RNase-L attenuates mitogen-stimulated gene expression via transcriptional and post-transcriptional mechanisms to limit the proliferative response

Sarah E. Brennan-Laun1,2, Xiao-Ling Li4, Heather J. Ezelle1,2,5, Thiagarajan Venkataraman2, Perry J. Blackshear6, Gerald M. Wilson1,3, and Bret A. Hassel1,2,5

1Marlene and Stewart Greenebaum Cancer Center, 2Department of Microbiology and Immunology, 3Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore MD, 4Genetics Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, 5Research Services, Baltimore Veterans Affairs Medical Center, Baltimore, MD, 6Laboratory of Signal Transduction, NIEHS-NIH, Research Triangle Park, NC

Running title: RNase-L attenuates the cellular response to mitogenic stimuli

ABSTRACT

RNase-L is an endoribonuclease that mediates diverse antiproliferative activities and tristetraprolin (TTP) is a mitogen-induced RNA binding protein that directs the decay of proliferation-stimulatory mRNAs. In light of their roles as endogenous proliferative constraints, we examined the mechanisms and functional interactions of RNase-L and TTP to attenuate a mitogenic response. Mitogen stimulation of RNase-L-deficient cells significantly increased TTP transcription and the induction of other mitogen-induced mRNAs. This regulation corresponded with elevated expression of serum response factor (SRF), a master regulator of mitogen-induced transcription. RNase-L destabilized the SRF transcript and formed a complex with SRF mRNA in cells providing a mechanism by which RNase-L downregulates SRF-induced genes. TTP and RNase-L proteins interacted in cells suggesting that RNase-L is directed to cleave TTP-bound RNAs as a mechanism of substrate specificity. Consistent with their concerted function in RNA turnover, the absence of either RNase-L or TTP stabilized SRF mRNA, and a subset of established TTP targets were also regulated by RNase-L. RNase-L deficiency enhanced mitogen-induced proliferation demonstrating its functional role in limiting the mitogenic response. Our findings support a model of feedback regulation in which RNase-L and TTP target SRF and SRF-induced transcripts. Accordingly, meta-analysis revealed an enrichment of RNase-L and TTP targets among SRF-

Background: Serum Response Factor (SRF) induces mRNAs that promote cell proliferation whereas RNase-L and tristetraprolin (TTP) degrade specific mRNAs to inhibit proliferation.

Results: RNase-L and TTP interact and downregulate SRF to attenuate mitogen-induced gene expression.

Conclusion: RNase-L and TTP are components of a regulatory network that limits the proliferative response to mitogens.

Significance: The RNase-L/TTP axis represents a target to inhibit cancer cell proliferation.
regulated genes suggesting that the RNase-L/TTP axis represents a viable target to inhibit SRF-driven proliferation in neoplastic diseases.

The cellular response to proliferative signals requires rapid reprogramming of gene expression, with transcripts encoding mediators of cell proliferation being induced within minutes of stimulation. Upon abrogation of the mitogenic stimulus, expression of the induced mRNAs are efficiently attenuated to restore differentiated tissues to a quiescent state (1,2). The stringent regulation of this response is essential for the homeostasis and viability of the organism. For example, failure to respond to mitogenic stimuli can result in defective tissue repair whereas failure to attenuate the response can lead to uncontrolled proliferation and neoplastic disorders including cancer (3). While the signaling pathways and gene products that are induced in response to mitogenic stimuli have been extensively studied, less is known about the mechanisms that attenuate this response. In this regard, early analyses of mitogen-induced genes revealed that antiproliferative effectors were induced in parallel with proliferation-stimulatory genes (4). These findings provided some of the first evidence of cell-encoded attenuators of the mitogenic response and suggested that they function in a negative feedback mechanism to limit proliferation. For example, platelet-derived growth factor induces the expression of interferon-β (IFNβ) and its downstream effector 2′,5′-oligoadenylate synthetase (OAS), both of which function in diverse antiproliferative activities (4). Similarly, tristetraprolin (TTP, zinc-finger protein 36 zfp36), an RNA binding protein (RNABP) that enhances the decay of proliferation-stimulatory transcripts to mediate multiple antiproliferative programs (5-7), was first discovered as a serum-induced transcript (8,9). The identification of these and other antiproliferative effectors established the critical function that endogenous constraints have on cell proliferation, regulating the mitogenic response, and inhibiting tumorigenesis (10). Accordingly, understanding the mechanisms by which these feedback regulatory pathways restrict cell proliferation may reveal novel therapeutic targets for malignant diseases. Towards this goal, we and others have reported that, in contrast to the transcriptional induction of mitogen-stimulated genes, post-transcriptional mechanisms occupy a central role in attenuating this response (6,7,11-13). Here we investigate the functional interaction of two pathways that limit the proliferative response following mitogen stimulation via mechanisms that regulate transcription and mRNA stability.

RNase-L is the terminal component of a RNA cleavage pathway that was discovered as a mediator of IFN-induced antiviral activities (14,15). However, it is now evident that this endoribonuclease has broader roles in the innate immune response and as a potent effector of diverse antiproliferative activities (16-22). For example, RNase-L-deficient cells exhibit an increased proliferative rate and a diminished response to cell cycle inhibition (23). Ectopic expression or activation of RNase-L can induce apoptosis or senescence in distinct cell types, whereas these responses are reduced in RNase-L-deficient cells and mice (24-26). Consistent with a role for RNase-L as an endogenous proliferative constraint, RNase-L expression inhibited tumorigenesis in nude mouse xenografts (27) and RNase-L knockout (KO) mice exhibited a greater tumor burden in a model of colitis-associated cancer (28). Furthermore, the RNASEL gene mapped to the hereditary prostate cancer susceptibility locus (HPC1) and mutations in RNASEL are associated with increased risk for prostate (29), head and neck (30), cervix (31), and breast cancers (32). The biologic activities of RNase-L are thought to occur primarily through the cleavage of its single-stranded RNA (ssRNA) substrates; consistent with this view, an RNase-L mutant with a deletion in its catalytic domain, acted as a dominant negative inhibitor of antiviral and antiproliferative activities (11). RNase-L is expressed at low basal levels in a latent, inactive form in most cell types (33-35). Activation of RNase-L is dependent on a family of OAS enzymes that require double-stranded RNA (dsRNA) for enzymatic activity to polymerize ATP into 2′,5′-linked oligoadenylates (2-5A, p,5′A(2′p5′A)n; n=1-3; n≥2) (14,33,34,36) RNase-L binds 2-5A with high affinity (Kd = 40 pM; (37)) and induces conformational changes to form a dimeric structure (38) as modeled from a recent crystallographic analysis (39,40). In the active dimeric form, the catalytic domain is exposed to mediate cleavage of ssRNA targets,
RNase-L attenuates the cellular response to mitogenic stimuli

including viral genomic and messenger RNAs (mRNAs), and cellular ribosomal, mitochondrial and specific mRNAs (17,41).

Microarray studies have identified distinct profiles of RNase-L-regulated RNAs in different cell types and physiologic settings (12,19,42-44). Among these, mRNAs that are stabilized in the absence of RNase-L, and are present in an RNase-L-mRNA protein (mRNP) complex, represent candidate substrate (45). The finding that RNase-L regulates a discrete subset of RNAs provides evidence of its capacity to selectively target specific transcripts, the regulation of which is thought to mediate its diverse biologic activities. However, the mechanisms underlying this substrate specificity are not known. A current model for the control of eukaryotic mRNA decay involves the interaction of specific RNABPs with cognate cis elements on target mRNAs. In turn, this interaction can recruit or exclude mRNA decay enzymes leading to transcript destabilization or stabilization respectively. In addition, regulatory RNAs such as microRNAs can play an important role to regulate mRNA stability via related mechanisms (46). By analogy to this paradigm, we hypothesized that targeted RNA cleavage by RNase-L may also require an RNABP. In this regard, the recent finding that RNase-L targeted the mRNA encoding the RNABP TTP (23) provided a clue to a potential RNA targeting mechanism. Specifically, the observation that RNase-L regulates TTP mRNA, and previous studies demonstrating that TTP autoregulates its own RNA (47,48), suggested that RNase-L and TTP may function in concert to degrade TTP mRNA and potentially other TTP targets. Consistent with this model, we determined that RNase-L and TTP proteins associate in an immunoprecipitable complex in cells (45). TTP mediates its antiproliferative activities by promoting the degradation of labile mRNAs containing A-U-rich elements (AREs) that are typically found in their 3'-untranslated regions (3'UTR) (49-51). Specifically, TTP binds AREs in target mRNAs and interacts with mRNA decay enzymes such as negative regulator of transcription (Not1)/ chromatin assembly factor (Caf1) (52), 5'-3' Exoribonuclease 1 (Xrn1) (53), chemokine c-c motif receptor 4 (Ccr4) (53), decapping protein (Dcp) (54), and components of the exosome (55), to stimulate their decay.

Established TTP targets include transcripts that encode mediators of cell proliferation such as c-Myc (56), hypoxia inducible factor 1, alpha (HIF-α) (57), cyclooxygenase 2 (Cox-2) (58), and proviral integration site 1 (Pim-1) (59). Increased TTP-mediated turnover of these transcripts results in proliferation-inhibitory activities including cell cycle arrest (60), senescence (61), apoptosis (57,62), and tumor suppression in vivo (5,63,64). A subset of TTP targets exhibit RNase-L-dependent regulation supporting a potential role for TTP in targeting RNase-L substrate cleavage (45). However, the functional significance of this regulation, and the biologic contexts in which it occurs, are not known. The major biologic functions attributed to TTP occur following the induction of its expression or activity by diverse stimuli including mitogens (7,64,65). In light of the overlapping roles for RNase-L and TTP in antiproliferative and tumor suppressor activities, we examined the regulation of TTP by RNase-L in the context of mitogen stimulation as a potential mechanism by which the proliferative response is attenuated.

Here we report that RNase-L post-transcriptionally downregulated TTP by destabilizing its mRNA in unstimulated conditions whereas it indirectly inhibited TTP transcription following mitogenic stimulation. This finding suggested that RNase-L targeted a transcription factor required for induction of TTP mRNA following serum stimulation. A binding site for serum response factor (SRF), a master transcriptional regulator of serum-induced genes, was identified in the ZFP36 gene promoter. Furthermore, SRF mRNA was previously reported to be downregulated by RNase-L in IFNγ-stimulated mouse embryo fibroblasts (MEFs) (66). Together these observations suggested that SRF mRNA is a direct target of RNase-L and that this regulation may account for the RNase-L-dependent regulation of TTP in mitogen-stimulated cells. Consistent with this prediction, RNase-L destabilized the SRF transcript and formed a physical complex with SRF mRNA in cells. We hypothesized that RNase-L and TTP function in concert to target SRF mRNA cleavage. Accordingly, we validated the association of RNase-L and TTP in cells and identified an essential role for the RNase-L pseudokinase domain in this interaction. SRF mRNA was...
RNase-L attenuates the cellular response to mitogenic stimuli

degraded in a TTP-dependent manner and contains a consensus ARE TTP binding site in its 3’UTR identifying SRF mRNA as a novel TTP target and RNase-L substrate. We further determined that established TTP-target mRNAs are also regulated by RNase-L providing evidence of a broader role for this mechanism of RNase-L-substrate targeting. Proliferation was enhanced following mitogen stimulation of RNase-L-deficient as compared to RNase-L competent cells, demonstrating its functional role in limiting the mitogenic response. Findings from this study thus identify a novel mechanism of RNase-L substrate specificity through its association with TTP. RNase-L functions via transcriptional and post-transcriptional mechanisms to attenuate the induction of TTP, and TTP target mRNAs, following mitogen stimulation. As important regulators of the cellular response to mitogenic stimuli that function to constrain cell proliferation, the RNase-L/TTP axis represents a potential therapeutic target for neoplastic diseases including cancer.

EXPERIMENTAL PROCEDURES

Cell culture and transfections- MEF, HeLa, and HEK 293-T cells were cultured in DMEM medium containing 10% FBS and 1X Antibiotic-Antimycotic (Invitrogen) and maintained at 37°C and 5% CO₂. Transfections in HeLa and 293-T cells were carried out using Lipofectamine 2000 according to supplier’s directions (Invitrogen). MEFs were derived from RNase-L +/- and TTP +/- mice, and WT mice of the same genetic background (25,67). All experiments with MEFs were performed with early and late passage cultures to insure that results were not due to passage-associated changes. SNAP-Control and SNAP-RNase-L stable HeLa cell lines were generated with by cloning full length RNase-L into pSNAP-tag plasmid downstream of SNAP tag.

Western Bloting and Immunoprecipitation- Preparation of cell lysates, measurement of protein concentration and Western blot (WB) analysis were performed as previously described (70). Antibodies were used at the following dilutions: Anti-Flag (Sigma) 1:1000, anti-Myc (Upstate Biotechnology,) 1:2000, anti-Actin (Sigma) 1:5000, anti-GAPDH (Thermo-Fisher Scientific) 1:1000, anti-SNAP(New England Biolabs) 1:500, anti-TTP (Abcam) 1:500, anti-SRF (Sigma) 1:2000, and anti-phospho-SRF (Sigma) 1:2000. For immunoprecipitation (IP), 500 µg of total protein was precleared for 1 hour at 4°C using 25µl of protein G-agarose beads (Santa Cruz). Protein that non-specifically bound to protein G-agarose was removed by centrifugation to generate the precleared cell lysate and primary antibodies or isotype control (IgG) were then added and incubated at 4°C for 2 hours with rotation. To IP the antibody complexes, 30 µl of protein G-agarose was added and incubated overnight at 4°C with rotation. The immunoreactive complex was pelleted by centrifugation at 10,000 rpm for 5 minutes. Supernatant was removed and the pellet was washed using 1ml of PBS four times. After the final wash, pellet was resuspended in 1x SDS loading buffer for WB analysis.

Measurement of mRNA: Purified total RNA from cells was isolated by TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. mRNA expression was measured by multiplex, quantitative real-time reverse-transcription-PCR (qRT-PCR) using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad) and the CFX96 (Bio-Rad). All mRNAs were measured with control mRNA primer sets (PGK1, HPRT, GAPDH) in parallel reactions. Each data point is taken as the mean ± standard deviation from triplicate qRT-PCR reactions for each RNA sample. Quality control of primer sets used for qPCR was performed using melt curve analysis vector (Clonetech) downstream of 3XFLAG-CMV-10 (Sigma) and generously provided by Gerald Wilson’s Lab. pcDNA-GFP-TTP and TTP deletion mutants were generated as described (69). SNAP-Control and SNAP-RNase-L stable cell lines were generated with by cloning full length RNase-L into pSNAP-tag plasmid downstream of SNAP tag.

Expression constructs- pcDNA3-FLAG-RNase-L and pCMV3B-Myc-RNase-L constructs and deletion mutants were subcloned from pGEN-GST-RNase-L constructs. RNase-L deletion constructs were kindly provided by Beihua Dong and Robert Silverman, The Cleveland Clinic Foundation (33,68). pcDNA-FLAG-TTP was generated by cloning TTP cDNA in pTRE2hyg

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
and appropriate controls, e.g. non-template and no reverse transcriptase. Primer sets are listed in table 1.

**Measurement of mRNA decay kinetics:**
Actinomycin D (actD) time course assays were used to determine the decay rates of SRF and TTP mRNAs. Briefly, 5µg/ml actD was added to cell culture media to inhibit transcription and total RNA was purified at specific times thereafter limited to 4 hours to avoid actD-enhanced apoptosis (71). SRF or TTP transcripts were measured by qRT-PCR and normalized to control mRNA as described above. To determine mRNA half-lives, first-order decay constants (k) were solved by nonlinear regression (PRISM) of the percentage of mRNA remaining versus the time of actD treatment. The half-lives were resolved by t1/2 = ln2/k and are based on the mean ± standard deviation of n (greater or equal to 3 or more) independent time course experiments.

**Ribonucleoprotein-Immunoprecipitations (RNP-IP) and analysis of bound RNA:** Analysis of mRNA in RNase-L RNP complexes was performed as adapted from a previously described protocol (72). HeLa cells stably expressing SNAP-control or SNAP-RNase-L were washed by scraping in 5 ml of ice-cold PBS and centrifuged at 500xg for 5 min. Cells were lysed in PLB buffer (10mM HEPES (pH 7.5), 50 mM KCl, 5mM MgCl2, 0.3% NP-40) containing 25x protease inhibitor cocktail/RNase-OUT (Invitrogen), then incubated on ice for 10 min. Supernatant was collected by centrifugation at 10,000xg for 15 minutes and the protein concentration was determined (BioRad); supernatants were stored at -80° or used directly for IP. For RNP-IP, 3mg of the supernatant was incubated with 70ul of 50% (v/v) suspension of protein-A Sepharose beads (Sigma) washed with NT2 buffer: (50 mM Tris, pH 7.4, 150 mM NaCl, 1mM MgCl2, 10 mM Hepes, pH7.0, 0.5% NP-40) and precoated with 15µg of SNAP antibody (NEB Biosciences) for 30mins at 4°C. Beads were washed 5 times with NT2 buffer and sequentially treated with DNase I (30°C for 15 minutes) and proteinase K (55°C for 15 minutes). The RNA was extracted with phenol/chloroform and precipitated in 0.3 M sodium acetate (pH 5.5) with 2 volumes ethanol at -20°C. RNA was pelleted at 10,000x g, washed in ethanol and resuspended in DEPC treated water for qPCR analysis to detect the presence of specific mRNAs in the immunoprecipitated complex. RNP-IPs in 293T cells were first co-transfected with RNase-L and TTP constructs then serum starved (DMEM + 0.05% FBS) for 20hrs; at 24 hours after transfection, medium was exchanged with DMEM containing 10% FBS and 200nM TPA for 1 hour, then cells were washed with ice-cold PBS and harvested. Anti-IgG1, anti-Myc-tag, or anti-FLAG antibodies were used to immunoprecipitate the respective proteins.

**Cell proliferation assay**- MEF WT and KO RNase-L cells were seeded into 96-well plates at 5,000 cells per well, allowed to attach for 18 hours, then serum starved for 20hours in DMEM containing 0.5% FBS. Cells were then treated with 200nM TPA and restored DMEM containing 10% FBS for 1 hour. Viable cells were quantified by MTT assay (Promega). Values from each time point were normalized to serum starved, or time 0 values. Each value is a mean of 4-6 identical wells +/- SD.

**Data analysis and statistics**- Values of data points in figures are the mean of at least three measurements +/- standard deviation unless otherwise indicated in the legend. P values given were determined by the unpaired t test. Differences yielding P < 0.05 were considered significant. Qualitative analyses shown (i.e. WB, IP) are representative of three independent experiments.

**RESULTS**
**RNase-L regulates TTP by distinct mechanisms in resting and mitogen-stimulated conditions.** ZNF36/TTP was originally discovered as a mitogen-induced transcript (8,9) and subsequent studies have demonstrated that it is also induced in response to diverse stress stimuli including proinflammatory cytokines (73-75) and microbial pathogens (7,65). In stimulated conditions, TTP is rapidly induced in parallel with immediate early genes and functions in a feedback loop to attenuate expression of stimulus-induced transcripts (59,76).
TTP expression and activity are tightly controlled by transcriptional and post-transcriptional mechanisms (7,77) and RNase-L was recently reported to contribute to this regulation (23). In light of the established antiproliferative activities of both RNase-L and TTP, we hypothesized that RNase-L-mediated regulation of TTP may be important in the context of mitogen stimulation that is relevant to their biologic roles as endogenous constraints on cell proliferation. To test this hypothesis, mouse embryonic fibroblasts (MEFs) derived from knockout (KO; RNase-L−/−) and wild type (WT; RNase-L+/+) mice (25) were cultured in 0.5% serum for 20 hours (serum starvation) and then treated with 10% serum/200nM 12-O-Tetradecanoylphorbol-13-acetate (TPA) to induce proliferation. This regimen of combining serum with a phorbol ester mitogen is a well-established system to study mitogen-induced gene expression that results in a robust induction of TTP and is referred to as ‘mitogen stimulation’ in the subsequent text (78,79). Mitogen stimulation resulted in a rapid induction of steady-state TTP mRNA in WT cells that was increased 8-9 fold over unstimulated cells by one hour post-stimulation and returned to basal levels by 4 hours (Fig. 1A). Strikingly, while the kinetics of TTP mRNA induction in KO MEFs paralleled those in WT MEFs, the magnitude of TTP mRNA induction in KO MEFs was much greater than that observed in WT MEFs, increasing 49-fold over its expression in unstimulated cells by one hour post-stimulation and returned to basal levels by 4 hours (Fig. 1A). These findings demonstrate an important role for RNase-L in the regulation of mitogen-induced TTP expression.

RNase-L-dependent regulation of gene expression may occur via direct cleavage of target RNAs or via indirect mechanisms that are downstream of the primary substrate. To investigate the mechanism by which RNase-L downregulates TTP, we determined if RNase-L directly targets TTP mRNA to decrease its half-life. Analysis of TTP mRNA turnover following inhibition of transcription by actinomycin-D (ActD) revealed a significant stabilization of TTP mRNA in RNase-L KO as compared to WT MEFs in unstimulated conditions (Fig. 1B). In further support of the direct targeting of TTP mRNA by RNase-L, TTP mRNA associated with RNase-L in transfected cells as analyzed by RNP immunoprecipitation (IP) (Fig. 1C). Specifically, TTP mRNA was enriched in RNP complexes isolated with RNase-L, but not isotype control antibody. In contrast, similar amounts of the abundant HPRT mRNA were present in both RNase-L and IgG IP complexes and served as a control for equivalent RNP precipitation and RNA yield in the different samples. In further support of this regulation, ectopic expression of RNase-L in HeLa cells, that express low endogenous RNase-L (23) resulted in a reduction in TTP mRNA (Fig 1B). These findings indicate that RNase-L directly targets the TTP transcript in basal conditions in agreement with a previous report (23). To determine if RNase-L-mediated destabilization of TTP mRNA could account for the increase in steady state TTP transcript observed in mitogen-stimulated MEFs, we determined the TTP mRNA half life in WT and RNase-L KO MEFs at one hour post-mitogen stimulation when TTP induction was maximal. However, TTP mRNA did not exhibit RNase-L-dependent destabilization in mitogen-stimulated conditions. In contrast, TTP mRNA half-life increased from 20 minutes in RNase-L KO MEFs to 1.2 hours in WT MEFs following serum stimulation (Fig. 1D). This discordance between the steady state expression of TTP mRNA and its half-life may reflect an enhanced autoregulation by TTP of its own mRNA turnover via an RNase-L-independent mechanism (47,48). Most strikingly, our results indicate that mitogen stimulation resulted in a dramatic 6-fold increase in steady-state TTP mRNA in RNase-L KO as compared to WT MEFs despite a decrease in its half-life. These findings suggested that the regulation of TTP by RNase-L in mitogen-stimulated conditions occurs through an indirect mechanism to downregulate TTP transcription.

RNase-L downregulates mitogen-induced TTP transcription via direct regulation of SRF. To investigate the impact of RNase-L on TTP transcription as a potential mechanism by which it downregulates steady state TTP mRNA following mitogen stimulation, unprocessed TTP transcripts were measured using primers that detect the single TTP intron as an indication of TTP transcriptional activity (80). Similar to the enhanced induction of steady-state TTP mRNA levels in RNase-L KO
RNase-L attenuates the cellular response to mitogenic stimuli

MEFs, TTP primary transcripts were also increased in RNase-L KO as compared to WT MEFs at 1 hour of mitogen stimulation and were elevated at all post-stimulation time points (Fig. 2A). This result demonstrated the RNase-L-dependent downregulation of TTP transcription in (81,82) mitogen stimulated MEFs and suggested that RNase-L directly targets a transcription factor that mediates TTP induction by mitogens. Consistent with this model, SRF is a master transcriptional regulator of serum-induced genes that was previously reported to be regulated by RNase-L in IFN-γ-stimulated smooth muscle cells (66). Furthermore, analysis of the ZFP36/TTP gene promoter revealed a serum response element (SRE), the site at which SRF binds to induce transcription of mitogen-induced genes, at positions -281->-264 relative to the ZFP36/TTP transcription start site (83). Additionally, TTP was identified in a genomic screen for actin-MAL-SRF induced genes in NIH 3T3 fibroblasts (84). Based on these findings, we hypothesized that RNase-L directly targets SRF mRNA to limit the induction of TTP, and potentially other serum induced transcripts, following mitogen stimulation. Consistent with this prediction, steady state SRF mRNA was induced to a higher level following mitogen stimulation of RNase-L KO as compared to WT MEFs (Fig. 2B). SRF mediates transcriptional induction in a complex with co-factors including the ternary complex factors (TCFs) (82,83) however, only SRF exhibited RNase-L-dependent regulation demonstrating the selective targeting of SRF mRNA in mitogen-stimulated conditions (data not shown). The RNase-L-dependent expression profiles of SRF and TTP in response to mitogen were similar and support our model in which RNase-L directly regulates SRF upstream of its indirect effect on TTP mRNA. Analysis of SRF mRNA stability revealed that its half-life was increased 2-fold in RNase-L KO as compared to WT MEFs following mitogen stimulation as predicted for a direct RNase-L target (Fig. 2C). RNase-L-dependent regulation of SRF mRNA was also observed in unstimulated cells (data not shown) and SRF mRNA was present in an RNP-IP complex with RNase-L that increased in response to mitogen stimulation (Fig. 2D). Together these data identify SRF mRNA as a direct target of RNase-L regulation and provide a potential mechanism by which it regulates TTP transcription in mitogen-stimulated conditions. However, we cannot rule out an alternate, indirect impact of SRF on TTP expression at this time.

RNase-L and TTP proteins functionally interact to target SRF mRNA turnover—The RNase-L-dependent regulation of SRF mRNA stability and the physical association of SRF mRNA and RNase-L protein provide evidence that SRF mRNA is an authentic RNase-L substrate. However, the mechanism by which SRF mRNA, and other candidate RNase-L substrates, is targeted for cleavage is not known. We previously determined that RNase-L and TTP proteins associate in cells suggesting that TTP may function to recruit RNase-L to its substrates, including SRF mRNA. To further examine the RNase-L-TTP interaction and its functional impact on SRF mRNA turnover, we first mapped domains required for their association in cells. Amino- and carboxyl-terminal deletions of RNase-L were expressed with full length TTP in cells and their association was analyzed by co-immunoprecipitation (68). This analysis indicated that deletion constructs that remove the amino-terminal 342 amino acids precipitated with TTP in proportion to their level of expression and were thus dispensable for TTP interaction (Fig. 3A). However, deletion of the carboxyl-terminus 399 amino acids abrogated RNase-L co-immunoprecipitation with TTP indicating that amino acids 343-662 of the pseudokinase domain were required for TTP interaction (Fig. 3A). Interestingly, the pseudokinase domain is also required for RNase-L dimerization (39) suggesting that it serves as an important platform for RNase-L-protein interactions. A complementary analysis of TTP deletion mutants revealed that no single deletion abolished immunoprecipitation with full length RNase-L and that the 77 amino acid RNA binding domain constituted a minimal interaction region (Fig. 3B). This finding suggested that multiple TTP domains contribute to its association with RNase-L; however further mapping is required to precisely define the interacting region. The low basal expression of RNase-L and TTP and the lack of antisera that efficiently immunoprecipitate these proteins precluded analysis of an interaction between both endogenous proteins, therefore we assessed the
RNase-L attenuates the cellular response to mitogenic stimuli

ability of transfected RNase-L or TTP to co-IP with endogenous TTP and RNase-L respectively. This approach demonstrated an interaction of either ectopically expressed component of this complex with their respective endogenous partner, providing further support for their interaction in cells (Fig. 3C). Together these results identify TTP as a novel component of an RNase-L-associated complex in cells and provide a potential mechanism for RNase-L-substrate targeting.

We hypothesized that TTP recruits RNase-L to cleave TTP-bound substrate RNAs. Therefore, in light of our data indicating that SRF mRNA is an RNase-L substrate (Fig. 2) and that RNase-L and TTP proteins form a complex in cells (Fig. 1C), we predicted that TTP will interact with SRF mRNA. Indeed, TTP formed an RNP-IP complex with SRF mRNA in cells (Fig. 4A). Consistent with the identification of SRF mRNA as a novel TTP target, a consensus ARE (CUUAAUUUAUU) TTP binding sequence was identified at bases 1929-1938 in the 3’UTR of SRF mRNA (85). In our model of TTP-mediated RNase-L substrate targeting, RNase-L and TTP function in concert to direct the degradation of SRF mRNA. As previously shown in Fig. 2C, deletion of RNase-L stabilized SRF mRNA; therefore, we hypothesized that a similar increase in SRF mRNA half-life will be observed in cells lacking TTP. To test this prediction, we analyzed the turnover of SRF mRNA in MEFs derived from WT and TTP KO mice. SRF mRNA half-life increased from 1.3 hours in WT MEFs to 3.1 hours in TTP KO MEFs (Fig. 4B) and closely matched its stabilization in RNase-L KO MEFs (Fig. 2C). Thus, consistent with our model, deletion of either RNase-L or TTP resulted in a comparable increase in SRF mRNA half-life, identifying SRF as a novel target of regulation by RNase-L and TTP.

RNase-L and TTP function to downregulate the expression of mitogen-induced transcripts and attenuate the proliferative response. In response to mitogen stimulation, SRF induces the transcription of many immediate early genes to promote cell proliferation (81,82). TTP is also induced by mitogens and functions to attenuate the expression of a subset of mitogen-induced ARE-containing transcripts (64,65). Our data suggest that RNase-L functions in association with TTP to degrade SRF mRNA providing a mechanism to limit SRF-induced transcription. In addition, we hypothesized that RNase-L, via its association with TTP, may act to post-transcriptionally downregulate a subset of TTP target mRNAs. To test this hypothesis, we examined a panel of established TTP target mRNAs for RNase-L-dependent regulation. Specifically, Cox-2 (58), vascular endothelial growth factor (VEGF) (86,87), tumor necrosis factor alpha (TNF-α) (67,74), and cyclin-dependent kinase inhibitor 1 (p21CIP) (23) mRNAs were expressed to higher levels in RNase-L KO as compared to WT MEFs following mitogen stimulation (Fig. 5A). However, interleukin beta (IL-1β) (88), HIF1-α (89), and PIM-1 (59) transcripts that are also known TTP target mRNAs (7,64) were not significantly affected by RNase-L deficiency (Fig. 5B). These findings indicate that RNase-L regulates a subset of TTP target mRNAs and suggest that additional components of an RNase-L/TTP mRNP complex or sequence determinants on mRNA targets may contribute to substrate selection (Table 2). RNase-L thus functions to limit the expression of mitogen-induced mRNAs via transcriptional and post-transcriptional mechanisms which, in turn, may contribute to its antiproliferative activity. Consistent with this prediction, RNase-L KO MEFs displayed a more robust proliferative response to mitogen stimulation as compared to that observed in WT MEFs (Fig. 5C). Together, our findings support a model in which RNase-L and TTP function in concert to regulate mitogen-induced transcripts and attenuate the proliferative response.

DISCUSSION

The cellular response to mitogenic stimulation must be tightly regulated to prevent the deleterious effects of uncontrolled proliferation. Therefore, understanding the mechanisms by which this response is regulated is essential to identify therapeutic targets for neoplastic disorders. RNase-L functions as an endogenous proliferative constraint (23-26) and TTP is induced by mitogenic stimulation as a feedback mechanism to attenuate the proliferative
RNase-L attenuates the cellular response to mitogenic stimuli

In light of recent findings demonstrating the regulation of TTP mRNA by RNase-L (23), and the interaction of RNase-L and TTP proteins (45), we sought to investigate the RNase-L-mediated regulation of TTP following mitogen stimulation that is relevant to their roles as antiproliferative effectors, and to their mechanisms of action in the post-transcriptional control of target mRNA turnover. Mitogen treatment of RNase-L KO MEFs resulted in a striking 6-fold increased induction of TTP mRNA as compared to that observed in WT MEFs (Fig. 1A). While RNase-L-dependent TTP mRNA decay has been previously reported (23), the increase in steady state TTP mRNA following mitogen stimulation did not reflect a stabilization of TTP mRNA but corresponded with an increase in TTP transcription (Fig. 2A). This finding indicated that RNase-L regulated TTP by distinct direct and indirect mechanisms in resting and mitogen-stimulated conditions respectively and suggested that RNase-L targeted a mitogen-induced regulator of TTP transcription.

SRF is a master transcriptional regulator of serum-induced genes (81,82,91) and RNase-L was previously reported to regulate SRF in IFN-γ-treated smooth muscle cells (66). Furthermore, a SRF binding site was identified in the ZFP36 gene promoter (84,85). These findings suggested that RNase-L may downregulate SRF mRNA to inhibit mitogen-induced transcription of TTP. Consistent with this hypothesis, the magnitude of SRF mRNA induction was higher in RNase-L KO as compared to WT MEFs (Fig. 2B). Moreover, RNase-L deficiency stabilized SRF mRNA and SRF mRNA was enriched in RNase-L immunoprecipitates indicating that it is an authentic RNase-L substrate (fig 2C,D). Interestingly, a recent study demonstrated that the induction of type-1 IFN stimulated genes was diminished in RNase-L deficient macrophages thus identifying a novel role for SRF in IFN signaling (92). In light of the established role of RNase-L as an IFN-regulated effector, and our data identifying SRF mRNA as an RNase-L target, RNase-L-mediated downregulation of SRF may serve as a feedback mechanism to attenuate expression of IFN-stimulated genes (ISG). As TTP is also induced by IFN (75), RNase-L and TTP may serve analogous functions to limit the expression of transcripts induced by mitogens and IFN. This broader role for RNase-L and TTP as feedback inhibitors of transcripts induced by diverse stimuli is the subject of ongoing investigation. Our results thus identified SRF as a novel RNase-L substrate and provided a mechanism by which it may indirectly impact the expression of mitogen-induced transcripts including TTP.

RNase-L-dependent regulation of SRF mRNA occurred in the absence of global changes in mRNA turnover, however, the mechanism(s) by which it selectively targeted the SRF transcript are not known. Considering our previous data indicating that RNase-L associated with TTP in cells (45), and published studies demonstrating that TTP autoregulates its own mRNA (47,48), we hypothesized that interaction with TTP may recruit RNase-L to specific mRNAs as a mechanism of substrate targeting. To further study this interaction, we used deletion mapping to identify the pseudokinase domain of RNase-L as a region that is required for TTP interaction (Fig. 3). The analysis of RNase-L and TTP protein interaction was done by immunoprecipitation to assess their association in a cellular context, however further studies using recombinant proteins are required to determine if the interaction is direct. Interestingly, the RNase-L pseudokinase domain is critical for coordinating 2-5A binding (39,40) and homodimerization (33,34) that occur upon its activation. Furthermore, the ATP competitive kinase inhibitor sunitinib was recently shown to bind the ATP pocket within this domain and inhibit RNase-L activation (93). Together, these studies establish a central role for the pseudokinase domain in RNase-L activation and suggest that heterologous proteins that interact in this domain may positively or negatively impact RNase-L activity as a novel mechanism to modulate its biologic functions. Future studies to determine the specific residues involved in these interactions, and their biophysical properties in the presence and absence of 2-5A, will provide insights into the mechanisms regulating RNase-L activity. In this regard, recent crystal structures of RNase-L suggest that multiple residues in the pseudokinase domain are exposed in the active dimeric conformation and may constitute a critical platform for regulatory interactions (39,40). Current modeling studies with RNase-L and TTP...
RNase-L attenuates the cellular response to mitogenic stimuli

will permit informed mutagenesis to test this hypothesis.

The interaction of RNase-L with TTP and the identification of a consensus ARE TTP binding site in the SRF mRNA 3'UTR suggested that the SRF transcript may be regulated by the coordinate action of RNase-L and TTP. Consistent with this prediction, SRF mRNA was stabilized to a nearly identical degree in MEFs lacking either RNase-L or TTP (Figs. 2C and 4B); furthermore, both RNase-L and TTP formed a complex with SRF mRNA in cells (Figs. 2D, 4A). The direct regulation of SRF by RNase-L and TTP was, in turn, predicted to indirectly impact the transcription of SRF-induced genes. In fact, a meta-analysis of SRF-regulated genes from four studies (94-97) revealed that 84% of their encoded transcripts contained an ARE (Table 2; (85)). This frequency of ARE-containing mRNAs represents a twelve-fold enrichment over their occurrence in the global mRNA population (~7% ARE positive; (98)) and suggests that ARE-mediated regulation is important for SRF-induced genes. Notably, 50% of the ARE-positive SRF-regulated transcripts are validated TTP targets or contain a consensus TTP binding site. This data points to a central role for TTP, as opposed to other ARE binding proteins, in control of the SRF transcriptome. In support of our model in which a subset of SRF mRNA and SRF-induced transcripts are regulated by the concerted action of RNase-L and TTP (Fig. 6), 16% of the SRF-regulated transcripts that contain a consensus TTP binding site or are validated targets are also predicted or validated RNase-L substrates (Table 2). Consistent with this analysis, four of seven established TTP targets tested were regulated by RNase-L in mitogen-stimulated MEFs (Fig 5A, B). Together, these data indicate that the regulation of mitogen-induced transcripts by RNase-L and TTP can occur through both transcriptional and post-transcriptional mechanisms. Accordingly, the extent to which one or both of these mechanisms function to regulate a given transcript will dictate its specific pattern of expression following mitogen stimulation. Indeed, two distinct expression profiles were observed for the TTP targets that we determined to be regulated by RNase-L. Specifically, whereas steady state expression of Cox2 and VEGF mRNAs continued to increase through eight hours post mitogen stimulation (Fig 5A), SRF, TNFα and p21CIP mRNAs peaked at 1-2 hours after mitogen stimulation and then declined (Fig 5B). As all of these transcripts are also SRF targets, failure to either attenuate transcription and, or increase turnover may account for the dysregulation observed in RNase-L KO MEFs. Future studies will focus on identifying the direct targets of RNase-L cleavage within the population of RNase-L-regulated transcripts to define the cis and trans-acting factors that mediate substrate recognition.

Our analysis (Table 2, Fig 5) and microarray data (12,17,42-44) indicated that only a subset of TTP targets exhibit RNase-L-dependent regulation (45). This lack of complete correspondence between RNase-L-regulated transcripts and TTP targets suggests that these transcripts are regulated by different mechanisms in distinct physiologic settings. Our data on TTP regulation in the presence and absence of mitogen stimulation provide an example of this context-specific regulation. Specifically, RNase-L-deficiency stabilizes TTP mRNA in unstimulated cells whereas the TTP transcript is destabilized following mitogen stimulation of RNase-L KO MEFs (compare panels B and D in Fig. 1). These distinct outcomes may reflect mitogen-induced changes in TTP activity and a corresponding impact on its autoregulation (23,99). In addition, alternative decay mechanisms mediated by distinct factors may contribute to the differential regulation observed in these settings. In agreement with this prediction, TTP interacts with established RNA decay enzymes to promote deadenylation and mRNA turnover (e.g. Ccr4/Caf1/Not1; (52,53)) that may represent a default mechanism when RNase-L is not expressed. In addition, the interaction of TTP AUFI was recently shown to influence target RNA selection (100) providing further evidence that the combination of RNABPs and decay factors present in different conditions are likely to influence TTP-directed mRNA degradation. Our results support a model in which RNase-L functions in a complex with TTP to regulate a subset of mitogen-induced transcripts (Fig. 6). A global comparison of transcripts and proteins bound to both RNase-L and TTP will provide a more complete picture of the factors that...
RNase-L attenuates the cellular response to mitogenic stimuli

RNase-L mediates diverse antiproliferative activities including quiescence (11), senescence (24), and apoptosis (25,26). Our results demonstrated that RNase-L downregulates mitogen-induced gene expression via transcriptional and post-transcriptional mechanisms suggesting that it plays a functional role to attenuate the proliferative response. Consistent with this prediction, proliferation was significantly increased following mitogen stimulation of RNase-L KO as compared to WT MEFs (Fig. 5C). The enhanced proliferation observed in mitogen-stimulated RNase-L KO MEFs corresponded with increased expression of TTP. This phenotype was unexpected as TTP expression is typically associated with antiproliferative activities (6,13). These data suggested that elevated TTP expression in the absence of RNase-L was insufficient to attenuate a proliferative response; however, the activity of TTP in this setting remains to be examined. For example, post-translational modifications and interacting proteins that impact the stability and RNA binding activity of TTP protein in response to inflammatory stimuli are well established (7,99) and similar regulation may occur following mitogen treatment. In addition, little is known about the regulation of RNase-L activity beyond its requirement for conversion to an enzymatically active dimer, as sensitive methods to detect its activity in cells are lacking. Given the critical nature of rapid induction and efficient attenuation in the physiologic outcome of a mitogenic response, the temporal regulation of RNase-L and TTP activities is likely to be particularly important and represents a key area for future investigation.

Taken together, our study identifies an important role for RNase-L in attenuating the mitogenic response through the transcriptional and post-transcriptional regulation of TTP and SRF. The physical association of RNase-L with TTP in cells, and its regulation of a subset of established TTP target mRNAs, supports a model in which the interaction of RNase-L with TTP, and possibly other RNABPs, dictates its substrate profile and hence biologic activity. Accordingly, this regulation and its associated impact on mitogen-induced proliferation, are reduced in RNase-L-deficient cells (Fig. 6). Both RNase-L and TTP mediate antiproliferative and tumor suppressor activities (6,7,20,23-26,64), accordingly, genetic disruption or inactivating mutations in either protein lead to a transformed phenotype in vitro and tumorigenesis in vivo (27,28,101). Furthermore, mutations in RNase-L and TTP are correlated with poor prognosis in several human cancers (6,29-32,102). Increased proliferative signaling is an established hallmark of cancer (103) and our data identify RNase-L and TTP as endogenous constraints on the proliferative response; therefore, strategies to enhance the activities of RNase-L and TTP represent a potential therapeutic approach for proliferative disorders.

REFERENCES

1. Lau, L. F., and Nathans, D. (1985) Identification of a set of genes expressed during the G0/G1 transition of cultured mouse cells. EMBO J 4, 3145-3151
2. Healy, S., Khan, P., and Davie, J. R. (2013) Immediate early response genes and cell transformation. Pharmacol Ther 137, 64-77
3. Chang, H. Y., Sneddon, J. B., Alizadeh, A. A., Sood, R., West, R. B., Montgomery, K., Chi, J. T., van de Rijn, M., Botstein, D., and Brown, P. O. (2004) Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds. PLoS Biol 2, E7
4. Zullo, J. N., Cochran, B. H., Huang, A. S., and Stiles, C. D. (1985) Platelet-derived growth factor and double-stranded ribonucleic acids stimulate expression of the same genes in 3T3 cells. Cell 43, 793-800
RNase-L attenuates the cellular response to mitogenic stimuli

5. Stoecklin, G., Gross, B., Ming, X. F., and Moroni, C. (2003) A novel mechanism of tumor suppression by destabilizing AU-rich growth factor mRNA. *Oncogene* 22, 3554-3561

6. Brennan, S. E., Kuwano, Y., Alkharouf, N., Blackshear, P. J., Gorospe, M., and Wilson, G. M. (2009) The mRNA-destabilizing protein tristetraprolin is suppressed in many cancers, altering tumorigenic phenotypes and patient prognosis. *Cancer Res* 69, 5168-5176

7. Sanduja, S., Blanco, F. F., Young, L. E., Kaza, V., and Dixon, D. A. (2012) The role of tristetraprolin in cancer and inflammation. *Front Biosci (Landmark Ed)* 17, 174-188

8. DuBois, R. N., McLane, M. W., Ryder, K., Lau, L. F., and Nathans, D. (1990) A growth factor-inducible nuclear protein with a novel cysteine/histidine repetitive sequence. *J Biol Chem* 265, 19185-19191

9. Lai, W. S., Stumpo, D. J., and Blackshear, P. J. (1990) Rapid insulin-stimulated accumulation of an mRNA encoding a proline-rich protein. *J Biol Chem* 265, 16556-16563

10. Klein, G. (1993) Genes that can antagonize tumor development. *FASEB J* 7, 821-825

11. Hassel, B. A., Zhou, A., Sotomayor, C., Maran, A., and Silverman, R. H. (1993) A dominant negative mutant of 2-5A-dependent RNase suppresses antiproliferative and antiviral effects of interferon. *EMBO J* 12, 3297-3304

12. Andersen, J. B., Mazan-Mamczarz, K., Zhan, M., Gorospe, M., and Hassel, B. A. (2009) Ribosomal protein mRNAs are primary targets of regulation in RNase-L-induced senescence. *RNA Biol* 6, 305-315

13. Sanduja, S., Kaza, V., and Dixon, D. A. (2009) The mRNA decay factor tristetraprolin (TTP) induces senescence in human papillomavirus-transformed cervical cancer cells by targeting E6-AP ubiquitin ligase. *Aging* 1, 803-817

14. Hovanessian, A. G. (2007) On the discovery of interferon-inducible, double-stranded RNA activated enzymes: the 2'-5'oligoadenylate synthetases and the protein kinase PKR. *Cytokine Growth Factor Rev* 18, 351-361

15. Silverman, R. H. (2007) A scientific journey through the 2-5A/RNase L system. *Cytokine Growth Factor Rev.* 18, 7

16. Malathi, K., Dong, B., Gale, M., Jr., and Silverman, R. H. (2007) Small self-RNA generated by RNase L amplifies antiviral innate immunity. *Nature* 448, 816-819

17. Li, X. L., Ezelle, H. J., Kang, T. J., Zhang, L., Shirey, K. A., Harro, J., Hasday, J. D., Mohapatra, S. K., Crasta, O. R., Vogel, S. N., Cross, A. S., and Hassel, B. A. (2008) An essential role for the antiviral endoribonuclease, RNase-L, in antibacterial immunity. *Proc Natl Acad Sci U S A* 105, 20816-20821

18. Ezelle, H. J., and Hassel, B. A. (2012) Pathologic effects of RNase-L dysregulation in immunity and proliferative control. *Front Biosci* 4, 767-786

19. Chakrabarti, A., Jha, B. K., and Silverman, R. H. New Insights into the Role of RNase L in Innate Immunity. *J Interferon Cytokine Res*

20. Jacobsen, H., Krause, D., Friedman, R. M., and Silverman, R. H. (1983) Induction of ppp(A2’p)nA-dependent RNase in murine JLS-V9R cells during growth inhibition. *Proc Natl Acad Sci U S A* 80, 4954-4958

21. Bourgeade, M. F., and Besancon, F. (1984) Induction of 2’,5’-oligoadenylate synthetase by retinoic acid in two transformed human cell lines. *Cancer Res* 44, 5355-5360

22. Krishnan, I., and Baglioni, C. (1980) Increased levels of (2’-5’)oligo(A) polymerase activity in human lymphoblastoid cells treated with glucocorticoids. *Proc Natl Acad Sci U S A* 77, 6506-6510

23. Al-Haj, L., Blackshear, P. J., and Khabar, K. S. A. (2012) Regulation of p21/CIP1/WAF-1 mediated cell-cycle arrest by RNase L and tristetraprolin, and involvement of AU-rich elements. *Nucleic Acids Res* 40, 7739-7752
RNase-L attenuates the cellular response to mitogenic stimuli

24. Andersen, J. B., Li, X. L., Judge, C. S., Zhou, A., Jha, B. K., Shelby, S., Zhou, L., Silverman, R. H., and Hassel, B. A. (2007) Role of 2-5A-dependent RNase-L in senescence and longevity. *Oncogene* **26**, 3081-3088

25. Zhou, A., Arumugam, J., Brown, T. L., Nie, H., Naik, S., Dong, B., Chang, A., Trapp, B., Fairchild, R., Colman, C., and Silverman, R. H. (1997) Interferon action and apoptosis are defective in mice devoid of 2',5'-oligoadenylate-dependent RNase L. *EMBO J* **16**, 6355-6363

26. Castelli, J. C., Hassel, B. A., Wood, K. A., Li, X. L., Amemiya, K., Dalakas, M. C., Torrence, P. F., and Youle, R. J. (1997) A study of the interferon antiviral mechanism: Apoptosis activation by the 2-5A system. *J Exp Med* **186**, 967-972

27. Liu, W., Liang, S. L., Liu, H., Silverman, R., and Zhou, A. (2007) Tumour suppressor function of RNase L in a mouse model. *Eur J Cancer* **43**, 202-209

28. Long, T. M., Chakrabarti, A., Ezelle, H. J., Brennan-Laun, S. E., Raufman, J. P., Pylypow, I., Silverman, R. H., and Hassel, B. A. (2013) RNase-L deficiency exacerbates experimental colitis and colitis-associated cancer. *Inflamm Bowel Dis* **19**, 1295-1305

29. Carpten, J., Nupponen, N., Isaacs, S., Sood, R., Robbins, C., Xu, J., Faruque, M., Moses, T., Ewing, C., Gillanders, E., Hu, P., Bujnowskiy, S., Makalowska, I., Baffoe-Bonnie, A., Fing, D., Smith, J., Stephan, D., Wiley, K., Brownstein, M., Gildea, D., Kelly, B., Jenkins, R., Hostetter, G., Matikainen, M., Schleutker, J., Klinger, K., Connors, T., Xiang, Y., Wang, Z., De Marzo, A., Papadopoulos, N., Kallioniemi, O. P., Burk, R., Meyers, D., Gronberg, H., Meltzer, P., Silverman, R., Bailey-Wilson, J., Walsh, P., Isaacs, W., and Trent, J. (2002) Germline mutations in the ribonuclease L gene in families showing linkage with HPC1. *Nature Genet* **30**, 181-184

30. Casey, G., Neville, P. J., Plummer, S. J., Xiang, Y., Krumroy, L. M., Klein, E. A., Catalona, W. J., Nupponen, N., Carpten, J. D., Trent, J. M., Silverman, R. H., and Witte, J. S. (2002) RNASEL Arg462Gln variant is implicated in up to 13% of prostate cancer cases. *Nature Genet* **32**, 582-583

31. Meyer, M. S., Penney, K. L., Stark, J. R., Schumacher, F. R., Sesso, H. D., Loda, M., Fiorentino, M., Finn, S., Flavin, R. J., Kurth, T., Price, A. L., Giovannucci, E. L., Fall, K., Stampfer, M. J., Ma, J., and Mucci, L. A. (2010) Genetic variation in RNASEL associated with prostate cancer risk and progression. *Carcinogenesis* **31**, 1597-1603

32. Madsen, B. E., Ramos, E. M., Boulard, M., Duda, K., Overgaard, J., Nordsmark, M., Wiuf, C., and Hansen, L. L. (2008) Germline mutation in RNASEL predicts increased risk of head and neck, uterine cervix and breast cancer. *Plos One* **3**, e2492

33. Dong, B., and Silverman, R. H. (1997) A bipartite model of 2-5A-dependent RNase L. *J Biol Chem* **272**, 22236-22242

34. Zhou, A., Hassel, B. A., and Silverman, R. H. (1993) Expression cloning of 2-5A-dependent RNAse: a uniquely regulated mediator of interferon action. *Cell* **72**, 753-765

35. Zhou, A. M., Molinaro, R. J., Malathi, K., and Silverman, R. H. (2005) Mapping of the human RNASEL promoter and expression in cancer and normal cells. *J Interf Cytok Res* **25**, 595-603

36. Clemens, M. J., and Williams, B. R. (1978) Inhibition of cell-free protein synthesis by ppA2'p5'A2'p5'A: a novel oligonucleotide synthesized by interferon-treated L cell extracts. *Cell* **13**, 565-572

37. Silverman, R. H., Jung, D. D., Nolan-Sorden, N. L., Dieffenbach, C. W., Kedar, V. P., and SenGupta, D. N. (1988) Purification and analysis of murine 2-5A-dependent RNase. *J Biol Chem* **263**, 7336-7341

38. Dong, B., and Silverman, R. H. (1995) 2-5A-dependent RNase molecules dimerize during activation by 2-5A. *J Biol Chem* **270**, 4133-4137

39. Huang, H., Zeqiraj, E., Dong, B., Jha, B. K., Duffy, N. M., Orlicky, S., Thevakanuraman, N., Talukdar, M., Pillon, M. C., Ceccarelli, D. F., Wan, L. C., Juang, Y. C., Mao, D. Y., Gaughan, C., Brinton, M. A., Perelygin, A. A., Kourinov, I., Guarne, A., Silverman, R. H., and Sicheri, F. (2014) Dimeric structure
of pseudokinase RNase L bound to 2-5A reveals a basis for interferon-induced antiviral activity. 
*Mol Cell* **53**, 221-234

40. Han, Y., Donovan, J., Rath, S., Whitney, G., Chitrakar, A., and Korennykh, A. (2014) Structure of human RNase L reveals the basis for regulated RNA decay in the IFN response. *Science* **343**, 1244-1248

41. Silverman, R. H. (2007) Viral encounters with 2',5'-oligoadenylate synthetase and RNase L during the interferon antiviral response. *J Virol* **81**, 12720-12729

42. Fabre, O., Salehzada, T., Lambert, K., Boo Seok, Y., Zhou, A., Mercier, J., and Bisbal, C. (2012) RNase L controls terminal adipocyte differentiation, lipids storage and insulin sensitivity via CHOP10 mRNA regulation. *Cell Death Differ* **19**, 1470-1481

43. Malathi, K., Paranjape, J. M., Bulanova, E., Shim, M., Guenther-Johnson, J. M., Faber, P. W., Eling, T. E., Williams, B. R., and Silverman, R. H. (2005) A transcriptional signaling pathway in the IFN system mediated by 2'-5'-oligoadenylate activation of RNase L. *Proc Natl Acad Sci U S A* **102**, 14533-14538

44. Domingo-Gil, E., Gonzalez, J. M., and Esteban, M. (2010) Identification of Cellular Genes Induced in Human Cells After Activation of the OAS/RNaseL Pathway by Vaccinia Virus Recombinants Expressing These Antiviral Enzymes. *J Interf Cytok Res* **30**, 171-188

45. Brennan-Laun, S. E., Ezel, H. J., Li, X. L., and Hassel, B. A. (2014) RNase-L control of cellular mRNAs: roles in biologic functions and mechanisms of substrate targeting. *J Interf Cytok Res* **34**, 275-288

46. Fabian, M. R., Sonenberg, N., and Filipowicz, W. (2010) Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem* **79**, 351-379

47. Brooks, S. A., Connolly, J. E., and Rigby, W. F. (2004) The role of mRNA turnover in the regulation of tristetraprolin expression: evidence for an extracellular signal-regulated kinase-specific, AU-rich element-dependent, autoregulatory pathway. *J Immunol* **172**, 7263-7271

48. Tchen, C. R., Brook, M., Saklatvala, J., and Clark, A. R. (2004) The stability of tristetraprolin mRNA is regulated by mitogen-activated protein kinase p38 and by tristetraprolin itself. *J Biol Chem* **279**, 32393-32400

49. Chen, C. Y., and Shyu, A. B. (1995) AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem Sci* **20**, 465-470

50. Lai, W. S., Carballo, E., Thorn, J. M., Kennington, E. A., and Blackshear, P. J. (2000) Interactions of CCCH zinc finger proteins with mRNA. Binding of tristetraprolin-related zinc finger proteins to Au-rich elements and destabilization of mRNA. *J Biol Chem* **275**, 17827-17837

51. Wilusz, C. J., Wormington, M., and Peltz, S. W. (2001) The cap-to-tail guide to mRNA turnover. *Nature Rev Mol Cell Biol* **2**, 237-246

52. Sandler, H., Kreth, J., Timmers, H. T., and Stoecklin, G. (2011) Not1 mediates recruitment of the deadenylase Caf1 to mRNAs targeted for degradation by tristetraprolin. *Nucleic Acids Res* **39**, 4373-4386

53. Hau, H. H., Walsh, R. J., Ogilvie, R. L., Williams, D. A., Reilly, C. S., and Bohjanen, P. R. (2007) Tristetraprolin recruits functional mRNA decay complexes to ARE sequences. *J Cell Biochem* **100**, 1477-1492

54. Lykke-Andersen, J., and Wagner, E. (2005) Recruitment and activation of mRNA decay enzymes by two ARE-mediated decay activation domains in the proteins TTP and BRF-1. *Genes Dev* **19**, 351-361

55. Chen, C. Y., Gherzi, R., Ong, S. E., Chan, E. L., Raijmakers, R., Pruijn, G. J., Stoecklin, G., Moroni, C., Mann, M., and Karin, M. (2001) AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell* **107**, 451-464
RNase-L attenuates the cellular response to mitogenic stimuli

56. Marderosian, M., Sharma, A., Funk, A. P., Vartanian, R., Masri, J., Jo, O. D., and Gera, J. F. (2006) Tristetraprolin regulates Cyclin D1 and c-Myc mRNA stability in response to rapamycin in an Akt-dependent manner via p38 MAPK signaling. *Oncogene* **25**, 6277-6290

57. Kim, H. K., Kim, C. W., Vo, M. T., Lee, H. H., Lee, J. Y., Yoon, N. A., Lee, C. Y., Moon, C. H., Min, Y. J., Park, J. W., and Cho, W. J. (2012) Expression of proviral integration site for Moloney murine leukemia virus 1 (Pim-1) is post-transcriptionally regulated by tristetraprolin in cancer cells. *J Biol Chem* **287**, 28770-28778

58. Boutaud, O., Dixon, D. A., Oates, J. A., and Sawaoka, H. (2003) Tristetraprolin binds to the COX-2 mRNA 3' untranslated region in cancer cells. *Adv Exp Med Biol* **525**, 157-160

59. Mahat, D. B., Brennan-Laun, S. E., Fialcowitz-White, E. J., Kishor, A., Ross, C. R., Pozharskaya, T., Rawn, J. D., Blackshear, P. J., Hassel, B. A., and Wilson, G. M. (2012) Coordinated Expression of Tristetraprolin Post-Transcriptionally Attenuates Mitogenic Induction of the Oncogenic Ser/Thr Kinase Pim-1. *Plos One* **7**

60. Lee, H. H., Vo, M. T., Kim, H. J., Lee, U. H., Kim, C. W., Kim, H. K., Ko, M. S., Lee, W. H., Cha, S. J., Min, Y. J., Choi, D. H., Suh, H. S., Lee, B. J., Park, J. W., and Cho, W. J. (2010) Stability of the LATS2 tumor suppressor gene is regulated by tristetraprolin. *J Biol Chem* **285**, 17329-17337

61. Masuda, K., Marasa, B., Martindale, J. L., Halushka, M. K., and Gorospe, M. (2009) Tissue- and age-dependent expression of RNA-binding proteins that influence mRNA turnover and translation. *Aging* **1**, 681-698

62. Johnson, B. A., Geha, M., and Blackwell, T. K. (2000) Similar but distinct effects of the tristetraprolin/TIS11 immediate-early proteins on cell survival. *Oncogene* **19**, 1657-1664

63. Rounbehler, R. J., Fallahi, M., Yang, C., Steeves, M. A., Li, W., Doherty, J. R., Schaub, F. X., Sanduja, S., Dixon, D. A., Blackshear, P. J., and Cleveland, J. L. (2012) Tristetraprolin impairs myc-induced lymphoma and abolishes the malignant state. *Cell* **150**, 563-574

64. Ross, C. R., Brennan-Laun, S. E., and Wilson, G. M. (2012) Tristetraprolin: Roles in cancer and senescence. *Ageing Res Rev* **11**, 473-484

65. Brooks, S. A., and Blackshear, P. J. (2013) Tristetraprolin (TTP): Interactions with mRNA and proteins, and current thoughts on mechanisms of action. *Bba-Gene Regul Mech* **1829**, 666-679

66. Shi, Z., and Rockey, D. C. (2010) Interferon-gamma-mediated inhibition of serum response factor-dependent smooth muscle-specific gene expression. *J Biol Chem* **285**, 32415-32424

67. Taylor, G. A., Carballo, E., Lee, D. M., Lai, W. S., Thompson, M. J., Patel, D. D., Schenkman, D. I., Gilkeson, G. S., Broxmeyer, H. E., Haynes, B. F., and Blackshear, P. J. (1996) A pathogenic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. *Immunity* **4**, 445-454

68. Dong, B., and Silverman, R. H. (1999) Alternative function of a protein kinase homology domain in 2', 5'-oligoadenylate dependent RNase L. *Nucleic Acids Res* **27**, 439-445

69. Lai, W. S., Carballo, E., Strum, J. R., Kennington, E. A., Phillips, R. S., and Blackshear, P. J. (1999) Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. *Mol Cell Biol* **19**, 4311-4323

70. Li, X. L., Andersen, J. B., Ezelle, H. J., Wilson, G. M., and Hassel, B. A. (2007) Post-transcriptional regulation of RNase-L expression is mediated by the 3' untranslated region of its mRNA. *J Biol Chem* **282**, 7950-7960

71. Suzuki, A., Tsutomi, Y., Akahane, K., Araki, T., and Miura, M. (1998) Resistance to Fas-mediated apoptosis: activation of caspase 3 is regulated by cell cycle regulator p21WAF1 and IAP gene family ILP. *Oncogene* **17**, 931-939

72. Lal, A., Mazan-Mamczarz, K., Kawai, T., Yang, X., Martindale, J. L., and Gorospe, M. (2004) Concurrent versus individual binding of HuR and AUF1 to common labile target mRNAs. *EMBO J* **23**, 3092-3102
73. Nakajima, K., and Wall, R. (1991) Interleukin-6 signals activating junB and TIS11 gene transcription in a B-cell hybridoma. Mol Cell Biol 11, 1409-1418

74. Suzuki, K., Nakajima, H., Ikeda, K., Maezawa, Y., Suto, A., Takatori, H., Saito, Y., and Iwamoto, I. (2003) IL-4-Stat6 signaling induces tristetraprolin expression and inhibits TNF-alpha production in mast cells. The J Exp Med 198, 1717-1727

75. Sauer, I., Schaljo, B., Vogl, C., Gattermeier, I., Kolbe, T., Muller, M., Blackshear, P. J., and Kovarik, P. (2006) Interferons limit inflammatory responses by induction of tristetraprolin. Blood 107, 4790-4797

76. Anderson, P. (2010) Post-transcriptional regulons coordinate the initiation and resolution of inflammation. Nature Rev Immunol 10, 24-35

77. Kyriakis, J. M., and Avruch, J. (2012) Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. Physiol Rev 92, 689-737

78. Lai, W. S., Thompson, M. J., and Blackshear, P. J. (1998) Characteristics of the intron involvement in the mitogen-induced expression of Zfp-36. J Biol Chem 273, 506-517

79. Schaljo, B., Kratochvill, F., Gratz, N., Sadzak, I., Sauer, I., Hammer, M., Vogl, C., Strobl, B., Muller, M., Blackshear, P. J., Poli, V., Lang, R., Murray, P. J., and Kovarik, P. (2009) Tristetraprolin is required for full anti-inflammatory response of murine macrophages to IL-10. J Immunol 183, 1197-1206

80. Clark, K. A., and Graves, B. J. (2014) Dual views of SRF: a genomic exposure. Genes Dev 28, 926-928

81. Treisman, R. (1994) Ternary complex factors: growth factor regulated transcriptional activators. Curr Opin Genetics Dev 4, 96-101

82. Matys, V., Kel-Margoulis, O. V., Fricke, E., Liebich, I., Land, S., Barre-Dirrie, A., Reuter, I., Chekmenev, D., Krull, M., Hornischer, K., Voss, N., Stegmaier, P., Lewicki-Potapov, B., Saxel, H., Kel, A. E., and Wingender, E. (2006) TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. Nucleic Acids Res 34, D108-110

83. Descot, A., Hoffmann, R., Shaposhnikov, D., Reschke, M., Ullrich, A., and Posern, G. (2009) Negative regulation of the EGFR-MAPK cascade by actin-MAL-mediated Mig6/Errfi-1 induction. Mol Cell 35, 291-304

84. Bakheet, T., Williams, B. R., and Khabar, K. S. (2003) ARED 2.0: an update of AU-rich element mRNA database. Nucleic Acids Res 31, 421-423

85. Cha, H. J., Lee, H. H., Chae, S. W., Cho, W. J., Kim, Y. M., Choi, H. J., Choi, D. H., Jung, S. W., Min, Y. J., Lee, B. J., Park, S. E., and Park, J. W. (2011) Tristetraprolin downregulates the expression of both VEGF and COX-2 in human colon cancer. Hepatogastroenterology 58, 790-795

86. Lee, H. H., Son, Y. J., Lee, W. H., Park, Y. W., Chae, S. W., Cho, W. J., Kim, Y. M., Choi, H. J., Choi, D. H., Jung, S. W., Min, Y. J., Park, S. E., Lee, B. J., Cha, H. J., and Park, J. W. (2010) Tristetraprolin regulates expression of VEGF and tumorogenesis in human colon cancer. Int J Cancer 126, 1817-1827

87. Chen, Y. L., Huang, Y. L., Lin, N. Y., Chen, H. C., Chiu, W. C., and Chang, C. J. (2006) Differential regulation of ARE-mediated TNFalpha and IL-1beta mRNA stability by lipopolysaccharide in RAW264.7 cells. Biochem Biophys Res Commun 346, 160-168

88. Kim, T. W., Yim, S., Choi, B. J., Jang, Y., Lee, J. J., Sohn, B. H., Yoo, H. S., Yeom, Y. I., and Park, K. C. (2010) Tristetraprolin regulates the stability of HIF-1alpha mRNA during prolonged hypoxia. Biochem Biophys Res Commun 391, 963-968
90. Volinsky, N., and Kholodenko, B. N. (2013) Complexity of receptor tyrosine kinase signal processing. *Cold Spring Harb Perspect Biol* 5, a009043
91. Treisman, R. (1992) The serum response element. *Trends Biochem Sci* 17, 423-426
92. Xie, L., Sullivan, A. L., Collier, J. G., and Glass, C. K. (2013) Serum response factor indirectly regulates type I interferon-signaling in macrophages. *J Interf Cytok Res* 33, 588-596
93. Jha, B. K., Polyakova, I., Kessler, P., Dong, B., Dickerman, B., Sen, G. C., and Silverman, R. H. (2011) Inhibition of RNase L and RNA-dependent protein kinase (PKR) by sunitinib impairs antiviral innate immunity. *J Biol Chem* 286, 26319-26326
94. Zhang, S. X., Garcia-Gras, E., Wycuff, D. R., Marriot, S. J., Kadeer, N., Yu, W., Olson, E. N., Garry, D. J., Parmacek, M. S., and Schwartz, R. J. (2005) Identification of direct serum-response factor gene targets during Me2SO-induced P19 cardiac cell differentiation. *J Biol Chem* 280, 19115-19126
95. Selvaraj, A., and Prywes, R. (2004) Expression profiling of serum inducible genes identifies a subset of SRF target genes that are MKL dependent. *BMC Mol Biol* 5, 13
96. Stritt, C., Stern, S., Harting, K., Manke, T., Sinske, D., Schwarz, H., Vinrion, M., Nordheim, A., and Knoll, B. (2009) Paracrine control of oligodendrocyte differentiation by SRF-directed neuronal gene expression. *Nature Neurosci* 12, 418-427
97. Sun, Q., Chen, G., Streb, J. W., Long, X., Yang, Y., Stoeckert, C. J., Jr., and Miano, J. M. (2006) Defining the mammalian CArGome. *Genome Res* 16, 197-207
98. Halees, A. S., El-Badrawi, R., and Khabar, K. S. (2008) ARED Organism: expansion of ARED reveals AU-rich element cluster variations between human and mouse. *Nucleic Acids Res* 36, D137-140
99. Johnson, B. A., Stehn, J. R., Yaffe, M. B., and Blackwell, T. K. (2002) Cytoplasmic localization of tristetraprolin involves 14-3-3-dependent and -independent mechanisms. *J Biol Chem* 277, 18029-18036
100. Kedar, V. P., Zucconi, B. E., Wilson, G. M., and Blackshear, P. J. (2012) Direct binding of specific AUF1 isoforms to tandem zinc finger domains of tristetraprolin (TTP) family proteins. *J Biol Chem* 287, 5459-5471
101. Sawaoka, H., Dixon, D. A., Oates, J. A., and Boutaud, O. (2003) Tristetraprolin binds to the 3'-untranslated region of cyclooxygenase-2 mRNA. A polyadenylation variant in a cancer cell line lacks the binding site. *J Biol Chem* 278, 13928-13935
102. Suswam, E. A., Shacka, J. J., Walker, K., Lu, L., Li, X., Si, Y., Zhang, X., Zheng, L., Nabors, L. B., Cao, H., and King, P. H. (2013) Mutant tristetraprolin: a potent inhibitor of malignant glioma cell growth. *J Neurooncol* 113, 195-205
103. Hanahan, D., and Weinberg, R. A. (2011) Hallmarks of cancer: the next generation. *Cell* 144, 646-674
104. Spencer, J. A., and Misra, R. P. (1999) Expression of the SRF gene occurs through a Ras/Sp/SRF-mediated-mechanism in response to serum growth signals. *Oncogene* 18, 7319-7327
105. Hausburg, M. A. (2010) Tristetraprolin regulation of MyoD mRNA stability commits quiescent adult muscle stem cells to myogenesis. Ph.D. thesis, University of Colorado at Boulder
106. Bisbal, C., Silhol, M., Laubenthal, K., Kaluza, T., Carnac, G., Milligan, L., Le Roy, F., and Salehzada, T. (2000) The 2'-5' oligoadenylate/RNase L/RNase L inhibitor pathway regulates both MyoD mRNA stability and muscle cell differentiation. *Mol Cell Biol* 20, 4959-4969
107. Guerri, A., Lahoute, C., Hebbrard, S., Collard, L., Graindorge, D., Favier, M., Cagnard, N., Batonnnet-Pichon, S., Precigout, G., Garcia, L., Tuil, D., Daegelen, D., and Sotiropoulos, A. (2012) Srf-dependent paracrine signals produced by myofibers control satellite cell-mediated skeletal muscle hypertrophy. *Cell Metab* 15, 25-37
108. Yi, X., Zeng, C., Liu, H., Chen, X., Zhang, P., Yun, B. S., Jin, G., and Zhou, A. (2013) Lack of RNase L attenuates macrophage functions. *Plos One* 8, e81269
RNase-L attenuates the cellular response to mitogenic stimuli

109. Bros, M., Wiechmann, N., Besche, V., Art, J., Pautz, A., Grabbe, S., Kleinert, H., and Reske-Kunz, A. B. (2010) The RNA binding protein tristetraprolin influences the activation state of murine dendritic cells. Mol Immunol 47, 1161-1170

110. Esnault, C., Stewart, A., Gualdrini, F., East, P., Horswell, S., Matthews, N., and Treisman, R. (2014) Rho-actin signaling to the MRTF coactivators dominates the immediate transcriptional response to serum in fibroblasts. Genes Dev 28, 943-958

111. Benson, C. C., Zhou, Q., Long, X., and Miano, J. M. (2011) Identifying functional single nucleotide polymorphisms in the human Cardgome. Physiol Genomics 43, 1038-1048

112. Treisman, R. (1995) Journey to the surface of the cell: Fos regulation and the SRE. EMBO 14, 4905-4913

113. Raghavan, A., Robison, R. L., McNabb, J., Miller, C. R., Williams, D. A., and Bohjanen, P. R. (2001) HuA and tristetraprolin are induced following T cell activation and display distinct but overlapping RNA binding specificities. J Biol Chem 276, 47958-47965

114. Kimura, Y., Morita, T., Hayashi, K., Miki, T., and Sobue, K. (2010) Myocardin functions as an effective inducer of growth arrest and differentiation in human uterine leiomyosarcoma cells. Cancer Res 70, 501-511

115. Stoceklin, G., Tenenbaum, S. A., Mayo, T., Chittur, S. V., George, A. D., Baroni, T. E., Blackshear, P. J., and Anderson, P. (2008) Genome-wide analysis identifies interleukin-10 mRNA as target of tristetraprolin. J Biol Chem 283, 11689-11699

116. Kratochvill, F., Machacek, C., Vogl, C., Ebner, F., Sedlyarov, V., Gruber, A. R., Hartweger, H., Vielmascher, R., Karaghiosoff, M., Rulicke, T., Muller, M., Hofacker, I., Lang, R., and Kovarik, P. (2011) Tristetraprolin-driven regulatory circuit controls quality and timing of mRNA decay in inflammation. Mol Syst Biol 7, 560

117. Brewer, B. Y., Malicka, J., Blackshear, P. J., and Wilson, G. M. (2004) RNA sequence elements required for high affinity binding by the zinc finger domain of tristetraprolin: conformational changes coupled to the bipartite nature of Au-rich mRNA destabilizing motifs. J Biol Chem 279, 27870-27877

FOOTNOTES

* This work was funded in part by NIH Grant AI077556 (B.A.H), a VA Merit Award (B.A.H.), and the NIH NCI T32 Cancer Biology Training Grant T32CA154274 (S.E.B-L.)

1 To whom correspondence may be addressed: Dr. Bret A. Hassel, Department of Microbiology and Immunology, University of Maryland School of Medicine, 685 W. Baltimore St., HSF-I 380, Baltimore, MD 21201 USA, Tel.: 410-328-2344; Fax: 410-706-6609, E-mail: bhassel@som.umaryland.edu

2 The abbreviations used are: IFNβ, Interferon-β; OAS, 2′,5′-oligoadenylate synthetase; TTP, Tristetraprolin; zfp36, zinc-finger protein 36; RNABP, RNA-binding protein; KO, knockout; HPC1, hereditary prostate cancer susceptibility locus; ssRNA, single-stranded RNA; dsRNA, double-stranded RNA; mRNA, messenger RNAs; mRNP, mRNA protein; ARE, A-U-rich elements; 3′UTR, 3′-untranslated regions, Not1, negative regulator of transcription; Caf1, chromatin assembly factor; Xrn1, 5′-3′ Exoribonuclease 1; Ccr4, chemokine c-c motif receptor 4; Dcp, decapping protein; c-Myc, v-myc avian myelocytomaosis viral oncogene homolog; HIF-α, hypoxia inducible factor 1, alpha; Cox-2, cyclooxygenase 2; Pim-1, provaliation integration site 1; SRF, serum response factor; qRT-PCR, quantitative real-time reverse-transcription-PCR; actD, Actinomycin D; RNP-IP, Ribonucleoprotein-Immunoprecipitations; MEF, mouse embryonic fibroblasts; WT, wild type; TPA, 12-O-Tetradecanoylphorbol-13-acetate; IP, immunoprecipitation; SRE, serum response element; TCF, ternary complex factors; VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor alpha; p21CIP,
RNase-L attenuates the cellular response to mitogenic stimuli

cyclin-dependent kinase inhibitor 1; ISG, IFN-stimulated gene; MyoD, myogenic differentiation 1; IL-6, interleukin 6; p21CIP, cyclin-dependent kinase inhibitor 1; Tera, ktera protein homolog; Junb, jun b proto-oncogene; Cyr6, cysteine-rich angiogenic inducer 61; Slc2a1, solute carrier family 2; Ereg, epiregulin; klf6, kruppel-like factor 6

FIGURE LEGENDS

FIGURE 1: RNase-L regulates TTP mRNA in basal and mitogen-stimulated conditions. A. Steady state TTP mRNA in WT and RNase-L KO MEFs was measured after mitogenic stimulation for the indicated times by qRT-PCR. Bars represent the mean ± SD of five independent experiments normalized to HPRT mRNA. Induced values are relative to expression in unstimulated conditions which was set to 1 for each cell type. * = p < 0.05, ** = p < 0.01. B. Steady-state RNase-L and TTP mRNA levels in HeLa cells stably transfected with control vector (SNAP) or epitope-tagged RNase-L expression vector (SNAP-RNase-L). Bars represent the mean ± SD of three independent experiments normalized to HPRT mRNA. C. TTP mRNA decay kinetics were measured by actD time courses in WT and RNase-L KO MEFs in resting (unstimulated) conditions. TTP mRNA half-lives were calculated from four independent experiments and representative data points from one experiment are shown (mean ± SD of three independent samples). D. The presence of TTP mRNA in a complex with RNase-L was analyzed by RNP-IP. RNase-L was IP from HeLa cells stably transfected with control vector (SNAP) or epitope-tagged RNase-L expression vector (SNAP-RNase-L) in resting (rest) and one hour mitogen induced (ind) conditions and RNA was isolated. IP with IgG was included as a control to detect nonspecific interaction with the antibody. TTP and control HPRT mRNAs in the IP complex were measured by qRT-PCR and normalized to PGK1 mRNA. Bars represent mean ± SD of three independent experiments. E. TTP mRNA decay kinetics were measured by act D time courses in WT and RNase-L KO MEFs at one hour post-mitogen stimulation. TTP mRNA half-lives were calculated from at least 6 independent experiments and representative data points from one experiment are shown (mean ± SD of three independent samples) (p < 0.01).

FIGURE 2: RNase-L directly regulates SRF mRNA to downregulate TTP transcription following mitogen stimulation. A. TTP primary transcripts were measured at the indicated times post-mitogen stimulation of WT and RNase-L KO MEFs by qRT-PCR using intronic primers to detect unspliced transcripts. Bars represent the mean ± SD of four independent experiments normalized to HPRT mRNA. B. SRF mRNA was measured at the indicated times post-mitogen stimulation of WT and RNase-L KO MEFs by qRT-PCR. Bars represent the mean ± SD of four independent experiments normalized to HPRT mRNA. C. SRF mRNA decay kinetics were measured by actD time courses in WT and RNase-L KO MEFs at one hour post-mitogen stimulation. SRF mRNA half-lives and SD shown were calculated from the indicated number independent experiments; representative data from one experiment are shown (mean ± SD of three independent samples) (p < 0.001). D. The presence of SRF mRNA in a complex with RNase-L was analyzed by RNP-IP as described in Figure 1C. Bars represent mean ± SD of three independent experiments, * = p < 0.05.

FIGURE 3: RNase-L and TTP co-immunoprecipitate in a complex from transfected cells. A. Myc-RNase-L deletion constructs (top panel) and full length FLAG-TTP were transfected into 293T cells. Myc-RNase-L proteins present in a FLAG-TTP IP complex were isolated at 24 hours post-transfection and measured by WB (lower panel; expression in 1/10 of input is shown below the IP). B. GFP-TTP deletion constructs (top panel) and full length FLAG-RNase-L were transfected into 293T cells. GFP-TTP proteins present in a FLAG-RNase-L IP complex were isolated at 24 hours post-transfection and measured by WB (lower panel; expression in 1/10 of input is shown below the IP). C. FLAG-TTP (left panel) or myc-RNase-L (right panel) was transfected into 293T cells and an antibody to the transfected protein was used for IP. The presence of endogenous RNase-L or TTP in the IP complex was measured by WB. KEN, Kinase Extension Nuclease domain; FL, Full Length; Nd, NH2-terminal amino acids
RNase-L attenuates the cellular response to mitogenic stimuli

depleted; Cd, COOH-Terminal amino acids deleted; NTD, NH2-Terminal Domain; RBD, RNA Binding Domain; CTD, COOH-Terminal Domain.

FIGURE 4: SRF mRNA is a novel target of TTP regulation. A. The presence of SRF mRNA in a complex with TTP was analyzed by RNP-IP in resting cells (rest) and at one hour post- mitogen stimulation (ind) as described in Figure 1C. IP with IgG is included as a control to detect nonspecific interaction with the antibody. Bars represent mean ± SD of three independent experiments. B. SRF mRNA decay kinetics were measured by actD time courses in WT and TTP KO MEFs at one hour post-mitogen stimulation. SRF mRNA half-lives and SD shown were calculated from the indicated numbers of independent experiments and representative data from one experiment are shown (mean ± SD of three independent samples) (p < 0.01).

FIGURE 5: RNase-L regulates a subset of TTP targets and reduces the proliferative response in mitogen-stimulated cells. Steady state mRNA expression of Cox-2, TNF-α, VEGF, and p21CIP mRNAs (A.) or HIF-1α, PIM-1, and IL-1β mRNAs (B.) were measured by qRT-PCR in WT and RNase-L KO MEFs at the indicated times post-mitogen stimulation. Bars represent the mean ± SD of three independent experiments normalized to HPRT mRNA. C. Proliferation of WT and RNase-L KO MEFs was determined by measuring the viable cells (MTT assay) at the indicated times after mitogenic stimulation. Each point represents the mean ± SD of three independent experiments.

FIGURE 6: Model depicting the roles of RNase-L and TTP in regulating mitogen-induced gene expression to attenuate a proliferative response. In WT cells (left panel), mitogen stimulation (1) induces the transcription of SRF-regulated genes that function in feedback regulation (2a) and to induce proliferation (2b). RNase-L and TTP function to downregulate SRF-induced transcription (3a) and destabilize proliferation-stimulatory transcripts (3b) to attenuate the proliferative response (4). In the absence of RNase-L (right panel), mRNAs encoding a subset of proliferation-stimulatory gene products, including SRF, are stabilized resulting in dysregulated proliferation. TTP targets regulated independent of RNase-L (e.g. HIF-1α, PIM-1) are unaffected in RNase-L-deficient cells. TTP autoregulation occurs by alternative mechanisms in the absence of RNase-L. Mitogen-induced phosphorylation and activation of SRF protein is not shown.
RNase-L attenuates the cellular response to mitogenic stimuli

Table 1: qRT-PCR primer sets in mouse and human.¹

| qRT-PCR primer sets           | Forward Primer         | Reverse Primer         |
|------------------------------|------------------------|------------------------|
| TTP mouse mRNA               | CAAGGCCATTCGCGCCAACCAT | CCTCCGTTGGTCGGATGACAGG |
| TTP mouse primary transcript | GACTGGCAAGCTGTGAAGT    | CAGTCAGGCGAGAGGTGA     |
| SRF mouse mRNA               | ATCGACAACAGCTGCGCGCG   | AATCGACGCGCTTGCGCGGCT  |
| TTP human mRNA               | CACCCCTCATGCAACCCTT    | GATGGCAGGCGACGTCAAGG   |
| SRF human mRNA               | ATCGACAACAGCTGCGCGCG   | AATCGACGCGCTTGCGCGGCT  |
| Cox-2 mouse mRNA             | CATCCCCCTCTGCGGAAGTT   | CATGGGAGTGTCGAGTCAT    |
| VEGF mouse mRNA              | AACTGGGAGAAGCCCTGAGTG  | GCGCTTCTCGTTTTTGACCT   |
| TNF-α mouse mRNA             | GACACCTCACACTGATCATTCT | CCACCTTTGGTGTGTGTACGA  |
| p21CIP mouse mRNA            | GCAGATCCACAGCGATATCCA  | CATGAGGCGCATCGCAATCAC  |
| IL-1β mouse mRNA             | GAGGCGCGATGATCTCTCA    | AGGCCACAGATTTTTGTCG    |
| HIF1-α mouse mRNA            | GGCGGCGAGACAGAGAGAAA   | GCTCACATTGTCGAGTGGCCA  |
| Pim-1 mouse mRNA             | AGGTGGGAGTGCTCCTTCCA   | TGCAAGTAGAAGCCAGCGA    |
| Pgk1 mouse mRNA              | CGGCTGGAGCTGTCCATCTGC  | TGCAAGTAGAAGCCAGCGA    |
| Pgk1 human mRNA              | GACGTGGGAGCTGGAGTGGTG  | GGACAGGAGCTTTAATCTCTGT |
| HpRt human mRNA              | AGACTTTGCTTTCCCTGTCCAG | CTTGGTGGGTCCTTTACCGA  |
| MyoD mouse mRNA              | CCATCCGCTACATCGAAGGT   | CACTGATGAGGCGGTGTCG    |

¹Sequences of forward and reverse primers used to test the specified mRNA levels in mouse and human samples.
Table 2: Shared targets of SRF, TTP, and RNase-L

| SRF target | TTP target | RNase-L target |
|------------|------------|---------------|
| *SRF* (104) | V*         | V*            |
| *TTP* (83,84) | V (47,48)  | V* (23)       |
| *MyoD* (85)  | V (105)    | V (12,106)    |
| *Cox-2* (107)| V (101)    | P (108)       |
| *IL-6* (107) | V (109)    | P (108)       |
| *Cyr61* (95) | P**        | P (12)        |
| *Slc2a1* (110)| P**        | P (43)        |
| *Ereg* (95)  | P**        | P (43)        |
| *Klf6* (111) | P**        | P (12)        |
| *cFos* (112) | V (113)    | X             |
| *p21CIP* (114)| V (23)     | X             |
| *Tera* (94)  | V (115)    | X             |
| *Junb* (95)  | V (116)    | X             |

(V) = Validated target  (P) = Predicted target  X = not a validated or predicted target

* Validated within this study  ** Predicted TTP target based on minimum consensus TTP target sequences of UAUUUAU  (117)
Figure 1

RNase-L attenuates the cellular response to mitogenic stimuli
Figure 2

RNase-L attenuates the cellular response to mitogenic stimuli

A. Relative TTP primary transcript levels

B. Relative SRF mRNA levels

C. % SRF mRNA remaining

D. Fold mRNA enrichment

KO RNase-L t1/2 = 2.5 ± 0.71 n = 7
WT RNase-L t1/2 = 1.3 ± 0.20 n = 6
Figure 3: RNase-L and TTP co-immunoprecipitate in a complex from transfected cells. 

A, Myc-RNase-L deletion constructs (top panel) and full length FLAG-TTP were transfected into 293T cells. myc-RNase-L proteins present in a FLAG-TTP IP complex were isolated at 24h post-transfection and measured by WB (lower panel; expression in 1/10 of input is also shown). 

B, GFP-TTP deletion constructs (top panel) and FLAG-RNase-L were transfected into 293T cells and GFP-TTP proteins that co-IP with FLAG-RNase-L were isolated at 24h post-transfection and measured by WB (lower panel; expression in 1/10 of input is also shown). 

C, FLAG-TTP (left panel) or myc-RNase-L (right panel) was transfected into 293T cells and an antibody to the transfected protein was used for IP. The presence of endogenous RNase-L or TTP in the IP complex was measured by WB. KEN, Kinase Extension Nuclease domain; FL, Full Length; Nd, NH$_2$-terminal 23 amino acids deleted etc.; Cd, COOH-Terminal 21 amino acids deleted etc.; NTD, NH$_2$-Terminal Domain; RBD, RNA Binding Domain; CTD, COOH-Terminal Domain.
Figure 4

RNase-L attenuates the cellular response to mitogenic stimuli.

A.

B.

Figure 4

RNase-L attenuates the cellular response to mitogenic stimuli.
Figure 5

RNase-L attenuates the cellular response to mitogenic stimuli
Figure 6

**WT**

**RNase-L KO**

**Attenuation of proliferative response**

**Uncontrolled proliferative response**

RNase-L-independent targets

TTP

SRF

m7G-polyA

Feedback regulation of TTP

mitogen-induced transcription attenuated

mitogen-induced transcription sustained

mitogen-induced transcripts degraded

mitogen-induced transcripts not degraded

proliferation stimulatory genes

Feedback regulation of TTP

SRF mRNA degraded

SRF mRNA stabilized

m7G-polyA

TTP mRNA

m7G-polyA

ZFP-36

SRF

Uncontrolled proliferative response

RNase-L attenuates the cellular response to mitogenic stimuli

by guest on March 24, 2020http://www.jbc.org/Downloaded from
RNase-L Attenuates Mitogen-stimulated Gene Expression via Transcriptional and Post-transcriptional Mechanisms to Limit the Proliferative Response
Sarah E. Brennan-Laun, Xiao-Ling Li, Heather J. Ezelle, Thiagarajan Venkataraman, Perry J. Blackshear, Gerald M. Wilson and Bret A. Hassel

J. Biol. Chem. published online October 9, 2014

Access the most updated version of this article at doi: 10.1074/jbc.M114.589556

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts