Published in final edited form as:
Mol Cell. 2011 July 8; 43(1): 97–109. doi:10.1016/j.molcel.2011.06.013.

Mammalian *hyperplastic discs* homolog EDD regulates microRNA-mediated gene silencing

Hong Su, **Shuxia Meng**, **Yanyan Lu**, **Melanie I. Trombly**, **Jian Chen**, **Chengyi Lin**, **Anita Turk**, and **Xiaozhong Wang***
Department of Molecular Biosciences, Northwestern University, Evanston, Illinois 60208, USA

SUMMARY

MicroRNAs (miRNAs) regulate gene expression through translation repression and mRNA destabilization. However, the molecular mechanisms of miRNA silencing are still not well defined. Using a genetic screen in mouse embryonic stem (ES) cells, we identify mammalian *hyperplastic discs* protein EDD, a known E3 ubiquitin ligase, as a key component of the miRNA silencing pathway. ES cells deficient for EDD are defective in miRNA function and exhibit growth defects. We demonstrate that E3 ubiquitin ligase activity is dispensable for EDD function in miRNA silencing. Instead, EDD interacts with GW182 family proteins in the Argonaute-miRNA complexes. The PABC domain of EDD is essential for its silencing function. Through the PABC domain, EDD participates in miRNA silencing by recruiting downstream effectors. Among the PABC-interactors, DDX6 and Tob1/2 are both required and sufficient for silencing mRNA targets. Taken together, these data demonstrate a critical function for EDD in miRNA silencing.

INTRODUCTION

MicroRNAs (miRNAs) modulate protein output of a large portion of the human transcriptome. miRNAs are therefore key regulators in diverse biological processes including development, proliferation, differentiation, apoptosis, host defense and cancer (Bushati and Cohen, 2007; Stefani and Slack, 2008). During miRNA biogenesis, two distinct ribonucleases Drosha and Dicer sequentially process long stem-loop containing primary transcripts and produce miRNA duplexes. The miRNA duplex is then unwound and loaded onto Argonautes (Ago) to form the core effector complexes, known as miRNA-induced silencing complexes (miRISCs). miRNAs act as a guide for miRISC to specifically regulate target mRNAs. Most animal miRNAs are partially complementary to their targets and miRNAs mediate their silencing effect by inhibiting translation and destabilizing mRNAs (Carthew and Sontheimer, 2009).

The molecular mechanisms by which miRISCs regulate target gene expression have been subject to intense debate [For review, see (Djuranovic et al., 2011; Huntzinger and Izaurralde, 2011)]. It has been reported that miRNAs inhibit mRNA translation at an

*Correspondence should be addressed to: Xiaozhong Alec Wang, Department of Molecular Biosciences, Northwestern University, 2205 Tech Drive, Hogan 2-100, Evanston, IL 60208-3500, Phone: Office: 847-467-4897, Fax: 847-467-1380, awang@northwestern.edu.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures, 7 figures and 1 table.

initiation step (Ding and Grosshans, 2009; Humphreys et al., 2005; Mathonnet et al., 2007; Pillai et al., 2005; Thermann and Hentze, 2007), whereas others indicate that miRISCs repress translation at post-initiation steps (Lytle et al., 2007; Olsen and Ambros, 1999; Petersen et al., 2006). Increasing evidence also demonstrates that deadenylation is a widespread mechanism associated with many miRNA targets (Behm-Ansmant et al., 2006; Eulalio et al., 2009a; Fabian et al., 2009; Wu et al., 2006). GW182 proteins, in worms, flies and mammals, have been shown to directly interact with Ago proteins and play essential roles in the miRNA silencing pathway (Behm-Ansmant et al., 2006; Chen et al., 2009a; Eulalio et al., 2008; Fabian et al., 2009; Liu et al., 2005; Tritschler et al., 2010; Zhang et al., 2007). More recently, genome-wide ribosome profiling studies demonstrate that changes in target mRNA abundance closely reflect the silencing effect of miRNAs on gene expression (Guo et al., 2010; Hendrickson et al., 2009). Therefore, miRNA-mediated translation repression and mRNA destabilization are likely coupled (Djuranovic et al., 2011).

To further understand downstream effectors of miRNA-Argonaute silencing complexes in mammalian cells, we devised a drug-selection based genetic screen to identify additional key components of the miRNA silencing pathway using mouse embryonic stem (ES) cells. Our design is based on a recent finding that tethering of Ago proteins to a reporter mRNA mimics the inhibitory effect of miRNAs on translation independently from miRNA biogenesis (Pillai et al., 2004). Using a reporter ES cell line in which λN22-tagged Ago2 represses a drug-selection reporter Hprt with 5 copies of BoxB in its 3′UTR, we performed an RNAi screen to isolate HAT-resistant ES cells that are defective in Ago-mediated translation repression. From this screen, we discovered that mouse *hyperplastic discs* homolog EDD, an E3 ubiquitin ligase, is a key component for Ago- and miRNA-mediated silencing. By exploring the protein-protein interaction network of EDD, we demonstrate that EDD interacts with multiple effector proteins to silence the expression of miRNA targets.

RESULTS

A genetic screen reveals a novel function for EDD in Ago2-mediated translational repression

Because miRNA biogenesis is relatively well understood, we designed a screening strategy focusing on the molecular mechanisms downstream of Ago-miRNA binding to its mRNA target. Interactions between λN22-Ago fusion proteins and BoxB sites in the 3′UTR of a reporter gene mimics miRNA-mediated translation repression independently from miRNA biogenesis (Pillai et al., 2004). Using a stable ES cell line (1A2) that expresses a Renilla (Rn) luciferase reporter with 5 copies of BoxB in its 3′UTR, we confirmed that tethering of any individual λN22-Ago1–4 is sufficient to inhibit the protein synthesis of Rn luciferase in stably transfected mouse ES cells with only a subtle effect on mRNA stability (Figure S1).

To establish a drug-selection based genetic screen, we chose hypoxanthine phosphoribosyl transferase *(Hprt)*, a reporter gene that allows both positive (HAT, hypoxanthine aminopterin thymidine) and negative (6-TG, 6-thioguanine) selection. First, we constructed an ES cell line that expresses *Hprt* with 5 copies of BoxB within its 3′UTR (“8–4” in Figure 1A). Second, we introduced a λN22HA-Ago2 transgene to silence Hprt expression, converting reporter cells to be HAT sensitive and 6-TG resistant (“H3” in Figure 1A). Third, we tested a positive control shRNA against λN22HA to down-regulate tagged Ago2, reverting the ES cells to a HAT resistant (HAT^R) and 6-TG sensitive ($6\text{-}TG^S$) phenotype (data not shown). Fourth, we carried out a genetic screen using shRNA libraries. After screening 40,000 independent puromycin resistant ES clones using the H3 reporter cell line, we identified and verified one positive shRNA that reproducibly gave rise to HAT^R clones (Figure 1B). This verified lentiviral shRNA specifically targets mouse E3 ubiquitin ligase EDD, the homolog of *Drosophila hyperplastic discs* gene, also known as Ubr5 (Callaghan et

al., 1998; Tasaki et al., 2005). Mouse EDD is a protein of 2792 amino acids that contains several distinct domains including UBA, UBR, PABC and HECT E3 ubiquitin ligase domains (Figure 1C). Drug resistance and sensitivity tests confirmed that EDD-shRNA expressing H3 cells were HAT^R. Surprisingly, these cells were also 6-TG^R, suggesting that RNAi mediated knockdown was not stable without a positive selection and the cells reverted to a Hprt negative status under 6-TG selection (Figure 1B). Nevertheless, in HAT^R H3 reporter cells expressing EDD-shRNA, Western blot analysis confirmed that endogenous EDD was significantly downregulated (Figure 1D). Taken together, these results suggest that EDD has a novel function in Ago2-mediated gene silencing.

EDD is a key effector for miRNA silencing

Next we used several independent assays to validate EDD function in Ago- or miRNA-mediated silencing. First, RT-qPCR analyses showed that the levels of mature mir-130a, mir-290, mir-19b and mir-21 remained unchanged in EDD knockdown ES cells (Figure S2B), confirming that EDD is not required for miRNA biogenesis. Using a stable reporter ES cell line (1A2E8) in which Rn-Luc reporter is repressed by binding of λN22HA-Ago2 to BoxB sequences at its 3′UTR (Figure 2A), we showed that knockdown of EDD by two different EDD shRNAs impaired Ago2-mediated repression of Rn luciferase (Figure 2B–2C). As seen in the Ago2 reporter cell line, Ago-mediated silencing was also compromised in Ago1, Ago3 and Ago4 tethering reporter cells upon EDD knockdown (Figure S2C–D) whereas EDD knockdown had no effect in control reporter cell lines (Figure S2E–F). Thus, EDD is required for the silencing effect of all Agos.

Similarly, in EDD knockdown mouse fibroblast STO cells, miRNA-mediated silencing was significantly compromised in both mir-CXCR and mir-30 reporter assays (Figure 2D–2E) (Doench et al., 2003; Zeng et al., 2002). Furthermore, we established inducible EDD knockdown HeLa cells using a modified *PiggyBac* transposon for Dox-inducible shRNA expression (Wiederschain et al., 2009) (Figure 2F). Upon Dox-induced knockdown of EDD in HeLa cells, previously identified miRNA targets p27Kip1 and HMGA2 were significantly upregulated (Figure 2G) (le Sage et al., 2007; Mayr et al., 2007). Let-7 negatively regulates HMGA2 expression through mRNA destabilization (Lee and Dutta, 2007). RT-PCR analyses on HMGA2 mRNA levels showed that HMGA2 mRNA was elevated in the absence of EDD (Figure 2H). To demonstrate that the abnormal HMGA2 expression seen in EDD knockdown HeLa cells was indeed due to a defect in let-7 mediated silencing, we performed a let-7 reporter assay using Rn-HMGA2-3′UTR-WT reporter which contains seven binding sites for the endogenous let-7 miRNA and a matching control with mutated sites (Rn-HMGA-3′UTR-m7) (Mayr et al., 2007). This assay confirmed that let-7 mediated silencing was compromised in EDD-knockdown HeLa cells, leading to increased HMGA2 expression (Figure 2I). Taken together, we conclude that EDD is a key effector of the miRNA silencing pathway.

EDD knockout impairs miRNA silencing and cell growth in ES cells

To investigate EDD function in ES cells, we examined whether EDD was required for silencing miRNA targets such as Bim, an endogenous miRNA target in ES cells (Spruce et al., 2010; Su et al., 2009; Ventura et al., 2008). In ES cells inducibly expressing EDD shRNA, Bim was upregulated after Dox treatment (Figure 3A). To confirm this finding, we generated Dox-inducible knockout ES cells ($EDD^{tet/tet}$) by introducing the tetracycline-regulated transcription unit at both endogenous EDD alleles (Figure 3B–C). Under normal growth condition, EDD was expressed at close to wild-type levels. Upon Dox induction, EDD expression quickly diminished in $EDD^{tet/tet}$ cells (Figure 3C, Western blot). Meanwhile, Bim protein expression was clearly elevated in inducible EDD knockout cells during a six-day interval (Figure 3D). The elevated Bim protein levels were more

pronounced at early time points whereas no obvious change in Bim levels was found after a prolonged (1 week) Dox treatment (data not shown). We reason this phenomenon is likely due to the negative effect of elevated Bim expression on ES cell growth (Su et al., 2009) and possibly other compensatory changes in these cells. Consistent with the previous observation in Ago-deficient ES cells (Su et al., 2009), EDD knockout induced an upregulation of Bim proteins without changing Bim mRNA levels (Figure 3D–E). Therefore, EDD is required for silencing Bim expression in ES cells.

Mouse ES cells lacking Dicer or all four Agos exhibit a severe proliferation defect (Murchison et al., 2005; Su et al., 2009). To evaluate EDD function in regulating ES cell proliferation, we measured the growth rate of EDD knockout cells. Upon EDD depletion, ES cells grew significantly slower than untreated control or wild type ES cells (Figure 3F). Furthermore, analysis of cell cycle profiles demonstrated that EDD knockout ES cells displayed a roughly 5% reduction in S-phase cells and a corresponding increase in G1 phase cells, indicating that the mutant ES cells underwent a G1 arrest (Figure 3G). Collectively, our data demonstrate that EDD is a key component of the miRNA silencing pathway and EDD play an important role in regulating ES cell proliferation.

EDD is indirectly associated with Ago-miRNA complexes

To further explore EDD function in the miRNA silencing pathway, we investigated whether EDD physically interacted with Ago proteins. Using sucrose gradient ultracentrifugation analysis, we found that endogenous Agos and EDD cofractionated as large protein complexes with a peak around 1,000 KDa in human 293T cells (data not shown). When overexpressed in 293T cells, EDD was associated with Ago2 complexes in co-immunoprecipitation (co-IP) experiments (Figure S3A–C). As expected, EDD interacted with all 4 Agos in 293T cells (Figure S3D). Furthermore, we found that Ago2-EDD interaction was resistant to RNase A or micrococcal nuclease (MN) treatment during cell lysis or after co-IP (Figure S3E–F). Therefore, EDD is in a complex with Ago2 either directly or indirectly through protein-protein interaction.

To demonstrate that Ago-EDD complexes exist at endogenous levels, we first performed a genetic rescue of inducible Ago-knockout ES cells (E7) with FLAG-Ago2 (Figure S4A) (Su et al., 2009). The rescued E7 cells expressed FLAG-Ago2 at levels similar to endogenous Ago2 and suppressed miRNA target Bim upregulation (Figure S4B). Anti-FLAG co-IP experiment showed that Ago2 interacted with endogenous EDD in the rescued E7 cells while no EDD interacted with HA-Ago2 in hypomorphic E7 cells (Figure S4C). In addition, using anti-Pan-Ago monoclonal antibody (Nelson et al., 2007), we immunoprecipitated endogenous Ago complexes from wild type ES cells and identified endogenous EDD in the complexes (Figure S4D). Furthermore, we purified and analyzed miRNA components in FLAG-EDD immune complexes from 293T cells. RT-qPCR showed that mir-19b and mir-221, two abundantly expressed miRNAs in 293T cells, were significantly enriched in EDD complexes, suggesting that Agos are miRNA-bound in EDD complexes (Figure S4E). Lastly, to test whether Ago2 and EDD directly interact with each other, we affinity-purified GST-Ago2 and FLAG-EDD from 2×10^8 stably transfected 293T cells (Figure S4F). Incubation of soluble GST-Ago2 with immobilized FLAG-EDD failed to retain significant amount of Ago2 directly associated with EDD (Figure S4F upper panels). This result showed that the interaction between Ago2 and EDD *in vivo* is indirect and is likely mediated through an additional protein.

EDD interacts with GW182 family proteins

GW182 family proteins directly bind to Agos and PABP proteins (Huntzinger et al., 2010; Jinek et al., 2010; Zekri et al., 2009). Importantly, EDD also shares a similar PABC domain

through which PABPs interact with GW182, thus raising the question whether EDD is associated with Ago through GW182 proteins. We first investigated whether EDD and GW182 genetically interacted with each other in the miRNA pathway using a robust artificial mir-CXCR reporter. Human 293T cells express EDD and 3 members of GW182 family proteins, also known as TNRC6A, 6B and 6C. Transient EDD knockdown induced a subtle silencing defect (about 10-fold repression remained) with mir-CXCR reporter in 293T cells (Figure S5A). Similarly, knockdown of TNRC6A/B/C in 293T cells led to a reduced silencing effect (about 10-fold repression remained) with mir-CXCR (Figure S5B–C). Simultaneous knockdown of EDD and TNRC6A/B/C proteins caused a more pronounced defect (a 4-5 fold repression remained) in mir-CXCR mediated silencing (Figure S5D). Taken together, the results suggest that EDD and GW182 family proteins genetically interact in the miRNA pathway.

To test whether GW182 proteins function as a bridge between Ago and EDD, we performed a series of protein-protein interaction assays. We first showed that overexpressed TNRC6A and EDD were complexed with each other in transiently transfected 293 cells (Figure 4A). To examine whether EDD interacts with endogenous TNRC6A, we established a stable HEK293 cell line that expressed a modest level of FLAG-EDD in comparison with endogenous EDD (Figure 4B, upper panels). FLAG-IP showed that EDD interacted with endogenous TNRC6A (Figure 4B, lower panels). Using *in vitro* GST pull down assay, we showed that the PABC domain of EDD interacted with the PAM2 motif of TNRC6A (Figure 4D). Because both EDD and GW182 proteins consist of UBA domains that potentially mediate dimerization (Kozlov et al., 2007; Su and Lau, 2009), we tested the role of UBA domains in EDD-GW182 interaction using two reciprocal *in vitro* GST pull down experiments, showing that the UBA domain of EDD interacted with the UBA domains of TNRC6A and TNRC6C (Figure 4E–F). Furthermore, using the EDD deletion mutant (EDDΔ) lacking both UBA and PABC domains, we showed that EDD bound to TNRC6A through both UBA hetero-dimerization and PABC-PAM2 interaction (Figure 4G). Finally, we tested whether GW182 proteins were required for *in vivo* association of Ago with EDD. To do so, we stably transfected FLAG-EDD-expressing 293T cells (described in Figure 4B) with a combination of shRNAs against TNRC6A, 6B and 6C (described in Figure S6B). In TNRC6A/B/C knockdown cells, FLAG-IP showed that Ago2 associated with EDD was significantly reduced (Figure 4H). Thus, these results demonstrate that EDD is associated with Ago-miRNA complexes through a protein-protein interaction with GW182 proteins in mammalian cells.

E3 ubiquitin ligase activity is not required for miRNA silencing

Since EDD is a known E3 ubiquitin ligase (Honda et al., 2002; Yoshida et al., 2006), we investigated whether ED regulated the stability of factors involved in miRNA silencing. We generated ES cells homozygous for a mutant EDD bearing an amino acid substitution from cysteine^{2761} to serine^{2761} (hereafter referred to *C→S*) (Figure 5A–B). Substitution of this conserved cysteine abolishes the E3 ubiquitin ligase activity of EDD (Honda et al., 2002). Homozygous $EDD^{C\rightarrow S/C\rightarrow S}$ ES cells are viable and morphologically indistinguishable from wild type or heterozygous ES cells. Abolishment of E3 ubiquitin ligase activity in EDD led to a significant increase of EDD protein level in both $EDD^{+/C\rightarrow S}$ and $EDD^{C\rightarrow S/C\rightarrow S}$ ES cells without any change in EDD mRNA abundance (Figure 5C–D). Thus, E3 ubiquitin ligase activity appears to be critical in regulating the EDD protein level itself. However, miRNA silencing was fully functional in $EDD^{C\rightarrow S/C\rightarrow S}$ ES cells in mir-CXCR reporter assay (Figure 5E). Furthermore, levels of the endogenous miRNA target Bim and EDD-interacting TNRC6A were not altered in $EDD^{+/C\rightarrow S}$ and $EDD^{C\rightarrow S/C\rightarrow S}$ ES cells (Figure 5F). Therefore, E3 ubiquitin ligase activity appears to be dispensable for EDD function in miRNA silencing.

The PABC domain is required for EDD function in miRNA silencing

Having ruled out the involvement of E3 ubiquitin ligase activity in miRNA silencing, we next examined the function of PABC domain in EDD. By gene targeting we generated a mutant $EDD^{\Delta PABC}$ allele that expressed a PABC domain-deleted EDD protein in ES cells (Figure 6A). The mutant protein is internally HA-tagged for easy detection. Although the targeting efficiency for heterozygous $EDD^{+/\Delta PABC}$ ES cells was approximately 25%, in an attempt to obtain homozygous $EDD^{\Delta PABC/\Delta PABC}$ ES cells, we failed to identify any homozygous ES cells after screening 300 clones, suggesting that PABC deletion might abolish EDD function and $EDD^{\Delta PABC/\Delta PABC}$ ES cells might exhibit similar growth defects as EDD knockout cells. Therefore, we generated an inducible EDD shRNA6 that targeted exon 51 in the wild type EDD allele (Figure 6A). As expected, the EDD shRNA6 specifically downregulated transcripts from the wild type allele without affecting mutant protein expression (Figure 6B). After downregulating wild type EDD proteins in $EDD^{+/\Delta PABC}$ ES cells, Bim protein was significantly upregulated as seen in EDD knockout cells (Figure 6C). Therefore, the PABC domain of EDD is required for EDD function in miRNA silencing.

To test whether the PABC domain is sufficient to repress target mRNA translation, we first introduced a λN22HA-GST-$PABC^{EDD}$ fusion protein into 1A2 reporter ES cells in which 5 copies of BoxB sequences are located in the 3′UTR of a Rn luciferase reporter. This assay showed that tethering of the PABC domain of either EDD or PABP to the 3′UTR was sufficient to repress Rn luciferase expression (Figure 6D) whereas it had no effect on a reporter lacking BoxB (Figure 6E), providing evidence for the importance of PABC domain in translation regulation. Furthermore, we performed a genetic rescue in $EDD^{tet/tet}$ ES cells by expressing a fusion protein that consists of both the Ago-binding domain of TNRC6A and the PABC domain of EDD. In this design, Ago-miRNA complexes can recruit the PABC domain into miRISCs. As predicted, expression of TNRC6A-GST-$PABC^{EDD}$ in $EDD^{tet/tet}$ ES cells restored the downregulation of Bim when EDD KO was induced (Figure 6F). Thus, the primary function for EDD in miRNA silencing appears to provide the PABC domain for miRISCs.

EDD interacts with downstream effectors important for miRNA silencing

Genetic evidence strongly supports a critical function for the PABC domain of EDD in miRNA silencing (Figure 6). In addition to GW182 proteins (Figure 4B), the PABC domains of EDD and PABPs have been shown to interact with PAM2 motifs in a number of proteins that are implicated in translation regulation and mRNA decay (Albrecht and Lengauer, 2004; Lim et al., 2006; Siddiqui et al., 2007). These proteins include PAIP1, PAIP2, ATXN2, ATXN2L, GSPT1/eRF3a, GSPT2/eRF3b, Tob1 and Tob2. Using GST fusion proteins that include the PABC domains from EDD and PABPC1, we first confirmed that all PAM2-containing proteins are associated with the PABC domain *in vitro* (Figure 7A). Through these proteins, EDD and PABPs may indirectly interact with several known translation regulator complexes including DDX6/p54/Rck (Nonhoff et al., 2007), eRF3-eRF1 (Alkalaeva et al., 2006), and CAF1-CCR4 deadenylase complexes (Mauxion et al., 2009; Siddiqui et al., 2007). We confirmed these reported or predicted protein-protein interactions in a series of co-IP experiments using epitope-tagged proteins in 293T cells (data not shown). To demonstrate that EDD interacts with these interactors at endogenous levels, we utilized the stable HEK293 cells expressing a modest level of FLAG-EDD (Figure 4B) for anti-FLAG IP experiments. The results showed that EDD interacted with endogenous Ago1, Ago2, GSPT1/2, ATXN2 and DDX6 (Figure 7B). We were not able to confirm other endogenous interactors because no working antibodies were available to us. Collectively, our data confirm all previously known or predicted protein-protein interactions summarized in Figure 7C. Consistent with the observation that EDD E3 ubiquitin ligase

activity does not regulate levels of some PABC-interacting protein in ES cells (Figure 5), we found that levels of EDD-interacting proteins including TNRC6A, ATXN2/2L and GSPT1/2 remained unchanged in both EDD knockdown HeLa cells and EDD knockout ES cells (Figure S6).

Both PABPs and EDD are components of Ago-TNRC6A/B/C protein complexes. Both share the same interactors through their PABC domains (Figure 7C). Therefore, Ago-TNRC6-EDD or Ago-TNRC6-PABP complexes are likely heterogeneous and they might have largely overlapping components. To further explore the function of this protein network in miRNA silencing, we constructed nine fusion proteins individually tagged with λN22HA and tested their ability to suppress the expression of Ff-luc reporter with 5 copies of BoxB in its 3′UTR (Figure 7D). Among these interactors, Tob1, Tob2 and DDX6 reproducibly repressed Ff-luc activity when tethered to the reporter mRNA (Figure 7D). Of note, DDX6/p54/Rck and CAF1-CCR4 that interact with Tob1/2 have been implicated in miRNA silencing (Behm-Ansmant et al., 2006; Chen et al., 2009a; Chu and Rana, 2006; Eulalio et al., 2007; Fabian et al., 2009; Minshall et al., 2009; Piao et al., 2010). However, the role of Tob1/2 in miRNA silencing has not been previously tested. To demonstrate the functional importance of Tob1/2, we asked whether they were required for Ago- and miRNA- mediated gene silencing. Using the 1A2E8 reporter ES cell line described in Figure 2A, we showed that knockdown of Tob1/2 and DDX6 significantly increased the activities of Ago2-repressed reporter Rn-luc whereas control shRNA did not release translation repression (Figure 7F). Similarly, using both mir-CXCR and mir-30 reporter assays in STO cells (Figure 7G–H), knockdown of Tob1/2 and DDX6 led to a compromised miRNA silencing function. Thus, these experiments provide evidence that EDD interactors are important components of the miRNA silencing pathway.

To test whether EDD is required for miRISCs to recruit downstream effectors such as DDX6, we measured the abundance of Ago2-DDX6 complexes in EDD knockdown cells. Co-transfection of FLAG-Ago2 and shRNA against endogenous EDD led to a decreased level of endogenous DDX6 associated with FLAG-Ago2, demonstrating that endogenous EDD has a role in maintaining the integrity of Ago-effector complexes (Figure S7). However, we failed to detect EDD-CNOT7 or Ago-CNOT7 complexes at the endogenous level in similar experiments (data not shown). Although we cannot rule out the possibility of technical failure due to the sensitivity of anti-CNOT7 antibody, this result might reflect the dynamic nature of Ago-CNOT7 or EDD-CNOT7 complexes. Consistent with this latter interpretation, other studies also failed to detect stable association of Tob1/2 or CAF1/CNOT7 with miRISCs (Fabian et al., 2009; Piao et al., 2010) despite its importance in miRNA-mediated silencing (Behm-Ansmant et al., 2006; Chen et al., 2009a). Taken together, our data demonstrate that through the PABC domain, EDD interacts with a network of proteins that are known to play critical roles in regulating translation efficiency and mRNA stability. By recruiting these effectors stably or transiently into miRISCs, EDD and its interactors play a critical function in miRNA-mediated gene silencing.

DISCUSSION

Accumulating evidence demonstrates that in animal species miRNAs regulate target gene expression through translation inhibition and mRNA destabilization (Djuranovic et al., 2011; Huntzinger and Izaurralde, 2011). In this study, we demonstrate that mammalian EDD is a key component of the miRNA silencing pathway. EDD family proteins, including EDD/Ubr5 in vertebrates and *hyperplastic discs (hyd)* in flies are highly conserved. EDD is essential for embryonic development in both mice and flies (Mansfield et al., 1994; Saunders et al., 2004). Genome-wide genetic screens in *Drosophila* S2 cells also identified

hyd as a gene important for miRNA silencing in flies (Zhou et al., 2008). Taken together, EDD function in miRNA silencing appears to be conserved in animals.

EDD is known as an E3 ubiquitin ligase that regulates the stability of the translation regulator PAIP2 (Yoshida et al., 2006). To our surprise, abolishment of E3 ubiquitin ligase function does not affect miRNA silencing in $EDD^{C\rightarrow S/C\rightarrow S}$ ES cells. In addition, EDD knockout does not affect overall levels of EDD interactors including TNRC6A, ATXN2, ATXB2L, GSPT1 and GSPT2. These findings suggest a novel EDD function in the miRNA pathway independent from its E3 ubiquitin ligase activity. By contrast, the PABC domain in EDD is not only essential for EDD function in miRNA silencing but also sufficient to mimic at least some aspects of translational regulation by EDD. Collectively, current data support at least two roles for the PABC domain in miRNA silencing. First, the PABC domain interacts with the PAM2 motif in GW182 proteins. Increasing evidence has demonstrated that GW182 family proteins are an integral component of functional miRISCs (Tritschler et al., 2010). Thus, GW182 protein can recruit PABC-containing proteins EDD and PABP into Ago-miRNA complexes (Fabian et al., 2009; Zekri et al., 2009). In addition to the PABC-PAM2 interface (Eulalio et al., 2009b), both EDD and PABP contain at least a second interface with GW182 proteins through either UBA dimerization for EDD or RRM interaction for PABP (Eulalio et al., 2009b; Huntzinger et al., 2010; Zipprich et al., 2009). Therefore, it is possible for the PABC domains of both proteins to be engaged with other interactors while maintaining an association with Ago-miRNA complexes. Second, data from *in vitro* and *in vivo* protein interaction experiments reveal that EDD and PABP share the same binding partners through PABC-PAM2 interactions although at slightly different affinities (Lim et al., 2006). These PAM2-containing proteins include PAIP1/2, ATXN2/2L ATXN2L, GSPT1/2 and Tob1/2. Through protein interaction with these proteins, an extended protein network includes DDX6/p54/Rck, eRF1, CAF1/CNOT7-CCR4 deadenylase complexes, all of which play key roles in regulating translation efficiency and mRNA stability. More importantly, knockdown of several components in this network also impairs miRNA silencing. These data clearly demonstrate the importance of the EDD protein network in the miRNA pathway.

Combined with other existing evidence (Djuranovic et al., 2011; Huntzinger and Izaurralde, 2011), we incorporate EDD into a working model for multimodal miRNA silencing mechanisms. In this model, miRNA-bound Agos recruit EDD and PABP through GW182 proteins at the 3′UTR of a target mRNA by protein-protein interaction. Once recruited, EDD and PABP interface with another set of downstream effectors to assemble either translation repressor complexes or mRNA destabilizing enzyme complexes. Translation repressor complexes may include DDX6 that inhibits translation at the initiation stage. Consistent with this notion, Dhh1p, the yeast homolog for DDX6, is known to function as a general translation repressor to block 48S preinitiation complex formation or to activate decapping enzymes (Coller and Parker, 2005). DDX6/p54/Rck has also been shown to be important for miRNA silencing function in both *Drosophila* and human cells (Chu and Rana, 2006; Eulalio et al., 2007). EDD and PABP can also interact with Tob1/2 and in turn recruit CCR4-CAF1 complexes to promote deadenylation of target mRNAs (Ezzeddine et al., 2007; Fabian et al., 2009; Zekri et al., 2009), resulting in mRNA destabilization and decoupling from translation. In line with this mechanism, many components of CAF1 deadenylase complexes including CAF1, NOT1 and Tob1/2 (this study) have been shown to be critical for miRNA silencing (Behm-Ansmant et al., 2006; Chen et al., 2009a; Fabian et al., 2009; Zekri et al., 2009). In this model, EDD and PABP function in parallel and have overlapping functions in miRNA silencing. Consistent with this view, the PABC domains from both proteins are sufficient to repress reporter activity and they share the same binding partners. Future experiments will be necessary to determine the relative contribution for EDD or PABP in the pathway.

EXPERIMENTAL PROCEDURES

Construction of reporter ES cell lines

To establish stable ES cell lines that express both control Ff-Luc and reporter Rn-Luc with 5 copies of Box B in the 3′UTR, we sequentially electroporated linearized *CMV-Ff-Luc-IRES-BSD-bPA* and *CMV-Rn-Luc-5BoxB-bPA::PGK-Hprt* into wild type AB2.2 ES cells. After sequential selection with Blasticidin (BSD) and HAT, individual BSD^R and HAT^R ES clones were picked and cultured for dual luciferase assays. These ES cell lines were further evaluated based on the stability of dual luciferase ratios during five consecutive passages. The cell line 1A2 was chosen for all subsequent studies. To investigate Ago-mediated translation repression, *PB-CAG-λN22HA-Ago::PGK-Puro* was integrated into 1A2 cells by PB-mediated transposition. Stable cell lines including 1A2E8 were established by Puro selection.

To establish a drug-selectable reporter ES cell line, a linearized *PGK-Hprt-5BoxB-bPA::PGK-Neo-bPA* vector was electroporated into a $DNMT1^{-/-}$ ES cell line (Guo et al., 2004) and stable clones including “8–4” were obtained by G418 and HAT selection. To maximize the efficiency of shRNA mediated knockdown, we chose $DNMT1^{-/-}$ ES cells as the starting point to construct our reporter cell line because siRNAs can induce transcriptional silencing in a DNA-methylation dependent manner in human cells (Morris et al., 2004). We then overexpressed λN22-Ago2 using a *PiggyBac* transposon that co-expresses a BSD resistant gene. After BSD and 6-TG selection, the H3 reporter cell line was chosen for subsequent genetic screens.

shRNA libraries and RNAi screen

A pooled retroviral pSM2-shRNA-mir30 library (Paddison et al., 2004) and a lentiviral pSIH-H1-Puro shRNA from System Biosciences were used in the screen. We transiently transfected shRNA library plasmids in 293T cells with helper plasmids or in Phoenix cells (a retroviral packaging cell line) to produce high-titer recombinant lentiviruses or retroviruses (Trombly et al., 2009), respectively. The “H3” reporter ES cells were infected with lentiviral or retroviral shRNA library and the infected cells were sequentially selected with puromycin (2 μg/ml) followed with HAT to isolate HAT^R ES colonies. HAT^R ES clones were then expanded to confirm their drug resistance and sensitivity and for isolating genomic DNA. Candidate shRNAs were identified by sequence analysis of PCR-amplified proviral fragments encoding gene-specific shRNAs (primer information see Table S1).

BAC recombineering and gene targeting

To generate EDD^{Tet}, $EDD^{C\rightarrow S}$ and $EDD^{\Delta PABC}$ ES cells, we constructed EDD gene targeting vectors using the BAC recombineering technique as previously described (Su et al., 2009). The original S129 BAC clones containing mouse EDD gene were obtained from the Welcome Trust Sanger Institute. The primers used for constructing retrieving vectors and recombination cassettes are summarized in Table S1. The sequences for individual targeted alleles including the synthesized EDD exon 1–3 for EDD^{Tet}, the cDNA encoding HA-tagged-ΔPABC EDD and the last exon for EDD (C→S) are available upon request. All targeting events were identified by long-range PCR screens and confirmed by Southern blot, RT-PCR for allele-specific transcripts and Western blot for allele-specific proteins. The primer sequences for screening and expression studies are listed in Table S1.

miRNA reporter and dual luciferase assays

mir-CXCR and mir-30 reporter and dual luciferase assays were described previously (Su et al., 2009). In brief, feeder-free ES cells or STO cells were transfected on a 24-well plate using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). For

mir-30 reporter assay, 0.1 μg *CMV-Ff-Luc-mir30x6*, 0.02 μg *CMV-Rn-Luc*, and 0.25 μg *U6-mir30A* were used for each well. For each well, 0.05 μg gene-specific shRNAs were used to knock down endogenous candidate genes for transient transfection experiments. For mir-CXCR assay, 0.1 μg *CMV-Ff-Luc*, 0.02 μg *CMV-Rn-Luc-CXCR*, and 15 nM dsRNA were used for each well. 0.025 μg gene-specific shRNAs were used for transient transfection experiments. For mir-CXCR assay in 293T cells, 2 ng *CMV-Ff-Luc*, 2 ng *CMV-Rn-Luc-CXCR*, and 1.5 nM dsRNA were used for each well. Gene-specific shRNAs were used at a concentration of 0.05 μg per well for transient transfection experiments. For tethering assays in Figure S1 and Figure 6D-E, 1A2 ES cells were transfected with 8 μg *PB-CAG-λN22HA-X::PGK-Puro-bPA* expression vector and 1 μg PBase by electroporation. Stable transfected cells were selected by puromycin (3 μg/ml). For tethering assay in 293T cells (Figure 7E), 5 ng *CMV-Ff-Luc-5BoxB*, 2 ng *CMV-Rn-Luc*, and 100 ng effectors were used for each well. Dual luciferase activity was measured 36 hours after transfection. For shRNA knockdown in the 1A2E8 reporter cell line, ES cells were transfected with *PB-U6-shRNA::PGK-Neo-bPA* and *PBase* by electroporation and stably transfected cells were selected by G418 (500 μg/ml) for 5 days. After releasing from selection for 2 days, different shRNA-expressing cells were plated at the same density (3×10^5 per well in a 24 well plate) and assayed for dual-luciferase activity the following day. Early passages of knockdown cells are necessary to avoid revertants or other genetic compensation in this assay. Dual luciferase assays were carried out with a dual-luciferase kit from Promega. For convenience of plotting, in some figures, the ratios of dual luciferases from control groups were set as 1 for comparison. The "fold of repression" in miRNA reporter assays is defined as dual luciferase ratio of a control group divided by that of an experimental group.

GST pull-down, co-immunoprecipitation and sucrose gradient ultracentrifugation

Bacterially expressed GST fusion proteins were constructed in *pGEX-KG* vectors, expressed and purified as previously described (Chen et al., 2009b). GST-EDD-PABC and GST alone were used in this study. For pull-down assays, aliquots of immobilized GST proteins (10 μg) were incubated with 293T cell lysates that expressed a variety of tagged EDD interactor proteins. The lysates were prepared in a lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1.0% NP-40, 10% glycerol, 1 mM EDTA, 1 mM PMSF, and Roche protease inhibitors). After binding and washing, the GST pulled-down proteins were detected by Western blot with different anti-tag antibodies.

Co-Immunoprecipitation of protein complexes from HEK293T and mouse ES cells was carried out as previously described (Chen et al., 2009b). For high-affinity and specific isolation of protein complexes, we used Flag M2 affinity-gel (Sigma) to purify a variety of FLAG-tagged proteins and their associated protein complexes. The same lysis buffer containing 1% NP-40 or 0.5% Triton X-100 described in GST pull-down assay was used for immunoprecipitation.

Sucrose gradient ultracentrifugation analysis was carried out under the condition for polysome profiling as previously described (Kiriakidou et al., 2007). Briefly, cycloheximide treated 293T cells (0.1 mg/ml) were lysed and pre-clarified at 14,000g for 10 min at 4°C. The supernatant was loaded onto 10%–50% or 5%-50% sucrose gradients with 70% sucrose cushion. Centrifugation was carried out in SW41 rotor at 36,000 rpm for 2 h or 18 h at 4°C. Sucrose fractions were collected using a Bio-Rad LP chromatography system and polysome profiles were monitored by UV absorption at 254 nm.

Cell cycle analysis and RT-qPCR analysis

FACScan analysis of cell cycle was performed using propidium iodide (PI) stained ES cells with a Beckman Coulter Epics XL-MCL at the Northwestern's Flow Cytometry Core Facility (Su et al., 2009).

Total RNA preparation, reverse transcription (RT) and qPCR analyses were performed using Trizol (Invitrogen), Superscript III (Invitrogen) and SYBR green kits (Applied Biosystems). All primer information is provided in Supplementary Table S1.

Statistical analysis

All data are presented as means with standard error of the means. Comparisons between two groups are analyzed using two-tailed unpaired student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, compared to the control groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank M.J. Henderson and C.K. Watts for human EDD cDNA, E. Izaurralde for various TNRC6A constructs, S. Krobitsch for human ATXN2 cDNA and M. Kiriakidou for anti-Ago (2A8) antibody. This work was supported by grants from NIGMS (5R21GM079528) and NU-PSOC (under 5U45CA143869) to X.W.

References

Albrecht M, Lengauer T. Survey on the PABC recognition motif PAM2. Biochem Biophys Res Commun. 2004; 316:129–138. [PubMed: 15003521]

Alkalaeva EZ, Pisarev AV, Frolova LY, Kisselev LL, Pestova TV. In vitro reconstitution of eukaryotic translation reveals cooperativity between release factors eRF1 and eRF3. Cell. 2006; 125:1125–1136. [PubMed: 16777602]

Behm-Ansmant I, Rehwinkel J, Doerks T, Stark A, Bork P, Izaurralde E. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes Dev. 2006; 20:1885–1898. [PubMed: 16815998]

Bushati N, Cohen SM. microRNA functions. Annu Rev Cell Dev Biol. 2007; 23:175–205. [PubMed: 17506695]

Callaghan MJ, Russell AJ, Woollatt E, Sutherland GR, Sutherland RL, Watts CK. Identification of a human HECT family protein with homology to the Drosophila tumor suppressor gene hyperplastic discs. Oncogene. 1998; 17:3479–3491. [PubMed: 10030672]

Carthew RW, Sontheimer EJ. Origins and Mechanisms of miRNAs and siRNAs. Cell. 2009; 136:642–655. [PubMed: 19239886]

Chen CY, Zheng D, Xia Z, Shyu AB. Ago-TNRC6 triggers microRNA-mediated decay by promoting two deadenylation steps. Nat Struct Mol Biol. 2009a

Chen J, Lu Y, Meng S, Han MH, Lin C, Wang X. alpha- and gamma-Protocadherins negatively regulate PYK2. J Biol Chem. 2009b; 284:2880–2890. [PubMed: 19047047]

Chu CY, Rana TM. Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54. PLoS Biol. 2006; 4:e210. [PubMed: 16756390]

Coller J, Parker R. General translational repression by activators of mRNA decapping. Cell. 2005; 122:875–886. [PubMed: 16179257]

Ding XC, Grosshans H. Repression of C. elegans microRNA targets at the initiation level of translation requires GW182 proteins. EMBO J. 2009; 28:213–222. [PubMed: 19131968]

Djuranovic S, Nahvi A, Green R. A parsimonious model for gene regulation by miRNAs. Science. 2011; 331:550–553. [PubMed: 21292970]

Doench JG, Petersen CP, Sharp PA. siRNAs can function as miRNAs. Genes Dev. 2003; 17:438–442. [PubMed: 12600936]

Eulalio A, Huntzinger E, Izaurralde E. GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. Nat Struct Mol Biol. 2008; 15:346–353. [PubMed: 18345015]

Eulalio A, Huntzinger E, Nishihara T, Rehwinkel J, Fauser M, Izaurralde E. Deadenylation is a widespread effect of miRNA regulation. RNA. 2009a; 15:21–32. [PubMed: 19029310]

Eulalio A, Rehwinkel J, Stricker M, Huntzinger E, Yang SF, Doerks T, Dorner S, Bork P, Boutros M, Izaurralde E. Target-specific requirements for enhancers of decapping in miRNA-mediated gene silencing. Genes Dev. 2007; 21:2558–2570. [PubMed: 17901217]

Eulalio A, Tritschler F, Buttner R, Weichenrieder O, Izaurralde E, Truffault V. The RRM domain in GW182 proteins contributes to miRNA-mediated gene silencing. Nucleic Acids Res. 2009b; 37:2974–2983. [PubMed: 19295135]

Ezzeddine N, Chang TC, Zhu W, Yamashita A, Chen CY, Zhong Z, Yamashita Y, Zheng D, Shyu AB. Human TOB, an antiproliferative transcription factor, is a poly(A)-binding protein-dependent positive regulator of cytoplasmic mRNA deadenylation. Mol Cell Biol. 2007; 27:7791–7801. [PubMed: 17785442]

Fabian MR, Mathonnet G, Sundermeier T, Mathys H, Zipprich JT, Svitkin YV, Rivas F, Jinek M, Wohlschlegel J, Doudna JA, et al. Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. Mol Cell. 2009; 35:868–880. [PubMed: 19716330]

Guo G, Wang W, Bradley A. Mismatch repair genes identified using genetic screens in Blm-deficient embryonic stem cells. Nature. 2004; 429:891–895. [PubMed: 15215866]

Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature. 2010; 466:835–840. [PubMed: 20703300]

Hendrickson DG, Hogan DJ, McCullough HL, Myers JW, Herschlag D, Ferrell JE, Brown PO. Concordant regulation of translation and mRNA abundance for hundreds of targets of a human microRNA. PLoS Biol. 2009; 7:e1000238. [PubMed: 19901979]

Honda Y, Tojo M, Matsuzaki K, Anan T, Matsumoto M, Ando M, Saya H, Nakao M. Cooperation of HECT-domain ubiquitin ligase hHYD and DNA topoisomerase II-binding protein for DNA damage response. J Biol Chem. 2002; 277:3599–3605. [PubMed: 11714696]

Humphreys DT, Westman BJ, Martin DI, Preiss T. MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proc Natl Acad Sci U S A. 2005; 102:16961–16966. [PubMed: 16287976]

Huntzinger E, Braun JE, Heimstadt S, Zekri L, Izaurralde E. Two PABPC1-binding sites in GW182 proteins promote miRNA-mediated gene silencing. EMBO J. 2010; 29:4146–4160. [PubMed: 21063388]

Huntzinger E, Izaurralde E. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat Rev Genet. 2011; 12:99–110. [PubMed: 21245828]

Jinek M, Fabian MR, Coyle SM, Sonenberg N, Doudna JA. Structural insights into the human GW182-PABC interaction in microRNA-mediated deadenylation. Nat Struct Mol Biol. 2010; 17:238–240. [PubMed: 20098421]

Kiriakidou M, Tan GS, Lamprinaki S, De Planell-Saguer M, Nelson PT, Mourelatos Z. An mRNA m7G cap binding-like motif within human Ago2 represses translation. Cell. 2007; 129:1141–1151. [PubMed: 17524464]

Kozlov G, Nguyen L, Lin T, De Crescenzo G, Park M, Gehring K. Structural basis of ubiquitin recognition by the ubiquitin-associated (UBA) domain of the ubiquitin ligase EDD. J Biol Chem. 2007; 282:35787–35795. [PubMed: 17897937]

le Sage C, Nagel R, Egan DA, Schrier M, Mesman E, Mangiola A, Anile C, Maira G, Mercatelli N, Ciafre SA, et al. Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. EMBO J. 2007; 26:3699–3708. [PubMed: 17627278]

Lee YS, Dutta A. The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. Genes Dev. 2007; 21:1025–1030. [PubMed: 17437991]

Lim NS, Kozlov G, Chang TC, Groover O, Siddiqui N, Volpon L, De Crescenzo G, Shyu AB, Gehring K. Comparative peptide binding studies of the PABC domains from the ubiquitin-protein

isopeptide ligase HYD and poly(A)-binding protein. Implications for HYD function. J Biol Chem. 2006; 281:14376–14382. [PubMed: 16554297]

Liu J, Rivas FV, Wohlschlegel J, Yates JR 3rd, Parker R, Hannon GJ. A role for the P-body component GW182 in microRNA function. Nat Cell Biol. 2005; 7:1161–1166. [PubMed: 16319968]

Lytle JR, Yario TA, Steitz JA. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5′ UTR as in the 3′ UTR. Proc Natl Acad Sci U S A. 2007; 104:9667–9672. [PubMed: 17535905]

Mansfield E, Hersperger E, Biggs J, Shearn A. Genetic and molecular analysis of hyperplastic discs, a gene whose product is required for regulation of cell proliferation in Drosophila melanogaster imaginal discs and germ cells. Dev Biol. 1994; 165:507–526. [PubMed: 7958417]

Mathonnet G, Fabian MR, Svitkin YV, Parsyan A, Huck L, Murata T, Biffo S, Merrick WC, Darzynkiewicz E, Pillai RS, et al. MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. Science. 2007; 317:1764–1767. [PubMed: 17656684]

Mauxion F, Chen CY, Seraphin B, Shyu AB. BTG/TOB factors impact deadenylases. Trends Biochem Sci. 2009

Mayr C, Hemann MT, Bartel DP. Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. Science. 2007; 315:1576–1579. [PubMed: 17322030]

Minshall N, Kress M, Weil D, Standart N. Role of p54 RNA helicase activity and its C-terminal domain in translational repression, P-body localization and assembly. Mol Biol Cell. 2009; 20:2464–2472. [PubMed: 19297524]

Morris KV, Chan SW, Jacobsen SE, Looney DJ. Small interfering RNA-induced transcriptional gene silencing in human cells. Science. 2004; 305:1289–1292. [PubMed: 15297624]

Murchison EP, Partridge JF, Tam OH, Cheloufi S, Hannon GJ. Characterization of Dicer-deficient murine embryonic stem cells. Proc Natl Acad Sci U S A. 2005; 102:12135–12140. [PubMed: 16099834]

Nelson PT, De Planell-Saguer M, Lamprinaki S, Kiriakidou M, Zhang P, O'Doherty U, Mourelatos Z. A novel monoclonal antibody against human Argonaute proteins reveals unexpected characteristics of miRNAs in human blood cells. RNA. 2007; 13:1787–1792. [PubMed: 17720879]

Nonhoff U, Ralser M, Welzel F, Piccini I, Balzereit D, Yaspo ML, Lehrach H, Krobitsch S. Ataxin-2 interacts with the DEAD/H-box RNA helicase DDX6 and interferes with P-bodies and stress granules. Mol Biol Cell. 2007; 18:1385–1396. [PubMed: 17392519]

Olsen PH, Ambros V. The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev Biol. 1999; 216:671–680. [PubMed: 10642801]

Paddison PJ, Silva JM, Conklin DS, Schlabach M, Li M, Aruleba S, Balija V, O'Shaughnessy A, Gnoj L, Scobie K, et al. A resource for large-scale RNA-interference-based screens in mammals. Nature. 2004; 428:427–431. [PubMed: 15042091]

Petersen CP, Bordeleau ME, Pelletier J, Sharp PA. Short RNAs repress translation after initiation in mammalian cells. Mol Cell. 2006; 21:533–542. [PubMed: 16483934]

Piao X, Zhang X, Wu L, Belasco JG. CCR4-NOT deadenylates mRNA associated with RNA-induced silencing complexes in human cells. Mol Cell Biol. 2010; 30:1486–1494. [PubMed: 20065043]

Pillai RS, Artus CG, Filipowicz W. Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. Rna. 2004; 10:1518–1525. [PubMed: 15337849]

Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basyuk E, Bertrand E, Filipowicz W. Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science. 2005; 309:1573–1576. [PubMed: 16081698]

Saunders DN, Hird SL, Withington SL, Dunwoodie SL, Henderson MJ, Biben C, Sutherland RL, Ormandy CJ, Watts CK. Edd, the murine hyperplastic disc gene, is essential for yolk sac vascularization and chorioallantoic fusion. Mol Cell Biol. 2004; 24:7225–7234. [PubMed: 15282321]

Siddiqui N, Mangus DA, Chang TC, Palermino JM, Shyu AB, Gehring K. Poly(A) nuclease interacts with the C-terminal domain of polyadenylate-binding protein domain from poly(A)-binding protein. J Biol Chem. 2007; 282:25067–25075. [PubMed: 17595167]

Spruce T, Pernaute B, Di-Gregorio A, Cobb BS, Merkenschlager M, Manzanares M, Rodriguez TA. An early developmental role for miRNAs in the maintenance of extraembryonic stem cells in the mouse embryo. Dev Cell. 2010; 19:207–219. [PubMed: 20708584]

Stefani G, Slack FJ. Small non-coding RNAs in animal development. Nat Rev Mol Cell Biol. 2008; 9:219–230. [PubMed: 18270516]

Su H, Trombly MI, Chen J, Wang X. Essential and overlapping functions for mammalian Argonautes in microRNA silencing. Genes Dev. 2009; 23:304–317. [PubMed: 19174539]

Su V, Lau AF. Ubiquitin-like and ubiquitin-associated domain proteins: significance in proteasomal degradation. Cell Mol Life Sci. 2009; 66:2819–2833. [PubMed: 19468686]

Tasaki T, Mulder LC, Iwamatsu A, Lee MJ, Davydov IV, Varshavsky A, Muesing M, Kwon YT. A family of mammalian E3 ubiquitin ligases that contain the UBR box motif and recognize N-degrons. Mol Cell Biol. 2005; 25:7120–7136. [PubMed: 16055722]

Thermann R, Hentze MW. Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation. Nature. 2007; 447:875–878. [PubMed: 17507927]

Tritschler F, Huntzinger E, Izaurralde E. Role of GW182 proteins and PABPC1 in the miRNA pathway: a sense of deja vu. Nat Rev Mol Cell Biol. 2010; 11:379–384. [PubMed: 20379206]

Trombly MI, Su H, Wang X. A genetic screen for components of the mammalian RNA interference pathway in Bloom-deficient mouse embryonic stem cells. Nucleic Acids Res. 2009; 37:e34. [PubMed: 19223321]

Ventura A, Young AG, Winslow MM, Lintault L, Meissner A, Erkeland SJ, Newman J, Bronson RT, Crowley D, Stone JR, et al. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. Cell. 2008; 132:875–886. [PubMed: 18329372]

Wiederschain D, Wee S, Chen L, Loo A, Yang G, Huang A, Chen Y, Caponigro G, Yao YM, Lengauer C, et al. Single-vector inducible lentiviral RNAi system for oncology target validation. Cell Cycle. 2009; 8:498–504. [PubMed: 19177017]

Wu L, Fan J, Belasco JG. MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci U S A. 2006; 103:4034–4039. [PubMed: 16495412]

Yoshida M, Yoshida K, Kozlov G, Lim NS, De Crescenzo G, Pang Z, Berlanga JJ, Kahvejian A, Gehring K, Wing SS, et al. Poly(A) binding protein (PABP) homeostasis is mediated by the stability of its inhibitor, Paip2. EMBO J. 2006; 25:1934–1944. [PubMed: 16601676]

Zekri L, Huntzinger E, Heimstadt S, Izaurralde E. The silencing domain of GW182 interacts with PABPC1 to promote translational repression and degradation of microRNA targets and is required for target release. Mol Cell Biol. 2009; 29:6220–6231. [PubMed: 19797087]

Zeng Y, Wagner EJ, Cullen BR. Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. Mol Cell. 2002; 9:1327–1333. [PubMed: 12086629]

Zhang L, Ding L, Cheung TH, Dong MQ, Chen J, Sewell AK, Liu X, Yates JR 3rd, Han M. Systematic identification of C. elegans miRISC proteins, miRNAs, and mRNA targets by their interactions with GW182 proteins AIN-1 and AIN-2. Mol Cell. 2007; 28:598–613. [PubMed: 18042455]

Zhou R, Hotta I, Denli AM, Hong P, Perrimon N, Hannon GJ. Comparative analysis of argonaute-dependent small RNA pathways in Drosophila. Mol Cell. 2008; 32:592–599. [PubMed: 19026789]

Zipprich JT, Bhattacharyya S, Mathys H, Filipowicz W. Importance of the C-terminal domain of the human GW182 protein TNRC6C for translational repression. RNA. 2009; 15:781–793. [PubMed: 19304925]

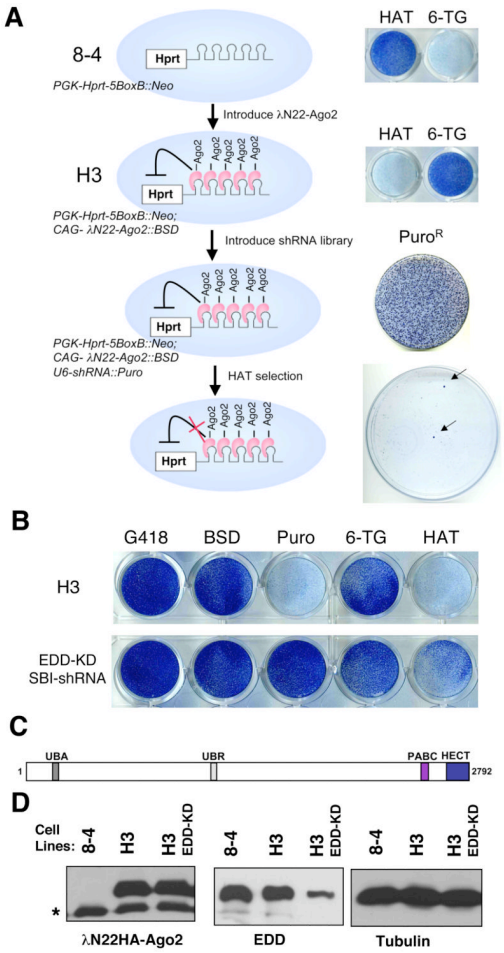

Figure 1. An RNAi screen revealed a novel function for EDD in Ago2-mediated translational repression

A. Experimental design for a drug selection based genetic screen to identify genes that are required for λN22HA-Ago2 mediated silencing on an Hprt-5BoxB reporter. A series of constructs were linked to various drug resistance genes: Neo (neomycin resistance gene), a PGK-Neo-bPA linked with Hprt minigene; BSD (Blasticidin S Deaminase), a PGK-BSD-bPA linked with λN22HA-Ago2; Puro (puromycin resistance gene), a PGK-Puro-bPA linked with shRNA. Shown are drug resistance and sensitivity of the reporter ES cells at different experimental stages. **B.** Confirmation of drug resistance and sensitivity for an identified EDD-shRNA-expressing ES cell clone. **C.** A schematic showing the known domain structure of EDD protein. **D.** Western blot analyses confirmed that EDD protein was downregulated in HAT resistant H3 cells expressing EDD-shRNA while no change of λN22HA-Ago2 was seen. * indicates a protein that cross-reacts with 12CA5 anti-HA antibody.

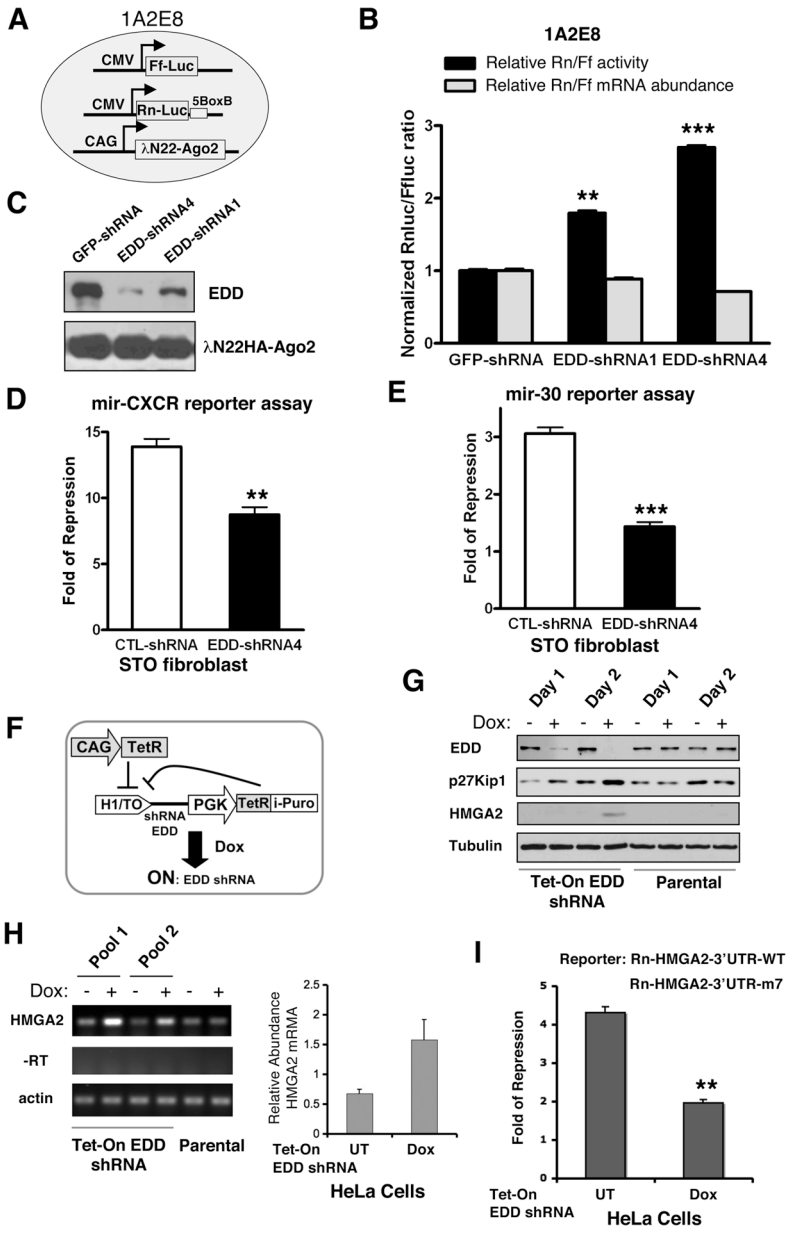

Figure 2. EDD is a key effector for miRNA silencing

A. In an ES cell reporter cell line (1A2E8), Rn-Luc activity is repressed by λN22HA-Ago2. Ff-Luc serves as an internal control. **B**. EDD is required for λN22HA-Ago2-mediated translation repression of Rn-Luc reporter. Shown are relative ratios Rn/Ff-luc activity and mRNA abundance (n=3). Rn-Luc activity was significantly increased in the reporter cells expressing different EDD shRNAs while no obvious change was seen for Rn-Luc mRNA. **C.** Western blot showing that EDD was significantly downregulated in EDD-shRNA-expressing 1A2E8 cells. **D–E.** miRNA silencing is significantly compromised in EDD knockdown STO cells. STO fibroblasts were transiently transfected with EDD shRNA-4 and dual luciferase reporters and effectors. Both mir-CXCR and mir-30 reporter assays demonstrated a defect in miRNA silencing in EDD knockdown cells. **F.** An inducible shRNA expression system in HeLa cells. HeLa cells are first stably transfected with a PiggyBac (PB) vector expressing Tet repressor (TetR) under the control of CAG promoter.

An inducible RNAi PB transposon is subsequently introduced to express EDD shRNA. The shRNA is under the control of a chimeric human H1 and TetO promoter. The integration of H1/TO-shRNA is selected with puromycin resistance conferred by PGK-TetR-IRES-Puro-bPA in the same PB transposon. In the absence of doxcycline (Dox), TetR suppresses shRNA expression. Upon Dox treatment, shRNA is induced. **G.** miRNA silencing defects in Dox-induced EDD knockdown HeLa cells. Endogenous miRNA targets p27Kip1 and HMGA2 were upregulated upon EDD depletion in HeLa cells. Due to the sensitivity of HMGA2 antibody and the expression level of HMGA2 in HeLa cells, endogenous HMGA2 was only detected 2 days after EDD knockdown. **H.** In EDD knockdown HeLa cells, HMGA2 mRNA was upregulated. RT-PCR and RT-qPCR showed an increased level of HMGA2 message 2 days after Dox induction. HMGA2 mRNA abundance was normalized to actin in each sample. For the left panel, 32 cycles were used for HMGA2 and 21 cycles were used for actin. **I.** The let-7 HMGA2-3′UTR reporter assay in HeLa cells confirmed that increased levels of HMGA2 protein and HMGA2 mRNA were due to a defect in miRNA-silencing. In the absence or presence of Dox, inducible EDD knockdown HeLa cells were transfected with Rn-HMGA2-3′UTR-WT or Rn-HMGA2-3′UTR-m7 (in which 7 let-7 binding sites had been mutated). Ff luciferase was used as an internal control. The let-7 mediated repression was measured by comparing normalized expression levels for Rn-HMGA2-3′UTR-WT versus Rn-HMGA2-3′UTR-m7. The result revealed a defect of let-7 mediated HMGA2 silencing in EDD-depleted HeLa cells. All results are shown as means ± SEM from 3–6 independent transfections and subjected to two-tailed *t*-tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

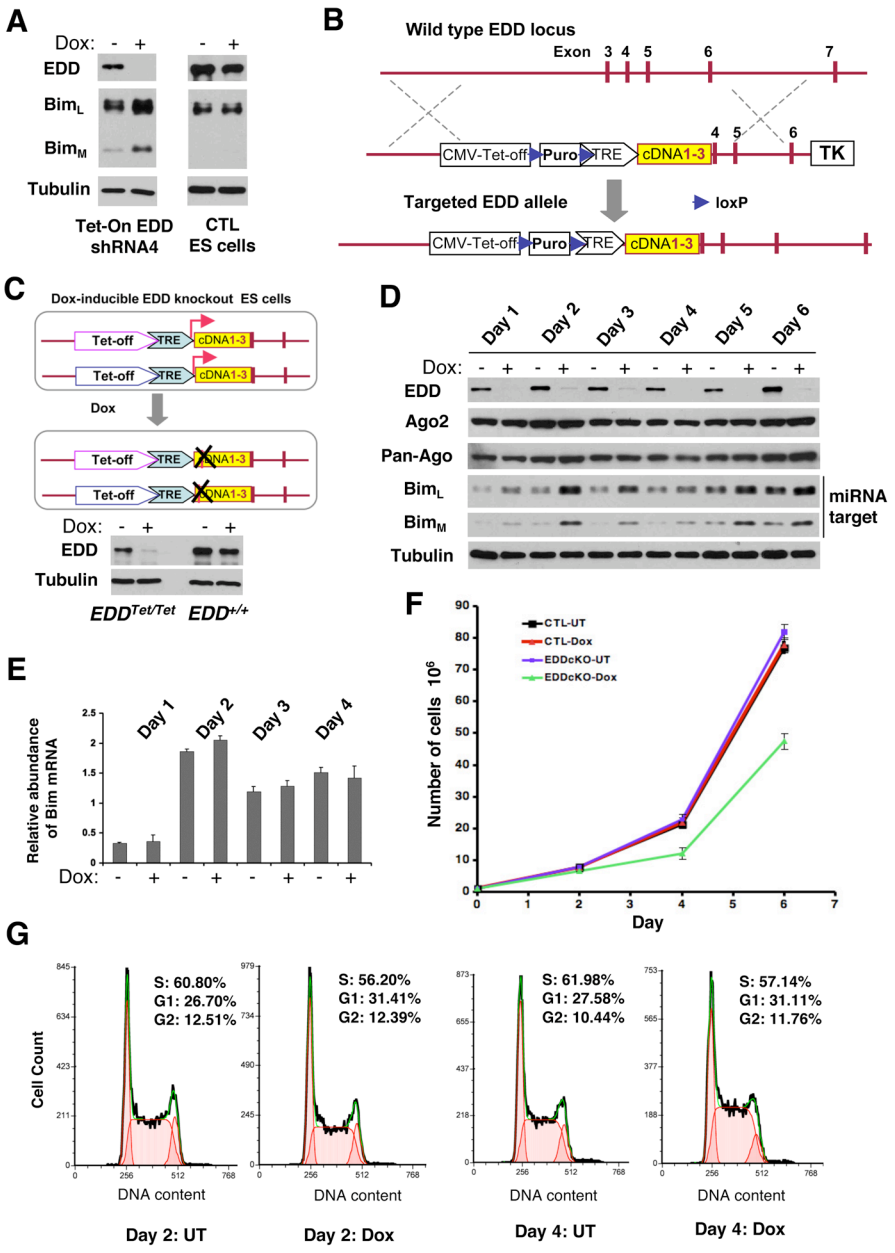

Figure 3. EDD knockout impairs miRNA silencing and cell growth in ES cells

A. shRNA-mediated EDD knockdown upregulates Bim, an endogenous miRNA target in ES cells. Using an inducible EDD knockdown strategy similar to that in Figure 2F, a known miRNA target Bim was upregulated in mouse ES cells after EDD knockdown. Two isoforms of Bim, Bim_L and Bim_M are shown. **B**. A diagram illustrating the design of Tet-controlled EDD knockout allele. In this allele, knock-in of tet-responsible element (TRE) at the endogenous EDD locus enables TetR-VP16 transactivator to regulate EDD transcription in a Dox-dependent manner. To achieve high-level EDD expression that is comparable to endogenous EDD, the gene targeting experiments were carried out in *PB-CAG-TetRVP16::PGK-Hyg*-transfected ES cells independent from *CMV-TetRVP16* linked to the targeted alleles. **C.** In *EDD*$^{tet/tet}$ homozygous ES cells, Western blots showed that EDD expression was under the control of Dox. **D–E.** Inducible EDD knockout led to increased

levels of Bim proteins without significant changes of Bim mRNA levels. The Western blots (in D) showed that Bim_L and Bim_M levels were significantly upregulated during a 6-day interval after Dox induction whereas the RT-qPCR analysis (in E) found no significant difference in Bim mRNA abundance. GAPDH was used to normalize relative Bim mRNA abundance. The cells were passaged at the end of Day 0, 2 and 4. **F.** EDD knockout impaired ES cell growth. A growth curve showing that ES cells grew significantly slower after EDD depletion (EDD cKO with Dox treatment). **G**. Cell cycle analysis showing that EDD knockout ES cells had a decrease in S phase population and an increase in G1 phase population, consistent with the overall slow growth phenotype observed in F. Error bars in E and F indicate mean ± SEM for three experiments.

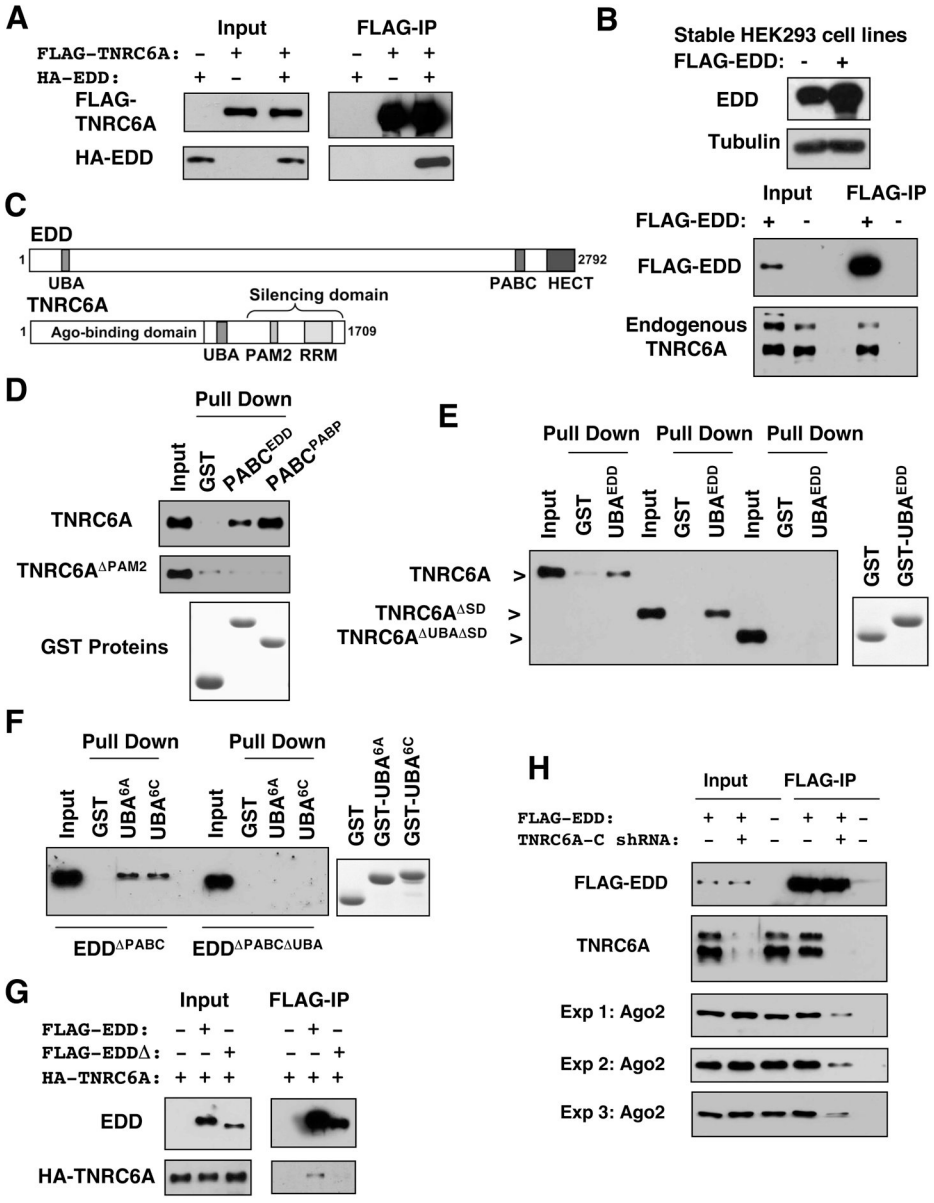

Figure 4. EDD interacts with GW182 family proteins

A. HA-EDD was associated with FLAG-TNRC6A in HEK 293T cells in a co-immunoprecipitation experiment. **B.** Stable 293T cells express a modest level of FLAG-EDD. Shown is a rabbit anti-EDD Western blot detecting both endogenous and FLAG-EDD in 293T cells (upper panels). A co-IP experiment showing that FLAG-EDD interacted with endogenous TNRC6A in stable FLAG-EDD expressing 293T cells (lower panels). **C.** A schematic showing functional domains of EDD and TNRC6A. **D.** The PABC domain of EDD interacted with the PAM2 motif in TNRC6A. GST-pull down assay showed that immobilized GST- $PABC^{EDD}$ as well as GST- $PABC^{PABP}$ but not GST alone interacted with full-length TNRC6A. No interaction with mutant TNRC6A without PAM2 motif was detected above the background signal. **E.** Interaction between the UBA domains of EDD and TNRC6A. GST-pull down assay mapped the UBA domain in TNRC6A was responsible for binding of TNRC6A with GST-UBA^{EDD}. **F.** A reciprocal experiment as shown in E showing that UBA domains from TNRC6A and TNRC6C interacted with the UBA domain

in EDD using EDD deletion mutants. **G.** A co-IP experiment in 293T cells showing that mutant EDD lacking both PABC and UBA domains (EDDΔ) failed to interact with HA-TNRC6A. **H.** GW182 family proteins are required for the association of EDD with Ago proteins. In 293T cells that stably express FLAG-EDD (shown in 4B), a combination of shRNAs against TNRC6A, 6B and 6C were stably transfected to down-regulate endogenous TNRC6 proteins. FLAG-IP was carried out to compare the abundance of endogenous Ago2 associated with EDD. In TNRC6-C knockdown samples, FLAG-EDD associated Ago2 was significantly decreased. Shown are three independent IP experiments.

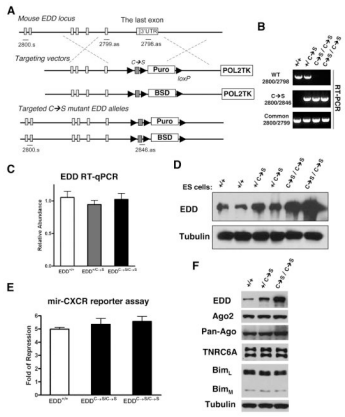

Figure 5. The E3 ubiquitin ligase activity is dispensable for EDD function in miRNA silencing

A. A gene targeting strategy to generate ES cells homozygous for C→S mutant EDD. Shown are part of wild type mouse *EDD* locus containing the last exon, EDD targeting vectors and targeted C→S mutant alleles. The mutant alleles contain the synthesized last EDD exon (C→S) marked in red. **B**. RT-PCR analyses showing the expression of allele-specific EDD transcripts. Primers are labeled on wild type and mutant alleles in A. **C**. RT-qPCR analysis showing similar levels of EDD mRNA in +/+, *+/C→S* and *C→S/C→S* ES cells. **D.** Western blots showing increased protein stability of EDD proteins in $EDD^{C\rightarrow S}$ ES cells. **E**. A luciferase-based mir-CXCR reporter assay showed a normal miRNA silencing function in $EDD^{C\rightarrow S/C\rightarrow S}$ ES cells. **F.** Western blot analyses showing that endogenous miRNA target Bim was not upregulated and EDD-interacting TNRC6A was not affected in $EDD^{C\rightarrow S/C\rightarrow S}$ ES cells. Error bars in C and E indicate mean ± SEM for three experiments.

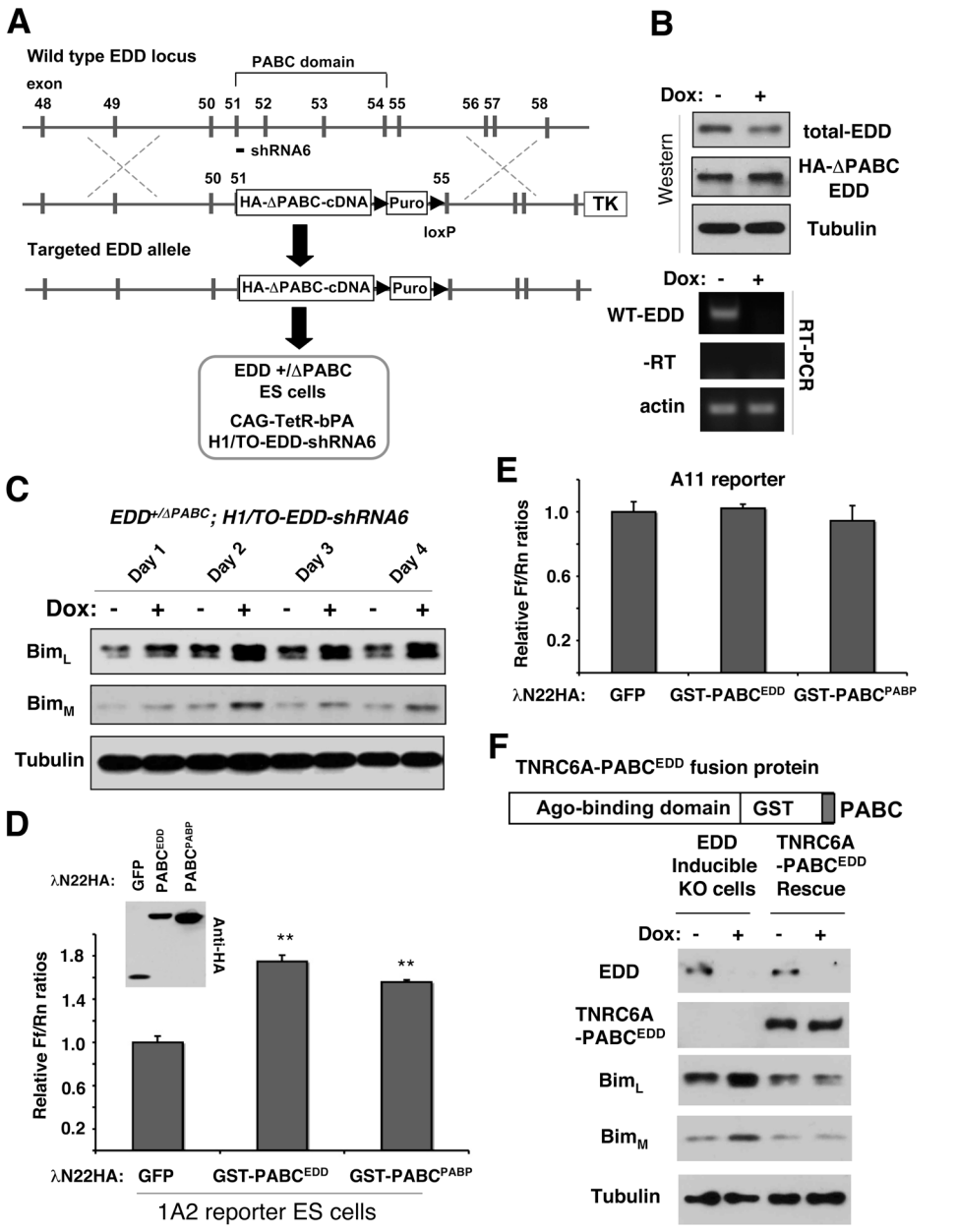

Figure 6. The PABC domain is required for EDD function in miRNA silencing

A. A gene targeting strategy to generate *EDD*$^{\Delta PABC}$ mutant allele that encodes a PABC domain-deleted EDD protein. Exons 51–54 from the wild type EDD allele encode the entire PABC domain. In the mutant allele, part of exon 51 and exons 52–54 are deleted and replaced with a cDNA fragment that is in-frame with part of exon 51. The cDNA fragment includes a HA-tag, the remaining EDD coding exons 55–60 and a poly-A termination signal of SV40 origin. An inducible wild type allele-specific shRNA (EDD-shRNA6) is designed to target the deleted portion of exon 51 in *EDD*$^{\Delta PABC/+}$ cells using the same strategy shown in Figure 2F. **B.** Western blot analysis confirmed that the HA-tagged mutant EDD protein was expressed (middle panel) and the total EDD level was slightly decreased upon depletion of wild-type EDD (upper panel). RT-PCR confirmed the knockdown of wild type EDD allele (27 cycles for EDD and 21 cycles for actin). **C.** The PABC domain is required for

EDD function in the miRNA silencing pathway. Knockdown of wild type EDD led to an upregulation of endogenous miRNA target Bim in heterozygous $EDD^{\Delta PABC/+}$ ES cells. **D.** The PABC domain is sufficient to repress reporter expression in a tethering assay. To test whether the PABC domain from EDD is capable of suppressing translation when tethered to the 3′UTR of a reporter mRNA, we stably expressed λN22 version of GST-$PABC^{EDD}$ and GST-$PABC^{PABP}$ in the 1A2 reporter ES cell line (see Figure S1A). Dual luciferase reporter assays showed that both PABC domains are sufficient to repress Rn expression when tethered to the 3′UTR of Rn mRNA. The results are shown as means ± SEM from 3 independent transfections and subjected to two-tailed *t*-tests. ** $p < 0.01$, *** $p < 0.0001$. **E.** As a negative control, when λN22-GST-PABC proteins were expressed in the A11 ES cells that express dual luciferase reporter lacking BoxB sequences (in Figure S1E), they did not alter the ratios of Ff/Rn. Error bars indicate mean ± SEM for three experiments. **F.** Genetic rescue of miRNA silencing defect in EDD knockout ES cells. A fusion protein was constructed to contain the Ago-binding domain from TNRC6A and GST-$PABC^{EDD}$ (TNRC6A-$PABC^{EDD}$). Expression of TNRC6A-$PABC^{EDD}$ rescued the miRNA silencing defect of Bim in $EDD^{tet/tet}$ homozygous ES cells in the presence of Dox.

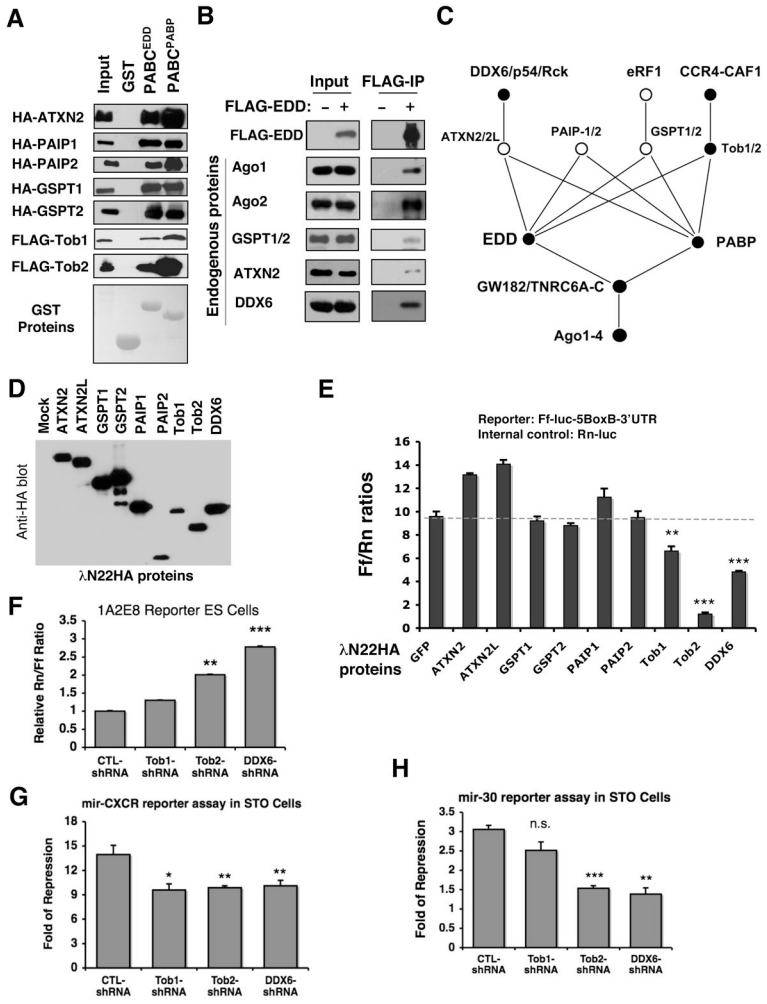

Figure 7. EDD interacts with multiple downstream effectors

A. GST-pull down assay showed that GST- PABC^{EDD} as well as GST- PABC^{PABP} interacted with tagged versions of ATXN2, PAIP1, PAIP2, GSPT1, GSPT2, Tob1 and Tob2 in 293T cell lysates. Shown are anti-Tag Western blots and a Coomassie stained gel picture (bottom panel). **B.** Co-IP experiments showed that EDD interacted with endogenous Ago1, Ago2, GSPT1/2, ATXN2 and DDX6 in stable FLAG-EDD expressing 293T cells (described in Fig. 4B). **C.** Summary of an EDD protein-protein interaction network. The proteins implicated in miRNA silencing are marked with closed circles whereas the proteins with open circles have not been tested for this function. **D.** Expression of λN22HA version of EDD-interacting proteins in 293T cells. **E.** A tethering assay using Ff-luc-5BoxB-3′UTR reporter showed that among these interactors, λN22HA-Tob1/2 and λN22HA-DDX6 repressed Ff-luc activity. **F.** Ago2 mediated Rn-luc silencing was defective in 1A2E8 reporter ES cells that expressed shRNAs against Tob1, Tob2 and DDX6, respectively. Shown are triplicates of Rn/Ff-luc ratios. Knockdown of Tob1, Tob2 and DDX6 was verified (data not shown). **G–H.** Knockdown of Tob1, Tob2 and DDX6 impairs miRNA silencing in STO cells. mir-CXCR assay and mir-30 assays are shown in D and E respectively. All results are shown as means ± SEM from at least 4 independent transfections. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.