A novel function of Tis11b/BRF1 as a regulator of Dll4 mRNA 3′-end processing

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ABSTRACT Tis11b/BRF1 belongs to the tristetraprolin family, the members of which are involved in AU-rich-dependent regulation of mRNA stability/degradation. Mouse inactivation of the Tis11b gene has revealed disorganization of the vascular network and up-regulation of the proangiogenic factor VEGF. However, the VEGF deregulation alone cannot explain the phenotype of Tis11b knockouts. Therefore we investigated the role of Tis11b in expression of Dll4, another angiogenic gene for which haploinsufficiency is lethal. In this paper, we show that Tis11b silencing in endothelial cells leads to up-regulation of Dll4 protein and mRNA expressions, indicating that Dll4 is a physiological target of Tis11b. Tis11b protein binds to endogenous Dll4 mRNA, and represses mRNA expression without affecting its stability. In the Dll4 mRNA 3′ untranslated region, we identified one particular AUUUA motif embedded in a weak noncanonical polyadenylation (poly(A)) signal as the major Tis11b-binding site. Moreover, we observed that inhibition of Tis11b expression changes the ratio between mRNAs that are cleaved or read through at the poly(A) signal position, suggesting that Tis11b can interfere with mRNA cleavage and poly(A) efficiency. Last, we report that this Tis11b-mediated mechanism is used by endothelial cells under hypoxia for controlling Dll4 mRNA levels. This work constitutes the first description of a new function for Tis11b in mammalian cell mRNA 3′-end maturation.

INTRODUCTION

Although underestimated for a long time, posttranscriptional regulation is now recognized as a key control mechanism of gene expression. Splicing, maturation, 3′-end processing, and stability represent major levels of regulation of mRNA levels. Thus RNA-binding proteins (RBPs) involved in the different steps of posttranscriptional regulation are key players in this process (Moore, 2005). Among them are members of the tristetraprolin family, nucleocytoplasmic shuttling proteins that were first characterized as mRNA-destabilizing proteins (Bao et al., 2009). In humans, the tristetraprolin family comprises three members: Tis11b/BRF1 (ZFP36-L1), Tis11d/BRF2 (ZFP36-L2), and the best studied, Tis11/TTP (ZFP36), also named tristetraprolin (TTP). All contain a unique CCCH tandem zinc finger (TZF) domain responsible for mRNA binding through short cis-acting sequences called AU-rich elements (AREs) present in the 3′ untranslated region (3′ UTR) of target mRNAs. It has been shown that TTP and Tis11b/BRF1 are able to activate and recruit the mRNA decay machineries onto target transcripts, and thus trigger mRNA degradation (Lykke-Andersen and Wagner, 2005). While in vitro overexpression studies suggest that all three family members have redundant RNA-destabilizing activities, the distinct phenotypes of knockout mice indicate that they might have more specific roles in the control of gene expression.

Indeed, mouse inactivation of Tis11b gene shows numerous vascular defects throughout the embryo, indicating the importance of...
the Tis11b protein in angiogenesis, which is the process of blood vessel growth from the preexisting vasculature. These defects were associated with an up-regulation of vascular endothelial growth factor (VEGF) protein (Bell et al., 2006). VEGF is such a fine regulator of angiogenesis that genetic inactivation of a single allele induces important vascular defects and subsequent embryonic lethality (Carmeliet et al., 1996; Ferrara et al., 1996). Thus VEGF expression levels are critical for physiology.

In a previous work, we were the first to show that Tis11b is involved in the regulation of VEGF expression, suggesting a role for Tis11b in angiogenesis (Chinn et al., 2002; Ciais et al., 2004). However, VEGF overexpression in transgenic mice induces embryonic lethality, mainly due to cardiovascular defects with no change in embryonic vessel density or caliber (Miquerol et al., 2000). Thus comparing the phenotype of homozygous Tis11b−/− knockout mice with that of VEGF-overexpressing transgenic mice indicated that overexpression of VEGF alone cannot account for the vascular defects observed in Tis11b knockouts. It is likely that Tis11b might repress other important genes involved in the control of angiogenesis.

Delta-like-4 (Dll4) is a transmembrane ligand of the Notch receptor family, which is involved in cell fate determination (Kume, 2009). Dll4 is specifically expressed in specialized endothelial cells, called “tip cells,” which lead the way for sprouting neo-vessels. Dll4 expression in the tip cells regulates angiogenic branching and density by repressing the ability of neighboring cells to respond to angiogenic stimulation (Williams et al., 2006; Hellstrom et al., 2007; Suchting et al., 2007). Dll4 haploinsufficiency and overexpression both result in embryonic lethality, strongly suggesting that finely tuned regulation of Dll4 expression is required during angiogenesis. Interestingly, we observed that the phenotypes of both transgenic mice overexpressing Dll4 and Tis11b knockout mice show strong similarities, as described in Supplemental Table S1. This suggested to us that Tis11b might also regulate Dll4 expression.

In this work, to further decipher the role of Tis11b in angiogenesis, we addressed the possibility that Tis11b controls Dll4 expression. In this paper, we present experimental evidence that Tis11b regulates Dll4 expression in endothelial cells and is involved in hypoxia-mediated regulation of Dll4. In addition, we observed that Tis11b does not control Dll4 mRNA stability, but modulates 3′ end maturation of the transcript through interaction with an ARE located in the polyadenylation (poly(A)) signal. Our work represents the first description of this novel function of Tis11b in mammalian mRNA 3′ processing.

RESULTS

Dll4 is a direct and physiological target of endogenous Tis11b in endothelial cells

To check for the involvement of Tis11b in Dll4 regulation, we performed a small interfering RNA (siRNA)-based experiment to inhibit endogenous Tis11b expression in primary endothelial cells, and we analyzed Dll4 expression. In Figure 1A, Western blot analysis shows a complete repression of Tis11b protein expression when human umbilical aortic endothelial cells (HuAEC) were transfected with either of two specific Tis11b-targeting siRNAs (T11b1 and T11b2), as compared with a negative control siRNA (CTL). The inhibition of Tis11b is accompanied by a substantial increase in Dll4 protein level (Figure 1A). As Tis11b controls mRNA turnover, we quantified Dll4 mRNA steady-state levels by reverse transcriptase quantitative real time PCR (RT-qPCR) in cells that were silenced for Tis11b expression or transfected with siCTL siRNA. As shown in Figure 1B, inhibition of Tis11b expression induced a significant two- to threefold increase in the Dll4 mRNA level in HuAEC cells. To exclude a cell type–dependent effect, the same experiment was performed in dermal human microvascular endothelial cells (HMVECs). As shown in Figure 1C, silencing of Tis11b in HMVECs also induced an increase in Dll4 mRNA expression.

To investigate whether Tis11b protein directly interacts with Dll4 mRNA, we performed RNP (ribonucleoprotein) immunoprecipitation experiments. After cross-linking of endogenous RNP complexes in living cells, we immunoprecipitated Tis11b-containing particles using a specific anti-Tis11b antibody (Ciais et al., 2004; Cherradi et al., 2006) and subjected them to RT-qPCR analysis. Figure 1D clearly shows that Dll4 mRNA was detected in the immunoprecipitated molecular complexes. No HPRT mRNA was detected in Tis11b-containing particles, demonstrating that Tis11b protein-Dll4 mRNA interaction was specific. Moreover, similar data were obtained using a commercial antibody (αBRF1/2) that targets a conserved sequence in both Tis11b and Tis11d proteins (unpublished data).

Altogether, these results indicate that Dll4 mRNA is a direct and physiological target of Tis11b in endothelial cells.

Tis11b does not alter Dll4 mRNA stability and is mainly localized in the nucleus of endothelial cells

Because Tis11b is known to regulate mRNA stability (Stoecklin et al., 2002), we tested whether Tis11b silencing had an effect on endogenous Dll4 mRNA turnover rate. Tis11b or CTL siRNAs were transfected in primary endothelial cells, and an inhibitor of RNA polymerase II (5,6-dichloro-1-β-d-ribofuranosylbenzimidazole [DRB]) was added. As shown in Figure 1D, knockdown of Tis11b did not alter Dll4 mRNA stability, suggesting that Tis11b is not involved in Dll4 mRNA stability. In addition, we performed RNA immunoprecipitation experiments. After cross-linking of endogenous RNP complexes in living cells, we immunoprecipitated Tis11b-containing particles using a specific anti-Tis11b antibody (Ciais et al., 2004; Cherradi et al., 2006) and subjected them to RT-qPCR analysis. Figure 1D clearly shows that Dll4 mRNA was detected in the immunoprecipitated molecular complexes. No HPRT mRNA was detected in Tis11b-containing particles, demonstrating that Tis11b protein-Dll4 mRNA interaction was specific. Moreover, similar data were obtained using a commercial antibody (αBRF1/2) that targets a conserved sequence in both Tis11b and Tis11d proteins (unpublished data).

Altogether, these results indicate that Dll4 mRNA is a direct and physiological target of Tis11b in endothelial cells.
Control of mRNA stability has been mainly described in the cytoplasm; we therefore analyzed the subcellular localization of endogenous Tis11b in HuAEC by immunofluorescence using an antibody that recognizes both Tis11b and Tis11d (αBRF1/2). Figure 2B clearly shows that Tis11b/d immunoreactivity was mainly present in the nucleus. Similar results were obtained in HMVECd (unpublished data).

Altogether, these results suggest that the induction of Dll4 mRNA observed in Tis11b-depleted endothelial cells is not due to changes in mRNA stability (a process occurring mainly in the cytoplasm), but rather to an unidentified mechanism of regulation of RNA processing that likely occurs in the nucleus.

Identification of potential binding sites for Tis11b protein in Dll4 mRNA
Tis11b has been shown to bind AREs, which often contain single or multiple copies of the core pentameric AUUUA motif. We therefore checked the human Dll4 cDNA sequence for consensus AUUUA motifs. We identified one such pentameric motif in close proximity with the cDNA 3′ end. However, we were surprised to observe that this deposited cDNA sequence (NM_019074.2) did not contain a poly(A) tail, and that its poly(A) site was not annotated. We examined the genomic sequences encoding the region around the poly(A) site, which appeared highly conserved between the human, mouse, rat, chicken, and zebrafish species. All contained a second pentameric motif located downstream of the 3′ end of the deposited human Dll4 cDNA sequence. We therefore assumed this cDNA sequence was incomplete. We decided to amplify it by 3′-RACE and to sequence it in order to check whether the second AUUUA motif was actually included in the Dll4 mRNA. We sequenced 12 amplified clones and identified heterogeneously located poly(A) tail–insertion sites. Five clones revealed an identical poly(A) insertion site defining a “hot spot,” whereas in the seven remaining clones, the poly(A) start sites were evenly located on both sides of this hot spot (Figure 3). The analysis of the complete human Dll4 3′ UTR cDNA sequences recovered in these experiments allowed us to confirm the presence of the two pentameric AUUUA motifs (named thereafter ARE1 and ARE2) identified in the conserved genomic sequences.

Tis11b regulates Dll4 expression through binding to AU-rich elements in its 3′ UTR
We next tested whether Tis11b exerted its regulation via direct binding to Dll4 AUUUA motifs. To address this hypothesis, we constructed luciferase reporter plasmids as described in Figure 4A, and transfected them with or without expression plasmids encoding...
β-galactosidase (β-Gal) or human Tis11b. We used NIH 3T3 cells for transfection experiments, as these cells express very low levels of Tis11b protein. Transfection of Tis11b protein in these cells had no or little effect on luciferase activity when the SV40 poly(A) signal was cloned downstream of the luciferase gene (Figure 4B). In contrast, Tis11b expression repressed luciferase expression by ~20–25% when the SV40 poly(A) signal was replaced by the 2000-nucleotide-long 3′ UTR (Dll4 3′ UTR plasmid). As we located potential binding sites for Tis11b in close proximity to the Dll4 poly(A) signal, we narrowed down the region of Dll4/Tis11b interaction by cloning only the Dll4 poly(A) signal downstream of the luciferase gene (DII4pA plasmid). Cotransfection of Tis11b with either DII4pA or Dll4 3′ UTR plasmid led to a very similar decrease in luciferase expression, indicating that the 125 nucleotides upstream of the poly(A) site are sufficient for Tis11b-mediated control of Dll4 expression. We further analyzed Tis11b interaction with the transcript by introducing mutations in the two pentameric motifs (AUUUA to AGGUA). Individual mutations of either the first AUUUA motif (ARE1) or the second AUUUA motif (ARE2) had no effect on Tis11b ability to reduce luciferase reporter expression. On the contrary, mutations of both motifs (ARE1/2 construction) completely abolished Tis11b-mediated control of luciferase activity. Altogether, these results indicate that the presence of either of the two ARE in this context is sufficient for control of Dll4 expression by Tis11b.

To assess direct binding of Tis11b to the two core pentameric sequences AUUUA, we next performed an electrophoretic mobility-shift assay (EMSA) experiment using recombinant Tis11b protein and radioactive RNA encoding 125 nucleotides upstream of the poly(A) site. As shown in Figure 4C, the addition of recombinant Tis11b induced a shift in the migration of the radiolabeled RNA probe. Moreover, this shift was abolished by the addition of cold competitor, which indicates specific binding of Tis11b protein to Dll4 mRNA 3′ UTR. In a separate set of in vitro UV-crosslinking experiments, we assessed the relative binding between Tis11b protein and Dll4 poly(A) RNA containing mutations on one, two, or none of the AUUUA pentamers. Figure 4D shows that Tis11b protein bound to wild-type RNA and to the ARE1 mutant, but not to the ARE2 mutant or to the ARE1/2 double mutant. These results indicate that ARE2 is the main pentamer responsible for in vitro Tis11b binding onto Dll4 3′ UTR.

The efficiency of the weak poly(A) signal of Dll4 can be modulated by Tis11b

Mammalian poly(A) signals (PAS) are composed of a canonical core A(A/U)UAUA hexamer (or a close variant) located between 10 and 35 nucleotides before a definite site of cleavage and a G/U-rich downstream element (Millevoi and Vagner, 2010). In addition to these signals, and especially in noncanonical PAS, it has been shown that an upstream UGUA element highly contributes to poly(A) signal efficiency via binding of CFI (Cleavage Factor I), thereby favoring the recruitment of the poly(A) machinery (Nunes et al., 2010). The second AUUUA motif is located 25 nucleotides upstream of the main poly(A) site of Dll4, suggesting that the Tis11b-binding site is embedded in the poly(A) signal, which directs the cotranscriptional recruitment of the cleavage/poly(A) machinery (Figure 3). Moreover, it overlaps one of the three UGUA elements present in this poly(A) signal. As we showed that Tis11b is present in the nucleus and that Tis11b can bind to ARE2, we next tested if Tis11b could decrease Dll4 mRNA by affecting its 3′-end processing in endothelial cells.

To address this question, we constructed a pDll4pA+SV40pA plasmid derived from the previously described pSV40pA and pDII4pA vectors, as illustrated in Figure 5A. pDII4pA+SV40pA...
plasmid contains the luciferase reporter gene cloned upstream of the Dll4 PAS, followed by the SV40 PAS. Figure 5B shows that pDll4pA luciferase activity is ~10-fold lower than luciferase activity obtained with pSV40pA. On the other hand, insertion of the SV40 PAS downstream of the Dll4 PAS (pDll4pA-Long+SV40pA plasmid) significantly increased the level of luciferase activity by threefold, indicating that a subpopulation of reporter transcripts is not properly cleaved at Dll4 PAS. The fact that luciferase activity triggered by pDll4pA-Long+SV40pA plasmid is not completely restored to the level of pDll4pA plasmid suggested that another level of regulation could be mediated by nucleotides downstream of Dll4 poly(A) signal. To assess this, we then shortened the pDll4pA-Long+SV40pA plasmid to make the pDll4pA-short+SV40pA, which contains the SV40 poly(A) signal in close proximity to the Dll4 PAS (Figure 5A). As shown in Figure 5B, luciferase activities triggered either by pDll4pA-Long+SV40pA or by pDll4pA-short+SV40pA plasmids are similar. Altogether, these results suggest that Dll4 PAS efficiency is weak and that it might be subjected to subtle regulation.

We next examined whether human Dll4 PAS is also weak in its genomic context. To this end, we designed reverse and forward primers located on both sides of the Dll4 PAS for RT-PCR amplification (rt2Dll4), as illustrated in Figure 5C. The 563-nucleotide-long rt2Dll4 amplicon thus encompassed the poly(A) cleavage sites and allowed us to detect the uncleaved transcript. Figure 5D shows detection of the reverse transcriptase (RT)-dependent rtDll4 transcript from HuAEC total RNA. This points out that at least part of Dll4 mRNA is not fully processed at the poly(A) signal. We further evaluated the possible involvement of Tis11b in cleavage and poly(A) efficiency of Dll4 PAS. Therefore we quantified total Dll4 mRNA expression by RT-qPCR analysis in Tis11b-depleted HuAEC cells. Positions of tDll4 and rtDll4 amplicons are shown in Figure 5C. As shown in Figure 5E, inhibition of Tis11b expression decreased the rtDll4/tDll4 ratio by approximately twofold, indicating that the Dll4 transcript is better processed at poly(A) sites in the absence of Tis11b.

**FIGURE 5:** Tis11b interferes with cleavage and poly(A) efficiencies. (A) Schematic representation of the plasmids used in NIH 3T3 cell transfections. All plasmids contain the luciferase gene under the control of the thymidine kinase promoter. The pSV40pA plasmid contains the SV40 PAS cloned downstream of the luciferase gene. For Dll4pA plasmid, the sequence inserted in place of the SV40 PAS begins 125 nucleotides before the expected Dll4 PAS and extends to the 475 downstream nucleotides. Plasmids pDll4pA-Long+SV40 and pDll4pA-Short+SV40 are derived from the pDll4pA plasmid with an SV40 PAS added at the end of the Dll4pA sequence or immediately after the Dll4 PAS, respectively. (B) NIH 3T3 cells were cotransfected with 250 ng of pSV40pA, pDll4pA, pDll4pA-Long+SV40pA, or pDll4pA-Short+SV40pA plasmid and 5 ng of pRL for normalization. Renilla and Firefly luciferase measurements were done 24 h after transfection. Data are the means of six independent experiments (**p < 0.01). (C) Representation of the organization of the Dll4 gene indicating the location of the poly(A) site and the PCR amplifications performed. rtDll4 fragment is encompassing Dll4 poly(A) sites. (D) Total HuAEC RNAs were reverse-transcribed and subjected to RT-PCR detection of the uncleaved Dll4 transcript (rt2Dll4). (E) RT-qPCR detection of total Dll4 transcript (tDll4) and read-through transcript (rtDll4) in Tis11b-depleted HuAEC cells (siTis11b) or control cells (siCTRL). The ratio of readthrough to total Dll4 transcript levels were normalized to the value measured in siCTRL-transfected cells (taken as 100%). Data are the mean of four independent experiments (**p < 0.01).
DISCUSSION

**Tis11b is a major modulator of angiogenesis**

In this work, we provide evidence that the AU-rich binding protein Tis11b/BRF1 is a direct regulator of Dll4 mRNA expression in endothelial cells. The identification of Dll4 as a Tis11b target was performed using a siRNA-based strategy to silence Tis11b protein expression. Except for the reporter gene studies used to confirm the AU-rich-dependent effect of Tis11b, we avoided overexpression experiments as much as possible in order to focus on physiological regulation of Dll4 expression. Even though it has been recently described that Tis11b and Tis11d in vivo functions can overlap (Hodson et al., 2010), the Tis11b silencing alone is sufficient to increase Dll4 mRNA in our study. Although we used two different siRNAs targeting the Tis11d sequence, we were not able to significantly suppress Tis11d protein to address its potential role in Dll4 expression. Moreover, TTP inhibition in endothelial cells had no effect on Dll4 RNA expression (Supplementary Figure S1), suggesting that physiological control of Dll4 expression by Tis11 family members is restricted to Tis11b.

Interestingly, Dll4 gene dosage has such a drastic effect on blood vessel development that both genetic haploinsufficiency and overexpression are lethal (Krebs et al., 2004; Trindade et al., 2008). Our work indicates that at least part of the Tis11b+/− phenotype might be due to Dll4 overexpression resulting from Tis11b depletion in endothelial cells. Moreover, we further illustrate Tis11b involvement in angiogenesis by showing that, besides VEGF, it regulates the expression of Dll4, a second key gene for which haploinsufficiency is known to trigger embryonic lethality.

Among various stimuli inducing VEGF and Dll4 expression, hypoxia plays a major role in inducing tumor angiogenesis and tumor growth. Therefore we examined the hypoxic regulation of Tis11b expression in endothelial cells. We established that hypoxia down-regulates Tis11b expression and that Tis11b is involved in hypoxia-mediated up-regulation of Dll4. This is in contrast with a previous work showing hypoxia up-regulation of Tis11b in human kidney tubular epithelial cells (Sinha et al., 2009). The authors also showed that hypoxia triggered an increase in Tis11b expression in human renal cell carcinoma (RCC), which was reversed by overexpression of the Von Hippel-Lindau (VHL) tumor suppressor gene. Altogether, these observations are in favor of a cell- and context-dependent regulation of Tis11b by hypoxia, which then contributes to modulation of angiogenesis.

**Mechanism of Tis11b activity**

Both pentamer AUUUA motifs identified in this study contributed to Dll4 gene control by Tis11b, although no specific interaction between Tis11b protein and ARE1 could be detected in our in vitro binding assay. Nuclear magnetic resonance structure of the Tis11d zinc finger domain (which is very similar to the Tis11b domain) in a complex with the UUAUUUAUU RNA-binding substructure showed that each zinc finger is in contact with a 5′-UAAU-3′ subsite (Hudson et al., 2004). Moreover, according to the AU-Rich Element-Containing mRNA Database (ARED; Bakheet et al., 2006), which clusters human and mouse AU-rich containing transcripts, ARE1 has a sequence of low consensus, with the only core AUUUA. It is possible that Tis11b needs a third partner to stabilize the binding to ARE1 allowing Tis11b-mediated regulation of Dll4 mRNA.

Tis11b family members are known to be shuttling proteins (Phillips et al., 2002). TTP and Tis11b proteins contain both nuclear localization and export signals and shuttling of the protein is mediated by CRM1-dependent nuclear export. Transfected TTP has been shown to be either nuclear or cytoplasmic, depending on the cell types examined (Johnson et al., 2002; Phillips et al., 2002). In endothelial cells, nuclear sequestration of TTP induced by TNFα treatment abrogates mRNA destabilization, indicating that subcellular localization of the Tis11 family members is part of their mechanism of action (Gringhuis et al., 2005). Here we show that Tis11b is mainly localized in the nucleus of endothelial cells, in contrast with previous reports showing cytoplasmic localization in various cell types (Benjamin et al., 2006; Cherradi et al., 2006). Surprisingly, Tis11b depletion in endothelial cells had no effect on Dll4 mRNA stability. Taken together, these findings suggest that Tis11b regulates Dll4 expression via a nuclear processing pathway that is independent of Dll4 mRNA turnover.

It recently has been reported that a yeast homologue of the tristetraprolin family interferes with mRNA 3′-end processing when AU-rich binding sites are located around the poly(A) signal (Prouteau et al., 2008). Interestingly, Dll4 mRNA PAS contains such a feature, with an efficient AU-rich binding site for Tis11b located less than 30 nucleotides upstream the main poly(A) site (ARE2). Thereby, we speculated that Tis11b binding to Dll4 pentameric motifs can interfere with 3′-end formation and thus modulate mRNA levels. Indeed, Tis11b depletion in endothelial cells decreases the ratio between Dll4 transcripts stopping at or read through the poly(A) signal, indicating that Tis11b can modulate 3′-end maturation efficiency. It is well known that poly(A) signals contain multiple binding sites for modulators of poly(A) efficiency and that processing of 3′-end maturation can be an important step of regulation (Millevoi and Vagner, 2010). For instance, it has been described that binding of polypyrimidine tract–binding protein (PTB) close to mRNA poly(A) signal could inhibit 3′-end cleavage and poly(A) leading to an increase in readthrough transcripts (Castelo-Branco et al., 2004). Moreover, readthrough transcripts resulting from defective poly(A) are subjected to rapid degradation by the nuclear surveillance pathway which therefore triggers down-regulation of gene expression (Milligan et al., 2005; West and Proudfoot, 2009).

Precise mechanisms of Tis11b modulation of 3′-end maturation remain to be identified. Tis11b binding to Dll4 AREs might alter the normal recruitment of the cleavage and poly(A) machinery and/or impede the maturation process as schematized in Figure 7. Resulting aberrant transcripts might be detected by the nuclear surveillance pathway, and subsequently degraded by the 3′-exonucleolytic

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**FIGURE 6:** Tis11b is involved in hypoxia-mediated Dll4 up-regulation. (A) Western blot analysis of Tis11d, Tis11b, Dll4, and tubulin protein expression in HMVECd and HuAEC cells grown under normoxia (Nx) and hypoxia (Hx, 1.5%O2). (B) HuAEC cells were cultured under normoxia (Nx, 21%O2) for 24 h after siRNA transfection and Dll4 mRNA transcripts were quantified by RT-qPCR analysis. Data are mean of four independent experiments. Values with distinct letters are statistically different (p < 0.05).
activity of the nuclear exosome (Houseley et al., 2006). Moreover, it has been recently described that improper cleavage and poly(A) of mRNA induce defects in RNA polymerase II recycling to the transcription initiation site, leading to down-regulation of gene expression (Mapandano et al., 2010).

To our knowledge, we present here the first evidence of a new function of Tis11b on 3′-end maturation in mammalian cells. These findings might be particularly relevant, considering that more than one-half of human transcripts contain multiple alternative poly(A) sites (Tian et al., 2005). Moreover, most of these poly(A) sites are surrounded by regions with high A and U content that can favor the presence of AREs.

MATERIALS AND METHODS

Cell culture

NIH 3T3 cells were grown in DMEM (Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum (FBS; Invitrogen). Primary endothelial cells (HMVECd and HuAEC) were purchased from LONZA (Basel, Switzerland) and cultivated according to the manufacturer's instructions. All cells were incubated at 37°C in a humid atmosphere in presence of 5% CO2/95% air atmosphere for normoxic conditions or in 1.5% air atmosphere for hypoxic conditions.

RNA ChIP assay

RNA ChIP assays were performed on HMVECd cells. Tis11b containing ribonucleoprotein complexes was immunoprecipitated with our monoclonal antibodies (Cherradi et al., 2006) and with commercial antibodies (#2119; Cell Signaling, Danvers, MA). Ribonucleoprotein complexes were cross-linked by addition of formaldehyde (1% final concentration for 10 min at 37°C), which was then quenched by glycine at 0.125 M for 5 min at 4°C. Cells pellets were resuspended in radioimmunoprecipitation assay (RIPA) buffer containing IP. After 30 min on ice, samples were centrifuged 10 min (14,000 rpm, 4°C). Incubation with 20 μl protein A agarose/salmon sperm DNA (50% slurry) for 1 h at 4°C removes nonspecific interactions. After a short centrifugation, the supernatant was immunoprecipitated overnight at 4°C with either antibodies to a peptide fragment (aa 49–63) of Tis11b protein (1:500; CovalAb, Lyon, France) or with αBRF1/2 antibodies (#2119; Cell Signaling) at a dilution of 1:250. A negative control was performed with non-immune rabbit immunoglobulin G (IgG). Antibody–RNA complexes were then collected by addition of 50 μl protein A agarose/salmon sperm DNA (50% slurry) for 1 h at 4°C, and underwent further centrifugation (1500 rpm for 5 min). After four washes with RIPA buffer, elution was done with 100 μl 50 mM Tris-HCl, 5 mM EDTA, 10 mM dithiothreitol (DTT), 1% SDS. Reversion of ribonucleoprotein cross-linking was performed by heating samples at 70°C for 45 min. After centrifugation, the supernatant contained RNA ready for reverse transcription.

siRNA silencing of Tis11b protein in primary endothelial cells

Primary endothelial cells were transfected with siRNA (final concentration: 20 nm/l) targeting human Tis11b sequence using Lipofectamine RNAiMAX from Invitrogen. Two different siRNAs were used to silence Tis11b protein expression to avoid off-target effects, and a nonrelevant siRNA was used to control of specificity. Protein and RNA extractions were done 48 h after siRNAs transfection. All siRNA were purchased from Applied Biosystems (Bedford, MA; #s2089, s2091, and AM4613).

Western blot assay

Two days after siRNA transfection, endothelial cells were lysed in RIPA buffer (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% Na deoxycholate, 1 mM EDTA, 1% Triton X100, and protease inhibitor cocktail (#P8340; Sigma, St. Louis, MO) for protein extraction. After fractionation of proteins on a 12% SDS–PAGE gel and blotting to a PVDF membrane, immunoblotting detection of Tis11b and Dll4 protein was done using Cell Signaling antibodies according to manufacturer’s instructions (#2119 and #2589, respectively). Anti-tubulin antibodies kindly provided by A. Andrieux (Grenoble Institute of Neuroscience, Grenoble, France) were used to check for equal protein loading.

RNA extractions and gene expression analysis by real-time PCR amplification

All RNA extraction was performed using RNAII or RNA XS extraction kits from Macherey-Nagel depending on sample sizes. cDNAs were generated from 1 μg total RNA by reverse transcription using the iScript system (Bio-Rad, Hercules, CA) as recommended by the manufacturer. Real-time PCR was performed using Bio-Rad CFX96 apparatus and qPCR Master Mix (Promega, Charbonnières Les Bains, France). For Dll4 mRNA half-life measurement, activity of the RNA polymerase II was inhibited by addition of DRB (10 μg/ml) and RNA was extracted at indicated time points. Specific primers used are listed in Supplemental Table S2.

Plasmid constructs

All constructs were cloned into pGL3-basic vector (Promega, Charbonnières Les Bains, France) containing the firefly luciferase gene reporter under the control of a thymidine kinase promoter. 3′ UTR Dll4 and Dll4pA were cloned by PCR on human genomic DNA using primers noted in Supplemental Table S2. The PCR reaction was performed using Advantage GC Genomic LA Polymerase Mix (Clontech, Mountain View, CA). The fragments obtained of 2000 (Dll4 3′-UTR) and 1000 bp (Dll4pA 3′-UTR) were subcloned into pGL3-basic vector (Promega, Charbonnières Les Bains, France) containing the firefly luciferase gene reporter under the control of a thymidine kinase promoter. 3′ UTR Dll4 and Dll4pA were cloned by PCR on human genomic DNA using primers noted in Supplemental Table S2. The PCR reaction was performed using Advantage GC Genomic LA Polymerase Mix (Clontech, Mountain View, CA). The fragments obtained of 2000 (Dll4 3′-UTR) and 1000 bp (Dll4pA 3′-UTR) were subcloned into pGL3-basic vector (Promega, Charbonnières Les Bains, France) containing the firefly luciferase gene reporter under the control of a thymidine kinase promoter.
PBS for 15 min at room temperature. After three washes with PBS, cells were permeabilized with 0.5% Triton X-100 for 15 min. Slides were then incubated 1 h at room temperature with rabbit anti-human BRF1/2 antibody (#2119; Cell Signaling) diluted at 1:50. After three rinses, slides were incubated for 1 h at room temperature with cyanine 3-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) diluted 1:500 and Phalloidin-Alexa Fluor 555 (A34055; Invitrogen). After washes in PBS, slides were counterstained with Hoechst 33258 (diluted 1:1000) and mounted in Fluorsave (Calbiochem, LaJolla, CA).

3′-end rapid amplification of cDNA ends (3′-RACE)

To determine the localization of the poly(A) site, 3′-RACE was performed as described by (Moucadel et al., 2007). Briefly, 2 μg human endothelial cell RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) in the presence of 500 ng of the V(T)17-AP oligo. cDNA was then amplified with a reverse adapter oligonucleotide (AP) and a forward oligonucleotide (Dll4-F701) located 300 nucleotides upstream of the known end of the human Dll4 3′ UTR. A 23-nucleotide downstream forward oligonucleotide and the same reverse AP primer were used to perform a nested PCR amplification. After gel purification, PCR products were cloned into pCRII-TOPO vector from Invitrogen and 12 clones were sequenced (GATC Biotech). Oligonucleotides are listed in Supplemental Table S2.

Statistical analysis

All results were analyzed by Kruskal-Wallis or Mann-Whitney test. Values of p < 0.05 (*) were considered statistically significant.

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UV cross-linking and EMSA experiments

EMSA and UV cross-linking experiments were performed as previously described (Ciais et al., 2004). Briefly, 5 × 10^5 cpm of 32P-labeled riboprobes were incubated with 250 ng of recombinant Tis11b protein in 10 mM HEPES (pH 7.6), 3 mM MgCl2, 40 mM KCl, 5% glyceral, 0.5% NP40, and 2 mM DTT. Yeast tRNA (50 ng/ml) and heparin (2 μg/ml) were then added for 10 min. RNA-protein complexes were then resolved by electrophoresis on a native 0.5X TBE-4% acrylamide gel and visualized with a β-Imager. For the UV cross-linking experiment, mixtures were prepared as for EMSA and exposed to UV light for 30 min at 4°C. Then, 100 U of RNase T1 (Invitrogen) was added for 20 min and RNA–protein complexes were analyzed by 10% SDS–PAGE.

Transient transfection and luciferase assay

Subconfluent NIH 3T3 cells were transfected using the Lipofectamine Reagent (Invitrogen). Briefly, for each experiment, 50 ng of plasmid encoding human Tis11b or β-galactosidase was cotransfected with 250 ng of either 3′ UTR Dil4, Dil4αPA, mutARE1, mutARE2, or mutARE1+2 plasmid and, for normalization purposes, 5 ng of plasmid encoding Renilla luciferase under the control of thymidine kinase promoter (pRL). At 24 h posttransfection, measurement of luciferase activities was performed using the Dual-Luciferase Reporter Assay System (Promega, Charbonnières Les Bains, France) according to the manufacturer’s instructions. Results are expressed as relative light units of firefly luciferase activity over relative light units of Renilla luciferase activity.
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