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TRIM/RBCC PROTEINS
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DEDICATION

To Eugenia and Franco,
For the privilege and delight of sharing your genes

PREFACE

The genomic 'golden age' has delivered the sequence of numerous novel genes while leaving us with many unanswered questions about their function. This is particularly true for gene families as, often, members are annotated based on homology rather than function. The tripartite motif family belonged to this category, although, during the last few years, the field boosted an important wealth of biochemical, cellular and physiological breakthrough data. In the first part of this book, we attempt to offer an overview of state-of-the-art basic findings on the tripartite motif (TRIM, also known as RBCC) family members and to deal in the second part with their relevant and growing physiological and pathological roles.

TRIM/RBCC Proteins begins with a general introduction on the genomic organization of the TRIM family and of its evolution, which produced one of the largest RING-containing protein families. TRIM proteins' conserved multi-domain structure is reviewed in Chapter 2 and translation into the ability to function as E3 ubiquitin ligases, the enzymes needed for the ubiquitin-modification of specific substrates, is dealt with in Chapter 3. The shared domain structure not only underscores the E3 ligase activity but also other common features, in particular TRIM proteins ability to often demarcate defined subcellular structures. Some are well-characterized compartments, such as the PML nuclear bodies, whereas others are still undefined cytoplasmic and nuclear structures (Chapter 4). Within the nucleus, some of the TRIM family members are involved in epigenetic control of transcription (Chapter 5). Moreover, a sub-class of TRIM members can also decorate cytoskeletal structures through their capacity to associate with the microtubular apparatus (Chapter 6).

TRIM family members implication in such an important process as ubiquitination makes them vulnerable to alteration in crucial physiological processes leading to a plethora of pathological conditions that are recapitulated in the second part of the book. Known for a long time, the acute promyelocytic leukemia t(15;17) translocation results in an oncogenic PML (a TRIM member)-retinoic acid receptor-α fusion protein. Since then, several other TRIM proteins have been implicated in tumorigenesis and are addressed in Chapter 7. However, one of the most recent arenas in which TRIM proteins were discovered to have a predominant and growing role is innate immunity, in which a good share of family

members are key players via direct interactions with pathogens, above all TRIM5 as one of the main HIV-1 restriction factors, and participating as sensors of 'danger' signaling pathways (Chapter 8). However, TRIM proteins can also be enriched in preferential tissues. This is the case of the skeletal muscle members, heavily implicated in trophic and metabolic muscle physiology as well as in genetic muscular dystrophies (Chapter 9). Other genetic disorders implicate the TRIM genes in developmental processes, and in this field the study of TRIM homologs in invertebrate and rodent models contributed to the understanding of their role in embryonic patterning and determination (Chapter 10).

Much of the TRIM family function has been discovered, but there is still a long way to go. I hope this volume provides the foundation to contribute to foster novel discoveries in the rapidly evolving field of TRIM proteins biology.

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GERMANA MERONI is a graduate of the University of Milan, Italy. She was a post-graduate fellow at the Department of Biotechnology of the San Raffaele Hospital in Milan, Italy, and then post-doctoral fellow at the Department of Human and Molecular Genetics of Baylor College of Medicine, Houston, TX (USA). She established her research group at the Telethon Institute of Genetics and Medicine (TIGEM) in Naples and then moved as leader of the Functional Genomics Laboratory at the Cluster in Biomedicine within AREA Science Park in Trieste, Italy. Her main research interest is the genetics and biochemistry of the Tripartite Motif family in health and disease.

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CHAPTER 1

GENOMICS AND EVOLUTION OF THE TRIM GENE FAMILY

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Abstract: The TRIM family comprises proteins characterized by the presence of the tripartite motif that is composed of a RING domain, one or two B-box domains and a Coiled-coil region. These proteins are implicated in a plethora of cellular processes such as apoptosis, cell cycle regulation, muscular physiology and innate immune response. Consistently, their alteration results in several pathological conditions emphasizing their medical relevance. The TRIM members domain structure underscores a common biochemical function as E3 ligases within the ubiquitylation cascade, which is then translated into diverse biological processes. The TRIM proteins represent one of the largest families in mammals counting in human almost 70 members. TRIM proteins are metazoan-specific and have been now identified in several species although the great increase in their number was generated in vertebrate species. The important expansion of the number of TRIM genes underlie the success of the tripartite module in ubiquitylation process. Furthermore, their massive diversification among species was achieved through fast evolution of the TRIM genes implicated in pathogen response.

INTRODUCTION

The TRIM family is composed of genes that encode proteins containing the TRIpartite Motif.[1] The tripartite motif is an integrated module composed of three different types of domains: RING domain (R), B-box domain (B), and a Coiled-coil (CC) region; from here also the name RBCC to refer to this class of proteins.[2] The first definition of the TRIM family as a whole dates back 10 years when the first 37 human and murine proteins recognized to share the tripartite motif were characterized.[1] Since then, many tripartite motif-containing genes have been identified in several species. The presence of a common proteic module underscores well-defined biochemical and structural properties, such as

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their ability to form homomultimers through the CC region and their aptitude to define definite sub-cellular compartments[1] (see Chapter 2 by Micale et al and Chapter 4 by Batty et al). The TRIM proteins also share the capability to act as E3 ligases, the enzymes that allow the transfer of the ubiquitin peptide to the specific target within the ubiquitylation cascade[3] (see Chapter 3 by Ikeda and Inoue). These common features are translated into a range of assorted biological processes such as regulation of transcription, regulation of cell cycle and division, control of apoptosis and other signaling cascades, to name some of them, and consequently in a plethora of different physiological roles (see Chapter 6 by Cambiaghi et al, Chapter 7 by Yap and Stoye, Chapter 8 by Cox, Chapter 9 by Mayans and Labeit, and Chapter 10 by Petrera and Meroni). The involvement in such a wide array of processes and pathways makes the TRIM members highly relevant in medicine for their implication in cancer onset and progression, in human Mendelian and autoimmune disorders, and in viral and microbial innate response.[3-8]

Especially thanks to the enormous genomic sequencing effort, in the last few years the identification and annotation of TRIM genes was completed in many vertebrate and invertebrate species with the human family counting 68 members.[4,9,10] This revealed that the TRIM members represent one of the largest families of proteins, certainly the largest RING-containing subfamily of E3 ubiquitin ligases, underlying the great success of the tripartite module during evolution.

THE HUMAN TRIM FAMILY

Structure of the TRIM Proteins

As better described in subsequent chapters of this book, the tripartite module is located in the most N-terminal portion of the TRIM proteins and contains, in the following order, a RING domain, one or two B-box domains and a CC region and it can be further associated with diverse domains in the C-terminal portion (Fig. 1) (see Chapter 2 by Micale et al). Whereas the tripartite motif is mainly involved in ubiquitylation mechanisms and in homo- and hetero-interactions, the C-terminal domains are principally concerned to direct specific partner interactions and TRIM subcellular localization. From this basic arrangement several variations are observed within the family. The RING is a cysteine-rich zinc-binding domain involved in the interaction with the ubiquitylation machinery and which confers the E3 ligase activity to the TRIM family members (see Chapter 3 by

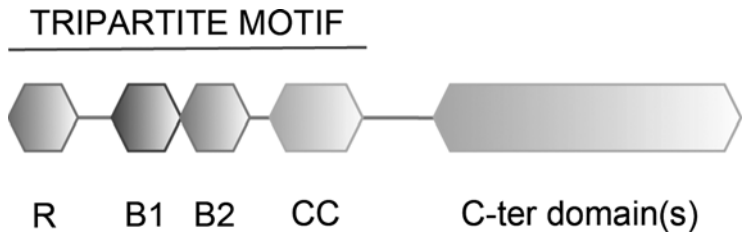

Figure 1. Schematic representation of TRIM protein domain organization. The scheme shows the N-terminal tripartite motif and its composing domains (R, RING domain; B1, B-box Type 1 domain; B2, B-box Type 2 domain; CC, Coiled-coil region) and the variable C-terminal (C-ter) domain.

Ikeda and Inoue). Six human family members represent incomplete non-orthodox TRIM proteins as they lack the N-terminal RING domain (Fig. 2). The B-box domains are zinc binding motif as well and they can be of two related but clearly different kinds: Type 1 and Type 2. They may be present in tandem (22 of the human TRIM members), in which case B-box Type 1 always precedes Type 2, or only one B-box can be present (46 of the human members) and in this case it is always Type 2 (Fig. 2). A CC region is following the B-box 2 domain and this intertwining of α-helices may differ in length and score prediction, and it can be either continuous or interrupted in the different TRIM members. Some TRIM proteins display an additional specialized α-helical domain downstream the canonical CC region, named COS, which assists their cytoskeletal interaction (Chapter 8 by Cox).[9] Within the tripartite motif, not only the domain but also the spacing between each of them is conserved in the family members. The maintenance of the domain scaffold, order and spacing clearly indicates that the tripartite structure is a functional module.

The most variable region within the TRIM family is the C-terminal portion. Each TRIM member displays specific domains and these domains can be present in combination (Fig. 2). The C-terminal domains found within the TRIM family are not an exclusive property of this family but may be present also in other otherwise unrelated proteins.[11-15] Given this variety, starting from the basic structure theme, a classification of the human TRIM family based on predicted domain arrangement has been proposed.[9] Following this scheme, the human TRIM family results sorted into 9 classes, from C-I to C-IX, to which 3 classes have been added in Figure 2: C-TM, including proteins with a putative trans-membrane region; C-IV-like, with proteins similar to those included in C-IV but consistently with both B-box domains; and UC, an unclassified TRIM protein resembling class IV but with the RING domain substituted by a PAAD domain.[16] Of these classes, mainly defined by the domain composition of the C-terminal portion associated with their tripartite motif, class C-IV showing the R/B2/CC/B-30-like arrangement represents the major group counting 30 members in human (Fig. 2). Interestingly, comparison of the TRIM protein complements of several vertebrate and invertebrate species confirmed that this domain-based classification has also solid evolutionary foundation. The TRIM proteins within the subgroups identified are phylogenetically related further substantiating that the addition of the C-terminal domains, chosen among a limited number of structures, occurred after the assembling of the tripartite motif. The latter evolved as a single unit and no swapping of domains occurred further during evolution. All the way through phylogenesis only loss of the RING domain in few members and possibly the decision of adopting either the single or the tandem arrangement of B-box domains in the tripartite motif have occurred.[10]

Genomic Organization of the Human TRIM Family

The human TRIM genes are distributed all over the genome and only few chromosomes do not host any TRIM genes[10] (Fig. 3). Some of the TRIM genes are present in clusters; in particular 8 genes are located on the short arm of chromosome 11 (11p15.4), 8 genes in the 6p21-22 region, and 3 TRIM genes are found on chromosome 5 (5q35.3) and 7 (7q11.23) (Fig. 3). As expected, several of these clustered genes are closely related and share high degree of similarity. Many of them possess the same genomic organization with 6-7 exons spanning a 10 kb region, likely suggestive of their recent origin through consecutive duplications.[1,10,17] It is noteworthy that the closest TRIM genes within the clusters belong to the C-IV class of R/B2/CC/B-30-like proteins. These clustered genes

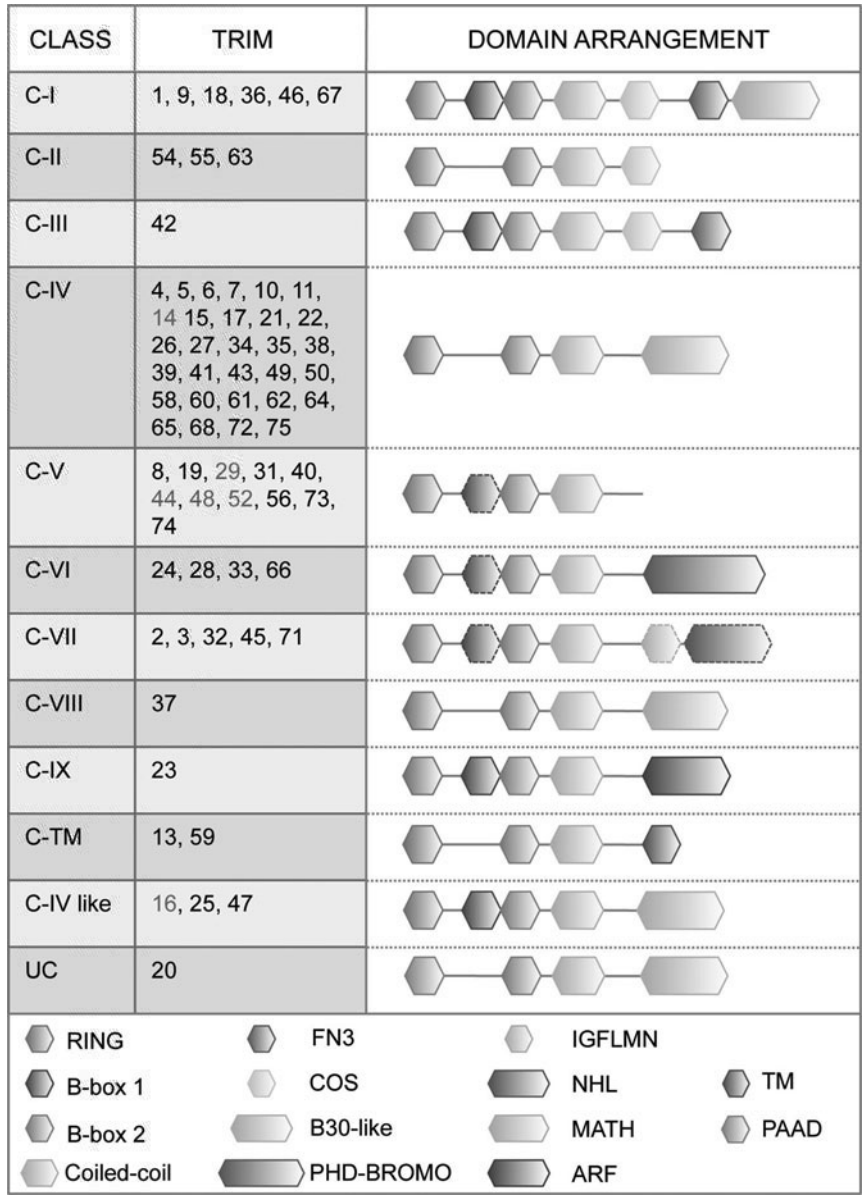

Figure 2. Classification of TRIM proteins. The TRIM proteins are classified into subgroups based on the C-terminal domain(s) associated with the tripartite motif as proposed.[9] The name of the class, the TRIM proteins that belong to each class, and the domain composition are indicated. Grey (red) numbers indicate RING-less TRIM proteins. Dashed domains indicate that they may be absent in some members of the subgroup. RING, B-box, CC, as in legend of Figure 1. FN3, fibronectin Type III repeat; COS, C-terminal subgroup one signature; B-30-like, also known as PRY-SPRY domain; PHD-BROMO, association of the Plant-Homeodomain and the Bromo domain; IGFLMN, filamin-type immunoglobulin domain; NHL, NCL-1, HT2A and Lin-41 domain; MATH, meprin and TRAF homology; ARF, ARF-like small GTPases; TM, transmembrane; PAAD, Pyrin, Aim, Asc, and death-domain-like. A color version of this image is available at www.landesbioscience.com/curie.

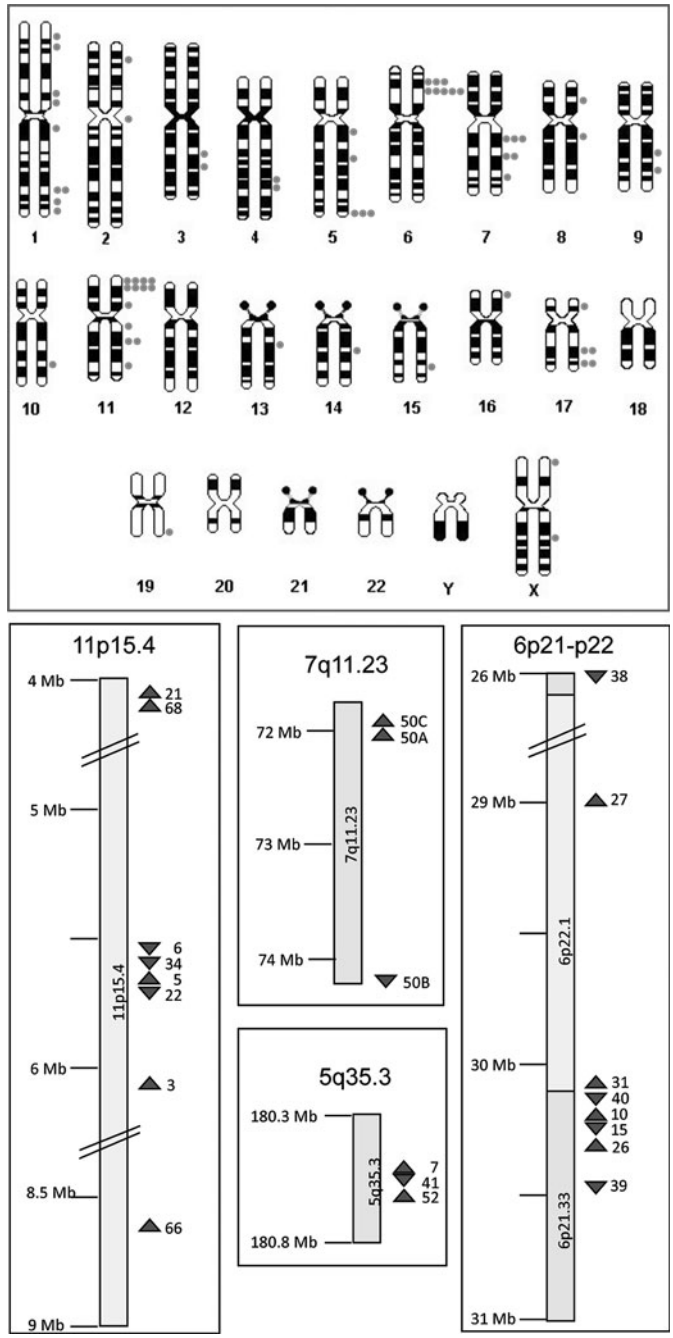

Figure 3. The TRIM genes in the human genome. Upper drawing, location of TRIM genes on human chromosomes. Gray (red) dots indicate TRIM genes. Lower panels, magnification of the chromosomal bands hosting clusters of TRIM genes (dark grey/red triangles); their orientation is also indicated. A color version of this image is available at www.landesbioscience.com/curie.

are also often involved in innate immune response and it is remarkable that the 6p21-22 TRIM cluster is located in the same chromosomal bands as the major histocompatibility complex (MHC) region which is known to play a key role in self/nonself recognition. This close preferential location of some TRIM genes in the MHC region is conserved in mammals, birds and fish suggesting co-evolution of the two immunity networks.[18-21] As for the nonclustered TRIM genes, the genomic organization is variable with some of the coding regions split in up to 20 exons and spanning genomic regions up to 150 kbp, while other smaller genes displaying 1 or few exons encompassing less than 10 kbp of the genome.[10] No matter the number of exons, the tripartite motif is often encoded by a single first exon further corroborating the evolution of this module *en bloc*.

Expression of the TRIM Genes

The expression of mammalian TRIM genes is generally constitutive and ubiquitous in adult tissues and, when investigated, this is also the case during embryonic development.[1] There are though some exceptions of TRIM genes expressed in multiple but defined tissues and other extremely tissue specific as, for example, TRIM9 whose expression is restricted to defined compartments of the central nervous system in both embryonic and adult tissues.[1,22] On top of their ubiquitous expression, many of the TRIM genes involved in innate immune cellular response are specifically induced upon interferon release.[23] This induction is observed also in other species, indeed a subset of teleost fish TRIM genes (finTRIM) have been also found to be up-regulated following viral infection.[24]

An important issue concerning the expression of the TRIM genes is the presence of several different TRIM transcripts originated from alternative splicing events and often leading to diverse predicted protein products.[1] These TRIM isoforms possess different biochemical properties and activities that are well studied in the case of *TRIM19* and *TRIM5*.[25,26] Though not yet well understood in the other family members, TRIM proteins ability to form homo-multimers makes it conceivable that the concomitant presence of different isoforms within the same cell might have important functional consequences.

TRIM FAMILY EVOLUTION

The tripartite motif is the characterizing module that defines the TRIM family. Its composing domains are present also in plant proteins but their ordered combination within the RBCC motif is an achievement of metazoans.[10] Indeed, plants possess B-box domains but they are differently arranged with respect to each other (tandem of B-box 1 are observed) and to other domains. Also unicellular eukaryotes do not display the tripartite motif combination. The B-box domains in animal species are present only within the tripartite motif. The assemblage of the tripartite module is witnessed by the presence of members of the TRIM family in invertebrate species, e.g., the fruitfly *Drosophila melanogaster* and the worm *Caenorhabditis elegans*, which possess 7 and 18 TRIM genes, respectively, and the urochordate *Ciona intestinalis* (10 TRIM genes)[10] (Table 1). In these species, distinct proximal and distal B-box domains (B-box 1 and 2), sharing with vertebrates the same *consensi*, are associated with a RING domain and a CC region in a tripartite motif as seen in mammals. The tripartite motif is therefore exclusive to metazoans despite the fact that its constitutive elements are not. Before invertebrate-vertebrate lineage split,

Table 1. TRIM gene complements in representative vertebrate and invertebrate species

	Species	TRIM Set
Mammals	*Homo sapiens* (man)	68
	Mus musculus (mouse)	67
	Rattus norvegicus (rat)	59
	Canis familiaris (dog)	58
	Bos Taurus (cow)	66
Fish	*Tetraodon nigroviridis* (pufferfish)	66
	Danio rerio (zebrafish)	208
Aves	*Gallus gallus* (chicken)	37
Invertebrate	*Ciona intestinalis*	10
	Drosophila melanogaster (fruit fly)	18
	Caenorhabditis elegans (worm)	7

the tripartite motif has been associated with a discrete number of C-terminal domains that have been maintained with little changes throughout evolution. From this on, this domain structure experienced a great success leading to large expansion of the number of TRIM genes in vertebrate species and in particular in mammals.

In other mammals, the number of TRIM genes is comparable to the human complement with approximately 60 to 70 genes in each species analyzed (e.g., mouse, rat, dog, cat, cow)[10] (Table 1). What is evident by comparing the sets of genes from different species is the presence of TRIM genes with clearly recognizable orthology relationship as well as other genes which do not share an orthologous counterpart. A private set of species-specific TRIM genes is consistently present in each of the species analyzed suggesting that a group of TRIM genes evolved driven by species-specific constraints.[10,27,28] This feature is even more striking when TRIM complements were analyzed in nonmammalian vertebrates. The chicken possesses 37 TRIM genes and the number is more variable in teleost fish, 66 in the pufferfish *Tetraodon negroviridis* and 208 in the zebrafish *Danio rerio*[21] (Table 1). Of these avian and fish complements, a massive number of genes specific for each of these species is detected strengthening the idea of a functional selected expansion of species-specific TRIM genes.[21,24]

The notion of a species-specific evolution of subsets of TRIM genes was first indicated by a pioneering study on *TRIM5α* gene evolution.[29] *TRIM5α* is a major HIV-1 restriction factor (see Chapter 7 by Yap and Stoye) and the rhesus monkey gene is much more effective than the human one in counteracting the virus. The majority of mammalian orthologous genes are subject to purifying selection devoted to maintain conservation of protein sequence/structure limiting the fixation of amino acid substitutions that can affect the original function. Molecular evolutionary studies showed that *TRIM5α* belongs to a primate lineage private TRIM set and that it has been subjected to several rounds of positive selection in the primate clade. This positive selection, very likely driven by the antagonistic conflict with the HIV-1 virus, led to the rapid fixation of amino acid changes at the site of viral capsid interaction. This analysis allowed indeed the identification of a stretch of residues within the B30-like domain of *TRIM5α* responsible for the species-specific anti-HIV-1 restriction activity. These residues are different between human and rhesus monkey accounting for the more efficient antiviral activity of the

latter.[29] Along the same line, comparison of human and murine TRIM gene sets revealed that the TRIM proteins follow different evolutionary paces and the family is split in two evolutionary distinct groups.[10] One group is highly conserved comprising genes subject to strong purifying selection and in which orthology is strictly maintained in mammalian species and easily traceable in nonmammal vertebrates. Consistently, all the invertebrate TRIM genes show high similarity with this group.[9,10,21] A second group is conversely evolutionary younger, more dynamic and fast-evolving and represents a *reservoir* for the development of novel TRIM functions. Not surprisingly, the species-specific subsets of TRIM genes belong to this group and duplication (or transposition) events generated novel TRIM proteins that have been, or will be, engaged to exert novel functions. Several of the genes belonging to this second group are subject, upon human-mouse comparison, to nonpurifying selection, i.e.,to a higher rate of nonsynonymous versus synonymous changes with respect to the first group of conserved TRIM genes.[10] It is therefore possible that, similarly to *TRIM5α*, some of these genes might have been positively selected at some points during evolution. Interestingly, the same evolutionary features have been observed in the subset of fish-specific TRIM genes where positive selection has been observed also within paralogues.[21] These dynamic and fast-evolving genes are mainly represented in the Class IV subgroup and include *TRIM5α* and several other genes implicated in the response to viral and microbial infections.

The success of the tripartite motif arrangement determined therefore the expansion of the TRIM family and the generation of novel members in several species. These newly originated TRIM genes represent the ground on which, through positive selection, evolution shaped novel functions via antagonistic interactions with pathogens.

CONCLUSION

The TRIM family represents the largest subgroup of RING-containing E3 ubiquitin ligases. The tripartite motif structure has been maintained throughout evolution and accorded an important expansion with the continuous generation of novel TRIM genes. The implication of several of these genes in important physiological and pathological processes calls the attention to the need of addressing and exploring important issues to achieve the definition of their native structure, biochemical activity, and biological mechanism of action.

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CHAPTER 2

THE TRIPARTITE MOTIF
Structure and Function

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Abstract: The TRIM/RBCC proteins belong to a family whom members are involved in a variety of cellular processes such as apoptosis and cell cycle regulation. These proteins are defined by the presence of a tripartite motif composed of three zinc-binding domains, a RING finger, one or two B-box motifs, a coiled-coil region and a highly variable C-terminal region. Interestingly, the preserved order of the tripartite motif from the N- to the C-terminal end of the protein and the highly conserved overall architecture of this motif throughout evolution suggest that common biochemical functions may underline their assorted cellular roles.
Here we present the structure and the proposed function of each TRIM domain including the highly variable C-terminal domain.

INTRODUCTION

The tripartite motif (TRIM) protein family (also known as the RBCC family) contains proteins composed of three zinc-binding domains, a RING finger (R), a B-box Type 1 (B1) and a B-box Type 2 (B2) followed by a coiled-coil region (CC) and a highly variable C-terminal region. The latter can be a Filamin-type immunoglobulin domain (IG-FLMN), a Ret finger protein domain (RFP-like), a NCL-1/HT2A/LIN-41 repeat domain (NHL), a meprin and tumor necrosis factor receptor-associated factor homology domain (MATH), a plant homeodomain finger (PHD-BROMO) or a ADP-ribosylation factor (ARF) domain.[1-4] These proteins show the same overall arrangement of the RING,

TRIM/RBCC Proteins, edited by Germana Meroni.

B-box and coiled-coil domains. The main divergences stand in the number of B-boxes and the nature of the C-terminal domain.[5]

The first TRIM domain was identified in the Xenopus transcriptional regulator XNF7.[6] Subsequently, the availability of genome sequences combined with functional genomics approaches allowed the identification of a large number of new members of the TRIM family.[5,7] To date, more than 70 *TRIM* genes have been recognized in the human genome and orthologues were found in numerous species, from primates to eels[8] and nematodes.[9,10]

Genes belonging to this family are involved in a variety of cellular processes, including regulation of cell cycle progression, differentiation, development, oncogenesis and apoptosis.[9] For instance, transcriptional intermediary factors 1α and 1β (TIF1α/TRIM24 and TIF1β/KAP1/TRIM28) modulate transcriptional machinery to control specific gene expression during cell proliferation, differentiation and development whereas TIF1, promyelocytic leukaemia protein (PML/TRIM19) and ret finger protein (RFP/TRIM27) acquire oncogenic activity when fused by chromosomal translocation to retinoic acid receptor α (RARα), RET or B-raf, respectively.[11-14] Interestingly, an expanding group of these proteins is considered to be a part of an innate and intrinsic immune system showing the ability to target retroviruses and prevent their replication.[10,15-17]

Finally, malfunctions of certain TRIM genes cause human genetic diseases. Mutations in Pyrin/TRIM20, MID1/TRIM18, MUL/TRIM37 and TRIM32 have been associated with familial Mediterranean fever, X-linked Opitz/GBBB syndrome, mulibrey nanism and Limb-girdle muscular dystrophy Type 2H, respectively.[18-23]

The order of the domains within the tripartite motif (RING, B-box1, B-box2 and Coiled-coil) is maintained from the N terminus to the C terminus; if one domain is absent, the order of the remaining ones is conserved. The spacing between adjacent domains is also maintained (Fig. 1).[5,24]

THE TRIPARTITE MOTIF

Ring

The key element of the tripartite motif is the RING domain, which with few exceptions is typically found within 10-20 aminoacids from the first methionine.[5,24] The basic sequence expression of the canonical RING is Cys-X_2-Cys-$X_{(9-39)}$-Cys-$X_{(1-3)}$-His-$X_{(2-3)}$-Cys-X_2-Cys-$X_{(4-48)}$-Cys-X_2-Cys (where X is any amino acid). RING fingers are subcategorized into RING-C2 and RING-H2 depending of whether a Cys or His residue occupies the fifth coordination site.[25] Three-dimensional structures of RING domains revealed that the conserved cysteine and histidine residues are buried within the domain core, where they help to maintain the overall structure through the binding of two atoms of zinc. Additional semi-conserved residues are implicated either in forming the domain hydrophobic core or in recruiting other proteins. Unlike zinc fingers, the zinc coordination sites in a RING "finger" are interleaved, yielding a rigid, globular platform for protein-protein interaction, hence RING domain.[26-28]

The RING domain participates in homo and heterodimerization.[29,30] Heterodimeric complex involving two proteins with RING domains, such as RAG1 and BRCA1, suggests that the interaction is governed largely by structural elements that flank the RING motifs.[30] The RING domains act in a variety of unrelated biochemical reactions through supramolecular RING domain self-assembly.[31,32] It was observed that purified RINGs from

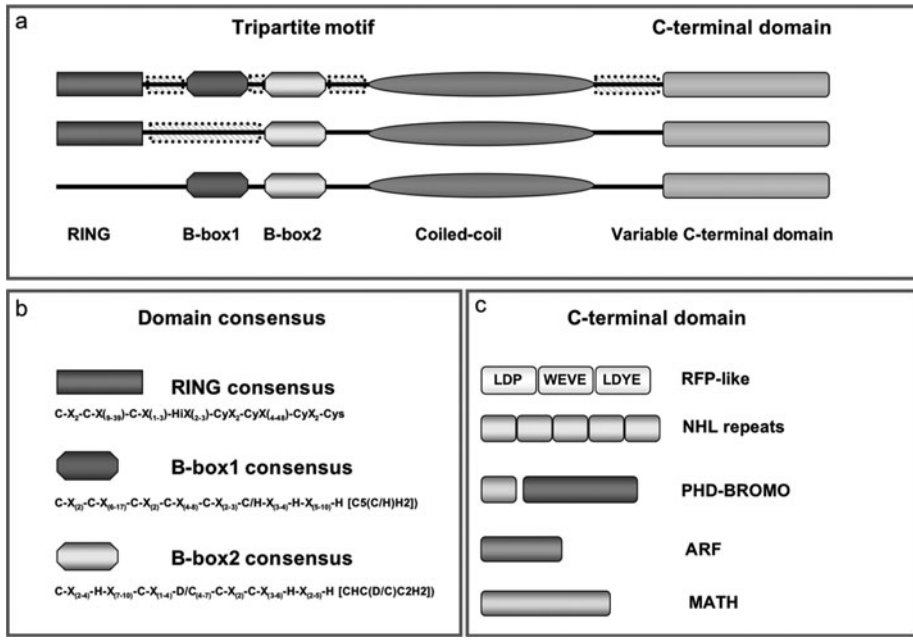

Figure 1. Schematic representation of the conserved domains of the TRIM/RBCC family members. a) Diagram of the domain composition in the tripartite-motif-containing proteins. The gray boxes represent the maintained spacing between adjacent domains. b) Consensus sequences for the RING, B-box1 and B-box2 domain within the tripartite motif. c) Variable C-terminal domain: RFP-like, B30.2/PRY-SPRY domain; NHL repeat; PHD-BROMO, plant homeodomain-bromodomain; ARF, ADP-ribosylation factor domain; MATH, meprin and TRAF homology domain.

a variety of functionally unrelated proteins, including PML, BRCA1 and BARD1, were able to self-assemble into supramolecular structures in vitro that resemble those they form in cells. RING bodies form polyvalent binding surfaces and scaffold multiple partner proteins. Interestingly, the formation of supramolecular structures by RINGs enhances specific activities of their partner proteins in two unrelated biochemical processes: first, reduction of 5' mRNA cap affinity of eIF4E by PML and second, E3 Ubiquitin conjugation activity of BRCA1:BARD1.[31,32] RING self-assembly creates bodies that act structurally as polyvalent scaffolds, thermodynamically by amplifying activities of partner proteins and catalytically by spatiotemporal coupling of enzymatic reactions. These supramolecular structures may serve in cells as a molecular mechanism of compartmentalization of biochemical reactions for subcellular organelles that are not membrane bound, such as those found in the nucleus. Moreover, insofar as PML-Nuclear Bodies are disrupted in the majority of cases of acute promyelocytic leukemia, RING self-assembly may be essential for normal cellular function, where it may serve to organize, control and integrate networks of biochemical reactions that collectively underlie biological phenomena.[31,32]

Bioinformatic analyses showed that ~300 human genes encode RING domain proteins. Most of these proteins are involved in the ubiquitination pathway as the RING domain mainly functions as an ubiquitin-protein isopeptide (E3) ligase.[33,34] Ubiquitination is a versatile posttranslational modification process mediated by ubiquitin,

a 76-residue polypeptide, used by eukaryotic cells to control protein level through the proteasome-mediated degradation or to control the activity of some proteins by posttranslational modification. The covalent attachment of several ubiquitin molecules in the form of a multiubiquitin chain on lysine residues of target proteins proceeds via three sequential steps: first the activation of the ubiquitin peptide by an activating E1 enzyme, second the transfer of the ubiquitin to an E2 conjugating enzyme and third its transfer to the substrate facilitated by an E3 ubiquitin ligase.[35] However, in some cases, multi-ubiquitylation requires the additional activity of certain ubiquitin-chain elongation factors termed E4 enzymes.[36]

E3s fall in two major classes: those containing a HECT domain and those containing a RING domain. HECT domain participates in the catalytic step by forming a thioester intermediate during ubiquitin transfer, whereas RING serves as a scaffold to bring together the substrate and the E2 and thus does not participate in the catalytic step per se. The crystallization of the E2 UbcH7 suggests interactions between RING fingers and E2s.[37] UbcH7 binds to the c-Cbl oncogene through contacts between a groove within the RING domain of c-Cbl and two loops in the E2 fold of UbcH7.[30,38] The interaction is largely due to van der Waals interactions involving hydrophobic residues in UbcH7 and the c-Cbl RING[37] (Fig. 2). Interacting residues in c-Cbl include a tryptophan (Trp) found in a number of active RING proteins, mutation of which abrogates E2 interaction and E3 activity.[39]

Not all RING domains possess intrinsic E3 activity. For example, the RING domains of Bard1,[40] Bmi1[41] and MdmX[42] do not exhibit E3 activity by themselves. In each of these cases, however, the RING domain interacts with a second RING domain protein (Brca1,

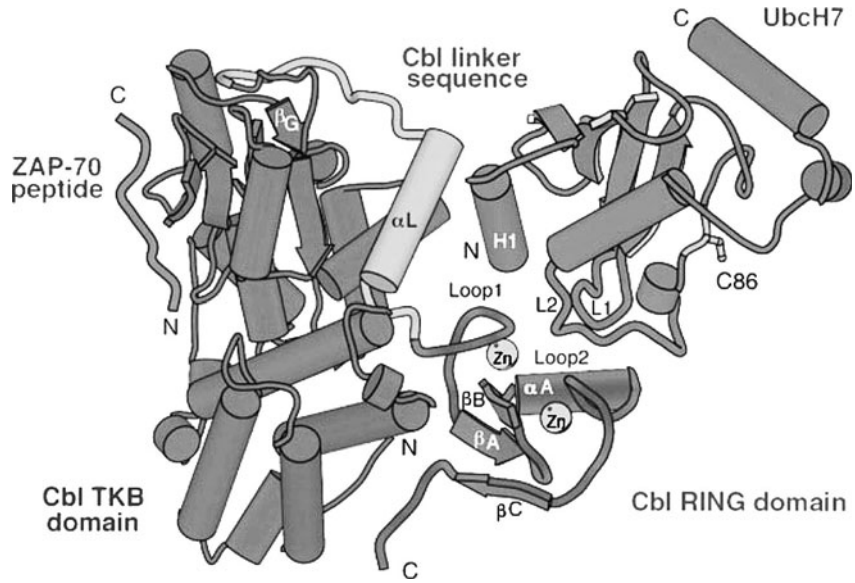

Figure 2. The E2 UbcH7-RING fingers domain interaction. UbcH7 binds to the RING domain of the c-Cbl oncogene through contacts between a groove within the RING domain of c-Cbl and two loops in the E2 fold of UbcH7. This interaction is largely due to van der Waals interactions involving hydrophobic residues in UbcH7 and the RING. Reprinted from: Zheng N et al. Cell 2000; 102(4):533-9,[37] ©2000 with permission from Elsevier.

Ring1b and Mdm2, respectively) and the heterodimer formation greatly stimulates E3 activity of the latter.

E3 ligase activity has been conclusively confirmed in some members of the TRIM family, including MID1/TRIM18, MURF1/TRIM63, Efp/TRIM25, TRIM32, TRIM22, TRIM44 and TRIM50[9,43] (see Chapter 3 by Ikeda and Inoue). Significantly, Kallijarvi et al. investigated the predicted ubiquitin ligase activity for the RING domain of TRIM37.[44] They showed that the TRIM37 full-length is highly polyubiquitinated when co-expressed with ubiquitin. The polyubiquitination was significantly reduced by converting two conserved cysteines to serines of the RING domain. Consistently, a point mutation (c.227T>C) that results to a Leu76Pro change, mapping in a predicted alpha helical region between the RING finger and the first B-box domain of TRIM37, is associated with a reduced polyubiquitination activity. It is likely that the change from a hydrophobic to a cyclic amino acid disrupts the integrity of the RBB motif and thus affects its function confirming the biological significance of RING domain in the E3 ligase activity.

The activity of RING domain ligases is controlled posttranslationally by covalent modifications such as phosphorylation or conjugation with ubiquitin-like proteins, by noncovalent binding of protein or small-molecule ligands, or by competition among substrates. All these posttranslational modifications could influence the action of these enzymes either through effects on the substrate, E2, or E3 itself.[28]

B-Box

B-box domains are important features of the TRIM proteins. B-boxes can have two different zinc binding motifs. Type 1 B-box (B-box1) contains the following zinc-binding consensus sequence C-$X_{(2)}$-C-$X_{(6-17)}$-C-$X_{(2)}$-C-$X_{(4-8)}$-C-$X_{(2-3)}$-C/H-$X_{(3-4)}$-H-$X_{(5-10)}$-H [C5(C/H)H2]), while the consensus for Type 2 B-box (B-box2) is C-$X_{(2-4)}$-H-$X_{(7-10)}$-C-$X_{(1-4)}$-D/$C_{(4-7)}$-C-$X_{(2)}$-C-$X_{(3-6)}$-H-$X_{(2-5)}$-H [CHC(D/C)C2H2].[5,45]

Around one-quarter of the human TRIM proteins possesses tandem B-boxes with the B-box Type 2 accompanied by an N-terminally positioned B-box Type 1. B-boxes can also be found as singleton in which case they are predominantly of the B-box 2 type.[5,6] Interestingly, the similarity in tertiary structures of the RING, B-box1 and B-box2 domains and the fact that B-box domains are associated predominantly with a RING domain suggested that these domains may have arisen as a result of gene duplication from a common ancestor.[45]

The structure of the B-box1 of TRIM18/MID1 was recently determined.[46] This domain consists of a three-turn α-helix, two short β-strands and three β-turns, encompassing Valine 117 to Proline 164, which binds two zinc atoms. One zinc atom is coordinated by cysteine residues 119, 122, 142 and 145, while cysteines 134 and 137 and histidines 15 and 159 coordinate the other (Fig. 3). The core of zinc-coordinating residues of B-box1 is conserved in all the other human TRIM proteins that contain tandem B-boxes as well as in those nonTRIM proteins in which the domain has been reported. However, the differences in sequence and size within the variable regions across the B-box1-containing proteins suggest that these regions may confer substrate specificity. Of note, the B-box1 structure closely resembles the folds of the RING, ZZ and U-box domains of E3 and E4 ubiquitin enzymes, raising the possibility that the B-box1 domain either has E3 activity by itself or enhances the activity of RING type E3 ligases (i.e., possibly confers E4 enzyme activity).

The B-box Type 2 domain is a prominent feature of the RBCC proteins but is also present in proteins beside TRIMs.[45] The first structure for a B-box2 was determined over

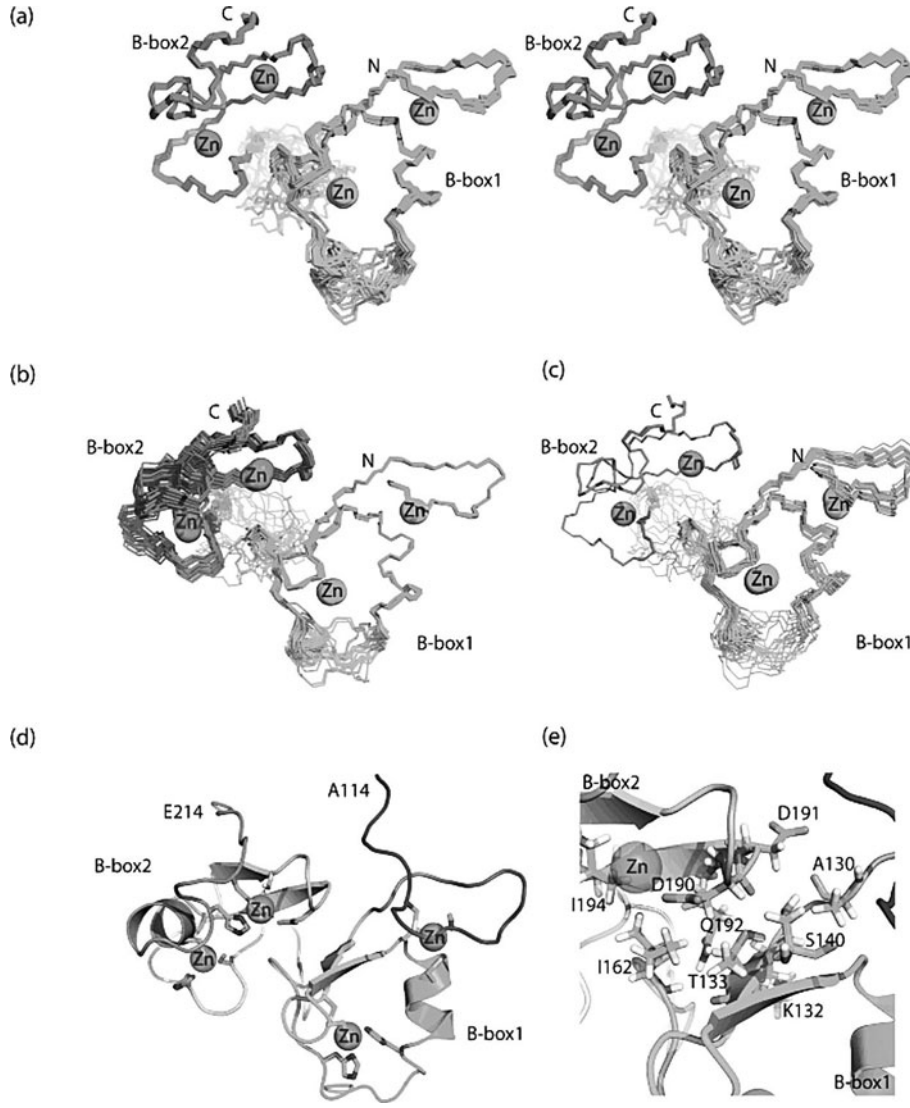

Figure 3. Solution structure of MID1 B-box1 and B-box2. a) Stereoview of 20 structures showing the superposition of backbone Ca, C and N atoms of the structured amino acid of both domains. The zinc atoms are shown as spheres and labeled. B-box1 is depicted in gray/green, B-box2 in dark gray/red and the unstructured residues in light gray/cyan. b) Superposition of the backbone atoms of the structured residues (Valine 117 to Histidine 150 and Histidine 159 to Leucine 161) of just the B-box1 domain. c) Superposition of the backbone atoms of the structured residues (Methionine 174 to Alanine 210) of the B-box2 domain only. The small increase in the precision of the positions of the nonsuperimposed domain indicates a small degree of flexibility between the B-box domains. Residues Valine 117-Glutamine 214 are shown for a-c. d) Ribbon representation of B1B2 (Alanine 114 to Glutamate 214) with the zinc ions shown as magenta spheres. Atom types of zinc-coordinating residues are color coded: yellow for sulfur, green for carbon, blue for nitrogen and red for oxygen. e) Close up view of the 10 residues located at the interface of the B-box domains. Reprinted from: Tao H et al. Biochemistry 2008; 47(8):2450-7, ©2008 with permission from the American Chemical Society. A color version of this figure is available online at www.landesbioscience.com/curie.

a decade ago for a 42 amino acid residue synthetic peptide encompassing the B-box2 domain from *Xenopus laevis* nuclear factor 7 (XNF7) using two-dimensional 1H-1H homonuclear NMR data.[6]

The XNF7 B-box, which contains four cysteine and three histidine residues, adopts a compact structure with two β-strands positioned perpendicular to each other, a helical turn and a number of small turns. It reportedly binds one zinc atom employing the Cys1, His1, Cys4 and His2 residues.[47] More recently the tertiary structure of the B-box2 domain from MID1 using multidimensional NMR spectroscopy has been solved.[45] It consists of a short α-helix and a structured loop with two short anti-parallel β-strands and adopts a tertiary structure similar to the B-box1 and RING structures, even though there is minimal primary sequence similarity between these domains. By mutagenesis, ESI-FTICR (electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry) and ICP (Inductively coupled plasma) mass spectrometry, it has been shown that the B-box2 domain coordinates two zinc atoms with a 'cross-brace' pattern (Fig. 3). The finding of a Cysteine 195 to Phenylalanine substitution identified in a patient with Opitz GBBB syndrome supports the importance of proper zinc coordination for the function of the MID1 B-box2 domain.[48] Finally, the recent release in Protein Data Bank of the structures of the B-box2 domains of MID2, TRIM63, TRIM39 and TRIM29 confirms the ββα-RING organization with two bound zinc atoms previously reported in MID1.

To date, no specific function has been assigned to the B-box2 domain. As the B-box2 domain is predominantly associated with RING and coiled-coil domains the possibility remains that it exerts some influence on the overall function of TRIM proteins.[10] It may have a role in the regulation of the RING domain function or in tandem with the B-box1 domain, possibly by influencing substrate recognition and/or ubiquitin ligase activity. Consistently, the MID1 B-box2 domain appears to impact the strength of the interaction between B-box1 and the Alpha 4 protein.[23,49] Alternatively, B-box2 may be important for the overall ability of these proteins to form homo-multimers and interact with other proteins.

Coiled-Coil

The coiled-coil motif of TRIM proteins is approximately 100 residues long and is frequently broken up in two or three separated coiled-coil sub-motifs. Their simple geometric organization, consisting of alpha helices wound together to form a "rope-like" structure stabilized by hydrophobic interactions, confers remarkable mechanical properties.

The primary sequence of the coiled-coil region within TRIM proteins, though rich in hydrophobic amino acids and frequently leucine residues, is not conserved. This domain is mainly involved in homo-interactions and in promoting the formation of high molecular weight complexes. Reymond and colleagues took advantage of the interaction-mating technique[5] and showed that a consistent number of TRIM members were able to homo-interact, whereas heterologous interactions were rare, indicating a high degree of specificity for homo-oligomerization, with few exceptions such as the interaction between TRIM1/MID2, TRIM18/MID1,[5] PML and TRIM27[50-55] and among TIF1 transcriptional cofactors.[2] Additionally, it was recently demonstrated that TRIM44 is able to heterodimerize with Terf (TRIM17) regulating its ubiquitination and stabilization. Results of in vivo co-immunoprecipitation assays by using TRIM44 and Terf deletion mutants suggest that the coiled-coil domains of these proteins are sufficient to sustain this protein-protein interaction.[56]

In order to map the structural determinants responsible for their self-association, TRIM mutants carrying individual deletions of relevant protein regions were analyzed by interaction mating and in vivo co-immunoprecipitation. Deletion of the CC region resulted in the loss of self-association, while removal of other regions only partially affected binding. Notably, isolated CC, but not isolated R or BB, was able to self-associate, suggesting that the CC region is necessary and sufficient for homo-interaction. In the same work the ability of CC to form higher order complexes was tested by gel filtration analysis of in vitro translated TRIM proteins. Each of the seven assayed TRIM proteins (TRIM6, 8, 11, 23, 28, 29 and 30) eluted in fractions corresponding to high molecular weight complexes.[5] Comparable results were gathered subsequently for TRIM18 (MID1), 19 (PML), 28 (TIF1beta) and 50.[2,43,51,54,57] Together, these results suggest that TRIM proteins form high molecular weight complexes in vivo as a consequence of the self-association properties of their CC region. Noticeably, the CC domain of TRIM proteins appears to be involved in the definition of discrete subcellular compartments within the cell.[5]

C-TERMINAL REGION

The TRIM motif is usually followed by one or more C-terminal domains of different length and nature. While the tripartite motif is restricted to the TRIM protein family, the C-terminal region domains are also present in otherwise unrelated proteins.[9]

Numerous TRIM proteins do not possess a defined C-terminal domain. In these cases, either their coding region is limited to the tripartite motif or the C-terminal portion is not similar to any other known domains or proteins. Recently, computational and/or functional investigations have permitted the subclassification of the entire human TRIM complement into nine subfamilies based on their varied C-terminal domain organization: COS-FN3-B30.2-like domain (C-I subfamily), COS-Acid Rich region (C-II), COS-FN3 (C-III), B30.2 like or SPRY-containing (C-IV), no identifiable C-terminal domain homology (C-V), PHD-BROMO domain (C-VI), immunoglobulin-NHL repeats (C-VII), MATH domain (C-VIII) and ARF domain (C-IX).[3]

Here we report the functional and molecular description of well-studied C-terminal domains of TRIM proteins.

Approximately two-third of the TRIM proteins possesses a B30.2 domain also known as RFP-like domain, first identified in TRIM27/RFP.[58,59] This 170 amino acids domain is found in two other protein families besides TRIMs: the receptor glycoproteins of the immunoglobulin superfamily (BTN)[60] and the stonutoxin (STNX), a secreted venomous protein product by stonefish *Synanceia horrida*.[61]

The description of the B30.2 domain was shortly followed by the description of the SPRY domain that has been identified in at least 10 distinct protein families.[62] In addition to this, a third domain called PRY is sometimes associated with SPRY. Homology investigations based on a hidden Markov Model (HMM) identified over 1800 sequences with B30.2, SPRY and PRY domains.[63] B30.2 domain sequences contain three blocks of conserved residues, the LDPD block (also known as PRY domain), the WEVE and the LDYE blocks (also known as SPRY domain). HMM analysis and evolutionary studies showed that the B30.2 domain is a fusion of PRY and SPRY domains. Thus B30.2 domain may be regarded as a sub-group of sequences referred to as SPRY, from which it has relatively recently evolved and expanded by incorporating PRY sequences at its amino terminus. The recently solved structure of several B30.2

indicates that the core is formed from a distorted 2-layer beta sandwich with the beta strands in an anti-parallel arrangement. Interspecies comparisons indicate that the protein sequence of the beta strand segments tends to be stable in length, in contrast to the many insertions and deletions in the putative loops connecting these strands.[64,65]

The existence of four regions within the B30.2 domain that exhibited substantial variation in length as well as extensive amino acid differences among the TRIM proteins was particularly noteworthy. Song has designated these variable regions v1, v2, v3 and v4. Because of the modest level of amino acid variation among the TRIM B30.2 domains, the boundaries of these variable regions are somewhat arbitrary.[65,66]

Collectively, proteins containing B30.2 domain cover a wide range of functions, including regulation of cytokine signalling (SOCS),[67,68] RNA metabolism (DDX1, hnRNPs),[69] intracellular calcium release (RyR receptors),[70] regulatory and developmental processes (HERC1, Ash2L).[71-73] In the TRIM family, B30.2 domain plays an important role in the regulation of immune response to retroviruses. For instance TRIM5α has been shown to be a cellular restriction factor for infection by HIV-1 on rhesus monkey cells.[74,75]

Differential splicing of the TRIM5 primary transcript gives rise to the expression of several isoforms of the protein product.[5] The TRIM5α isoform is the largest product (493 amino acid residues in humans) and contains the B30.2/SPRY domain. The other TRIM5 isoforms (γ and δ are the best substantiated of these) lack an intact B30.2/SPRY domain.[74]

The TRIM5γ_{rh} isoform does not inhibit HIV-1 or SIV$_{mac}$ infection. In fact, TRIM5γ_{rh} has been shown to exhibit a weak dominant-negative activity, repressing the ability of wild-type TRIM5α_{rh} to inhibit HIV-1 infection.[74] Recognition of HIV-1 viral capsid by the B30.2 domain of TRIM5α is critical and the site of interaction has been narrowed down to a patch of 13 amino acids located in the PRY region of TRIM5α.[64]

It was recently found that the B-box 2 domain mediates higher-order self-association of TRIM5 oligomers. This self-association increases the efficiency of TRIM5 binding to the retroviral capsid, thus potentiating restriction of retroviral infection. Additionally, the potentiation of capsid binding that results from B-box 2-mediated self-association is essential for restriction when B30.2 (SPRY) domain-mediated interactions with the retroviral capsid are weak. Thus, B-box 2 dependent higher-order self-association and B30.2 (SPRY)-dependent capsid binding represent complementary mechanisms whereby sufficiently dense arrays of capside-bound TRIM5 proteins can be achieved.[34]

Beside TRIM5, a number of other TRIM proteins have been shown to possess antiviral activities[9,10,76] (see Chapter 7 by Yap and Stoye). For example, TRIM1 moderately restricts infections with N-MLV,[77] TRIM34 can moderately inhibit SIV$_{mac}$ infection[78] and overexpression of TRIM19 mediates resistance against the human cytomegalovirus, the vesicular stomatitis virus, herpes simplex virus and influenza virus A.[79-81] Moreover, TRIM28 restricts MLV in cells of germline origin by inhibiting LTR-driven transcription[76] and TRIM32 was reported to attenuate transcription of the HIV LTR.[82,83] Most TRIM proteins, which were reported in an antiviral context (e.g., TRIM1, 5, 22, 26 and 34), possess a B30.2 domain at the C-terminus.

Additionally, the B30.2 domain is also involved in subcellular localization of some TRIM proteins. It was recently demonstrated that the B30.2/SPRY domain of the antiviral TRIM22 protein is important to localize the protein in distinct nuclear bodies. Particularly, it was observed that Val493 and Cys494 in the B30.2/SPRY domain are critical for the presence of TRIM22 within nuclear bodies.[84] Interestingly, the C-terminus

deletion mutant containing only the B30.2/SPRY domain also localized as cytoplasmic protein suggesting that this motif may collaborate with other sequences such as the putative bipartite NLS to import TRIM22 into the nucleus.[84,85] Similarly, mutations in the B30.2 domain of TRIM9 and TRIM18 also lead to changes in their subcellular localization, while both proteins are normally located in microtubuli-associated structures, the mutated forms localize into cytoplasmic speckles.[3]

In silico approaches helped to define a subfamily that shares an identical C-terminal domain arrangement including MID1, MID2, TRIM9, TNL, TRIM36, TRIM46. Significantly, it was showed that all the analyzed members of this subfamily associate with the microtubule cytoskeleton, suggesting that subcellular compartmentalization is determined by a unique domain architecture that may in turn reflect basic functional similarities. This region called COS box and adjacent to the coiled-coil consists of two alpha-helical coils. It is also present within the MURF family (muscle specific RING finger) (see Chapter 8 by Cox and Chapter 9 by Mayans and Labeit). MURF1 family members have established themselves as excellent candidates for linking myofibril components (including the giant, multi-functional protein, titin/connectin) with microtubules, intermediate filaments and nuclear factors. Consistently with its role in linking microtubules, mutations in the COS box result in a complete loss of microtubule localization but not dimerization ability. Conversely, inclusion of the COS box into a nonmicrotubule binding TRIM protein redirects the fused protein to a microtubule-associated localization.[3]

A role for the coiled-coil/COS box region in dimerization and microtubule association is also supported by the observation that mutations in Murf3 removing amino acids either from the N-terminal end to the coiled-coil or from the C-terminal end of the COS box maintain their microtubule association and dimerization properties.[86] However, the presence of coiled-coil domains throughout the generally nonmicrotubular TRIM superfamily suggests that a coiled-coil alone is not sufficient to trigger association with the microtubule scaffold. It also supports the notion that the COS box conferrs microtubule binding capacity assisted by the coiled-coil.

A less-frequent C-terminal domain is the NHL domain that consists of 5 or 6 repeats that usually end with a tryptophane and an aspartate residue.[4] Each repeat is approximately 40-residues long and resembles the WD40-repeat. Similarly to NHL proteins, WD40 proteins have four to eight repeating units flanked by Glycine-Histidine (GH) and Tryptophane-Aspartate (WD) at both termini and fold into a beta-propeller.[4] The neural activity-related RING finger protein (NARF) predominantly expressed in the brain (especially in the hippocampus) is a TRIM protein containing the NHL domain. This motif is involved in the interaction with myosin V, one of the most abundant myosin isoforms in neurons. Several observations indicated that NARF contributes to the alteration of neural cellular mechanisms along with myosin V.[87] Another brain-expressed TRIM-NHL protein, TRIM2 binds to and regulates the neurofilament light subunit (NF-L) by ubiquitination. Deletion of the NHL repeats reduces the binding and deletion of both the central region and RBCC domains abolishes the NF-L–TRIM2 interaction. Interestingly, *Trim2* knockout mice were born without any obvious brain defects but develop progressive neurodegeneration, suggesting that TRIM-NHL proteins may also serve important functions in the adult brain.[88] Recently, it has been shown that the NHL domain of TRIM32 protein, both in *Caenorhabditis elegans* and in mice positively regulates miRNA activity and plays roles in developmental timing and asymmetric cell division.[89,90]

The TRIM proteins containing an additional Cys/His cluster (PHD finger) and a bromo-related domain represent a more homogeneous subfamily composed of four members, the TIF1 proteins with function as transcriptional intermediary factors, being either co-activators or corepressors. The intrinsic transcriptional repression functions are localized in their COOH-terminal PHD and bromodomain.[2,91,92]

ADP-ribosylation factor domain protein 1 (ARD1) is a multifunctional protein belonging to the TRIM family, that encodes a 64-kDa protein with a structure comprising at its C-terminus a 18-kDa ADP-ribosylation factor (ARF) domain. The ARF family contains three classes of typical mammalian ARFs that are grouped by similarities of amino acid sequence and gene structure, as well as by phylogenetic relationships.[93] ARFs play major roles in regulating intracellular vesicular trafficking through interaction with coat proteins, as well as in cytoskeletal and membrane remodelling through activation of phosphatidylinositol 4-phosphate 5-kinase and phospholipase D.[94,95] ARD1, like all ARFs, alternates between active GTP-bound and inactive GDP-bound states.[96] Via its C-terminal ARF domain, recombinant ARD1 binds to guanine nucleotides, in a GTP-dependent manner.

CONCLUSION

Despite the growing importance of the TRIM family of protein and their role on different essential cellular processes, the overall function of the highly conserved tripartite structure remains to be elucidated. Interestingly inspection of the whole family reveals conserved residues spacing between the RING, B-boxes and coiled-coil domains suggesting that the overall architecture of the tripartite motif is highly conserved throughout evolution and that the tripartite motif serves as an integrated functional structure. These features support the idea that the tripartite motif may serve as an integrated functional structure, rather than a collection of separate modules.

Experimental evidences have implicated the RING domain in ubiquitin-ligase function, the coiled-coil domain in multimerization and the B30.2 domain in the regulation of immunity response to retroviruses, whereas little is known about the function of B-box domains. The propensity of these proteins to define specific and often novel cellular compartments and to facilitate protein aggregation suggests that they might act as a protein scaffold for the assembly of higher order molecular weight structures to recruit proteins involved in several distinct processes.

We are persuaded that in the near future a comprehensive analysis of multiple (all) TRIM proteins will consent to further clarify the many and different function of this interesting protein family.

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CHAPTER 3

TRIM PROTEINS AS RING FINGER E3 UBIQUITIN LIGASES

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Abstract The tripartite motif (TRIM) proteins harboring the RING finger, B-box and coiled-coil (RBCC) domain motifs form a large protein family. The members of this family are involved in various biological processes, including growth, differentiation, apoptosis and transcription and also in diseases and oncogenesis. Recent studies have revealed that TRIM proteins play key roles in innate antiviral immunity. An accumulating body of evidence has demonstrated that some TRIM proteins function as E3 ubiquitin ligases in specific ubiquitin-mediated protein degradation pathways; however, the precise mechanisms underlying this function have not been fully elucidated. In this chapter, we focus on the TRIM family of proteins specially with regard to E3 ligase.

INTRODUCTION

A large number of proteins (more than 17,000) harboring the RING finger domain have been identified in diverse eukaryotes in the simple modular architecture research tool (SMART) database. The RING finger motif is defined as a unique linear series of conserved cysteine and histidine residues, i.e., Cys-X_2-Cys-X_{11-16}-Cys-X-His-X_2-Cys-X_2-Cys-X_{7-74}-Cys-X_2-Cys (C_3HC_4 type), where X can be any amino acid. Three-dimensional analyses of RING domains have confirmed that the RING finger motif is composed of a unique "cross-brace" arrangement with 2 zinc ions and it folds into a compact domain comprising a small central β sheet and an α helix.[1,2] Frequently, the RING domain is associated with cysteine-rich B-box domains followed by a predicted coiled-coil domain. This ensemble of a RING domain, 1 or 2 B-box domains and a coiled-coil domain are called RBCC or TRIM.[3]

TRIM/RBCC Proteins, edited by Germana Meroni.

Each domain of the TRIM protein may operate as a functional unit, either independently or in combination with other domains and play important roles in the recognition of specific interacting partners or the formation of oligomers. Studies have shown that the RING finger proteins play crucial roles in growth, differentiation, transcription, signal transduction and oncogenesis.[4-10] These studies have revealed some essential roles played by the RING finger domains in the function of these proteins. Recently, it was revealed that the RING finger proteins are often involved in the ubiquitin-mediated protein degradation pathway.

TRIM PROTEINS ARE INVOLVED IN PROTEIN MODIFICATION PATHWAY BY UBIQUITIN

Ubiquitin-dependent protein degradation is a specific and elaborate mechanism in which the target protein to be destroyed is tagged with ubiquitin. Ubiquitination is accomplished by a complex process involving the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) (Fig. 1).[11] Ubiquitin ligase mediates the transfer of ubiquitin from E2 to a substrate, enabling degradation of the latter by the 26S proteasome. The C_3HC_4-type RING finger domain is found in several E3 proteins, including Cbl,[12] BRCA1,[13] estrogen-responsive finger protein (Efp)[14] and murine double minute 2 (Mdm2) [15] and so on. The RING-H2 subtype in which Cys5 is substituted with histidine is found in RING box protein 1 (Rbx1) and anaphase promoting complex (APC) subunit 11 (Apc11), which are components of the Skp1-Cullin-F-box (SCF) and APC E3 complexes,[16] respectively and in several other ubiquitin ligases. E3 enzyme is thought to be important for specific recognition of the substrate in the ubiquitination pathway. In the

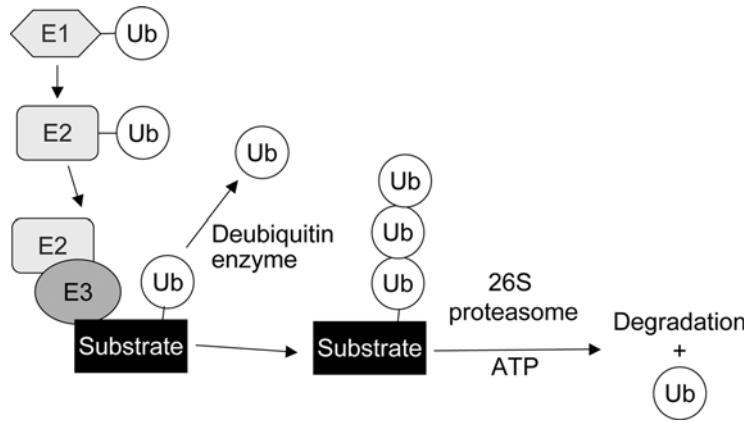

Figure 1. The ubiquitin proteolytic pathway. In the ubiquitin-proteasome degradation pathway, ubiquitin (Ub) is first covalently ligated to target proteins via a multienzymatic system consisting of ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligating (E3) enzymes. E1 activates a ubiquitin monomer at its C-terminal cysteine residue to create a high-energy thioester bond; it is then transferred to a reactive cysteine residue of the E2 enzyme. The final transfer of ubiquitin to a reactive lysine residue of the substrate protein is brought about by the E3 enzyme. E3 enzymes function as substrate-recognition molecules and are capable of interacting with both E2 and the substrate. The ubiquitinated protein is then conveyed to the 26S proteasome, where it undergoes final degradation and the ubiquitin is finally released and recycled.

ubiquitination model established many years ago, the first ubiquitin moiety is anchored via its COOH-terminal Gly residue to an -NH2 group of an internal Lys residue in the target substrate. This is followed by the generation of a polyubiquitin chain in which additional ubiquitin moieties are linked to one another via Gly-76-Lys-48 isopeptide bonds.[17] During recent years, other modes of ubiquitination have been discovered. One such noncanonical conjugation reaction involves the anchoring of ubiquitin to Lys residues other than Lys-48 in the previously conjugated ubiquitin moiety. The ubiquitination of Lys-63 appears to play a role in a variety of processes, including the endocytosis of cell surface receptors,[18,19] postreplicative DNA repair,[20] stress responses,[21] ribosomal functioning,[22] and activation of the IB signaling complex.[23] This type of modification does not appear to involve proteolysis of the target substrates but plays a role in the activation/inactivation of the target protein.

EFP/TRIM25 FUNCTIONS AS E3 LIGASE FOR BOTH UBIQUITINATION AND ISGYLATION

In the RING finger protein family, Efp is a member of the RING-finger, B1- and B2-box, coiled coil and SPRY (RBCC-SPRY) subfamily. Efp was isolated as an estrogen-responsive gene by the genomic binding-sites cloning method utilizing a recombinant estrogen receptor (ER) protein.[24] The estrogen-responsive element (ERE) to which ER can bind is found at the 3′-untranslated region (UTR) in the Efp gene and the gene's expression is predominantly detected in female reproductive organs, including the uterus, ovary and mammary gland,[25] and in breast and ovarian cancers.[26] Estrogen-induced Efp expression is found in the uterus, brain and mammary gland cells. Efp-knockout mice have underdeveloped uteri and estrogen responses are markedly attenuated in uteri of knockout mice; this suggests that Efp is necessary for estrogen-induced cell growth.[27] Moreover, the growth of MCF7 breast cancer cells implanted in female athymic mice is reduced by treatment with the Efp antisense oligonucleotide. In contrast, Efp-overexpressing MCF7 cells in ovariectomized athymic mice generate tumors in the absence of estrogen.[14] These findings indicate that Efp mediates estrogen-dependent growth in breast cancer cells. We identified 14-3-3σ, which is responsible for reduced cell growth, as a factor that interacts with Efp and detected 14-3-3σ accumulation in the embryonic fibroblasts of Efp-knockout mice. Furthermore, it has been revealed that Efp is an E3 ubiquitin ligase that targets the proteolysis of 14-3-3σ (Fig. 2).

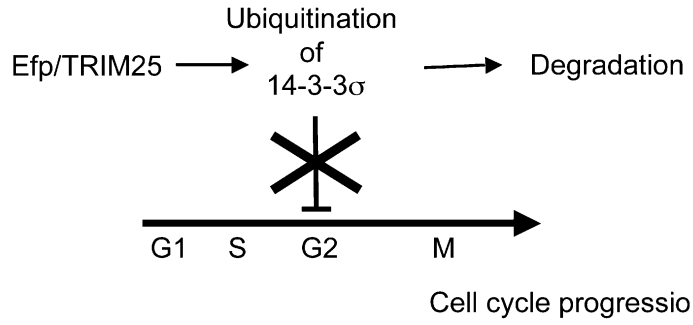

Figure 2. Model for tumor growth controlled by Efp. Efp stimulates tumor growth by targeting 14-3-3σ (a negative regulator of the cell cycle) for proteolysis as an E3 ubiquitin ligase.

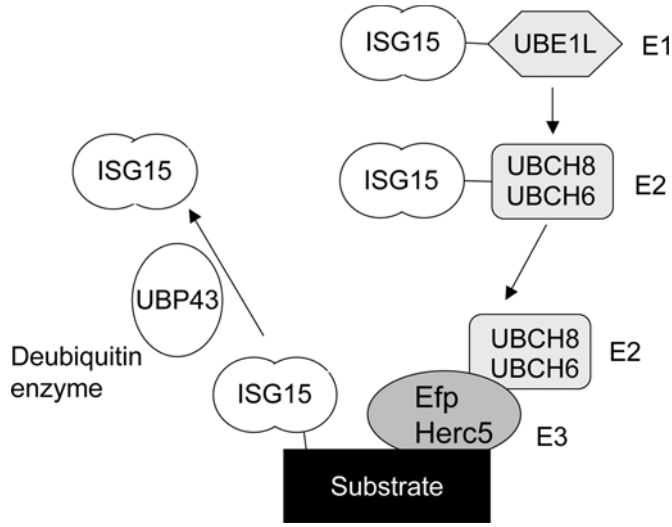

Figure 3. Pathway of ISGylation. ISG15, a ubiquitin-like molecule containing 2 conserved ubiquitin domains, is conjugated to proteins; this conjugation is similar to ubiquitination. In this system, the E1 (UBE1L), E2 (UbcH8) and E3 (Efp and Herc5) enzymes required for ISGylation have been hitherto identified.

In particular, the RING domain that preferentially binds to E2 UbcH8 is essential for the ubiquitination of 14-3-3σ. The 14-3-3σ degradation after ubquitination coupled with Efp as an E3 lagase, followed by activation of the cyclin-Cdk complexes, leads to cell cycle progression, cell proliferation and tumor growth; these findings provide an insight into the cell-cycle machinery and tumorigenesis of breast cancer cells.

In addition, we showed that Efp expression is up-regulated by Type I interferon (IFN) through an IFN-stimulated response element (ISRE) located in the first intron.[28] Electrophoretic mobility shift assays and chromatin immunoprecipitation assays showed that the ISRE binds to signal transducer and activator of transcription 1 (STAT1). Moreover, we showed that the Efp protein could be conjugated with not only ubiquitin but also ISG15, a ubiquitin-like molecule.[28] The IFN-stimulated gene 15 (ISG15) has been originally identified as a Type I IFN-stimulated gene encoding a 15-kD protein.[29] The ISG15 protein contains 2 conserved ubiquitin-like domains and is conjugated to target proteins or ISGylated along with E1, E2 and E3 by a process similar to ubiquitination (Fig. 3).[30] Thus far, Efp and Herc5 have been identified as E3 ligases that undergo ISGylation.[28,31,32] In particular, Efp can conjugate with both ISG15 and ubiquitin and can also conjugate these molecules to 14-3-3σ.[14,28,32] ISG15 expression and protein ISGylation are strongly induced by viral infection;[33,34] this finding indicates that the ISGylation system is a novel pathway that transduces the antiviral response in IFN-stimulated cells. It has been reported that ISG15 can inhibit the release of human immunodeficiency virus Type 1 (HIV-1) virions[35] and attenuate Sindbis virus infection.[36] ISG15$^{-/-}$ mice are more susceptible to infection with the influenza virus, herpes simplex virus Type 1 and Sindbis virus.[37] These data suggest that Efp is an IFN-responsive gene that potentially mediates the functions of IFN and is involved in the ISGylation and ubiquitination of proteins, including Efp itself.

Recently, we reported that TRIM25 induces ubiquitination of retinoic acid-inducible gene I product (RIG-I), which is a cellular sensor of RNA virus infection and induces IFN-β

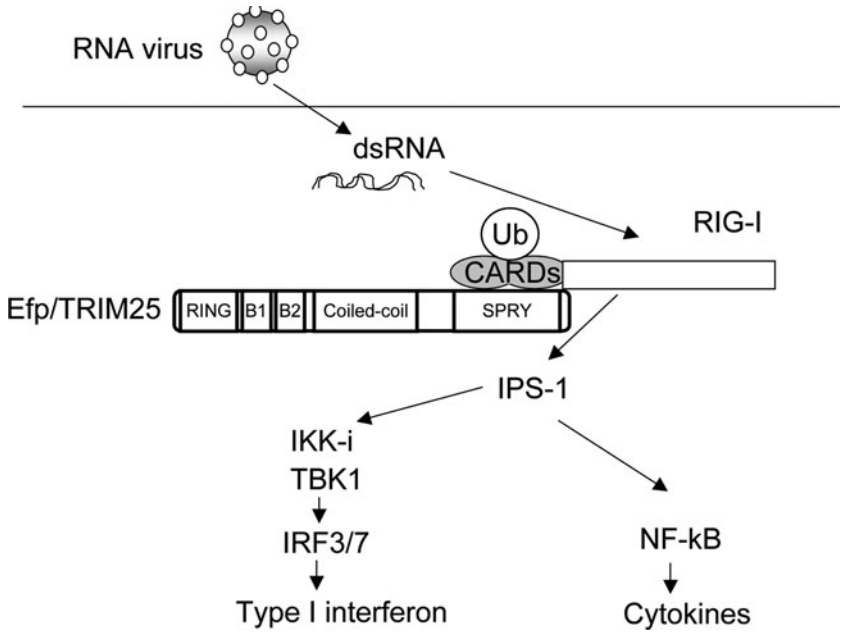

Figure 4. Efp blocks RNA virus infection. Efp can bind the CARDs of RIG-I, an RNA virus sensor, through its C-terminal SPRY domain. This interaction stimulates the Lys-63-mediated conjugation of ubiquitin to the CARDs, resulting in a marked increase in the downstream signaling activity of RIG-I.

expression (Fig. 4).[38] RIG-I encodes two caspase recruitment domains (CARDs) at the N terminus and an RNA helicase domain at the C terminus. The helicase domain recognizes viral dsRNA and the CARDs directly transmits a signal that activates both IRF-3 and NFκB to induce the expression of IFN-β and the antiviral cytokine gene.[39] The C-terminal SPRY domain of TRIM25 interacts with the N-terminal CARDs of RIG-I; this interaction effectively delivers the Lys-63-linked ubiquitin moiety to the CARDs of RIG-I, resulting in a marked increase in the downstream signaling activity of RIG-I. The Lys-172 residue of RIG-I is critical for efficient TRIM25-mediated ubiquitination, mitochondrial antiviral signaling protein (MAVS) binding and RIGI-mediated induction of antiviral signal transduction. TRIM25$^{-/-}$ mouse embryonic fibroblasts (MEFs) showed that TRIM25 is essential not only for RIG-I ubiquitination but also for RIG-I-mediated IFN-β production and antiviral activity in response to RNA virus infection.[38,40] Thus, TRIM25 E3 ubiquitin ligase induces the Lys-63-linked ubiquitination of RIG-I, which is crucial for innate host antiviral immunity elicited by the RIGI signaling pathway. Moreover, the influenza A virus nonstructural protein 1 (NS1) inhibits ubiquitination of the TRIM25-mediated RIG-I CARD and RIG-I signal transduction. TRIM25 is therefore a key host factor targeted by influenza virus infection.[41]

E3 UBIQUITIN LIGASES IN TRIM/RBCC PROTEINS

The presence of RING finger domains in E3 ubiquitin ligases implies that the members of this TRIM family are potential targets for specific regulators/adopters in ubiquitin-dependent protein degradation. In fact, some genes belonging to the TRIM

family have been shown to have E3 ligase activity. Next, we discuss such TRIM family members, focusing on the findings of recent studies.

MID1/TRIM18

A positional cloning approach has revealed that MID1 is involved in the Opitz GBBB syndrome (OS).[42-44] Further, MID1 associates with microtubules, whereas its mutant forms do not.[45] These findings suggest that MID1 plays a physiological role in microtubule dynamics. The α4 protein, a regulatory subunit of protein phosphatase 2A (PP2A)[46] was isolated using yeast two-hybrid screening with MID1 as bait. The cellular localization of MID1 and α4 is coincident with cytoskeletal structures. The expression of MID1 with a mutation at the C terminus, which mimics the mutant protein in some individuals with OS, causes the formation of cytoplasmic clumps containing both proteins. A cytosolic PP2A was identified as the substrate for the E3 ligase activity of MID1. In contrast, the addition of a proteasome inhibitor to OS-derived fibroblasts expressing dysfunctional MID1 does not upregulate either PP2A or the enzyme's polyubiquitinated forms;[47] this suggests that MID1 mutations result in decreased proteolysis of the C subunit of PP2A in individuals with OS.

TRIM11

TRIM11 belongs to a protein family composed of a RING finger domain, which is a putative E3 ubiquitin ligase; a B-box domain; a coiled-coil domain; and an SPRY domain. A recent experiment with yeast two-hybrid screening has revealed that TRIM11 can interact with humanin,[48] which is a newly identified anti-apoptotic peptide that specifically suppresses Alzheimer's disease (AD)-related neurotoxicity. Humanin binds with Bax and prevents the translocation of Bax to mitochondria, thus preventing the release of cytochrome c.[49] Thus, humanin seems to exert its anti-apoptotic effects by interfering with Bax function. The intracellular level of humanin was drastically reduced in the presence of TRIM11 and mutation of the RING finger domain or treatment with a proteasome inhibitor attenuates the effect of TRIM11 on the intracellular level of humanin.[48] These results suggest that TRIM11 may act as an E3 ligase in the ubiquitin-mediated degradation of humanin.

SSA/Ro (SSA1/TRIM21)

TRIM21 has been identified as an autoantigen in Sjögren syndrome.[49,50] A recent study reported that TRIM21 exhibits E3 activity and interacts with the human IgG1 heavy chain. The IgG1 heavy chain is polyubiquitinated by TRIM21 and degraded through the ubiquitin-proteasome system; this suggests that it plays a significant role in the quality control of IgG1.[51] In addition, TRIM21 ubiquitinates IRF-8 and enhances IL-12p40 expression in IFN-γ/TLR-stimulated macrophages. These findings imply that TRIM21 contributes to the elicitation of innate immunity in macrophages.[52]

TRIM32

TRIM32 belongs to the TRIM protein family and possesses 6 C-terminal NHL domains. A point mutation in 1 NHL domain (D487N) has been linked to 2 forms of muscular dystrophy—limb girdle muscular dystrophy Type 2H and sarcotubular myopathy.

TRIM32 is primarily expressed in skeletal muscles and its expression is significantly elevated in muscles undergoing remodeling and during myogenic differentiation. TRIM32 ubiquitinates actin and thus probably participates in myofibrillar protein turnover, especially during muscle adaptation.[53]

MuRF1/TRIM63

MuRF1/TRIM63 and 2 other members of the MuRF family, namely, MuRF2/TRIM55 and MuRF3/TRIM54, all belong to the TRIM family. MuRF proteins localize to the sarcomere,[54] and MuRF1 associates with titin in the M band of the sarcomere, thus possibly maintaining the stability of the sarcomeric M band.[55,56] MuRF-1 is an E3 ubiquitin ligase that acts on troponin I and is upregulated in atrophic muscles.[57,58] Muscular atrophy, which is associated with various diseases and is a side effect of treatment with synthetic glucocorticoid treatment, is characterized by accelerated protein degradation via the ubiquitin proteasome system. MuRF1$^{-/-}$ mice exhibit increased resistance to muscular atrophy and significant muscle preservation after denervation; this suggests that MuRF-1 plays a critical role in muscle turnover.[58,59]

TRIM5

TRIM5α, which is a member of the TRIM-SPRY subfamily, has recently been identified as a cellular factor that is essential for retroviral restriction and targets incoming retroviral capsids after viral penetration[60] or the Gag assembly during HIV-1 production by rapidly degrading HIV-1 Gag polyproteins (Fig. 5).[61] HIV-1 infection

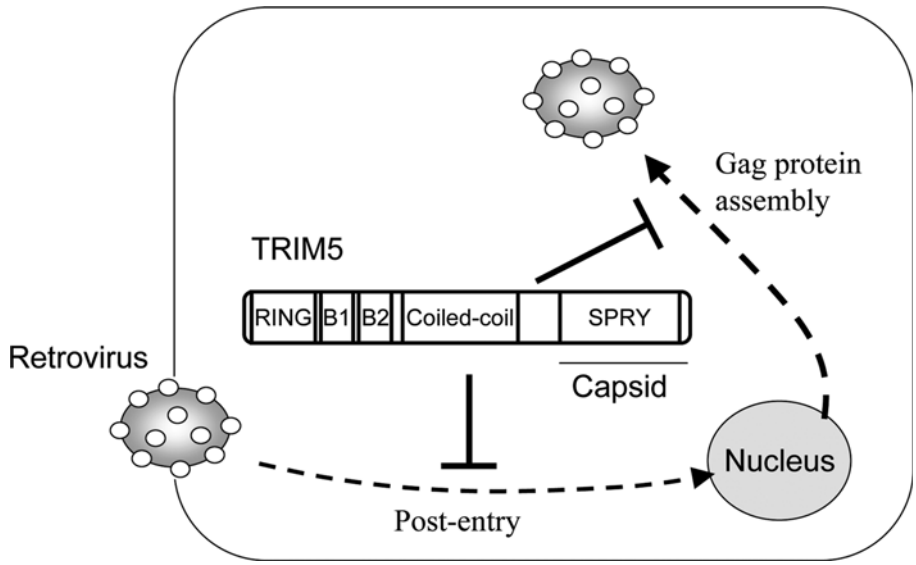

Figure 5. Model for retroviral restriction by TRIM5α. TRIM5α functions as a cellular factor for retroviral restriction that targets incoming retroviral capsids after viral penetration (post entry) or the Gag assembly during retrovirus production. TRIM5 may be involved in viral degradation of the Gag protein.

is blocked by rhesus monkey TRIM5α but not by human TRIM5α, whereas N-tropic murine leukemia virus (N-MLV) infection is blocked by both rhesus monkey TRIM5α and human TRIM5α.[62,63] These findings imply that species-specific variations in TRIM5α govern its ability to block infection by diverse retroviruses. The RING and SPRY domains are required for this restrictive activity and variations in the latter determine the specificity of retroviral restriction.[64] Indeed, the SPRY domain of TRIM5α appears to associate with the major core protein CAp30 of N-MLV but not with that of B-MLV.[65] Polyubiquitylation and rapid degradation of TRIM5α require intact RING and B-box domains; however, rapid turnover of TRIM5α is not required for its antiretroviral activity.[66] Our study revealed that the mRNA and protein expression of human TRIM5α is induced by IFN and that the transcription factor STAT1 is bound to the ISRE in the gene promoter.[67] The human TRIM5α gene is located at the chromosomal position 11p15, clustering with other TRIM genes, namely, TRIM21, TRIM6, TRIM34 and TRIM22, which are also known as IFN-inducible genes. This suggests that TRIM5α activity could be modulated by IFN.

CONCLUSION

We have summarized the structural characteristics and functions of TRIM family proteins, specifically with regard to E3 ligase activity. However, relatively few proteins have been proven to be E3 ligases and the function of most of the members of this family remains unclear. Investigation of RING finger proteins as possible novel E3 ligases would uncover important mechanisms underlying cellular protein degradation and modification and provide new insights into the physiological and pathophysiological roles of these family members. In particular, functional analysis of the RING finger proteins involved in innate antiviral immunity can open up new fields of research. In addition to TRIM5 and TRIM25, some members of the TRIM family are known to be regulated by IFN. These proteins may play a role in viral infection by inducing posttranscriptional modifications in their target proteins. In order to understand the functions of TRIM family, it is necessary to identify the substrate of TRIM E3 ligase. We then need to clarify how the substrates are modified by ubiquitine and/or ubiquitin-like molecules and play roles in cells and in vivo.

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CHAPTER 4

PML NUCLEAR BODIES AND OTHER TRIM-DEFINED SUBCELLULAR COMPARTMENTS

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Abstract: Tripartite motif (TRIM) proteins are defined by their possession of a RING, B-box and predicted coiled coil (RBCC) domain. The coiled-coil region facilitates the oligomerisation of TRIMs and contributes to the formation of high molecular weight complexes that show interesting subcellular compartmentalisations and structures. TRIM protein compartments include both nuclear and cytoplasmic filaments and aggregates (bodies), as well as diffuse subcellular distributions. TRIM19, otherwise known as promyelocytic leukaemia (PML) protein forms nuclear aggregates termed PML nuclear bodies (PML NBs), at which a number of functionally diverse proteins transiently or covalently associate. PML NBs are therefore implicated in a wide variety of cellular functions such as transcriptional regulation, viral response, apoptosis and nuclear protein storage.

INTRODUCTION

The family of tripartite motif (TRIM) containing proteins are defined by a conserved motif that contains a RING (really interesting new gene) domain, one or two B-box domains and a predicted coiled coil region.[1] TRIM proteins often show interesting subcellular compartmentalisations and structures, which is in part due to the oligomerization abilities of the TRIM motif facilitating both homo- and hetero-oligomerization of TRIM proteins. Whilst some of the TRIM component domains are not always present, their linear sequence order is always preserved (RING domain—B1—B2—coiled coil) and the motif is mostly evolutionarily conserved.[2] Therefore the various sub-domains of the tripartite motif likely

TRIM/RBCC Proteins, edited by Germana Meroni.

Table 1. Summary of known cellular localizations of TRIM proteins. Most of the cellular localizations are for exogenously expressed protein. Examples of interactions are taken from the Human Protein Reference Database (HPRD)

Name	Aliases	Domains	Cellular Localization
TRIM1	FXY2, MID2, RNF60		Filaments (C)
TRIM2	RNF86		Filaments (C)
TRIM3	BERP, RNF22, RNF97, HAC1		Diffuse (C) Speckles (C) Filaments (C)
TRIM4	RNF87		Speckles (C)
TRIM5	RNF88		Speckles (C)
TRIM6	RNF89		Speckles (C) Tracks (N)
TRIM7	GNIP, RNF90		Diffuse (C and N)
TRIM8	GERP, RNF27		Speckles (N)
TRIM9	RNF91		Speckles (C)
TRIM10	RFB30, RNF9, HERF1		Aggregates (C) Speckles (C)
TRIM11	RNF92		Diffuse (C and N)
TRIM13	RNF77, RFP2, LEU5		Speckles (C)
TRIM14	-		Speckles (C)
TRIM16	EBBP		Diffuse (C)
TRIM17	RBCC, RNF16, TERF		Speckles (C)
TRIM18	FXY, MID1, RNF59, XPRF		Filaments (C)
TRIM19	PML, MYL, RNF71		Bodies (N)
TRIM20	PYRIN, MEFV		Diffuse (C)

continued on next page

function in an integrated manner rather than as separate domains that happen to be adjacent to each other.[2] In terms of biochemical activity, many TRIM proteins that contain the RING domain also have ubiquitin (Ub) E3 ligase activity[3] (which is discussed in Chapter 3 by Ikeda and Inoue). A summary of TRIM proteins with known cellular localizations (mostly of exogenously expressed protein) can be found in Table 1, together with their aliases.

One of the best-characterised TRIM proteins is TRIM19, otherwise known as promyelocytic leukaemia (PML) protein. PML is necessary for the formation of nuclear protein aggregates termed PML nuclear bodies (PML NBs). A number of functionally distinct proteins associate either transiently or covalently with PML NBs and thus PML is implicated in a variety of cellular functions. In this chapter, we will review current knowledge on the tripartite motif with a specific focus on PML NBs.

Table 1. Continued

Name	Aliases	Domains	Cellular Localization
TRIM21	RNF21, RO52, SSA1		Speckles (C and N)
TRIM22	RNF94, STAF50		Diffuse, Speckles (C)
TRIM23	ARD1, ARFD1, RNF46		Speckles (C and N)
TRIM24	RNF82, TIF1, TIF1A		Speckles (C), Bodies (N)
TRIM25	EFP, RNF147, ZNF147		Diffuse and aggregates (C)
TRIM26	AFP, RNF95, ZNF173		Diffuse and aggregates (C)
TRIM27	RFP, RNF76		Speckles (C), Bodies (N)
TRIM28	KAP1, RNF96, TIF1B		Chromatin domains
TRIM29	ATDC		Filaments (C)
TRIM31	C6orf13, RNF		Diffuse (C and N)
TRIM32	HT2A		Diffuse and speckled (C and N)
TRIM33	PTC7, RFG7, TIF1G		Diffuse and speckled (N)
TRIM35	HLS5		Speckled (C and N)
TRIM36	RBCC728, RNF98, HAPRIN		Filaments (C)
TRIM37	MUL, POB1, TEF3		Peroxisome
TRIM41	RINCK		Speckles (C and N)
TRIM45	RNF99		Diffuse (C and N)
TRIM46	TRIFIC, GENEY		TRIM46
TRIM47	GOA, RNF100		TRIM47
TRIM54	MURF, MURF3, RNF30		TRIM54
TRIM55	MURF2, RNF29		TRIM55
TRIM63	IRF, MURF1, RNF28, SMRZ		TRIM63
TRIM69	HSD34, RNF36, Trif, Trimless		TRIM69

Localisation:
N = Nuclear C = Cytoplasmic

Domains:
RING, B-Box 1, B-box 2, Coiled coil, COS box, NHL, ARF, WD40, Fibronectin Type-III, B30.2/SPRY, PHD, Filamin, BROMO, MATH

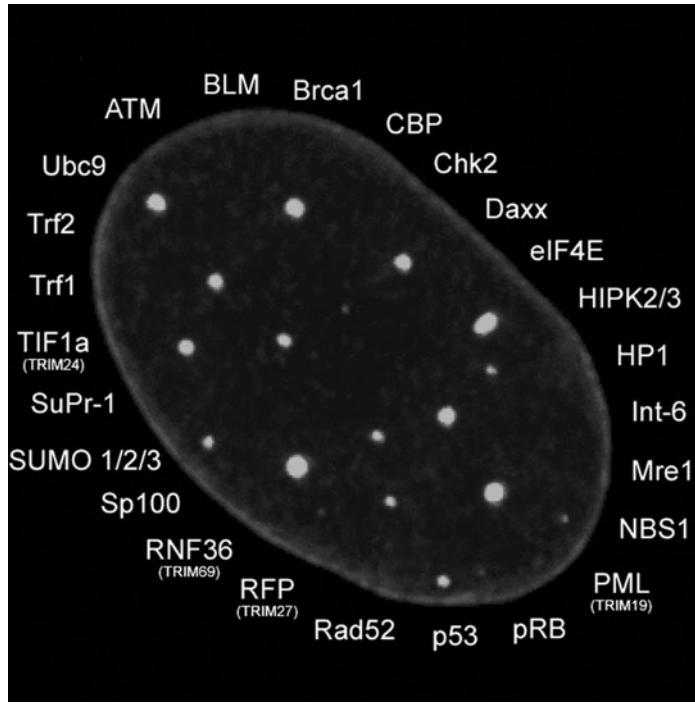

Figure 1. Proteins that show associations with PML nuclear bodies. The major constituent proteins of PML NBs are PML and Sp100. All the other proteins show transient localization to the bodies, or are recruited under certain conditions.

PML NUCLEAR BODIES AND ASSOCIATED PROTEINS

Also known as ND10, PODs and Kremer bodies, PML NBs are spherical bodies typically found within the mammalian cell nucleus. They range from 0.3 to 1.0 μm in diameter and usually number between 10 and 30 per cell, but this varies according to cell line and cell cycle phase.[4] PML and SUMO- (small ubiquitin-like modifier) modified PML are the nucleating components of PML NBs.[5,6] The main constituent protein of the bodies is PML, which is thought to provide a scaffold around which the other protein components accumulate.[6] The aggregation of sumoylated PML protein into PML NBs is thought to prompt the recruitment of other associated proteins to the scaffold. The other known constituent PML NB protein is Sp100.[7] Transiently associated proteins, or proteins recruited to PML NBs under certain conditions include CBP,[8] BLM,[5] Daxx (a transcriptional repressor),[5,9] p53 (an activator of transcription), pRB[10] and SUMO.[11] Since these proteins function in diverse cellular pathways it has been proposed that PML NBs act as storage depots where localised protein concentrations can be regulated.[12] Figure 1 lists some of the known PML NB-associated proteins.

The specific function of PML NBs is unclear, although given the diversity of proteins that are associated with PML, functional roles in tumour suppression,[13] apoptosis,[14] DNA replication and repair,[15] response to viral infection,[16] nuclear protein storage[12] and gene regulation and transcription[17] have been suggested.

THE TRIPARTITE MOTIF IN PML

The tripartite motif in PML plays an integral role in the formation of PML NBs, PML-homo and hetero interactions as well as PML growth suppressor,[13,18] apoptotic[14,19] and anti-viral activities.[20,21] The influences of each component domain have been investigated thoroughly.

RING Domain

The RING finger motif is a cysteine-rich zinc-binding module and was first discovered in the human protein RING1.[22] It is found in many cellular and viral proteins and mediates E3 ubiquitin ligase activity.[3,23] Mutations of the conserved zinc-binding cysteines in the RING finger motif of PML disrupt PML NB formation in vivo and results in a nuclear diffuse localization of PML.[24] These mutations correlate with loss of PML growth suppression,[25,26] apoptotic and anti-viral activities.[27,28] Furthermore, mutagenesis studies of surface residues that form part of the PML RING domain resulted in very large nuclear aggregates of PML protein in vivo.[11] The requirement of an intact RING domain for PML NB formation may relate to specific protein-protein interactions mediated by the RING and thereby playing an essential role in supramolecular assembly.[29] There are a number of examples of heterodimer RING-RING interactions (Brca1/Bard), although these are usually associated with the formation of complexes that mediate E3 Ub ligase activity. Although speculative, one cannot discount an indirect role for the PML RING domain in mediating E3 SUMO or Ub ligase activity.

B Boxes in PML

B boxes are also cysteine/histidine rich protein sequence motifs.[30] The B boxes in PML (B1 and B2) are small (42 and 46 residues respectively) and have been shown to bind zinc.[31] Substitution of conserved zinc ligands in B1 or B2 in PML disrupts PML body formation in vivo but does not prevent multimerization.[31] Together with the RING finger the B-boxes, B1 and B2, exert PML growth suppressor activity.[32] Interestingly B1 SUMO modification has not only been shown to be critical for recruitment of the 11S proteasomal subunit to PML NBs[33] but also necessary for recruitment of Daxx to PML NBs.[34] A recent structure of the human MID1 B-box 1 domain shows that the B-box has a fold similar to the RING domain providing another potential interface for mediating E2 interactions and conferring either E3 or E4 ligase activities.[35]

Coiled-Coil Domain of PML

PML possesses a weakly predicted α-helical coiled-coil domain, which is essential for PML homodimerization and heterodimerization with PML-RARα without the need for the RING and B-box domains.[26,36-38] The coiled-coil domain is also essential for PML NB formation and full growth suppressor activity in vivo.[32] Removal of the coiled-coil prevents formation of high molecular weight complexes. Recently it has been shown that SIAH-1/2 binds and targets the coiled-coil domain in PML for proteasome-mediated degradation, leading to a loss of PML NBs.[39] These results involve the coiled-coil region in PML as a novel structural determinant for targeted degradation.

THE FORMATION AND DISSOLUTION OF PML NUCLEAR BODIES

PML protein is found in the nucleoplasm in a soluble fraction that is not modified by SUMO-1.[40] PML NB genesis is thought to be a two-stage process, beginning with the formation of primary aggregates of nonsumoylated PML protein[33] and driven by homodimerization via its coiled-coil domain, part of the tripartite motif.[41] Formation of the mature bodies is mediated by the covalent modification of PML protein by SUMO-1,[33] polymeric SUMO-2[42] and SUMO-3[43] at up to three lysine residues (amino acid positions 65, 160 and 490;[44,45] The structure of the primary and mature bodies differs—mature bodies visualised using electron microscopy possess a shell of PML protein that surrounds other PML NB-associated proteins.[33,46] A PML mutant unable to be SUMO-modified forms aberrant nuclear aggregates and fails to recruit proteins typically associated with PML NBs, such as Daxx and Sp100.[6] Recently the nuclear localization of PML has been shown to be regulated by SUMO-3,[43] one of the least abundant human SUMO isoforms.[47]

PML protein also possesses a SUMO binding motif, known as a SUMO interacting motif or SIM, which is independent of its sumoylation sites and is required for PML NB formation.[48] SUMO is noncovalently bound to PML at the SIM motif and it is proposed that SIM's can confer the ability to aggregate SUMO labelled proteins as well as promote sumoylation of assembled complexes.[49] The proposed model of PML NB formation predicts that PML protein is aggregated via interactions between RBCC motifs. The PML RING domain is in turn required for efficient PML sumoylation.[45] Upon sumoylation in interphase cells, noncovalent binding of PML to SUMO-modified PML through the SIM is the nucleation event for the later recruitment of other sumoylated proteins or proteins containing SIMs to PML NBs.[48] The regulation of PML sumoylation including SUMO polymer chain length is not yet clear, although recent studies now suggest the existence of specific SUMO-targeted E3 ubiquitin ligases linking sumoylation to ubiquitin-mediated degradation.[50] Among these proteins are the RNF4 family of RING proteins that contain SIMs.[51] It will be interesting to see how these newly defined proteins might regulate sumoylated-PML and by inference PML NB formation.

PML NUCLEAR BODY DYNAMICS—NUMBER, MOVEMENT AND MORPHOLOGY

Levels of endogenous PML expression are variable within a cell-line population,[52] as determined by studying labelling intensity and number of PML NBs and the presence of a nonspeckled diffuse nuclear staining. In HeLa and Hs27 cells, late G1 cells show strong labelling in around 15 bodies. In S phase cells there are many smaller dots in addition to nuclear diffuse staining and in G2 PML NB number decreases until two to three are present in mitotic cells. PML NBs reappear between mitosis and late G1, with cytoplasmic bodies found in addition. Thus it appears that PML nuclear bodies show significant variation within the cell cycle.[52]

PML NB number is also a reflection of cell cycle status, especially at S phase when the number of bodies increases twofold.[4] In general, PML NBs are positionally and structurally stable over extended periods of interphase, with the exception of some smaller bodies that show limited localized or ATP-dependent rapid movement.[53] Stress may also induce the fission of PML microstructures, which also show fast movement, from parental NBs.[54] Also, as cells enter S phase PML NB structural stability is lost which

is exemplified by distortions in shape and by fission and fusion events. The association of fission products with chromatin implies that PML NBs are responding to changes in chromatin organization and topology. A redistribution of PML protein and not de novo synthesis was found to be responsible for the increase in PML NB number with the highest PML protein levels found in G1, dropping by 10 and 30% respectively in S phase and G2.[4]

Perhaps not unexpected, PML NB components like Sp100 and SUMO-1 also undergo changes in a cell cycle-dependent manner. Both are conjugated to SUMO-1 during interphase, but are deconjugated during mitosis.[55] Mitotic PML is stabilised by phosphatase inhibitors; treatment of interphase cells with such inhibitors induces production of PML similar to the mitotic species. Therefore phosphorylation is also important in the differential modification of PML during the cell cycle.[55] Although showing tight colocalization throughout interphase, PML and Sp100 become separated in early prophase of mitosis.

At mitosis PML protein partitions via mitotic accumulations of PML protein (MAPPs), which differ in dynamics, biochemistry and structure to PML NBs.[56] Unlike PML NBs they do not contain Sp100, SUMO-1 or Daxx, their loss occurring prior and concurrent to chromatin condensation and nuclear membrane breakdown respectively.[56] MAPPs contribute to the re-establishment of PML NBs in G1 where some MAPPs remain in association with the mitotic chromosomes, potentially forming nucleation sites for PML NB formation at G1.[56]

Other Body Morphologies

Up until this point we have discussed typical PML NBs, however, there are some other observed morphologies of PML NBs which are found under conditions of a particular cell cycle phase (giant PML NBs) or senescence (senescence associated nuclear bodies or SANB). There are also cytoplasmic PML bodies, which play a different role in the cell to their nuclear counterparts.

Giant PML Nuclear Bodies

PML protein may also be involved in heterochromatin remodelling at G2.[57] In immunodeficiency, centromeric instability and facial dysmorphy (ICF) syndrome patients heterochromatin HP1 proteins (HP1α, β and γ) accumulate in a giant body (only present in G2) over the 1qh and 16qh juxtacentromeric heterochromatins. PML protein is also present within this body and as such it is proposed to be a giant PML nuclear body. It contains a core of satellite DNA, although controversially, several studies have suggested that PML NBs do not typically contain DNA. By analogy to normal PML NBs , G2 PML NBs may contain PML and Sp100 organized in ordered concentric spherical layers around satellite DNA. These large PML containing structures would function in the re-establishment of a condensed heterochromatic state on late-replicated satellite DNA through an unknown mechanism.[57]

Senescence-Associated Nuclear Bodies (SANB)

Spontaneous or oncogene retrieval-induced senescence is associated with formation of large PML NBs (SANB) that also contain nucleolar components.[58] SANB appear to associate with the periphery of the nucleolus and perhaps play a role in the ubiquitin-proteasome pathway.

Cytoplasmic PML Bodies

Cytoplasmic PML bodies have been observed by indirect immunofluorescence in a number of cell lines.[41,59-61] As all PML isoforms comprise a nuclear localisation signal in exon 6,[41] these bodies could represent PML protein that has become trapped outside of the reformed nuclear membrane and lamina. Alternatively, the cytoplasmic bodies could comprise of the PML I isoform since it contains a nuclear export sequence (NES),[62] encoded within exon 9 of the *PML* gene.[63] (The isoforms of PML are discussed further in the following section). The ability of PML to become SUMO-modified and induce poly-PML aggregates leading to PML bodies could be utilised in important cytoplasmic functions. Indeed, it has recently been shown that cytoplasmic PML and PML-I bodies play a key role in the TGF-ß signalling which links cell regulation to a nonnuclear form of PML.[61]

ISOFORMS OF PML PROTEIN

TRIM family proteins often comprise a number of isoforms and PML protein is the good example of this. The *PML* genomic locus is found on chromosome 15, extends over 35 Kb and consists of nine exons, from which a range of transcripts are produced via alternative splicing. This leads to the production of a number of PML proteins ranging between forty-eight to ninety-seven kDa. There are seven isoforms of PML, PML I to VII, which all share the N-terminal tripartite motif (found within exons one to three), but show C-terminal sequence differences.[2,41] The different isoforms show different cellular localisations; either nuclear, or both nuclear and cytoplasmic.

Since TRIM proteins share a common N-terminal motif, C-terminal differences (such as the presence of other motifs including the BROMO, PHD and SPRY domains) may explain other diverse functionalities. These differences may also result from alternative splicing, such as in the case of PML protein where different PML isoforms have the potential to facilitate isoform-specific cellular activities. The existence of several PML isoforms may also explain the high number of cellular and viral proteins that interact with PML protein.[21] This could also account for the diversity of functionality accredited to PML within the cell.

The expression of specific PML isoforms is not limited to particular primary cells or cell lines.[63] PML III, IV and V were found to be minor isoforms when compared to PML I and II, indicating differential isoform-dependent expression levels. Interestingly stable expression of each of the isoforms (I-V) in a *pml*-null background produces distinct subcellular localizations. PML I has both a nuclear and cytoplasmic distribution (unsurprising since it contains an NES). PML II has a thread-like distribution, PML IV has a large number of small irregular shaped bodies throughout the nucleus and PML V produces large, dense bodies. As in other TRIM proteins, the PML isoforms' C-terminal domains may therefore be involved in interactions with specific cellular compartments.[63] However, co-expression of several isoforms leads to their colocalization, highlighting the ability of TRIM proteins to heterodimerize. Below are examples of proposed isoform-specific functions.

Upon different types of stress (UV-C or γ-irradiation, chemical inhibition of transcription or DNA synthesis and proteasome inhibition) endogenous PML protein forms nucleolar caps that eventually surround nucleolar components.[58] It was observed

that the PML I isoform contains a nucleolar targeting domain within the evolutionarily conserved C-terminus, which contains a predicted exonuclease III fold, both of which are essential for targeting PML-1 to nucleolar fibrillar centres. The function of PML-I at the nucleolus is unclear but the isoform specific localisation implies that PML isoforms can have distinct biochemical functions.

Another PML isoform, PML III, has been demonstrated to localize to the centrosome and the pole of the mitotic spindle through the use of isoform-specific antibodies.[64] PML III is implicated in the control of centrosome duplication as its specific knock-down leads to amplification of centrosomes, (also seen in a significant number of PML -/- MEFs). PML III may regulate this by repression of Aurora A kinase activity, which ultimately leads to the inhibition of Cdk2/cyclin E in its role of regulating centrosome duplication. However this could not be confirmed by others.[63]

It is established that PML protein and NBs play an important role in the regulation of multiple apoptotic pathways.[65,66] To further understand the role of PML, the PML IV isoform was found to specifically bind to the tumour suppressor p53 and regulate its activity.[67] Interestingly, PML IV was shown to recruit p53 to PML NBs implicating PML it in a p53-dependent apoptotic pathway.[68]

PML NUCLEAR BODIES AND DISEASE

PML protein has shown to be involved in both oncogenesis and response to viral infection. The involvement of TRIM proteins in cancer is explored in Chapter 6 by Cambiaghi et al..

PML Nuclear Bodies and Acute Promyelocytic Leukaemia

PML protein was first identified as part of the reciprocal chromosomal translocation at t(15;17) q(22;21) found in acute promyelocytic leukaemia (APL). The resulting fusion protein comprises PML fused to retinoic acid receptor α (RARα).[69-72] Interestingly, normal PML protein is unable to form PML NBs, instead showing a microspeckled pattern as a result of the heterodimerization of PML with the PML-RARα fusion protein.[73-75] APL is characterised by a block in the differentiation of promyelocytes which can be unblocked by treatment with all-trans retinoic acid (ATRA). The restoration of a normal PML NB pattern[76] correlates with the release of the differentiation block in promyelocytes.[77] Patients with APL are often able to go into remission after differentiation therapy.

Influence of the Coiled-Coil in PML Localization in APL

The PML-RARα fusion protein has reduced intranuclear mobility and shows mislocalisation when compared with RARα.[78] Using Fluorescence Recovery After Photobleaching (FRAP), PML-RARα sumoylation site mutants were shown to have the same intracellular localization and reduced mobility as wild type PML-RARα, indicating that sumoylation of PML-RARα (at each of three different sites) does not contribute to the altered distribution and reduced mobility of PML-RARα. Instead a coiled-coil deletion mutant PML-RARα indicated that the coiled-coil domain of PML-RARα is responsible for its immobilization and thus mislocalization within the nucleus.[79]

PML and the Antiviral Response

Some TRIMs display antiviral properties, such as TRIM1, TRIM5α, TRIM19 and TRIM22,[21] with evidence also for influence over viral replication. TRIMs may act in both the cytoplasm and nucleus, at multiple viral life cycle stages and target various viral proteins.[21] PML protein (with its various antiviral activities) and TRIM5α (possessing Lv1 and Ref1 antiretroviral activities) are the best-characterized TRIM proteins in terms of their involvement in viral infection responses.[20,21]

In early DNA viral infections, PML NBs become associated with the parental genomes and early replication compartments.[80-82] Type I IFNs are known to induce an anti-viral state. Interestingly, interferon induction also induces the expression of PML, producing an increase in the size and number of PML NBs.[83,84] Over-expression of PML also provides resistance to vesicular stomatitis virus and influenza A virus.[85] Infection with adenovirus causes a redistribution of PML nuclear bodies into thread-like structures associated with viral replication centres. The adenovirus Type 5 (Ad5) early region 4 open reading frame (E4 ORF3) product is responsible for this reorganization, colocalizing with the PML threads.[86,87] E4 ORF3 was also found to specifically interact with PML II.[88]

The RBCC containing protein TRIM5α, containing the C-terminal SPRY domain, was found to block HIV-1 infection prior to reverse transcription in the cells of Old World monkeys.[89] Interestingly, TRIM5α also localises to cytoplasmic speckles[21,89,90] which are highly mobile showing a dynamic morphology[91] indicating a functional role for TRIM5α cytoplasmic bodies.

NUCLEAR COMPARTMENTS SHARING ASSOCIATIONS WITH PML NUCLEAR BODIES

PML nuclear bodies have been shown to specifically associate with a variety of other nuclear compartments. In some cases it is unclear whether these associations are of functional importance or occur through stochastic processes. In the following section, these associations are discussed in the context of PML NB function.

Transcriptional Compartments

The DNA damage response in cells results in PML sequestration of Mdm2, a p53 ubiquitin-ligase, to the nucleolus thereby potentiating p53 stability.[92] There is little evidence to suggest that PML NBs show association with the nucleoli of normal primary cell lines that have not undergone stress or are not tumour lines. Recently a novel PML compartment in association with nucleolar structures in human mesenchymal stem cells has been observed in growth permitting conditions[93] indicating that PML may be involved in nucleolar functions of normal nontransformed cells, as this compartment is not present in rapidly-growing tumour-derived cells. However, it does lack the morphology of normal PML NBs.

A cell cycle-dependent association of PML NBs with RNA polymerase II active transcription sites has been demonstrated.[94] In this study, 30% of PML NBs in unsynchronized cells associated with active transcription sites containing $RNAPII_O$ (RNA

polymerase II with a hyperphosphorylated CTD, which forms part of the elongation complex). This increased to 70% of PML NBs in G1 cells and 80% upon exposure to IFNγ suggesting a correlation between alteration of PML NB spatial associations with increased transcriptional activity. However the specific functional role of PML NBs in transcription is still unclear.

PML and CREB binding protein (CBP), a transcriptional coactivator and histone acetyl transferase, are found to colocalize at the PML NB.[8,46] PML, CBP and RNA polymerase II have also been found together at a subset of PML NBs, supporting the idea that transcription takes place at PML NBs. This alludes to a possible function for PML in the regulation of transcription.

Chromatin Associations

There is some evidence for a cell-cycle dependent PML NB-chromatin association, in particular the observed dynamic link between PML NBs and centromeres in G2 that is stabilised upon proteasome-mediated proteolysis inhibition.[95] An observation that Vmw100 (Herpes simplex virus Type 1 regulatory protein) induces proteasome-dependent degradation of both principal PML NB component proteins PML and Sp100 and CENP-C (a centromeric protein) indicated a connection between PML NBs and centromeres in uninfected cells. Proteasome inhibitor treatment revealed an association between the two compartments in a significant number of G2 Hep2 cells and subsequent reassessment of unsynchronised cells revealed rare cells in late G2 that had considerable PML NB-centromere associations.[95]

A subset of PML NBs, known as alternative lengthening of telomeres (ALT) associated PML NBs (APBs), are found to colocalize with telomeres in certain immortalized cell lines. Such cells seek to perpetuate or surpass existing telomeric length but are telomerase (hTERT) negative and thus employ a different method involving homologous recombination.[96,97] APBs contain telomeric DNA and telomere-specific binding proteins TRF1 and TRF2, plus other DNA recombination and replication proteins suggesting a role of PML NBs in regulating telomere length.[98]

The first study of the 3D spatial organization of PML NBs and gene-rich and gene-poor chromosomal regions revealed a highly nonrandom association of the gene-rich major histocompatibility complex (MHC) on chromosome 6 with PML NBs.[99] Interestingly, this association remained when a subsection of this chromosomal region is integrated into another chromosome in a mutant cell line.[99] An extension of this study revealed that the distance between a genomic locus and its nearest PML NB correlates with the transcriptional activity and gene density around that specific locus.[100] Locus-PML NB mean minimum distances for four different loci (6p24, Histone cluster 6p22, TAP/LMP and the centromere of chromosome six were compared between S phase and G1/G0 cells and it was found that only the histone-encoding cluster (transcribed in S phase alone) was more strongly associated with PML NBs in S phase than in G1/G0. These studies provided the first rigorous and statistical meaningful study of PML NB associations with specific genomic regions establishing a direct link between PML NB association with gene-rich and transcriptional active regions. They also alluded to the possibility that PML NBs associations are directly linked to the functionality of the specific gene region such that the insertion of a gene-rich and active region into a different chromosome maintained the PML NB association.

TRIM PROTEINS THAT LOCALISE TO PML NUCLEAR BODIES

Other members of the TRIM protein family are known to interact with PML protein and thus localise to PML NBs. These are TIF1α (also known as TRIM24), RFP (TRIM27 or RET finger protein) and RNF36 (TRIM69 or Trif). Interestingly like PML, both TIF1 and RFP become oncogenic as the result of chromosomal translocations producing aberrant fusion proteins that contain RFP and TIF1.[101-103]

TIF1α (TRIM24)

TIF1α is fused to *Braf*, resulting in the TIF1α-B-Raf (T18) oncoprotein in mouse hepatocellular carcinomas.[103] PML, TIF1α and RXRα/RARα have been shown to function together in a transcription complex dependent on retinoic acid (RA).[104] PML acts as the ligand-dependent coactivator of RXRα/RARα and interacts with TIF1α and CBP which localise to PML NBs.[104] In *Pml*$^{-/-}$ cells the ability of TIF1α and CBP to act as transcriptional coactivators on RA is impaired. T18 oncoprotein disrupts the RA-dependent activity of the complex in a dominant-negative manner resulting in a growth advantage.[104] TIF1α also fuses to the RET receptor tyrosine kinase in human childhood papillary thyroid carcinomas.[102] Like PML, it was shown that TIF1α is SUMO-1 modified[105] by two SUMO-1 moieties per TIF1α. By analogy to PML and Sp100, TIF1α is also chromatin associated and was shown to interact with HP1 similar to Sp100.[106] Interestingly, PML protein has been implicated as a cofactor in TIF1α-dependent enhancement of RARα-mediated transcriptional activation.[104] Like other TRIM proteins, TIF1α is able to heteromultimerize via the TRIM motifs and can bind TIF1γ.[107]

As mentioned previously, the adenovirus Type 5 E4 ORF3 product is able to cause the reorganization of PML bodies in to tracks within the nucleus.[86,87] Recently, TIF1α has also been identified as a novel E4 ORF3-interacting partner and is also found reorganized into PML-track structures upon E4 ORF3 expression.[108] E4 ORF3 binds TIF1α in vivo and directs this PML NB reorganization via the TRIM domain.

RFP (TRIM27 or Ret Finger Protein)

Like other TRIM proteins, the subcellular localization of RFP can be nuclear, as detected by a monoclonal antibody (RFP-1) generated against amino acids 148 to 163.[109] In HL-60 cells fractionated into nuclear and cytoplasmic components, the protein reactive with RFP-1 is detected only in the former. Over 90% of nuclei of human spermatogenic cells (but not mature spermatozoon or human testicular tumour cells) were positive for RFP and so were up to 60% of cells in other human adult tissues, which suggests a functional role for RFP in spermatogenesis. In contrast to these cell-specific studies, it has been observed that RFP shows differential nuclear and cytoplasmic distributions in different cell types.[110] RFP is expressed in the nuclei of cells ranging from peripheral and central neurones and hepatocytes to adrenal chromaffin cells and also at high levels in male germ line cells (such as round spermatids, also forming a perinuclear cap in primary spermatocytes). However in some plasma cells, solitary plasmacytoma and multiple myeloma it shows high cytoplasmic expression levels. This cell-specific differential RFP distribution must relate to the different functions of RFP perhaps in the regulation of growth or differentiation of different cell types.

Like PML, RFP, (also known as *Ret* finger protein), is sumoylated and localizes to PML NBs. RFP functions as a transcriptional repressor by interacting with Enhancer of Polycomb 1 (EPC1), (a Polycomb group member that plays a role in heterochromatin formation).[111,112] In this context, RFP shows either a cytoplasmic or nuclear localization depending upon cell type.[113] Within its coiled-coil domain is a NES and in NIH3T3 cells this results in a cytoplasmic localization for RFP, but conversely in HepG2 cells the NES is masked. Treatment with 12-*O*-tetradecanoylphorbol-13-acetate or overexpression of constituently active protein kinase Cα (PKCα) removes this masking and RFP is able to relocate to the cytoplasm. NIH3T3 cells treated with PKC inhibitors show blocked NES function, leading to nuclear localization of RFP. Therefore RFP export appears to be positively regulated by PKC activation, however it is not a direct substrate.[113]

RFP, SUMO-1 and PIASy (protein inhibitor of activated STAT y, an E3 SUMO ligase[114]) were found to localize in a characteristic nuclear structure juxtaposed with the inner nuclear membrane (XY body) of primary spermatocytes in mouse testis.[112]

As previously described, TRIM proteins can homodimerize through their coiled-coil domains.[41] For RFP this interaction requires an intact B box (despite it not being an interacting interface itself), but not the RING finger.[115] The coiled-coil domain of RFP is required for its heterodimerization with PML.[116] Interestingly, RING finger and B box mutations affect RFP subcellular localisation suggesting that homo-oligomerization of RFP and its specific subcellular localisation depends upon an intact and complete TRIM motif.[115] In this context, ectopic expression of RFP in HEK293 cells causes extensive apoptosis with an intact tripartite motif required for this pro-apoptotic function. Interestingly, sole expression of the tripartite motif (without the C-terminal RFP domain) actually results in a further increase in pro-apoptotic activity.[117] Since the RING domain is involved this could suggest that many of the TRIM family proteins play a role in the control of cell survival.

In primary lymphocytes Int-6 (a subunit of the eukaryotic translation initiation factor eIF3), RFP and certain PML bodies colocalise, with RFP shown to trigger translocation of Int-6 into PML NBs in HeLa cells.[118] The interaction of RFP with Int-6 is mediated via its C-terminal Rfp (B30.2) domain (unlike the interaction between RFP and PML, which is coiled-coil dependent).

RNF36 (TRIM69 or Trif)

The gene for Trif (testis-specific ring finger) was isolated from a mouse testis cDNA library[119] and the protein contains the tripartite motif and C-terminal B30.2/SPRY domain. Later renamed RNF36, the protein is expressed in germ cells at round spermatid stages during spermatogenesis.[120] Expression of full-length GFP-RNF36 in HEK293 cells produces a speckled nuclear localization. However, truncated RNF36 (containing only the RING and B-box domains and retaining a putative NLS) localizes to the nucleus but not in a speckled pattern.[120] From immunofluorescence and coimmunoprecipitation studies full-length RNF36 and PML protein were found to colocalize and interact. Since p38 (a mitogen-activated protein kinase) inhibition leads to its cytoplasmic translocation, RNF36's nuclear localization may be phosphorylation-dependent. Interestingly, over-expression of full-length RNF36 induced about 50% cell death in transfected cells as a result of apoptosis.[120] Given PML's involvement in the regulation of apoptotic pathways and its interaction with RNF36, RNF36's induction of apoptosis may be via a PML-dependent (but possibly p53-independent) pathway.

OTHER TRIM-DEFINED SUBCELLULAR COMPARTMENTS

The coiled-coil domain of the tripartite motif allows TRIM proteins to oligomerize into large subcellular structures, such as the PML nuclear bodies. Many TRIMs are found to localize to different types of cytoplasmic or nuclear thread-like, filamentous and speckle or body aggregates. These structures can represent endogenous localisation patterns but more commonly are a result of overexpression studies. The ability of TRIM proteins to oligomerize is an interesting biochemical property, that can infer an ability to partition between soluble and insoluble high molecular weight complexes as part of function. An extensive investigation of the subcellular localization of all TRIMs using GFP, found most to localize to discrete cytoplasmic or nuclear structures, occasionally associated with diffuse background staining.[2]

Table 1 lists the currently known cellular localizations of TRIM proteins. Cytoplasmic TRIMs include those that form filaments (TRIM1, 2, 18, 36, 46, 54, 55), speckles (TRIM4, 5, 9, 13, 14, 17) or are diffuse in the cytoplasm (TRIM16 and 20). Other TRIMs show a number of cytoplasmic localizations such as TRIM3 which has cytoplasmic speckles, filaments and a diffuse component, TRIM10 which forms cytoplasmic speckles and aggregates, TRIM22 with its cytoplasmic speckles and diffuse component and TRIMs 25 and 26 with their cytoplasmic aggregates and diffuse component. Nuclear TRIMs can be found in speckles (TRIM8) or nuclear bodies (TRIM19 and 69). TRIM33 localizes to nuclear speckles and shows a diffuse nuclear distribution.

TRIM proteins may also show any other combination of cytoplasmic and nuclear subcellular localizations. TRIM 21, 23, 35 and 41 all form both cytoplasmic and nuclear speckles, whilst TRIM6 and TRIMs 27 and 24 have cytoplasmic speckles, but differ by also localizing to nuclear tracks and nuclear bodies respectively. TRIM63 forms cytoplasmic filaments and nuclear speckles. TRIM7, 11, 31 and 45 all show both cytoplasmic and nuclear diffuse components.

The subcellular localization (and possibly functions) of TRIM proteins can often be correlated with particular domain architecture or C-terminal features. Examples of this include the C-terminal differences in PML isoforms that allow nuclear localization and export and TRIMs containing a PHD or BROMO domain (TRIMs 24, 28 and 33) which can associate with specific chromatin regions.[2,121] Another example of this is that of a subfamily of TRIMs (including TRIM1 (MID2), TRIM9, TRIM18 (MID1), TRIM36 and TRIM46 (TRIFIC)) in which all analyzed members associate with the microtubule cytoskeleton.[122] All subfamily members share an identical domain arrangement and contain a novel motif, the COS box, found adjacent to the coiled-coil. As expected most of the COS box-containing proteins in Table 1 show a cytoplasmic filamentous subcellular localization. Often the presence of the fibronectin Type III (fnIII) domain correlates with the presence of the COS box. 13% of the TRIMs described in Table 1 possess a COS box. Mutations in this motif abolish microtubule-binding ability and if incorporated into a nonmicrotubule-binding TRIM, the protein is redirected to the microtubules.[122] The majority of TRIM proteins (just over 60% of TRIM proteins described in Table 1) contain a SPRY/B30.2 domain. B30.2 is actually a SPRY domain plus an additional PRY domain and is found in TRIMs, having been evolutionarily selected and maintained, speculatively as part of the innate immune recognition of retroviruses.[123] This tallies with current thinking that many TRIM proteins possess antiviral functionality, such as in the example of TRIM5α, which in fact does contain a SPRY/B30.2 domain.[21] However this domain is noticeably absent from PML.

As seen with PML protein in APL, delocalization from expected subcellular localizations is often an indicator of disease. A further example of this is TRIM37, which shows a particularly interesting subcellular localization and is mutated in the autosomal recessive genetic disorder Mulibrey (muscle, liver, brain and eye) nanism syndrome[124] to produce a nonfunctional truncated protein. TRIM37 is normally targeted to the peroxisome, however this localization is compromised upon transient expression of *TRIM37* containing the major mutation found in the Finnish population, becoming homogeneously distributed throughout the cytoplasm and the nucleus.[125] TRIM37 has also been shown to be an E3 ubiquitin ligase,[126] perhaps indicating that defective ubiquitin-dependent degradation plays a role in the pathogenesis of this disease.

CONCLUSION

The tripartite motif is present in a large number of proteins. The RBCC domain of TRIMs is essential for their subcellular compartmentalization, as illustrated by the formation of PML NBs which are the best characterized of TRIM subcellular compartments. The coiled-coil domain of the tripartite motif confers an ability to oligomerize, thus mediating the aggregation of large multi-protein complexes and the partitioning of soluble and insoluble protein fractions. The ability for both homo- and hetero-oligomerisation gives rise to increased functional diversity amongst TRIMs. TRIM subcellular compartments may be found in both nuclear and cytoplasmic locations and are able to form both transient and stable complexes with other proteins in the cell, again potentiating functionality. In addition, the RING finger plays a role in the mediation of E3 ubiquitin ligase activity, suggesting that all TRIMs are potential E3 ubiquitin ligases.

Whilst the tripartite motif allows oligomerisation of TRIM proteins, it is the domain structure and composition of the rest of the protein that tends to influence the functions and subcellular locations of TRIM-defined compartments. This makes unambiguous definition of TRIM functionality difficult. There is redundancy in the C-terminal domain composition of TRIM proteins, the most common domain of which is SPRY/B30.2. Others of interest are the PHD and BROMO domains (involved in localization to chromatin) and the COS domain (often correlating with the presence of the fnIII domain and required for localization to microtubules). Interestingly a number of TRIM proteins including PML are associated with human disease, often resulting in their delocalization from their subcellular compartments.

Undoubtedly as more TRIM proteins are investigated in depth the link between subcellular localization and domain structure will become clearer. Within the field of PML, future research will certainly extend insight at both a biochemical and cellular level into the formation of PML nuclear bodies and the interactions between PML and its associated proteins.

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CHAPTER 5

TRIM INVOLVEMENT IN TRANSCRIPTIONAL REGULATION

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Abstract: Members of the tripartite motif (TRIM) protein family are found in all multicellular eukaryotes and function in a wide range of cellular processes such as cell cycle regulation, differentiation, development, oncogenesis and viral response. Over the past few years, several TRIM proteins have been reported to control gene expression through regulation of the transcriptional activity of numerous sequence-specific transcription factors. These proteins include the transcriptional intermediary factor 1 (TIF1) regulators, the promyelocytic leukemia tumor suppressor PML and the RET finger protein (RFP). In this chapter, we will consider the molecular interactions made by these TRIM proteins and will attempt to clarify some of the molecular mechanisms underlying their regulatory effect on transcription.

INTRODUCTION

Transcription factors that associate with DNA sequences in promoters and enhancers often recruit coregulators that modulate positively or negatively their activity. Many of these coregulators exist as components of large multisubunit complexes and act either through chromatin remodeling and histone modification, or at steps involving subsequent preinitiation complex formation or function (for a review see refs. 1 and 2). Recently, several tripartite motif (TRIM) proteins, also known as RBCC (N-terminal RING finger/B-box/coiled coil) proteins, have been described as dedicated coregulators in *Drosophila*, *C. elegans* and mammals.[3-7] Although the precise mechanisms by which these TRIM proteins influence transcription are still under investigation, consistent evidence

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is accumulating for a role at the chromatin level. The activity of the transcriptional intermediary factors TIF1α/TRIM24, TIF1β/KAP1/TRIM28 and TIF1δ/TRIM66 as chromatin-related cofactors is well documented.[8-12] There is also evidence that TRIM19 and TRIM27, better known respectively as promyelocytic leukemia (PML) and RET finger (RFP) proteins, can regulate transcription through interaction with chromatin modifiers.[13,14] These findings are reviewed here and the possibility that TRIMs influence transcription by other mechanisms is also considered.

THE TIF1 FAMILY PROTEINS IN GENE-SPECIFIC REGULATION

TIF1s are members of a conserved subfamily of TRIM proteins, with orthologs present from *Drosophila* (Bonus)[3] to mammals (TIF1α to δ)[5,6,8,12,15,16] and playing crucial roles in (patho)physiological processes as diverse as organ formation and tumorigenesis (Table 1).[3,17-24] All family members have an N-terminal TRIM/RBCC motif with potential self-assembly properties[25,26] and a C-terminal bromodomain preceded by a PHD finger, two well-conserved signature motifs widely distributed among nuclear proteins acting at the chromatin level (see Chapter 2 by Micale et al).[27,28] They also have intrinsic kinase activity[9,29] and repress transcription when tethered to a promoter.[3,6,9,12,16] In the case of TIF1α, -β and -δ, a mechanistic link between repression and histone modification has been established with the demonstration that deacetylase inhibitors such as Trichostatin A can interfere with repression.[9,12] Consistent with this, TIF1β has been reported to be an intrinsic component of the histone deacetylase complex N-CoR1 and to interact both physically and functionally with the Mi2-α/CHD3 subunit of the nucleosome remodeling and deacetylase (NuRD) complex.[10,30] In addition, TIF1α, TIF1β and TIF1δ have been demonstrated to interact directly with the heterochromatin protein 1 (HP1) family proteins, a class of non-histone chromosomal proteins that serve as dose-dependent regulators of higher-order chromatin structures and contribute to the regulation of euchromatic genes (for a review see refs. 31 and 32).[8,9,12,33] It is currently assumed that the TIF1s function to target chromatin modifying complexes to specific sites in the genome through their interaction with sequence-specific DNA binding transcription factors (see Table 1).

TIF1α/TRIM24 in Nuclear Receptor-Mediated Transcription

Nuclear receptors (NRs) comprise a superfamily of transcription factors that regulate transcription in a ligand-dependent manner and play a crucial role in many aspects of vertebrate development, cell differentiation, proliferation and homeostasis.[34] Like other DNA binding transcription factors, they control transcription by recruiting different coregulator complexes.[35] TIF1α, also known as TRIM24, was among the first coregulators identified as interacting with nuclear receptors and it was shown to regulate either positively or negatively their transcriptional activity in a ligand-dependent fashion.[5,18,19,36,37] Of the four TIF1s described in mammals, TIF1α is the only member known to interact with liganded NRs.[8,12,16] Interaction is mediated through contacts with residues within the AF-2 activation domain of NRs [e.g., retinoic acid (RAR), vitamin D3 (VDR), thyroid (TR) and estrogen (ER) receptors] by means of a single LxxLL motif or NR box located in the middle region of TIF1α.[5,8] This interaction conserved in evolution is also found in *Drosophila* with the fly ortholog of the TIF1 family, Bonus

(Bon).[3] Specifically, it has been demonstrated, both biochemically and genetically, that Bonus is able to interact with the nuclear receptor βFTZ-F1 to downregulate its transcriptional activity.[3] In support of a similar effect for TIF1α in vivo, an enhanced RA induction of well-established RA target genes such as *Cyp26a1*, *Rbp1*, *Tgm2* and *Stra6* was observed in *TIF1α*-null compared to wild-type MEF cell lines.[18] Moreover, chromatin immunoprecipitation and transient transfection assays showed that, upon RA induction, TIF1α directly targets the retinoic acid (RA) responsive elements (RARE) in the *CYP26A1* promoter and can repress in a dose-dependent manner RAR-mediated transactivation on a RARE-responsive promoter.[18]

A fundamental physiological role for TIF1α in repressing a molecular pathway involving the RA receptor isotype α (RARα), which functions to prevent liver tumor formation, was recently demonstrated by genetic studies in mice lacking TIF1α.[18] In *TIF1α*-null mice, hepatocytes fail to execute proper cell cycle exit during the neonatal-to-adult transition and continue to cycle in adult livers, becoming prone to a continuum of cellular alterations that progress towards metastatic hepatocellular carcinoma (HCC). Not surprisingly, analysis of gene expression profiles revealed aberrant expression of numerous RA responsive genes in the liver tumors from *TIF1α* knockout mice. More importantly, it was shown that deletion of a single *RARα* allele in a *TIF1α*-null background was enough to suppress HCC development and to restore the wild-type expression of RA-responsive genes in the liver.[18] Altogether, these results define TIF1α as a potent liver-specific tumor suppressor in mice and provide genetic evidence that TIF1α and RARα act in opposition to each other in liver cancer.[18,38]

More recently, it has been shown that, in addition to hepatic tumors, *TIF1α* knockout mice spontaneously develop pathological calcifications in arterial vessels, lung alveoli and vibrissae.[19] Importantly, these ectopic calcifications were correlated to an increase in expression of several vitamin D receptor (VDR) direct target genes involved in calcium homeostasis (e.g., *Casr*, *Trpv5* and *Trpv6*, *Calb1* and *S100g*). Their increased expression in *TIF1α*-deficient kidneys provides evidence of the importance of TIF1α in repressing the VDR pathway in the kidney.[19] TIF1α appears therefore to act as a negative regulator of multiple NR-dependent pathways in vivo.

TIF1β/TRIM28 in KRAB-ZFP-Mediated Repression

KRAB-zinc finger proteins (ZFPs), in which a potent repressor domain called the Krüppel-associated box (KRAB) is attached to a tandem array of zinc finger motifs of the Krüppel Cys_2-His_2 type, are specific to tetrapod vertebrates and represent one of the largest family of transcriptional repressors in mammals; it has been estimated that more than 400 human loci are capable of encoding KRAB-ZFPs.[39] Their importance is inferred from their recent origin and subsequent rapid expansion in vertebrate lineages, but their role in vivo remains largely unknown. At a mechanistic level, however, it is well understood how KRAB-ZFPs operate to silence transcription; they mediate silencing through association with the corepressor protein TIF1β (also called TRIM28 or KAP1).[6,40] The tripartite motif of TIF1β binds directly to the KRAB domain of KRAB-ZFPs.[25] This binding requires integrity of all three sub-domains of the TIF1β tripartite motif with each sub-domain contributing to the formation of an oligomer that is obligatory for KRAB interaction.[25] This interaction is entirely specific for the TIF1β tripartite motif since it was not observed with the tripartite motifs from related proteins such as TIF1α, TIF1γ, TIF1δ or MID1.[12,26,40]

Table 1. The TIF1 family members in transcriptional regulation

	Transcriptional Interactor				
Family Member	DNA Binding Transcription Factor	Non DNA Binding Transcriptional Cofactor	Transcriptional Effect, Mechanism of Action	Biological Function	Refs.
Human or mouse					
TIF1α/TRIM24	Nuclear receptors	HP1, GRIP1, CARM1	Modulates ligand-dependent transactivation by nuclear receptors	Acts in liver tumor suppression and prevents arterial calcification	5, 8, 9, 18, 19, 36-38
TIF1β/TRIM28/ KAP1	KRAB-ZFPs	HP1, Mi-2α/CHD3, SETDB1/ESET	Mediates KRAB-ZFP repression through recruitment of chromatin modifiers	Regulates progression through differentiation and retrovirus silencing	8-11, 40-48
	p53*	MDM2	Inhibits p53 acetylation by interacting with MDM2 and stimulating p53-HDAC1 interaction	Regulates p53-mediated apoptosis	50
	E2F1	nd	Inhibits E2F1 activity by promoting E2F1-HDAC1 interaction	Regulates E2F1-mediated apoptosis	51
TIF1γ/TRIM33/ RFG7	Smad2/3	nd	Binds receptor-activated Smad2/3 in competition with Smad4	Stimulates TGFβ-dependent erythroid differentiation	22
TIF1δ/TRIM66	nd	HP1	Represses transcription when targeted to DNA	May regulate postmeiotic germ cell gene expression	12
Xenopus laevis Ectodermin (xTIF1γ)	Smad4	nd	Induces Smad4 ubiquitination and degradation	Acts in early embryonic development as a a general inbibitor of TGFβ and BMP signaling	23

continued on next page

Table 1. Continued

Family Member	Transcriptional Interactor: DNA Binding Transcription Factor	Transcriptional Interactor: Non DNA Binding Transcriptional Cofactor	Transcriptional Effect, Mechanism of Action	Biological Function	Refs.
Danio rerio moonshine (DrTIF1γ)	nd	nd	nd	Regulates hematopoiesis	24
Drosophila melanogaster Bonus	Nuclear receptors	nd	Binds to and inhibits βFTZ-F1 transcriptional activity	Is required for viability, molting and numerous events in meta-morphosis	3

*: no direct interaction; nd: not determined; CARM1: coactivator-associated arginine methyltransferase 1; CHD3: chromodomain helicase DNA binding, protein 3; E2F1: E2F transcription factor 1; GRIP1: glucocorticoid receptor-interacting protein 1; HP1: heterochromatin protein 1; KRAB-ZFP: Krüppel, associated box-zinc finger protein; MDM2: mouse double minute 2; SETDB1/ESET: SET domain bifurcated 1/ERG-associated protein with SET domain.

To repress gene transcription by KRAB-ZFPs, TIF1β recruits HP1 proteins through a PxVxL motif located in its middle region.[9,33] Additionally, TIF1β associates with the component of the NuRD histone deacetylase complex Mi-2α/CHD3 and the histone H3 lysine 9 (H3K9) methyltransferase SETDB1/ESET via its C-terminal PHD-bromodomain unit.[10,11] The PHD domain binds to the SUMO E2 protein Ubc9 and directs SUMO conjugation of the adjacent bromodomain. Once modified by SUMO, the bromodomain of TIF1β recruits Mi-2α/CHD3 and SETDB1/ESET and their associated proteins.[41] It is currently believed that TIF1β assembles these proteins onto KRAB-ZFPs to coordinate histone deacetylation and methylation, as well as HP1 deposition, all of which cooperatively result in heritable gene silencing through the formation of condensed, transcriptionally inactive heterochromatin-like structures and/or spatial relocalization to pericentric heterochromatin domains.[42-44]

The results discussed above point to a link between TIF1β-mediated corepression and pericentric heterochromatin. Further supporting evidence for this link came from the fact that, during cell differentiation, TIF1β undergoes a dramatic redistribution, the protein moving from euchromatic nuclear compartments to heterochromatic compartments.[45] This differentiation-induced heterochromatin association of TIF1β was not observed with a PxVxL-motif mutant that fails to interact with HP1.[45] Importantly, genetically engineered F9 embryonic carcinoma cells producing this mutant form of TIF1β were able to differentiate into primitive endoderm-like cells after exposure to retinoic acid, but were unable to further differentiate into parietal endodermal cells upon addition of Bt_2cAMP, thus indicating that interaction between TIF1β and HP1 is an absolute requirement for progression through cell differentiation.[46] Importantly, interaction with HP1 was also shown to be essential in regulating TIF1β-mediated silencing of provirus in embryonic carcinoma (EC) and stem (ES) cells.[47,48]

Recently, a comprehensive study of the genomic regions bound by TIF1β in human Ntera2 cells revealed that a fourth of the TIF1β bound promoters are also enriched for trimethylated histone H3 lysine 9, indicating that many but not all TIF1β target sites are occupied by the selective histone mark for HP1.[49] This strongly suggests that HP1 may not be required for all the actions of TIF1β. In support of this, it has recently been reported that TIF1β inhibits p53 transcriptional regulation by a mechanism that is independent of its interaction with HP1.[50] This inhibitory function of TIF1β is executed in concert with MDM2, a RING domain ubiquitin E3 ligase which binds to p53, inhibits p53 acetylation and promotes p53 ubiquitination and degradation (see ref. 50 and refs. therein). TIF1β binds directly MDM2 via its coiled coil domain.[50] It cooperates with MDM2 to stimulate p53-HDAC1 complex formation, thus promoting p53 deacetylation and then p53 ubiquitination and degradation.[50] Interestingly, a similar mechanism of TIF1β transcriptional inhibition by promoting deacetylation was also described for the E2F1 transcription factor.[51]

Analyses of TIF1β deficient mice have provided evidence that TIF1β exerts cellular function(s) essential for early embryogenesis[20] and spermatogenesis.[21] An important future task will be to identify the transcriptional targets of TIF1β that mediate these functions. Recently, TIF1β was also found to be important for the maintenance of ES self-renewal.[52,53] More than 3000 genes whose promoter regions are occupied by TIF1β in mouse ES cells were identified.[53] A consensus binding motif was deduced to be GCCGCGXX and, importantly, a total of 326 target genes were found to be occupied by not only TIF1β, but also by three other pluripotency-associated transcription factors, CNOT3, C-MYC and ZFX. These common target genes are enriched for genes involved in cell cycle and cell survival, suggesting that TIF1β together with CNOT3, C-MYC and ZFX control self-renewal by regulating these processes.[53]

TIF1γ/TRIM33 in Smad-Mediated Transcription

Human TIF1γ (also known as TRIM33 and RFG7) was initially identified by virtue of its sequence homology with TIF1α[15,16] and, similarly to TIF1α, it was found in the context of a fusion oncoprotein with the tyrosine kinase domain of Ret from childhood papillary thyroid carcinomas.[17] Through their respective tripartite motifs, TIF1α and TIF1γ can hetero-oligomerize as efficiently as they homo-oligomerize, thus suggesting some possible cross-talk between the signaling pathways regulated by these two proteins.[26]

Two recent studies have implicated TIF1γ in the control of the signaling and gene responses triggered by members of the transforming growth factor-β (TGFβ) family.[22,23] TGFβ family members bind two types of membrane serine/threonine kinases, the Type I and Type II receptors, forming an heteromeric receptor complex. The Type II receptor then phosphorylates and activates the Type I receptor, which in turn phosphorylates Smad transcription factors (Smad2 and Smad3), which then form complexes with Smad4 and regulate the transcription of specific genes (see ref. 22 and refs. therein). He et al identified human TIF1γ as a protein that selectively binds receptor-activated Smads 2 and 3.[22] This binding is specific for TIF1γ and occurs via the middle region of TIF1γ. Of interest, it was shown that, in agreement with the fact that *moonshine*, the closest homolog of TIF1γ in the zebrafish, is an essential gene in hematopoiesis,[24] TIF1γ associates with Smad2/3 to stimulate erythroid differentiation in response to TGFβ.[22] These findings have been extended in an independent study by Dupont et al, who provided evidence that TIF1γ, as well as its *Xenopus* counterpart (called Ectodermin), also interfere with the Smad responses by binding to Smad4 and causing Smad4 ubiquitination and degradation.[23] This selective control of the protein level of Smad4 by TIF1γ appears to be needed to limit the TGFβ-growth arrest response in epithelial cells and was shown to play an important role in germ layer specification during the early development of *Xenopus* embryos.[23] It was demonstrated to rely on an enzymatically active TIF1γ RING finger domain and therefore provides the first evidence for a TRIM protein of the TIF1 subfamily acting as an E3 ubiquitin ligase to cause transcription factor degradation.[23]

PML/TRIM19 IN THE CONTROL OF TRANSCRIPTION

The *PML* gene was originally identified as the t(15; 17) chromosomal translocation partner of *RARα* in acute promyelocytic leukemia (APL). The PML protein acts as a negative growth regulator and tumor suppressor and as a specific regulator of hematopoietic differentiation.[54,55] Various PML isoforms have been identified that share the same N-terminal TRIM motif with variable C terminal lengths generated by alternative splicing (for a review see ref. 56). PML nuclear isoforms are typically found concentrated in discrete nuclear speckles called PML-Nuclear Bodies (PML-NBs), which recruit critical regulators of cell proliferation, apoptosis, genome stability and posttranslational modifications (see Chapter 4 by Batty et al). PML is not only the major component of PML-NBs, but also a key determinant of their formation; no PML-NB is observed in $PML^{-/-}$ cells[54] and any mutations in critical RING finger or B box cysteine residues of the PML TRIM motif disrupt PML-NB formation (see ref. 57 and refs. therein). Although the exact function(s) of PML-NBs remains still largely unknown, several lines of evidence support a role in transcriptional regulation. These include the colocalization of PML-NBs with many transcription factors and cofactors such as

CBP, HP1, Sp100, Daxx, Rb and p53, the detection of nascent RNA in the immediate periphery of PML-NBs, the association of PML-NBs with regions of high transcriptional activity and their interaction with specific genomic loci.[57-63] Moreover, PML by itself displays the properties expected for a coregulator playing a role in transcription, as either an activator or a repressor depending on the gene under consideration (see Table 2).[7]

PML as a Positive Regulator of Transcription

In agreement with a role of PML in transcriptional activation, it has been shown that PML interacts and colocalizes with the transcriptional co-activator and histone acetyltransferase CBP in the PML-NBs.[58,60] PML can potentiate the transcriptional activation function of CBP and serve as a co-activator for nuclear receptors.[36,64,65] Deletion analyses indicated that both the activation domains (AF-1 and AF-2) of the progesterone receptor (PR) as well as the tripartite motif of PML were required for the PML effect on PR-mediated transactivation.[64] These findings were supported by the analysis of *PML*$^{-/-}$ cells, showing that in the absence of PML, retinoic acid receptor-dependent transactivation was impaired as well as retinoic acid-induced myeloid differentiation and growth inhibition.[54] Moreover, PML has been reported to interact with and potentiate transcriptional activation by the p53 family members, i.e., p53, p63 and p73.[66-68] PML enhances p53 activity by several means: by recruiting p53 to PML-NBs and promoting its acetylation by CBP,[69] by interacting with p53 and MDM2 and preventing p53 ubiquitination,[70] by sequestering MDM2 to the nucleolus,[71] by promoting p53 phosphorylation by Chk2 and CK1 and blocking p53-MDM2 interaction,[72,73] or yet by promoting p53 deubiquitination by the ubiquitin protease HAUSP.[74] Similarly, PML increases p73 acetylation in a PML-NB-dependent manner, thus preventing its ubiquitinylation and subsequent degradation.[68] On the basis of a number of cotransfection and interaction data, PML was also shown to co-activate Fos and the hematopoietically expressed GATA2 transcription factor through a mechanism that requires an intact PML tripartite motif.[75,76] Moreover, a physical and functional link was described between a specific PML isoform (PMLI) and the leukemia-associated transcription factor AML-1; PMLI interacts with AML-1 through their respective C-terminal region, targets AML-1 into PML-NBs together with its co-activor p300, enhances AML-1-mediated transcription and stimulates differentiation of myeloid cells.[77] An unexpected role for a cytoplasmic isoform of PML (cPML) as an essential modulator of TGF-β-induced gene expression has recently been discovered; cPML physically interacts with Smad2/3 and SARA (Smad anchor for receptor activation) and is required for association of Smad2/3 with SARA and the accumulation of SARA and TGF-β receptor in the early endosome—a process that is crucial for TGF-β signal transduction.[78] Finally, a link between PML, higher order chromatin organization and gene regulation has been established by the demonstration that PML functionally and physically interacts with the matrix-attachment (MAR)-binding protein SATB1 to regulate chromatin-loop architecture and transcription of the major histocompatibility complex (MHC) class I locus.[79] On the other hand, PML was found to inhibit Daxx-mediated transcriptional repression by promoting recruitment of Daxx to the PML bodies,[80] while the specific isoform PMLIV can re-activate Myc-repressed target genes such as the cell cycle inhibitors *CDKN1A*/*p21* and *CDKN2B*/*p15* by mediating Myc degradation in a manner dependent on the RING domain of PML.[81]

PML as a Negative Regulator of Transcription

Evidence for a role of PML as a negative regulator of transcription is supported by the early findings that PML can inhibit transcription by itself when tethered to DNA, possibly through interaction with histone deacetylases.[13,82] In addition, PML was shown to interact through its coiled coil domain with the chromatin related-corepressors N-CoR and mSin3a and to mediate the transcriptional repression function of the tumor suppressor MAD.[83] Furthermore, a direct downregulatory effect of PML on the transcriptional activity of a variety of sequence-specific transcription activators has been described (see Table 2). It was found that PML interacts directly with the DNA binding domain of the Sp1 transcription factor through its coiled coil domain and inhibits the transactivation activity of Sp1 on the epidermal growth factor receptor (*EGFR*) gene promoter by preventing it from binding to DNA.[84] A similar mechanism of repression was described in the case of Nur77 and NF-κB, two potent transcriptional activators involved in induction of apoptosis.[85,86] Recently, PML was also shown to inhibit IFN-γ-mediated STAT-1α DNA binding and transcriptional activity, thus leading to a downregulation of numerous IFN-γ-regulated genes.[87] Finally, PML can form stable complexes with the retinoblastoma protein pRB within PML-NBs, interact with the pocket region of pRB through its B boxes and abolish activation of glucocorticoid receptor (GR)-mediated transcription by pRB.[88]

Overall, these studies define PML as a unique coregulator that in addition to interacting with various sequence-specific transcription factors, can influence their transcriptional activity by different biochemical means leading to up- or downregulation of gene expression. In the future, it will be of great importance to determine whether PML exerts all of its transcriptional activities in the PML-NBs or throughout the nucleoplasm.

RFP/TRIM27 IN THE CONTROL OF TRANSCRIPTION

The RET finger protein (RFP), also designated TRIM27, was originally identified in the context of a fusion protein with the RET tyrosine kinase that possesses transforming activity.[89] In addition to a N-terminal tripartite motif, RFP contains a specific C-terminal RFP or B30.2 domain (see Chapter 2 by Micale et al). RFP is widely expressed and, depending on the cell type or tissue, is localized either to the cytoplasm or nucleus.[90] In the nucleus, a portion of RFP associates with the nuclear matrix and localizes into the PML-NBs, where RFP binds directly to PML and Int-6.[91,92] Although no biological function has yet been ascribed to RFP, it has been shown to cause extensive apoptosis when overexpressed in human embryonic kidney 293 cells.[93] From a molecular point of view, this pro-apoptotic function of RFP may rely on its ability to control transcription. Indeed, as mentioned for PML, RFP is a protein that exhibits a potent transcriptional repressive activity when tethered to DNA through fusion to a heterologous DNA binding domain.[14] This repressor activity is regulated by sumoylation[94] and resides mainly in the coiled-coil domain, which represents a binding site for several proteins involved in chromatin-based gene silencing such as Enhancer of Polycomb 1 (EPC1), methyl-CpG binding proteins MBD2/4 and Mi-2β/CHD4, the main component of the NuRD complex (see Table 3).[95,96] Importantly, a direct inhibitory effect of RFP on the transcriptional activity of basic helix-loop-helix (bHLH) transcription factors has been described; RFP binds to the bHLH domain of

Table 2. Role of PML (TRIM19) in transcriptional regulation

PML Role	Transcription (co)Factor	Interaction Domain	Mechanism of Action	Biological Function	Refs.
Co-activation	RARα	No direct interaction	Stabilizes CBP-RAR complex	Growth inhibition, cellular differentiation	36, 54, 65
	PR	No direct interaction	nd	nd	64
	p53	DNA binding domain of p53, C-ter region of PML	Recruits p53 to the PML-NBs, promotes p53 acetylation, phosphorylation and deubiquitin-ation, sequesters MDM2 in the nucleolus	Apoptosis, senescence, growth inhibition	66, 69-74
	p63	nd	Recruits p63 to the PML-NBs	nd	67
	p73	nd	Increases p73 acetylation	nd	68
	Fos	No direct interaction	nd	nd	75
	GATA-2	Zinc finger region of GATA-2, B-box domain of PML	nd	nd	76
	AML-1	C-ter regions of AML-1 and PML I	Recruits AML-1 to the PML-NBs together with p300	Myeloid cell differentiation	77
	CBP	Aa 311-521 of CBP, coiled coil domain of PML	Recruits CBP to the PML-NBs	Cell growth control	36, 58, 60, 65
Co-repression	MAD	No direct interaction	Increases MAD-mediated repression via direct interaction with multiple corepressors (c-Ski, N-CoR and mSin3A)	Suppression of cell proliferation	83
	HDAC1	C-ter aa (447-633) of PML	nd	nd	82, 83
	c-Ski	Aa 261-330 of c-Ski, coiled coil domain of PML	Recruits c-Ski to the PML-NBs	Suppression of cell proliferation	83

continued on next page

Table 2. Continued

PML Role	Transcription (co)Factor	Interaction Domain	Mechanism of Action	Biological Function	Refs.
	N-CoR	Aa 1502-1581 of N-CoR, coiled coil domain of PML	Recruits N-CoR to the PML-NBs	nd	83
De-activation	Sp1	DNA binding domain of Sp1, coiled coil domain of PML	Inhibits Sp1 transactivation activity by preventing it from binding to DNA	Repression of *EGFR* transcription	84
	Nur77/NR4A1	DNA binding domain of Nur77, coiled coil domain of PML	Interferes with Nur77 DNA binding	Cell growth, apoptosis	85
	NFκB	nd	Interferes with NFκB DNA binding	Repression of *A20* transcription, apoptosis	86
	STAT-1α	nd	Inhibits STAT-1α DNA binding	Negative regulation of IFN-γ signaling	87
	pRB	The pocket region of pRB, the RING and B1-B2 regions of PML	nd	Inhibition of glucocorticoid receptor (GR)-mediated transcription by pRB	88
De-repression	Daxx	nd	Recruits Daxx to the PML-NBs and inhibits Daxx-mediated repression	Apoptosis	80
	Myc	N- and C-ter domains of Myc, C-ter region of PML	Induces Myc destabilization	Granulocytic differentiation	81

nd: not determined; Aa: amino acids; AML-1: acute myeloid leukemia 1 protein; CBP: CREB-binding protein; c-Ski: cellular Sloan-Kettering viral oncogene homolog; Daxx: Fas death domain-associated protein; Fos: FBJ osteosarcoma oncogene; HDAC1: Histone deacetylase 1; Myc: myelocytomatosis oncogene; N-CoR: nuclear receptor corepressor; Nur77/NR4A1: nuclear receptor subfamily 4, group A, member 1; PR: progesterone receptor; pRB: retinoblastoma protein; RAR: retinoic acid receptor; STAT-1: signal transducer and activator of transcription 1.

Table 3. Role of RFP (TRIM27) in transcriptional regulation

RFP Role	Transcription (co)Factor	Interaction Domain	Cellular Function	Ref.
Co-repression	Mi-2β/CHD4	C-ter of Mi-2β, coiled coil domain of RFP	Mi-2β enhances the repressing activity of RFP	96
	EPC1	EPcA and C-ter domains of EPC1, coiled coil domain of RFP	nd	14
	MBD2/4	Aa 413-580 of MBD4, coiled coil domain of RFP	Enhances MBD2- and MBD4-dependent repression	95
Co-activation	ERα	No direct interaction	Interacts directly with the C-ter Glu/Arg-rich region of the ERα repressor SAFB1 and positively regulates a subset of ERα target genes (e.g., *CCND1* and *PR*) in MCF-7 cells	99
	Mi-2β/CHD4	C-ter of Mi-2β, coiled coil domain of RFP	Associates with Mi-2β, MCRS1 and UBF in the nucleolus and up-regulates rDNA transcription	100
De-activation	SCL	bHLH domain of SCL, B box and coiled coil motif of RFP	Inhibits transactivation by SCL and by other bHLH transcription factors (E47, MyoD, mASH-1)	97
	pRB	Coiled coil and B30.2 domains of RFP	Inhibits Rb-mediated transactivation by preventing the degradation of the EID-1 inhibitor of histone acetylation	98

nd: not determined; Aa: amino acids; CHD4: chromodomain helicase DNA binding protein 4; EPC1: enhancer of polycomb 1; ERα: estrogen receptor alpha; MBD2/4: methyl-CpG-binding domain protein 2/4; MCRS1: microspherule protein 1; pRB: retinoblastoma protein; SAFB1: scaffold attachment factor B1; SCL: stem cell leukemia protein; UBF: upstream binding transcription factor, RNA polymerase I.

the Stem Cell Leukemia gene product (SCL) through its B box and coiled coil domain and specifically inhibits transactivation by SCL and three other bHLH proteins, E47, MyoD and mASH-1, via a mechanism that requires histone deacetylation activity.[97] Recently, RFP was also shown to inhibit transcription activation by the Retinoblastoma protein pRb; RFP binds to pRB through its coiled coil and C-terminal B30.2 domain, provokes stabilization of the histone acetyltransferase inhibitor EID-1 and, through this, inhibits pRB gene-activating function.[98]

Supporting the notion that RFP could play a dual role in transcription, being involved in both repression and activation, RFP was also reported to regulate positively estrogen receptor α (ERα)-mediated transcription, through a mechanism that may involve a direct interaction with the ERα repressor SAFB1 (Scaffold attachment factor B1).[99] Moreover, RFP and Mi-2β/CHD4, known to be involved in transcriptional repression in the nucleus, have been reported to form a complex with the nucleolar protein MCRS1 (microspherule protein 1) and the rRNA transcription factor UBF in the nucleolus, where they play a direct transactivating role on ribosomal gene transcription.[100]

OTHER TRIM FAMILY MEMBERS IN TRANSCRIPTIONAL REGULATION

Besides the TIF1s, PML and RFP, a few other TRIM proteins have been associated with transcription in different organisms (see Table 4). These include RPT-1/TRIM30, Pub/TRIM14 and TRIM45 in human and mouse, Xnf7 in *X. laevis* and TAM-1 in *C. elegans*. RPT-1 (Regulatory protein, T-lymphocyte, 1) is selectively expressed by resting inducer T cells and was shown to downregulate gene expression directed by the long terminal repeat (LTR) promoter region of human immunodeficiency virus Type 1 (HIV-1) or by the promoter region of the gene encoding the α chain of the interleukin 2 receptor (*IL-2Rα*).[101] Pub, also designated TRIM14, was originally identified based on its

Table 4. Other TRIM proteins in transcriptional regulation

TRIM	Transcription Factor	Cellular Function	Refs.
Human or mouse			
RPT-1/TRIM30	nd	Downregulates *IL-2Rα* and HIV-1 transcription	101
Pub/TRIM14	Pu.1/Spi-1	Inhibits Pu.1 transcriptional activity	103
TRIM45	nd	Represses transcription by ELK-1 and AP-1	104
Other species			
Xnf7 (*X. laevis*)	nd	Regulates pre-mRNA maturation	105, 106
TAM-1* (*C. elegans*)	PHA-4/FoxA	Cooperates with PHA-4 to repress ectodermal genes in pharyngeal precursor cells	4

*: lacks the coiled coil domain; nd: not determined; PHA-4/FoxA: defective pharyngeal development protein 4/forkhead box A; Pub: PU.1 binding protein; RPT-1: Regulatory protein, T-lymphocyte, 1; Spi-1: spleen focus forming virus (SFFV) proviral integration oncogene; TAM-1: Tandem Array expression Modifier 1.

ability to interact with the human transcription factor PU.1/Spi-1, an Ets family protein which plays a central role in the differentiation and proliferation of macrophages and B cells during hematopoiesis.[102,103] In addition to a tripartite motif, Pub contains a B30.2 domain and was shown, in transient transfection assays, to inhibit PU.1 transcriptional activity in a B box integrity-dependent manner.[103] TRIM45 is a widely expressed TRIM protein, harboring in its C-terminal region a filamin-type immunoglobin (IG-FLMN) domain.[104] In forced expression studies, TRIM45 selectively inhibits the transcriptional activity of EIK-1 and AP-1, suggesting that it may act as a negative modulator of the mitogen-activated protein kinase (MAPK) signaling pathway.[104] Xnf7 is one of the first TRIM proteins described and was characterized in two amphibian species, *Xenopus laevis* and *Pleurodeles waltl*.[105] In addition to the tripartite motif, XNF7 has a chromodomain and a B30.2 domain in its N-terminal and C-terminal regions, respectively. It was found that during oogenesis, XNF7 associates through its B box and the coiled coil with the elongating RNA polymerase II transcripts on the loops of the lampbrush chromosomes, thus suggesting a role in pre-mRNA transcription and/or processing.[105,106] Biochemical and genetic studies in *C. elegans* have identified TAM-1, a RING finger/B box protein lacking the coiled coil domain, as a corepressor interacting with and mediating via association with the NuRD complex the repressive activity of the PHA-4/FoxA transcription factor.[4] It was found that TAM-1 and NuRD cooperate with PHA-4 to repress ectodermal genes in pharyngeal precursor cells and thereby promote specification to pharyngeal fate.[4]

CONCLUSION

Although up to now only a relatively small subset of TRIM proteins has demonstrated transcriptional regulatory activity, it appears that these TRIMs control transcription by means of a wide and varied range of activities and play critical roles in a plethora of cellular processes such as apoptosis, cell cycle regulation, differentiation and retrovirus restriction. This functional diversity relies on the intrinsic properties of the concerned proteins and their ability to interact with distinct classes of sequence-specific transcription factors and cofactors. Through these interactions, they can either positively or negatively regulate target gene expression. Prominent among the mechanisms of action is the potential to cooperate with chromatin modifiers, to recruit transcription factors and cofactors to specialized nuclear compartments and to regulate directly or indirectly their posttranslational modification. Exactly how TRIMs operate in these alternative mechanisms and how they are regulated should receive great deal of attention in the coming years.

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CHAPTER 6

TRIM PROTEINS IN CANCER

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Abstract: Some members of the tripartite motif (TRIM/RBCC) protein family are thought to be important regulators of carcinogenesis. This is not surprising as the TRIM proteins are involved in several biological processes, such as cell growth, development and cellular differentiation and alteration of these proteins can affect transcriptional regulation, cell proliferation and apoptosis. In particular, four TRIM family genes are frequently translocated to other genes, generating fusion proteins implicated in cancer initiation and progression. Among these the most famous is the promyelocytic leukaemia gene *PML*, which encodes the protein TRIM19. *PML* is involved in the t(15;17) translocation that specifically occurs in Acute Promyelocytic Leukaemia (APL), resulting in a PML-retinoic acid receptor-α (PML-RARα) fusion protein.

Other members of the TRIM family are linked to cancer development without being involved in chromosomal re-arrangements, possibly through ubiquitination or loss of tumour suppression functions.

This chapter discusses the biological functions of TRIM proteins in cancer.

INTRODUCTION

The RBCC/TRIM (tripartite motif) protein family is involved in a wide range of biological processes, such as cell growth, development and cellular differentiation. Thus, alteration of these proteins has repercussion for several pathological conditions, from hereditary genetic diseases to cancer development and viral infections. Their striking feature is the tripartite or RBCC motif that is invariably present at the N-terminal region of these proteins, while the C-termini can have different domains. The tripartite motif is

TRIM/RBCC Proteins, edited by Germana Meroni.

composed of three cysteine-rich zinc-binding domains, a RING-finger and two B-boxes (B1 and B2), followed by a α-helical coiled-coil region.

Within the vast family of TRIM proteins, few have been well characterized or have established physiological roles. Four family members, TRIM19/PML, TRIM24/TIF1α, TRIM33/TIF1γ and TRIM27/RFP, have been described to acquire oncogenic activity upon chromosomal translocations: in these genetic alterations, the RBCC motif is fused to truncated products of other genes, suggesting that the RBCC motif may play a crucial role in cell transformation. Other members of the TRIM family are linked to cancer development without being involved in chromosomal re-arrangements.

Several data indicated that some RBCC/TRIM proteins may be implicated in ubiquitination, probably because the tripartite module represents the ideal scaffold for such a process. Since the conjugation of ubiquitin proteins to target substrates might require higher order structures, it has been proposed that this large protein family represents a novel class of "single protein RING finger" E3-ubiquitin (Ub) ligases. Considering the huge number of substrates that can be targeted by an E3 ligase, it is not surprising that mutations in *RBCC/TRIM* genes can result in several pathological conditions, including cancer.

More recently, it was suggested that some TRIM proteins such as PML, RFP and TRIM32 may be a new class of SUMO protein ligases (SUMO E3), their SUMO activity depending on an intact tripartite motif. Finally, several TRIM proteins are involved in the regulation of p53-pathways.

This chapter focuses on those TRIM proteins whose involvement in cancer development and progression has been more fully described in literature.

TRIM PROTEINS IN CHROMOSOMAL TRANSLOCATIONS

PML

Included in this group is the ProMyelocytic Leukaemia protein PML, probably the best-known among the TRIM proteins, which becomes oncogenic when its RBCC motif recombines with the product of the retinoic acid receptor (*RARα*) gene.[1-3] The *PML* gene (*PML*) fuses to the retinoic acid receptor α (*RARα*) gene, leading to the production of two fusion genes that encode for the chimera proteins PML-RARα and RARα-PML. Both proteins co-exist in leukemic cells, but, to date, work has mainly focused on PML-RARα, since it retains most of the functional domains of the parental proteins, while little is known about RARα-PML.

PML was identified by cloning the breakpoint sites of the t(15:17) chromosome translocation associated with Acute Promyelocytic Leukaemia (APL); the majority (95%) of APL patients present this translocation.[4] RARα is a member of the nuclear hormone receptor superfamily of transcription factors. In physiological conditions, upon retinoic acid (RA) binding, *RARα* transactivates target genes involved in hematopoietic differentiation. In contrast, in the absence of the ligand, transcription is repressed by the recruitment of histone deacetylases (HDACs) through direct interaction between RARα and the corepressors N-CoR and SMRT. In APL, the fusion protein PML-RARα acts as a potent dominant negative inhibitor of the wild-type (WT) RARα, due to its increased affinity for transcriptional corepressors and HDACs. This strong interaction, in turn, leads to repression of transcription and consequent block of differentiation. As a result, in

the presence of low or even physiological concentrations of RA ($1 \times 10^{-9} - 1 \times 10^{-7}$ M), RARα is no longer able to release HDACs. However, pharmacological doses of RA ($1 \times 10^{-6} - 2 \times 10^{-5}$ M) cause the dissociation of the PML-RARα/HDAC complex, restoring the normal differentiation pathway.[5] The clinical treatment of APL patients with RA represents the first example of "differentiation therapy": the drug treatment degrades PML/RARα and restores the normal function of PML. Nevertheless, treatment of APL with RA as a single agent results in remission only in a small percentage of patients.[6,7] However, addition of the DNA damaging agent arsenic trioxide (As_2O_3) to the RA treatment increases the percentage of APL eradication to more than 90%.[8] As_2O_3 binds directly to cysteine residues in zinc fingers located within the RBCC domain of PML-RARα and PML. This binding induces PML oligomerization with consequent increased affinity for the small ubiquitin-like protein modifier (SUMO)-conjugating enzyme UBC9, resulting in enhanced SUMOylation and degradation. The loss of PML-RARα through degradation enables terminal differentiation of APL cells explaining the efficacy of the combined treatment.[7,8]

PML-RARα associates physically with PML, potentially interfering with its function. Accordingly, transgenic mice expressing PML-RARα in the myeloid compartment develop leukaemia with APL features, while dominant negative RARα mutants do not (as the mutation does not interfere with PML functions), highlighting the importance of PML functional disruption for leukemogenesis.[9]

PML, as most of the TRIM family members, forms high molecular weight complexes in vivo as a consequence of self-association properties of the coiled-coil region.[10] The coiled-coil region is responsible for the formation of stable PML homodimers and for PML/PML-RARα hetero-dimerization,[5] whereas the RING-finger domain is involved in protein-protein interaction.[11] The tripartite motif seems to be essential for the correct localization of PML and, indeed, key to the growth suppressor activity of the protein.[12]

PML genomic locus spans approximately 35 Kb and is subdivided in nine exons. Due to alternative splicing of the gene transcript, which always involves coding exons, PML exists in a number of different isoforms.[13] The N-terminal region is common to all PML isoforms (amino acids 1-394) and contains the region homologous to the DNA-binding finger domain of numerous transcriptional factors. The C-terminus instead differs in several isoforms and it is variably encoded by exons 7, 8 and 9.[13] All PML isoforms are almost equally expressed in the different cell lines and have the potential to act as transcription factors and to form homo- or hetero-dimers.[13] The function of the complex splicing pattern of PML is not known, however, the role of the different PML isoforms might be to provide several distinct surfaces for protein interactions.

In APL, the PML locus on chromosome 15 can be alternatively disrupted at three different sites (breakpoint cluster regions-bcrs): bcr3 in intron 3, bcr1 intron 7 and bcr2 in exon 6. The resulting PML-RARα proteins contain the N-terminal part and the tripartite motif, but invariably lose the C-terminal domain. In this domain resides a putative phosphorylation site with a serine/proline-rich region, potentially involved in the regulation of the normal protein. Therefore, the PML-RARα chimera protein could be under a different regulatory mechanism and this could contribute to the pathogenesis of APL.

In addition to splicing, posttranslational modifications may increase the level of complexity, either by directly affecting protein-protein interactions (the PML sequence contains SUMO-1 modification sites and phosphorylation sites) or by interfering with the ability of PML to form Nuclear Bodies. In fact, physiological localization of PML

is observed both in the nucleoplasm and in the discrete subnuclear matrix-associated compartments known as PML-Nuclear Bodies (PML-NBs).[15] In APL cells, instead, the fusion protein PML/RARα disrupts PML-NBs and relocalises PML into hundreds of microspeckles in the nucleus and in the cytoplasm. This aberrant localization results from a coiled-coil domain interaction between PML and PML/RARα heterodimers.[15-17] Treatment with RA leads to clinical remission associated with NB re-organization, strongly suggesting that PML-NB integrity could be critical for normal cellular functions. Genetic evidence indicates that the structure of PML-NBs depends on the presence of PML.[18] In cells derived from PML-knockout mice, PML-NBs do not exist and all the proteins normally targeted to these organelles are nuclear diffused or concentrated in distinct domains. The product of the *BLM* gene, for instance, which is inactivated in the Bloom syndrome, normally colocalizes with PML in the NBs, but it is no longer found in these structures in PML-null cells.[19]

A cytoplasmic PML isoform, in which a nuclear export sequence (NES) in exon 9 is retained, is also generated as a consequence of alternative splicing. This isoform can shuttle between nucleus and cytoplasm and it is not localized at NBs. It has been implicated in the modulation of TGF-β signalling[20] and calcium metabolism regulation at the endoplasmic reticulum (ER).[21]

A number of tumours and tumour-derived cell lines are characterized by a novel form of PML-NBs, termed ALT-associated PML-Bodies (APBs), in which PML localizes at the telomeres, together with other telomere binding proteins, to maintain telomere length.[22]

The study of mice in which PML has been inactivated by homologous recombination has led to the conclusion that the *PML* gene is not required for viability, at least in the mouse. PML- knockout mice develop normally and do not get spontaneous cancers but are more sensitive to tumour promoting agents.[23] PML can therefore antagonize the initiation, promotion and progression of tumours of different histological origins, behaving in vivo as a tumour suppressor. Consistent with these findings, the overexpression of PML inhibits the transformation of rat embryo fibroblasts induced by Ha-Ras expression in combination with oncogenic mutants of p53 or c-Myc,[24] and causes a dramatic drop in the capacity of cell lines to form colonies in a typical colony formation assay.[12]

PML can exert its growth suppression activity either through the induction of p53-dependent/independent apoptosis or replicative senescence.

The first evidence that PML is involved in the p53-dependent apoptotic pathway came from experiments carried out in PML-knockout mice. These animals are resistant to the lethal effects of ionizing radiations.[25] Similarly, PML –/– splenocytes are resistant to γ-irradiation-induced apoptosis.[26] In these cells, DNA-damage induced apoptosis depends entirely on the presence of a normal p53 function. P53 –/– cells, in fact, are completely insensitive to irradiation, while PML –/– cells display an intermediate phenotype, suggesting that PML may be only required to support p53 pro-apoptotic functions. Upon γ-irradiation p53 becomes stabilized and is activated by posttranslational modifications that take place at several residues. The DNA-damage checkpoint kinase Chk2 is responsible for serine 20 modification. PML recruits Chk2 into PML-NBs and enhances p53 phosphorylation.[27] Similarly, the homeodomain-interacting protein kinase-2 (HIPK2) was found to colocalize with PML within the NBs.[28,29] HIPK2 is responsible for phosphorylating p53 on serine 46 upon ultraviolet irradiation, an event that has been linked to induction of apoptosis.[30] The herpes virus associated ubiquitin-specific protease (HAUSP) is another enzyme affecting p53 activity and localizing at the NBs.

MDM2, the main regulator of p53, was also found to be associated with PML-NBs, in particular after the inhibition of the nuclear export.[31] More recently, it was shown that PML and MDM2 interact[32] and that upon DNA-damage PML protects p53 from MDM2-mediated ubiquitination and degradation.[33] In fact, it appears that following UV irradiation two different complexes, PML/p53 and PML/MDM2, are formed, leading to p53 stabilization.[34]

As regards the role of PML in p53-independent apoptosis, it was reported that splenocytes and hepatocytes from PML-knockout mice have defective Fas- and TNF-induced apoptosis. These two agents induce activation of extrinsic apoptotic pathways, considered p53-independent.[25] The Fas-interacting protein DAXX was found to act as a positive mediator of Fas- and TNF-induced apoptosis. Two models have been proposed to explain the co-operation between PML and DAXX in regulating apoptosis.[35] According to the first one, upon Fas-ligand binding, DAXX moves to the nucleus where it directly interacts with PML. DAXX is a transcriptional repressor and PML is thought to antagonize its transcriptional function by sequestering it into PML-NBs. The second model proposes that the PML/DAXX complex is released from the nuclear bodies upon PML deSUMOylation by Supr-1, or upon DAXX phosphorylation by the homeodomain-interacting protein kinase-1 (HIPK-1). The PML/DAXX complex would have then transcriptional functions and activate pro-apoptotic genes.

Recently, PML was found to be a critical factor for mitochondrial damage linked to ER stress-induced cell death.[36] In the cytoplasm, in fact, PML was found to localize to the ER and specific membrane structures (mitochondrial-associated membranes, MAMs) involved in ER-to-mitochondria calcium ion (Ca^{2+}) transport.

The existence of a link between premature senescence, PML-NBs and p53 is now generally accepted. In primary fibroblasts, PML overexpression induces premature senescence in a p53-dependent manner, since p53-knockout mouse embryo fibroblasts (MEFs) are protected from this effect. PML itself is upregulated during replicative or Ras-induced senescence.[37] The resulting increase in PML levels leads to a concomitant increase in number and size of PML-NBs. Expression of the activated form of Ras (Ras^{VAL12}) also induces relocalization of p53 within the PML-NBs and promotes its acetylation at lysine 382. Acetylation is essential for p53 biological function and is profoundly impaired in PML –/– MEFs. Although PML does not possess intrinsic acetyltransferase activity, it directly interacts with the acetyltransferase CBP/p300, recruiting it to the NBs. Therefore, PML, p53 and CBP/p300 form a three-complex that localizes to PML-NBs, facilitating p53 acetylation by CBP/p300. Collectively, these observations suggest that PML is required for p53 acetylation and senescence upon oncogene expression. However, conclusive evidence that PML, like p53, is essential for the induction of cellular senescence upon oncogenic transformation comes from the observation that Ras^{VAL12}-induced senescence is drastically impaired in PML –/– cells.

The NAD-dependent deacetylase SIRT1, a negative regulator of p53, has also been found in NBs. SIRT1 binds to p53 and specifically deacetylases lysine 382, resulting in decreased p53-dependent transcription and PML-induced senescence.[38]

In conclusion, PML is involved in a great variety of processes, most of them closely linked to its primary localization into the NBs, where a plethora of different proteins are also assembled and anchored. Thus, the oncogenicity of PML/RARα, which appears to involve the RBCC motif, may result from an abnormal subnuclear organization that diverts subsets of proteins from their natural functions.

TIF1α

Like PML, the Transcriptional Intermediary Factor 1 α (TIF1α), or TRIM24, seems to be involved in the RA-dependent activation pathway.[3]

TIF1α is a nonhistone chromosomal protein (E. Remboutsika, R. Losson and P. Chambon, unpublished results) found to interact specifically and in a ligand-dependent manner with several nuclear receptors, including RARs, the Retinoic X Receptors, the vitamin D3 receptor (VDR), and the oestrogen (ER) and progesterone receptors (PR), and to positively or negatively regulate their transcriptional activity.[39] TIF1α is maintained at high levels in myeloid progenitor cells when granulocyte differentiation is induced by RA, indicating that RARα-mediated TIF1α expression is required for myeloid differentiation.[40] TIF1α can also function as a liver-specific tumour suppressor by attenuating RARα-mediated transcription in mice. TRIM24-knockout mice are viable and fertile, but homozygous deletion of TRIM24 induces a high incidence of hepatic tumour development by 9 months of age. Deletion of a single RARα allele in a *Trim24*-null background suppresses hepatocellular carcinoma (HCC) development and restores wild-type expression of RA-responsive genes in the liver.[41] TIF1α, moreover, can repress transcription in transient transfections when tethered to template DNA through chromatin association, which is mediated by its peculiar C-terminal domain. This domain, called PHD-BROMO domain, contains a polycomb homology domain (PHD) finger and a bromodomain,[42-44] which are characteristic motifs of proteins known to function as transcriptional coregulators at chromatin level.[45,46] Recently, it has been demonstrated that TIF1α recognizes acetylated histone 3 at K23 (h2K23) and unmodified histone H3K4 through a noncanonical histone signature and that its regulation is associated with cellular proliferation and tumour development.[47]

TIF1α, like PML, can operate as a transcriptional intermediary factor, regulating the ability of RXRα/RARα to activate transcription.[3] A putative functional interaction between these two proteins has been suggested in PML –/– cells, where the ability of TIF1α to act as a transcriptional co-activator upon RA is impaired. Moreover, PML and TIF1α co-immunoprecipitate when they are both transiently transfected, but no binding is observed when assayed in yeast two-hybrid assays, suggesting that no direct interaction occurs between them. Recently, Tisserand and colleagues have associated *Trim24* with a negative regulation of the IFN/STAT pathway through RARα inhibition, suggesting that this repression may prevent liver cancer.[48]

Similarly to PML, TIF1α also occurs in chromosomal re-arrangements leading to the formation of the oncoprotein T18, which retains the N-terminal moiety of TIF1α fused to a truncated C-terminal portion of the mouse homologue B-raf proto-oncogene.[3,49] By analogy with PML/RARα, the RBCC domains of TIF1α may potentiate the oncogenicity of B-raf C-terminal through two different mechanisms, not mutually exclusive. In the first, formation of TIFα/TIF-B-raf heterodimers involving the coiled coil domain may have a dominant negative effect, relocating TIF1α and/or TIF1α-associated proteins from the nucleus to the cytoplasm. TIF1α delocalization inactivates its transcriptional function, lending a selective growth advantage to the transformed cells and abrogating their capacity to respond to RA. In addition, because TIF1α associates with nuclear receptors (ER and VDR),[39] T18 may interfere with other hormone-dependent pathways. In the second, the truncated B-raf, associated with the RBCC motif of TIF1α, may acquire a different intracellular localization, thereby increasing its oncogenic potential.

TIF1α is also found fused with RET (Re-arranged during Transfection) receptor tyrosine kinase (tk) in the papillary thyroid carcinomas (PTC). The resulting oncoprotein

displays the C-terminal ret tk domain fused to the RBCC of TIF1α.[50] A modified TIF1α protein, in concert with the altered ret tk activation, might be important for tumour induction and/or progression.

TIF1γ/TRIM33

In contrast with TIF1α, the ubiquitous nuclear protein Transcriptional Intermediary Factor 1 gamma, TIF1γ/TRIM33, does not interact with nuclear receptors but exhibits a strong silencing activity when tethered to a promoter.[51] The *TIF1γ* gene has been mapped to the 1p13 locus. Interestingly, a nonrandom chromosomal translocation t(1;22)(p13;q13) involving this region has been reported in acute megakaryocytic leukaemia [Mitelman F. (1993). In: *Human Gene Mapping*, pp.773-812]. *TIF1γ* was shown involved in the control of haematopoietic cell fate by the TGFβ/Smad pathway.[52] More recently, *TIF1γ* has been proposed as an epigenetically regulated tumour suppressor gene in hematopoietic cells. The authors suggest that in chronic myelomonocytic leukemia changes in *TIF1γ* expression may be considered as biomarkers of response to the demethylating agents and other chromatin structure modifiers (e.g., HDAC inhibitors) currently being developed for the treatment of this disease.[53]

RFP

The last TRIM family member acquiring a transforming activity when its RBCC motif is fused to another protein is the Ret Finger Protein (RFP), also known as TRIM27. Together with other family members, RFP also has, in addition to the tripartite domain, a highly conserved 185 to 195 amino acid motif at the C-terminal region, called the rfp domain or B30.2 motif.[54] Interestingly, *RFP* was originally identified as a gene which becomes oncogenic following its fusion with the *RET* proto-oncogene. This translocation is generated by DNA re-arrangement, which occurs during transfection of the NIH 3T3 cells with human T-cell lymphoma DNA. The resulting transforming protein RFP/RET shows the fusion of the tripartite motif of RFP with the C-terminal tyrosine kinase domain of the RET protein.[55-57] RFP/RET triggers cellular transformation through a mechanism which is still unknown, but two options seem likely: (i) the RFP RBCC motif causes ligand-independent dimerization of the fusion protein resulting in constitutive kinase activation and subsequent cell transformation; (ii) RFP nuclear localization signal leads the fusion protein into the nucleus where it dimerizes with the wild-type RFP, interfering with its normal functions. However, it is also possible that the normal amino-terminal domain of the tyrosine kinase contains regulatory elements that become lost during gene fusion causing abnormal enzymatic activity leading to cellular transformation. Indeed, this mode of activation is frequent with tyrosine kinases.

RFP is a component of PML-NBs through direct interaction with PML, which is mediated by the B-box and the distal α-helical coiled-coil domains of RFP.[58] In APL-derived NB-4 cells, which cannot differentiate, RFP is found dispersed in microgranules, retaining, however, its colocalization with PML and/or PML/RARα. After treatment with All Trans Retinoic Acid (ATRA), the block in differentiation is released and RFP and PML colocalize together with the other components in the reformed PML-NBs. Further studies demonstrated that RFP exhibits transcriptional repressive activity.[59-62] Recently, an interesting study showed evidence for physical and functional interactions between the retinoblastoma tumour suppressor protein (Rb) and RFP. It has been

proposed that RFP may inhibit *Rb* activating function by inducing stabilization of the histone acetyl-transferase (HAT) inhibitor EID-1 (E1A-like inhibitor of differentiation).[63] EID-1 is involved in the transcriptional regulation of genes that are required for cellular differentiation through the block of the activity of the histone acetyltransferase p300.[64] TRIM27 expression also correlates with ERBB2 protein expression and ERBB2 gene amplification in breast cancer.[65]

All these data suggest that RFP may have a role in growth regulation and that, in the appropriate context, could act as an oncogene.[58]

Finally, a recent study suggests that TRIM27/RFP functions as a SUMO E3 ligase toward MDM2 and that TRIM27 acts on MDM2 independently of PML, with whom it directly interacts.[66]

TRIM PROTEINS AS E3-Ub LIGASES

EFP

A striking example of an E3-Ub ligase involved in oncogenesis is EFP (oestrogen-responsive finger protein), also called TRIM25, which was found to be highly expressed in breast cancer. Since the discovery that the ovarian hormone oestrogen stimulates breast tumour growth, many efforts have been directed to determine an endocrine therapy which can inhibit the synthesis or the action of oestrogen.[67] The direct effect of oestrogen on oestrogen-responsive tissues requires two oestrogen receptors (ERα and ERβ): these are ligand-dependent transcription factors found in low levels in the normal mammary gland tissue and in higher concentration in most human breast cancers.[68] Downstream target genes of ERs have an important role in mediating oestrogen action in breast cancer; among these, *EFP* is a target gene of ERα and, besides breast cancer, it is also expressed in a variety of other female organs.[69] Urano et al, analysed the effect of EFP antisense oligonucleotides on tumour formation from breast cancer MCF7 cells implanted in female athymic mice. Administration of antisense EFP oligonucleotides inhibited the tumour growth generated by MCF7 cells. Moreover, EFP-overexpressing MCF7 cells in ovariectomized athymic mice generated tumours in the absence of estrogen.[70] More detailed studies demonstrated that EFP directly degrades the cell cycle regulatory protein 14-3-3σ through an ubiquitin-dependent pathway in which EFP functions as an E3-Ub ligase. 14-3-3σ degradation is followed by dissociation of the protein from the cyclin-Cdk complexes, leading to cell cycle progression and tumour growth. Thus, EFP may contribute to the deregulated proliferation of breast cancer cells *via* the accelerated destruction of the cell cycle regulator 14-3-3σ. In MEFs, loss of TRIM25 causes accumulation of 14-3-3σ, which is responsible for reduced cell proliferation. In agreement with these findings, TRIM25 immunoreactivity is significantly correlated with poor prognosis in patients with breast cancer.[71] It has been reported that even in the absence of hormone-stimulation, TRIM25 physically interacts with ERα leading to ERα ubiquitylation. In the presence of oestrogen, TRIM25 overexpression enhances ERα-mediated transcription, suggesting that TRIM25 overexpression functions as a cofactor for ERα-induced transcription.[72]

Recently, the accumulation of experimental evidences regarding oestrogen-responsive genes such as EFP has opened the way for the development of potential molecular targets of therapeutic intervention. In particular, it has been shown that DNA-modified siEfps

(chimeric siEfps) have good potential in clinical applications inducing fewer off-target effects or immune responses in mammalian cells.[73]

TRIM32

TRIM32, like TRIM25, is linked to cancer without being an oncogenic fusion protein. The C-terminal portion of the protein contains NHL repeats: the domain consists of repeats of a ~44-residue sequence that is rich in glycine and hydrophobic residues, and contains a cluster of charged residues near its C-terminal end. This domain is known by the name of NHL because was first identified in three proteins, NCL-1,[74] HT2A,[75] and LIN-41.[76]

TRIM32 accumulates in characteristic cytoplasmic speckles.[10,77]

Among TRIM family members, TRIM32 is unique in being involved both in hereditary developmental syndromes and in carcinogenesis. In fact, TRIM32 is mutated in the limb-girdle muscular dystrophy Type 2H (LGMD2H) and, more recently, a second mutation has been linked to the Bardet-Biedl syndrome (BBS).[78,79] In addition, deficiency of TRIM32 in mice results in several features observed in human LGMD2H.[80]

TRIM32 was originally identified in the mouse clonal epidermal model of carcinogenesis. In this model, nontransformed keratinocytes, treated with 7,12-dimethylbenz[*a*]anthracene (DMBA), gave rise to three independently initiated clones; these clones, once inoculated in mice, produced papillomas and squamous cell carcinomas (SCCs), used to derive the tumorigenic cell lines.[81] The clonality of the model permits to dissect the process of carcinogenesis in normal, initiated and tumorigenic cells. Initiated and tumorigenic cells from this model exhibit a 2-5 fold increase in TRIM32 expression compared to normal cells, indicating a role for TRIM32 in tumour initiation but also during progression and transformation.[77]

TRIM32 level is also elevated in a fraction of human head and neck squamous cell carcinoma (HNSCC) samples and in the adjacent mucosa. HNSCC represents the sixth most common cancer in the world and can affect different anatomical regions: the oral cavity, oropharynx, larynx and hypopharinx. The prognosis of HNSCC is influenced by many factors: tumour, node, metastasis staging and pathologic grading of differentiation. TRIM32 expression is elevated early in HNSCC development and, as in the clonal keratinocyte model, maintained in malignant progression. Interestingly, in vitro ectopic expression of TRIM32, at levels comparable to those detected in sporadic cancer, increases transformation frequency and is able to block UV-induced apoptosis in keratinocytes.[77] Increased TRIM32 expression has also been detected in murine skin tumours, induced after exposure to UVB or to a chemical [12-*O*-tetradecanoylphorbol-13-acetate (TPA) + DMBA] two-step carcinogenesis protocol.[77] TRIM32 imparts a survival phenotype to epidermal cells responding to UVB/TNFα-induced stress, whereby these epidermal cells persist and can accumulate additional UVB-induced DNA damage or other oncogenic events, leading to cancer development.

TRIM32 has the hallmarks of an E3-Ub ligase, including self-ubiquitination and interaction with ubiquitinated proteins in response to combined UVB/TNFα (Tumour Necrosis Factor α).[77] A direct target of TRIM32 degradation through ubiquitylation is Piasy, a member of the PIAS (protein inhibitors of activated STATs) family: this protein is a E3-SUMO ligase, involved in the control of apoptosis, senescence and Nuclear Factor-Kappa β (NF-Kβ) activation.[82] Piasy promotes keratinocyte apoptosis in response to UVB and TNFβ-induced stress through the inhibition of NF-Kβ survival function. The interaction between TRIM32 and Piasy, therefore, appears to modulate the balance

between survival and apoptosis in response to specific stress conditions. An interesting open question is whether this interaction is also important in the development of human HNSCC since this mechanism may have important clinical implications.

A role for TRIM32 as tumour suppressor has been also suggested. In mammalian cells, TRIM32 induces tumour necrosis factor (TNF)-mediated apoptosis through its interaction and ubiquitylation of XIAP (X-linked inhibitor of apoptosis). Degradation of XIAP prevents its inhibition of pro-apoptotic caspases.[83]

Recently, TRIM32 was found to interact with RARα enhancing its transcriptional activity in the presence of RA. TRIM32 overexpression in mouse neuroblastoma cells and embryonal carcinoma cells promoted stability of RARα, resulting in enhancement of neural differentiation. These findings suggest that TRIM32 could be a therapeutic target for developmental disorders and RA-dependent leukaemia.[84]

TRIM PROTEINS WITH TUMOUR SUPPRESSOR FUNCTIONS OR INVOLVEMENT IN p53 PATHWAYS

TRIM8/GERP

TRIM8/GERP (glioblastoma expressed RING finger protein) has been implicated in a variety of neoplastic transformations. TRIM8 is highly conserved between human and mouse and contains two B-box domains. The gene is expressed in several tumours, including anaplastic oligodendroglioma, and maps to chromosome 10q24.3, a region showing frequent deletions or loss of heterozygosity in glioblastomas,[85] which suggests the presence of a tumour suppressor gene at that site.

A study of primary tumour samples from patients undergoing surgery for laryngeal squamous cell carcinoma (LSCC), the most frequent neoplasia of the head and neck region, supports TRIM8 tumour suppressor role.[86] Microarrays screening, performed to identify genes regulated during tumour progression, showed that TRIM8 inversely correlates with metastatic potential. The anti-metastatic and anti-proliferative involvement of TRIM8 was also tested in an in vitro colony formation assay: human cell lines stably transfected with TRIM8 cDNA produce significantly less colonies than control cells.[86]

TRIM8 is a suppressor of cytokine signalling 1 (SOCS1)-interacting protein that is induced by IFNγ.[87] TRIM8 was also shown to interact with the protein inhibitor of activated STAT3 (PIAS3). PIAS3 inhibits IL-6-dependent activation of STAT3, a signalling pathway that is known to be important for cancer development and progression.[88] Ectopic expression of TRIM8 can block PIAS3 action on STA3 either by inducing its degradation or by excluding PIAS from the nucleus.[88] Moreover, a recent study has shown that the translocation of phosphorylated STAT3 into the nucleus is modulated by the interaction between TRIM8 and the heat shock protein 90β (HSP90β) and consequently regulates transcription of Nanog in embryonic stem cells, suggesting a role for TRIM8 in the regulation of stem cell self-renewal or differentiation.[89]

More recently, TRIM8 has been described as a new modulator of p53-mediated tumour suppression mechanisms.[90] Under stress conditions, such as UV exposure, p53 induces the expression of TRIM8, which in turn impairs p53 interaction with MDM2 leading to p53 stabilization, cell cycle arrest and reduction of cell proliferation. Conversely, silencing of TRIM8 reduces the capacity of p53 to activate genes involved in cell-cycle arrest and DNA-repair.

Other TRIM proteins have been shown involved in p53 regulation, for instance TRIM24/TIF1α and TRIM19/PML which, respectively, target p53 for degradation (TRIM24 deletion in human breast cancers leads to p53-dependent apoptosis, suggesting that TRIM24 could constitute a potential therapeutic target in breast cancer that expresses wild-type p53) or facilitate p53-Thr18 phosphorylation in response to DNA-damage.[91-93]

TRIM28 interacts and co-operates with MDM2 to promote p53 ubiquitylation and degradation;[94] MAGE proteins, which are upregulated in many cancers, were reported to function as cofactors in TRIM28-mediated p53 suppression.[95]

TRIM29/ATDC (ataxia telangiectasia group D-complementing) binds to p53, exporting it out of the nucleus and thus blocking p53-mediated transcription.[96] This interaction is tightly regulated by TRIM29 acetylation.[96] Histone deacetylase 9 deacetylates TRIM29, preventing its association with p53, and inhibiting its cell growth-promoting activity.[97] TRIM29 can also suppress UV-induced apoptosis in HCT116 cells through inhibition of p53acetylation. Thus,TRIM29 might be involved in DNA-damage response and act as an oncogene promoting tumour growth.[98]

Increased levels of **TRIM13**, upon ionizing radiation, can induce the ubiquitylation and degradation of MDM2 with consequent p53 protein stabilization and induction of apoptosis,[99] suggesting that TRIM13 functions as a tumour suppressor.

Finally,**TRIM22** expression is also found increased in response to p53; ectopic expression of TRIM22 in U-937 cells resulted in reduced clonogenic growth, while TRIM22 mRNA levels correlated with ATRA-induced differentiation, suggesting that TRIM22 might be involved in the differentiation of leukemic cells.[100]

CONCLUSION

In summary, TRIM proteins participate in several mechanisms important for the maintenance of cellular homeostasis but acquire oncogenic potential when altered. Some members of this family can be present as fusion proteins due to chromosomal re-arrangements; these chimera proteins have a dominant negative effect on both wild type partners, interfering with their physiological functions. Conversely, other TRIMs, although not involved in genetic aberrations, are linked to oncogenesis either because they alter the ubiquitination pathways of key cell-cycle proteins or because they lose their tumour suppression function. Moreover, a few members of the TRIM family have a dual role in tumour suppression or tumour promotion by modulating p53 activation.

Thus, further understanding of TRIM protein role in tumorigenesis, in particular their involvement in ubiquitination and SUMOylation, may provide novel targets for effective cancer therapies of the future.

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CHAPTER 7

TRIM PROTEINS AND THE INNATE IMMUNE RESPONSE TO VIRUSES

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Abstract: Many TRIM proteins are up-regulated by interferons, suggesting that they might be involved in the innate immune response against viruses. Indeed, some members of the family have been shown to be either regulators of the interferon pathways or to be directly involved in the restriction of viruses. While the mechanisms of actions are varied, the modular organization of these proteins seems to be important for their activities, many of which are linked to the ubiquitination/proteasomal degradation system. The different domains enable the TRIM proteins to interact with either viral components or signaling molecules in the interferon induction pathways.

INTRODUCTION

A number of possible functions have been ascribed to members of the TRIM family of proteins but little certainty exists about their true physiological roles. One possibility attracting increasing attention is an involvement in the innate immune system designed to provide first line defense against pathogen invasion.[1] The innate immune response to viral infection is mediated primarily by interferons, cytokines that induce an anti-viral state through mechanisms involving complex signal cascades.[2] There is increasing evidence that many members of the TRIM family of proteins are involved either in the induction of these pathways or as effectors of the anti-viral state. This evidence includes (a) studies showing that the expression levels of *TRIM* genes are up regulated upon viral infection in an interferon-dependent manner,[3-5] (b) genetic analyses indicating that at least 2 *TRIM* genes, *TRIM5* and *TRIM22*, have evolved under positive selection, which is a hallmark of genes that provide resistance to infections[6,7] and (c) studies of individual TRIM proteins

TRIM/RBCC Proteins, edited by Germana Meroni.

demonstrating direct interactions with different viruses. These include TRIMs 1,[8,9] 5,[8,10-13] 19,[14] 22,[4,15] 25,[16] 28,[17] 32,[18] and 34.[9]

Some of the proteins now referred to as TRIM proteins were first isolated in screens for interferon inducible proteins.[15,19] A number were independently shown by transcriptional profiling to be up-regulated following influenza virus infection of lung epithelial cells.[3] Most recently, close correspondence of TRIM expression was shown following influenza infection of mouse macrophages and treatment of human macrophages with LPS and interferon-γ[5]. In almost every case TRIM up-regulation required receptor mediated interferon signaling. However, it should be noted that other *TRIM* genes were constitutively expressed in dendritic cells; these do not appear to be interferon regulated.[5]

Antagonistic interactions between host defense systems and virus lead to rapid fixation of genetic mutations that alter the interaction. Such interactions can be detected by determining the ratio of coding sequence changes leading to amino acid change (dN) with those that do not (dS). A ratio greater than one provides evidence for an antagonistic interaction. Studies of *TRIM5* and *TRIM22* provide evidence for multiple episodes of positive selection involving protein domains likely important for virus binding as indicated by functional studies.[6,7]

In the following sections, the anti-viral activities of TRIM 25, 22, 28 and 5 will be reviewed in greater detail. Other TRIMs that have been implicated in the anti-viral response but whose mechanisms are less clearly defined will then be briefly described. This will be followed by a discussion of TRIM 21 and 30, which negatively regulate the response to virus infection. Finally, we will consider how the known properties of TRIM proteins might be utilized as part of an anti-viral response.

TRIM25 AND INTERFERON β INDUCTION

Interferons, which are cytokines secreted during viral infections, form a vital part of the innate immune response by inducing genes that inhibit viruses.[2] The interferons themselves are induced when viral components, e.g., ds-RNA, are detected by the infected cell. Different parts of the virus are recognized by different cellular receptors depending on the route of entry into the cell. Through a cascade of signaling molecules, interferon production is then induced (Fig. 1).

Viral RNA in the cytoplasm is detected by the RNA helicases RIG-I (retinoic acid inducible gene I) and mda-5 (melanoma differentiation associated gene 5),[20-23] which contain two caspase recruitment domains (2CARD) in the N-terminal region and potential ATP-dependent RNA helicase activities in the C-terminal region.[24,25] RIG-I is ubiquitinated in its 2CARD by TRIM25, which interacts with RIG-I via its B30.2 domain.[16] The addition of ubiquitin to RIG-I requires the RING motif of TRIM25 and increases upon infection by Sendai virus, an RNA virus. Ubiquitination of RIG-I occurs at residue K172 of its 2CARD and is crucial for function as it influences the binding to MAVS,[16] the next molecule in the signaling pathway for interferon β induction (Fig. 1).[26-29] In the mutant K172R where the target lysine for ubiquitin is changed to arginine, ubiquitination is reduced with an accompanying decrease in ability to induce interferon β. TRIM25 null cells have an increase susceptibility to VSVG and Newcastle disease virus while over-expression of TRIM25 leads to increased resistance to infection

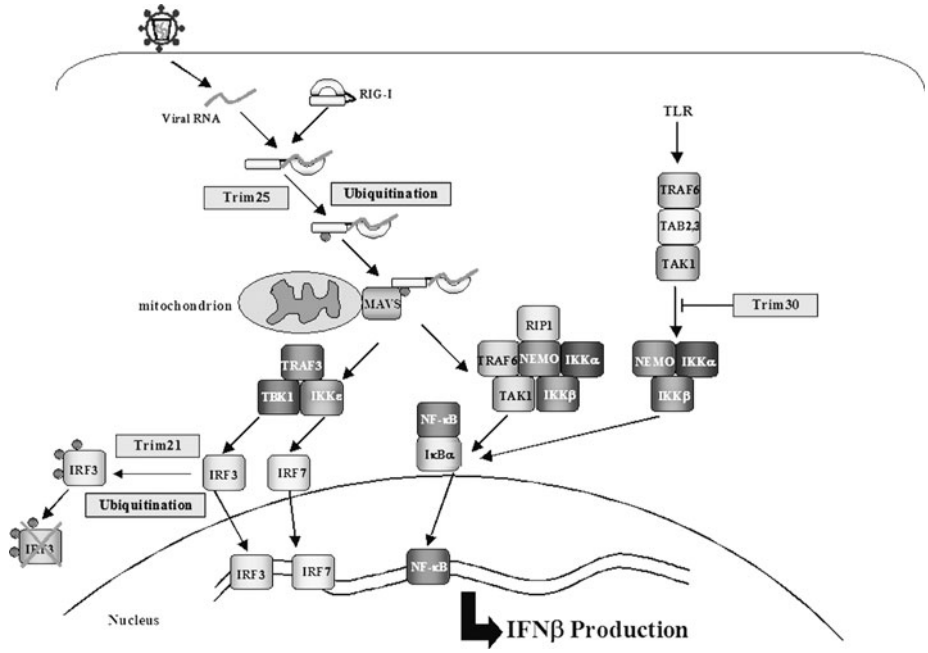

Figure 1. Regulation of interferon β by TRIMs21, 25 and 30. Interferon β production is induced upon viral infection by a cascade of signaling molecules. TRIM25 ubiquitinates RIG-I, enabling it to interact with MAVS, the next molecule in the pathway. TRIM21 negatively regulates interferon β by targeting its transcription factor, IRF3, for proteasomal degradation through ubiquitination, while Trim30 negatively regulates NF-κB activtion by targeting TAB2 and TAB3.

with VSVG. These results confirm the vital anti-viral role of TRIM25 in interferon β induction during viral infection via the RIG-I signal transduction pathway.

TRIM22 AND INHIBITION OF HIV-1 PRODUCTION

HIV-1 replication is blocked by interferons in many cell types and numerous studies have been initiated to isolate the effector genes.[30] TRIM22 was originally isolated from a cDNA library of human lymphoblastoid Duadi cells that had been treated with α/β interferon.[15] It is highly upregulated upon interferon stimulation,[4,15,31] with expression levels increasing by at least 86 fold in a recent study.[4] Over-expression of TRIM22 inhibits HIV-1 replication in several cell lines, including monocyte-derived macrophages, HOS, U2OS, 143B and HeLa cells.[4,31]

Initial studies where TRIM22 was cotransfected with a luciferase gene that was under the control of the HIV-1 LTR suggested that the block occurred at the level of transcription from the viral LTR (Fig. 2).[15] Indeed, this seems to occur in many cell lines tested. Unlike TRIM5, TRIM22 does not affect the early steps of HIV-1 infection, as measured by VSVG-pseudoyped HIV-1 vectors. However, in a recent report, it was found to affect release of viral particles, even in cell lines where the effect on transcription is minimal (Fig. 2).[4]

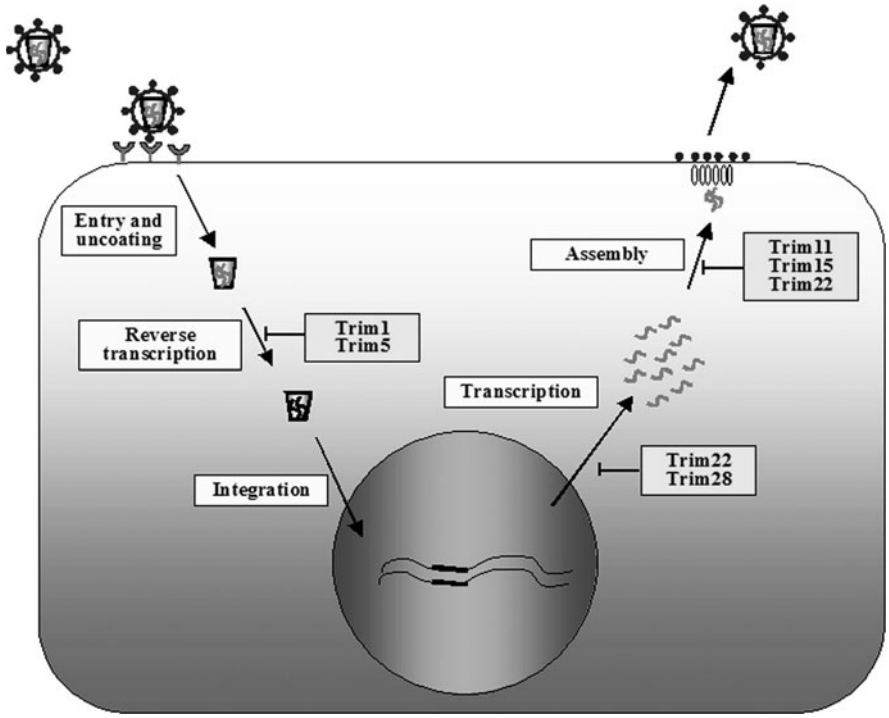

Figure 2. Retroviral life-cycle showing the action of restricting TRIM proteins. TRIMs 1 and 5 act on reverse transcription while TRIMs 22 and 28 represses viral transcription. TRIMs 11, 15 and 22 affect viral assembly resulting in reduced viral production.

Knock down of TRIM22 with shRNAs increased HIV-1 particle production in the presence of interferon β, suggesting that TRIM22 could be a downstream effector of the interferon β response to HIV-1 infection.[4] The block by TRIM22 seems to be specific for HIV-1, as it had no effect on budding of MLV or other lentiviruses such as EIAV. This was consistent with the finding that TRIM22 interacted with HIV-1 Gag but not the Gag from MLV or EIAV.[4] Fluorescence microscopy with GFP tagged Gag revealed that TRIM22 reduced the trafficking of HIV-1 Gag to the plasma membrane. Myristic acid is added to HIV-1 Gag posttranslationally to target it to the host cell membranes.[32-34] However, myristoylation is not affected by TRIM22.[4] The decrease in trafficking was also not due to the increased turnover of Gag. Although the precise mechanism of the block remains to be elucidated, it seemed to be dependent on the presence of an intact RING domain in TRIM22.[4]

TRIM28 AND RETROVIRAL SILENCING

The eukaryotic genome is littered with copies of retroelements, an indication that is it under constant attack by foreign genetic elements.[35] Retroviral expression is silenced in embryonic murine cells, a mechanism that probably evolved to protect the germ line from invasion by endogenous retroviruses and retrotransposons.[36,37] This effect is

dependent on the repressor binding site (RBS) downstream of the retroviral promoter.[36,38,39] A point mutation in this site can relieve this block, suggesting that it could be bound by a corepressor complex. The complex was purified by sequential chromatography and mass spectrometry revealed the presence of TRIM28.[17] TRIM28 seems to be an integral component of the repression complex as its levels corresponded to the ability to bind RBS (Fig. 2). This activity decreases in embryonic cells that were induced to differentiate, which was accompanied by a reduction in TRIM28 expression and a loss of RBS activity, together with a lack of ability to restrict MLV. The involvement of TRIM28 was confirmed by the observation that MLV silencing was also attenuated by RNAi to knock down TRIM28 in embryonic cells. In addition, TRIM28 was shown to bind specifically to the RBS in vivo in repressing cells but not in nonrepressing cells that contained a point mutation in the RBS.[17]

TRIM28 itself lacks DNA-binding ability[17] and is probably recruited to the RBS by a KRAB-ZNF protein, which can recognize and bind to asymmetric DNA sequences that are the size of the RBS.[40] TRIM28 interacts with the KRAB-ZNF protein via its RBCC domain.[41,42] It in turn recruits other effector proteins, which include SETDB1 and CHD3,[43,44] both of which contain a SIM motif that is recognized by the sumoylated bromodomain of TRIM28.[45] Sumoylation of the bromodomain is an absolute requirement for TRIM28 mediated repression and is effected by the adjacent PHD domain on TRIM28, which acts as an E3 SUMO ligase that recruits ubc9.[45] In addition, TRIM28 can also bind and recruit HP1, which recognizes the H3-K9 methyl mark.[45] The presence of the effector molecules in the vicinity of the RBS results in chromatin reorganization near the promoter region, producing a silent chromatin state.

TRIM5 AND RETROVIRAL RESTRICTION

TRIM5 restricts a wide range of retroviruses including MLV,[8,11-13] HIV-1,[10] HIV-2,[46] SIV,[11] EIAV[11,12] and foamy viruses.[47] Restriction occurs post entry into the host cell as viruses with identical envelope proteins differ in susceptibility to the factor (Fig. 2). A late block to replication has also been described[48] but this remains a controversial claim.[49] In most cases, the process of reverse transcription is markedly reduced. Exceptions include the restriction of SIV by squirrel monkey TRIM5[46] and the block to foamy viruses,[47] where events after reverse transcription are affected. The target of TRIM5 has been identified as the capsid protein of the virus,[10,50-52] in particular, residues that are exposed on the exterior of the viral core.[8,53] The specificity determinants on TRIM5 lie in the B30.2 domain,[6,54-56] which is under positive selection,[6] a hallmark of genes involved in protection against pathogens. Residues in the three variable regions of the B30.2 domain all contribute the recognition of the viral capsid.[57] Binding of the restricting TRIM5 to HIV-1 capsid (CA) protein has been documented.[58] An alternative binding domain has also arisen twice, independently, in primates, where the B30.2 domain has been replaced with cyclophilin A (CypA), another HIV-1 CA binding moiety.[59] This occurred through the insertion of CypA in the TRIM5 gene, between exons 7 and 8 in the owl monkey[60,61] and after exon 8 in macaque monkeys,[62-66] resulting in a product called TRIM5CypA where the RBCC is fused to CypA.

While there is general agreement about the contribution of the B30.2 domain to recognizing and binding retroviral CA, the function of the RBCC in restriction is less clear. The coiled coil is essential for activity as it enables the protein to multimerize, a process that could increase the avidity of the TRIM5 binding to the viral CA.[67-69]

Mutants that lack a coiled coil fail to mulimerize[67,70] and are unable to bind viral CA[58] or restrict.[69-71] The contributions of the RING and B-BOX are less well defined. While the RING and B-BOX motifs are clearly required for activity in TRIM5,[10,70,71] deletion of either of these motifs from TRIM5CypA, do not abolish restriction when the mutants were expressed from a retroviral LTR.[69,72] The E3 ligase activity of the RING motif has been reported and TRIM5 is known to self-ubiquitinate.[73-75] Although it has been tempting to invoke mechanisms where ubiquitination of either viral CA by TRIM5, or binding of ubiquitinated TRIM5 to a viral CA targets it for proteasomal degradation, inhibition of proteasomal function does not abolish restriction of retroviral replication.[76,77] However, it relieves the block in reverse transcription, indicating that the virus is inhibited at a later stage in the presence of the proteasome inhibitor.[77,78] Hence, there is probably more than one mechanism of restriction of retroviruses by TRIM5, one of which is dependent on the integrity of the proteasomal machinery and the other occurring as a result of virus sequestration in TRIM5 cytoplasmic bodies.[79]

OTHER TRIMs ASSOCIATED WITH VIRAL RESISTANCE

Other TRIM proteins have been associated with viral infections. However, the biological implications or mechanism of actions are less clear. TRIM32 was found to interact with the activation domain of lentiviral Tat proteins but had no effect on Tat function.[18] TRIM19, otherwise known as PML, has been much studied.[14] It may contribute to defence against a wide range of viruses including HSV-1,[80] Ebola virus,[81] LCMV,[82-85] Lassa virus,[84] influenza,[86] VSV,[85,86] Rabies,[87,88] HIV-1[89] and HFV.[90] How TRIM19 interferes with so many viruses with widely differing replication strategies is still unclear. It is likely that a number of different activities are involved. For example, TRIM19 was reported to act against HFV by repressing viral transcription through the binding and sequestering of the viral transactivator, tas, an interaction involving the RING motif.[90] By contrast, antiviral activities against VSV and influenza seemed to be dependent on the coiled coil of TRIM19.[86] Some viruses, e.g., HSV-1, encode proteins that degrade TRIM19, adding weight to the idea that TRIM19 has anti-viral activity.[91]

More recently, TRIM1 and TRIM34 were reported to have weak activities against N-MLV[8] and lentiviruses[9] respectively (Fig. 2). In TRIM1, the specificity was mapped to the B30.2 domain.[56] A study investigating the antiretroviral effects of TRIM proteins identified TRIM11 and TRIM15 as potential retroviral restriction factors in human cells (Fig. 2).[92] While the E3 ligase function of TRIM11 seemed to be crucial for its antiretroviral function, TRIM15 was found to interact with the MLV Gag precursor through its B-box.

TRIM PROTEINS THAT NEGATIVELY REGULATE THE INNATE IMMUNE RESPONSE

Following the response to viral infections, the genes that are induced to produce the anti-viral state have to be down regulated, as their sustained over-expression could lead to pathological conditions that are seen in autoimumune diseases.[93] IFNβ production can be turned off by the ubiquitination and proteasomal degradation of IRF3, the transcription factor that controls its expression.[94-96] This process has recently been found to involve TRIM21, which inhibited IFNβ expression in a dose-dependent manner (Fig. 1).[97] The

RING motif and SPRY domain were both important for its function as deletion mutants lacked the inhibitory activity. IRF3 interacted with TRIM21 in pull-down experiments and the binding requires the presence of an intact SPRY domain.[97] TRIM21 is able to increase the ubiquitination of IRF3 when overexpressed in cells, leading to a reduction in the levels of IRF3. However, treatment of the cells with MG132, which inhibits the proteasome, rescued IRF from degradation and restored IFNβ promoter activity, showing that TRIM21 targets IRF3 for proteasomal degradation by promoting its ubiquitination (Fig. 1).[97] In cells where TRIM21 was knocked down by shRNA, there was an acculmulation of IRF3, accompanied by a higher level of IFNβ gene expression and an increase in IFNβ-induced chemokine RANTES, thus confirming the role of TRIM21 as negative regulator of IFNβ.[97]

In mice, TRIM30 provides a similar function by negatively regulating Toll-like receptor signaling (Fig. 1).[98] Toll-like receptors detect the presence of invading microbes and initiate a cascade of signaling that results in the activation of NF-κB, which leads to the induction of genes involved in both the innate and adaptive immunity.[99] TRIM30 interacts with TAK1, TAB2 and TAB3, which are molecules in the signaling pathway, resulting in the degradation of TAB2 and TAB3 (Fig. 1).[98] This leads to a lack of autoubiquitination of TRAF6 and reduces phosphorylation of IκBα, two processes which are required for the activation of NF-κB.[100] Hence, TRIM30 interferes with NF-κB activation, resulting in the inhibition of cytokines like IL-6 and TNF. The RING motif is required for its activity as RING mutants failed to degrade TAB2 and cannot block NF-κB activation.[98] TRIM30 expression is induced by NF-κB, suggesting that it is acting through a negative feedback mechanism.[98] TRIM30 is absent in humans and it will be interesting to find the gene that carries out it function.

DISCUSSION AND CONCLUSION

The TRIM proteins associated with viral infections seem to have different mechanisms of actions, responding to a range of viruses at various stages of their life cycles. However, they are united by a few common themes.

In many cases expression of the TRIM genes is closely associated with activation of the interferon system. TRIMs 21, 25 and 30 regulate the induction of cytokines/ interferon β[16,97,98] whereas TRIM5 and TRIM22, among many others, are up- regulated by interferons.[4,5,15,31,101,102] Hence, they may act as regulators or downstream effectors of the anti-viral interferon pathway.

Binding to the target molecule is another important feature of the TRIM proteins. Different TRIM domains can contribute to the binding activity (Fig. 3). The C-terminal region of the protein, which may carry at least nine different structural motifs,[1] is often involved. Thus, TRIM5,[54-56] TRIM25[16] and TRIM21[97] attach to retroviral CA, RIG-I and IRF3 respectively using the B30.2 domain, while TRIM28 interacts with a variety of molecules using its RBCC, PHD and bromodomain.[17] Binding of TRIM15 to the MLV Gag precursor is mediated by the B-Box[92] while the interaction between TRIM22 and HIV-1 Gag is less well defined.[4] In the case of TRIM5, binding to the target retroviral CA is also apparently dependent on the multimerization state of the molecule; the interaction is abolished in mutants where the coiled coil that provides this function is deleted (Fig. 3).[58] Multimerization could increase avidity, permitting functional interactions even where initial binding events are relatively weak.[69] This might facilitate recognition of multiple targets and generate a broadly acting defence system. Another role for the coiled coil

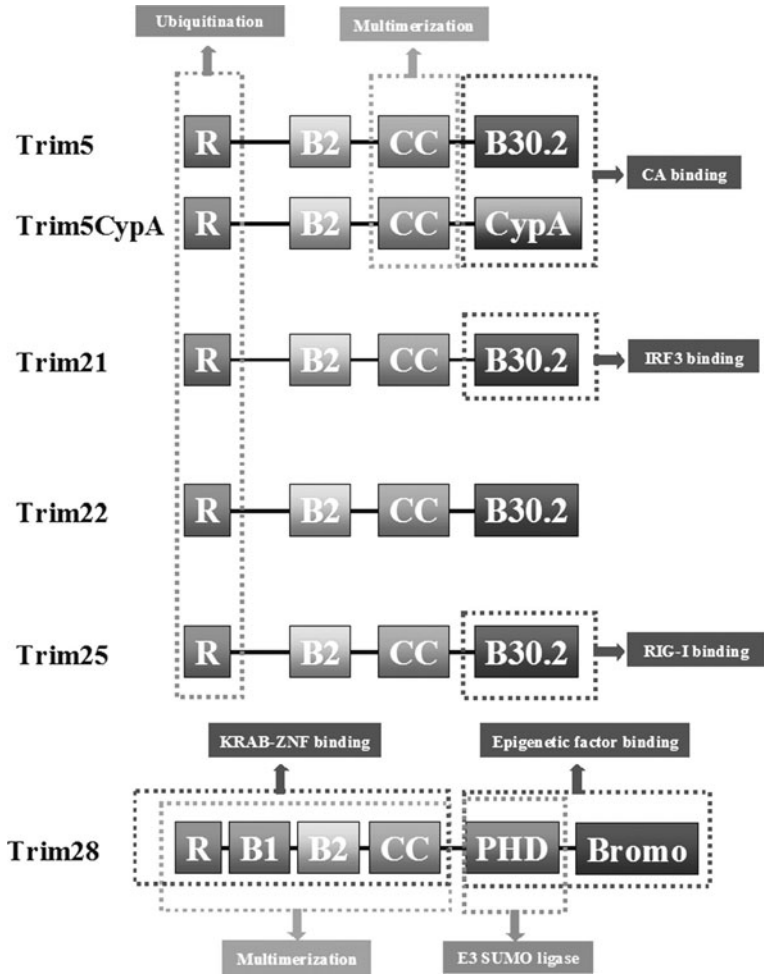

Figure 3. Domains in TRIM proteins involved in anti-viral activities. A schematic representation of the modular organization of TRIM proteins is shown with interaction domains boxed in darkest gray (blue), while those involved in ubiquitination and multimerization are boxed in medium grays (red and green) respectively. A color figure is available online at www.landesbiosciene.com/curie.

domain might be to facilitate the formation of cytoplasmic bodies in which virus could be sequestered.[79]

Alternatively, binding might represent a means of bringing an effector function into proximity to its target. One example is the case of TRIM28 where the presence of E3 sumo ligase activity in the PHD domain results in the sumoylation of the bromodomain, a requirement for silencing, by recruiting ubc9.[17] More commonly, E3 ubiquitin ligase activity and/or the related proteasomal degradation system are often associated with the anti-viral activity (Fig. 3). In TRIM11 and 22, the evidence is circumstantial, where lack of activity results from the removal of the RING motif.[4,92] More direct involvement of the E3 ligase activity is observed in TRIM25, where the RING motif is required for the

ubiquitination of the target, RIG-I,[16] in TRIM21, which promotes the ubiquitination and subsequent proteasomal degradation of IRF3,[97] and also in TRIM5,[77] though the precise role of ubiquitination is less clear (Fig. 3). While there is agreement that the RING motif of TRIM5 possesses E3 ligase activity and that TRIM5 is ubiquitinated, how this contributes to restriction remains unknown.[73-75] Retroviral CA lacking lysine residues that are the targets for ubiquitin conjugation are still restricted, suggesting that the target for the E3 ligase activity might not be the retroviral CA.[47] Indeed, recent evidence seem to suggest that TRIM5 is targeted for proteasomal degradation in the presence of the restricted virus, implying that a possible mechanism could involve the targeting of the TRIM5/viral complex to the proteasome through the ubiquitination of TRIM5, rather than the viral target.[103]

It is therefore tempting to speculate that the anti-viral activity of TRIM proteins can be explained in terms of the modular organisation of these proteins. It will be interesting to correlate the structural organization of the TRIM proteins with their antiviral activities as the mechanisms of more members of the family are unravelled.

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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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CHAPTER 9

MuRFs

Specialized Members of the TRIM/RBCC Family with Roles in the Regulation of the Trophic State of Muscle and Its Metabolism

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Abstract: MuRFs, brief for muscle specific RING finger proteins, correspond to a subfamily of the TRIM/RBCC protein family. Here, we review recent progress on the structural biology of MuRF1, the MuRF family member being most clearly associated with muscle diseases. The emerging understanding of the structural biology of MuRFs and their interaction with their numerous myocellular proteins, at least in part representing ubiquitination targets for MuRFs, is likely to provide future rationales to modulate their activity, thus affecting their roles in muscle disease progression.

INTRODUCTION: SKELETAL MUSCLE PLASTICITY AND MuRFs

Skeletal muscles are formed by an intriguingly regular assembly of protein filaments that, organized into contractile units or sarcomeres, exhibit a high crystalline-like order. In mature muscle, these myofilaments develop force along their longitudinal axis by the synergistic action of millions of actin and myosin interactions that result in a sliding mechanism of contraction (for review, see Clark et al, 2002).[1] Although the stroke of each individual actin-myosin interaction only amounts to a few picoN force and nanometer displacement, the global result is voluntary mobility at the organismic level. In addition to the scientific fascination of the dynamics and synchronization of its molecular motors, the skeletal muscle system is central in medicine because multiple chronic disease states in humans carry associated the perturbation of its trophic state (for review, see Potthoff et al,

TRIM/RBCC Proteins, edited by Germana Meroni.

2007).[2] For example, skeletal muscle trophicity is impaired in systemic diseases such as sepsis, diabetes, chronic obstructive pulmonary disease, heart failure and cancer. While the above disorders develop into distinct clinical conditions, their associated dysfunctions of the skeletal muscle system are similar, namely impaired contractility, reduced protein synthesis and enhanced protein degradation.[3,4] Secondary myopathies in chronic disorders are a major cause of morbidity due to the loss of muscle strength and/or mass that delays the remobilization of patients. Moreover, these associated myopathies often deteriorate further the underlying metabolic disorders that caused the primary disease in the first place, thus initiating a vicious cycle that results in a metabolic myopathy syndrome.

The mechanisms that link patient immobilization or metabolic disorders with the loss of muscle strength are varied, comprising processes that range from enhanced proteolysis (and thereby loss of contractile proteins)[5,6] to disruption of contractile functions without the overt loss of muscle protein or ultrastructural alterations. When protein degradation occurs, this is mediated by distinct proteolytic systems, namely the acidic-lysosomal, the calcium-dependent, or the ATP-ubiquitin dependent pathways.[7,8] Yet, the exact molecular mechanisms that enhance proteolysis and down-regulate protein synthesis in muscle loss remain unclear. In this regard, the identification of several so-called atrogins, proteins that become induced in a skeletal muscle undergoing atrophy, has greatly advanced our understanding of catabolic events in muscle. Several members of the TRIM family have emerged as important players during muscle remodeling, in particular TRIM32 and TRIM63 (also referred to as MuRF1, see below). Mutations in *TRIM32* cause a Bardet-Biedl syndrome[9] and limb girdle muscular dystrophy Type 2H.[10,11] Functionally, TRIM32 has been implicated in the degradation of myosin, actin[12] and dysbinding.[13] In addition, TRIM32 activates specific set of micro-RNAs by its interaction with Argonaute-1.[14] For more details on TRIM32 and its emerging roles in neuromuscular diseases (Chapter 10 by Petrera et al).

Within the family of muscle-specific ubiquitin E3 ligases, MuRF1 and MAFbx are emerging as central factors in muscle plasticity. These have been shown to be robustly up-regulated by a variety of muscle atrophy inducing conditions (e.g., starvation, unloading, the action of TNF-α and the synthetic anti-inflammatory and immunosuppressant steroid dexamethasone)[15-17] (Fig. 1). Here, we focus on the TRIM family members MuRF1 (TRIM63) and the closely related MuRF2 and MuRF3 (TRIM55 and TRIM64, respectively) since recent studies have shed light on their functional roles,[17-20] their molecular targets[21] and structural characteristics.[15,22]

DISCOVERY AND CONSERVED FEATURES OF THE MuRF GENE FAMILY

The term MuRF (muscle-specific RING finger protein) was coined by Spencer et al,[23] when they discovered the first member of this family, now termed MuRF3. This protein is specific of striated muscle, associates with microtubules and becomes up-regulated during late differentiation of cultured C2C12 cells, thus acting as a developmental, differentiation-dependent regulator of microtubule stability. In separate studies, two other MuRF proteins were identified, MuRF1[24] and MuRF2,[25] that bind to the M-line region of the titin muscle filament. The three MuRFs are coded by different genes located in distinct loci of the mouse and human genomes. Despite, they share remarkably high levels of sequence similarity (Fig. 2a), constituting a distinct and closely related protein family.[24]

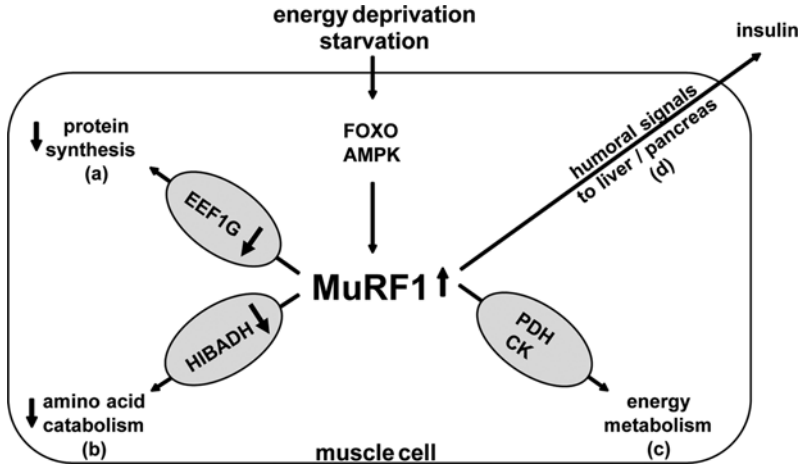

Figure 1. Model of the catabolic action of the ubiquitin ligase MuRF1. Its induction by starvation, muscle unloading, or catabolic hormones (e.g., via the AMP kinase and FOXO pathways) leads to ubiquitination of specific targets and their degradation. Combined, this leads to a coordinated downregulation of muscle protein, carbohydrate and energy metabolism. Figure modified from: Hirner S et al. J Mol Biol 2008; 379(4):666-677;[41] ©2008 Elsevier.

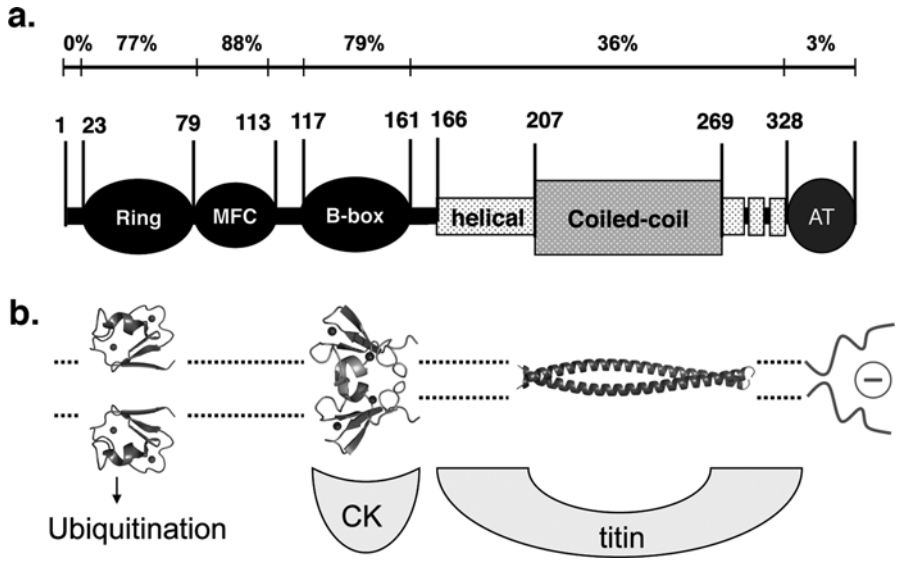

Figure 2. Domain composition of MuRF1 as family representative. N-terminally, two different start codons are present as a conserved feature in MuRF1 and MuRF2, raising the possibility of alternative initiation. The structure of the RING finger and CC domains have been predicted from sequence data, the structure of the B-box domain has been elucidated experimentally.[22] The acidic C-terminal tail can be expected to be intrinsically disordered. The degree of sequence identity (%) per domain is given in the upper bar. The proposed binding partners of each domain are shown in yellow (creatine kinase is indicated as CK, proposed to link MuRF1 to regulation of energy metabolism). A color version of this image is available at www.landesbioscience.com/curie.

MuRFs are multidomain proteins with a characteristic tripartite core structure known as TRIM (tripartite motif), which typically contains up to three sequentially conserved, N-terminal zinc-binding domains—commonly a RING finger, a B-box Type 1 (B1) and a B-box Type 2 (B2)—followed by a coiled coil fraction (CC).[26] The tripartite motif fold variation of MuRFs comprises a RING domain in N-terminal position, a unique MuRF-family specific motif (MFC), a single B2 box, a CC domain and a C-terminal acidic tail (Fig. 2a). RING, MFC and B2 domains share exceptionally high levels of sequence conservation within the MuRF family (approx. 81% seq.id.), while the CC fraction is reasonably well conserved (36% seq.id.). The most divergent parts in MuRFs are their N- and C-termini that differ in residue length and lack any detectable similarity (Fig. 2). In TRIMs, RING motifs are often associated with ubiquitination,[5] B-box domains appear involved in establishing heterologous protein interactions[21,27-30] and the CC domain is generally thought to govern self-association.[23,30-32] This functional demarcation is, however, not strict. CC domains in TRIMs also bind cellular targets, as in the ret finger protein (rfp) whose distal CC binds to promyelocytic leukemia protein (PML),[33] MuRF3 where it arbitrates its association with microtubules[23] and MuRF1 where it binds to titin.[15,24] Similarly, B-boxes and RING motifs can self-associate as, for example, the B2 of MuRF1.[22]

Given that MuRFs form a distinct subset within the TRIM and RING protein families, they have received different names depending on their classification as part of these groups. As members of the RING family, MuRF1, MuRF2 and MuRF3 have been termed RNF28, RNF29 and RNF30, respectively. While within the TRIM family they are named TRIM63, TRIM55 and TRIM54. The nomenclature is further complicated by different splice isoforms that have been described for MuRF2.[25,34] The differences in the biological roles of these splice isoforms, if any, are presently unknown.

THE MOLECULAR STRUCTURE OF MuRFs

MuRFs mediate a wide variety of protein-protein interactions, but it is unknown which specific domains in these proteins are responsible for the diverse contacts. To date, structural data have only become available for the B2 domain of MuRF1,[22] a representative of the CHC_2C_2HC B2 subtype and a member of the RING-like family. MuRF1 B2 folds into a β/α architecture, where a α-helix (α1) packs against a three-stranded anti-parallel β-sheet (β1-β3), supporting three loop regions (L1, L2, L3) involved in the binding of two zinc ions (Fig. 3a). Zinc coordination follows a classical "cross-brace" topology, where the binding site I (ZnI) is formed by the first and third protein ligand pairs and site II (ZnII) by the second and fourth ligand sets. MuRF1 B2 forms robust, high affinity dimers as shown by crystallographic, NMR and analytical ultracentrifugation data.[22] In these, the α-helix of one subunit docks into a concave depression formed by the α-helix and β-sheet of the other (Fig. 3b), a unique association pattern among RING-like folds. The dimer interface contains clusters of hydrophobic and polar interactions. Polar residues are contributed by the L1 loop, while hydrophobic groups are provided by strands-β1 and -β3 in one subunit and the amphipathic helix α1 in the other. The interacting groups are relatively well conserved in the B2 family, suggesting a potential for dimerization of other B2 boxes.

An analysis of the surface topography of MuRF1 B2 reveals that each protomer exhibits a long, shallow groove that encircles the C-terminal metal site ZnII formed by loops L2

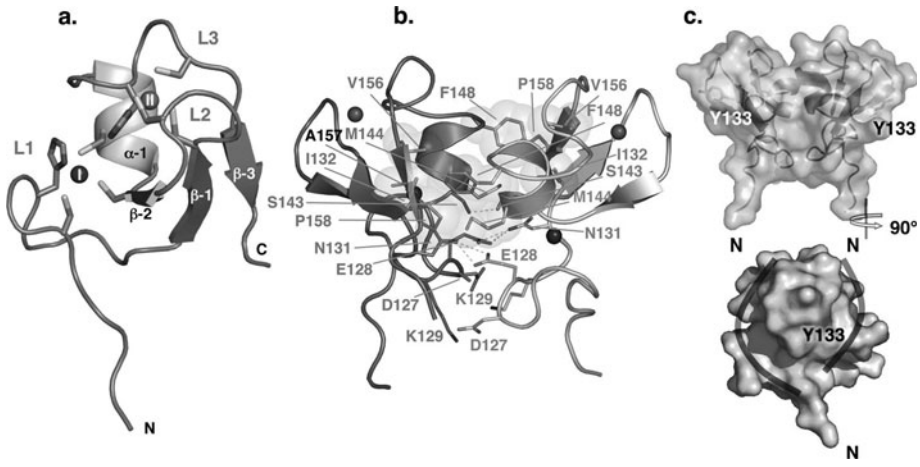

Figure 3. Crystal structure of MuRF1 B2. a) A ββα RING-like architecture binds two zinc ions at a mutual distance of ~10.7 Å; b) The MuRF1 B2 dimer. Interface contacts include hydrophobic groups (shown in green with their corresponding van der Waals spheres) and polar interactions (given as dashed lines). Residue F148 is central to the dimeric interaction, establishing 15 hydrophobic contacts; c) Surface representation. The groove encircling the metal site ZnII is indicated. The exposed side chains of hydrophobic residues are coloured green and the conserved aromatic Y133 is labelled. A color version of this image is available at www.landesbioscience.com/curie. Panels (a) and (b) are reproduced from Mrosek M et al. Biochemistry 2008; 47(40):10722-30,[22] with permission from the American Chemical Society.

and L3 (Fig. 3c). The groove hosts a hydrophobic cluster contributed by the surface of the β-sheet and including the highly conserved aromatic residue Y133. This groove does not resemble those defining ubiquitin ligase activity in clefts of RING domains that are shaped by long loop insertions absent in B2 motifs. Thus, MuRF1 B2 is unlikely to act in ubiquitination. Instead, its hydrophobic features, which are conserved in B2 boxes, can be expected to mediate the heterologous interactions established by this motif.

In MuRFs, the B2 box is followed by a large α-helical domain, which at least in part can be predicted to form a dimeric, parallel coiled-coil motif (Fig. 2b). These properties were identified through a structure prediction analysis based on the sequence of MuRF1 and experimentally supported by CD data as well as an analysis of its oligomeric state.[15] In addition to inducing self-association, the α-helical domain of MuRF1 mediates binding to titin,[15,35] an intra-sarcomeric filament system of vertebrate striated muscle that has elastic and signaling properties and is central to mechano-transduction. The MuRF1 binding site in titin has been identified as a conserved set of three Ig and FnIII modules (Ig^{A168}-Ig^{A169}-$FnIII^{A170}$, hereby A168-A170) directly preceding a kinase domain in the sarcomeric M-line. The crystal structure of A168-A170 has been elucidated and its interaction with MuRF1 characterized.[15] A168-A170 shows an extended, rigid architecture, characterized by a shallow surface groove that spans its full length and is defined by the long-range domain arrangement of the tandem (Fig. 4). The dimensions and geometry of this groove fit well a α-helix along its length, particularly when the helix exhibits an axial bending as that of dimeric coiled-coils. Thus, this groove has been predicted to accommodate the helical domain of MuRF1. Effectively, isothermal calorimetry data have shown that the helical fraction of MuRF1 is sufficient to secure a high affinity interaction ($K_d \approx 35$ nM) with A168-A170. Even though the interacting

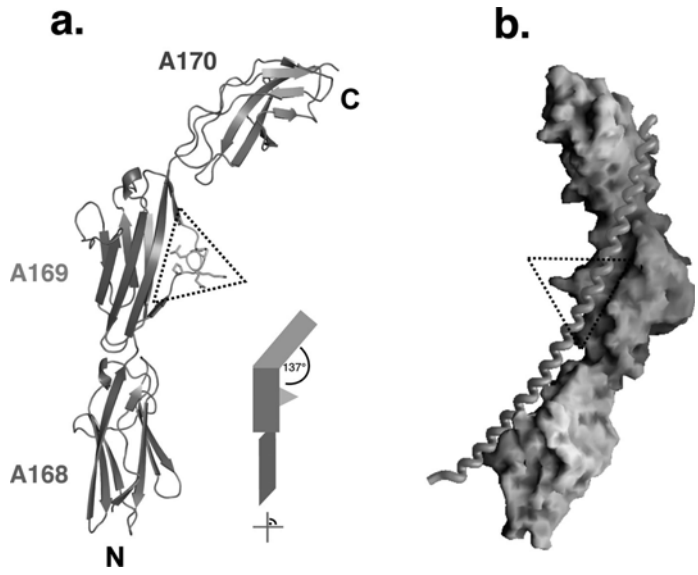

Figure 4. Crystal structure of A168-A170, MuRF1 binding locus in M-line titin. a) Ribbon representation. The insertion loop in Ig^{A169} is indicated by a dash line. The inset shows the molecular conformation schematically; b) Surface representation where dark gray/green emphasizes the local curvature. A ridge is present in the concave side of the molecule. The distinct loop of Ig^{A169} is marked; c. Modeling of a α-helical peptide in coiled-coil conformation onto the surface of A168-A170 according to shape complementarity. A color version of this image is available at www.landesbioscience.com/curie. Figure adapted with permission from Mrosek M et al. FASEB J 2007; 21(7):1383-92.[15]

surface in the MuRF1/titin complex appears extensive, pull-down assays and site-directed mutagenesis have shown that an insertion motif, $_{102}$KTLE$_{105}$, in Ig A169 at the middle point of the surface groove is a primary determinant of MuRF1 recognition (Fig. 4). This motif is part of a unique 9-residue loop insertion in A169 that occurs between β-strands A and A′ and is not present in any other domain of titin (composed of ~300 Ig-like domains). It is yet to be established whether A168-A170 or the B2 box of MuRF1 could serve as potential therapeutic targets against MuRF-1 mediated muscle atrophy.

PHYSIOLOGICAL ROLES AND CELLULAR TARGETS OF MuRF1

Transcriptional Induction and Upstream Regulation of MuRF1 Expression

A variety of signals, all associated with myopathy, induce MuRF1 transcription in skeletal muscles. Namely, diabetes, microgravity, denervation, immobilization, TNF-α and dexamethasone, among others.[3,5,36] A recent study that compared the effectiveness of inhibitors of MuRF1 induction by TNF-α suggested that the ERK MAP kinase pathway might be involved in turning on MuRF1.[37] Metabolic factors, such as starvation and, more specifically, amino acid deprivation, also induce MuRF1 transcription in skeletal muscle.[21] In summary, a large variety of stimuli linked to catabolic states have been shown to elevate

MuRF1 (and MAFbx) levels. This highlights the need to characterize the regulatory elements of the MuRF1 gene and to identify the specific components that mediate its induction.

Myocellular Targets of MuRF1

Depending on the system used (cultured tissue or live mice) and the mode of MuRF1 induction (e.g., creatine kinase [CK] driven expression, nonphysiological cytomegalo-virus driven overexpression; stress-induction caused by TNF-α or starvation) different preferential targets have been reported. When overexpressing MuRF1 in C2C12 cells, troponin-I and myosin heavy chain become dramatically down-regulated.[6,38] Consistent with the idea that myosin heavy chain is a conserved target recognized by the MuRF-family, Fielitz and coworkers observed a downregulation of myosin in MuRF1/MuRF2 double KO mice.[19] Therefore, both components of the thick and the thin filament were suggested to represent MuRF1 targets during atrophy response and myofibrillar degradation. In a more recent study, Goldberg and coworkers therefore systematically compared the degradation of troponin-I, myosin heavy chain and other thick filament components that occurs after denervation in wildtype mice and compared this to mice with conditionally induced loss of MuRF1 RING finger domain. Their results indicate that MuRF1 RING finger is required for degradation of thick filament but not thin filament components. Moreover, MuRF1 appears to be required for the degradation (directly or indirectly) of the regulatory myosin light chain-2 and of C-protein.[39] Consistent with this study, Carrier and coworkers observed the MuRF1-dependent ubiquitination of C-protein in myocardium ("cardiac C-protein, My-BP-C).[40] Future studies will need to clarify the functional consequences of thick-filament component ubiquitination, i.e., if this triggers degradation e.g., by recruiting protease machineries to the thick filament.

Within the myofibrillar protein apparatus, MuRF1 and MuRF2 also interact with the titin muscle filament.[24] Immuno-localization studies show that MuRF1 is primarily located in the sarcomeric M-line,[41] suggesting that the anchoring of MuRF1 onto M-line titin is a predominant event in vivo (Fig. 5). Although it is attractive to speculate that titin might be an ubiquitination target of MuRF1, which either labels it for degradation or modulates its biomechanical signaling properties, no evidence that support this view has been gathered to date. This included a study in MuRF1-KO mice that showed that the ubiquitination status of titin in vivo was not affected.[37] Similarly, the posttranslational modification of MuRF1 by titin kinase has not been identified so far. Thus, the cellular function of this interaction remains elusive. Alternatively, titin might sequester MuRF1 from the cytoplasm, preventing it from acting on its cellular targets in unchallenged muscles.

Technically, it is difficult to identify MuRF1 targets in skeletal muscle using KO models, because MuRF1 is not expressed at significant levels before challenge (making it perhaps difficult to distinguish primary and secondary MuRF1 mediated effects). Therefore, in a different study MuRF1 was over-expressed under the control of a skeletal muscle-specific CK promoter. In this mouse model, effects on metabolic enzymes dominated, including the down-regulation of pyruvate dehydrogenase that contributes to linking the glycolysis metabolic pathway to the citric acid cycle.[41] An important role for MuRF1 in muscle metabolism regulation was also raised by studies on amino-acid starved wildtype and MuRF1-KO mice: Amino acid deprivation suggested that HIBADH (an enzyme connecting the pentose shunt pathway with valine catabolism) is a MuRF1 target.[21] The absence of muscle atrophy and intactness of sarcomeres in this mouse model suggests that MuRF1 alone is not sufficient to induce muscle atrophy.[41]

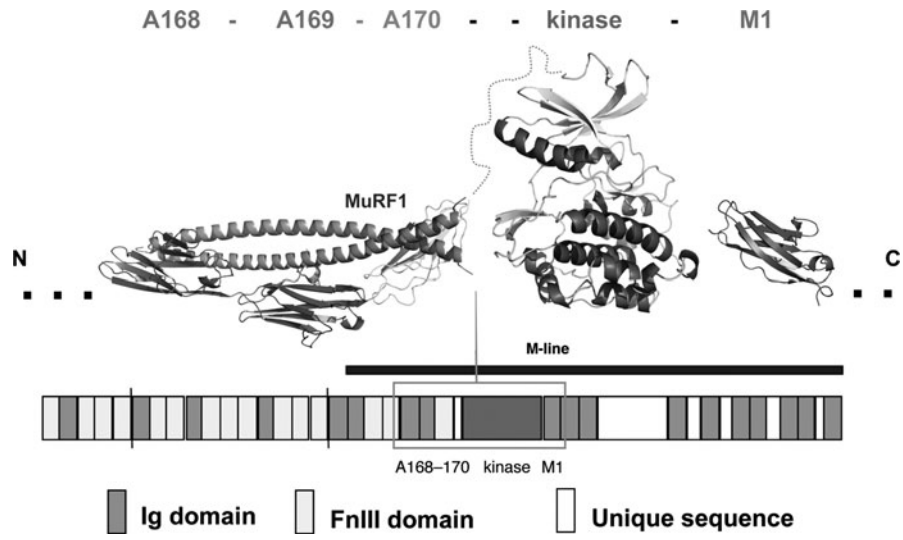

Figure 5. Molecular reconstruction of M-line titin. The structures of A168-A170, titin kinase and M1 have been elucidated independently using X-ray crystallography. The coiled-coil domain of MuRF1 has been modeled.

Taken together, the above data suggest that MuRF1-dependent poly-ubiquitination is an intricate process and using an appropriate assay is critically important for the identification of physiologically preferred targets. In particular, future studies will be required to unravel the relation between mechano-signalling, titin kinase and the role of MuRF1 in muscle remodeling. This ultimately may also lead to an understanding how the complex interactom of the previously identified MuRF1 ligands including components of the thick filament, isopeptidase T-3, GMEB-1, CARP, SQSTM1, EEF1G and creatine kinase are integrated into a coordinated physiologically meaningful stress signaling.

OUTLOOK: EMERGING CONCEPTS ON THE REGULATION OF THE TROPIC STATE OF MYOCYTES AND THEIR METABOLISM BY UBIQUITIN LIGASES

Several key issues remain unclear at present. For example, an important aspect is whether the several MuRF ubiquitin ligases target different signaling pathways or similar atrophy responses in a redundant fashion. Thus, there is a need for future integrated models that (i) account for the action of site-specific proteases (e.g., calpains) as potential initial rate-limiting proteolytic step;[23,42] (ii) consider that MAFbx and MuRF1 may target different myocellular pathways and that (iii) include the possibility of MuRFs degrading synergistically the same targets, as suggested by the action of MuRF1 and MuRF3 on myosin[43] or the degradation of CARP and EEF1G jointly by MuRF1 and MuRF2.[17]

A striking difference between MuRF1 and its close homologs MuRF2/3 is its tightly regulated expression at the transcriptional level. In contrast to MuRF2 and MuRF3, MuRF1 is strikingly induced by myopathic stimuli. However, in vitro studies show that MuRFs

interact with highly related proteins. All three MuRFs have been found to interact with FHL2.[17,19] In addition, a comparison of MuRF2 and MuRF1 identified a set of 35 proteins jointly recognized by them.[17] Thus, it is unclear how MuRF1 activities (virtually absent in an unchallenged skeletal muscle) are integrated with MuRF2/3 signaling (already present in skeletal muscles in basal unchallenged states). Also, the recent observation that overexpression of MuRF1 in mice does not cause myopathy or muscle wasting per se clearly points to the need of understanding what regulates MuRF1 target recognition and potential proteolysis. In these studies, most of the potential interacting partners of MuRF1 (as identified by yeast two-hybrid studies) were not affected by MuRF1 overexpression but a highly limited set of muscle proteins including pyruvate dehydrogenase alpha subunit (PDH-A) was targeted instead[41] (Fig. 6). This suggests that posttranscriptional modifications, e.g., phosphorylation,

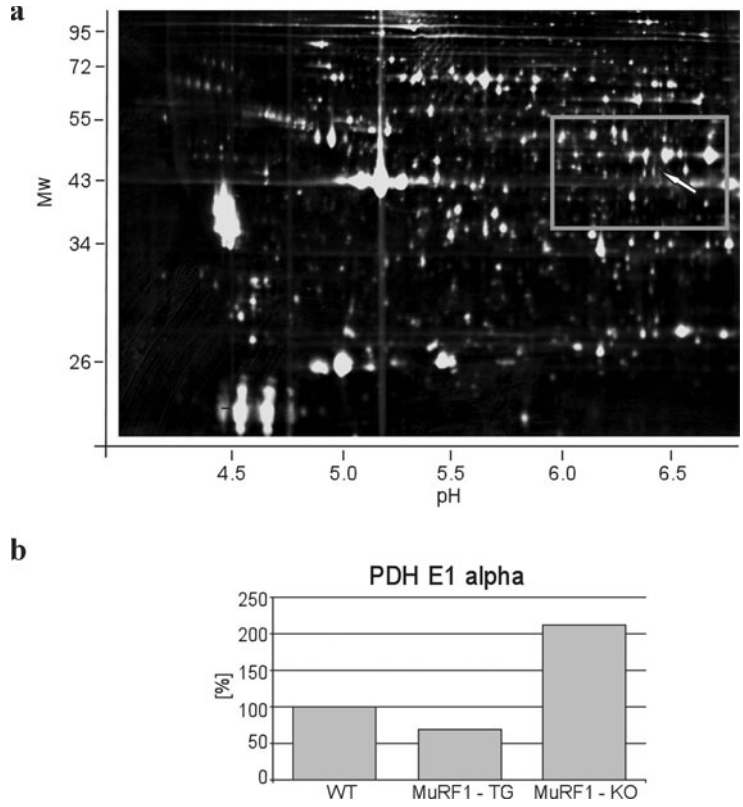

Figure 6. Proteome studies on MuRF1-TG mice in search of proteins degraded by MuRF1. MuRF1 was expressed in skeletal muscles under the control of a creatine-kinase promoter. The proteomes expressed in wildtype and MuRF1-TG mouse M. quadriceps muscles were extracted and labeled with the DIGE system with green or red dyes (GE Healthcare). a) Comparison by 2D gelelectrophoresis revealed highly similar proteomes, as indicated by the merge appearing in yellow (a). Only a very limited number of proteins showed shifts to green, consistent with their downregulation by MuRF1 overexpression (for an example, see arrow). b) Mass spectrometry and quantification of the green-shifted spot indicated down-regulation of pyruvate-dehydrogenase E1 alpha subunit in while other structural muscle proteins appeared unaltered. A color version of this image is available at www.landesbioscience.com/curie. Figure modified from: Hirner S et al. J Mol Biol 2008; 379(4):666-677;[41] ©2008 Elsevier.

might be required to activate MuRF1. The preferential in vivo targeting of PDH by MuRF1 also raise the possibility that the regulation of muscle metabolism, rather than muscle wasting, is an important physiological role of MuRF1.

CONCLUSION

MuRF1-3 are a muscle-specific group of three closely related ubiquitin ligases. Recent studies have indicated their crucial role for muscle cell trophicity, myocellular integrity and metabolism. Consistent with these diverse functions, the interactome of MuRFs compromises >40 different partners. Therefore, the highly complex physiological interplay of MuRFs in the myocell will need to be unraveled to realize the potential of MuRF activity modulation as a therapeutic principle for the treatment of muscle disorders. Knowledge of the structural biology of MuRFs together with their myocellular key interactants such as titin will be mandatory for this.

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CHAPTER 10

TRIM PROTEINS IN DEVELOPMENT

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Abstract: TRIM proteins play important roles in several patho-physiological processes. Their common activity within the ubiquitylation pathway makes them amenable to a number of diverse biological roles. Many of the TRIM genes are highly and sometimes specifically expressed during embryogenesis, it is therefore not surprising that several of them might be involved in developmental processes. Here, we primarily discuss the developmental implications of two subgroups of TRIM proteins that conserved domain composition and functions from their invertebrate ancestors. The two groups are: the TRIM-NHL proteins implicated in miRNA processing regulation and the TRIM-FN3 proteins involved in ventral midline development.

INTRODUCTION

As summarized in previous chapters, the proteins containing the tripartite motif (RING, B-box, Coiled-coil) and known as TRIM or RBCC are involved in several important biological and physiological processes.[1] Among them, many have important repercussions on development. Clear-cut separation between involvement in other processes and development is not always possible and it is well known, for example, that genes important for cell cycle regulation, and for that implicated in cancer, are often key players in embryonic processes. In this chapter, we will summarize experimental data and findings that corroborate the developmental involvement, considered as broad meaning, of some TRIM family members.

Basic cellular and developmental functions have been conserved throughout evolution especially in some of the subgroups that compose the TRIM family. Within each of these subgroups, the TRIM members are characterized by having similar

TRIM/RBCC Proteins, edited by Germana Meroni.

domain composition and by sharing similarity with members of the tripartite motif family present in invertebrate species.[2,3] Here, we will recapitulate the findings of mainly two subgroups of vertebrate and invertebrate TRIM proteins. The function of the mammalian developmental TRIM genes has been mostly deduced form either genetic diseases in which they are involved as causative genes or from the generation and characterization of transgenic mouse models. Often, though, the breakthrough on their function comes from the study of their related invertebrate TRIM gene mutants in classical experimental models, such as the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. It is clear from the analysis of the TRIM subgroups presented here that several gene functions, though maintained in their basic fundament, may have split, diversified, and sharpened during the evolution from invertebrates to mammals.

THE TRIM-NHL PROTEIN GROUP

The TRIM family members of the NHL subgroup are characterized by the presence of a series of NHL repeats downstream the tripartite motif.[2,3] In Figure 1, the phylogenetic relatedness among members of this subgroup in human (as representative of the mammalian clade), worm, and fruit fly, together with their domain composition is shown (Fig. 1). Besides the tripartite motif and the NHL repeats, some of the members also display an Immunoglobulin Filamin-type (IG_FLMN) domain (Fig. 1). The characterizing region of this group is however the NHL repeat domain that was named after its identification comparing the sequence of members of the TRIM-NHL family, *ncl-1*, HT2A (former TRIM32 name), *lin-41*.[4] The NHL repeats present similarity to the WD-40 repeats and form a β-propeller structure as well, as demonstrated by the solved structure of the fruit fly *brat* protein.[5] Experimental data and occurrence of natural mutations point to the NHL as the crucial domain to exert the role played by this subgroup of TRIM proteins.

The human members of this subgroup include TRIM2, 3, 32, 71 and two proteins presenting evolutionary relationship but with no obvious NHL region, TRIM45 and 56 (Fig. 1). Mammalian TRIM2 and TRIM3 are more similar to each other than to the remaining TRIM-NHL paralogues. They are both predominantly expressed in brain compartments and interact with Myosin V.[6,7] TRIM3 is involved in endosomal trafficking possibly in correlation with the recycling of receptors to the cell membrane.[8] Whether TRIM2 might also be implicated in endosomal transport and regulation is presently not known. However, data on *Trim2* gene-trap mouse line indicate a role in adult central nervous system since mutant brains present abnormal accumulation of neurofilaments and axon swelling ultimately leading to loss of cerebellar and retina neurons.[9]

TRIM32 has pleiotropic functions and is involved in two human hereditary pathological conditions. Limb Girdle Muscular Dystrophy Type 2H (LGMD2H) is caused by mutations in the NHL domain of TRIM32 whereas a specific amino acid substitution in the B-box 2 domain has been associated to Bardet-Biedl Type 11, a genetic disorder characterized by obesity, retinal dystrophy, and renal abnormalities.[10,11] Generation of a *Trim32* knock-out allele as well as of a mouse line carrying the most common single amino acid substitution occurring in human LGMD2H patients recapitulates the human myopathy phenotype and revealed a neurological component.[12,13] The latter is in full accord with a role recently ascribed to TRIM32 in the regulation of cortical neurogenesis.

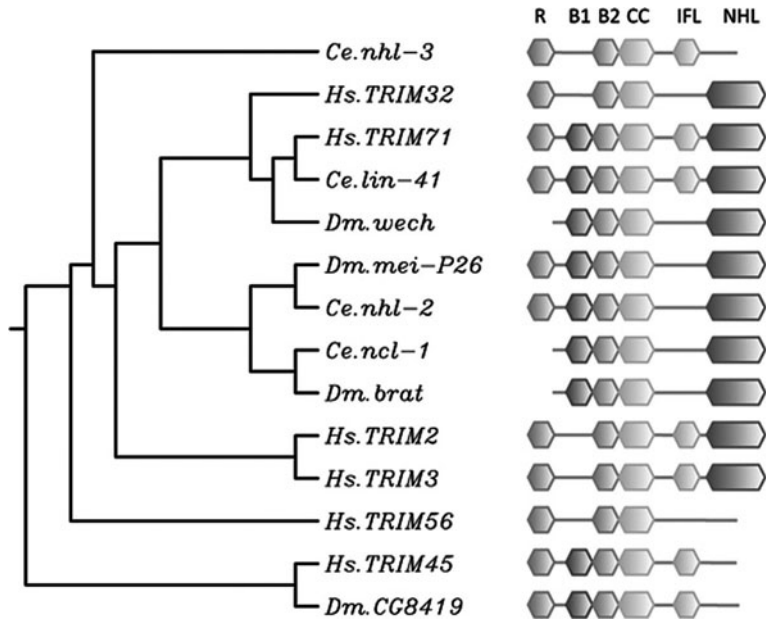

Figure 1. Phylogenetic tree showing the clade of TRIM-NHL proteins. *Hs*, *Homo sapiens*; *Ce*, *Caenorhabditis elegans*; *Dm*, *Drosophila melanogaster*. At the side of each member the domain composition is schematized. Related members not displaying the NHL repeats but sharing close relationship are also shown. R, RING domain; B1, B-box1 domain; B2, B-box2 domain; CC, Coiled-coil region; IFL, Immunoglobuline Filamin type domain (IG_FLMN); NHL, NHL repeat.

During specific neurogenetic phases, TRIM32 is asymmetrically distributed during neural progenitor cell division favoring cell cycle exit and neurogenesis of the cells which it resides in.[14] In addition, a role in carcinogenesis and apoptosis in keratinocytes, related to its ability to affect cell cycle, c-Myc, and other oncogenes, has been reported.[15,16] This wide range of functional involvements correlates with the large number of targets identified as assessed or putative substrates of TRIM32 ubiquitin ligase activity.[17-19]

TRIM32 close members are human TRIM71 and its orthologues in *C. elegans* and *D. melanogaster*, *lin-41* and *wech,* respectively (Fig. 1). *lin-41* belongs to the class of heterochronic genes involved in the *C. elegans* developmental timing pathway.[20] In the worm, its expression is timely and inversely regulated by one of the first discovered miRNA molecules, *let-7*, and is mainly detected in neurons, gonads and muscles.[20] Gain-of-function *lin-41* mutations lead to reiteration of the larval state whereas null mutations cause precocious expression of adult fates at larval stages, in particular during vulval development, oocyte production, and timing of cell cycle exit and differentiation of hypodermis blast cells.[20] Expression studies showed that the murine and human orthologues of *lin-41*, *mLin-41/Trim71* and *LIN-41/TRIM71*, are likewise temporally and dynamically regulated by the let-7 class of miRNAs in limb and in tail buds, muscle tissue, and eyes thus recapitulating the expression pattern observed in worm.[21,22] To confirm a conserved role in development, mouse *Lin-41* null mutants display an embryonic phenotype characterized by craniofacial abnormalities and neural tube closure defects although full characterization is not yet reported.[23] The *wech* gene in the fruit

fly is expressed in the nervous system and is involved in sensory organs development. Differently from what reported studying the orthologues in other species, loss-of-function *wech* mutants interfere with the correct link between integrins and the actin cytoskeleton at focal adhesion points leading to defective muscle-tendon attachment.[24]

A separate clade within the TRIM-NHL proteins includes two well-studied *drosophila* genes, *brat* and *mei-P26*, and apparently no vertebrate homologues (Fig. 1). These two genes are implicated in embryonic development, in male and female infertility, and in tumor formation, indeed *brat* stands for *bra*in *t*umor for its main role as tumor suppressor in the larval brain.[25-27] Both *brat* and *mei-P26* are implicated in regulation of cell proliferation and growth, cell cycle exit, and cell differentiation.[26] Although there is no close evolutionary relationship, *brat* and TRIM32 share asymmetric distribution in progenitor cells to support cell cycle exit and favor neuronal fate. Furthermore, both proteins control, among other cell cycle genes, the c-Myc oncogene.[14,28]

Despite the above somewhat different and species specific roles, there is growing evidence that TRIM-NHL proteins coordinate the transition between the steps of stem cell renewal, commitment and terminal differentiation through the regulation of miRNA activity in different systems (Fig. 2). TRIM-NHL family members act at

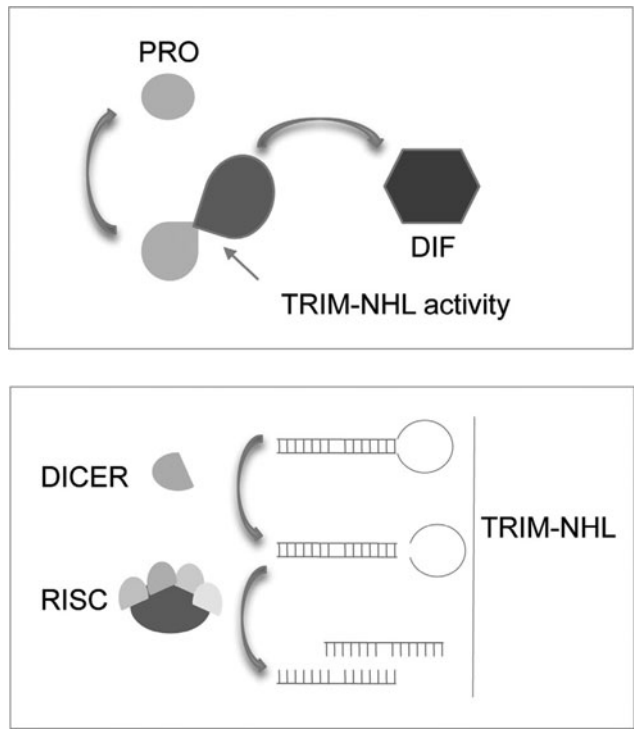

Figure 2. Schematic representations of general TRIM-NHL functional roles (see text). Upper panel, asymmetric distribution of some TRIM-NHL proteins during cell division of progenitor cells. The daughter cell with higher level of TRIM-NHL (blue/dark gray) will proceed towards differentiation (DIF) whereas the one with lower level (light blue/light gray) will repopulate the pool of progenitor cells (PRO). Bottom panel, simplified representation of the miRNA processing cascade and its component (DICER and the RISC complex) in which several TRIM-NHL members participate.

different levels of miRNA processing cascade and cross-regulation of these genes with the miRNA pathway has been observed.[29] Several TRIM-NHL proteins interact with components of the RISC core complex and localize to the P-bodies; *mei-P26*, *brat*, *nhl-2*, TRIM32, and mLin41 interact with Argonaute 1 (Ago 1 or the correspondent gene in the other species) and some of them also with Dicer and other Ago family members.[14,27,30,31] In particular, mLin41 has been shown to promote the degradation of Ago2. These interactions exert different effects, in some cases with opposite outcomes, on the expression, processing and regulation of miRNA products. *nhl-2* is able to enhance miRNA-mediated silencing but without increasing miRNA expression levels whereas *mei-P26* inversely impacts on global miRNA expression.[27,32] Thus, although phenotypes and physiological roles are not fully comparable due to lack of strict orthologous relationship and involvement in species specific processes, TRIM-NHL proteins exert roles that have been shared and diversified in the different species and among the different members.

THE TRIM-FN3 PROTEIN GROUP

The group of TRIM proteins containing the Fibronectin Type III (FN3) domain often also shares the PRY-SPRY C-terminal domain (Fig. 3). The PRY-SPRY domain is also present alone downstream the tripartite motif in the majority of the TRIM family members.[2,3] The TRIM-FN3 group comprises proteins that possess, downstream the Coiled-coil region, the COS domain that is responsible for their association with the microtubular cytoskeleton (see Chapter 4 by Batty et al). Three proteins, TRIM54, 55, and 63, although related, do not possess the FN3 and PRY-SPRY domain; they are implicated in muscular development and homeostasis and are described elsewhere (see Chapter 9 by Mayans and Labeit) (Fig. 3). Little is known for some members of this subgroup, e.g., TRIM42 and 46, whereas for *Xenopus trim36* a role upstream the Wnt/β-catenin pathway during early development has been demonstrated.[33] The frog *trim36* is involved in dorsal axis formation through the regulation of microtubule polymerization during cortical rotation, possibly determining proper distribution and stabilization of Wnt11 and/or β-catenin.[33]

Loss-of-function mutations in the *TRIM18/MID1* gene are responsible for a developmental genetic disorder, X-linked Opitz syndrome.[34] Patients with the disease present with abnormalities of the body midline structures, such as cleft of lip and palate, laryngo-tracheo-esophageal defects, cardiac abnormalities, hypospadias, and hypoplasia of cerebellar vermis.[35] These defects occur during embryonic development though the pathogenetic mechanisms are still unclear. A murine *Mid1* gene knock-out mouse line recapitulates the midline neurological defect showing anterior cerebellar hypoplasia caused by a defective antero-posterior definition of the dorsal midbrain-cerebellar boundary.[36] At the cellular level, TRIM18/MID1 binds alpha4 to ubiquitinate and regulate the level of its catalytic partner, protein phosphatase 2A (PP2A).[37] Recently, the TRIM18/MID1-PP2A complex has been shown to participate in mTORC1 signaling.[38,39] Opitz syndrome patient fibroblasts show decreased mTOR/Raptor complex formation and mTORC1 signaling displaying reduced protein translation and cell size.[38] Consistently, TRIM18/MID1 effect on protein translation by means of its association with specific motif on messenger RNAs has been recently shown.[40] The highly variable expressivity of the Opitz syndrome clinical spectrum

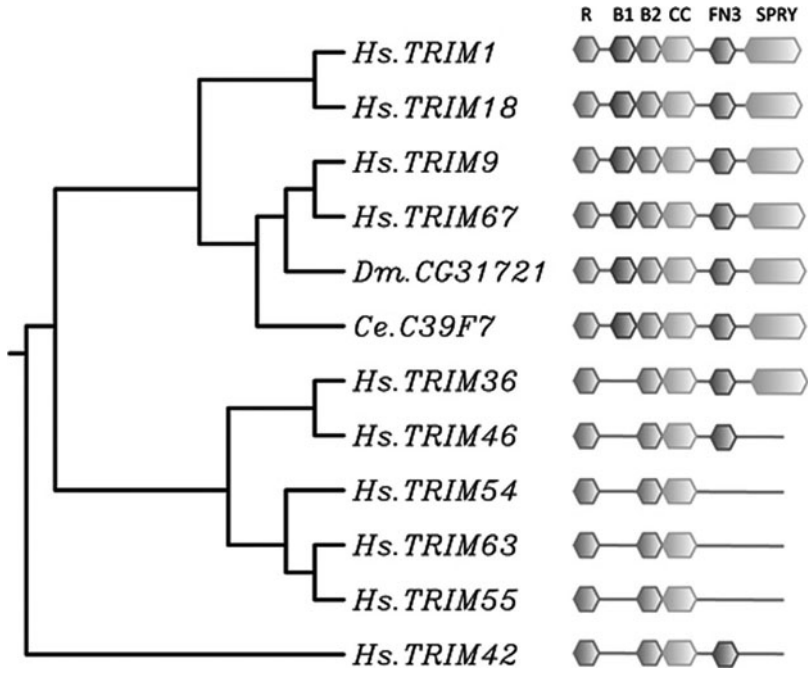

Figure 3. Phylogenetic tree showing the clade of TRIM-FN3 proteins. *Hs*, *Homo sapiens*; *Ce*, *Caenorhabditis elegans*; *Dm*, *Drosophila melanogaster*. Beside each member the domain composition is schematized. Related members not displaying the FN3 and PRY-SPRY domains but sharing close relationship are also shown. R, RING domain; B1, B-box1 domain; B2, B-box2 domain; CC, Coiled-coil region; FN3, fibronectin Type III repeat; SPRY, PRY-SPRY domain.

and the incomplete phenotypic recapitulation of the mouse model suggest that other genes might act as TRIM18/MID1 modifiers during body midline development. A candidate for this role is TRIM1/MID2, a close TRIM18/MID1 paralogue, which shares with the latter high degree of similarity and biochemical features.[41,42] The two genes show redundancy in the establishment of a gene expression cascade leading to left-right asymmetry determination in early chicken embryos.[43] Such redundancy has been observed also in the frog where the *Xenopus* orthologues of the two genes cooperate in the stabilization of the microtubular apparatus during the morphological remodeling of the epithelial neural plate necessary for neural tube closure and likely for the morphogenesis of other organs epithelia.[44] Thus, studies in several experimental models suggest that TRIM18/MID1, possibly in cooperation with TRIM1/MID2, is involved in the mTOR pathway to regulate microtubule dynamics and ultimately morphogenesis of midline epithelia.

The other TRIM-FN3 cluster important for developmental processes is the group of TRIM9-related members (Fig. 3). This includes two genes in human, *TRIM9* and *TRIM67*, and two invertebrate genes showing no univocal orthology with any of the mammalian above, the worm C39F7.2, now named *madd-2*, and the fruit fly CG31721, recently published with the name of *asap* (Fig. 3). *madd-2* stimulates ventral axon

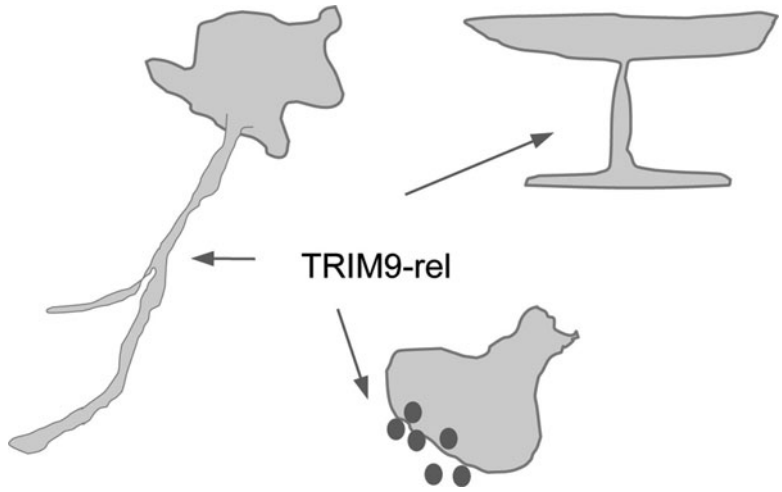

Figure 4. Schematic representation of the roles of TRIM9-related proteins in neuromuscular systems: axon guidance and branching (left); muscle arm guidance (top right); and synaptic vesicle release (bottom right). See text.

branching and promotes attractive axon guidance of *C. elegans* sensory neurons through the *unc-6* (netrin) and *unc-40* (DCC) guidance pathway by regulating actin dynamics.[45] Moreover, *madd-2,* by way of the same signaling partners is implicated in directing muscle arms, specialized worm membrane extensions, to the ventral midline for proper formation of neuromuscular junctions (from here the name *m*uscle *a*rm *d*evelopment *d*efective mutants).[46] Similarly, the *a*nomalies in *s*ensory *a*xon *p*atterning (*asap*, CG31721) drosophila product determines the patterning of ventral spinal cord sensory neurons. In particular, *asap* is implicated in netrin guidance of projections through the ventral midline by regulating contralateral axonal branches.[47] Although no clear orthology with either TRIM9 or TRIM67 is discernible, the data on TRIM9 suggest analogous roles, and possibly even more sophisticated ones, in the mammalian central nervous system. While for TRIM67 no information is available, TRIM9 expression has been thoroughly analyzed. Murine *Trim9* is expressed in the developing cerebral cortex, in the dorsal thalamus, and also in regions of the midbrain, hindbrain and spinal cord during embryogenesis. *Trim9* is highly restricted and preferentially expressed in proliferating compartments of the basal central nervous system regions and differentiating ones of alar regions.[48] In adult, *Trim9* is also specifically expressed in Purkinje cells, in the hippocampus, and cortex.[48] Biochemical data indicate that TRIM9 is involved in the modulation of calcium-dependent synaptic vesicle exocytosis through interaction with SNARE complexes thus indicating a role in the activity of mature neurons.[49] Along the same line, TRIM9 has been shown to be severely decreased in affected brain areas in Parkinsons's disease and dementia patients.[50] Thus, expression and functional data suggest that TRIM9 might exert roles during neurogenesis, possibly similar to those observed in invertebrates, as well as in adult neuronal physiology (Fig. 4).

OTHER TRIM PROTEINS INVOLVED IN DEVELOPMENTAL PROCESSES

Those described in the previous paragraphs are the main classes of TRIM proteins implicated in development, however, other family members play roles during embryogenesis. Among them, TRIM11 is involved in the modulation of Pax6, a paired- and homeo-box-containing neurogenic transcription factor that regulates eye and brain organogenesis.[51] TRIM11 interacts directly with Pax6 and induces its degradation. TRIM11 expression is also controlled by Pax6 thus establishing a self-regulatory feedback loop to maintain a balance of the two proteins during cortical neurogenesis.[52]

TRIM33/TIF1γ is a member of the PHD-BROMO class also known as transcription intermediary factor 1 (TIF1) genes implicated in transcriptional regulation and frequently implicated in tumorigenesis (see Chapter 6 by Cambiaghi et al and Chapter 7 by Yap and Stoye). Like several genes implicated in cancer, TRIM33/TIF1γ exert a role also during development acting as a key player in TGFβ signaling pathway that comprises a complex network of molecules activated/inhibited by ligands of the superfamily (TGFβ, BMPs, Activin, Nodal, etc.).[53] TRIM33/TIF1γ acts at the level of R-Smad/Smad4 transcriptional complex formation and activity through different proposed mechanisms. One report suggests that TRIM33/TIF1γ competes with Smad4 for the binding to activated R-Smads and that both complex can promote TGFβ signaling in hematopoiesis.[54] However, mutations in TRIM33/TIF1γ zebrafish orthologue, *moonshine* (*mon*), inhibits hematopoiesis. Hematopoietic progenitor *mon* mutant cells express reduced amounts of transcription factors required for differentiation and undergo apoptosis.[55] Consistent with the involvement in contrasting mesodermal determination, depletion of TRIM33/TIF1γ in *Xenopus* embryos promotes mesendoderm induction, hence the name of *Ectodermin* in this species.[56] In mice, knock-out of *Trim33*/Tif1γ results in embryonic lethality due to excessive Nodal signaling.[57] This activity is exerted through its ubiquitin E3 ligase action on the Smad4 protein. Monoubiquitination of Smad4 impairs R-Smad/Smad4 complex formation and hence activation of TGFβ signaling target genes transcription.[58] Recent studies suggest that this inhibition occurs at the sites of Smad Binding Elements on the chromatin where TRIM33/TIF1γ is recruited through binding of its PHD-BROMO domain to specifically modified H3 histone tail.[59] This mechanism offers the explanation for dynamic formation and disruption of the active complex for the finely tuned regulation of TGFβ signaling response during development and malignancies.

CONCLUSION

Even though here we addressed examples of family members with assessed implications in development, the number of TRIM genes concerned in such processes may be likely higher. Experimental findings, mainly through the generation and characterization of novel animal model mutants and the detection of novel TRIM genes implicated in congenital disorders, will reveal their roles and their potential relevance in human embryonic development.

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