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Trim65: A cofactor for regulation of the microRNA pathway

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Abbreviations: TRIM65, Tripartite Motif-Containing 65; MiRNA, microRNA; AGO, Argonaute; RISC, RNA-induced silencing complex; TNRC6, Trinucleotide repeat containing 6; DGCR8, DiGeorge syndrome critical region gene 8; TARBP2, TAR (HIV-1) RNA binding protein 2; HCIP, High confidence interacting protein; MOV10, Moloney leukemia virus 10; AP-MS, Affinity purification coupled with mass spectrometry; IMP-1, IGF2 mRNA-binding protein 1; PDCD4, Programmed cell death 4; PTEN, Phosphatase and tensin homolog

MicroRNA (miRNA) comprise a large family of non-protein coding transcripts which regulate gene expression in diverse biological pathways of both plants and animals. We recently used a systematic proteomic approach to generate a protein interactome map of the human miRNA pathway involved in miRNA biogenesis and processing. The interactome expands the number of candidate proteins in the miRNA pathway and connects the network to other cellular processes. Functional analyses identified TRIM65 and at least 3 other proteins as novel regulators of the miRNA pathway. Biochemical studies established that TRIM65 forms stable complexes with TNRC6 proteins and these molecules co-localize in P-body-like structures. Gain of function and RNAi analyses reveal that TRIM65 negatively regulates miRNA-driven suppression of mRNA translation by targeting TNRC6 proteins for ubiquitination and degradation. The potential molecular mechanisms which regulate TRIM65 catalytic activity are discussed.

Introduction

RNA-mediated gene silencing is an evolutionarily conserved mechanism in which small RNAs induce inactivation of cognate mRNA sequences. MicroRNA (miRNA) represents a class of small RNAs found in plants, animals and some viruses. MiRNAs function like rheostats to rapidly and reversibly fine tune transcription ensuring proper development and homeostasis. MiRNAs have emerged as key regulators of diverse aspects of biology, including differentiation, cell death, metabolism and cellular defense.[1-3] Some miRNAs regulate their mRNA target through direct cleavage, however in mammals they most often cause translational repression and mRNA destabilization. Disruption of miRNA function has consequences on cellular homeostasis, thereby contributing to disease pathogenesis, including neurodegenerative, cardiovascular or inflammatory disorders and cancer.[4-7]

MiRNAs are transcribed from endogenous genes by RNA polymerase II. A nuclear microprocessor complex containing a catalytic RNase III-type enzyme (DROSHA) crops primary miRNA transcripts to release a pre-miRNA hairpin structure of roughly 70 nucleotides. The pre-miRNA is exported to the cytosol where it is processed into a mature miRNA by another RNase III enzyme known as DICER, which is also the core of a large protein complex. The single-stranded mature miRNA contains about 22 nucleotides and is loaded into an effector RNA-induced silencing complex (RISC) where miRNAs silence complementary target mRNA. The RISC ribonucleoprotein complex contains Argonaute (AGO) and TNRC6 (also known as GW182) family members. The miRNA-loaded AGO complex serves as an address label for complementary or partially complementary mRNAs that become silenced by RISC-associated executioner complexes. The effector machinery controlling translational inhibition by miRNA can involve mRNA decapping and deadenylation but the mechanisms are still debated.[8] The central constituents of the canonical miRNA-mediated gene silencing pathway are now outlined, but these components are incorporated in large protein complexes containing sets of incompletely defined cofactors. The molecules and molecular mechanisms which regulate miRNA function remain incompletely understood.

MicroRNA Interactome

Most proteins function by collaborating with other proteins in molecular networks that form dynamic machine-like structures.[9,10] Methods to purify and identify the components of these protein-protein complexes have improved greatly in recent years

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and computational tools to organize and map the data evolved in parallel. Proteomic strategies have been successfully applied to define interaction networks and detect novel interactions or unexpected relationships which predict new players in a variety of fundamental biological processes.[11-14] Recently, we described a discovery-type affinity purification mass spectrometry (AP-MS) approach to identify additional cofactors which regulate miRNA function in human cells.[15] AP-MS relies on the affinity of a bait protein with its interaction partners to pull down protein complexes which are subsequently identified by high resolution tandem mass spectrometry. To facilitate protein purification, epitope tagged molecules are stably expressed in the cells of interest. Then protein complexes are eluted from antibody-conjugated resins under standardized conditions. Developments in computational mass spectrometry have allowed automated analysis of large amounts of MS data. Thus, AP-MS based proteomics has become one of the most versatile techniques for delineating biological pathways on a global scale.[16]

Several AP-MS studies have provided important insights to advance our understanding of multimolecular complexes and allowed initial characterization of nodes along the miRNA pathway. Gregory, et al[17] used AP-MS to identify the double-stranded-RNA-binding protein, DGCR8 as a key component of a multiprotein microprocessor complex. Subsequently, epitope tagged DICER was shown to associate with TARBP2 and AGO proteins in human HEK293 cells.[18,19] Using tagged TARBP2 as bait established reciprocal associations with DICER. The network was then extended to include EIF6 and MOV10.[18,20] Similar studies examined mammalian AGO clade proteins as baits; these efforts identified associations with DICER, MOV10, TNRC6, and TARBP2 among others.[19,21-24] Thus, there have been multiple efforts to examine one or a small set of proteins to characterize the network of protein associations in the miRNA pathway. To investigate how miRNA associated proteins are coordinated into a systematic circuit, we extended this approach to include 40 genes with known or suspected involvement in various phases of the miRNA pathway.[15] Each gene was fused with the FLAG epitope and stably expressed in HEK293 cells (**Fig. 1**). Protein complexes were retrieved by elution from anti-FLAG immunoaffinity columns. Following gel digestion with trypsin, peptides were analyzed by LC-MS/MS. For statistical comparisons controls included datasets from >100 proteins isolated under identical conditions. Computational analyses of MS profiles were used to exclude non-specific binding molecules and assemble a model network of high confidence interacting proteins (HCIP). Filtering out false positive associations depends on adequate reference control data sets. However, the rigorous algorithms used to limit false positives often result in a high false negative rate. Our recent studies characterized a human miRNA pathway interactome comprising 499 unique interactions between 40 baits and 363 high confidence interacting proteins.[15] Comparing with community databases (BioGRID, IntAct and STING plus curated literature), we found 55 known interactors and 444 interactions which were not present in these databases. 93% (50 of 54) of the novel associations tested were validated by co-immunoprecipitation in HEK293T cells or by reciprocal interaction in AP-MS.[15] However, this physical map does not represent a complete interaction network. Interactors may be omitted due to the high stringency of the algorithm, cell-type dependence and the low concentrations of some interacting proteins. Yet, the list of interacting proteins serves as a community resource and a guide for future efforts to identify miRNA cofactors. The interactome includes candidates which may directly or indirectly participate in miRNA activity and suggests how the miRNA machinery may be interwoven with other cellular processes. Additional functions of bait proteins which are unrelated to miRNA may be overlaid upon the miRNA circuit.

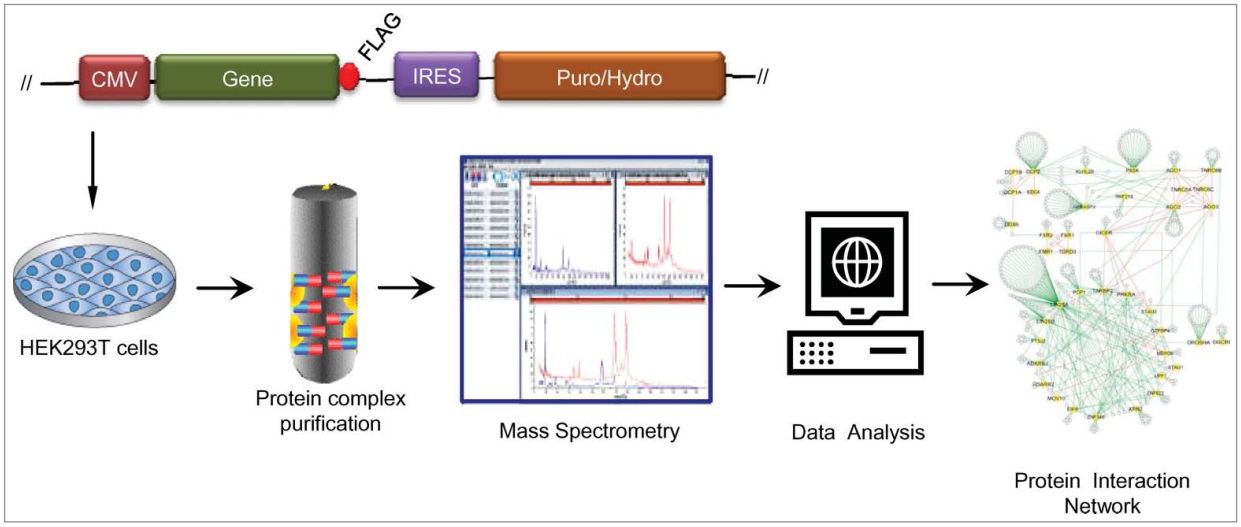

Figure 1. Construction and analysis of the miRNA pathway interactome. Schematic illustration describing the experimental pipeline including production of FLAG-tagged gene constructs, selection of HEK293T stable cell lines, protein complex affinity purification, identification of protein interactors by mass spectrometry, statistical data analysis and integration of high confidence interacting proteins into an interaction map. Details of the 499 protein interactions in the miRNA pathway interactome are presented in Li, et al. [15]

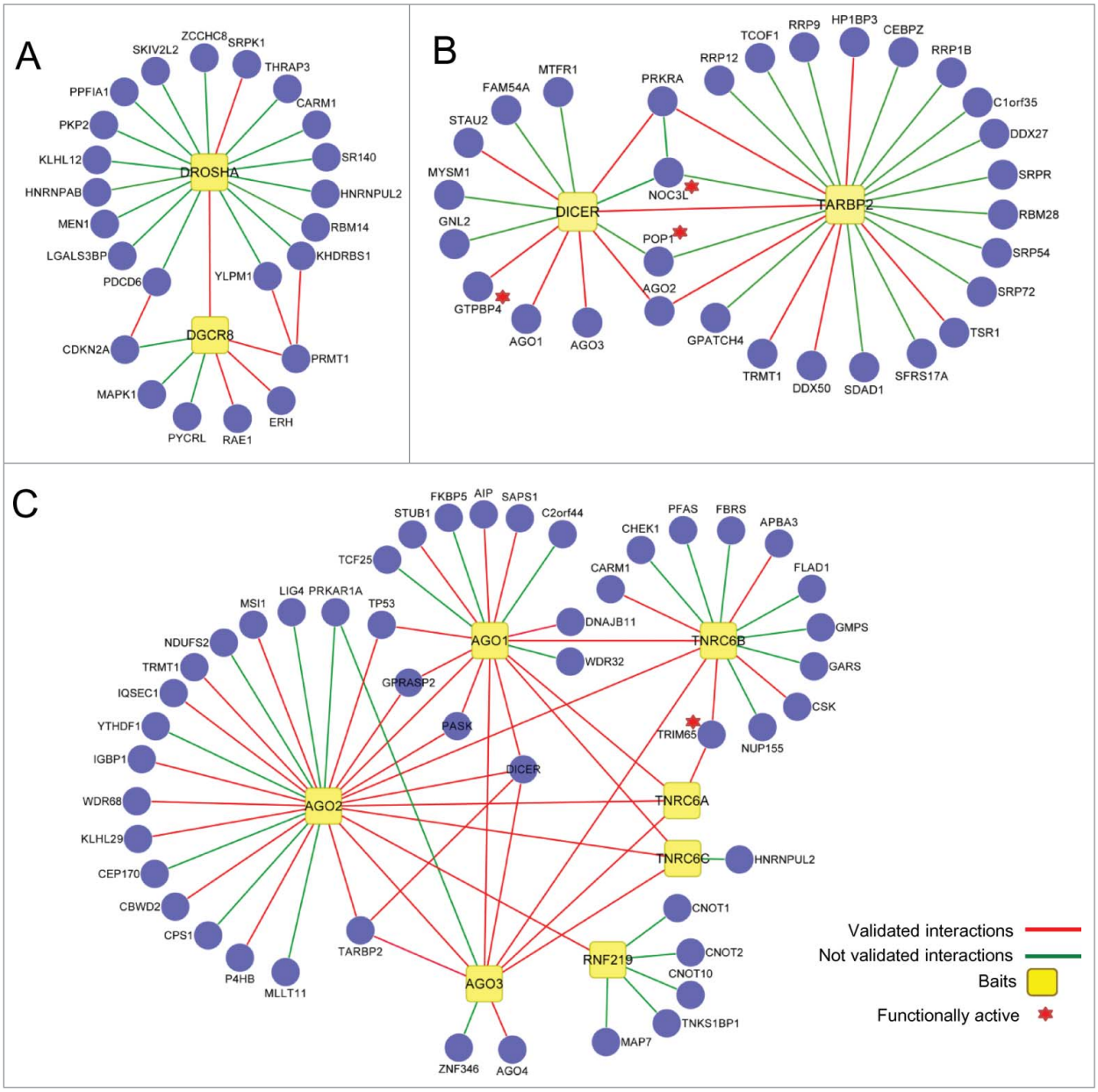

Figure 2. Subnetwork maps of the miRNA pathway interactome. (**A**) DROSHA-DGCR8 microprocessor module. (**B**) DICER-TARBP2 segment. A complete listing of HCIP for PRKRA is supplied in reference 15. (**C**) AGO-TNRC6 effector network. Line colors indicate validated interactions. Stars denote novel functionally validated targets.

The landscape view of the miRNA interactome revealed extensive connectivity.[15] Distinct subnetworks are also observed. For example, DROSHA and DGCR8 form an isolated microprocessor subnetwork consistent with their predominant nuclear localization (**Fig. 2A**). Five out of 7 HCIP associated with DGCR8 (CDKN2A, MAPK1, DROSHA, RAE1, PRMT1) and 16 out of 18 DROSHA interacting HCIP (SRPK1, THRAP3, CARM1, SR140, HNRNPUL2, RBM14, KHDRBS1, DGCR8, MEN1, HNRNPAB, PDCD6, PKP2, PPFIA1, SKIV2L2, YLPM1, ZCCHC8) have predominant nuclear localization, while the other proteins are more broadly distributed. The reported functions of these HCIP cover transcriptional regulation, RNA splicing, and RNA export.

The cytosolic endoribonuclease, DICER is located in the hub of the miRNA pathway. DICER along with its cofactor, TARBP2 process pre-miRNA and help assemble and load mature miRNAs into AGO complexes.[18,25,26] Thirteen HCIP associate with DICER in the network, 7 of which (GTPBP4, POP1, NOC3L, MTFR1, FAM54A, GNL2, MYSM1) were not previously reported (**Fig. 2B**). Five DICER interactors (NOC3L, POP1 and 3 previously known interactors, AGO2, PRKRA and TARBP2) form a highly interconnected subnetwork. TARBP2

associates with at least 23 distinct partners, although it is not clear how many of these interactions are direct or if some depend upon RNA bridging.

The CCR4-NOT deadenylase associates with the RISC,[27] but the precise connections between the complexes are not well understood. RNF219, an unstudied ring finger protein, bridges 4 subunits of the CCR4-NOT deadenylase complex (CNOT1, CNOT2, CNOT10, TNKS1BP1) to the AGO subnetwork (**Fig. 2C**). Taken together, the MPI comprises at least 3 major clusters corresponding to the well-established core components, DROSHA, DICER and AGO.

RNAi knockdown was used for screening the effects of 66 HCIP, which lacked known roles in the miRNA-pathway, on let-7a miRNA function in HeLa cells.[28] Six candidate genes with multiple hits were identified. Knockdown of 4 genes (POP1, NOC3L, PURA and TRIM65) also regulated let-7a reporter activity in A549 cells.[15] POP1 associates with DICER and TARBP2 in the miRNA pathway interactome (**Fig. 2B**). POP1 is a subunit of the RNase P and RNase MRP endoribonuclease complexes. Eukaryotic POP1 coordinates with other subunits including RPP38 and RPP30 to bind predicted RNA stem loop structures.[29] These ribonuclease complexes are involved removing the 5' leader sequences from tRNAs and may also participate in generation of miRNAs by a non-canonical biogenesis pathway.[30,31]

RNAi screening indicated NOC3L (also known as FAD24) contributes to miRNA biogenesis or processing. NOC3L is an oligonucleotide binding protein involved in adipogenesis and glucose metabolism.[32,33] Let-7 family miRNAs also play crucial roles in adipose tissue differentiation and glucose homeostasis,[34,35] but potential links between NOC3L and miRNAs were previously unknown. NOC3L interacts with PRKRA (also termed PACT), DICER and TARBP2. PRKRA is structurally and functionally related to TARBP2. PRKRA and TARBP2 molecules have similar size, share domain structure, bind double stranded RNA and both are present in ~500 kDa complexes that contain DICER and AGO.[36,37] PRKRA enhances strand selection for selected miRNAs[37] and depletion of PRKRA strongly affects accumulation of mature miRNA *in vivo*.[36] However, the underlying mechanisms for these activities are not understood. DICER may differentially employ PRKRA and TARBP2 proteins to form or stabilize discrete RISC assemblies.[38] NOC3L is uniquely positioned to coordinate miRNA processing (**Fig. 2B**).

PURA interacts with LIN28A and LIN28B in our network.[15] LIN proteins were first characterized as controllers of developmental timing in C. elegans.[39] LIN family proteins act as suppressors of let-7 miRNA biogenesis in undifferentiated cells. In humans, LIN28 is selectively activated in a subset of poorly differentiated tumors.[40] PURA is an evolutionarily conserved cellular protein which binds single-stranded oligonucleotides with purine-rich repeats. PURA participates in processes of transcription and RNA transport,[41-43] but there were no prior reports connecting PURA with the miRNA pathway. Interestingly, NOC3L and PURA primarily display nuclear localization, suggesting they may participate in a nuclear RISC-like complex or in pre-miRNA processing.[44] In contrast, to the above positive regulators of miRNA activity, TRIM65 negatively regulates let-7a activity. The molecular basis for TRIM65 activity is discussed below. Finally, RNAi depletion of GTPBP4 and PRKRIR increased let-7a reporter levels in HeLa cells, but they do not significantly regulate reporter activity in the A549 human lung cell line.[15] Further screening of additional cell types and miRNA systems is required to validate these results and identify other cofactors involved in miRNA pathways. In summary, a sampling of HCIP (66 of 363) identified 6 genes capable of regulating let-7a miRNA activity, 2 of which may be cell type dependent.

TRIM65 and the miRNA Pathway

TRIM65, a previously unidentified regulator of miRNA processing, is a member of the tripartite motif family. TRIM family members share a characteristic N-terminal tripartite motif composed of an ordered combination of 3 domains consisting of a zinc finger of the RING type followed by one or 2 B-box segments and a coiled coil (CC) region (**Fig. 3**). The RING segment is a rigid cross-braced structure in which the zinc coordination sites are interleaved.[45] B-box domains also adopt a cross-brace conformation and bind zinc atoms.[46,47] TRIM65 possesses only one B-box, a B-box2 type domain.[48] The coiled coil domain contains hyper-helical regions, which are common building blocks in protein chemistry. The CC domain is often associated with protein-protein interactions and oligomerization. Each domain within the TRIM motif also exists independently in many proteins. The patterned conjunction of domains within the TRIM motif has been preserved from worms to humans. Not only has the TRIM motif been evolutionarily conserved, but the success of the tripartite motif architecture is witnessed by the large expansion of TRIM genes in vertebrate species. Humans encode about 70 members of the TRIM family. The success of the TRIM motif in metazoan evolution suggests that a common biochemical function underlies this structure. The TRIM module is followed by a diverse group of domains in the C-terminal segment. At least 11 types of C-terminal domains are found among human TRIM proteins[49]; the variability in these C-terminal segments is predicted to contribute to function by recruiting unique classes of binding partners.

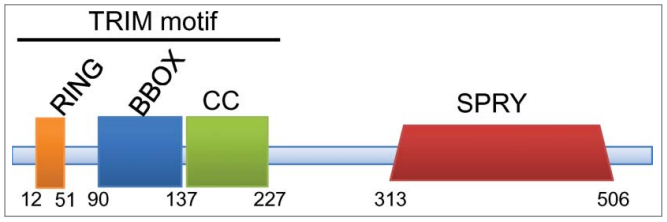

Figure 3. Schematic representation of TRIM65. The full length TRIM65 peptide contains 517 amino acids. The N-terminus includes the multi-domain tripartite (TRIM) motif. Diagram shows positions of the RING finger, B box zinc finger, coiled coil and SPRY domains. The RING domain imparts E3 ubiquitin ligase activity.

Gene expression profiling and immunohistochemistry indicate *trim65* genes are universally expressed in human tissues. Anti-TRIM65 antibodies display diffuse staining patterns on human cell lines with concentrations in P-body-like structures.[15] The human *trim65* gene (NCBI reference sequence NM 173547) consists of 6 exons and is located on chromosome 17q25.1. Expression of TRIM family proteins is often complicated by the occurrence of multiple alternate splice transcripts including some which display opposing function.[50–52] The dominant expression of a single TRIM65 isoform in most cell lines tested should simplify analysis. However, we recently cloned an additional TRIM65 isoform which lacks exon 5 (predicting a 22 amino acid deletion of residues 307 through 328) from the T98 glioblastoma cell line. The distribution and function of this shortened isoform are unknown. Genes homologous to *trim65* exist among mammals, chicken and pufferfish but orthologs were not found in invertebrates.[48] Genome-wide association studies of cerebral white matter lesions link *trim65* to subcortical vascular dementia, a common cause of disability in the elderly.[53-55] Aside from these linkage studies little was known about TRIM65.

Comparisons of genetic relatedness based on sequence similarities within the core TRIM motif, indicate TRIM65 branched from an ancient subgroup of TRIM proteins, including many which express β-propeller-like NHL structures at their C-terminus.[48] This ancient family of TRIM-NHL proteins includes several molecules which physically interact with the RISC and mediate a regulatory role on miRNA activity. TRIM-NHL proteins are expressed in worms (NHL-2 and LIN41), flies (Mei-P26) and vertebrates (TRIM32 and TRIM71).[56] The Drosophila *mei-P26* gene was the first TRIM homolog implicated in regulation of the miRNA pathway.[57] Mei-P26 and a RING-less TRIM-NHL-like protein termed BRAT interact directly or indirectly with AGO proteins and control miRNA expression in the Drosophila ovarian stem cell lineage.[57] TRIM71 regulates translational repression and colocalizes with RISC associated molecules including AGO, MOV10 and TNRC6B.[58] TRIM71 interactions with RISC-associated molecules are largely RNA-dependent. [58] Mammalian TRIM71 mediates AGO ubiquitination, but the functional consequences of TRIM71-mediated AGO ubiquitination are unclear.[58-61] LIN41 is the C. elegans ortholog of TRIM71; LIN41 was shown to immunoprecipitate with DICER.[62] While Mei-P26 and TRIM71/LIN41 inhibit miRNA-mediated repression, NHL-2 and TRIM32 enhance silencing of miRNA target genes. TRIM32 inhibits proliferation and induces differentiation of murine neural progenitor cells.[63] The mechanistic basis for this activity may include regulation of miRNA activity, as TRIM32 interacts with AGO proteins and enhances the activity of the known stem cell regulator, let-7a miRNA.[63] The C. elegans NHL-2 protein also regulates let-7 miRNAs and interacts with the CGH-1 helicase which in turn associates and colocalizes with AGO and TNRC6 orthologs in P-body-like cytoplasmic structures.[64] However, the molecular mechanisms by which these evolutionarily conserved TRIM proteins negatively or positively modulate miRNA activity are still largely unknown.

TRIM65 is the latest member of the TRIM family shown to regulate miRNA activity. Unlike the TRIM family members described above, TRIM65 possesses a SPRY domain (domain in SPla and the RYanodine receptor) at the C-terminus (**Fig. 3**). The SPRY domain structure is characterized as a bent β-sandwich formed by 2 anti-parallel β-sheets.[65] The SPRY domain is thought to serve as a protein interaction module.[66] Several TRIM family members carrying the SPRY domain were found to participate in innate immunity.[52] The role of the SPRY domain in TRIM65 activity has not been determined.

Studies of the miRNA interactome noted that TRIM65 bound to TNRC6 proteins. Domain mapping experiments demonstrated that the TRIM65 CC domain is sufficient for TNRC6A and TNRC6B association.[15] This association was stable in the presence of RNase, suggesting direct protein-protein interactions. TNRC6A and its paralogs are part of the multimeric RISC effector complexes.[27,67-69] Endogenous TRIM65 is found in punctate cytosolic structures which partially co-localize with endogenous TNRC6A in HeLa and HEK293 cells. After treatment with MG132 these TRIM65-containing P-body-like structures appear enlarged and concentrated.[15] It is unknown if TRIM65 participates in assembly/disassembly of the RISC.

Based on its intact RING domain, we predicted TRIM65 was an ubiquitin E3 ligase and that TNRC6 proteins were substrates. *In vitro* ubiquitination assays demonstrated bacteria-derived TRIM65-GST effectively delivered ubiquitin to TNRC6A. In the contrast, a TRIM65 ligase defective mutant containing a disrupted RING domain failed to catalyze ubiquitination.[15] To examine the role of TRIM65 in ubiquitination of endogenous TNRC6, TRIM65 and its RING mutant were transfected into HeLa cells treated with the MG132 proteasome inhibitor. Overexpression of TRIM65 enhances TNRC6A ubiquitination *in vivo*, while the TRIM65 mutant had little effect on TNRC6A ubiquitination.[15] Furthermore, TRIM65 overexpression results in degradation of endogenous TNRC6A protein when MG132 proteasome inhibitor is absent.[15] In contrast, TNRC6A protein levels show no substantial change when cells are transfected with the TRIM65 ligase mutant. TNRC6B or TNRC6C protein levels are also reduced when co-expressed with TRIM65.[15] The effects on TNRC6 were specific as the level of other proteins in the RISC machinery was not affected. Knockdown with siRNA was used to corroborate the role of TRIM65. TNRC6A ubiquitination is reduced and its half-life is prolonged after silencing TRIM65.[15] Furthermore, there is evidence of constitutive TNRC6A ubiquitination,[15,70] which may be due to endogenous TRIM65 activity. The combined experiments suggest TRIM65 is a cognate E3 ligase which selectively targets TNRC6 proteins for ubiquitin-mediated degradation by the proteasome. Yet details of the ubiquitination process including the type of ubiquitin linkage, the E2 conjugating enzymes involved and the TNRC6 ubiquitin acceptor sites remain unidentified.

TRIM65 Relieves miRNA Mediated Translational Repression

As TRIM65 regulates the ubiquitination and stability of TNRC6 proteins it was predicted that it should have a

corresponding role in regulating miRNA activity. Silencing TRIM65 with siRNA reduces let-7a reporter activity; ectopic expression of TRIM65 has the reciprocal effect. In contrast, a mutant TRIM65 construct in which the RING domain was disrupted to destroy E3 ubiquitin ligase activity, failed to modulate reporter activity.[15]

TRIM65 knockdown with siRNAs was also used to evaluate the effect of TRIM65 depletion on the endogenous miRNA-guided mRNA silencing machinery. Two miR-21 targeted genes, PDCD4 and PTEN[71,72] and one let-7-targeted gene, IMP-1[73] were examined after knockdown of TRIM65. Depletion of TRIM65 increases TNRC6A protein levels resulting in concomitant reduction of PDCD4, PTEN and IMP-1 (**Fig.** 4). Real-time PCR quantification was used to monitor mRNA decay rates of PDCD4, PTEN and IMP-1 after transcription was stopped with Actinomycin D. Their mRNA abundance and half-life are not altered by silencing TRIM65, indicating TRIM65 regulates the expression of protein-coding genes at the post-transcriptional level.[15] As expected, knockdown of TNRC6A or/and TNRC6B leads to increases of PDCD4, PTEN and IMP-1 expression (**Fig.** 4). Collectively, the data suggest a model in which TRIM65 relieves miRNA-mediated translational repression by ubiquitination and degradation of TNRC6 (**Fig.** 5).

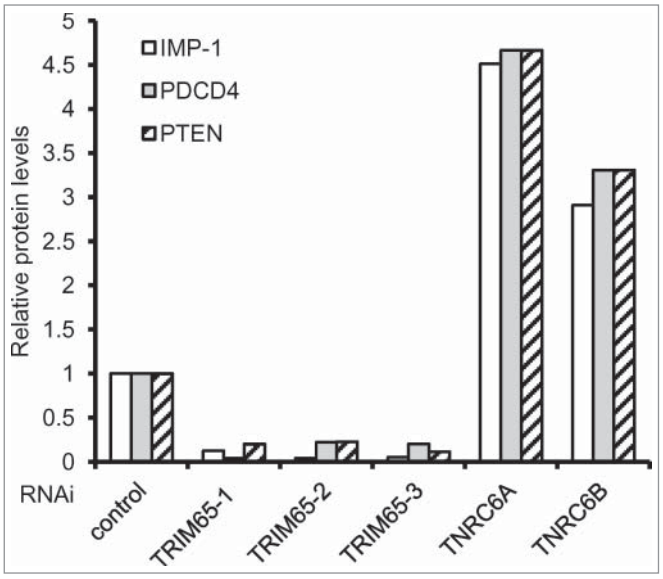

Figure 4. TRIM65 regulates gene expression. Control or validated siRNAs against TRIM65 (3 independent sequences), TNRC6A and TNRC6B were transfected into HeLa cells. After 48 hr endogenous IMP-1, PDCD4, PTEN and β-actin levels were examined by Western blot. Band intensity was quantitated and normalized to β-actin.

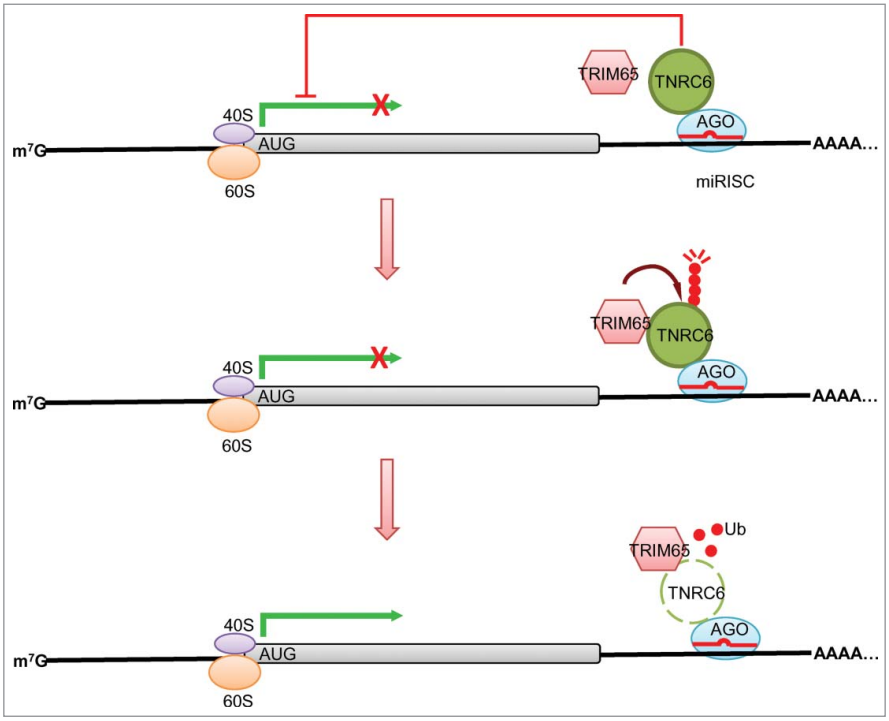

Figure 5. Model of the successive steps in TRIM65-mediated regulation of the miRNA pathway. MiRNA effector complexes containing AGO and TNRC6 direct mature miRNAs to recognize and repress expression of target genes. TRIM65, an E3 ubiquitin ligase, associates with TNRC6 proteins in AGO containing miRNA effector complexes. The signals which control assembly of TRIM65-TNRC6 complexes or activation of E3 ligase activity remain unknown. TRIM65-mediated ubiquitination of TNRC6 substrates results in their degradation by the proteasome. Without the proper set of cofactors AGO effector complexes have impaired silencing function and translation proceeds in the absence of inhibition by miRNAs.

Proteins of the TNRC6 family are central components of the RISC and coordinate downstream silencing events. The molecular mechanisms directing assembly, stability and remodeling of TNRC6 complexes are incompletely understood.[74,75] Unrestricted TRIM65 activity could destroy TNRC6 stability, in turn impairing RISC stability, miRNA activity and perturbing cellular homeostasis. Thus, the molecular mechanisms which switch TRIM65 E3 ligase activity on or off are critical for understanding miRNA regulation. To investigate the relationship among TNRC6, TRIM65 and other components of the RISC, cell lysates from cells stably expressing FLAG-TRIM65 were fractionated by centrifugation over a sucrose gradient. AGO and TNRC6 proteins sediment across a broad range of sucrose densities,[19,23] FLAG-TRIM65 was primarily located in a low sucrose density fraction with a subset of TNRC6 and AGO proteins. In contrast, FXR1 and PABP1 did not sediment with TRIM65, but were found in the higher density AGO and TNRC6 containing fractions.[15] The results emphasize the heterogeneity of RISC effector complexes; it is not clear if TRIM65 containing AGO/TNRC6 complexes are active or inactive in miRNA-mediated gene silencing. In addition to TRIM65 monomers, more

slowly migrating oligomeric structures were observed by SDS-PAGE under reducing conditions. The oligomeric TRIM65 bands survive heating to 95°C for 5 minutes in SDS sample buffer. TRIM65 oligomers were shown to sediment in the same fractions as monomeric TRIM65. Co-immunoprecipitation suggests that TNRC6A interacts with both TRIM65 monomers and oligomers.[15] Co-expression of HA- and FLAG-tagged TRIM65 demonstrates the capacity for TRIM65 self-association. The apparent molecular weight of the TRIM65 oligomers is consistent with a homo-trimer.[15] Although most TRIM proteins form dimers, TRIM21 is an example of another SPRY containing TRIM protein which forms homo-trimers.[76] The domain(s) responsible for TRIM65 oligomerization is unknown. Examination of GFP fusion proteins containing isolated RING, B-box, CC or SPRY domains failed to identify an individual region sufficient for self-association.[15]

Close inspection of the monomeric TRIM65 band revealed the appearance of a doublet. Digestion with a serine/threonine phosphatase, calf-intestinal alkaline phosphatase, suggested the presence of TRIM65 phosphoproteins. To date phosphorylated TRIM65 was only detected in the monomeric fraction of cell derived TRIM65. The apparent reciprocal relationship between phosphorylation and oligomerization requires further examination. Since non-phosphorylated bacterially derived TRIM65 can conjugate ubiquitin on TNRC6A in vitro,[15] phosphorylation may not be required for TRIM65 enzyme activity. It remains possible that phosphorylation inhibits TRIM65 catalytic activity or that phosphorylation participates in regulating the balance of TRIM65 monomers and trimers. As the activity and stability of many proteins are regulated posttranslationally, further biochemical analysis is required to understand whether oligomerization or posttranslational modifications modulate TRIM65 E3 ligase and miRNA activity.

Conclusions and Future Perspectives

Development of mass spectrometry-based proteomics coupled with improvements in computational analyses allow system-wide studies of protein interactions. The resulting lists of interacting proteins include potential new members of system pathways and suggest avenues of communication with other signaling modules. Results can depend on cell type and additional evidence is required to integrate novel interaction partners into the overall organization of miRNA circuits. Applying this screening approach to the human miRNA pathway (Version 1.0) identified a repertoire of ~500 protein-protein interactions. Unraveling which candidates participate in canonical or alternative miRNA pathways remains a challenge. The components which process and present miRNA often overlap with molecules involved in regulating processing of other non-coding RNAs including siRNA and piRNA. Thus, candidates identified in this screen may include constituents of related pathways. Future directions include deciphering the crosstalk between the miRNA network and other cellular processes. Identification of TRIM65 as a new regulator of miRNA activity established the utility and power of the proteomic discovery approach.

The past decade witnessed discovery of multiple molecules critical for miRNA biogenesis and processing. We are beginning to appreciate that dysregulation of miRNA expression or function contributes to aberrant gene expression patterns associated with various diseases including cancer.[39,77-81] Thus, there are many translational motives for unraveling the molecular mechanisms which regulate miRNA pathways. TRIM65 represents a previously unidentified regulator of miRNA processing. TRIM65 is a ubiquitously expressed ubiquitin E3 ligase that selectively targets TNRC6 family proteins for proteasomal degradation. As TNRC6 proteins are essential components of the RISC and miRNA effector machinery, their degradation negatively regulates miRNA-mediated gene silencing. Tight control of miRNA activity is required for cellular homeostasis. Thus, TRIM65 activity cannot proceed in an uncontrolled manner. Future research will elucidate whether TRIM65 functions to regulate specific miRNAs or if it has a more global effect on the miRNA pathway. The fundamental mechanisms regulating assembly of TRIM65 complexes and TRIM65 E3 ligase activity remain to be determined. Understanding the molecular mechanisms underlying TRIM65 E3 ligase activity and identifying the intrinsic and extrinsic factors which regulate TRIM65 activity in different contexts are critical outstanding issues. Exploring these unresolved issues may offer opportunities to regulate miRNA activity in disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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