A biosensor for MAPK-dependent Lin28 signaling

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INTRODUCTION

Lin28a is a pluripotency-associated RNA-binding protein that exerts posttranscriptional effects to govern many developmental and cellular signaling pathways (Jiang and Baltimore, 2016). Lin28a promotes progrowth gene expression in part by repressing maturation of the Let-7 family of microRNAs (miRNAs), and undergoes reciprocal translational repression by Let-7 miRNAs (Rybak et al., 2008). Lin28a also controls gene expression through regulation of mRNA translation (Cho et al., 2012). Appropriate control of Lin28a protein levels is necessary for correct organismal development. Mice deficient in Lin28a suffer perinatal lethality (Shinoda et al., 2013). Conversely, Lin28a overexpression can lead to abnormal growth and metabolic phenotypes (Zhu et al., 2010, 2011; Shyh-Chang et al., 2013), and Lin28a is up-regulated in numerous human cancers (Viswanathan et al., 2009; Shyh-Chang and Daley, 2013). Despite a growing appreciation for the necessity of tight control of Lin28a in producing appropriate gene expression programs, the signaling that regulates dynamic changes in Lin28a protein levels remains incompletely understood.

Recently, we reported rapid signal-dependent induction of Lin28a through posttranslational regulation of Lin28a protein stability. MAPK pathway activation was shown to enhance phosphorylation and binding of the Dicer cofactor, TRBP (TAR RNA-binding protein), as well as Dicer itself, to Lin28a in a stabilizing interaction.
with binding and increase of Lin28a by the TRBP-B segment of TRBP, but not by the N-terminal or C-terminal segments TRBP-A and C.

TRBP phosphorylation at S152 potentiates Lin28a elevation

TRBP phosphorylation has been shown to increase its stability (Chen et al., 2015) and enhance binding to Dicer and other binding partners (Paroo et al., 2009; Kim et al., 2014). In addition, we previously showed that a phosphomimic TRBP mutant, with four serines mutated to aspartates, exhibited enhanced binding to Lin28a in purified protein interactions (Amen et al., 2017). These findings implicated MAPK pathway-mediated phosphorylation of TRBP in Lin28a binding, without indicating which of the four serine residues was involved. We identified an "FXF" putative Erk docking motif (Gonzalez et al., 1991) within TRBP-B, beginning at aa 112. Two of the serines mutated in the full-length phosphomimetic construct, S142 and S152, are present in TRBP-B; both serines are candidates for proline-directed kinase phosphorylation, but S152 is a closer match to an Erk phosphorylation consensus sequence (Figure 2A, PXSP; Jacobs et al., 1999).

Immunoblot of lysates from 293T cells expressing myc-TRBP-B revealed a multibanding pattern between 20 and 25 kDa when probed with an antibody raised and purified against phospho-S152 TRBP (Supplemental Figure S2A). Consistently, exposure of myc-TRBP-B lysates to lambda phosphatase treatment collapsed the myc-reactive bands to a singlet at the expected molecular weight of TRBP-B (Figure 2B). Phorbol 12-myristate 13-acetate (PMA) can increase TRBP phosphorylation through the MAPK signaling cascade (Paroo et al., 2009). Treating 293T cells expressing myc-TRBP-B with PMA led to an increase in phospho-S152 TRBP immunoreactivity, which was reduced in lysates from cells treated with MAPK/Erk kinase (MEK) inhibitor U0126 (Figure 2C and additional representative blot Supplemental Figure S2B). We conclude that TRBP-B can be phosphorylated within living cells in a MEK-dependent manner. To evaluate the role of this TRBP phosphorylation site in producing Lin28a elevation, we immunoblotted lysates from 293T cells which were coexpressing FL-Lin28a and myc-TRBP-B, and treated with either vehicle control, PMA, or PMA plus U0126. Immunoblot showed that FL-Lin28a protein levels were increased by myc-TRBP-B expression, and that PMA treatment further enhanced Lin28a elevation in a manner blocked by U0126 (Figure 2, C and D).

We next conducted experiments in TRBP KO TEFs to specifically evaluate the role of the putative Erk phosphorylation site TRBP S152 in conferring stimulus-mediated Lin28a elevation by TRBP-B. A phosphomutant myc-TRBP-B with S152 mutated to alanine was generated and we observed that reactivity of the TRBP-B band with anti-phospho-TRBP antibody was abolished in cells expressing TRBP-B S152A (Figure 2E and Supplemental Figure S2C). In TRBP KO TEFs coexpressing FL-Lin28a and either myc-TRBP-B or phosphomutant myc-TRBP-B S152A (at similar levels; Supplemental Figure S2D), TRBP-B or TRBP-B S152A expression could each produce some basal Lin28a elevation, but cells expressing TRBP-B S152A were unable to respond to PMA (Figure 2F). PMA produced a modest Lin28a induction in the absence of TRBP-B expression, an effect which might be due to direct Lin28a phosphorylation (Liu et al., 2017; Tsanov et al., 2017) or an alternative unknown interacting protein. In addition, we observed that purified GST-TRBP-B protein could specifically coassociate with purified Lin28a, compared with GST alone, and that this association was decreased by the phosphomutation TRBP-B S152A (Supplemental Figure S2E). Decreased binding of Lin28a by TRBP-B S5A, in comparison to wild-type TRBP-B, may be due to reduced phosphorylation in bacteria (Macek and Mijakovic, 2011; Pereira et al., 2011; Cousin et al., 2013) or to
Lin28a-3 interacts with full-length TRBP and TRBP-B

To address which region of Lin28a protein interacted with TRBP, we generated three domain-based truncations of Lin28a (Figure 3A). Collectively, these experiments indicate that the TRBP-B region is sufficient to bind and elevate Lin28a protein levels, and support a role for TRBP S152 in transducing MAPK pathway activation through TRBP to regulate Lin28a.

In the absence of the hydroxyl group on residue 152. This result is consistent with previous work showing that a purified recombinant full-length phosphomimic TRBP protein interacts more strongly in vitro with Lin28a in comparison to recombinant wild-type TRBP (Amen et al., 2017). Collectively, these experiments indicate that the TRBP-B region is sufficient to bind and elevate Lin28a protein levels, and support a role for TRBP S152 in transducing MAPK pathway activation through TRBP to regulate Lin28a.
treatment with PMA (24 h) in 293T cells coexpressing TRBP-B. FL-Lin28a-1 levels were not significantly changed either by coexpression of TRBP-B alone or by TRBP-B plus PMA stimulation, while FL-Lin28a-3 levels were robustly increased by TRBP-B coexpression and further enhanced by PMA treatment (Figure 3, E and F).

In addition to TRBP phosphorylation, Lin28a has also been reported to undergo MAPK-dependent phosphorylation at serine 200, which can regulate its stability (Liu et al., 2017; Tsanov et al., 2017). To evaluate the participation of Lin28a-3 phosphorylation, compared with TRBP-B phosphorylation (Figure 2, E and F), in Lin28a-3 protein up-regulation, we assessed the fold stabilization of wild-type and phosphomutant FL-Lin28a-3 (Lin28a-3 S200A) by immunoblot of functional domains of Lin28a revealed by its crystal structure (Nam et al., 2011; Mayr et al., 2012). Lin28a-1, aa 1–112, included the structurally uncharacterized amino terminus and the cold-shock domain (CSD). Lin28a-2, aa 75–154, encompassed half of the CSD, extending to the end of the CCHC Zn knuckles. Lin28a-3 began at the amino terminus of the CCHC, extending to the structurally uncharacterized carboxy terminus of the protein (Figure 3A).

Full-length FL-Lin28a and FL-Lin28a-3 levels were each elevated above baseline by myc TRBP coexpression in 293T cells (Figure 3, B and C). In some experimental replicates, we observed mild increases in FL-Lin28a-1 by TRBP. We tested whether, like full-length FL-Lin28a, either FL-Lin28a-1 or FL-Lin28a-3 could be elevated by treatment with PMA (24 h) in 293T cells coexpressing TRBP-B. FL-Lin28a-1 levels were not significantly changed either by coexpression of TRBP-B alone or by TRBP-B plus PMA stimulation, while FL-Lin28a-3 levels were robustly increased by TRBP-B coexpression and further enhanced by PMA treatment (Figure 3, E and F).

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**FIGURE 2:** TRBP-B phosphorylation at S152 enhances binding to Lin28a. (A) Putative ERK substrate sequence of TRBP at serine 152, including FSP docking site. Weaker candidate ERK substrate at serine 142. (B) Immunoblot from 293T cells expressing TRBP-B shows two or more phosphorylated species exist and can be collapsed by lysate treatment with lambda phosphatase. (C, D) Representative blot and quantitation showing levels of FL-Lin28a, phospho-TRBP, and myc-TRBP-B proteins from 293T cells pretreated with U0126 (20 uM), with or without PMA treatment (50 ng/ml; n = 15). (E, F) Representative blot and quantitation showing effect of PMA on FL-Lin28a level in Tarbp2−/− MEF cells coexpressing TRBP-WT or S152A and treated with PMA (n = 8). (D, F) *, p < 0.05, ANOVA; #, p < 0.05, paired t test.
Lin28a-3 interacts with TRBP-B in a phosphorylation-inducible manner

The identification of Lin28a-3 and TRBP-B as interacting truncations which reproduce the induction observed with full-length TRBP and Lin28a proteins, presented an opportunity to create a physiological sensor for this pathway. We assayed the efficiency of fluorescence resonance energy transfer (FRET) between TRBP constructs tagged with the CFP variant, Cerulean, and Lin28a constructs tagged with a 293T cell lysates in the presence or absence of TRBP-B coexpression or epidermal growth factor (EGF) treatment (Figure 3, G and H). Levels of wild-type FL-Lin28a-3 protein were significantly elevated by coexpression with TRBP-B, and this effect was further enhanced by 293T stimulation with EGF (one-way analysis of variance [ANOVA] compared with wild-type Lin28a-3 alone). In contrast, levels of FL-Lin28a-3 S200A protein could be increased by TRBP-B coexpression but were not further affected by EGF treatment (one-way ANOVA; pairwise t tests revealed that there was a significant effect of EGF treatment with wild-type Lin28a-3 (Figure 3H). These results indicate that phosphorylation of both TRBP S152 and of Lin28a S200 participate in the induction of Lin28a protein levels by TRBP expression and stimuli producing MAPK pathway activation.

293T cell lysates in the presence or absence of TRBP-B coexpression or epidermal growth factor (EGF) treatment (Figure 3, G and H). Levels of wild-type FL-Lin28a-3 protein were significantly elevated by coexpression with TRBP-B, and this effect was further enhanced by 293T stimulation with EGF (one-way analysis of variance [ANOVA] compared with wild-type Lin28a-3 alone). In contrast, levels of FL-Lin28a-3 S200A protein could be increased by TRBP-B coexpression but were not further affected by EGF treatment (one-way ANOVA; pairwise t tests revealed that there was a significant effect of EGF treatment with wild-type Lin28a-3 (Figure 3H). These results indicate that phosphorylation of both TRBP S152 and of Lin28a S200 participate in the induction of Lin28a protein levels by TRBP expression and stimuli producing MAPK pathway activation.

Lin28a-3 interacts with TRBP-B in a phosphorylation-inducible manner

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FIGURE 3: A C-terminal Lin28a truncation sufficient to respond to TRBP and TRBP-B. (A) Domain map of Lin28a, with truncations Lin28a-1, 2, and 3. (B, C) Coexpression of FL-Lin28a and truncations with full-length TRBP in 293T cells increases Lin28a and Lin28a-3. (*, p < 0.05, ANOVA, n = 4). (D–F) Effect of coexpression of TRBP-B and treatment with PMA on protein level of FL-Lin28a-1 (D) and FL-Lin28a-3 (E) (*, p < 0.01, two-way ANOVA, n = 3–5). (G, H) Effect of coexpression of TRBP-B and treatment with EGF on protein level of WT or S200A FL-Lin28a-3 (*, p < 0.01, ANOVA; *, p < 0.05, paired t test; n = 9).
high FRET efficiency in our imaging system, we used a FRET sensor for calmodulin (Supplemental Figure S3A; Romoser et al., 1997). We confirmed YFP bleaching to 75% or greater reduction of signal in each cell (Supplemental Figure S3, B–D).

We tested the effects of coexpressing CFP-tagged full-length TRBP and TRBP truncations, TRBP-A and TRBP-B, with a panel of YFP-tagged Lin28a constructs in 293T cells. When coexpressed with full-length TRBP, both Lin28a-1 and Lin28a-3 showed positive FRET efficiency, indicative of significant interaction (Figure 4A). In congruence with our previous findings, FRET efficiency of Lin28a-3-YFP with CFP-TRBP was highest, followed by Lin28a-1-YFP, with no FRET between Lin28a-2-YFP and CFP-TRBP (Figure 4A). Consistent with its failure to elevate Lin28a protein levels, TRBP-A displayed no FRET-detectable interaction with any Lin28a-YFP truncation (Figure 4B). In contrast, TRBP-B showed significant CFP recovery when coexpressed with Lin28a-1-YFP, Lin28a-3-YFP, or full-length Lin28a-YFP (Figure 4C). CFP-TRBP-AB showed FRET intermediate between full-length and TRBP-B (Supplemental Figure S3, E and F), and was not pursued further as the more effective and shorter TRBP-B truncation was also less likely to mimic or preclude endogenous TRBP function. We noted that full-length Lin28a, known to be capable of direct binding to TRBP (Amen et al., 2017; Supplemental Figure S2E), did not show significant CFP recovery after photobleaching (Figure 4A). This result is consistent with a risk of false-negative results from FRET analyses based on fluorophore orientation (Miyawaki, 2011). On the basis of these results, we concluded that TRBP-B can interact with Lin28a-3, and also to a lesser degree with Lin28a-1. Further, observed FRET efficiency of the Lin28a-3-YFP and CFP-TRBP-B was higher than between full-length Lin28a and TRBP, indicating that the truncated constructs produce a preferred conformation for a FRET biosensor.

To determine whether TRBP binding by Lin28a-1 or Lin28a-3 could be increased by phosphorylation, we treated 293T cells expressing CFP-TRBP-B and either Lin28a-1-YFP or Lin28a-3-YFP with either EGF (a MAPK pathway activator) or vehicle and measured donor dequenching 60 min after treatment (Figure 4D). FRET between CFP-TRBP-B and Lin28a-3-YFP increased significantly after EGF treatment (Figure 4D), consistent with the stimulus-dependent increase in protein levels observed in Figure 3, E and F. In contrast, EGF treatment did not alter FRET efficiency of Lin28a-1-YFP and CFP-TRBP-B. Notably, there was no detectable FRET between Lin28a-3-YFP and circular permutation of the Venus YFP, cpVenusE172. FRET was measured in living 293T cells by bleaching the FRET acceptor, YFP, and measuring dequenching of the donor, CFP. As a benchmark for
the S152A phosphomutant CFP-TRBP-B S152A, indicating a critical role for this serine residue in interaction of the Lin28a/TRBP FRET sensor (Figure 4E). In cells treated with EGF for variable times before acceptor bleaching, CFP-TRBP-B and Lin28a-3-YFP demonstrated a time-dependent increase in FRET with a maximal plateau by 60–90 min (Figure 4E). This corresponded with a similar time-dependent increase in the ratio of phosphorylated TRBP-B to total myc-TRBP-B observed by immunoblot after EGF treatment (Figure 4, F and G).

The importance of phosphorylation for TRBP-B and Lin28a-3 interactions was further evaluated by testing the effects of phosphomutants, CFP-TRBP-B S152A and Lin28a-3 S200A-YFP, on FRET efficiency. We conducted a multicomponent FRET comparison assessing basal FRET with the following pairs: TRBP-BWT/Lin28a-3WT, TRBP-BS152A/Lin28a-3WT, TRBP-BWT/Lin28a-3S200A, and TRBP-BS152A/Lin28a-3S200A (Figure 4H). Significant positive FRET was observed only with the wild-type (TRBP-BWT/Lin28a-3WT) pair. FRET efficiency of both FRET pairs containing phosphomutant TRBP-B (TRBP-BS152A) were significantly reduced from the wild-type FRET pair. A FRET pair containing wild-type TRBP-B and phosphomutant Lin28a-3 (Lin28a-3 S200A) failed to show positive FRET, but was also not significantly different from zero or from the wild-type FRET pair. We note that a necessitated change in imaging system (Figure 4H compared with the system used in Figure 4, A–E) contributed to mild CFP photodamage that underlies reduced basal FRET values in Figure 4H but does not impact the validity of comparisons, which are made within the experiment. Collectively, results from our experiments using phosphomutants of TRBP-B (Figures 2, E and F, and 4, E–H) and Lin28a-3 (Figures 3G and 4H) indicate that phosphorylation of both S152 on TRBP and of S200 on Lin28a can contribute to MAPK-dependent co-up-regulation of these proteins, although the impact of TRBP S152 phosphorylation may marginally predominate in our assay system.

Optimization and validation of a sensor for Lin28a/TRBP binding

On the basis of the observation that FRET between TRBP-B and Lin28a-3 was increased within 90 min by EGF, and absent with phosphomutant TRBP-B, we performed time-lapse experiments to characterize the stimulation kinetics in more detail. We expressed CFP-TRBP-B and Lin28a-3-YFP in 293T cells and calculated the ratio of sensitized YFP fluorescence emission, determined using the NFRET formula, to CFP fluorescence (Xia and Liu, 2001). Baseline emission ratios were measured in individual cells for 10 min, followed by imaging for 35 min after EGF treatment. EGF induced a modest average change in NFRET (Figure 5, A and B; "Cer/E172"), indicating that further optimization might generate a more useful sensor for TRBP-Lin28a interaction. To enhance the dynamic range of the sensor, we tested a panel of FRET pairs, a strategy that has improved previous sensors (Figure 5A; Zhou et al., 2015). Significant positive FRET was observed only with the wild-type (TRBP-BWT/Lin28a-3WT) pair. Consistent with previous Lin28a/TRBP interaction resulted in a marked improvement, with the new FRET pair improved previous sensors (Figure 5A; Zhou et al., 2015). Significant positive FRET was observed only with the wild-type (TRBP-BWT/Lin28a-3WT) pair. This slower kinetics than Erk activity (Figure 5F, black curve). This slower Lin28a/TRBP FRET response compared with Ekar was reproduced in cells treated with PMA (Supplemental Figure S5, A and B). We noted that cells with stronger Ekar activity also showed greater increases in Lin28a/TRBP NFRET, and analyzed the correlation between response magnitude in the two signals. Comparing the increase in Ekar intensity at 30 min of EGF, to the increase in NFRET at the same time point, we observed a positive correlation in these two readouts (Figure 5G). We conclude that this sensor is a reliable tool for imaging ongoing changes in Lin28a/TRBP binding in real time within cells.

Compared with bimolecular FRET sensors, unimolecular FRET-based biosensors offer advantages in that they do not require equivalent coexpression of constructs and, due to tethering, they produce virtually complete colocalization of the fluorescent biosensor components. Prompted by the efficacy of the bimolecular Lin28a/TRBP sensor, we developed a unimolecular version by introducing a linker domain between Cer3-TRBP-B and Lin28a-3-YFP (Figure 4C). After 30 min of EGF treatment (100 ng/ml) in 293T cells, the expressed unimolecular sensor exhibited a twofold increase in FRET efficiency (Supplemental Figure S4D), compared with a 1.5-fold increase over baseline FRET efficiency in the bimolecular sensor at this same 30 min time point (Figure 4E). As expected, CFP/YFP colocalization was also improved in the unimolecular compared with the bimolecular sensor (Supplemental Figure S4E). In future studies, this optimized unimolecular Lin28a/TRBP biosensor may also facilitate tagging for targeting to discrete subcellular locations to visualize variability and temporal dynamics in signal-dependent regulation of miRNA biogenesis.

Signal-responsive regulation of miRNA biogenesis factors allows rapid changes in miRNA content in response to dynamic cellular and environmental contexts (Baek et al., 2008; Selbach et al., 2008). In this work, we dissected a MAPK-dependent mechanism for enhancing the interaction between two miRNA-binding proteins, Lin28a and TRBP, which can selectively modulate mature miRNA production. We found that expression of a midregion of TRBP (TRBP-B, amino acids 98–234), containing the second dsRBM, was sufficient to elevate levels of Lin28a protein in a manner indistinguishable from full-length TRBP, whereas a TRBP truncation lacking this midregion failed to increase Lin28a (Figure 1, E–G). TRBP-B, as well as a larger TRBP truncation containing the same middle region (TRBP-AB), could each associate with Lin28a in cellular lysates (Figure 1H), while a truncation lacking this region did not. Interestingly, another TRBP-binding protein, Dicer, has been shown to associate with the carboxy terminus of TRBP by crystal structure (using TRBP amino acids 258–366 only; Wilson et al., 2015) and by biochemical mapping (TRBP amino acids 298–366; Daniels et al., 2009); this may perhaps enable the simultaneous association by different regions of full-length TRBP with both Dicer and Lin28a as has been
data suggest that the C-terminal Lin28a-3 truncation contains a minimal unit for signal-inducible binding to TRBP. Our finding that the signal-inducible binding region of Lin28a includes regions of lower homology with vertebrate paralog Lin28b, such as the C-terminal 20 amino acids of Lin28a-3, is consistent with previous work showing that full-length Lin28a can coassociate with TRBP, whereas the highly homologous Lin28b does not (Amen et al., 2017).

Collectively, our data support a model in which phosphorylation of both Lin28a and TRBP contribute to their signal-induced interaction. We identified a role for TRBP phosphorylation at serine 152, an Erk consensus site proximal to a putative Erk docking site, in enhancing Lin28a interactions after EGF or PMA. Although phosphomutant TRBP S152A remained able to stabilize full-length Lin28a over baseline (Figure 2, D and F), it was unable to participate in signal-enhanced elevation of Lin28a protein or FRET efficiency. These findings highlight a role for TRBP S152 in signal-dependent enhancement of TRBP-Lin28a interactions.
interactions and induction of Lin28a protein. The dsRBMs of TRBP, including the motif within TRBP-B, participate in homodimerization and binding to other proteins, most notably PACT and PKR (Daher et al., 2001; Laraki et al., 2008), and could also play a role in basal binding to Lin28a. Binding to RNA has previously been shown to facilitate the interaction of full-length Lin28a and TRBP in cell extracts (Amen et al., 2017), and might enhance intracellular interaction of TRBP-B and Lin28a-3 in the biosensor. In addition to TRBP phosphorylation, we found that phosphorylation of Lin28a-3 participated in potentiating interaction with TRBP-B and elevating Lin28a-3 levels. Lin28a serine 200 phosphorylation has been previously reported to regulate Lin28a stability (Liu et al., 2017; Tsanov et al., 2017). Data from our FRET experiments using S-to-A phosphomutants (Lin28a-3 S200A and TRBP-B S152A) showed that phosphorylation of both TRBP and Lin28a were required for maximal interaction, and phosphomutants of either Lin28a-3 or TRBP-B reduced FRET-detectable interaction. Direct binding of activated ERK to TRBP has been previously reported (Paroo et al., 2009) and the TRBP-B truncation contains a putative ERK docking site (Figure 2A), making it interesting to speculate whether this could participate in recruiting activated ERK to the proximity of a Lin28a/TRBP complex.

The developed Lin28a/TRBP FRET sensor leverages the utility of FRET imaging approaches for probing complex protein/protein interactions to provide spatiotemporal information about specific binding interactions. We observed that FRET signal, indicative of Lin28a and TRBP binding, exhibited slower kinetics relative to Erk phosphorylation readout from the EKAR sensor. We did not find a condition that induced return of the FRET signal to baseline levels, suggesting that signal-induced binding of the sensor may persist despite dephosphorylation, or that the truncation constructs included in the sensor lack a sequence involved in dissociation or dephosphorylation. The TRBP literature implicates multiple proline-dependent MAPks, chiefly Erk and JNK, in TRBP regulation (Kim et al., 2014; Chen et al., 2015; Warner et al., 2016), including additional residues within TRBP-B. This FRET sensor may be of future use in probing the function of these phosphoregulatory sites, as well as investigation of compartmentalized TRBP and Lin28a interactions in a variety of physiological contexts. For example, both TRBP and Lin28a have roles in protein translation and silencing in the perinuclear reticulum compartment (Cho et al. 2012; Stalder et al., 2013). Similarly, the sensor could be used to gain spatiotemporal information regarding TRBP interaction with Lin28a occurring downstream from neurotrophins (Huang and Ruiz et al., 2012; Ruiz et al. 2014; Amen et al., 2017), which may exert effects on synaptic plasticity and protein synthesis in a variety of neuronal compartments.

MATERIALS AND METHODS

Cloning
TRBP truncations (as published [Daher et al., 2001]) were N-terminally tagged with myc (MEEQKLISETD), or fluorescent proteins Cerulean (Rizzo et al., 2004), Cerulean-3 (Markwardt et al., 2011), or ECFP (Llopis et al., 1998) in a pcDNA3.1+ vector backbone. Lin28a truncations were derived from FL-Lin28a (Amen et al. 2017) by PCR and tagged with flag (DYDDDKD) at the amino terminus or YFP variants Venus (Nagai et al. 2002), Citrine (Griesbeck et al., 2001), cpVenus-E172, or cpVenus-L194 (Nagai et al. 2004) at the carboxy terminus. Phosphomimic and phosphomutant versions of all TRBP and Lin28a constructs were derived from wild-type versions of these constructs, using a QuickChange kit (Agilent) per manufacturer protocols. A unique molecular sensor was developed using Gibson assembly to fuse Cer3-TRBP-B and Lin3-L194 with a flexible linker from the EKAR sensor (Ding et al., 2015) in a pcDNA 3.1+ backbone. EKAR ratiometric Erk sensor was a gift from Robert Campbell (University of Alberta, Edmonton, Canada) (Addgene; plasmid #60974).

Cell culture
HEK 293T cells were cultured and transfected as described (Amen et al., 2017). When multiple truncations of the same protein were expressed for the same experiment, transfection levels were optimized to equalize affinity tag level in lysates from different truncations. Total DNA transfected was equalized between conditions using empty pcDNA vector. Mouse tail epithelial fibroblast cells (TEFs) lacking endogenous murine TRBP (Tarbp2 KO; gift of Anne Gatignol, McGill University, Montreal, QC, Canada [Daher et al., 2009]) were cultured in DMEM with 10% fetal bovine serum supplemented with penicillin and streptomycin. Cells were seeded at 15,000 cells per well in 24-well plates, followed by lipofectamine LTX Plus transfection per manufacturer’s protocol.

Immunoblotting
Cultured cells were washed in cold phosphate-buffered saline (PBS) and harvested on ice with lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1% Triton X-100, 0.2% SDS) plus protease inhibitor cocktail (Roche; 11836170001) and phosphatase inhibitors (0.2 mM sodium orthovanadate, 1 mM sodium pyrophosphate). Protein concentration was determined by bicinchoninic acid (BCA) assay and equal protein amounts resolved on SDS–PAGE gels and electrotransferred to polyvinylidene fluoride membrane. Membrane was blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) tween 20 (TBST 0.1%) for 1–3 h and probed with primary antibodies: c-Myc (Life Technologies; 132500), FLAG (Sigma; M2, F3165), TRBP (Abcam; ab72110, or Proteintech; 15753-1-AP), Phospho-TRBP (custom), Hsc70 (Santa Cruz; sc7298), GST (Pierce; PA1-982A), and Lin28a (Cell Signaling; A177 3978S). All immunoblots were scanned and quantified without image adjustment. For representative image figures, image levels were uniformly and minimally adjusted for visual clarity in some instances.

Protein purification
GST-tagged proteins were purified from BL21 Escherichia coli using glutathione sepharose resin (GE Healthcare) according to the manufacturer’s protocol, eluted with 10 mM glutathione at pH 7.4, and dialyzed to remove glutathione. MBP-Lin28a was purified using amylose resin (New England Biolabs) and MBP was cleaved with Factor XA (New England Biolabs) according to the manufacturer’s protocols. In vitro pull down was carried out by binding GST proteins equimolar to 50 μg GST-TRBP-B overnight to glutathione resin, blocking with 5% BSA in PBS, then mixing with 25 μg purified Lin28a and rotated for 3 h at 4°C in PBS. The mixture was washed with 20x bead volume and then eluted by boiling in SDS gel loading buffer.

Immunoprecipitation
For FL-Lin28a coimmunoprecipitation of TRBP and its truncations, mouse anti-flag M2 antibody (Sigma) was adhered to protein G sepharose beads overnight after blocking with 5% BSA for 1 h. HEK 293T cells coexpressing the constructs of interest were harvested in colP lysis buffer (100 mM KCl, 4 mM MgCl2, 10 mM HEPES [pH 7.3], 50 μM ZnCl2, 0.5% NP-40) with protease inhibitor cocktail (Roche), and phosphatase inhibitors (Sigma; phosphatase inhibitor cocktail 2 and 3). Insoluble material was first removed by centrifugation (10,000 × g) and lysates precleared by rotation (4°C, 1 h) with
unblocked sepharose beads. Equal masses of protein in precleared lysates were brought to equal volume, added to flag antibody-coated beads, and rotated (4°C, 3–4 h). After three washes with cold PBS wash buffer (150 mM NaCl, 1 mM MgCl2, 50 mM HEPES [pH 7.8], 50 μM ZnCl2, 0.05% NP-40), immunoprecipitated material was eluted at room temperature using 1× flag peptide (Sigma; F3290) diluted in cold PBS wash buffer.

**Epifluorescence imaging**

Live cell fluorescence and FRET imaging were conducted on a Zeiss Axiovert 200M microscope controlled by MetaFluor 6.2 software. Cells seeded on poly(lysine)-coated glass-bottom imaging dishes (MatTek Corporation) were incubated at 37°C in Hank’s balanced salt solution (HBSS) and imaged at 40× magnification with 50% neutral density filters, illuminated by an arc lamp and captured on a cooled charge coupled device (Photometrics). Fluorescence emission was collected from cyan fluorescent protein (420DF20 excitation filter, 500 ms excitation, 475DF40 emission filter); yellow fluorescent protein (420DF20 excitation for 50 ms, 535DL25 emission filter); sensitized YFP emission (CFP excitation and YFP emission); and red fluorescent protein (568DF55 excitation filter, 50 ms, 600DRLP dichroic mirror, 653DF95 emission filter). For Figure 4H only, FRET efficiency imaging was conducted on an Olympus IX71 microscope controlled by MetaMorph software and illuminated by a pE300 LED light source (CoolLED) at 40× magnification.

For endpoint FRET imaging, cells expressing CFP-tagged TRBP or a truncation and YFP-tagged lin28a or a truncation were serum starved for approximately 15 min in imaging media. Several images were taken to establish a baseline, followed by 90 s, 3 min, and 5 min illumination at 504 nm to bleach YFP. Images were quantified using MetaFluor 6.2 software (Universal Imaging). FRET efficiency was calculated based on the recovery in background-corrected CFP brightness after YFP bleaching, using the formula FRET efficiency = \( 1 - \frac{F_{\text{da}}}{F_d} \) where \( F_{\text{da}} \) is the CFP fluorescence observed when both donor and acceptor are active and \( F_d \) is the fluorescence observed after YFP photobleaching. To confirm complete photobleaching, percent change in YFP intensity was calculated, using the formula

\[
\% \text{ photobleaching} = \frac{[\text{Intensity final} - \text{Intensity initial}]}{\text{intensity initial}} \times 100\%
\]

Only dishes with an average reduction of 80% or more in YFP signal were used for FRET efficiency calculation in epifluorescence experiments.

For time-course FRET imaging, cells expressing CFP-tagged TRBP-B with YFP-tagged Lin28a-3 were serum starved (0.5–2 h) in imaging media, then imaged every 30 s. CYFRET, CFP, YFP, and RFP intensity in each region of interest (ROI) were measured over time using MetaFluor software and, after background subtraction, used to calculate the normalized FRET emission ratio, a measure that adjusts for expression level and spectral bleedthrough of donor and acceptor fluorophores (Xia and Liu, 2001):

\[
\text{NFRET} = \text{FRET intensity} - [\text{YFP intensity x a}] - [\text{CFP intensity x b}]
\]

\[
\text{NFRET ratio} = \frac{\text{NFRET}}{\text{CFP}}
\]

Bleedthrough value “a” was determined using cells expressing only YFP and then imaged in both the CYFRET and YFP channels. “a” was defined as the signal in the YFRET channel as a percent of YFP signal; on this system, the value used was 0.14. Bleedthrough value “b” was calculated using the same approach with CFP alone, and came to 0.32. Representative images shown in Figure 5D have had the NFRET value calculated using the Image Calculator function in ImageJ to perform the same series of calculations (i.e., mean background subtraction in each channel, followed by subtraction of estimated bleedthrough from CFP and YFP direct channels, applied to CYFRET channel).

**Statistical analyses**

All quantified data represent mean ± SEM. Statistical analysis included one-way ANOVA for independent samples with a Bonferroni post hoc test, \( \alpha = 0.05 \), comparing with Lin28a alone or a comparable condition. Where noted, two-tailed Student’s t tests were used for pairwise comparison of untreated and treated conditions (Figure 2, C and F). Linear regression analysis and slope significance testing were carried out using a Graphpad Prism protocol equivalent to ANCOVA. Before linear regression was performed, independent variables were subjected to a Grubb’s test with \( \alpha = 0.05 \). The result was used to justify removing one statistically significant outlier from TRBP-full regression analysis (Figure 1H).

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