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ORIGINAL ARTICLE

The novel tumor suppressor NOL7 post-transcriptionally regulates thrombospondin-1 expression

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Thrombospondin-1 (TSP-1) is an endogenous inhibitor of angiogenesis whose expression suppresses tumor growth *in vivo*. Like many angiogenesis-related genes, TSP-1 expression is tightly controlled by various mechanisms, but there is little data regarding the contribution of post-transcriptional processing to this regulation. NOL7 is a novel tumor suppressor that induces an antiangiogenic phenotype and suppresses tumor growth, in part through upregulation of TSP-1. Here we demonstrate that NOL7 is an mRNA-binding protein that must localize to the nucleoplasm to exert its antiangiogenic and tumor suppressive effects. There, it associates with the RNA-processing machinery and specifically interacts with TSP-1 mRNA through its 3′UTR. Reintroduction of NOL7 into SiHa cells increases luciferase expression through interaction with the TSP-1 3′UTR at both the mRNA and protein levels. NOL7 also increases endogenous TSP-1 mRNA half-life. Further, NOL7 post-transcriptional stabilization is observed in a subset of angiogenesis-related mRNAs, suggesting that the stabilization of TSP-1 may be part of a larger novel mechanism. These data demonstrate that NOL7 significantly alters TSP-1 expression and may be a master regulator that coordinates the post-transcriptional expression of key signaling factors critical for the regulation of the angiogenic phenotype.

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INTRODUCTION

Angiogenesis is one of the hallmarks of cancer and is required for tumors to grow beyond a diffusion gradient.[1,2] It is regulated by a balance of pro-and antiangiogenic factors, and, in many cancers, the differential regulation of these factors is a precursor to the angiogenic switch and subsequent malignant transformation.[3–5] Current therapies aimed at disrupting this process have been hindered by off-target effects owing to the diversity of angiogenic molecules and their downstream signaling pathways.[6–9]

This diversity is mediated in part by the post-transcriptional processing of many of these angiogenesis-related transcripts, where alternative splicing, polyadenylation, stability, and translational control contributes to their expression level and functionality. In some cases, as in the alternative splicing of vascular endothelial growth factor (VEGF) and its receptor, VEGF receptor 1, these processes can change the function of the molecule itself.[10–13] In other cases, these processes can influence the bioavailability through modulation of secretory domains or mRNA stability.[14–18] The post-transcriptional regulation of mRNA stability is particularly critical for the rapid cellular response to internal and external stimuli and is therefore a crucial mechanism in the control of growth factors, cytokines, and angiogenic molecules.[19]

While advances in our understanding of the post-transcriptional regulation of proangiogenic factors have emerged, considerably less is known about the post-transcriptional processing of antiangiogenic molecules. The first endogenous antiangiogenic molecule reported was thrombospondin-1 (TSP-1).[20,21] TSP-1 suppresses angiogenesis by inhibiting cell migration, inducing apoptosis, and modulating the signaling of growth factors.[22–24] TSP-1 expression is downregulated in a number of cancers, and reintroduction of TSP-1 has been shown to significantly suppress tumor growth.[25–28] Owing to its central role in physiological and pathological angiogenesis, expression of TSP-1 mRNA is tightly controlled, with no known alternative splicing or polyadenylation isoforms. TSP-1 mRNA is post-transcriptionally regulated by TGF-β stimulation, hypoxia, or heat shock, but the underlying mechanism is poorly understood.[29–34]

Post-transcriptional regulation is achieved in part through interaction with RNA-binding proteins (RBPs) such as HuR. HuR regulates a number of angiogenesis-related transcripts, including VEGF, COX-2, and HIF-1α[35–38] and recently has been shown to bind to the 3′UTR of TSP-1.[39] While many of the well-characterized mRNA-stabilizing RBPs such as HuR function in the cytoplasm, the existence of significant parallel mechanisms for nuclear RNA turnover argues for similar stabilizing roles in this compartment. Many of the decay pathways active in the nucleus are tied to processing events such that degradation of aberrant transcripts and further processing of mRNAs occur co-transcriptionally and are tied to the RNA polymerase II machinery in ribonucleoprotein (RNP) complexes.[40–43] These RNPs, including nuclear-processing factors such as XRN2, EXOSC10, TRAMP, and others are critical for appropriate expression tied to transcriptional termination, 3′ end processing, and mRNA quality control.[44–55] Within the nucleus, RBPs have a central role in coordinating mRNA quality control through alternative decay, stabilization, and export.[56–59]

NOL7 is a novel tumor suppressor that induces an antiangiogenic phenotype through differential regulation of VEGF and TSP-1.[60] However, the mechanism underlying this function is unknown. In this study, we demonstrate that NOL7 must reside in the nucleoplasm to upregulate TSP-1 and induce an antiangiogenic and tumor suppressive phenotype. There, it functions as an RBP that associates with the RNA-processing machinery. NOL7 interacts with polyadenylated transcripts, specifically with TSP-1 mRNA through its 3′UTR. Reintroduction of NOL7 increases

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reporter gene expression through this interaction at both the mRNA and protein levels. This is also observed in endogenous TSP-1, where reintroduction of NOL7 significantly increases the TSP-1 transcript half-life. NOL7 post-transcriptional stabilization is limited to a distinct subset of mRNAs through a novel mechanism. Together, these data demonstrate that NOL7 is a critical regulator of TSP-1 expression and may participate in the coordination of cellular signaling pathways through post-transcriptional mRNA regulation to control the angiogenic process.

RESULTS

NOL7 must reside in the nucleoplasm to induce an antiangiogenic phenotype and suppress tumor growth

NOL7 is a highly basic protein that suppresses tumor growth and induces an antiangiogenic phenotype in part through upregulation of TSP-1 expression.[60] NOL7 is localized exclusively to the nucleus and nucleolus of cells and completely absent from the cytoplasm (Supplementary Figure S1), but it is not known if NOL7 is sequestered or functional within these compartments. To determine the compartment in which NOL7 must reside for its function, we made a series of mutational constructs that targeted NOL7 to the nucleolus (NOL7 wild-type), nucleoplasm (N23(−)) or cytoplasm (N123(−)) (Zhou *et al*[61]; Figure 1a). In SiHa cells, which essentially lack endogenous NOL7,[60,62] reintroduction of wild-type nucleolar NOL7 was able to significantly upregulate endogenous TSP-1 expression compared with GFP controls, while cytoplasmic NOL7 had no effect on TSP-1 levels (Figure 1b). However, nucleoplasmic NOL7 was still able to upregulate endogenous TSP-1 to the same levels as wild-type NOL7 (Figure 1b). In the same fashion, there was no difference in endothelial cell migration between cells treated with conditioned media from GFP control or cytoplasmic NOL7 (Figure 1c). However, conditioned media from wild-type NOL7 and nucleoplasmic NOL7 were able to suppress

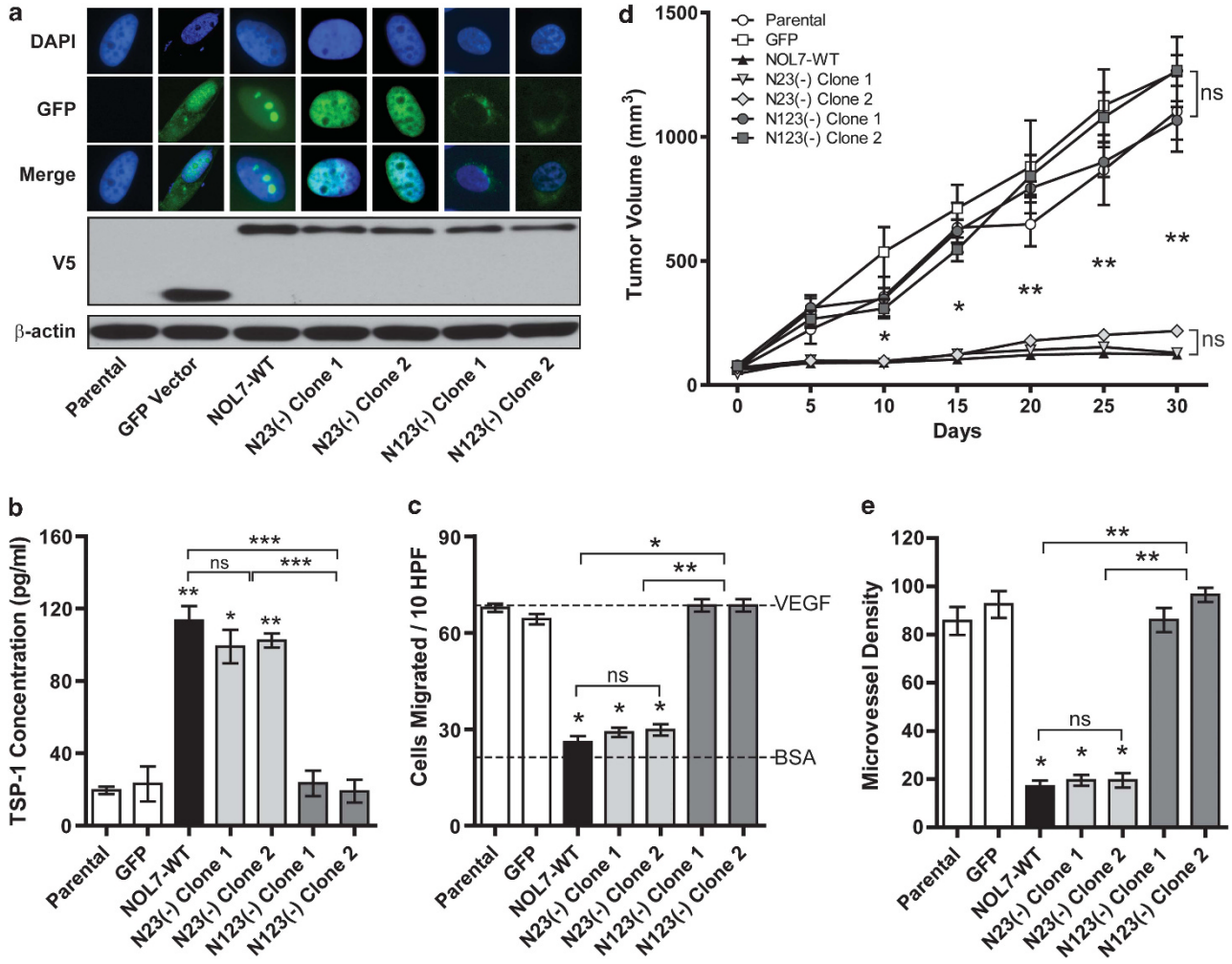

Figure 1. NOL7 must reside in the nucleus to modulate TSP-1 expression, inhibit endothelial cell migration and suppress tumor growth. (**a**) Cells were transfected with GFP-tagged wild-type NOL7 or mutants that target NOL7 to the nucleoplasm (N23(−), clones 1 and 2) or the cytoplasm (N123(−), clones 1 and 2). Cell nuclei were counterstained with DAPI and imaged by fluorescence microscopy. (**b**) Conditioned media from transfected cells was analyzed by enzyme-linked immunosorbent assay and concentration calculated from a standard curve. Data are represented as mean ± s.e.m. Significance was calculated using Student's *t*-test from three independent experiments. $*P<0.005$; $**P<0.002$; $***P<0.001$; n.s., not significant. (**c**) Serum-free conditioned media from parental SiHa, GFP and NOL7 wt- and mutant-transfected clones were functionally tested for their ability to stimulate endothelial cell migration. Data are represented as mean ± s.e.m. Significance was calculated using Student's *t*-test from four independent experiments. $*P<1\times10^{-5}$; $**P<1\times10^{-8}$; n.s., not significant. (**d**) SiHa parental, GFP and NOL7 wt- and mutant-transfected cells were subcutaneously injected into nude mice and monitored over a period of 30 ($n=6$ animals per group). Data are represented as mean ± s.e.m. Significance was calculated using two-way analysis of variance. $*P<0.001$; $**P<0.0001$; n.s., not significant. (**e**) Tumor angiogenesis was assessed by CD31 staining, followed by microvessel density quantification. Data are represented as mean ± s.e.m. Significance was calculated using Student's *t*-test ($n=6$ animals per group). $*P<2\times10^{-6}$; $**P<1\times10^{-10}$; n.s., not significant.

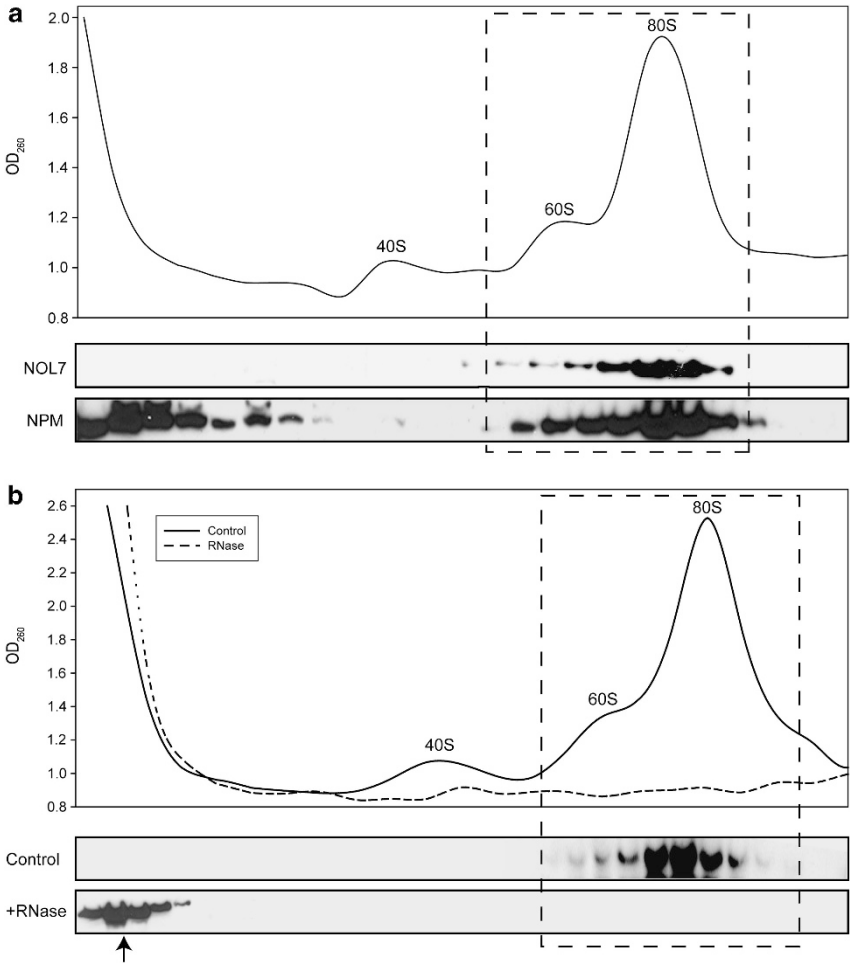

Figure 2. NOL7 interacts with a large RNP complex in an RNA-dependent manner. NOL7-transfected lysate was separated on a 10–30% gradient. Individual fractions were collected and plotted on the basis of their absorbance at 260 nm. Individual fractions were also subjected to western blotting for NOL7. The co-migration of NOL7 with a large RNP complex is marked with a box. (**a**) Total lysate was evaluated for association of NOL7 into RNP complexes. (**b**) Lysate was either mock-treated or digested with RNase A before separation.

endothelial cell migration to the same degree (Figure 1c). This correlated with a significant suppression in tumor growth, where both nucleoplasmic and nucleolar NOL7 demonstrated 90% less tumor volume than GFP control or cytoplasmic NOL7 (Figure 1d). Tumor angiogenesis, as assessed by CD31 microvessel density, revealed that wild-type and nucleoplasmic NOL7 suppressed tumor angiogenesis, whereas GFP control and the cytoplasmic NOL7 mutant displayed robust angiogenesis in these tumors (Figure 1e). Taken together, this suggests that NOL7 must reside in the nucleoplasm to induce an antiangiogenic phenotype and suppress tumor growth.

NOL7 interacts with a large ribonucleoprotein complex in an RNA-dependent manner

To determine the potential mechanism by which NOL7 suppresses tumor growth and induces an antiangiogenic phenotype in the nucleoplasm, we hypothesized that NOL7 may be part of a nucleic-acid-interacting complex. To test this, we performed sucrose gradient ultracentrifugation of NOL7-expressing lysate (Figure 2a). NOL7 co-migrated with a large, ~70S complex (Figure 2a, box). To determine if the association of NOL7 was dependent upon RNA, lysate was either mock-treated or treated with RNase, and separated as before (Figure 2b). Treatment with RNase caused NOL7 to revert completely to the soluble fraction (Figure 2b, arrow). This demonstrates that NOL7 interacts with a large RNP complex in an RNA-dependent manner.

NOL7 interacts with mRNA-processing factors

To identify the RNP complex and protein cofactors of NOL7, mass spectroscopic (MS) analysis was performed on comigrating NOL7 immunoprecipitates (Figure 3a). This sample was highly enriched for RNA-processing factors, particularly those involved in mRNA maturation. To confirm the putative cofactors identified by mass spectroscopy, coimmunoprecipitation of NOL7 or controls was performed and analyzed by western blot against the endogenous MS-identified proteins. To determine if these putative cofactors interact in an RNA-dependent or -independent manner, lysates were mock-treated or digested with RNase A before immunoprecipitation (IP). SR proteins SF2-ASF and SRp40, RNA-processing factor NCL, and the 3′-processing and decay factors XRN2, CNOT3, and CPSF2 were found to specifically associate with NOL7 in either the presence of absence of RNA (Figure 3b). In addition, the multifunctional RBPs HuR, NPM, and EXOSC10 associated with NOL7 only in the presence of RNA. These data demonstrate that NOL7 interacts with mRNA-processing complexes and specifically associates with proteins involved in mRNA maturation.

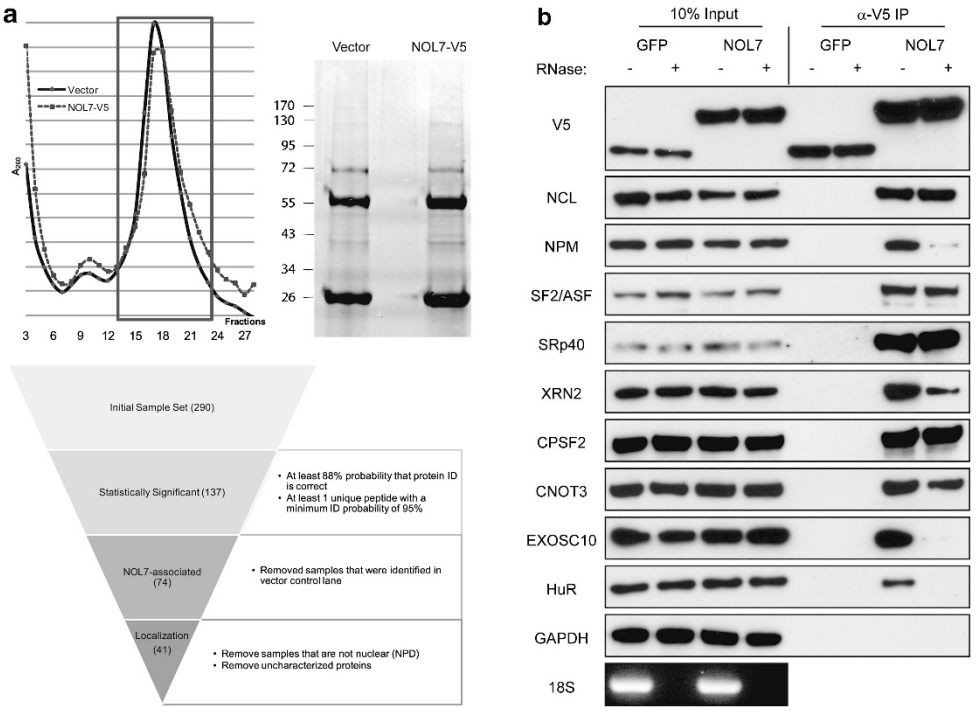

Figure 3. NOL7 interacts with 3′ end-processing proteins. (**a**) Lysate from SiHa cells stably expressing GFP-V5 or NOL7-V5 was separated by gradient ultracentrifugation and the large 70S fractions were pooled, immunoprecipitated and separated by SDS–polyacrylamide gel electrophoresis and stained with Coomassie before analysis by mass spectroscopy. Data was curated from the mass spectroscopy results to identify putative functional cofactors of NOL7. (**b**) GFP-V5 or NOL7-V5 lysate was mock-treated (−) or digested with RNase (+). Lysates were immunoprecipitated using α-V5-conjugated beads and coimmunoprecipitating proteins were analyzed by western blot. As a control for RNase digestion, RNA was extracted from the lysates after treatment, reverse transcribed and RT–PCR against the 18S rRNA was performed.

NOL7 interacts specifically with mRNA

The protein cofactors, identified suggested that NOL7 may be involved in mRNA processing. To determine if NOL7 interacts with mRNA, total lysate overexpressing GFP control or NOL7 was incubated with oligo(dT) beads to precipitate polyadenylated transcripts and coprecipitated proteins were visualized by western blotting. NOL7, but not GFP, interacted with the polyA transcripts, demonstrating that this was not an artifact of overexpression (Figure 4, lanes 3 and 4). This was not due to nonspecific binding to the beads, as treatment of the lysate with RNase before incubation completely abolished NOL7 binding (Figure 4, lane 5). Finally, to demonstrate that this interaction was mRNA-specific and not a random, charge-based interaction, the competition of the bound proteins with either polyA or polyC at five or ten times the input RNA was performed. NOL7 binding was lost in the polyA treatments (Figure 4, lanes 6 and 8) but unaffected by polyC competition (Figure 4, lanes 7 and 9), indicating that NOL7 interacts specifically with mRNA.

NOL7 interacts with TSP-1 mRNA through its 3′UTR

NOL7 induces an antiangiogenic phenotype in the nucleus through upregulation of TSP-1 (Figure 1). To determine if NOL7 interacts with TSP-1 mRNA, total lysate overexpressing GFP, NOL7, or the TSP-1 mRNA-binding protein HuR was immunoprecipitated and the associated mRNAs were analyzed by northern blotting (Figure 5a). TSP-1 mRNA was significantly associated with both NOL7 and HuR, and absent from GFP (Figure 5a). This demonstrates that NOL7 is capable of specifically interacting with TSP-1 mRNA. The association of NOL7 with polyadenylated transcripts and 3′-processing factors suggested that NOL7 might interact with TSP-1 through its 3′UTR. To determine if NOL7 might bind in this region, the 3′UTR of TSP-1 or R01, a random, nongenic sequence of the same length were *in vitro* transcribed and biotinylated. Total lysate from cells overexpressing GFP, NOL7, or HuR were incubated with increasing amounts of biotinylated transcript, precipitated using streptavidin beads, and analyzed by western blotting (Figure 5b). GFP did not bind to either transcript at any of the concentrations assayed. Both NOL7 and HuR bound to TSP-1 3′UTR in a dose-dependent manner, but was not detected in the negative-control lanes (Figure 5b). Together, this demonstrates that NOL7 interacts specifically with TSP-1 mRNA through its 3′UTR.

The 3′UTR of TSP-1 is sufficient for NOL7-mediated post-transcriptional upregulation

Interaction of NOL7 with the 3′UTR of TSP-1 suggested it may have a role in its post-transcriptional regulation. To determine if this interaction can affect downstream expression levels, luciferase reporters were cloned in-frame with the 3′UTR of TSP-1, positive-control SV40 late polyA signal (EMP), or a negative-control nongenic (R01) sequence. SiHa cells express extremely low levels of endogenous NOL7, such that reintroduction in stable cell lines restored NOL7 to near-endogenous levels observed in 293T cells, and expression of exogenous NOL7 or HuR did not affect endogenous levels of either gene (Supplementary Figure S2). Luciferase levels for clones bearing the TSP-1 3′UTR were significantly increased in NOL7- and HuR-expressing cells (Figure 6). No difference was observed between GFP, NOL7, or HuR in EMP or R01 constructs, indicating that the increase in luciferase was due specifically to regulation through the TSP-1 3′UTR. To determine if this upregulation was due to an increase in mRNA levels or through an increase in translational rate or protein stability, luciferase levels were analyzed by real-time PCR. The upregulation of luciferase was observed at the mRNA as well as protein levels, suggesting that the NOL7 regulates expression at the level of mRNA abundance.

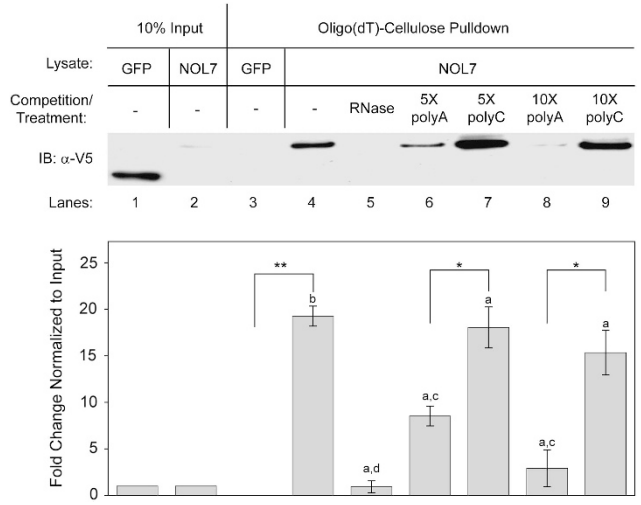

Figure 4. NOL7 specifically interacts with mRNA. HEK293T cells were transfected with GFP control or NOL7. Proteins associated with poly(A) mRNA were pulled down using oligo(dT) cellulose. Input lanes represent 10% of total input. As controls, lysate was digested with RNase A before incubation or bound proteins were competed using five or ten times input RNA of polyA or polyC. Coprecipitation of GFP and NOL7 was evaluated by western blot. Data are represented as mean ± s.e.m. Significance was calculated using Student's *t*-test relative to NOL7 input (**a, b**) and relative to the NOL7-bound (**c, d**) fractions. $^{a}P<0.002$; $^{b}P<4\times10^{-4}$; $^{c}P<0.001$; $^{d}P<0.0001$. Statistical significance between groups was also calculated (bars). $*P<0.001$, $**P<1\times10^{-4}$. Data are represented as mean ± s.e.m. from three independent assays.

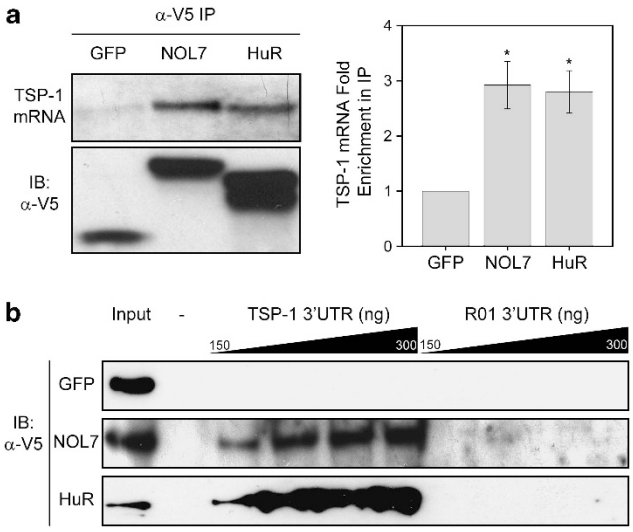

Figure 5. NOL7 coimmunoprecipitates TSP-1 mRNA. (**a**) Lysate from cells expressing GFP, NOL7 or HuR were immunoprecipitated with α-V5 beads and RNA was extracted. RNA was then subjected to northern blotting using a probe against the 3′UTR of TSP-1. Western blotting to confirm equivalent protein IP was performed. Densitometry scanning was performed on northern bands and normalized to protein IP lanes. Data are represented as mean ± s.e.m. from three independent experiments. $*P<0.01$. (**b**) The 3′UTR of TSP-1 and a negative-control sequence R01 were *in vitro* transcribed and biotinylated. Increasing amounts of transcript were incubated with lysate expressing GFP, NOL7 or HuR. Transcripts were precipitated using streptavidin beads and coprecipitating proteins were analyzed by western blot.

NOL7 post-transcriptionally regulates TSP-1 expression

To determine if NOL7 could post-transcriptionally upregulate endogenous TSP-1, two clones each from SiHa cells stably expressing GFP, NOL7 or HuR were treated with α-amanitin to block RNA Pol II-mediated mRNA transcription. TSP-1 expression levels were measured by real-time PCR for increasing durations of α-amanitin treatment. In cells expressing GFP, TSP-1 expression fell to almost half its original level within 1 hour. However, cells expressing NOL7 or HuR decreased only slightly throughout the timecourse (Figure 7a). Reintroduction of NOL7 doubled the half-life of endogenous TSP-1 mRNA (Figure 7b), suggesting that NOL7 regulates TSP-1 expression by enhancing the post-transcriptional stability of its mRNA.

NOL7 may stabilize a specific subset of angiogenesis-related mRNAs

To determine if NOL7 was capable of post-transcriptionally stabilizing other transcripts in addition to TSP-1, we assessed steady-state and post-transcriptional mRNA levels in a panel of angiogenesis-related mRNAs. SiHa cells stably expressing GFP or NOL7 were left untreated or transcriptionally inhibited with α-amanitin for 4 hours, when the maximal effect of NOL7 post-transcriptional stabilization was observed for TSP-1 (Figure 7). Using the TaqMan 384-well Human Angiogenesis Array, we found approximately one-third of the mRNAs analyzed were differentially expressed at a statistically significant level between the untreated samples, suggesting that NOL7 can modulate the steady-state expression of angiogenesis-related genes at the mRNA level (Figure 8a). Within these genes, a specific subset of mRNAs was post-transcriptionally stabilized upon reintroduction of NOL7 (Figure 8b). Further, over half of the post-transcriptionally stabilized genes were also upregulated and functionally associated with antiangiogenic and antitumorigenic cell phenotypes (Figure 8c). Together, this indicates that NOL7 is capable of modulating the expression of a specific subset of mRNAs post-transcriptionally, while others may be differentially regulated as a consequence of NOL7 modulation of upstream targets in linked signaling pathways. Finally, it indicates that for some genes, such as *TSP-1*, this post-transcriptional regulation may significantly alter the available mRNA pool and influence downstream function and phenotype.

DISCUSSION

NOL7 is a novel tumor suppressor that significantly suppresses *in vivo* tumor growth and induces an antiangiogenic phenotype in part through upregulation of TSP-1. In this work, we characterize NOL7 as a novel RBP that increases TSP-1 expression through post-transcriptional mRNA stabilization. NOL7 must reside in the nucleoplasm to exert its anticancer phenotype. Within the nucleus, NOL7 is component of a large, RNA-dependent RNP comprised of mRNA-processing factors. NOL7 specifically interacted with a number of these putative cofactors as well as polyadenylated transcripts. NOL7 was shown to interact specifically with the TSP-1 transcript through binding to its 3′UTR. Reporter constructs bearing the TSP-1 3′UTR were significantly upregulated at both the mRNA and protein level, and endogenous TSP-1 mRNA was stabilized in cells re-expressing NOL7. Finally, this post-transcriptional regulation was demonstrated to be specific to a subset of angiogenesis-related mRNAs. Taken together, this demonstrates that NOL7 is a novel RBP that post-transcriptionally upregulates TSP-1 through an increase in mRNA stability. Further, this suggests that NOL7 may regulate the antiangiogenic phenotype and suppress tumor growth through post-transcriptional modulation of gene expression.

Interaction of NOL7 with components of the RNA-processing machinery in a large RNP suggests that NOL7 may contribute to

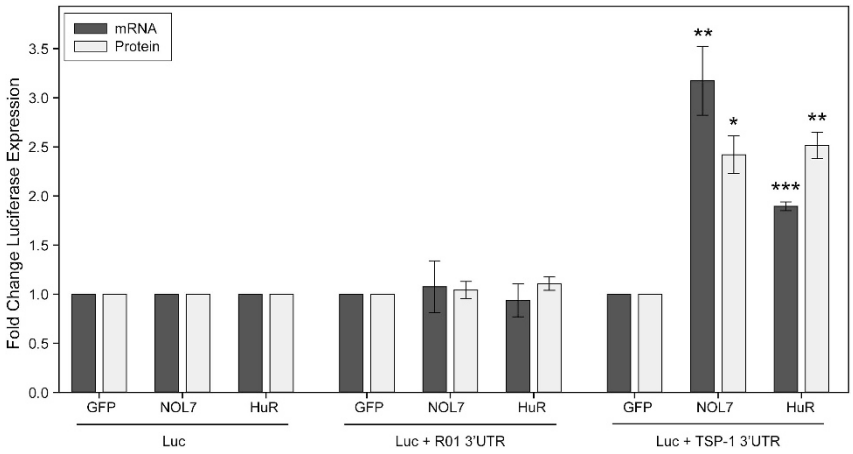

Figure 6. The 3′UTR of TSP-1 is sufficient for NOL7-mediated post-transcriptional upregulation. Two clones each from SiHa cells stably co-expressing GFP, NOL7 or HuR and luciferase-EMP, -R01 or –TSP-1 were assayed for luciferase expression at the mRNA (dark-gray bar) or protein (light-gray bar). To control for vector expression artifacts, values were normalized to luciferase-EMP and reported as a percentage of GFP expression for each set of constructs. Data are represented as mean ± s.e.m. from four independent experiments. $*P < 3 \times 10^{-5}$; $**P < 9 \times 10^{-5}$; $***P < 2 \times 10^{-7}$.

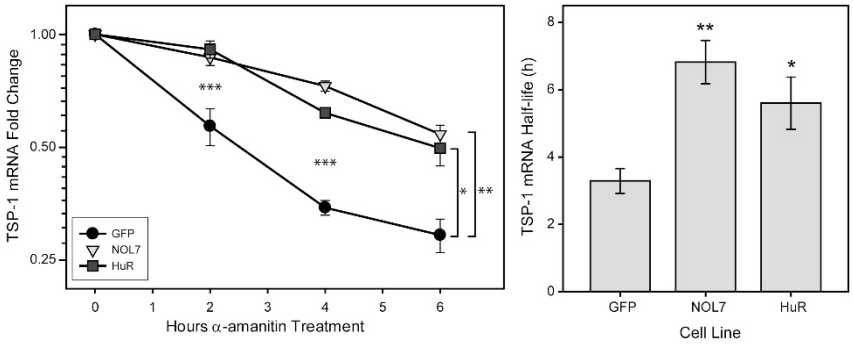

Figure 7. NOL7 post-transcriptionally stabilizes TSP-1 mRNA. SiHa cells stably expressing GFP, NOL7 or HuR were treated for 0, 2, 4 or 6 with 5 μg/ml α-amanitin. Endogenous TSP-1 levels were assayed by real-time PCR and calculated via $\Delta\Delta C_t$ method. Half-life calculations were calculated from the nonlinear regression of the exponential decay curve $N_0 = N(t)e^{-\lambda t}$, where the TSP-1 mRNA half-life $t_{1/2} = -\ln(2)/\lambda$. Data are represented as mean ± s.e.m. from four independent experiments. $*P < 0.03$; $**P < 0.001$; $***P < 0.0001$.

the co-transcriptional processing of mRNA at multiple levels. These data characterize the role of NOL7 during the 3′ end processing and maturation of TSP-1 mRNA, but do not exclude the possibility that NOL7 may contribute to other aspects of mRNA metabolism. It has been demonstrated that the efficacy of downstream processing steps can form feedbacks that influence upstream transcriptional initiation and elongation.[63,64] In addition, some nuclear mRNA complexes are active both in late-maturation and early-initiation steps of processing. One such complex, the CCR4-Not complex, has roles in mRNA transcription and degradation and its activity can influence cellular signaling pathways.[40,41] NOL7 was recently identified in a proteomic study of the CCR4-Not complex as a factor that interacts specifically with multiple core subunits of the complex,[65] validated in this work by evidence that NOL7 specially interacts with the subunit CNOT3 (Figure 3). Similarly, NOL7 also interacts specifically with the 5′→3′ endonuclease complex and nuclear exosome via its binding with XRN2 and EXOSC10. While it is unclear if NOL7 functions as a part of these complexes, it does suggest that NOL7 could be playing a role as a 'master regulator' of gene expression through regulation of mRNA maturation and degradation, and subsequent control of critical signaling molecules on multiple levels. This may be particularly significant in its regulation of the angiogenic phenotype, as feedback mechanisms and integrated signaling can have a major role in driving the angiogenic switch.

Angiogenesis is critical in cancer development and represents a promising target for therapy. However, the diversity and redundancy of many angiogenic molecules, coupled with the complexity of angiogenic signaling, have hindered progress in the field. Particularly, the ability of post-transcriptional regulation to rapidly and significantly alter the signaling capability of many of these factors, and in some cases change the functionality of these molecules entirely has been overlooked.[12,66–70] Proangiogenic molecules such as fibroblast growth factor 2 (FGF-2), VEGF and COX-2 all undergo alternative post-transcriptional processing that results in modulation of their half-life and downstream functionality.[17,18,71–75] Importantly, some major angiogenic signaling factors such as TGF-β have been found to modulate gene expression through a dual regulation of transcriptional activity and mRNA turnover.[68] Coupled with constitutive low-level expression of many of these mRNAs, rapid changes in mRNA stability can significantly alter the steady-state pool of a given mRNA in response to stimulus[19] and represent a key target for modulating the angiogenic phenotype in cancer.

While post-transcriptional regulation of proangiogenic factors has been described in the literature, evidence regarding alternative processing and stability and angiogenic inhibitors is lacking. Despite decades of research focused on TSP-1, few reports address the issue of its post-transcriptional regulation. The evidence available suggests that post-transcriptional regulation is tied to conditions that promote or suppress tumorigenesis *in vivo*, such as

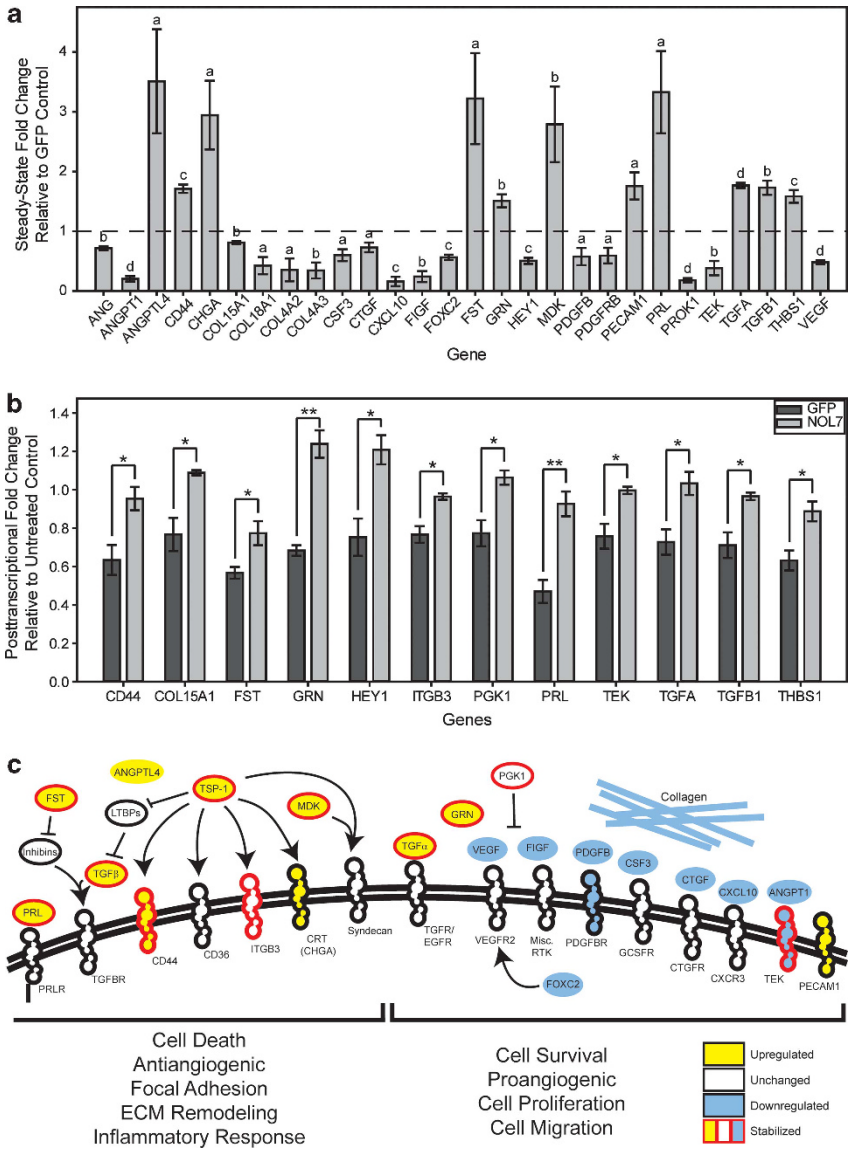

Figure 8. NOL7 specifically regulates distinct subset of angiogenic transcripts. Total complementary DNA from GFP- or NOL7-expressing cells untreated or transcriptionally inhibited with α-amanitin were analyzed by real-time PCR on TaqMan angiogeniesis array cards. Data are represented as mean ± s.e.m. from three independent experiments. (**a**) Steady-state expression (untreated NOL7 normalized to untreated GFP). Dashed line indicates the GFP control normalization. $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$; $^{d}P<0.0001$. (**b**) Post-transcriptional expression (α-amanitin-treated GFP/NOL7 normalized to untreated controls). $^{*}P<0.05$, $^{**}P<0.01$. (**c**) Genes significantly upregulated (yellow), unchanged (white), or downregulated (blue) are represented schematically based on their functional annotations, ligand-receptor interactions, and signaling. Those genes that were post-transcriptionally stabilized by NOL7 are outlined in red.

hypoxia or oncogene expression. For example, TSP-1 mRNA is destabilized by overexpression of the oncogene Myc or hypoxic conditions, while its half-life is increased by heat shock or TGF-β stimulation.[29–34] Interestingly, TGF-β is activated by TSP-1, indicating that the post-transcriptional stabilization may be part of a feedback loop to suppress tumor growth. To the best of our knowledge, no studies have investigated any tumor-associated somatic mutations within the TSP-1 3′UTR. The contribution of 3′UTR *cis*-elements to the overall regulation of TSP-1 expression and the *trans*-factors that bind to them, such as AUF1 and HuR, are also not well characterized. Here, we demonstrate that NOL7 is a novel TSP-1 3′UTR-interacting protein that stabilizes the TSP-1 transcript.

NOL7 may represent a novel type of RBP in cancer, because of its unique domain structure and role as a tumor suppressor *in vivo*. NOL7 lacks any sequence similarity to known proteins or domains, suggesting NOL7 may employ a novel method of interaction with its targets. While the data here are insufficient to identify a NOL7-interacting *cis*-element, it does rule out classic binding elements such as AREs, as VEGF, FGF-2, and interleukin 8 are not post-transcriptionally regulated by NOL7. Nonetheless, these data suggest that NOL7 binds and regulates a specific subset of mRNAs, and for some of these transcripts, that stabilization may significantly alter the steady-state expression level. In addition, some genes were differentially regulated only at the steady-state, suggesting that for some genes the effect of NOL7 expression may be propagated through signaling pathways or be secondary effects of NOL7's function on upstream targets. Assessment of mRNA levels over a transcriptional inhibition timecourse will be necessary to confirm the modulation of mRNA half-life and

expansion of these results to include more genes and pathways is necessary. For example, a major angiogenic transcription factor, HIF-1α, was not profiled on this array, and could contribute significantly to the differential expression of NOL7 targets, including downstream genes affected only at the steady state. The differentially regulated genes further suggest that NOL7 may be having a role in mediating focal adhesion, remodeling of the extracellular matrix and epithelial to mesenchymal (EMT) transition through its post-transcriptional mechanisms, pathways that are critical for tumor growth suppression, metastasis and antiangiogenic therapy. Acquisition of an EMT phenotype is associated with acquired resistance to angiogenic therapies, metastasis, and has recently been tied to global enrichment of ARE-containing mRNAs.[76–78] Therefore, the role of NOL7 in post-transcriptional regulation of these phenotypes may be critical in avoiding acquired resistance and increasing the efficacy of current angiogenic therapies.

In conclusion, NOL7 is a novel tumor suppressor that must reside in the nucleoplasm to suppress *in vivo* tumor growth. Reintroduction of NOL7 induces an antiangiogenic phenotype drive in part by the post-transcriptional stabilization of TSP-1. This is achieved through specific interaction with the TSP-1 3′UTR, which is sufficient to post-transcriptionally upregulate gene expression at the mRNA and protein levels. Finally, this post-transcriptional regulation was demonstrated to be specific to a small subset of mRNAs. Taken together, this demonstrates that NOL7 is a novel RBP that post-transcriptionally upregulates TSP-1 through an increase in mRNA stability. Further characterization of the mechanism underlying this function and the phenotypic consequences will illustrate the potential role of NOL7 as a master regulator of the angiogenic phenotype through post-transcriptional modulation of gene expression.

MATERIALS AND METHODS

Further details and additional methods can be found in the Supplementary section.

Cell culture and fluorescence microscopy

HEK293T and SiHa cells were obtained from the ATCC (Manassas, VA, USA) and cultured, as previously described.[60,61] Fluorescence microcopy was performed as described.[61]

In vivo tumor studies, ELISA and migration assay

Conditioned media from the SiHa parental, GFP control, NOL7 wild-type or NOL7 mutant cells were assayed for TSP-1 and VEGF by enzyme-linked immunosorbent assay (ELISA), as described by the manufacturer (R&D Systems, Minneapolis, MN, USA). The migration assay was performed, as previously described.[79] For tumor studies, 10^7 cells in PBS were injected subcutaneously into 6–8-week-old female nu/nu mice (Charles River Laboratories, Wilmington, MA, USA) ($n = 6$ per group). Tumor growth was monitored with a caliper. Statistics were calculated using two-way analysis of variance. Microvessel density was calculated from CD31 staining, as previously described.[60]

Northern and western blotting

Northern blotting was performed using the NorthernMax kit (Ambion, Austin, TX, USA). The TSP-1-specific RNA probe was *in vitro* transcribed and labeled with [α-^{32}P]-UTP (Perkin–Elmer, Waltham, MA, USA) using the MAXIscript kit, according to the manufacturer's instructions (Ambion). For westerns, proteins were separated on SDS–polyacrylamide gel electrophoresis gels, transferred to ImmunoBlot PVDF membrane (Bio-Rad, Hercules CA, USA), blocked in 5% milk-TBST and probed overnight. Secondary was probed at 10 ng/ml for 1 h at room temperature. Blots were visualized using Pierce SuperSignal West Dura substrate.

Sucrose gradient ultracentrifugation

Lysate was separated on 10–30% continuous gradients prepared manually in sucrose gradient buffer (50 mM Tris–HCl, 80 mM KCl, 5 mM $Mg(C_2H_3O_2)_2$, 2% sucrose, protease inhibitors). RNA digestion was performed by incubation with 500 μg/ml RNase A and 50 μg/ml EDTA at 37 °C for 1 h. All gradients were separated at 27 500 rpm at 4 °C on Beckman LM-80 Centrifuge (Beckman-Coulter, Inc., Brea, CA, USA) for 8 h. Equivalent fractions were collected manually measured for absorbance at 260 nm.

Immunoprecipitation and pulldowns

Cells stably expressing GFP-V5 or NOL7-V5 were resuspended in sucrose lysis buffer and lysed by freeze–thaw. RNase digestion was performed, as described above. For IP, 250 μg total protein was mock-treated or RNase digested and incubated with 25 μl α-V5 agarose beads (Sigma–Aldrich, St Louis, MO, USA). Purified complementary DNA was reverse transcribed using the Superscript III one-step RT-PCR kit and amplified with 18S specific primers (Invitrogen, Carlsbad, CA, USA). For oligo(dT) pulldowns, 500 A_{260} units were bound to 10 mg oligo(dT) cellulose beads (Ambion) and incubated with buffer alone or buffer containing 2500 or 5000 A_{260} units of polyadenylic or polycytidylic acid (Sigma–Aldrich). For 3′UTR pulldowns, the 3′UTR of TSP-1 or R01 was *in vitro* transcribed using the MEGAscript kit from Ambion (Ambion/Applied Biosystems, Austin, TX, USA). RNA was incubated with 100 μg lysate and bound to streptavidin Dynabeads (Invitrogen). In all cases, beads were washed thoroughly and bound proteins were eluted by boiling in SDS sample buffer. Results were quantified using Bio-Rad QuantityOne software (Bio-Rad) and normalized to input. Statistical significance was determined from three independent assays using Student's *t*-test.

3′UTR luciferase assays

Two clones each of SiHa cells stably co-expressing GFP, NOL7, or HuR and EMP, R01, or TSP-1 3′UTR luciferase reporter constructs were measured using the Steady-Glo luciferase assay system (Promega, Madison, WI, USA), according to manufacturer's instructions. Values for GFP, NOL7 and HuR clones were averaged, normalized to luciferase control, and reported as a percentage of GFP. Luciferase mRNA levels were measured by quantitative PCR. Statistical significance was calculated using Student's *t*-test from four independent assays against log values to control for normalization bias.

Quantitative real-time PCR

RNA levels were measured by real-time quantitative PCR using the Ag-Path One Step RT–PCR kit (Ambion/Applied Biosystems). For each, 30 ng total RNA was amplified on the CFX-1000 (Bio-Rad) and detected using TaqMan probes against target transcripts (Applied Biosystems). Relative expression levels were calculated using the $\Delta\Delta C_t$ method relative to 18S. Statistical differences were calculated as indicated.

Measurement of post-transcriptional mRNA abundance

Two clones each of SiHa cells stably co-expressing GFP, NOL7 or HuR were plated in six-well plates, washed with PBS and transcriptionally inhibited in complete media containing 5 μg/ml α-amanitin for 0, 2, 4 or 6 hours. After treatment, RNA was collected from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Post-transcriptional TSP-1 abundance was measured by real-time quantitative PCR. TSP-1 mRNA levels were normalized to time zero and plotted as a function of α-amanitin treatment duration such that $N_0 = N(t)e^{-\lambda t}$. Half-life was calculated as $t_{1/2} = -\ln(2)/\lambda$. Statistical significance was calculated using two-way analysis of variance and Student's *t*-test from four independent assays.

TaqMan Array

SiHa cells stably expressing GFP or NOL7 were untreated or transcriptionally inhibited with 5 μg/ml α-amanitin for 4 h. After treatment, RNA was collected from cells using the RNeasy Mini Kit (Qiagen) and reverse transcribed with Superscript VILO (Invitrogen). 100 ng complementary DNA from treated and untreated GFP and NOL7 expressing cells was combined with TaqMan Gene Expression Master Mix, loaded onto the Human Angiogenesis Array, and analyzed on the 7900HT Fast real-time PCR system (Applied Biosystems). For steady state, expression was calculated between untreated samples relative to GFP. For post-transcriptional expression, mRNA levels were normalized to untreated samples and compared directly. Statistical significance was calculated using Student's *t*-test from an average of three independent assays.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57–70.
2 Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (Research Support, NIH, Extramural Review) 2011; **144**: 646–674.
3 Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000; **407**: 249–257.
4 Folkman J. Role of angiogenesis in tumor growth and metastasis. *Semin Oncol* 2002; **29**(6 Suppl 16): 15–18.
5 Carmeliet P. Angiogenesis in health and disease. *Nat Med* 2003; **9**: 653–660.
6 Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer* 2008; **8**: 592–603.
7 Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. *Nature* 2005; **438**: 967–974.
8 Heath VL, Bicknell R. Anticancer strategies involving the vasculature. *Nat Rev Clin Oncol* 2009; **6**: 395–404.
9 Quesada AR, Medina MA, Alba E. Playing only one instrument may be not enough: limitations and future of the antiangiogenic treatment of cancer. *Bioessays* 2007; **29**: 1159–1168.
10 Nowak DG, Woolard J, Amin EM, Konopatskaya O, Saleem MA, Churchill AJ *et al.* Expression of pro- and anti-angiogenic isoforms of VEGF is differentially regulated by splicing and growth factors. *J Cell Sci* 2008; **121**(Pt 20): 3487–3495.
11 Rennel E, Waine E, Guan H, Schuler Y, Leenders W, Woolard J *et al.* The endogenous anti-angiogenic VEGF isoform, VEGF165b inhibits human tumour growth in mice. *Br J Cancer* 2008; **98**: 1250–1257.
12 He Y, Smith SK, Day KA, Clark DE, Licence DR, Charnock-Jones DS. Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. *Mol Endocrinol* 1999; **13**: 537–545.
13 Robinson CJ, Stringer SE. The splice variants of vascular endothelial growth factor (VEGF) and their receptors. *J Cell Sci* 2001; **114**(Pt 5): 853–865.
14 Harper SJ, Bates DO. VEGF-A splicing: the key to anti-angiogenic therapeutics? *Nat Rev Cancer* 2008; **8**: 880–887.
15 Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. *Endocr Rev* 1997; **18**: 4–25.
16 Yamazaki Y, Morita T. Molecular and functional diversity of vascular endothelial growth factors. *Mol Divers* 2006; **10**: 515–527.
17 Hall-Pogar T, Zhang H, Tian B, Lutz CS. Alternative polyadenylation of cyclooxygenase-2. *Nucleic Acids Res* 2005; **33**: 2565–2579.
18 Lukiw WJ, Bazan NG. Cyclooxygenase 2 RNA message abundance, stability, and hypervariability in sporadic Alzheimer neocortex. *J Neurosci Res* 1997; **50**: 937–945.
19 Hargrove JL, Schmidt FH. The role of mRNA and protein stability in gene expression. *FASEB J* 1989; **3**: 2360–2370.
20 Rastinejad F, Polverini PJ, Bouck NP. Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. *Cell* 1989; **56**: 345–355.
21 Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA *et al.* A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc Natl Acad Sci USA* 1990; **87**: 6624–6628.
22 Volpert OV. Modulation of endothelial cell survival by an inhibitor of angiogenesis thrombospondin-1: a dynamic balance. *Cancer Metastasis Rev* 2000; **19**: 87–92.
23 Ren B, Yee KO, Lawler J, Khosravi-Far R. Regulation of tumor angiogenesis by thrombospondin-1. *Biochim Biophys Acta* 2006; **1765**: 178–188.
24 Lawler J, Detmar M. Tumor progression: the effects of thrombospondin-1 and -2. *Int J Biochem Cell Biol* 2004; **36**: 1038–1045.
25 Volpert OV, Stellmach V, Bouck N. The modulation of thrombospondin and other naturally occurring inhibitors of angiogenesis during tumor progression. *Breast Cancer Res Treat* 1995; **36**: 119–126.
26 Bleuel K, Popp S, Fusenig NE, Stanbridge EJ, Boukamp P. Tumor suppression in human skin carcinoma cells by chromosome 15 transfer or thrombospondin-1 overexpression through halted tumor vascularization. *Proc Natl Acad Sci USA* 1999; **96**: 2065–2070.
27 Streit M, Velasco P, Brown LF, Skobe M, Richard L, Riccardi L *et al.* Overexpression of thrombospondin-1 decreases angiogenesis and inhibits the growth of human cutaneous squamous cell carcinomas. *Am J Pathol* 1999; **155**: 441–452.
28 Weinstat-Saslow DL, Zabrenetzky VS, VanHoutte K, Frazier WA, Roberts DD, Steeg PS. Transfection of thrombospondin 1 complementary DNA into a human breast carcinoma cell line reduces primary tumor growth, metastatic potential, and angiogenesis. *Cancer Res* 1994; **54**: 6504–6511.
29 Kang JH, Kim SA, Hong KJ. Induction of TSP1 gene expression by heat shock is mediated via an increase in mRNA stability. *FEBS Lett* 2006; **580**: 510–516.
30 Okamoto M, Ono M, Uchiumi T, Ueno H, Kohno K, Sugimachi K *et al.* Up-regulation of thrombospondin-1 gene by epidermal growth factor and transforming growth factor beta in human cancer cells--transcriptional activation and messenger RNA stabilization. *Biochim Biophys Acta* 2002; **1574**: 24–34.
31 Phelan MW, Forman LW, Perrine SP, Faller DV. Hypoxia increases thrombospondin-1 transcript and protein in cultured endothelial cells. *J Lab Clin Med* 1998; **132**: 519–529.
32 Janz A, Sevignani C, Kenyon K, Ngo CV, Thomas-Tikhonenko A. Activation of the myc oncoprotein leads to increased turnover of thrombospondin-1 mRNA. *Nucleic Acids Res* 2000; **28**: 2268–2275.
33 Laderoute KR, Alarcon RM, Brody MD, Calaoagan JM, Chen EY, Knapp AM *et al.* Opposing effects of hypoxia on expression of the angiogenic inhibitor thrombospondin 1 and the angiogenic inducer vascular endothelial growth factor. *Clin Cancer Res* 2000; **6**: 2941–2950.
34 Penttinen RP, Kobayashi S, Bornstein P. Transforming growth factor beta increases mRNA for matrix proteins both in the presence and in the absence of changes in mRNA stability. *Proc Natl Acad Sci USA* 1988; **85**: 1105–1108.
35 Levy NS, Chung S, Furneaux H, Levy AP. Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. *J Biol Chem* 1998; **273**: 6417–6423.
36 Sengupta S, Jang BC, Wu MT, Paik JH, Furneaux H, Hla T. The RNA-binding protein HuR regulates the expression of cyclooxygenase-2. *J Biol Chem* 2003; **278**: 25227–25233.
37 Sheflin LG, Zou AP, Spaulding SW. Androgens regulate the binding of endogenous HuR to the AU-rich 3'UTRs of HIF-1alpha and EGF mRNA. *Biochem Biophys Res Commun* 2004; **322**: 644–651.
38 Dixon DA, Tolley ND, King PH, Nabors LB, McIntyre TM, Zimmerman GA *et al.* Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells. *J Clin Invest* 2001; **108**: 1657–1665.
39 Mazan-Mamczarz K, Hagner PR, Corl S, Srikantan S, Wood WH, Becker KG *et al.* Post-transcriptional gene regulation by HuR promotes a more tumorigenic phenotype. *Oncogene* 2008; **27**: 6151–6163.
40 Collart MA, Timmers HT. The eukaryotic Ccr4-not complex: a regulatory platform integrating mRNA metabolism with cellular signaling pathways? *Prog Nucleic Acid Res Mol Biol* 2004; **77**: 289–322.
41 Denis CL, Chen J. The CCR4-NOT complex plays diverse roles in mRNA metabolism. *Prog Nucleic Acid Res Mol Biol* 2003; **73**: 221–250.
42 Azzouz N, Panasenko OO, Colau G, Collart MA. The CCR4-NOT complex physically and functionally interacts with TRAMP and the nuclear exosome. *PLoS ONE* 2009; **4**: e6760.
43 Garneau NL, Wilusz J, Wilusz CJ. The highways and byways of mRNA decay. *Nat Rev Mol Cell Biol* 2007; **8**: 113–126.
44 West S, Gromak N, Proudfoot NJ. Human 5' --> 3' exonuclease Xrn2 promotes transcription termination at co-transcriptional cleavage sites. *Nature* 2004; **432**: 522–525.
45 Teixeira A, Tahiri-Alaoui A, West S, Thomas B, Ramadass A, Martianov I *et al.* Autocatalytic RNA cleavage in the human beta-globin pre-mRNA promotes transcription termination. *Nature* 2004; **432**: 526–530.
46 Kim M, Krogan NJ, Vasiljeva L, Rando OJ, Nedea E, Greenblatt JF *et al.* The yeast Rat1 exonuclease promotes transcription termination by RNA polymerase II. *Nature* 2004; **432**: 517–522.
47 Kawauchi J, Mischo H, Braglia P, Rondon A, Proudfoot NJ. Budding yeast RNA polymerases I and II employ parallel mechanisms of transcriptional termination. *Genes Dev* 2008; **22**: 1082–1092.
48 El Hage A, Koper M, Kufel J, Tollervey D. Efficient termination of transcription by RNA polymerase I requires the 5' exonuclease Rat1 in yeast. *Genes Dev* 2008; **22**: 1069–1081.
49 Richard P, Manley JL. Transcription termination by nuclear RNA polymerases. *Genes Dev* 2009; **23**: 1247–1269.
50 Hilleren P, McCarthy T, Rosbash M, Parker R, Jensen TH. Quality control of mRNA 3'-end processing is linked to the nuclear exosome. *Nature* 2001; **413**: 538–542.
51 de Almeida SF, Garcia-Sacristan A, Custodio N, Carmo-Fonseca M. A link between nuclear RNA surveillance, the human exosome and RNA polymerase II transcriptional termination. *Nucleic Acids Res* 2010; **38**: 8015–8026.
52 Anderson JT, Wang X. Nuclear RNA surveillance: no sign of substrates tailing off. *Crit Rev Biochem Mol Biol* 2009; **44**: 16–24.
53 Glaunsinger BA, Lee YJ. How tails define the ending: divergent roles for polyadenylation in RNA stability and gene expression. *RNA BIOL* 2010; **7**: 13–17.

54 Mukherjee D, Gao M, O'Connor JP, Raijmakers R, Pruijn G, Lutz CS *et al.* The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements. *EMBO J* 2002; **21**: 165–174.
55 Houseley J, LaCava J, Tollervey D. RNA quality control by the exosome. *Nat Rev Mol Cell Biol* 2006; **7**: 529–539.
56 Moore MJ. Nuclear RNA turnover. *Cell* 2002; **108**: 431–434.
57 Vasudevan S, Peltz SW. Nuclear mRNA surveillance. *Curr Opin Cell Biol* 2003; **15**: 332–337.
58 Brennan CM, Gallouzi IE, Steitz JA. Protein ligands to HuR modulate its interaction with target mRNAs *in vivo*. *J Cell Biol* 2000; **151**: 1–14.
59 Gallouzi IE, Brennan CM, Stenberg MG, Swanson MS, Eversole A, Maizels N *et al.* HuR binding to cytoplasmic mRNA is perturbed by heat shock. *Proc Natl Acad Sci USA* 2000; **97**: 3073–3078.
60 Hasina R, Pontier AL, Fekete MJ, Martin LE, Qi XM, Brigaudeau C *et al.* NOL7 is a nucleolar candidate tumor suppressor gene in cervical cancer that modulates the angiogenic phenotype. *Oncogene* 2006; **25**: 588–598.
61 Zhou G, Doçi CL, Lingen MW. Identification and functional analysis of NOL7 nuclear and nucleolar localization signals. *BMC Cell Biol* (Research Support, NIH, Extramural Research Support, Non-US Govt) 2010; **11**: 74.
62 Doçi CL, Mankame TP, Langerman A, Ostler KR, Kanteti R, Best T *et al.* Characterization of NOL7 gene point mutations, promoter methylation, and protein expression in cervical cancer. *Int J Gynecol Pathol* (Research Support, NIH, Extramural Research Support, Non-US Govt) 2012; **31**: 15–24.
63 Neugebauer KM. On the importance of being co-transcriptional. *J Cell Sci* 2002; **115**(Pt 20): 3865–3871.
64 Zorio DA, Bentley DL. The link between mRNA processing and transcription: communication works both ways. *Exp Cell Res* 2004; **296**: 91–97.
65 Lau NC, Kolkman A, van Schaik FM, Mulder KW, Pijnappel WW, Heck AJ *et al.* Human Ccr4-Not complexes contain variable deadenylase subunits. *Biochem J* 2009; **422**: 443–453.
66 Ladomery MR, Harper SJ, Bates DO. Alternative splicing in angiogenesis: the vascular endothelial growth factor paradigm. *Cancer Lett* 2007; **249**: 133–142.
67 Qiu Y, Hoareau-Aveilla C, Oltean S, Harper SJ, Bates DO. The anti-angiogenic isoforms of VEGF in health and disease. *Biochem Soc Trans* 2009; **37**(Pt 6): 1207–1213.
68 Dibrov A, Kashour T, Amara FM. The role of transforming growth factor beta signaling in messenger RNA stability. *Growth Factors* 2006; **24**: 1–11.
69 Hashimoto-Uoshima M, Yan YZ, Schneider G, Aukhil I. The alternatively spliced domains EIIIB and EIIIA of human fibronectin affect cell adhesion and spreading. *J Cell Sci* 1997; **110**: Pt 18 2271–2280.
70 Wang ET, Sandberg R, Luo S, Khrebtukova I, Zhang L, Mayr C *et al.* Alternative isoform regulation in human tissue transcriptomes. *Nature* 2008; **456**: 470–476.
71 Touriol C, Roussigne M, Gensac MC, Prats H, Prats AC. Alternative translation initiation of human fibroblast growth factor 2 mRNA controlled by its 3′-untranslated region involves a Poly(A) switch and a translational enhancer. *J Biol Chem* 2000; **275**: 19361–19367.
72 Claffey KP, Shih SC, Mullen A, Dziennis S, Cusick JL, Abrams KR *et al.* Identification of a human VPF/VEGF 3′ untranslated region mediating hypoxia-induced mRNA stability. *Mol Biol Cell* 1998; **9**: 469–481.
73 Levy AP, Levy NS, Goldberg MA. Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. *J Biol Chem* 1996; **271**: 2746–2753.
74 Levy AP, Levy NS, Wegner S, Goldberg MA. Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J Biol Chem* 1995; **270**: 13333–13340.
75 Levy NS, Goldberg MA, Levy AP. Sequencing of the human vascular endothelial growth factor (VEGF) 3′ untranslated region (UTR): conservation of five hypoxia-inducible RNA-protein binding sites. *Biochim Biophys Acta* 1997; **1352**: 167–173.
76 Kanies CL, Smith JJ, Kis C, Schmidt C, Levy S, Khabar KS *et al.* Oncogenic Ras and transforming growth factor-beta synergistically regulate AU-rich element-containing mRNAs during epithelial to mesenchymal transition. *Mol Cancer Res* 2008; **6**: 1124–1136.
77 Chung JH, Rho JK, Xu X, Lee JS, Yoon HI, Lee CT *et al.* Clinical and molecular evidences of epithelial to mesenchymal transition in acquired resistance to EGFR-TKIs. *Lung Cancer* 2011; **73**: 176–182.
78 Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009; **139**: 871–890.
79 Lingen MW. Endothelial cell migration assay. A quantitative assay for prediction of *in vivo* biology. *Methods Mol Med* (Research Support, US Govt, PHS) 2003; **78**: 337–347.

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