

Title: A Biosensor for MAPK-Dependent Lin28 Signaling

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Running head: Biosensor for Lin28/TRBP interaction

Highlight Summary: Elucidation of the molecular interactions enabling rapid post-translational Lin28a induction by protein stabilization with TRBP downstream of MAPK/Erk pathway activation is leveraged to generate a biosensor for dynamic visualization of Lin28a upregulation.

Abbreviations:

TRBP: Tar RNA Binding Protein
miRNA: microRNA
MAPK: Mitogen-activated protein kinase
EKAR: Erk Kinase Activity Reporter
FSP/ FXP: amino acid sequence of Erk docking motif
FL-Lin28a: Flag tagged Lin28a
dsRBM: double-stranded RNA binding motif

Abstract:

Intracellular levels of the RNA-binding protein and pluripotency factor, Lin28a, are tightly controlled to govern cellular and organismal growth. Lin28a is extensively regulated at the post-transcriptional level, and can undergo mitogen-activated protein kinase (MAPK)-mediated elevation from low basal levels in differentiated cells by phosphorylation-dependent stabilizing interaction with the RNA-silencing factor HIV TAR-RNA-binding protein (TRBP). However, molecular and spatio-temporal details of this critical control mechanism remained unknown. In this work, we dissect the interacting regions of Lin28a and TRBP proteins and develop biosensors to visualize this interaction. We identify truncated domains of Lin28a and of TRBP that are sufficient to support co-association and mutual elevation of protein levels, and a requirement for MAPK-dependent phosphorylation of TRBP at putative ERK-target serine 152, as well as Lin28a serine 200 phosphorylation, in mediating increase of Lin28a protein by TRBP. The phosphorylation-dependent association of Lin28a and TRBP truncated constructs is leveraged to develop FRET-based sensors for dynamic monitoring of Lin28a and TRBP interaction. We demonstrate response of bimolecular and unimolecular FRET sensors to growth factor stimulation in living cells, with coimaging of Erk activation to achieve further understanding of the role of MAPK signaling in Lin28a regulation.

Introduction:

Lin28a is a pluripotency-associated RNA-binding protein that exerts post-transcriptional effects to govern many developmental and cellular signaling pathways (Jiang et al., 2016). Lin28a promotes pro-growth 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., 2011, Shyh-Chang et al., 2011), and Lin28a is upregulated in numerous human cancers (Viswanathan et al., 2009, Shyh-Chang & 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 post-translational regulation of Lin28a protein stability. MAPK pathway activation was shown to enhance phosphorylation and binding of the Dicer cofactor, TRBP, as well as Dicer itself to Lin28a in a stabilizing interaction (Amen et al., 2017). This complex exerts selectivity in miRNA biogenesis, allowing Lin28a to reduce the level of Let-7 miRNAs while Dicer enhances maturation of many miRNAs not targeted by Lin28 (Huang & Ruiz et al., 2012). A Lin28a/TRBP/Dicer protein complex was described initially in cultured neurons, but MAPK-dependent TRBP

phosphorylation and Lin28a elevation were also observed downstream of trophic factors in other primary cell types, indicating a widespread role for this interaction in regulating growth responses (Amen et al., 2017). However, molecular details of the Lin28a/TRBP interaction, including its timescale and spatial distribution in cells, were relatively unexplored. In this work we characterize the biology of Lin28a and TRBP interacting domains. We identify a TRBP phosphorylation site crucial for signal responsive interaction with Lin28a and leverage this characterization to develop a genetically-encoded fluorescent biosensor for Lin28a/TRBP binding, allowing examination of the magnitude and spatiotemporal visualization of Lin28a/TRBP binding in living cells.

Results:

A TRBP truncation sufficient to interact with full-length Lin28a.

To investigate TRBP-induced elevation of Lin28a protein levels, we first identified the region of TRBP required for this response. Truncation mutants of TRBP, TRBP A (1 – 105), TRBP B (98 – 234), TRBP AB (1 – 234) and TRBP C (228 – 366) (Figure 1A), developed by the Gatignol lab (Daher et al., 2009), were tagged with the myc epitope. When co-expressed in HEK 293T cells with flag-tagged lin28a (FL-Lin28a), TRBP-A and TRBP-B both elevated FL-Lin28a, while TRBP-C had no effect. (Figure 1B-C). Expression of TRBP-C was low, even with high DNA transfections (Figure S1A). Dose titration showed that elevation of FL-Lin28a protein by TRBP-A and TRBP-B was most readily detected at low FL-

Lin28a expression levels (Figure S1B-C). Expressed TRBP-B could also elevate levels of endogenous TRBP protein (Figure 1D), in line with a previous demonstration that dsRNA binding motifs (dsRBMs) in these two truncations mediate TRBP dimerization (Kok et al., 2007). To distinguish effects on Lin28a elevation due to the TRBP truncation alone from effects that might result from heterodimerization of a TRBP truncation with endogenous TRBP, we next carried out experiments in TRBP knockout cells.

Coexpression of TRBP-A and TRBP-B with FL-Lin28a in mouse tail epithelial fibroblast cells (TEFs) lacking endogenous murine TRBP (*Tarbp2* KO) (Daher et al., 2009) revealed that TRBP-B, but not TRBP-A, could elevate FL-Lin28a levels; TRBP-B increased FL-Lin28a comparably to full length TRBP expression (Figure 1E-G). Across a range of expression levels in TRBP KO TEFs, full length TRBP and the TRBP-B truncation produced approximately linear FL-Lin28a protein elevations, while no increase in FL-Lin28a was observed with TRBP-A (Figure 1G). These results strongly suggested that TRBP-B retained the capacity to interact with Lin28a, while effects of TRBP-A were absent when endogenous full-length TRBP could not be recruited.

In previous work, we demonstrated that full-length TRBP can directly bind Lin28a in a stabilizing complex (Amen et al., 2017). Thus, we next compared the association of truncations TRBP-A, TRBP-B, and TRBP-AB with FL-lin28a. Immunoprecipitation of FL-lin28a from 293T cells showed that TRBP-AB coimmunoprecipitated with FL-Lin28a, as did TRBP-B more weakly, while TRBP-A did not coimmunoprecipitate with FL-Lin28a (Figure 1H). Since TRBP-B

expression is sufficient to elevate Lin28a levels (Figure 1B-G), we speculate that reduced stability during the process of immunoprecipitation may contribute to relatively modest coimmunoprecipitation with Lin28a (Figure 1H). Collectively, these results are consistent 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 phosphomimic 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 multi-banding pattern between 20 and 25 kDa when probed with an antibody

raised and purified against phospho-S152 TRBP (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 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 co-expressing 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 2C-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,S2C). In TRBP KO TEFs co-expressing FL-Lin28a and either myc-TRBP-B or phosphomutant myc-TRBP-B-S152A (at

similar levels, 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 (Tsanov et al., 2017; Liu et al., 2017) or an alternative unknown interacting protein. In addition, we observed that purified GST-TRBP-B protein could specifically co-associate with purified Lin28a, compared to GST alone, and that this association was decreased by the phosphomutation TRBP-B S125A (Figure S2E). Decreased binding of Lin28a by TRBP-B SA, in comparison to wildtype TRBP-B, may be due to reduced phosphorylation in bacteria (Pereira et al., 2011; Cousin et al., 2013; Macek & Mijakovic, 2011) or to the absence of the hydroxyl group on residue 125. 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 wildtype 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.

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). Since Lin28b, a paralog with 86% amino acid identity between Lin28A amino acid 19 and 177,

fails to bind to TRBP (Amen et al., 2017), we reasoned that amino acid sequences unique to Lin28a at the amino- or the carboxyl- terminus of the protein might participate in TRBP interaction. Truncations were designed to correspond to functional domains of Lin28a revealed by its crystal structure (Nam et al., 2011, Mayr et al., 2013). Lin28a-1, aa 1-112, included the structurally uncharacterized N-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 N-terminus of the CCHC, extending to the structurally uncharacterized C-terminus of the protein (Figure 3A).

Full-length FL-Lin28a and FL-Lin28a-3 levels were each elevated above baseline by myc TRBP co-expression in 293T cells (Figure 3B-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 hours) in 293T cells co-expressing TRBP-B. FL-Lin28a-1 levels were not significantly changed either by co-expression of TRBP-B alone or by TRBP-B plus PMA stimulation, while FL-Lin28a-3 levels were robustly increased by TRBP-B co-expression and further enhanced by PMA treatment (Figure 3E, F).

In addition to TRBP phosphorylation, Lin28a has also been reported to undergo MAPK-dependent phosphorylation at serine 200, which can regulate its stability (Tsanov et al., 2017, Liu et al., 2017). To evaluate the participation of Lin28a-3 phosphorylation, compared to TRBP-B phosphorylation (Figure 2E,F), in Lin28a-3 protein upregulation, we assessed the fold stabilization of wildtype

and phosphomutant FL-Lin28a-3 (Lin28a-3 S200A) by immunoblot of 293T cell lysates in the presence or absence of TRBP-B co-expression or EGF treatment (Figure 3G-H). Levels of wildtype FL-Lin28a-3 protein were significantly elevated by co-expression with TRBP-B, and this effect was further enhanced by 293T stimulation with EGF (one-way ANOVA compared to wildtype Lin28a-3 alone). In contrast, levels of FI-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 wildtype 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

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 FRET efficiency between TRBP constructs tagged with the CFP variant, Cerulean, and Lin28a constructs tagged with a 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 high FRET efficiency in our imaging system, we used a FRET

sensor for calmodulin (Figure S3A) (Romoser et al., 1997). We confirmed YFP bleaching to 75% or greater reduction of signal in each cell (Figure S3B-D).

We tested the effects of co-expressing 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 (Figure S3E-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, and 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). Based on 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 epidermal growth factor (EGF, a MAPK pathway activator) or vehicle and measured donor dequenching 60 minutes 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 3E 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 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 time prior to acceptor bleaching, CFP-TRBP-B and Lin28a-3-YFP demonstrated a time-dependent increase in FRET with a maximal plateau by 60-90 minutes (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 4F-G).

The importance of phosphorylation for TRBP-B and Lin28a-3 interactions was further evaluated by testing the effects of phosphomutant constructs, CFP-TRBP-B S152A and Lin28a-3 S200A-YFP, on FRET efficiency. We conducted a multi-component 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 wildtype (TRBP-BWT / Lin28a-3WT) pair. FRET efficiency of both FRET pairs containing phosphomutant TRBP-B (TRBP-BS152A) were significantly reduced from the wildtype FRET pair. A FRET pair containing wildtype TRBP-B and phospho-mutant Lin28a-3 (Lin28a-3S200A) failed to show positive FRET, but was also not significantly different from zero or from the wildtype FRET pair. We note that a necessitated change in imaging system (Figure 4H compared to 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 (Figure 2E,F; Figure 4E-H) and Lin28a-3 (Figure 3G, Figure 4H) indicate that phosphorylation of both S152 on TRBP and of S200 on Lin28a can contribute to MAPK-dependent co-upregulation 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

Based on the observation that FRET between TRBP-B and Lin28a-3 was increased within 90 minutes 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 & Liu, 2001). Baseline emission ratios were measured in individual cells for ten minutes, followed by imaging for thirty-five minutes after EGF treatment. EGF induced a modest average change in NFRET (Figure 5A,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 (Zhou et al., 2015) (Figure 5A). Optimization resulted in a marked improvement, with the new FRET pair showing higher average response to EGF (Figure 5B, “Cer3/L194”). We tested the dependence of the Cer3/L194 Lin28a/TRBP FRET sensor on MEK activity. Consistent with previous Lin28a/TRBP interactions, MEK inhibition with U0126 (30 min) blocked EGF-induced NFRET increases, while 293T cells pretreated with vehicle retained an EGF-stimulated increase in NFRET (Figure 5C).

To investigate the temporal relationship between Erk activation and Lin28a/TRBP binding, we co-expressed an intensitometric Erk activity sensor (EKAR) based on dimerization dependent RFP (Ding et al., 2015) with the optimized Lin28a/TRBP sensor. The spectral separation between Cerulean3/Venus and RFP permitted us to monitor signal-induced changes in single cells at these two different steps within the EGF response pathway. Representative images of cells before and for 80 minutes after EGF treatment are shown in Figure 5D. Consistent with our earlier demonstration that S152A TRBP-B failed to show detectable FRET with Lin28a-3, we observed that NFRET/CFP ratio between the phosphomutant and Lin28a-3 did not increase

after EGF treatment, although EKAR activity indicates rapidly peaking Erk activation (Figure 5E). In contrast, 293T cells expressing the sensor using WT-TRBP-B demonstrate NFRET response to EGF (Figure 5F). Erk activity reached a plateau within 15 minutes of EGF on average, and remained at peak activity for the duration of imaging (Figure 5F, red curve). The NFRET signal from the optimized Lin28a/TRBP sensor increased more gradually, showing slower kinetics than Erk activity (Figure 5F, black curve). This slower Lin28a/TRBP FRET response compared to EKAR was reproduced in cells treated with PMA (Figure S5A-B). We noted that cells with stronger EKAR response 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 minutes of EGF, to the increase in NFRET at the same timepoint, 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 to bimolecular FRET sensors, unimolecular FRET-based biosensors offer advantages in that they do not require equivalent co-expression 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 minutes of EGF treatment (100ng/ml) in 293T cells, the expressed unimolecular sensor exhibited a two-fold increase in FRET efficiency (Figure

S4D), compared to a 1.5 fold increase over baseline FRET efficiency in the bimolecular sensor at this same 30 minute timepoint (Figure 4E). As expected, CFP/YFP colocalization was also improved in the unimolecular compared to bimolecular sensor, (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.

Discussion

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 mid-region 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, while a TRBP truncation lacking this mid-region failed to increase Lin28a (Figure 1E-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 C-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 previously demonstrated (Amen et al., 2017). TRBP binding increases the stability of Dicer (Paroo et al., 2009; Wilson et al., 2015) and TRBP phosphorylation at serines 142, 152, 283 and 286 has been previously reported to increase TRBP stability and elevate levels of both TRBP and Dicer (Paroo et al., 2009).

Of the three domain-based Lin28a truncations tested, only Lin28a-3, which begins at the CCHC zinc knuckles and terminates at the C terminus of the protein (amino acids 136-209), exhibited elevated protein levels when co-expressed with full length TRBP or with TRBP-B (Figure 3B,C), and signal-induced elevation in cells co-expressing TRBP-B (Figure 3D-H). While FRET pairs containing either the amino-terminal Lin28a truncation (Lin28a-1) or Lin28a-3 with TRBP-B exhibited FRET, FRET efficiency with Lin28a-3 was not only strongest at baseline but also capable of stimulus-mediated increase (Figure 4A-E). Low basal FRET between Lin28a-1 and TRBP-B could occur due to a secondary interface between the two proteins not detected by an immunoprecipitation approach. These 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

co-associate with TRBP, while 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 cellular MAPK pathway activation with EGF or PMA. While phospho-mutant TRBP-B S152A remained able to stabilize full-length Lin28a over baseline (Figure 2D,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 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 (Tsanov et al., 2017; Liu 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 read out 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 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 (Warner et al., 2016, Chen et al., 2015; Kim et al., 2014), 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 peri-endoplasmic reticulum region (Stalder et al., 2013; Cho et al. 2012). Similarly, the sensor could be used to gain spatio-temporal information regarding TRBP

interaction with Lin28a occurring downstream of neurotrophins (Huang & 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 & Methods

Cloning

TRBP truncations (as published (Daher et al., 2001)) were N-terminally tagged with myc (MEEQKLISEEDL), or fluorescent proteins Cerulean (Rizzo et al., 2004), Cerulean-3 (Markwadt 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 (DLYDDDDKD) at the N 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 C 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. Unimolecular 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 R. Campbell (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 A. Gatignol, (Daher et al., 2009)) were cultured in DMEM with 10% FBS 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 protocol.

Immunoblotting

Cultured cells were washed in cold 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 (sodium orthovanadate 0.2 mM, sodium pyrophosphate 1 mM). Protein concentration was determined by Bicinchoninic acid (BCA) assay and equal protein amounts resolved on SDS-PAGE gels and electrotransferred to PVDF membrane. Membrane was blocked with 5% BSA in Tris-buffered saline tween 20 (TBST 0.1%) for 1-3 hr and probed with primary antibodies: c-Myc (Lifetech 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 *E. coli* using glutathione sepharose resin (GE Healthcare) according to manufacturer 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 (NEB) according to manufacturer protocols. *In vitro* pulldown 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 hours at 4 °C in PBS. Mixture was washed with 20x bead volume and then eluted by boiling in SDS gel loading buffer.

Immunoprecipitation

For FL-Lin28a co-immunoprecipitation 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 hour. HEK 293T cells coexpressing the constructs of interest were harvested in coIP lysis buffer (100 mM KCl, 4 mM

MgCl₂, 10 mM HEPES (pH 7.3), 50 μ M 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 x g) and lysates pre-cleared by rotation (4 °C, 1 hr) with unblocked sepharose beads. Equal masses of protein in pre-cleared lysates were brought to equal volume, added to flag antibody-coated beads, and rotated (4 °C, 3-4 hr). After washing 3x with coIP wash buffer (150 mM NaCl, 1 mM MgCl₂, 50 mM HEPES (pH 7.8), 50 μ M ZnCl₂, 0.05% NP-40), immunoprecipitated material was eluted at room temperature using 1x flag peptide (Sigma F3290) diluted in coIP 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(L)lysine coated glass-bottom imaging dishes (MatTek Corporation) were incubated at 37 °C in Hank's Balanced Salt Solution (HBSS), and imaged at 40x 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, 535df25 emission filter); sensitized YFP emission (CFP excitation and YFP emission); and red fluorescent protein (568DF55 excitation filter, 50ms, 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 40x 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 fifteen minutes in imaging media. Several images were taken to establish a baseline, followed by 90 second, 3 minute and 5 minute 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 following formula:

$$FRET\ efficiency = 1 - F_{da}/F_d$$

where F_{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:

$$\% photobleaching = [(intensity\ final - intensity\ initial) / intensity\ initial] * 100\%$$

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 hrs) in imaging media, then imaged every 30 s. CYFRET, CFP, YFP and RFP intensity in each 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 & Liu, 2001).

$$NFRET = FRET\ intensity - [YFP\ intensity \times a] - [CFP\ intensity \times b]$$

$$NFRET\ ratio = NFRET / CFP$$

Bleedthrough value “a” was determined using cells expressing only YFP, 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 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 $\pm$ SEM. Statistical analysis included one-way ANOVA for independent samples with a Bonferroni *post hoc* test, $\alpha = 0.05$, comparing to Lin28a alone or comparable condition. Where noted, two-tailed student's *t* tests were used for pairwise comparison of untreated and treated conditions (Figure 2C, 2F). 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).

Author Contributions

MKM & JZ conceived of the project and supervised its execution. LO planned and carried out experiments and analyses in fig 1-4, 5a-b and e-f. KG carried out experiments in 5c-d and g, S4A-B, and assisted with image preparation. WM performed cloning in fig S4C. LO, MKM & JZ wrote the manuscript.

Acknowledgements/Notes

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FIGURE 1

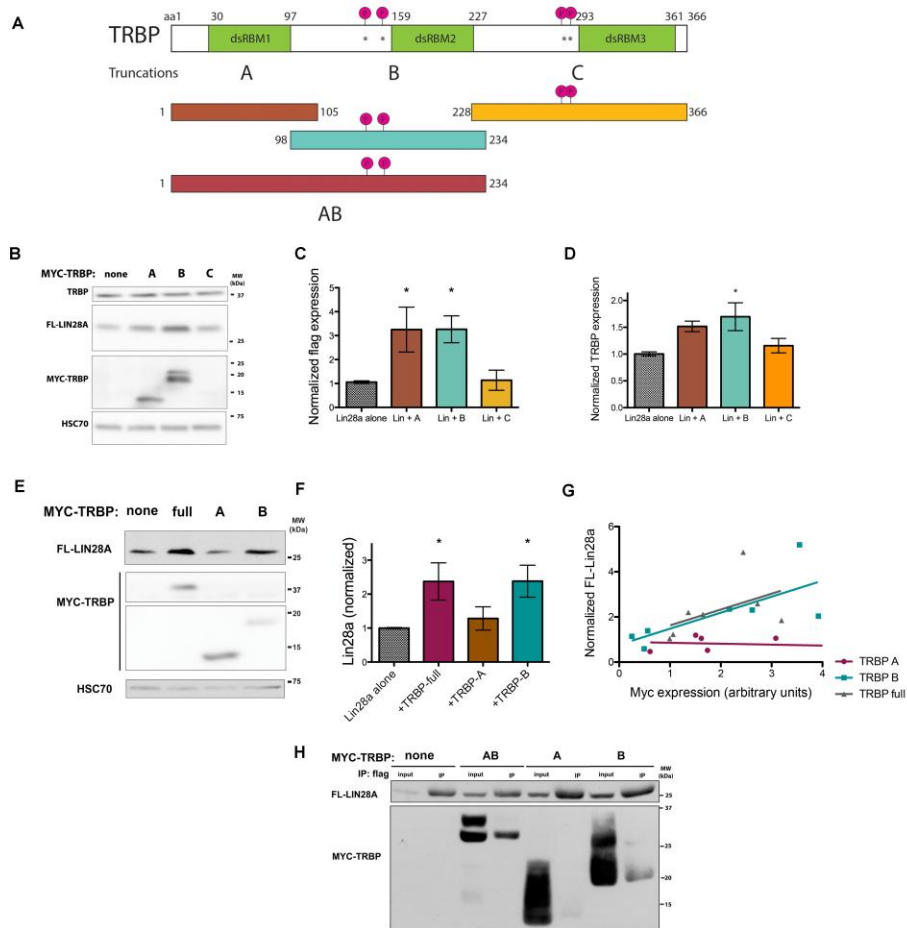


Figure 1. A TRBP truncation sufficient to bind and elevate Lin28a levels. (A) Domain map of TRBP, with truncations TRBP A, B, C and TRBP AB. Phosphorylation sites (pink) as described (Paroo et al., 2009), at serine residues 142, 152, 283 and 286. (B,C) TRBP A and B stabilize FL-Lin28a in 293T cells. Representative immunoblot (B) of lysates of 293T cells expressing Lin28a alone or coexpressing Lin28a with TRBP truncations, and quantified data normalized to HSC70 loading control (C)(n=7-14). (D) Increase in endogenous TRBP level, normalized to HSC70, quantified from immunoblots of lysates of 293T cells expressing Lin28a alone or coexpressing

Lin28a with TRBP truncations (n=5-8). (E,F) Full length TRBP (TRBP full) and TRBP B, but not TRBP A, elevate Lin28a levels from baseline in Tarbp2^{-/-} MEF cells. Representative immunoblot (E) and quantification (F) of FL-Lin28a normalized to HSC70 and plotted relative to the Lin28a alone condition set as 1.0 (n=9). (G) Scatterplot of the FL-Lin28a protein levels relative to co-expression level of full length TRBP, TRBP A, or TRBP B proteins in Tarbp2^{-/-} cells. FL-Lin28a levels were positively correlated with increasing expression TRBP full and TRBP B. The regression line for TRBP full has a correlation coefficient (r) of 0.51 (p=0.114 for deviation from zero slope). One statistically significant outlier was removed (Methods). Myc TRBP A regression r=0.06 (p=0.638) and Myc TRBP B regression r=0.62 (p=0.06) (H) Coimmunoprecipitation of FL-Lin28a with myc-TRBP-AB and B, but not TBRP-A. (A-F) * p<0.05, ANOVA

FIGURE 2

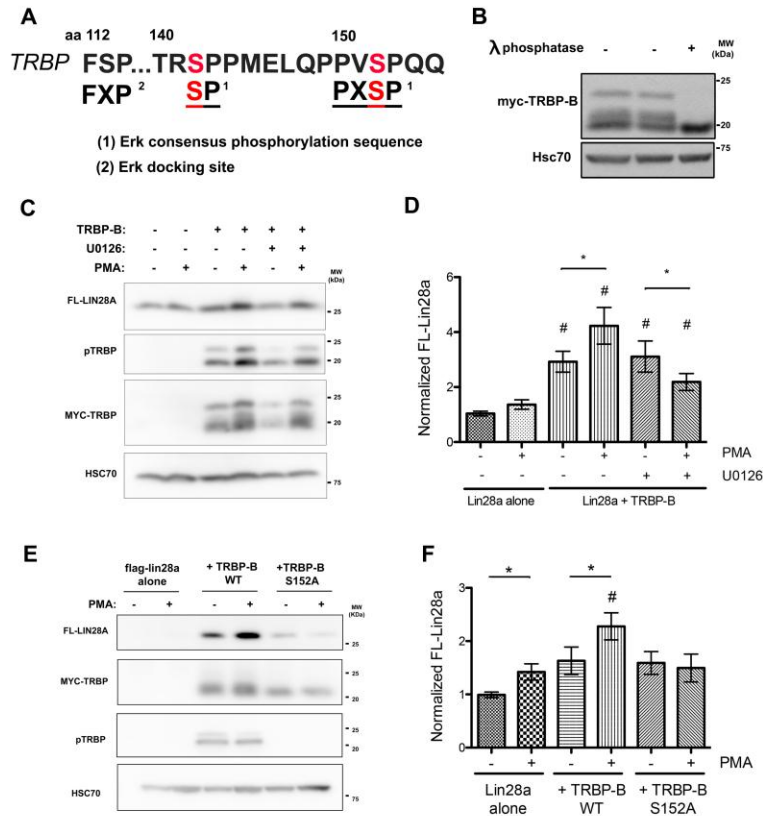


Figure 2. TRBP-B phosphorylation at S152 enhances binding to Lin28a. (A) Putative ERK substrate sequence of TRBP at serine 152, including FSP docking site. Weaker candidate ERK substrate at serine 142 (B) Immunoblot from 293T cells expressing TRBP-B shows two or more phosphorylated species exist and 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.

FIGURE 3

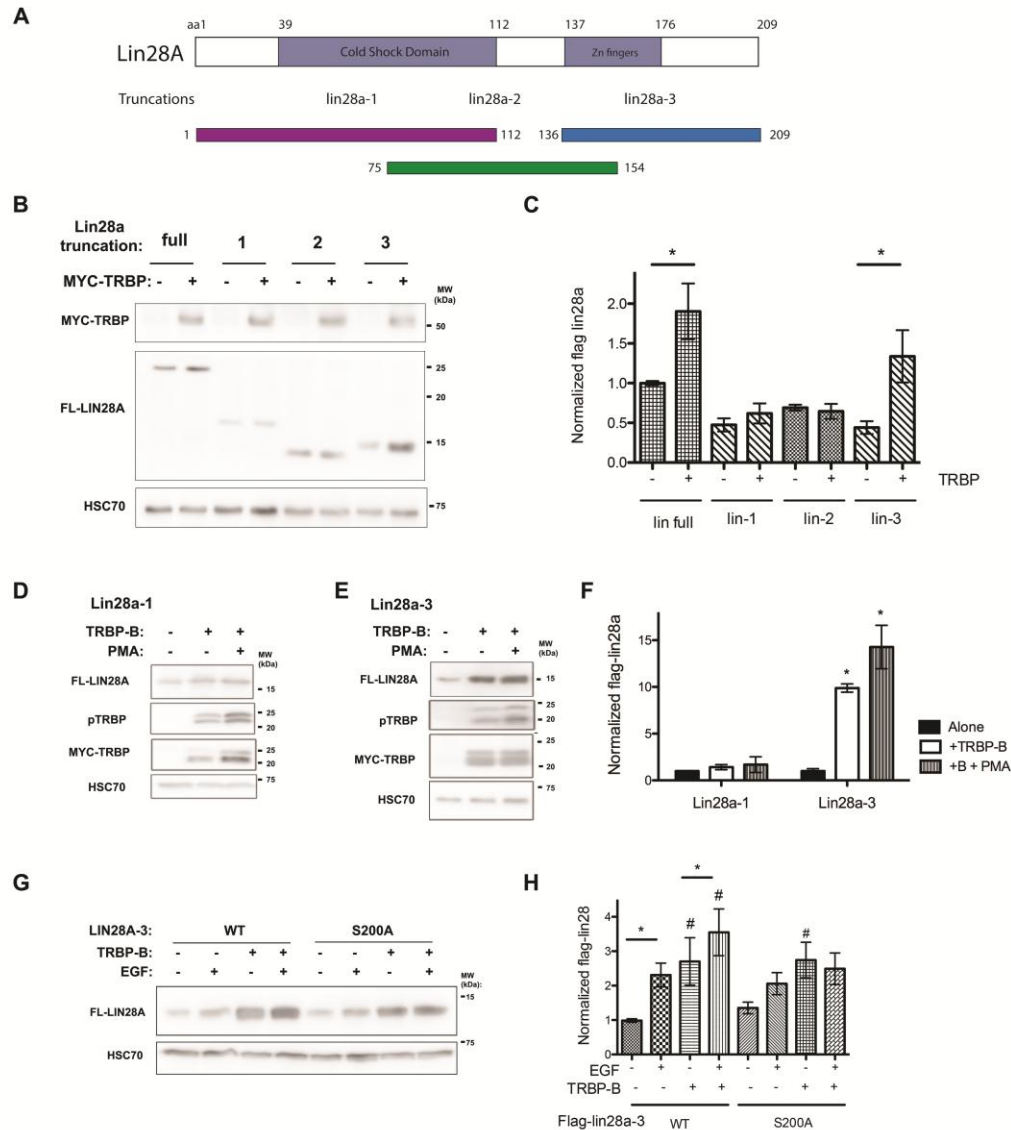


Figure 3. A C-terminal Lin28a truncation sufficient to respond to TRBP and TRBP-B. (A) Domain map of Lin28a, with truncations Lin28a-1, 2 and 3. (B,C) Coexpression of FL-Lin28a and truncations with full length TRBP in 293T cells increases Lin28a and Lin28a-3. (* $p < 0.05$, ANOVA, $n = 4$) (D-F) Effect 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, $n=9$).

FIGURE 4

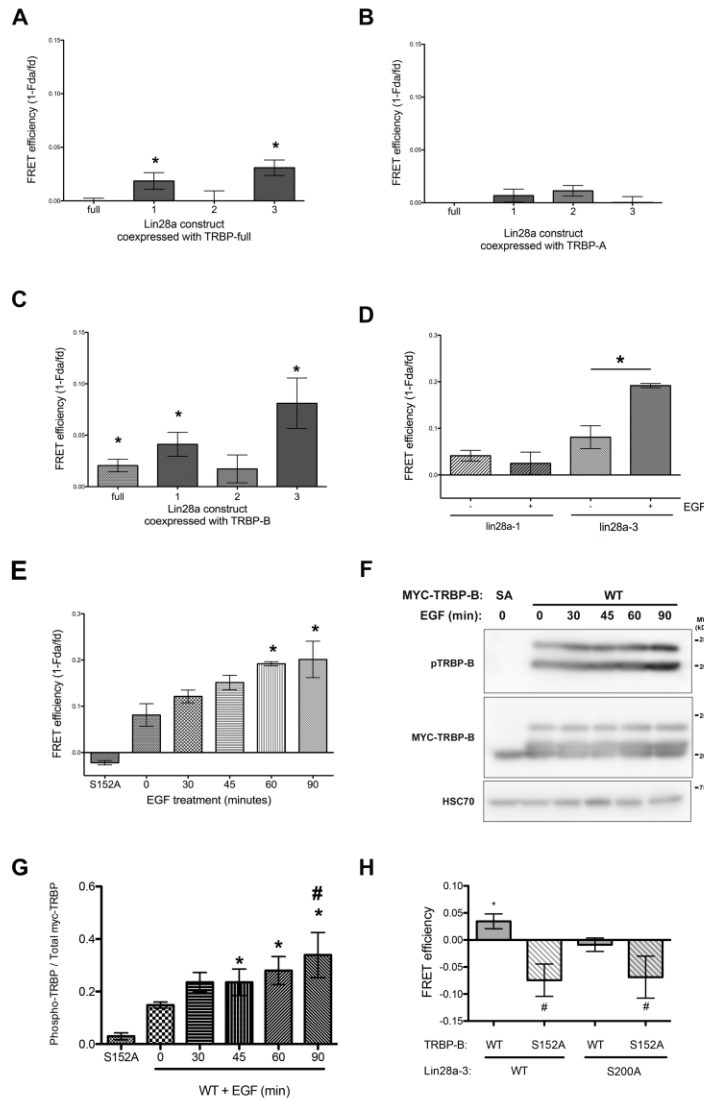


Figure 4. Lin28a-3 interacts with TRBP-B in a phosphorylation inducible manner. (A) FRET efficiency of C-terminal cpVenusE172-tagged Lin28a and truncations with full-length N-terminal Cerulean3-tagged TRBP (B-C) FRET efficiency of Lin28a and truncations with TRBP-A and B. (A-C) * $p < 0.05$ vs. 0, t test (D) FRET efficiency of Lin28a-1 and 3 with TRBP-B after treatment with 100 ng/mL EGF for 60 minutes. (E) FRET efficiency of Lin28a-3 with TRBP-B after EGF treatment. "S152A" indicates efficiency of Lin28a-3 with phosphomutant TRBP-S152A (D-E, * $p < 0.05$ vs. untreated, ANOVA) (A-E) $n = 15-75$ cells per column from at least two dishes. (F, G)

Increase in phospho-TRBP-B, measured by immunoblot, after EGF treatment. (# $p < 0.05$, ANOVA, * $p < 0.05$, paired t test; $n=6$) (H) FRET efficiency, measured with a different instrument, for Lin28a-3 WT and S200A with TRBP-B WT and S152A. (* $p < 0.05$ vs. 0, t test, # $p < 0.05$ vs WT/WT pair, ANOVA).

FIGURE 5

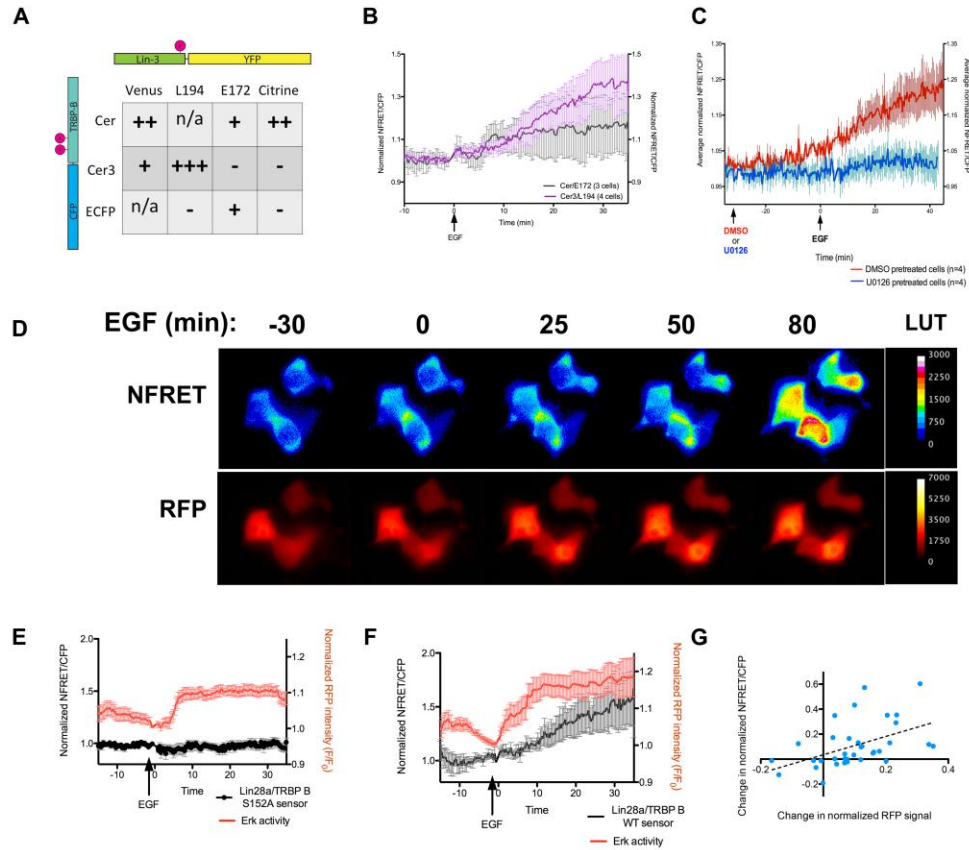


Figure 5. Optimization of a sensor for phosphorylation-induced Lin28a/TRBP binding. (A) Qualitative assessment of Lin28a/TRBP FRET pairs, scored based on flat baseline and magnitude of response (n=2-6 dishes/pair). (B) Comparison of NFRET ratio change, normalized to T0, between representative 293T cells expressing sensor with original FRET pair (“Cer/E172”) or optimized sensor (“Cer3/L194”) after EGF treatment. (C) Optimized sensor FRET response to EGF in 293T cells pretreated with vehicle (red) or U0126 (blue) (n=4 cells per condition). (D)

Pseudocolor image series of Cer3/L194 sensor signal ("NFRET") and EKAR sensor intensity ("RFP") in 293T cells pretreated with DMSO and then treated with EGF. (E-F) Representative curves showing change in NFRET (black) coimaged with increase in Erk activity (red) in response to EGF in cells expressing S152A sensor (E, n=7) or WT sensor (F, n=6) (G) Regression analysis of EKAR intensity change and NFRET intensity change, measured 30 minutes after EGF treatment (n=35 cells from 7 dishes). Regression line $r = 0.23$ ($p=0.004$).