CHAPTER 8

THE MICROTUBULE-ASSOCIATED C-I SUBFAMILY OF TRIM PROTEINS AND THE REGULATION OF POLARIZED CELL RESPONSES

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Abstract: TRIM proteins are multidomain proteins that typically assemble into large molecular complexes, the composition of which likely explains the diverse functions that have been attributed to this group of proteins. Accumulating data on the roles of many TRIM proteins supports the notion that those that share identical C-terminal domain architectures participate in the regulation of similar cellular processes. At least nine different C-terminal domain compositions have been identified. This chapter will focus on one subgroup that possess a COS motif, FNIII and SPRY/B30.2 domain as their C-terminal domain arrangement. This C-terminal domain architecture plays a key role in the interaction of all six members of this subgroup with the microtubule cytoskeleton. Accumulating evidence on the functions of some of these proteins will be discussed to highlight the emerging similarities in the cellular events in which they participate.

INTRODUCTION

TRIM proteins are believed to share a number of basic properties, including the ability to multimerize and facilitate the transfer of ubiquitin on to partner proteins.[1] TRIM proteins generally form large scaffolding complexes and can be found in almost any part of the cell, including in the nucleus, free in the cytoplasm, or tethered to a particular organelle or intracellular structure.[2] As inferred by the TRIM nomenclature, this family of proteins is defined by the existence of a full N-terminal tripartite, or RBCC, domain complement (RING, one or two B-box(es), and a Coiled Coil).[1] Of note, bona fide or classic TRIM

TRIM/RBCC Proteins, edited by Germana Meroni.

proteins (i.e., those with the complete RBCC complement) are not found in unicellular eukaryotes such as *Saccharomyces* species but only true multicellular organisms suggesting this tripartite domain has evolved to perform functions specific to multicellularity.[3]

In an attempt to help understand the cellular roles of different members of the large TRIM family, we and others have subclassified the TRIMs based on the unique combination of motifs present in each of their C-terminal regions.[2,3] The subfamily categories used hereafter are those designated by Short and Cox[3] and are referred to as C-terminal subgroup I (C-I) through subgroup 9 (C-I, C-II, C-III … C-IX), although Lerner et al[4] suggested division of the C-V subgroup based on the prediction of a transmembrane region at the C-terminus of two members of this original group.

This chapter will focus on TRIM proteins of the C-I subfamily as this highly related group performs their functions tethered to the microtubule cytoskeleton. This association with the microtubule network is conferred by a small yet unique central motif, termed the COS box.[3] Although also found in a number of other non-TRIM microtubule associated proteins, two other TRIM subfamilies also possess this motif: the C-II subfamily, which consists of the three MURF proteins that also associate with the microtubule cytoskeleton, and the single member of the C-III subfamily, TRIM42, which remains largely uncharacterized. The members of the C-I and C-II subfamilies are distinguished by their unique C-terminal domain composition and are therefore likely to regulate distinct intracellular processes.[3] The function of the C-II MURF subfamily will be covered elsewhere in this book.

Overview of the C-I Subfamily

In humans, the C-I TRIM subfamily consists of six members: MID1 (TRIM18), MID2 (TRIM1), TRIM9, TNL (TRIM67), TRIM36 (HAPRIN) and TRIFIC (TRIM46). Each of these proteins not only possess an RBCC domain containing two B-boxes but also the C-terminal COS box, Fibronectin Type III motif, and SPRY or B30.2-like domains. Within this subfamily, the proteins cluster into three separate groups based on the degree of primary sequence identity. For example, MID1 shares 76% identity with MID2 but less than 25% primary sequence identity with the other members. Likewise, TRIM9 is most similar to TNL (65% identity) while HAPRIN is the most closely related to TRIFIC (43% identity).[3] Based on these similarities and the existence of only a single C-I subfamily member in both *D. melanogaster* and *C. elegans* that most closely matches TRIM9, it is likely this subfamily arose from two separate gene duplications of an ancestral *TRIM9*-like gene to generate three such genes, then subsequent individual gene duplication and further divergence of each of these three lineages.

MID1/TRIM18

Mid1 (or *Fxy* [for RING Finger gene on the X and Y] as it was then known) was first identified serendipitously in the mouse through sequencing efforts aimed at characterizing the major pseudoautosomal region (PAR) at the distal tip of the X chromosome.[5] The PAR is a stretch of sequence identity between the X and Y chromosomes in mammals that serves to facilitate pairing of the sex chromosomes during cell division. As its original name implies, the mouse *Fxy/Mid1* gene uniquely spans the PAR boundary such that the first 3 coding exons are X chromosome-specific, while the remainder are found

on both the X and Y chromosomes.[5] Using a positional cloning or 'reverse genetics' strategy, *MID1* was identified shortly thereafter in humans as the gene responsible for the X-linked form of Opitz G/BBB syndrome,[6] which is diagnosed by its characteristic facial dysmorphology and specific anogenital anomalies, as well as variably penetrant, laryngeotracheal and cardiac defects.[7,8]

The mutations in *MID1* that underlie Opitz syndrome fall in to all mutation classes: missense, nonsense, small in-frame and frame-shift insertions and deletions, as well as whole gene deletions, and interruptions due to chromosome translocations. Although numerous studies have investigated the possibility of genotype-phenotype correlations to explain the highly variable phenotypic presentation, there has been little convincing data to support such correlations at this time. These outcomes are consistent with the findings that all coding mutations tested to date have the same general consequence, that is, to reduce or disrupt the protein's association with the microtubule cytoskeleton[9] and likely result in mis-targeting of its ubiquitin ligase activity.[10-12] These data support the notion that Opitz syndrome is due to loss of function of MID1.[9] As mentioned above, it may be that other genetic variants (e.g., in MID2, Alpha4 or other partner proteins) and/or specific epigenetic factors determine the ultimate severity of presentation in patients although none have been definitively identified as yet.

In humans, the *MID1* gene is subject to X-inactivation and its expression is influenced by the presence of endogenous retroviral elements within the locus, which may contribute to the highly variable presentation.[13] In contrast, in the mouse, the gene escapes inactivation due in part to its unique position across the PAR boundary and, as a result, has undergone considerably more evolutionary tinkering than most other genes on the mouse X chromosome.[5] Aside from the reduced evolutionary selection pressure on the murine *Mid1* gene, the *MID1* gene is otherwise very well conserved amongst other vertebrates. However, like most of the C-I subfamily members, *MID1* does not have an ortholog in invertebrates suggesting it has evolved to perform a critical regulatory role in vertebrate biology.[3] Although widely expressed in early to mid-gestational tissues in all species, *MID1* expression is prominent in most tissues typically affected in Opitz syndrome, including the face, urogenital region, and heart.[6,14-17] One prominent exception is the developing CNS. In this tissue, expression of *MID1* is very strong yet associated anomalies such as mental retardation, autistic features and/or structural brain defects are usually relatively mild in patients and IQ is typically normal.[8] These mild CNS phenotypes may be due to the compensatory role of MID2 (see below). Expression of *MID1* has also been detected in the early gastrulating embryo in chicks, where its expression becomes transiently asymmetric around Hensen's node, and plays a role, together with *MID2*, in establishing left-right asymmetry.[18,19]

MID2/TRIM1

MID2 (also known as *TRIM1*) was identified by two groups based on its high degree of primary sequence similarity to *MID1*.[20,21] Like *MID1*, the *MID2* gene localizes to the X chromosome (Xq22). Analysis of the genes flanking both *MID2* and *MID1* supports the notion that the two genes arose by a recent intrachromosomal duplication event.[20,21] *MID2* is expressed in many of the same tissues as *MID1* during development, although in general at lower levels than *MID1*, with the exception of the heart which is a prominent site of expression for *MID2*.[17,19-21]

Consistent with their high level of amino acid sequence identity, MID2 and MID1 can readily heterodimerize via their coiled coil domains, with dimerization stabilizing their association, via their COS boxes, with the microtubule cytoskeleton.[11] Both proteins also bind the same two protein partners (see below). The heterodimerization of MID2 and MID1 is likely to have functional significance as endogenous levels of mutant MID1 protein in a cell line derived from an Opitz syndrome patient is sufficient to perturb the intracellular localization of ectopically expressed MID2, and ectopically expressed mutant MID2 can also disrupt the localization of endogenous and ectopically expressed MID1 (unpublished data). Granata et al[19] provided the first evidence in direct support of the functional implications for this interdependency by demonstrating that expression of either MID1 or MID2 was sufficient to rescue the perturbation in left-right asymmetry resulting from knockdown of both genes in chick embryos. More recently in Xenopus, depletion of both *MID1* and *MID2* together but not either by themselves was found to disrupt epithelial morphology in the neural plate, a site of high expression of both genes. The specific disruption of both apical markers and the basal lamina in the absence of obvious effects on cell fate indicate a requirement of the MID proteins for apico-basal polarity.[17] It is therefore believed that variation in the level of expression or activity of MID2 could contribute to the phenotypic variation seen in patients carrying *MID1* mutations.[9,11] That said, no definitive causative mutations have been found in *MID2* in *MID1*-mutation negative Opitz syndrome cases to suggest that its loss might also be sufficient to cause similar developmental anomalies.

Cellular Function(s) of MID1 and MID2: Clues from Their Binding Partners

Even though most of the available functional data has been generated with respect to MID1, the following sections should be considered as applying to both MID1 and MID2 because they both exhibit the capacity to interact with the same two protein partners. At the same time, though, this has hindered the progress on understanding their functions in early development, especially in the mouse as evidenced by the failure of Mid1 knockout lines to reproduce any of the corresponding human phenotype.[22,23] As a result, the identification of their protein partners, most notably the phosphoprotein Alpha4 (also known as IGBP1), has been the main driver of functional studies in recent years. Each of these interactors are discusses briefly below.

MIG12

Using a yeast two-hybrid assay, Berti et al[24] identified MIG12 as a novel interacting factor of MID1. This *M*ID1 *i*nteractor derives its name from the sequence homology with a protein of unknown function, called *G12*, that was first identified in a screen for genes expressed during zebrafish gastrulation.[25] Like *MID1* (Xp22.3), *MID2* (Xq22) and *Alpha4* (Xq13; see below), the *MIG12* gene also resides on the X chromosome, at band p11.4. The existence of the genes encoding all these interacting proteins on the X chromosome may be just co-incidence but could suggest the importance of gene dosage or coregulated expression to facilitate proper control of cellular activity.

MIG12 is expressed in a subset of the tissues that express *MID1*, including the developing CNS, limbs, the thyroid and parathyroid, the phallic part of the urogenital sinus, the anal canal, and the bladder lumen epithelium.[24] Hayes et al[26] also identified *MIG12* in a screen for genes expressed differentially in mucus-secreting cells and ciliated cells.

At the protein level, MIG12 is recruited to the microtubules by binding to the coiled coil region of MID1, where it appears to stabilize the microtubule network.[17,24] In ciliated epithelia, MIG12 was found to localize strongly to the base of cilia. Through knockdown with antisense morpholinos, Hayes et al[26] found MIG12 to be required for ciliogenesis, a process dependent on microtubules, and more globally participates with both MID1 and MID2 to facilitate closure of the neural tube.[17] MIG12 has also been implicated in the regulation of fatty acid synthesis by numerous studies,[27-29] although how and whether this role ties in with its interaction with MID1 and MID2, if at all, is currently unknown.

Alpha4

Both MID1 and MID2 tightly bind Alpha4, recruiting it to the microtubule network.[10-12] Alpha4 is a novel partner of the protein phosphatase 2 family, which includes the major cellular serine/threonine phosphatase, PP2A, and the structurally related phosphatases, PP4 and PP6.[30-32] PP2A, the best-characterized member of this family, is predominantly found as a heterotrimeric complex consisting of the catalytic (C) subunit, a constant regulatory A subunit, and a variable regulatory B subunit.[33] The variable B subunits are thought to direct the phosphatase complex to its phosphorylated targets. Alpha4, however, is an atypical regulator of PP2A in that it binds directly to the catalytic subunit of PP2A (PP2Ac), displacing the A and B subunits.[31] Although numerous studies suggest Alpha4 binding inhibits PP2A activity, Prickett and Brautigan[34] have provided evidence to suggest that Alpha4 may sterically alter, rather than occlude, the catalytic site of PP2-type enzymes thereby altering their substrate specificities. This alteration of PP2-type phosphatase activity by Alpha4 likely serves three purposes: (1) the refocusing of phosphatase activity to specific substrates, (2) the protection of PP2Ac from ubiquitin-mediated degradation, a process regulated by MID1 (and MID2) and (3) the sequestration of a stable reserve of PP2Ac, allowing for rapid adaptive responses.[30,34,35] In this regard, binding of the MID proteins to Alpha4 involves a region distinct from that binding PP2Ac, and co-immunoprecipitations as well as yeast two-hybrid and three-hybrid assays have confirmed that Alpha4 forms a complex involving both PP2Ac and MID1/MID2.[11,12,22,35,36] These observations and the fact that RBCC/TRIM proteins typically possess E3 ubiquitin ligase activity raised the possibility that Alpha4-PP2Ac complex was either a target for MID-mediated ubiquitylation and degradation or regulated the activity of the MID proteins against other targets, or indeed both. Evidence to date supports the former as the primary mode of action; that Alpha4 protects PP2Ac from MID1-dependent ubiquitylation and degradation.[12,35] Under normal growth conditions, this would serve to maintain numerous PP2A targets, particularly those associated with the microtubule cytoskeleton, in a basal state of dephosphorylation.[30] Under certain stimuli (e.g., conditions of stress) this pool could either be transiently activated, assembled into a functional trimeric complex, or the excess degraded via the ubiquitin-proteasome system.

More recently, two studies have shown that Alpha4 is also modified by ubiquitin.[35,37] McConnell and colleagues observed a moderate mobility shift in Alpha4 on immunoblots of extracts from cells cotransfected with differentially tagged ubiquitin and Alpha4. Although additional bands of lighter intensity were evident, they concluded that Alpha4 is primarily mono-ubiquitylated.[35] In contrast, Han et al[37] found that MID1 is able to interact with multiple E2 enzymes to mono- and poly-ubiquitylate Alpha4 on its C-terminus. Interestingly, in most cases mono- and di-ubiquitylated Alpha4 predominated although some E2 enzymes resulted in higher levels of poly-ubiquitylation, raising the possibility

of some degree of cell-type-specificity in ubiquitin ligase activity. In addition to being ubiquitylated, Alpha4 has been reported to have a Ubiquitin Interacting Motif, or UIM, at its N-terminus (residues 46-60) that has been proposed to 'cap' the ubiquitin in the mono-ubiquitylated form of PP2Ac and thus protect the phosphatase catalytic subunit from poly-ubiquitylation and degradation.[35] This model is consistent with the findings of Han and colleagues[37] and support the idea that ubiquitylation of PP2Ac by MID1 is a two-step process: mono-ubiquitylation by the RING domain, then poly-ubiquitylation facilitated by the B-box domains. It would then follow that 'dissociation' of Alpha4 from the complex, or alternatively a triggered conformational change in bound Alpha4, perhaps in response to phosphorylation, might uncap the mono-ubiquitylated PP2Ac and free the B-boxes of MID1 to facilitate poly-ubiquitylation.

The MID1-Alpha4-PP2Ac Complex and mTOR Signaling

MID-Alpha4-PP2Ac complexes represent only a portion of the total cellular Alpha4, and to a lesser extent the PP2A pool.[34] Nevertheless, new data indicates that these complexes play critical roles in the regulation of a number of important signaling pathways, most notably via the mammalian Target Of Rapamycin (mTOR) kinase.[38] mTOR is a highly conserved serine/threonine kinase that exists as two distinct complexes, mTORC1 and mTORC2, in both yeast and mammals. mTOR signaling was originally characterized as a key complex controlling cell growth in response to nutrients and growth factor signaling. However, since then, mTOR has also been directly linked to autophagy and other stress-related cellular responses, the regulation of mRNA translation, cell-cell adhesion and cell motility, re-organization of the cytoskeleton, metabolism, and aging.[39] As it's name implies, TOR was initially identified from a yeast mutant that exhibited resistance to rapamycin, a bacterial antifungal agent. Rapamycin, or siromilus as it is commercially known, is no longer used as an antifungal treatment as it was found to exhibit powerful immunosuppressive and anti-proliferative properties.[39] On the flip side, because of these other properties, it has found widespread use in the field of organ transplantation and the treatment of certain cancers.[40-42] When present intracellularly, rapamycin binds to the ubiquitously expressed FKBP12 protein which then complexes with TOR to inactivate the kinase and potently suppress mRNA translation and cell growth.[43]

PP2A and Alpha4 have long been implicated in the regulation of mTOR signaling, with much of the recent focus on their involvement being on the mTOR-dependent regulation of mRNA translation. In fact, in vertebrates, both PP2Ac and Alpha4 have been found to physically complex with mTOR to regulate the downstream effects of mTORC1, just as they do in yeast. In vivo, Alpha4 is directly phosphorylated on one or more N-terminal serine residues that, based on studies of its yeast homolog Tap42, may be mediated by mTOR.[44] This association with TOR occurs at the plasma membrane and, at least in yeast, the complex is rapamycin-sensitive and influenced by phosphorylation.[44,45] It remains contentious as to whether Alpha4 in vertebrates is similarly responsive. However, it has been shown that phosphorylation of Alpha4 is not required for binding to PP2Ac or to regulate its activity per se.[31] It is nevertheless tempting to speculate that this phosphorylation may modulate some other key function of Alpha4 such as its interaction with mTOR, increasing accessibility for MID1/MID2 to act on PP2Ac, or to disrupt its interaction with, or signaling to, downstream targets.

Two well-characterized targets of the Alpha4-PP2A-mTOR axis are 4EBP1 and the p70/S6 kinase, which both play key roles in the initiation of translation, particularly

of mRNAs containing 5' terminal oligopyrimidine tracts (5'TOP sequences).[46] In high nutrient conditions or growth factor stimulation, mTOR is activated and directly phosphorylates 4EBP1 and p70/S6K to increase translation, while Alpha4-PP2A opposes this response by binding to and de-phosphorylating these targets.[47] More recently, the Alpha4-PP2Ac complex has also been shown to physically interact with the poly(A) binding protein (PABP) which is required for circularization of mRNAs and efficient translation.[48] These observations indicate that the Alpha4-PP2Ac complex plays numerous roles in the regulation of mRNA translation by mTOR. To further complicate the story, Liu et al[38] found that knockdown of *MID1* or *Alpha4* expression, which results in elevated levels of PP2Ac, not only suppressed p70/S6K phosphorylation but also led to disruption of mTORC1 (but not mTORC2) suggesting MID1-Alpha4-PP2Ac acts both upstream and downstream of mTORC1 to regulate translation. These data nevertheless indicate that higher levels of expression of MID1 likely correlate with increased mTORC1 activity.

Interestingly, Aranda-Orgillés and colleagues[49] also reported that microtubule-bound MID1-Alpha4-PP2Ac were part a larger multiprotein complex containing active polyribosomes and mRNAs. However, their unexpected finding was that 5'TOP-containing mRNAs were not enriched in this complex. Instead, the associated mRNAs contained purine-rich sequences that conferred increased stability and translational efficiency.[50] It will be of interest to determine whether the MID1-Alpha4-PP2Ac complex sequesters these purine-rich mRNAs away from mTOR-responsive 5'TOP targets to enable independent translation regulation of each population.

Regulation of MID1 and MID2 Binding to the Microtubules

Although most efforts have focused on the regulation of PP2Ac turnover by MID1, a role for the complex in regulating MID1 function is also plausible. Both Liu et al[10] and Short et al[11] demonstrated that MID1 is phosphorylated by a serine/threonine kinase, raising the possibility of a regulatory role for phosphorylation in either the control of E3 ligase activity, the interaction with the microtubule network, or binding to its partners. Short et al[11] noted numerous putative and conserved serine and threonine phosphorylation sites that were shared between MID1 and MID2. Using various deletion mutants, they found that the linker region between the RING and B-box domains was the only site of serine phosphorylation. Notably, this region harbors only two invariant serine residues, at positions 92 and 96, within consensus sites for GSK3 and the MAP kinase, ERK2 (P-N-S/T-P), respectively.[51,52]

Using live cell imaging and two different MAP kinase inhibitors, Liu and colleagues provided data supporting the involvement of MAPK activity in the association of MID1 with the microtubule network.[10] Consistent with this notion, they also showed that co-expression of Alpha4 reduced the overall level of phosphorylation of MID1 as well as decreased the filamentous appearance of MID1. Aranda-Orgillés and colleagues, however, could not reproduce this result, although differences in culture conditions during the live cell imaging may explain this discrepancy: serum-containing media[53] versus serum-free media;[10] the latter condition frequently used to improve the sensitivity of detection of phosphorylation and dephosporylation events. Instead, Aranda-Orgillés and colleagues reported that substitution of S96 with Aspartic or Glutamic Acid, which can act as phosphomimics, affected the ability of MID1 to be transported along the microtubules, but not its binding to microtubules per se. This transportation was similarly dependent on

both Alpha4 and PP2A,[53] a fact that argues against direct de-phosphorylation of MID1 by the Alpha4-PP2A complex. These data can however be reconciled if the phosphatase inactivates the MAPK responsible for phosphorylating MID1, e.g., MAP2K or ERK2, which are known targets of PP2A. Such a mode of action could provide a feedback mechanism to regulate microtubule binding and/or specific downstream signaling events controlled by the interplay of these kinases and phosphatase.

TRIM9

TRIM9 was identified by two independent groups: by Reymond et al[2] using a systematic genomic screen for TRIM family members, and by Li et al[54] using a yeast two hybrid system to search for binding partners of SNAP25, an essential component of the SNARE complex that mediates docking and fusion of neurotransmitter-containing vesicles to the plasma membrane. Li and colleagues reported that rat TRIM9 (known then as Spring, for SNAP25 associated RING finger protein) is predominantly expressed in the fetal and adult brain and enriched at synaptic terminals in both soluble and synaptic vesicle-associated forms.[54] The interaction between these two proteins involved the coiled-coil domain of TRIM9 and the N-terminus of SNAP25.[54]

The N-terminal region of SNAP25 required for TRIM9 interaction encodes a coiled coil t-SNARE domain. Similar t-SNARE domains are also found in other components of the SNARE complex, including syntaxin1 and VAMP2/synaptobrevin, and are critical for the assembly of the final trans-SNARE complex.[55] Consequently, Li and colleagues investigated the binding relationship between these proteins and TRIM9. Using a series of in vitro binding assays, they found that TRIM9 individually competed with these proteins for binding to SNAP25, preventing the assembly of the ternary SNARE complex.[54] It is not known whether SNAP25 binds to monomers or dimers of TRIM9 but, given the importance of the coiled coil in dimerization and stabilization of TRIM binding to the microtubule cytoskeleton,[3] this could have implications for the kinetics of vesicle exocytosis. Indeed, when TRIM9 was ectopically expressed in PC12 cells, which typically promotes dimerization and microtubule association, it significantly inhibited Ca2+-dependent exocytosis.[54] Expression of just the coiled coil domain of TRIM9, but not the RING domain, also inhibited exocytosis. Similar inhibition of Ca2+-dependent vesicle exocytosis has been reported following inhibition or knockdown of SNAP25[56] suggesting that TRIM9 functions to regulate synaptic vesicle exocytosis by modulating the amount of SNAP25 available for formation of SNARE complexes.

Like MID and MID2, TRIM9 possess numerous putative phosphorylation sites. Li et al[54] proposed that phosphorylation of TRIM9 may therefore be important for the regulated binding of SNAP25. Such a mechanism could control: 1) release of SNAP25 thus increasing its availability to form the SNARE complex, 2) the E3 ligase activity of TRIM9 and thus determining the rate of turnover of SNAP25 (or perhaps even TRIM9 itself), or 3) the amount of either protein associating with the synaptic vesicle. Alternatively, TRIM9 phosphorylation could regulate its association with, and movement along, the microtubule cytoskeleton in much the same way as seen for MID1,[53] and therefore represent a means by which to control the polarized localization of SNAP25 to the axonal plasma membrane.[57]

The importance of TRIM9 function in regulating vesicle exocytosis is further highlighted by the observation that TRIM9 along with other SNARE proteins is significantly

repressed in brains of mice following infection with Rabies virus, which is associated with increased accumulation of presynaptic vesicles.[58] A similar repression of TRIM9 was also seen in neurons of patients with Parkinson's disease and dementia with Lewy bodies.[59] Interestingly, in the latter cases, most of the TRIM9 in these patients was found in the Lewy bodies themselves, which contain aggregates of ubiquitin-modified proteins including the structurally distinct E3 ligases, Parkin and SIAH1, and their targets such as α-synuclein.[60,61] Although a role for TRIM9-mediated ubiquitylation in synaptic transmission has not been directly investigated, Tanji et al[59] did report that TRIM9 preferentially binds the UbcH5b E2 conjugating enzyme and could poly-ubiquitylate itself. They therefore suggested that TRIM9 might regulate its own turnover and that this was important for proper neuronal function.[59] Fanelli et al[62] however, previously provided evidence to suggest that many TRIM proteins, including TRIM9, are targeted for ubiquitin-mediated turnover by the SIAH E3 ubiquitin ligases, SIAH1 and SIAH2. Further work is therefore needed in order to elucidate the specific role TRIM9 E3 ligase activity plays in regulating neurotransmitter vesicle exocytosis.

More recent studies in *C.elegans* have revealed additional roles for TRIM9. In independent genetic screens, two research groups identified the nematode TRIM9 ortholog (also called Madd2) as a key regulator of muscle arm extension[63] and axon branching,[64] respectively. Like axons, muscle arms are plasma membrane extensions from the body wall muscles that ultimately establish the neuromuscular junctions with motor axons.[63] A further study by Song et al[65] also identified a role for TRIM9 in ventral guidance of hermaphrodite-specific and touch neurons in *C.elegans* and sensory neurons in *Drosophila*. Significantly, all studies revealed that Madd2/TRIM9 functions within the evolutionarily conserved UNC-40 pathway. UNC-40 is a transmembrane receptor that regulates the cells response to signaling cues provided by the secreted ligand, UNC-6/Netrin. Netrins were first identified as attraction cues for axon guidance but have since been shown to have roles in both attraction and repulsion (depending on whether its coreceptor, UNC-5, is also expressed), as well as in cell-cell and cell-ECM interactions that impact cell migration.[66] In humans, there are two orthologs of UNC-40: DCC (Deleted in Colorectal Cancer) and Neogenin, and five Netrin ligands. Data from these groups suggest Madd2 potentiates UNC-40 pathway activity to regulate attraction-mediated guidance but has no role in the response to repulsive cues.[63-65]

Notably, all the Madd2/TRIM9 mutants identified in the three studies were either truncating mutations or harbored deletions within the SPRY/B30.2 domain and therefore would be predicted to form intracellular aggregates rather than 'coating' the microtubule cytoskeleton.[3] Using yeast two-hybrid assays, Alexander and colleagues[63] showed that Madd2/TRIM9 directly bound both the cytoplasmic domain of UNC-40 as well as the Rho/Rac-GEF,UNC-73. This binding, which in the case of UNC-40 was mediated by the SPRY domain of Madd2/TRIM9,[64] enhanced the interaction between UNC-40 and UNC-73.[63] Using different TRIM9 constructs in rescue experiments, both Alexander et al[63] and Song et al[65] also demonstrated a requirement for the TRIM9 RING domain, and therefore likely ubiquitin ligase activity, in axon guidance. Although the exact molecular nature of the TRIM9-UNC-40/DCC interaction remains unclear, the normally asymmetric membrane localization of the phospholipid-binding protein, MIG-10/lamellipodin, which regulates actin polymerization and lamellipodial growth,[67,68] is disrupted in TRIM9 mutants.[65] Of note, the asymmetric localization of MIG-10 is also determined by its interaction with the CED-10/Rac1 GTPase.[69] Together these data suggest that TRIM9 functions to either directly or indirectly promote Rac1 activation following Netrin stimulation of UNC-40 to

drive polarized growth of axons. Whether this involves ubiquitin-mediated degradation or ubiquitin controlled localization of TRIM9 target proteins requires further investigation.

A recent report by Tcherkezian et al[70] adds a further possible intriguing twist to the story. These researchers found that the translational initiation machinery associates with the cytoplasmic domain of DCC and that binding of Netrin to DCC promoted the release of the initiation complex and formation of active polysomes.[70] They proposed that these polysomes remain in close proximity to DCC, probably tethered to the cytoskeleton, to allow spatial precision of translation and rapid responses to extracellular cues that direct processes such as adhesion, guidance, and synaptic plasticity. It will be very interesting to determine if the potentiation of DCC activity by TRIM9 also involves regulation of this local translational response given the recently discovered role of MID1 in regulating translation initiation.

TRIM36/HAPRIN

TRIM36 was first identified by Kitamura and colleagues[71] as a haploid germ cell-specific RBCC containing protein (hence its original name, Haprin). The expression of Haprin/TRIM36 in sperm is highly regulated, starting at the late steps of spermatid differentiation when elongated spermatids appear. An important clue to the function of Haprin/TRIM36 was gleaned following its antibody-mediated inhibition in permeabilized sperm. In this experiment, inhibition of Haprin resulted in a specific block of the acrosome reaction, an essential prerequisite event for mammalian fertilization.[71] The acrosome is a large Golgi-derived vesicle containing enzymes required for digestion of the egg's protective coating, the zona pellucida. The release of the acrosomal contents requires fusion of the acrosomal outer membrane with the plasma membrane via a process involving SNARE complex components, similar to that described for synaptic exocytosis.[72,73] Therefore, Haprin/TRIM36 may play a similar role in regulating the formation of the acrosomal SNARE complexes as shown for TRIM9 in SNARE complex formation at the neuronal synapse. It would seem unlikely, given the level of amino acid identity between the Haprin/TRIM36 and TRIM9 coiled coil domains, that Haprin would also bind SNAP25, but further studies should elucidate whether there are similarities in binding partners or complexes regulated by these two factors.

However, at least in Xenopus, expression of TRIM36 is not restricted to the maturing sperm, but is expressed widely throughout development including in the neural tube, the posterior region of somites, the eyes and craniofacial mesenchyme, and, as expected, in male germ cells.[74,75] Both Yoshigai et al[75] and Cuykendall and Houston[74] employed antisense approaches to knockdown expression of TRIM36 early in development. Yoshigai and colleagues[75] reported marked disorganization of somites following suppression of TRIM36, while Cuykendall and Houston[74] also described more pronounced effects including defects in ventralization. The latter phenotype was associated with reduced nuclear beta-catenin and marked loss of expression of Wnt target genes in the organizer region. These gene expression and phenotypic effects could be rescued with ectopic expression of wild-type TRIM36 mRNA, Wnt11 mRNA (which shows a similar vegetal localization as TRIM36 mRNA within the germ plasm during Xenopus oogenesis), or tipping of the early embryos, the latter a classic phenomenon associated with disruption of the microtubule cytoskeleton.[74] Despite the impact on Wnt signaling, they concluded that TRIM36 was not regulating Wnt signaling directly but rather was essential for positioning

of the dorsalizing Wnt signal. Importantly, TRIM36 carrying mutations in the B-box2 domain were unable to rescue the phenotype or autoubiquitylate itself, suggesting that TRIM36 ubiquitin ligase activity is required for these early morphogenetic events.[74] As an interesting aside, these investigators noted that ubiquitous expression of high doses amounts of TRIM36 led to disaggregation of the embryos during gastrulation and to epidermal lesions suggesting perturbed cell-cell and cell-ECM adhesions. Whether this is related in any way to disruptions in signaling via small GTPases such as Rac, as suggested with TRIM9[63] and MID1-Alpha4,[76] is currently unknown.

In another study, Miyajima and colleagues[77] somewhat unexpectedly identified the centromeric protein, CENP-H, as a potential interactor of Haprin/TRIM36. While these authors showed that ectopic expression of TRIM36 slowed the cell cycle and attenuated cell growth, a specific role for the CENP-H-TRIM36 interaction could not be demonstrated, and TRIM36 did not appear to ubiquitylate CENP-H.[77] Additional validation of this interaction is therefore needed before any firm conclusions can be drawn about the significance of this detected interaction. The identification of other interacting partners of Haprin should greatly aid our understanding of the role of this TRIM protein is early development and the regulation of male fertility.

TRIFIC/TRIM46 AND TNL/TRIM67

TRIFIC (TRIM46) and TNL (TRIM67) represent the remaining members of the C-I TRIM subfamily. Although these two proteins were identified based on sequence similarity and a domain architecture resembling MID1 and MID2, they are most closely related to Haprin/TRIM36 and TRIM9, respectively.[3] To date, there is little known about their tissue distribution of expression or function, aside from their association with the microtubule cytoskeleton.[3,78] However, based on their high degree of primary sequence identity with the more well-characterized C-I members, both proteins most likely will play some role in intracellular signaling at the cytoskeletal-plasma membrane interface.

CONCLUSION

The first appearance of bona fide TRIM proteins in multicellular organisms and the marked expansion of particular subfamilies in vertebrates likely corresponds with the increasing complexity and need for tissue-specific regulation of basic cellular processes in these organisms. Consistent with this notion, a single 'archetypical' member of the C-I TRIM subfamily exists in invertebrate species such as *C.elegans* and *D.melanogaster*, while the subfamily has expanded to six members with the same conserved domain architecture in humans. Emerging data on multiple C-I members point to important roles in regulating key cell signaling events at the plasma membrane, be it through transient activation and/or turnover of signaling components, control of endo- and exocytosis, or via localized translational responses. These key processes ultimately contribute to a cell's response to changes in their local tissue environment, which in turn may directly impact cell-cell and cell-ECM communication that underpins morphogenetic processes and higher tissue function. Further investigation in to the functions of C-I subfamily members is sure to yield exciting results and illuminate the intricacies of some of the key signaling pathways involved in these processes.

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