# LIM-domain proteins, LIMD1, Ajuba, and WTIP are required for microRNA-mediated gene silencing

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**In recent years there have been major advances with respect to the identification of the protein components and mechanisms of microRNA (miRNA) mediated silencing. However, the complete and precise repertoire of components and mechanism(s) of action remain to be fully elucidated. Herein we reveal the identification of a family of three LIM domain-containing proteins, LIMD1, Ajuba and WTIP (Ajuba LIM proteins) as novel mammalian processing body (P-body) components, which highlight a novel mechanism of miRNA-mediated gene silencing. Furthermore, we reveal that LIMD1, Ajuba, and WTIP bind to Ago1/2, RCK, Dcp2, and eIF4E in vivo, that they are required for miRNA-mediated, but not siRNA-mediated gene silencing and that all three proteins bind to the mRNA 5′ m$^7$GTP cap–protein complex. Mechanistically, we propose the Ajuba LIM proteins interact with the m$^7$GTP cap structure via a specific interaction with eIF4E that prevents 4EBP1 and eIF4G interaction. In addition, these LIM-domain proteins facilitate miRNA-mediated gene silencing by acting as an essential molecular link between the translationally inhibited eIF4E-m$^7$GTP-5′cap and Ago1/2 within the miRISC complex attached to the 3′-UTR of mRNA, creating an inhibitory closed-loop complex.**

argonaute | eIF4E | tumor suppressor | P-bodies | m$^7$GTP cap

MicroRNAs (miRNAs) are small noncoding RNAs that play important roles in a wide range of biological processes including development, cellular differentiation, proliferation, apoptosis, and cancer (1). To execute their regulatory functions miRNAs assemble together with the Argonaute (Ago) proteins into miRNA induced silencing complexes (miRISCs) (2). miRNAs within these complexes guide the bound Ago proteins to fully or partially complementary mRNA target sequences, resulting in mRNAs that are then silenced posttranscriptionally (2). Despite major advances in identifying key components of this pathway the complete protein repertoire and mechanism(s) of miRNA-mediated silencing remain to be fully elucidated. Here we show a distinct mechanism of miRNA-mediated silencing through the identification of three unique mammalian processing body (P-body) associated proteins, LIMD1, Ajuba, and WTIP (Ajuba LIM proteins) (3). The Ajuba LIM proteins are part of the larger Zyxin family, characterized by triple tandem arrayed c-terminal zinc-finger LIM domains. We demonstrate that these three LIM-domain containing proteins (4) interact with components of RNA induced silencing complexes (RISC) as well as eIF4E and the mRNA m$^7$GTP cap–protein complex and are required for miRNA-mediated gene silencing. We propose a model whereby the LIMD1, Ajuba, and WTIP proteins enable miRNA-mediated silencing by facilitating a simultaneous association between the translationally repressed m$^7$GTP cap structure and an active miRISC complex attached to the 3′-UTR of mRNA. We propose this role in miRNA-mediated regulation represents a tumor suppressive function of Ajuba LIM proteins, specifically LIMD1, which has previously been validated as a bona fide tumor suppressor in lung cancer. This function is in keeping with current evidence implicating the disruption of the miRNA pathway with cancer initiation and development (5).

## Results and Discussion

**LIMD1 Is a Component of Cytoplasmic P-bodies.** LIMD1 and its family members have been shown to localize to sites of cell adhesion where their activity regulates cell–cell adhesion, migration, and intracellular signaling via a nucleo-cytoplasmic shuttling function (4, 6). However, there is a cytoplasmic reservoir of LIMD1 not associated with the cytoskeleton the function of which is unclear (7). We performed a series of experiments to characterize the localization and thus infer possible functions of cytoplasmic LIMD1. Enhanced green fluorescent protein-tagged (EGFP) LIMD1 was expressed in U2OS cells and we observed a variety of subcellular localization patterns (Fig. S1). Of specific interest was the presence of discrete cytoplasmic foci, a pattern that showed remarkable similarity to that described for mammalian P-bodies. P-bodies are currently believed to form as a consequence of miRNA-mediated silencing and are sites where repressed mRNAs accumulate and are subject to degradation or storage (3, 8, 9). P-bodies are identified by the composition of proteins that function in cap-dependent translation (eIF4E), translation suppression (RCK), RNA interference-mediated posttranscriptional gene silencing (Ago2, GW182), and decapping of mRNA (Dcp2) (10–12). To determine if these LIMD1 associated cytoplasmic foci also contain P-body associated proteins, YFP-tagged Ago2, RCK/p54, Dcp2, and eIF4E were coexpressed in U2OS cells with mTangerine-tagged LIMD1 (mTan-LIMD1) (Fig. 1 *A* and *B*). We observed colocalization between mTan-LIMD1 and YFP-eIF4E and YFP-Dcp2 and to a lesser extent (but still significant) between mTan-LIMD1 and YFP-RCK/p54 and YFP-Ago2 (Fig. 1 *A* and *B*). The quantified colocalization coefficient values confirmed the degree of

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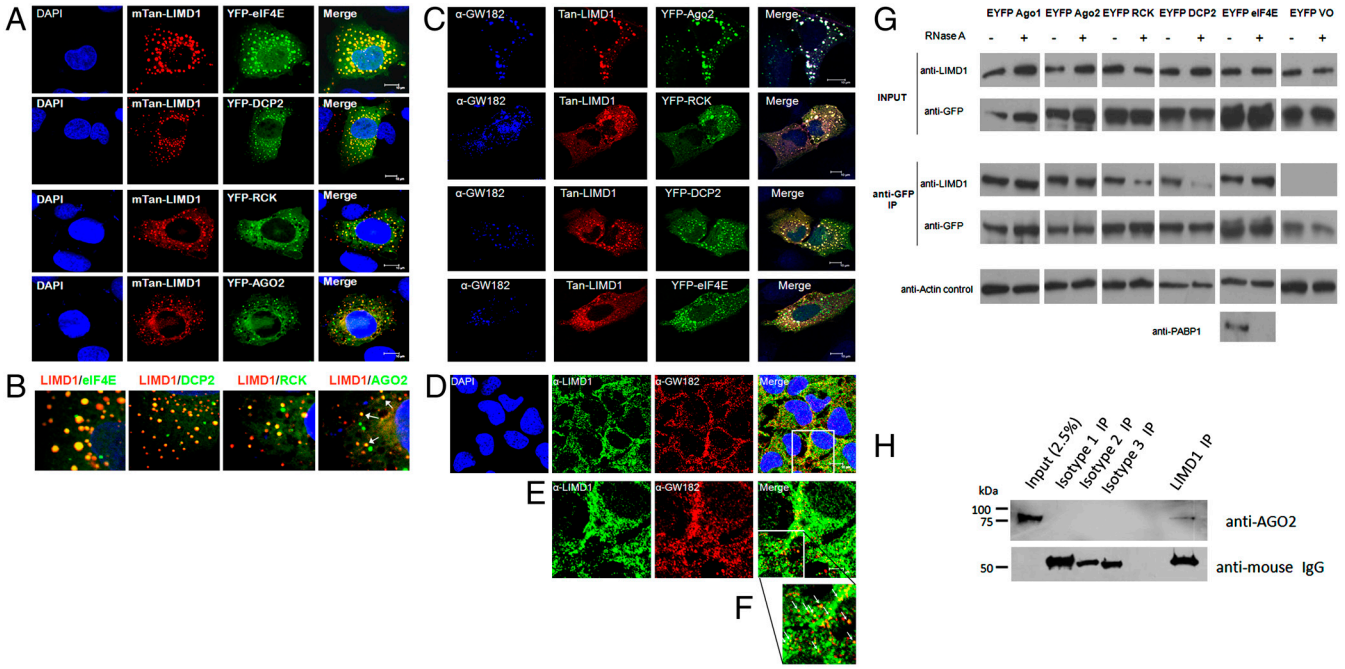


**Fig. 1.** In vivo colocalization and binding of LIMD1 to eIF4E, Dcp2, Ago2, and RCK within P-bodies. (*A*) U2OS cells transiently cotransfected to express mTAN-LIMD1 and the indicated YFP-tagged P-body proteins, were fixed 48 h posttransfection and proteins visualized by confocal microscopy. (*B*) Digital zoom showing greater detail of the degree of colocalization for the indicated merged panels. (*C*) Endogenous LIMD1 and GW182 colocalize in U2OS cells. U2OS cells were cotransfected with mTan-LIMD1 and the indicated EYFP-P-body marker proteins. At 48 h posttransfection, cells were fixed and then immunocytochemistry performed using anti-GW182 (human serum #18033). Triple colocalization is indicated by white coloration in merged panel. Scale bar = 10 μm. (*D*) U2OS cells were fixed and then immunocytochemistry performed using anti-LIMD1 (monoclonal 3F2/C6) and anti-GW182 (human serum #18033 ) to detect endogenous proteins. Scale bar = 10 μm. (*E*) Higher magnification of boxed region in (*D*), Scale bar = 5 μm and (*F*) digital zoom of boxed region in (*E*) to highlight colocalized (yellow) LIMD1/GW182 positive foci/P-bodies (white arrows). (*G*) Extracts derived from U2OS cells expressing Xpress®-tagged LIMD1 together with the indicated YFP-tagged proteins, were incubated in the absence or presence of RNAse A and proteins recovered by immunoprecipitation with anti-GFP antibodies. (*H*) Endogenous coprecipitation of Ago2 with LIMD1. 2.5 μg of anti-LIMD1 (monoclonal 3F2/C6) or isotype specific controls were used for immunoprecipitation. Proteins detected by Western blotting with anti-Ago2 (C34C6) antibody and antimouse IgG shown as input control.

colocalization visualized for the mTan-LIMD1 and YFP-P-body component pairs (Fig. S2).

GW182 is reported to be required for P-body formation and in conjunction with Ago2 essential for miRNA-mediated gene silencing (13). We therefore, performed additional colocalization studies to visualize endogenous human GW182 with mTan-LIMD1 and other YFP-tagged P-body components Ago2, RCK, Dcp2, and eIF4E. As expected we observed localization of the know RISC protein components into P-body structures (Fig. 1*C*) (13). Notably, we also found specific colocalization of endogenous LIMD1 and GW182 (Fig. 1 *D–F*). Interestingly, both endogenous LIMD1 and GW182 formed smaller P-body structures in U2OS cells (approximately 5–10 nm) (Fig. 1*F*) compared to the range in size of P-bodies (100–300 nm) detected upon ectopic expression of YFP-Ago2/eIF4E and EGFP-LIMD1 (Fig. 1*C*).

**LIMD1 Interacts with RISC and the m$^7$GTP Cap Complex.** To further investigate whether LIMD1 interacted with components of RISC Xpress-tagged LIMD1 was coexpressed with YFP-tagged Ago2, RCK, Dcp2, and eIF4E in U2OS cells and the protein complexes immunoprecipitated (IP). Western blotting of the immunopurified complexes revealed that Ago2, RCK, Dcp2, and eIF4E formed complexes with LIMD1 (Fig. S3*A*). To determine whether these were protein–protein interactions or via an RNA component, we also performed the IP in the presence of RNase A. Interaction of LIMD1 with Ago1, Ago2, and eIF4E was insensitive to RNase A, in contrast RCK and Dcp2 were found to be partially and significantly dependent upon RNA, respectively (Fig. 1*G*). To map the binding interface we used GST fusion proteins of full-length LIMD1, the pre-LIM region (GST-Δ472–676) and LIM domains (GST-Δ1–467) and incubated these with U2OS cell extracts transfected with either HA-tagged Ago1, Ago2, YFP-tagged RCK, Dcp2, or eIF4e. Fig. S3*B* shows that all of these proteins interacted with full-length LIMD1. Ago1, Ago2, and eIF4E bound to the pre-LIM region, whereas the RNA-mediated interaction of RCK/p54 and Dcp2 were dependent on the context of full-length LIMD1. None of the proteins tested were able to interact with the LIM domains (Fig. S3*B*). To confirm the validity and biological significance of the above interaction in vivo we showed that endogenous LIMD1 coimmunopurified endogenous Ago2 from U2OS cells extracts (Fig. 1*H*).

**LIMD1 Directs P-body Assembly.** To determine whether LIMD1 was essential for P-body formation and integrity we depleted endogenous LIMD1 from U2OS cells using lentiviral shRNA-mediated RNAi and examined the effects on P-body formation. We developed U2OS cell lines that stably express scrambled shRNA (negative control), a LIMD1-5′UTR targeted shRNA (sh-LIMD1 5′) and a LIMD1-targeted shRNA, allowing concurrent expression of an RNAi resistant Flag-His-tagged LIMD1 wild-type protein (rrΔLIMD1-FL) or a LIM domains deletion mutant protein (rrΔ472–676) (Fig. 2*A*). Cells were transiently transfected with each of the four indicated YFP-P-body markers Ago2, RCK, Dcp2, and eIF4E. For all four markers in the scrambled shRNA cells we detected the characteristic P-body pattern and distribution (Fig. 2*B*, first column). Loss of LIMD1 expression (sh-LIMD1-5′) resulted in a significant decrease in the number of eIF4E or Dcp2 associated P-bodies (Fig. 2*B*, second column), indicating that endogenous LIMD1 is required for a specific subset of eIF4E/Dcp2 (but not Ago2/RCK) enriched P-bodies. Rescued-expression of wild-type LIMD1 (rr-LIMD1 FL) resulted in the reformation of eIF4E and Dcp2 P-bodies

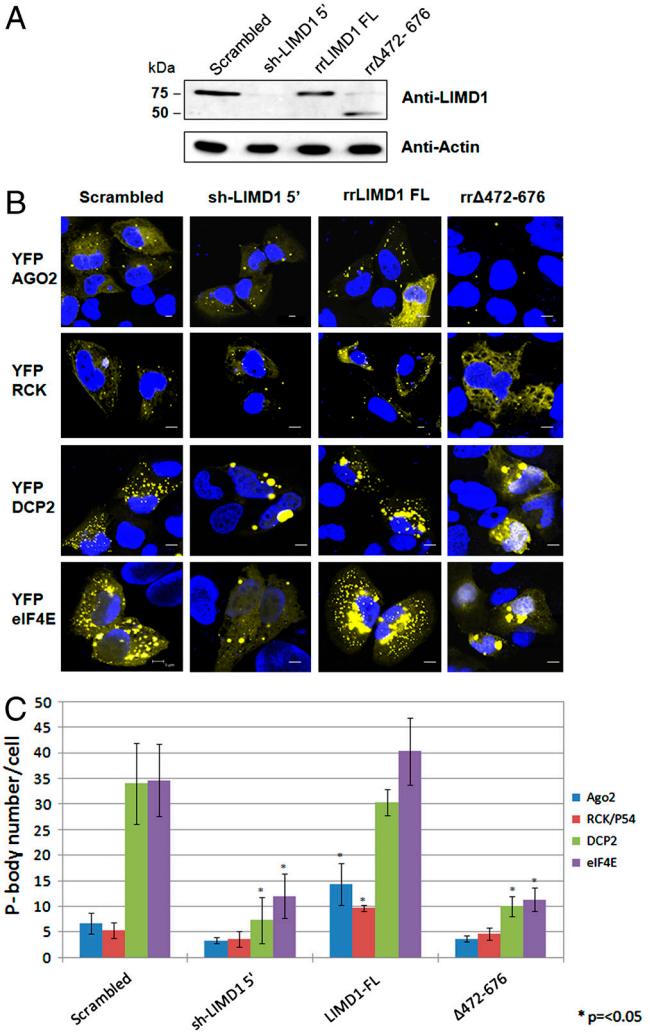


**Fig. 2.** Depletion of endogenous LIMD1 decreases P-body number. U2OS cells were transduced with lentivirus to stably express scrambled shRNA (a negative control), LIMD1-5′-UTR targeted shRNA (sh-LIMD1 (5′) shRNA) or a LIMD1 targeted shRNA, with concurrent expression of an RNAi resistant Flag-His-tagged LIMD1 protein (LIMD1-FL). (*A*) Stable knockdown of endogenous LIMD1 and expression of the RNAi resistant protein, LIMD1-FL confirmed by Western blotting. The band at approximately 75 kDa in the rrΔ472–676 lane represents residual endogenous LIMD1. (*B*) YFP fused P-body protein markers were overexpressed in these cell lines and visualized by confocal microscopy. (*C*) P-body numbers were quantified for all cells in the field of view (×63 magnification) and data from at least 3 random fields were collected and analyzed using Prism 4 software.

to the level observed in the scrambled shRNA control cell line (Fig. 2*B*, compare first and third column; quantified in Fig. 2*C*). In contrast, reexpression of the LIMD1 deletion mutant (rrΔ472–676-FL) did not reverse this effect (Fig. 2*B*, rightmost column). These findings demonstrated a critical function for full-length LIMD1 in the formation of P-bodies, specifically those rich in Dcp2 and eIF4E. Furthermore, the three terminal LIM domains are likely to be integral to this function. In light of the above findings we postulated that LIMD1 might be a key regulatory component of miRNA-mediated gene silencing.

**LIMD1 Is a Critical Component of miRNA-Mediated Silencing.** The majority of proteins critical for miRNA silencing localize to P-bodies (13–15) and their depletion often results in disruption of P-body formation as well as loss of miRNA-mediated silencing (14–16). The loss of ability for eIF4E and Dcp2 to localize to P-bodies following LIMD1 depletion (Fig. 2) suggests LIMD1 may have an early association with miRISC/mRNP silenced complexes and a function therein (10, 17). Furthermore, the predominant disruption of eIF4E/Dcp2 containing P-bodies but not Ago2/RCK-bodies by LIMD1 depletion is also suggestive of LIMD1 being preferentially involved in the translational initiation and decapping mechanism of miRNA silencing rather than the siRNA-mediated process that requires Ago2 and RCK (16, 18).

We utilized the shRNA-directed knockdown and rescue cell lines to determine whether LIMD1 functions as a critical effector protein that specifically enables miRNA-mediated gene silencing. Cell lines were transfected with Ren/Luc reporters containing complementary and partially complementary *Let-7a* sites to distinguish between siRNA and miRNA-mediated silencing. In agreement with our hypothesis, stable depletion of LIMD1 caused a specific derepression of miRNA but not siRNA-mediated silencing (Fig. 3*A*). Furthermore, this effect could be reversed in the rrLIMD1-FL knockdown/rescue cell line where an RNAi-resistant wild-type LIMD1 was expressed simultaneously with depletion of the endogenous protein (Fig. 3*A*). Rescue with the LIMD1Δ472–676 deletion mutant was unable to reverse the derepressive effect (Fig. 3*A*). To further corroborate the role of LIMD1 in miRNA-mediated silencing we repeated the reporter assay using wild-type and *Limd1*$^{-/-}$ null mouse embryonic fibroblasts (MEFs) (19). These data confirmed that loss of LIMD1 expression results in a specific loss of miRNA-mediated silencing (Fig. 3*B*). We therefore hypothesized that disruption of LIMD1 expression and therefore miRNA-mediated silencing, could be one of the mechanisms by which LIMD1 contributes to cellular transformation and tumorigenesis, in keeping with the role of miRNA-mediated gene silencing as a tumor suppressive regulatory pathway (5, 20–22). The specific loss of miRNA-mediated silencing in LIMD1$^{-/-}$ MEFs supports a link between the disruption of this pathway and the protumorigenic phenotype reported in these mice (19). This theory was further supported by the findings that LIMD1$^{-/-}$ MEFs had translation rates significantly higher (40%) than wild-type MEFs as determined by [$^{35}$S]-methionine incorporation assays (Fig. 3*C*). Ectopic expression of LIMD1 that increased P-body numbers and size (Fig. 1) also enhanced *Let-7a* specific miRNA-mediated silencing (Fig. S4). This is in agreement with published data where increased P-bodies represent more repressed mRNA multimerized into a P-body (3). The finding that LIMD1 represents a critical component of the miRNA silencing pathway prompted us to compare the ability of LIMD1 to specifically regulate miRNA silencing with other validated proteins implicated in RNAi. To this end we employed siRNA-targeted knockdown of Ago1, Ago2, RCK/p54, GW182, Dcp2, and eIF4E in addition to LIMD1 and compared the levels of specific siRNA and miRNA-mediated repression (Fig. 3*D*). In corroboration with our previous data, siRNA-targeted depletion of LIMD1 caused an attenuation of miRNA-mediated silencing (Fig. 3*D*). This was similar to that observed with ablation of GW182. In comparison, loss of Ago2 resulted in a specific derepression of siRNA-mediated silencing (Fig. 3*D*). The degree of siRNA-targeted depletion for each of the indicated RNAi components and LIMD1 were confirmed via qRT-PCR (Fig. S5*A*). Quantification of luciferase mRNA by qRT-PCR showed that at least for *Let-7a* targets, loss of LIMD1 did not affect reporter mRNA levels (Fig. S5*B*). This implies that LIMD1 exerts its physiological effects during miRNA silencing at the level of translation inhibition and not at the level of mRNA half-life or rates of degradation.

To determine if LIMD1-regulated silencing occurs with an endogenous 3′UTR *Let-7* target sequence we utilized a pMIR-CMV luciferase reporter fused to the full-length 3′UTR of High Mobility Group AT Hook 2 (HMGA2) (23). Depletion of LIMD1 by targeting siRNA resulted in a derepression of silencing similar to that seen previously (Fig. S6*A*). The use of 2′OMe inhibitors against *Let-7a* demonstrated these reporters were regulated via

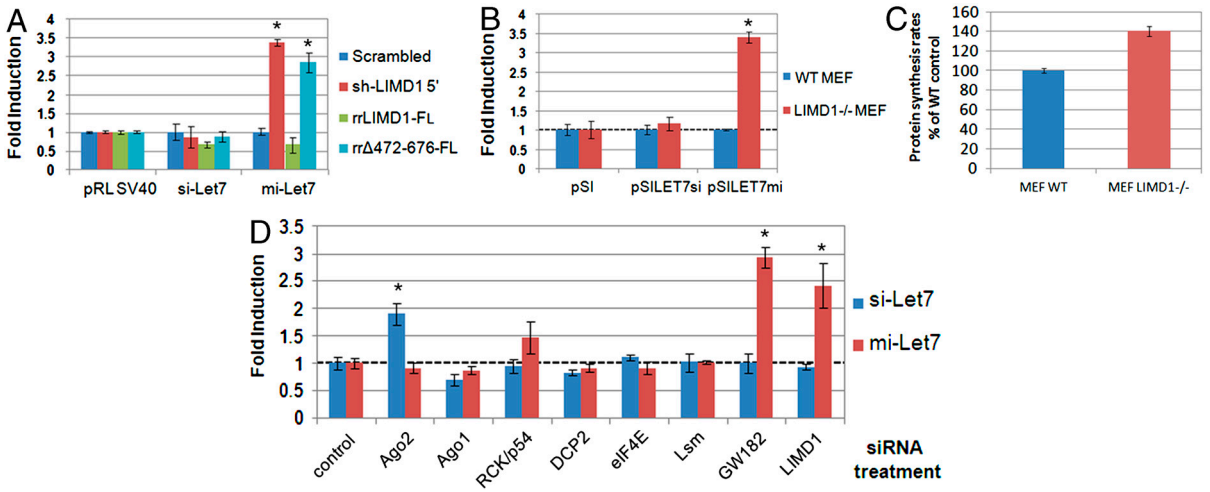


**Fig. 3.** LIMD1 is specifically involved in miRNA-mediated gene silencing. Release of gene repression by siRNA or miRNA-targeted events are presented as fold induction of RL over FFL (see *Methods* for details). (*A*) Depletion of endogenous LIMD1 derepresses miRNA-mediated gene silencing. The stable cell lines described in Fig. 2 expressing scrambled, sh-LIMD1-5′, rrLIMD1-FL or rrΔ472–676-FL were transfected with the si-Let7 and mi-Let7 RL/FFL reporters. After 24 h, total cell extracts were analyzed for dual-luciferase activity. and changes in relative luciferase activities presented as fold induction of RL over FFL (see *Methods* for details). (*B*) miRNA-mediated gene silencing in Limd1$^{-/-}$ mouse embryonic fibroblasts (MEFs). si-Let7 and mi-Let7a-pSiCheck reporters were transfected into wild-type and Limd1$^{-/-}$ derived MEFs. After 24 h luciferase activities determined, as described. (*C*) Rates of protein synthesis for wild-type MEF and LIMD1 null$^{-/-}$ MEFs were determined via $^{35}$S-Met incorporation assay. Values shown as % of wild type control set to 100%. (*D*) U2OS cells were transfected with the indicated siRNAs. At 48 h posttransfection, cells were further transfected with si-Let7 and mi-Let7 RL/FFL luciferase reporters; following a further 24 h cell extracts were assayed for luciferase activity as described above.

*Let-7* in agreement with published studies (23) and the derepressive effect was equivalent to that observed after depletion of LIMD1 (Fig. S6*A*). Interestingly, when we combined LIMD1 depletion with inhibition of *Let-7* we did not observe any additional enhancement of luciferase expression (Fig. S6*A*). Therefore, indicating LIMD1 activity is unlikely to be via a pathway that is independent of *Let-7* mediated silencing. The degree of LIMD1 knockdown was confirmed via qRT-PCR (Fig. S6*B*). To confirm these findings we extracted protein from U2OS cells targeted with siRNA against LIMD1 or 2′OMe inhibitors of *Let-7*. The extent of LIMD1 depletion was quantified by qRT-PCR (Fig. S6*E*). Western blotting of endogenous HMGA2 demonstrated an increase in protein level after inhibition of *Let-7* or depletion of LIMD1 (Fig. S6*C*). Analysis of HMGA2 mRNA by qRT-PCR showed no significant changes in mRNA levels (Fig. S6*D*) indicating the increase in HMGA2 protein is likely dependent on release of translational repression.

**Ajuba and WTIP Associate with LIMD1 in P-bodies and Have A Critical Role in miRNA-Mediated Silencing.** The six Zyxin family members can be divided into two subgroups based on a phylogenetic relationship at the genetic level (Fig. 4*A*). We therefore examined the possibility of whether the closely related subgroup members Ajuba and WTIP, also associate with LIMD1 into P-bodies and whether they too play a role in miRNA-mediated gene silencing. U2OS cells transfected with vectors expressing Asred-tagged-LIMD1 and EGFP-tagged Ajuba, LIMD1, or WTIP showed a high degree of colocalization (Fig. 4 *B* and *C*). In contrast, there was little colocalization of LIMD1 with TRIP6, LPP, or Zyxin (Fig. 4 *B* and *C*). Because we had established the LIMD1 cytoplasmic foci to be P-bodies these data indirectly suggest that like LIMD1, Ajuba, and WTIP also represented unique components of P-bodies. Ajuba and WTIP were also found to immunoprecipitate with P-body components Ago1, Ago2, RCK, Dcp2, or eIF4E in vivo (Fig. S7). We next determined if the association of Ajuba and WTIP with P-bodies was an indication of a specific role in miRNA gene silencing. siRNA-targeted depletion of all six Zyxin family members was compared with depletion of Ago2 and GW182. Fig. 4*D* shows that like LIMD1, depletion of Ajuba or WTIP had similar specific derepressive affects on miRNA-mediated silencing. However, the more distantly related family member proteins Zyxin, Trip6, and LPP had no significant effects on either miRNA or siRNA-mediated silencing (Fig. 4*D*). Therefore, these data establish LIMD1, Ajuba, and WTIP as a unique family of P-body associated components required for miRNA-mediated silencing. The degree of siRNA-targeted depletion for each of the indicated RNAi-mediated silencing components and Ajuba LIM protein family were confirmed via qRT-PCR (Fig. S8). Relative expression of LIMD1, Ajuba, and WTIP in U2OS and HEK293 cell lines was determined by Western blot (Fig. S9*A*).

**LAW Proteins Interact with the m$^7$GTP Cap Complex.** The precise mechanism of mammalian miRNA-mediated silencing of gene expression is still under debate with one fundamental issue being whether repression occurs at translation initiation or postinitiation (3, 24). However, there is growing evidence favoring an interaction between the 5′-m$^7$GTP cap structure and the 3′UTR bound Ago2/GW182 miRISC complex, facilitating inhibition of either translation initiation or elongation (25, 26). For human *Let-7a*, the ability to inhibit translation initiation of target mRNA is dependent on the presence of an m$^7$GTP cap structure (27). We therefore reasoned that due to the ability of LIMD1 to bind eIF4E and regulate endogenous *Let-7a*-mediated silencing, LIMD1, Ajuba, and WTIP proteins might function in miRNA silencing by simultaneously binding eIF4E and/or m$^7$GTP cap and the miRISC complex in a possible inhibitory mRNA closed-loop complex. To examine this, we investigated the association of Xpress-tagged Zyxin family proteins with the m$^7$GTP cap. Fig. 5*A* shows that LIMD1, Ajuba, and WTIP could be specifically copurified from U2OS cell extracts with endogenous eIF4E using m$^7$GTP-Sepharose but not control GTP-Sepharose beads (Fig. 5*A*), whereas the less related subgroup Zyxin, LPP, and Trip6 were not (Fig. 5*B*). The interactions between LIMD1, Ajuba, and WTIP proteins and m$^7$GTP-sepharose could be inhibited by addition of the inhibitory cap-analog, m$^7$GpppG (Fig. 5*A*), but were not affected by RNase A treatment (Fig. 5*B*).

To further dissect out how LIMD1 binding at the m$^7$GTP cap might function to prevent translation initiation we performed further binding studies with GST-eIF4E, GST-LIMD1, and GST-LIMD1 deletion mutants. These experiments (Fig. 5*C*) revealed

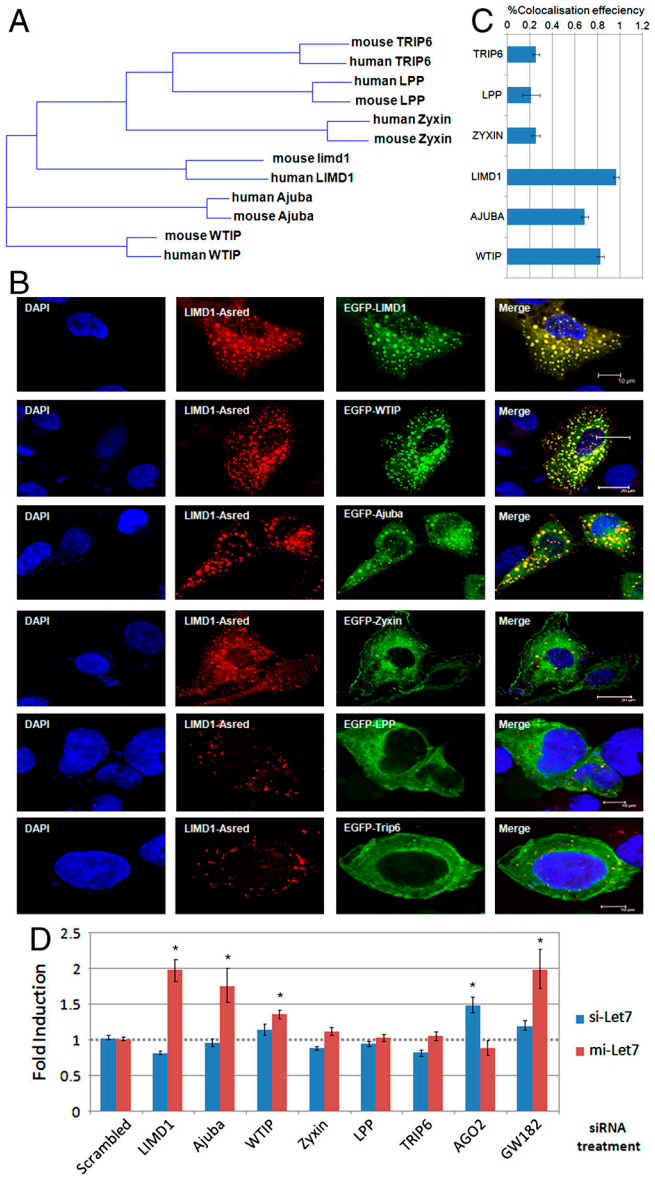


**Fig. 4.** LIMD1, Ajuba, and WTIP are a new family of positive regulators of miRNA-mediated gene silencing. (*A*) Phylogenetic analysis based on amino acid homologies between the six established members of the Zyxin family of LIM domain containing proteins. This figure shows a clear division into two subfamilies containing LIMD1, Ajuba, and WTIP (LAW) or Zyxin, LPP, and TRIP6 (ZLiP). gi_13517496 mouse TRIP6, gi_2558592 human TRIP6, gi_1537030 human LPP, gi_55154563, mouse LPP, gi_58530845, human zyxin; gi_6756085 , mouse zyxin; gi_7305237 , mouse LIMD1; gi_7657307 , human LIMD1; gi_29470380, human Ajuba; gi_31981662 , mouse Ajuba; gi_46402183 , mouse WTIP; gi_89057404 , human WTIP (*B*) Ajuba and WTIP colocalize with LIMD1 to form P-bodies. Vector encoding a LIMD1-Asred fusion protein was cotransfected into U2OS cells with EGFP fusion proteins of all six family members. At 48 h posttransfection cells were fixed and visualized via confocal microscopy. (*C*) Percentage colocalization efficiency of the indicated EGFP-fusion proteins with LIMD1-Asred fluorescent fusion protein. (*D*) Comparative analysis of siRNA-targeted depletion of all six LIM domain family members together with Ago2 and GW182 and affects on siRNA and miRNA-mediated repression. U2OS cells were transiently transfected with the indicated siRNA. At 48 h posttransfection, cells were further transfected with si-Let7 and mi-Let7 RL/FFL reporter constructs and following a further 24 h, luciferase activity assayed as described Fig. 3. * indicates $p$-value ≤0.01

that GST-LIMD1 bound endogenous eIF4E, eIF4A, and PABP but not 4EBP1 or eIF4G, whereas GST-eIF4E bound LIMD1, eIF4A, 4EBP1, eIF4G, and PABP (Fig. 5*C*). This suggests LIMD1 may act in a similar manner to eIF4G by binding eIF4E, 4A, and PABP, but with the additional ability of interacting with the translationally inhibitory miRISC via direct and indirect interactions with Ago1/2, and RCK/DCP2 respectively. Furthermore, the consistent binding of eIF4A suggests this initiation factor might play a role in miRNA-mediated translational repression together with LIMD1.

**LIMD1 Enhances the Association of eIF4E and Ago2.** We next sought evidence to show that LIMD1 could simultaneous interact with miRISC and the eIF4E/$m^7$GTP cap complex to form/stabilize a possible translationally inhibited closed-loop complex. U2OS cells were transfected with Xpress-LIMD1, EYFP-eIF4E, and HA-Ago2 (representative of miRISC) (Fig. 5*D*). These experiments showed an increase in the coimmunoprecipitation of Ago2 with eIF4E upon the additional coexpression of LIMD1 (Fig. 5*D*, arrow).

In summary, we have demonstrated that LIMD1, Ajuba, and WTIP localize to P-bodies and that LIMD1 is a required component for the formation of foci that are rich in eIF4E and Dcp2 and may represent a distinct subset of P-bodies where mRNA is processed in a specific manner not requiring the constant presence of Ago2 and RCK. Like other components of P-bodies, LIMD1, Ajuba, and WTIP also function in miRNA-mediated silencing and thus represent a unique family of essential proteins involved in this pathway. LIMD1 interacts with several miRISC components as well as factors of the translation initiation complex including eIF4E, facilitating the interaction between Ago2 and eIF4E. This supports a model where by LIMD1 acts in a role similar to eIF4G, binding eIF4E-$m^7$GTP cap and other initiation complex factors (including eIF4A), but with the additional ability of recruiting the bound miRISC generating a translationally inhibitory closed-loop complex thus enabling silencing (proposed model Fig. S9*B*).

In conclusion, we propose the involvement of LIMD1 in miRNA-mediated silencing is consistent with its tumor suppressive role and may reflect the protumorigenic phenotype reported in LIMD1$^{-/-}$ mice (19). This would be consistent with the enhanced cellular transformation and tumorigenesis reported when components of the miRNA biogenesis pathway are aberrantly expressed (5, 20–22). To date, tumor suppressor activity associated with RNAi has been attributed to proteins that regulate miRNA biogenesis (such as TRBP2, Lin28, and p53). Thus our findings that LIMD1 is associated with RISC and the 5′-cap-complex suggests a unique mechanism for tumor suppressive activity. One in which LIMD1 is intimately involved in the miRNA-mediated effector silencing function and is distinct from miRNA biogenesis.

## Methods

**$m^7$GTP-5′CAP Pull Down Assay.** Cells were suspended in 100 μL CAP buffer. 100 μg protein was incubated with $m^7$GTP Sepharose or control GTP–Sepharose beads (GE Healthcare) when required. 200 μM of $m^7$GpppG analog was added. The complex was incubated on ice for 15 min in 3 × 200 uL of CAP buffer. Proteins detected by Western blot analysis.

**RNA Interference, Let7 Inhibition and Dual-Luciferase Assay for let-7 siRNA and miRNA-Mediated Gene Silencing.** SMARTpool siRNAs (Dharmacon) were obtained against LIMD1, Ajuba, WTIP, Zyxin, LPP, Trip6, RCK/p54, GW182, Ago1, Ago2, Lsm1, and a scrambled control. Cells were transfected with 20 nM siRNA using Dharmafect Duo Transfection Reagent (Dharmacon). 2′ OMe inhibitors were transfected at 80 nM 16 h prior to the subsequent transfection of reporter constructs. Subsequent transfection with reporter constructs was carried out 48 h post siRNA transfection using 100 ng/well PsiCheck-2, PsiCheck-2-Let7asi1 or PsiCheck2-mi3 plasmid DNA or 33.3 ng/well pRLSV40L74/siTar2, pRLSV40l78/miTar4, or pRLSV40 with 4.125 ng PGL3 or 50 ng/well pMIR-CMV VO or HMGA2 3′UTR and 5 ng pRLSV40 DNA using GeneJuice transfection reagent (Merck Biosciences). After 24 h cells were lysed in 150 μL passive lysis buffer (Promega) and the luciferase activities determined with the Promega Dual-luciferase reporter assay system.

For more detailed information, see *Methods* in *SI Text*.

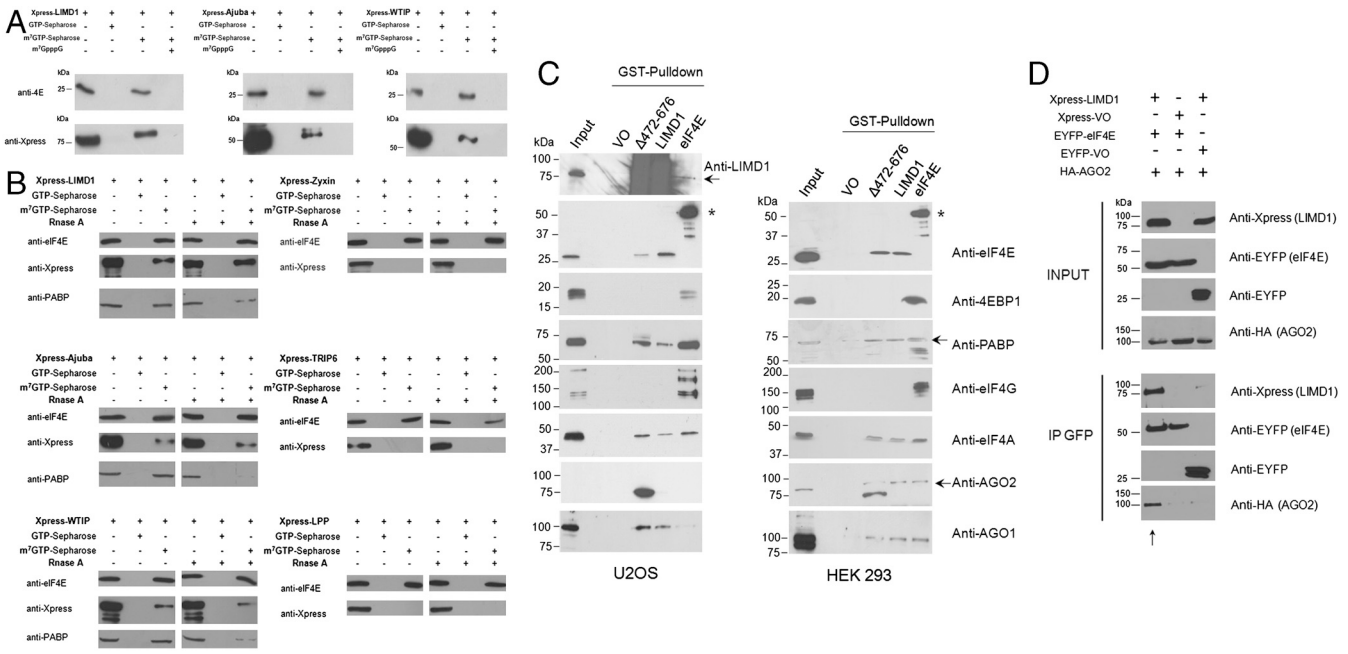


**Fig. 5.** LIMD1, Ajuba, and WTIP are recovered specifically with the m$^7$GTP cap structure in association with eIF4E. (*A*) Extracts derived from cells transfected with Xpress-tagged LIM-domain proteins were incubated with m$^7$GTP-Sepharose or GTP-Sepharose beads, in the absence (–) and presence (+) of the m$^7$GpppG cap analog. Inputs (5%) and bound fractions (40%) were analyzed by Western blotting using anti-Xpress antibody (LIM family member proteins) or a polyclonal anti-eIF4E antiserum. (*B*) m$^7$GTP-Sepharose binding assays were carried out as in panel (*A*) but with the inclusion of incubation with RNase A where indicated. (*C*) GST-VO, GST-Δ472–676, GST-LIMD1, or GST-eIF4E proteins were incubated with U2OS and HEK 293 cell extracts. The GST-tagged proteins were recovered on Glutathione-Sepharose and the recovery of the indicated endogenous proteins determined by Western blotting. (*D*) LIMD1 influences the level of Ago2 that immunoprecipitates with eIF4E. Equal amounts Xpress-tagged LIMD1, Xpress-VO, HA-AGO2, EYFP-eIF4E, or EYFP-VO were transfected into U2OS cells in the combinations shown. EYFP-tagged proteins were recovered by IP and the association with the indicated proteins determined by Western blotting; the Xpress vector served as a negative control.

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