypothetical model is proposed in which the cytoplasmic location of ASF/SF2 would hamper the binding and function of the spliceosome component snRNP-U12, thus retaining the intron and giving rise to the short isoform of endoglin up-regulation during endothelial senescence (Fig. 9). Interestingly, it has been reported that alterations in the snRNAs stoichiometry of the minor spliceosome are associated with the physiopathology of a rare autosomal recessive disease named spinal muscular atrophy (SMA), which is attributable to mutations in the *SMN1* gene involved in the correct spliceosome assembly (Zhang *et al.*, 2008). Accordingly, some, but not all, of the so-called U12-type introns are differentially spliced in cells derived from patients with SMA (Boulisfane *et al.*, 2010). Whether the S/L ratio of endoglin isoforms is altered in these patients remains to be investigated.

Mutations in the human *ENG* gene are responsible for HHT-1, an autosomic dominant vascular disease whose symptoms increase and become worse with age (Plauchu *et al.*, 1989). One can speculate that this age-dependent penetrance of the disease may involve the ASF/SF2-induced expression of S-endoglin. Haploinsufficiency of the predominantly expressed L-endoglin isoform is widely accepted as the pathogenic mechanism of this disease (Abdalla & Letarte, 2006; Lopez-Novoa & Bernabeu, 2010). Moreover, we have shown that S-endoglin is up-regulated in aged mice as well as during senescence of endothelial cells and exerts an antagonistic role to that of L-endoglin (Blanco *et al.*, 2008; Velasco *et al.*, 2008). Consequently, the increased S-endoglin expression during aging would increase the functional L-endoglin haploinsufficiency in HHT-1 and could explain why the symptoms become worse with aging.

Alternative splicing factor or splicing factor-2 exerts important biological functions, as revealed by gene suppression studies: genetic inactivation in DT40 cells gives rise to genomic instability (Li & Manley, 2005) and systemic ASF/SF2 knockout mice show an early embryonic lethality before day 7.5 (Xu *et al.*, 2005). In addition, the heart-specific deletion of ASF/SF2 causes the death of animals at around 6-8 weeks after birth owing to a dilated cardiomyopathy that fails to maintain proper blood circulation, and a disturbed Ca^{2+} metabolism leads to failure of the contractile apparatus (Xu *et al.*, 2005). Given the involvement of endoglin in vascular pathology (Bernabeu *et al.*, 2007; Blanco *et al.*, 2008; Lebrin & Mummery, 2008), it would be interesting to investigate the phenotype and the IR regulation of endoglin in endothelium-specific ASF/SF2 knockout mice. Unfortunately, this animal model is not available as yet. The involvement of ASF/SF2 in cellular senescence is in line with the fact that ASF/SF2 plays essential functions that are tissue- or developmental stage-specific (Moroy & Heyd, 2007) and that some post-translational modifications determine not only its subcellular localization but also its function. Indeed, a low phosphorylation state of the RS domain in ASF/SF2 has been associated with translation machinery in the cytoplasm, influencing the nuclear and cytoplasmic processing of specific mRNAs, including the splicing and translational control of endogenous mRNA targets (Sanford *et al.*, 2005). One of these targets is the vascular endothelial growth factor A (VEGF) mRNA. Vascular endothelial growth factor A is a key regulator of physiological and pathological angiogenesis. Alternative splice-site selection in the terminal exon of *VEGF* gene gives rise to two families of isoforms with distinct angiogenic activities. Proximal splice-site selection in exon 8 results in proangiogenic VEGFxxx isoforms (xxx is the number of amino acids), whereas distal splice-site selection results in antiangiogenic VEGFxxxb isoforms. The balance between proangiogenic and antiangiogenic VEGF isoforms is regulated by ASF/SF2 that favors proximal splice-site selection in the nucleus. Conversely, TGF-β1 induces the distal splice-site selection leading to antiangiogenic activity of VEGF (Nowak *et al.*, 2008). In addition, the inhibition of the ASF/SF2 phosphorylation promotes its cytoplasmic localization associated with increased expression levels of the antiangiogenic isoform VEGF165b (Nowak *et al.*, 2010). As shown in this work, ASF/SF2 targets the endoglin mRNA in the cytoplasm to promote intron retention and expression of the TGF-β co-receptor S-endoglin that displays antiangiogenic activity (Perez-Gomez *et al.*, 2005). This is in agreement with the induced expression of S-endoglin during aging/senescence (Blanco *et al.*, 2008) and with the fact that senescent endothelial cells do not form neoangiogenic webs on matrigel assays (Chang *et al.*, 2005). Because aging is associated with decreased angiogenesis (Brandes *et al.*, 2005; Erusalimsky, 2009), these data further support the connection between ASF/SF2 and senescence. They also suggest the existence of a common genetic program involving alternative splicing of certain genes whose resulting variants orchestrate the same biological function.

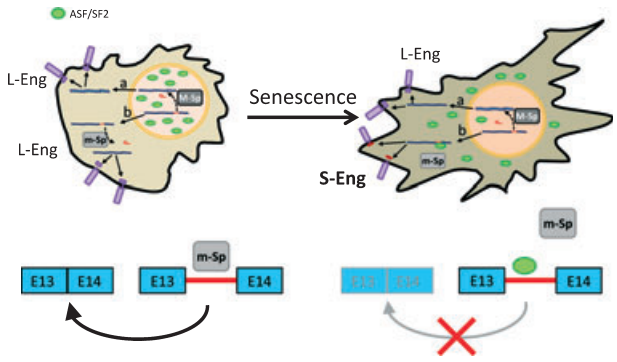

Fig. 9 Hypothetical model S-endoglin regulation during senescence. In young highly proliferating endothelial cells (left), the last intron of the *ENG* gene is removed in the mature mRNA, leading to the predominantly expressed L-endoglin isoform. This maturation process involves not only by the nuclear major spliceosome (M-Sp) but also by the minor spliceosome in the cytoplasm (m-Sp). While the M-Sp processes most of the constitutive and alternative splicing, the m-Sp processes certain mRNAs that still retain small introns such as intron #14 of *ENG*. In proliferating cells, alternative splicing factor or splicing factor-2 (ASF/SF2) (green oval) localizes in the cell nucleus (left). However, when the endothelial cell reaches the senescence stage (right), ASF/SF2 translocates to the cytoplasm. Then, ASF/SF2 binds to the *cis*-element overlapping the branch point of the S-endoglin mRNA forming a stable RNA/protein complex that interferes with the accessibility of the minor spliceosome to the branch point. Consequently, ASF/SF2 stabilizes the intron retention, thus upregulating the levels of S-endoglin mRNA.

Experimental procedures

In silico analysis of genomic sequences

Genomic sequences for the endoglin gene from different species were obtained from the Ensembl database (http://www.ensembl.org) or UCSC Genome Bioinformatics (http://genome.ucsc.edu/). Then, the FSPLICE and FGENESH tools were used to predict exons and introns in the DNA sequences (http://linux1.softberry.com/berry.phtml). The Transeq tool at the European Bioinformatic Institute (http://www.ebi.ac.uk) was used to analyze the reading frame of the intron retained. Multiple sequence alignments were performed using ClustalW2 software on the EBI Web page and analyzed with BioEdit Sequence Alignment Editor software (Hall, 1999). The prediction of human putative intronic and exonic *cis*-elements for splicing regulating factors was carried out using the SpliceAid database (Piva *et al.*, 2009) (http://www.introni.it/splicing.html), which

takes into account data from ESEfinder (Cartegni *et al.*, 2003) and RESCUE-ESE (Fairbrother *et al.*, 2002), among others.

Engineering of the MinigEND constructs and expression vectors

Human genomic DNA was obtained from a primary culture of human umbilical vein endothelial cells (HUVECs) using the QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany). The DNA sequence from exon #13 to the stop codon present in exon #14 of *ENG*, including the intervening intron, was amplified by PCR. The 385-bp product was subcloned in the pCR2.1 vector using the TOPO-TA Cloning kit (Invitrogen, Carlsbad, CA, USA) and then inserted into *SalI*/*BglII* unique restriction sites of the pDisplay vector (Invitrogen). The resulting construct was named pDisplay/MinigEND. Site-directed mutagenesis was performed on the pDisplay/MinigEND vector in order to destroy the predicted ASF/SF2 binding sites (Liu *et al.*, 1998; Smith *et al.*, 2006), giving rise to the MinigEND-(+23) and (−36), as well as the double mutant (+23/−36). In the first case, the change introduced was a silent mutation that does not affect the corresponding amino acid codified in the short variant of the MinigEND. The mutation in the second case does not break the branch point consensus. The sequence of primers used is available (Table S1). Correct constructions were verified by sequencing. The cDNA sequences of SC35 (NM_003016) and ASF/SF2 (NM_006924) were obtained from human total RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). The sequences were amplified by PCR with the specific V5 primers for SC35 and ASF/SF2 as detailed in the supplementary material (Table S1). The PCR products were TA-cloned in the pcDNA3.1/V5-His-TOPO vector (Invitrogen), introducing V5 and 6xHis epitopes in the C-terminal of the protein. The cytoplasmic T7-tagged ASF/SF2-KS (Cazalla *et al.*, 2002) and ASF/SF2-$A_1A_2A_3$ (Sinha *et al.*, 2010) mutants in pCGT7 have been described. Cell transfections with expression vectors were carried out using SuperFect Reagent (Qiagen), according to the manufacturer's instructions.

Cell culture

Human embryonic kidney 293T cells were grown in DMEM medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and 100 U mL^{-1} penicillin/streptomycin. The human endothelial cell line HMEC-1 was maintained in MCDB 131 medium supplemented with 10% FCS, 1 μg mL^{-1} hydrocortisone, 1 ng mL^{-1} epidermal growth factor, 200 mM L-glutamine, and 100 U mL^{-1} penicillin/streptomycin. Primary cultures of human umbilical vein endothelial cells (HUVECs) were maintained in EBM-2 medium plus EGM-2 SingleQuots supplement (Lonza, Walkersville, MD, USA). Replicative senescence of HUVECs was achieved by culturing the cells for at least 14 continuous passages (Blanco *et al.*, 2008). Oxidative stress-induced senescence experiments were carried out using hydrogen peroxide (H_2O_2) under two different experimental conditions (Chen *et al.*, 1998; Haendeler *et al.*, 2004). Thus, HUVECs were incubated with 20 μM H_2O_2 for 24 h or with 150 μM H_2O_2 for 1 h, then fresh medium was added, and the cells were incubated for an additional period of 23 h. The cells were maintained in a NAPCO incubator at 37°C in a humidified atmosphere with 5% CO_2.

Western blot analysis

The cells were lysed at 4°C for 30 min with a lysis solution containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% digitonin, the Phosphatase inhibitors cocktail set II (Calbiochem, Merck KGaA, Darmstadt, Germany), and Complete (Roche Diagnostics GmbH, Mannheim, Germany). Lysates were centrifuged at 20 000 *g* for 10 min, and then the supernatants were separated and tagged as cytosolic extracts. The pellets were washed three times with the previous lysis buffer and lysed again with RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.05% SDS, the Phosphatase inhibitors cocktail set II and Complete) to obtain nuclear extracts. Aliquots of lysates containing equivalent amounts of total protein were separated by SDS–PAGE and electrotransferred to a PVDF membrane using an iBlot gel transfer system (Invitrogen). Immunodetection was carried out with the mouse monoclonal antibodies anti-HA (12CA5; Roche), anti-V5 (R960-25; Invitrogen), or the rabbit polyclonal antibody anti-ASF/SF2 (ab38017; Abcam, Cambridge, UK), followed by incubation with the appropriate secondary antibody, anti-mouse or anti-rabbit IgG, both coupled to HRP (Dako, Glostrup, Denmark). Protein bands were revealed using the SuperSignal chemiluminescent substrate (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Senescence and immunofluorescence microscopy

Senescence was induced by culturing HUVECs along passages until this stage was reached. Alternatively, HUVECs were incubated with H_2O_2, as described above. Cellular aging was monitored by senescence-associated β-galactosidase activity (SA-β-Gal), as previously described (Haendeler *et al.*, 2004; Blanco *et al.*, 2008), and by RT-PCR with the indicated primer pair (Table S1). In parallel, cells grown on gelatin-coated glass coverslips were fixed, permeabilized with 3.5% formaldehyde and 100 ng mL^{-1} L-α-lysophosphatidylcholine (Sigma Aldrich, St. Louis, MO, USA) in cold PBS, and then blocked with 1% BSA. To analyze the subcellular distribution of ASF/SF2 during senescence, samples were incubated for 1 h at 4°C with a rabbit polyclonal antibody, anti-ASF/SF2 (ab38017; Abcam), followed by incubation with Alexa-488 green-conjugated goat anti-rabbit IgG (Invitrogen). Nuclei were visualized by incubation with 4′-6-diamidino-2-phenylindole, DAPI (Sigma Aldrich). To monitor the subcellular localization of ASF/SF2 cytosolic mutants, cells were incubated with the anti-T7-Tag mouse monoclonal antibody (69522; Novagen, EMD Chemicals Inc., San Diego, CA, USA), followed by the secondary Alexa-488-conjugated goat anti-mouse IgG (Invitrogen). The samples were mounted with ProLong Gold (Invitrogen) and observed using a Leica TCS SP5 AOBS confocal spectral microscope.

siRNA-mediated knockdown of the U6atac snRNP

To abolish the minor spliceosome in the cell, the expression of the *RNU6ATAC* gene was blocked by siRNA. This gene encodes the small RNA molecule U6atac that is an essential component of the minor spliceosome. Three different specific siRNAs were obtained from Sigma Aldrich (SASI_Hs02_00381692, SASI_Hs02_00381693, and SASI_Hs02_00381694). As a negative control, a scrambled FAM-labeled sequence of siRNA (siSC) (Ambion Inc., Austin, TX, USA) was used, which also allowed the assessment of transfection efficiency. Cells were seeded on 6-well plates and transfected with 25 pmoles of siRNA using Lipofectamine 2000 (Invitrogen), following the manufacturer's protocol.

Reverse transcription-polymerase chain reaction (RT-PCR) and RNA immunoprecipitation (RIP)

For the RT-PCR experiments, total RNA was isolated using the RNeasy mini kit (Qiagen). First-strand cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories) and amplified by PCR using the HotMaster Taq DNA polymerase (5 Prime GmbH; Hamburg, Germany)

with the specific primer pairs detailed in the supplementary material (Table S1). RNA immunoprecipitation was carried out according to the protocol described (Peritz *et al.*, 2006) with some modifications. Briefly, cells were lysed and pre-cleared with protein G-sepharose (GE Healthcare Bio-Science AB, Uppsala, Sweden). The lysates were incubated with anti-V5 antibodies and protein G-sepharose to allow the formation of complexes. Protein G-sepharose alone or with irrelevant IgGs from the ×63 hybridoma supernatant were used as negative controls. Unbound proteins were washed off, and immunoprecipitated RNA was isolated with Trizol reagent (Invitrogen). The RNA was retrotranscripted with the iScript cDNA synthesis kit (Bio-Rad Laboratories) and then analyzed by PCR with primers to the human MinigEND or endoglin intron (Table S1).

Statistical analysis

Results are represented as mean ± SEM. Data were subjected to statistical analysis Student's t-test. In the figures, the statistically significant values are referred to control condition, except when indicated (*$P < 0.05$; NS = not significant).

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Authorship contributions

FJB conducted experiments. FJB and CB participated in research design, data analysis and writing of the manuscript. CB acquired funding for the research.

References

Abdalla SA, Letarte M (2006) Hereditary haemorrhagic telangiectasia: current views on genetics and mechanisms of disease. *J. Med. Genet.* **43**, 97–110.

Bellon T, Corbi A, Lastres P, Cales C, Cebrian M, Vera S, Cheifetz S, Massague J, Letarte M, Bernabeu C (1993) Identification and expression of two forms of the human transforming growth factor-beta-binding protein endoglin with distinct cytoplasmic regions. *Eur. J. Immunol.* **23**, 2340–2345.

Bernabeu C, Conley BA, Vary CP (2007) Novel biochemical pathways of endoglin in vascular cell physiology. *J. Cell. Biochem.* **102**, 1375–1388.

Bernabeu C, Lopez-Novoa JM, Quintanilla M (2009) The emerging role of TGF-beta superfamily coreceptors in cancer. *Biochim. Biophys. Acta* **1792**, 954–973.

Black DL (2003) Mechanisms of alternative pre-messenger RNA splicing. *Annu. Rev. Biochem.* **72**, 291–336.

Blanco FJ, Santibanez JF, Guerrero-Esteo M, Langa C, Vary CP, Bernabeu C (2005) Interaction and functional interplay between endoglin and ALK-1, two components of the endothelial transforming growth factor-beta receptor complex. *J. Cell. Physiol.* **204**, 574–584.

Blanco FJ, Grande MT, Langa C, Oujo B, Velasco S, Rodriguez-Barbero A, Perez-Gomez E, Quintanilla M, Lopez-Novoa JM, Bernabeu C (2008) S-endoglin expression is induced in senescent endothelial cells and contributes to vascular pathology. *Circ. Res.* **103**, 1383–1392.

Boulisfane N, Choleza M, Rage F, Neel H, Soret J, Bordonne R (2010) Impaired minor tri-snRNP assembly generates differential splicing defects of U12-type introns in lymphoblasts derived from a type I SMA patient. *Hum. Mol. Genet.* **20**, 641–648.

Brandes RP, Fleming I, Busse R (2005) Endothelial aging. *Cardiovasc. Res.* **66**, 286–294.

Burge CB, Tuschl TH, Sharp PA (1999) Splicing of precursors to mRNAs by the spliceosomes. In *The RNA World*. (Gesteland RF, Cech TR, Atkins JF, eds). Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, pp. 525–560.

Caceres JF, Kornblihtt AR (2002) Alternative splicing: multiple control mechanisms and involvement in human disease. *Trends Genet.* **18**, 186–193.

Caceres JF, Misteli T (2007) Division of labor: minor splicing in the cytoplasm. *Cell* **131**, 645–647.

Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR (2003) ESEfinder: A web resource to identify exonic splicing enhancers. *Nucleic Acids Res.* **31**, 3568–3571.

Cazalla D, Zhu J, Manche L, Huber E, Krainer AR, Caceres JF (2002) Nuclear export and retention signals in the RS domain of SR proteins. *Mol. Cell. Biol.* **22**, 6871–6882.

Chang MW, Grillari J, Mayrhofer C, Fortschegger K, Allmaier G, Marzban G, Katinger H, Voglauer R (2005) Comparison of early passage, senescent and hTERT immortalized endothelial cells. *Exp. Cell Res.* **309**, 121–136.

Chen QM (2000) Replicative senescence and oxidant-induced premature senescence. Beyond the control of cell cycle checkpoints. *Ann. N Y Acad. Sci.* **908**, 111–125.

Chen QM, Bartholomew JC, Campisi J, Acosta M, Reagan JD, Ames BN (1998) Molecular analysis of H2O2-induced senescent-like growth arrest in normal human fibroblasts: p53 and Rb control G1 arrest but not cell replication. *Biochem. J.* **332** (Pt 1), 43–50.

Comi P, Chiaramonte R, Maier JA (1995) Senescence-dependent regulation of type 1 plasminogen activator inhibitor in human vascular endothelial cells. *Exp. Cell Res.* **219**, 304–308.

Crovato TE, Egebjerg J (2005) ASF/SF2 and SC35 regulate the glutamate receptor subunit 2 alternative flip/flop splicing. *FEBS Lett.* **579**, 4138–4144.

ten Dijke P, Goumans MJ, Pardali E (2008) Endoglin in angiogenesis and vascular diseases. *Angiogenesis* **11**, 79–89.

Erusalimsky JD (2009) Vascular endothelial senescence: from mechanisms to pathophysiology. *J. Appl. Physiol.* **106**, 326–332.

Fairbrother WG, Yeh RF, Sharp PA, Burge CB (2002) Predictive identification of exonic splicing enhancers in human genes. *Science* **297**, 1007–1013.

Faughnan ME, Palda VA, Garcia-Tsao G, Geisthoff UW, McDonald J, Proctor DD, Spears J, Brown DH, Buscarini E, Chesnutt MS, Cottin V, Ganguly A, Gossage JR, Guttmacher AE, Hyland RH, Kennedy SJ, Korzenik J, Mager JJ, Ozanne AP, Piccirillo JF, Picus D, Plauchu H, Porteous ME, Pyeritz RE, Ross DA, Sabba C, Swanson K, Terry P, Wallace MC, Westermann CJ, White RI, Young LH, Zarrabeitia R; HHT Guidelines Working Group (2011) International guidelines for the diagnosis and management of hereditary haemorrhagic telangiectasia. *J. Med. Genet.* **48**, 73–87.

Gao K, Masuda A, Matsuura T, Ohno K (2008) Human branch point consensus sequence is yUnAy. *Nucleic Acids Res.* **36**, 2257–2267.

Graveley BR (2000) Sorting out the complexity of SR protein functions. *RNA.* **6**, 1197–1211.

Graveley BR (2001) Alternative splicing: increasing diversity in the proteomic world. *Trends Genet.* **17**, 100–107.

Haendeler J, Hoffmann J, Diehl JF, Vasa M, Spyridopoulos I, Zeiher AM, Dimmeler S (2004) Antioxidants inhibit nuclear export of telomerase reverse transcriptase and delay replicative senescence of endothelial cells. *Circ. Res.* **94**, 768–775.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser.* **41**, 95–98.

Hall SL, Padgett RA (1996) Requirement of U12 snRNA for *in vivo* splicing of a minor class of eukaryotic nuclear pre-mRNA introns. *Science* **271**, 1716–1718.

Holaska JM, Kowalski AK, Wilson KL (2004) Emerin caps the pointed end of actin filaments: Evidence for an actin cortical network at the nuclear inner membrane. *PLoS Biol.* **2**, 1354–1362.

Ibrahim EC, Schaal TD, Hertel KJ, Reed R, Maniatis T (2005) Serine/arginine-rich protein-dependent suppression of exon skipping by exonic splicing enhancers. *Proc. Natl. Acad. Sci. U S A* **102**, 5002–5007.

Jurica MS, Moore MJ (2003) Pre-mRNA splicing: awash in a sea of proteins. *Mol. Cell* **12**, 5–14.

Karni R, de Stanchina E, Lowe SW, Sinha R, Mu D, Krainer AR (2007) The gene encoding the splicing factor SF2/ASF is a proto-oncogene. *Nat. Struct. Mol. Biol.* **14**, 185–193.

Konig H, Matter N, Bader R, Thiele W, Muller F (2007) Splicing segregation: the minor spliceosome acts outside the nucleus and controls cell proliferation. *Cell* **131**, 718–729.

Kwan T, Benovoy D, Dias C, Gurd S, Serre D, Zuzan H, Clark TA, Schweitzer A, Staples MK, Wang H, Blume JE, Hudson TJ, Sladek R, Majewski J (2007) Heritability of alternative splicing in the human genome. *Genome Res.* **17**, 1210–1218.

Lamond AI, Spector DL (2003) Nuclear speckles: a model for nuclear organelles. *Nat. Rev. Mol. Cell Biol.* **4**, 605–612.

Lareau LF, Green RE, Bhatnagar RS, Brenner SE (2004) The evolving roles of alternative splicing. *Curr. Opin. Struct. Biol.* **14**, 273–282.

Lebrin F, Mummery CL (2008) Endoglin-mediated vascular remodeling: mechanisms underlying hereditary hemorrhagic telangiectasia. *Trends Cardiovasc. Med.* **18**, 25–32.

Ledee DR, Chen J, Tonelli LH, Takase H, Gery I, Zelenka PS (2004) Differential expression of splice variants of chemokine CCL27 mRNA in lens, cornea, and retina of the normal mouse eye. *Mol Vis.* **10**, 663–667.

Li X, Manley JL (2005) Inactivation of the SR protein splicing factor ASF/SF2 results in genomic instability. *Cell* **122**, 365–378.

Liu HX, Zhang M, Krainer AR (1998) Identification of functional exonic splicing enhancer motifs recognized by individual SR proteins. *Genes Dev.* **12**, 1998–2012.

Long JC, Caceres JF (2009) The SR protein family of splicing factors: master regulators of gene expression. *Biochem. J.* **417**, 15–27.

Lopez-Novoa JM, Bernabeu C (2010) The physiological role of endoglin in the cardiovascular system. *Am J Physiol Heart Circ Physiol.* **299**, H959–H974.

McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J, McCormick MK, Pericak-Vance MA, Heutink P, Oostra BA, Haitjema T, Westerman CJJ, Porteous ME, Guttmacher AE, Letarte M, Marchuk DA (1994) Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nat. Genet.* **8**, 345–351.

Meshorer E, Soreq H (2002) Pre-mRNA splicing modulations in senescence. *Aging cell.* **1**, 10–16.

Moore MJ, Silver PA (2008) Global analysis of mRNA splicing. *RNA.* **14**, 197–203.

Moroy T, Heyd F (2007) The impact of alternative splicing *in vivo*: mouse models show the way. *RNA.* **13**, 1155–1171.

Nott A, Meislin SH, Moore MJ (2003) A quantitative analysis of intron effects on mammalian gene expression. *RNA.* **9**, 607–617.

Nowak DG, Woolard J, Amin EM, Konopatskaya O, Saleem MA, Churchill AJ, Ladomery MR, Harper SJ, Bates DO (2008) Expression of pro- and anti-angiogenic isoforms of VEGF is differentially regulated by splicing and growth factors. *J. Cell Sci.* **121**, 3487–3495.

Nowak DG, Amin EM, Rennel ES, Hoareau-Aveilla C, Gammons M, Damodoran G, Hagiwara M, Harper SJ, Woolard J, Ladomery MR, Bates DO (2010) Regulation of vascular endothelial growth factor (VEGF) splicing from pro-angiogenic to anti-angiogenic isoforms: a novel therapeutic strategy for angiogenesis. *J Biol Chem.* **285**, 5532–5540.

Ong SE, Mittler G, Mann M (2004) Identifying and quantifying *in vivo* methylation sites by heavy methyl SILAC. *Nat Methods.* **1**, 119–126.

Patel AA, Steitz JA (2003) Splicing double: insights from the second spliceosome. *Nat. Rev. Mol. Cell Biol.* **4**, 960–970.

Perez-Gomez E, Eleno N, Lopez JM, Ramirez JR, Velasco B, Letarte M, Bernabeu C, Quintanilla M (2005) Characterization of murine S-endoglin isoform and its effects on tumor development. *Oncogene* **24**, 4450–4461.

Perez-Gomez E, Villa-Morales M, Santos J, Fernandez-Piqueras J, Gamallo C, Dotor J, Bernabeu C, Quintanilla M (2007) A role for endoglin as a suppressor of malignancy during mouse skin carcinogenesis. *Cancer Res.* **67**, 10268–10277.

Peritz T, Zeng F, Kannanayakal TJ, Kilk K, Eiriksdottir E, Langel U, Eberwine J (2006) Immunoprecipitation of mRNA-protein complexes. *Nat. Protoc.* **1**, 577–580.

Piva F, Giulietti M, Nocchi L, Principato G (2009) SpliceAid: a database of experimental RNA target motifs bound by splicing proteins in humans. *Bioinformatics* **25**, 1211–1213.

Plauchu H, de Chadarévian J-P, Bideau A, Robert J-M (1989) Age-related clinical profile of hereditary hemorrhagic telangiectasia in an epidemiologically recruited population. *Am. J. Med. Genet.* **32**, 291–297.

Sakabe NJ, de Souza SJ (2007) Sequence features responsible for intron retention in human. *BMC Genomics.* **8**, 59.

Sanford JR, Ellis JD, Cazalla D, Caceres JF (2005) Reversible phosphorylation differentially affects nuclear and cytoplasmic functions of splicing factor 2/alternative splicing factor. *Proc. Natl. Acad. Sci. U S A* **102**, 15042–15047.

Sanford JR, Coutinho P, Hackett JA, Wang X, Ranahan W, Caceres JF (2008) Identification of nuclear and cytoplasmic mRNA targets for the shuttling protein SF2/ASF. *PLoS ONE* **3**, e3369.

Sanford JR, Wang X, Mort M, Vanduyn N, Cooper DN, Mooney SD, Edenberg HJ, Liu Y (2009) Splicing factor SFRS1 recognizes a functionally diverse landscape of RNA transcripts. *Genome Res.* **19**, 381–394.

Shen H, Kan JL, Green MR (2004) Arginine-serine-rich domains bound at splicing enhancers contact the branchpoint to promote prespliceosome assembly. *Mol. Cell* **13**, 367–376.

Shovlin CL (2010) Hereditary haemorrhagic telangiectasia: pathophysiology, diagnosis and treatment. *Blood Rev.* **24**, 203–219.

Sinha R, Allemand E, Zhang Z, Karni R, Myers MP, Krainer AR (2010) Arginine methylation controls the subcellular localization and functions of the oncoprotein splicing factor SF2/ASF. *Mol. Cell. Biol.* **30**, 2762–2774.

Smith PJ, Zhang C, Wang J, Chew SL, Zhang MQ, Krainer AR (2006) An increased specificity score matrix for the prediction of SF2/ASF-specific exonic splicing enhancers. *Hum. Mol. Genet.* **15**, 2490–2508.

Solis AS, Peng R, Crawford JB, Phillips III JA, Patton JG (2008) Growth hormone deficiency and splicing fidelity: two serine/arginine-rich proteins, ASF/SF2 and SC35, act antagonistically. *J Biol Chem.* **283**, 23619–23626.

Tacke R, Manley JL (1995) The human splicing factors Asf/Sf2 and Sc35 possess distinct, functionally significant Rna-binding specificities. *EMBO J.* **14**, 3540–3551.

Tarn WY, Steitz JA (1996) Highly diverged U4 and U6 small nuclear RNAs required for splicing rare AT-AC introns. *Science* **273**, 1824–1832.

Thomas CP, Andrews JI, Liu KZ (2007) Intronic polyadenylation signal sequences and alternate splicing generate human soluble Flt1 variants and regulate the abundance of soluble Flt1 in the placenta. *FASEB J.* **21**, 3885–3895.

Torrado M, Iglesias R, Nespereira B, Centeno A, Lopez E, Mikhailov AT (2009) Intron retention generates ANKRD1 splice variants that are co-regulated with the main transcript in normal and failing myocardium. *Gene* **440**, 28–41.

Velasco S, Alvarez-Munoz P, Pericacho M, Dijke PT, Bernabeu C, Lopez-Novoa JM, Rodriguez-Barbero A (2008) L- and S-endoglin differentially modulate TGFbeta1 signaling mediated by ALK1 and ALK5 in L6E9 myoblasts. *J. Cell Sci.* **121**, 913–919.

Verduci L, Simili M, Rizzo M, Mercatanti A, Evangelista M, Mariani L, Rainaldi G, Pitto L (2010) MicroRNA (miRNA)-mediated interaction between leukemia/lymphoma-related factor (LRF) and alternative splicing factor/splicing factor 2 (ASF/SF2) affects mouse embryonic fibroblast senescence and apoptosis. *J Biol Chem.* **285**, 39551–39563.

Voglauer R, Chang MW, Dampier B, Wieser M, Baumann K, Sterovsky T, Schreiber M, Katinger H, Grillari J (2006) SNEV overexpression extends the life span of human endothelial cells. *Exp. Cell Res.* **312**, 746–759.

Wang J, Smith PJ, Krainer AR, Zhang MQ (2005) Distribution of SR protein exonic splicing enhancer motifs in human protein-coding genes. *Nucleic Acids Res.* **33**, 5053–5062.

Will CL, Luhrmann R (2005) Splicing of a rare class of introns by the U12-dependent spliceosome. *Biol Chem.* **386**, 713–724.

Wu Y, Zhang Y, Zhang J (2005) Distribution of exonic splicing enhancer elements in human genes. *Genomics.* **86**, 329–336.

Xu XD, Yang DM, Ding JH, Wang W, Chu PH, Dalton ND, Wang HY, Bermingham JR, Ye Z, Liu F, Rosenfeld MG, Manley JL, Ross J, Chen J, Xiao RP, Cheng HP, Fu XD (2005) ASF/SF2-Regulated CaMKII delta alternative splicing temporally reprograms excitation-contraction coupling in cardiac muscle. *Cell* **120**, 59–72.

Zhang Z, Lotti F, Dittmar K, Younis I, Wan L, Kasim M, Dreyfuss G (2008) SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. *Cell* **133**, 585–600.

Zhou Z, Licklider LJ, Gygi SP, Reed R (2002) Comprehensive proteomic analysis of the human spliceosome. *Nature* **419**, 182–185.

Zielinski J, Kilk K, Peritz T, Kannanayakal T, Miyashiro KY, Eiriksdottir E, Jochems J, Langel U, Eberwine J (2006) *In vivo* identification of ribonucleoprotein-RNA interactions. *Proc. Natl. Acad. Sci. U S A* **103**, 1557–1562.

Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1 Sequence of the different primers used in PCR reactions

Fig. S1 Confocal microscopy analysis of the alternative splicing factor or splicing factor-2 cytoplasmic mutants.

Fig. S2 siRNA-mediated knock-down of U6atac in HUVECs.

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