Review

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Emerging RNA-binding roles in the TRIM family of ubiquitin ligases

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Abstract: TRIM proteins constitute a large, diverse and ancient protein family which play a key role in processes including cellular differentiation, autophagy, apoptosis, DNA repair, and tumour suppression. Mostly known and studied through the lens of their ubiquitination activity as E3 ligases, it has recently emerged that many of these proteins are involved in direct RNA binding through their NHL or PRY/SPRY domains. We summarise the current knowledge concerning the mechanism of RNA binding by TRIM proteins and its biological role. We discuss how RNA-binding relates to their previously described functions such as E3 ubiquitin ligase activity, and we will consider the potential role of enrichment in membrane-less organelles.

Keywords: NHL domains; PRY/SPRY domains; RNA-binding; TRIM proteins; TRIM25; ubiquitination.

Introduction

The control of gene products at the RNA and protein levels is an essential mechanism governing cellular fate. This post-transcriptional regulatory layer determines the location, quantity and time constraints of effector molecules required to respond to stimuli. Perturbations in the levels of these molecules caused by mutations in RNA-binding

proteins (RBPs) often lead to diseases such as neurological disorders and cancer (Cooper et al., 2009). In recent decades, systems biology and the ‘omics’ revolution have greatly advanced our understanding of the global post-transcriptional changes that occur during various biological processes, including cell development and immune responses (Ahmad and Lamond, 2014; Angerer et al., 2017). In addition, methodological advances in the RNA field have expanded the number of putative RBPs (Baltz et al., 2012; Castello et al., 2012; Gerstberger et al., 2014; Dominguez et al., 2018; Hentze et al., 2018) and put a spotlight on low-affinity and multivalent protein-RNA interactions at the core of many important regulatory mechanisms (Jankowsky and Harris, 2015; Falkenberg et al., 2017). Among the newly identified RBPs, many lack classical RNA-binding domains (RBDs) such as the RNA recognition motif (RRM), the cold shock domain (CSD) and K-homology (KH) domain (Hentze et al., 2018). Instead, these proteins feature domains hitherto only known to be involved in protein-protein and protein-DNA interactions and include both structured and disordered regions (Hentze et al., 2018). The mechanisms of RNA association for the large number of newly identified RBPs remain to be elucidated, creating exciting new questions about the basis of RNA recognition in the cell. Several of the newly identified RBPs are members of the tripartite motif (TRIM) protein family, which will be the focus of this review.

From a biochemical point of view, TRIM proteins constitute one of the largest subfamilies of ubiquitin E3 ligases found in the animal kingdom (Crawford et al., 2018) (Figure 1). Although first thought to be metazoan-specific, members of this family have now been identified in plants and fungi, highlighting its long evolutionary history (Sardiello et al., 2008; Marín, 2012; Crawford et al., 2018). The TRIM family is characterised by the presence of three distinct N-terminal domains: the RING domain, one or two B-boxes, and a coiled-coil region (RBCC), invariably ordered in this sequence from N- to C-terminus (Reymond et al., 2001) (Figure 2). To date, at least 77 TRIM proteins have been identified in humans, some of which lack components of the RBCC region but are evolutionarily closely related (Hatakeyama, 2017). In addition, 18 TRIM family

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Figure 1: Phylogeny of human TRIM proteins.

A phylogenetic tree based on the tripartite motif alone mostly reflects the natural classification of TRIM proteins based on the C-terminal domains. Proteins with an incomplete tripartite motif (except for loss of B-box1) were excluded from the analysis. Within the PRY/SPRY family, branches coloured in brown represent those where B-box1 is lost while it is maintained within those coloured in red. *Note that TRIM45 has a Filamin domain but no NHL domain.

members have been identified in *Caenorhabditis elegans*, seven in *Drosophila melanogaster* and more than 200 in zebrafish, underscoring the functional adaptability of this tripartite domain arrangement across species (Reymond et al., 2001; Sardiello et al., 2008; Langevin et al., 2019).

RNA related functions have long been known for certain TRIM proteins (Fridell et al., 1995; Frank and Roth, 1998; Slack et al., 2000; Sonoda and Wharton, 2001) with the C-terminal domain playing a key role in these interactions (Sonoda and Wharton, 2001; Loedige et al., 2013). In 2014, *in vitro* evidence of direct RNA binding by TRIM proteins was presented for BRAT (BRAIn Tumour), a member of the TRIM-NHL subfamily in *Drosophila* (Loedige et al., 2014). A few months earlier, mRNA interactome capture studies of HEK293, HeLa, and mouse embryonic stem cells (mESCs) had identified TRIM25, TRIM28, TRIM56 and TRIM71 as being RBPs (Baltz et al., 2012; Castello et al., 2012; Kwon et al., 2013). Recently, a more unbiased protein-RNA crosslinking capture study also added TRIM33,

TRIM44 and TRIM26 to those above (Trendel et al., 2019, see Table 1). Mounting evidence of RNA binding in this family, in combination with the ubiquitination function of TRIM proteins, points to an involvement in systems that rapidly alter protein levels, stability and function in response to the presence of regulatory and scaffolding RNA molecules.

TRIM proteins have roles in a wide range of biological processes such as cellular differentiation, autophagy, apoptosis, DNA repair and tumour suppression (Hatakeyama, 2017). Notably, over 20 members have been implicated in cell signalling pathways involved in innate immunity, having both positive and negative regulatory roles in the transcriptional activation of nuclear factor kappa-B (NF- κ B) and interferon regulatory factors (IRFs) 3/7 required for the production of antiviral cytokines and interferons (van Gent et al., 2018). Some TRIM proteins can also target viral particles directly, as has been shown for TRIM5 α in response to HIV infection (Pertel et al.,

Figure 2: The TRIM family includes more than 70 proteins in humans. Shown are the RBCC (A) and BCC (B) motif-containing TRIM proteins, with numbers corresponding to individual TRIM genes. Members of the RBCC group were structurally classified into 11 subfamilies (I–XI) by Ozato et al. (2008) and are shown schematically. Asterisks (*) indicate the NHL and PRY/SPRY subfamilies discussed in this review that have clear RNA-binding evidence. (1): TRIM19 has isoform specific C-terminal domains (Nisole et al., 2005). (2): TRIM56 does not contain a canonical NHL domain (Liu et al., 2016).

2011), or indirectly, in the case of TRIM21/Ro52 through association with antibody-coated virions (McEwan et al., 2011). The latter has implications in diseases such as Sjögren’s syndrome and congenital heart block in which autoantibodies against TRIM21/Ro52 are pathogenic (Ambrosi et al., 2014; Ambrosi and Wahren-Herlenius, 2015).

In addition, a number of family members are crucial in neuronal development, with specific roles in neurite growth (Hung et al., 2010), as well as axon guidance and polarisation (Khazaei et al., 2011; Menon et al., 2015; van Beuningen et al., 2015). These include members of the TRIM-NHL subfamily TRIM2, TRIM3 and TRIM32, as well as the SPRY-containing TRIM9, TRIM46 and TRIM67

Table 1: Table of all candidate RNA-binding human TRIM proteins covered in this review with relevant orthologs, biological functions and RNA binding domains.

Name	Relevant orthologs	RNA binding domain	C-terminal domain	Biological functions	Key references
TRIM2	BRAT, Mei-P26 (<i>D. melanogaster</i>), NHL-2, NCL-1 (<i>C. elegans</i>)	Unknown, NHL by homology	NHL	Neuronal development, long-term potentiation	Ohkawa et al. (2001); Balastik et al. (2008); Khazaei et al. (2011); Thompson et al. (2011); Chen et al. (2015); Tocchini and Ciosk (2015)
TRIM3	BRAT, Mei-P26 (<i>D. melanogaster</i>), NHL-2, NCL-1 (<i>C. elegans</i>)	Unknown, NHL by homology	NHL	Neuronal development, long-term potentiation	Cheung et al. (2010); Chen et al. (2014); Schreiber et al. (2015); Tocchini and Ciosk (2015)
TRIM25	N/A	PRY/SPRY and/or coiled coil	PRY/SPRY	Innate immunity, cell differentiation, morphogenesis, microRNA regulation	Orimo et al. (1999); Gack et al. (2007); Kwon et al. (2013); Choudhury et al. (2014); Sanchez et al. (2018)
TRIM26	N/A	Unknown	PRY/SPRY	Innate immunity, DNA damage response	Wang et al. (2015); Treiber et al. (2017); Williams and Parsons (2018); Trendel et al. (2019)
TRIM28	N/A	Unknown	Bromodomain	Regulation of histone modification, regulation of autophagy, DNA damage response, pluripotency maintenance	Czerwinska et al. (2017); Trendel et al. (2019)
TRIM32	Abba (<i>D. melanogaster</i>), NHL-1 (<i>C. elegans</i>)	Unknown, NHL by homology	NHL	Cell differentiation, microRNA regulation	Frosk et al. (2002); Kudryashova et al. (2005, 2009); Schoser et al. (2005); Schwamborn et al. (2009); Tocchini and Ciosk (2015); Kumari et al. (2018)
TRIM33	N/A	Unknown	Bromodomain	Cell differentiation, immune response, cell cycle regulation, DNA damage response	Kulkarni et al. (2013); Pommier et al. (2015); Gallouet et al. (2017); Tanaka et al. (2018); Ali et al. (2019); Trendel et al. (2019)
TRIM44	N/A	Unknown	None	Ocular development, innate immunity	Yang et al. (2013); Zhang et al. (2015b); Trendel et al. (2019)
TRIM56	N/A	Unknown, NHL by homology	NHL-like	Innate immunity	Shen et al. (2012); Kwon et al. (2013); Tocchini and Ciosk (2015); Liu et al. (2016); Garcia-Moreno et al. (2018); Kumari et al. (2018); Trendel et al. (2019)
TRIM65	N/A	Unknown	PRY/SPRY	microRNA regulation, innate immunity	Li et al. (2014); Lang et al. (2017)
TRIM71	Wech (<i>D. melanogaster</i>), LIN41 (<i>C. elegans</i>)	NHL domain	NHL	Cell differentiation, miRNA regulation	Slack et al. (2000); Rybak et al. (2009); Loedige et al. (2013); Zou et al. (2013); Tocchini et al. (2014); Mitschka et al. (2015); Tocchini and Ciosk (2015); Nguyen et al. (2017); Kumari et al. (2018)

(Schwamborn et al., 2009; Boyer et al., 2018). Transcriptional regulation has also been shown to be mediated by several TRIM proteins, specifically TRIM19/PML, TRIM24/TIF1 α , TRIM28/TIF1 β /KAP1, TRIM33 and TRIM66 (Cammass et al., 2012). Thus, the conserved RBCC motif is rather versatile and involved in a wide array of cellular pathways. Not surprisingly, members of the TRIM family have been implicated in both congenital and hereditary diseases. Mutations in TRIM18/MID1, for example, cause X-linked Opitz syndrome (De Falco et al., 2003). In addition, many different TRIM proteins have been connected to inflammatory diseases and cancer [reviewed in Hatakeyama (2011)]. In some cases, increased protein expression is associated with malignancy, while other TRIM proteins (e.g. TRIM3)

promote cellular differentiation and have tumour suppressor roles (Hatakeyama, 2011; Chen et al., 2014).

The RBCC motif mediates ubiquitination

The most well-established biochemical function of TRIM proteins is the catalysis of the third step in the ubiquitination cascade, acting as RING E3 ligases to covalently modify protein substrates with ubiquitin (Ub) (Chu and Yang, 2010; Buetow and Huang, 2016) (Figure 3A, B). The RING domain mediates this reaction by binding the Ub-loaded E2 conjugating enzyme and positioning the Ub moiety in the correct orientation (Buetow and

Figure 3: Ubiquitination in the TRIM family.

(A) The ubiquitination cascade involves an E1 activating enzyme that binds ATP and catalyses the formation of a thioester bond (represented by ~) with ubiquitin (Ub). The E2 conjugating enzyme transfers Ub to its cysteine active site to form a second thioester linkage. In the presence of a substrate (S) and a RING-containing TRIM protein, the latter can act as an E3 ligase to modify residues such as lysine on the substrate. (B) Different types of Ub linkages result in a range of chain lengths and topologies, which can have different downstream effects [see Akutsu et al. (2016) for a comprehensive review]. (C) RING-containing E3s associate with the Ub-conjugated E2 to aid catalysis. Shown is the crystal structure of the RING domain of TRIM25 in complex with Ub ~ UBCH5A (E2) (Koliopoulos et al., 2016) (PDB:5FER).

Huang, 2016) (Figure 3C). Upon association of the RING domain and the E2 Ub conjugate, a conserved cysteine in the E2 is poised for catalysis. The reaction typically results in the formation of an isopeptide bond between the carboxy-terminal glycine residue of Ub and a lysine residue on the substrate, although other residues can be involved (Buetow and Huang, 2016). In addition, TRIM proteins have been shown to become auto-ubiquitinated in a functionally relevant manner, such as in the case of TRIM5 α and TRIM25 (Diaz-Griffero et al., 2006; Choudhury et al., 2017).

With multiple ligation cycles, the target protein can become multi-mono-ubiquitinated and poly-ubiquitinated, resulting in chains of various topologies and lengths (Figure 3B) [see Akutsu et al. (2016) for a comprehensive review]. The diversity in the locations and types of ubiquitin linkages makes this post-translational modification highly versatile. Lysine 48 (K48) ubiquitination, for instance, normally targets proteins for degradation through the proteasome system. Sequence motifs present in a protein substrate that regulate its stability through ubiquitin-mediated degradation or ubiquitin-independent pathways are called degrons. These are short linear motifs that are important for substrate targeting but are not themselves ubiquitinated (Meszaros et al., 2017). Due to the lack of sufficient data it is difficult, however, to define a degron motif for TRIM family members at this point. In contrast to K48 linkages, ubiquitination through lysine 63 (K63) forms more linear ubiquitin chains with roles in DNA repair and endocytosis (Akutsu et al., 2016). Additionally, a relatively high number of TRIM members can add the Small Ubiqutin-like Modifier (SUMO) through interaction with the corresponding conjugating enzyme UBC9 (Chu and Yang, 2010). TRIM28, for instance, is able to modify IRF7 with SUMO, thereby inhibiting its activity (Liang et al., 2011). Additionally, TRIM25 can ligate the ubiquitin-like ISG15 protein to several substrates in response to viral infection (Martin-Vicente et al., 2017). Therefore, the TRIM family is able to catalyse a wide variety of modification and linkage types, consistent with its extensive involvement in many different biological pathways. Notably, some TRIM proteins like *Drosophila* BRAT do not contain a RING domain and it is unclear whether these can act as E3 ligases (Figure 2). However, in the case of TRIM16, co-immunoprecipitation studies showed that it can homo- and hetero-oligomerize with other TRIM members and have ubiquitination activity despite lacking a classical RING domain (Bell et al., 2012).

Higher order oligomerisation of the RING finger may be a general feature of this type of domain (Kentsis

et al., 2002a,b). In the case of TRIM proteins, RING self-association has been demonstrated to be important for TRIM5 α , TRIM25, TRIM32 and TRIM19/PML catalytic function (Ganser-Pornillos et al., 2011; Koliopoulos et al., 2016; Fletcher et al., 2018; Wang et al., 2018). In contrast to the cullin RING ligases that form multi-subunit ubiquitination complexes (Buetow and Huang, 2016), the TRIM family has been referred to as ‘single protein RING fingers’, alluding to the fact that substrate recruitment and catalysis occur through domains located on the same polypeptide chain (Meroni and Diez-Roux, 2005). However, the formation of multimeric structures observed with monomers of TRIM5 α and TRIM32, for example, indicates that ordered aggregation may be necessary for full activity (Albor et al., 2006; Ganser-Pornillos et al., 2011). This is consistent with the observations that many TRIM members form subcellular compartments (Reymond et al., 2001).

Bioinformatic studies on the RBCC motif showed that it evolved as a functional unit rather than three separate domains, strongly suggesting that the RING domain is intricately linked structurally and functionally to the B-box (BB) and coiled-coil (CC) regions (Sardiello et al., 2008). The conservation of linker residues separating the different domains and the observation that the RBCC motif is often encoded by a single exon support this idea (Hennig et al., 2008; Sardiello et al., 2008). In addition, although the RING and first BB motifs have been lost in some TRIM members, the second BB and CC regions are highly conserved (Sardiello et al., 2008). The BB and CC domains are thought to enhance ubiquitination by promoting multimerisation and aiding in substrate recognition, as has been shown for TRIM5 α and TRIM21 (Diaz-Griffero et al., 2006; Li and Sodroski, 2008). However, multimerisation may be context dependent: recent preprints describe the characterisation of the RBCC motif of TRIM28 in its entirety (Lim et al., 2018; Stoll et al., 2018). In both structural models, the RING and BB domains are in close proximity to the CC domain, which mediates dimerisation. One of the studies found that although the first BB of TRIM28 promoted oligomerisation, this was not essential for transcriptional silencing of retrotransposons in cell-based assays (Stoll et al., 2018). Thus, high order self-association through the RING and BB regions may only be necessary in some contexts and/or depend on additional factors (e.g. viruses) to occur (Ganser-Pornillos et al., 2011).

Diversity in the C-terminal domains

Although the N-terminal RBCC architecture is highly conserved and specific to the TRIM family, the C-terminal

region contains a variety of domain organisations not specific to the group. These domains are involved in substrate recruitment by association with targets through both protein-protein and protein-RNA interactions, thereby localising ubiquitination substrates to the Ub-conjugated E2 bound by the RING domain (Gack et al., 2007; Biris et al., 2013; Zhang et al., 2017). Several classifications of the TRIM proteins have been conducted based on structural similarities and sequence homology of the RBCC and C-terminal regions, resulting in the establishment of up to 11 distinct subclasses (I to XI) (Figure 2) (Reymond et al., 2001; Short and Cox, 2006; Hennig et al., 2008; Ozato et al., 2008; Sardiello et al., 2008; Marín, 2012).

The most common C-terminal domain, found in subfamilies I and IV, is the *Sp1A* kinase and *Ryanodine* receptors (SPRY) domain present in ~50% of the family members (Figure 2). This region comprises ~140 residues that fold into a β -sandwich structure (Ozato et al., 2008). In many cases, N-terminal to this domain is the SPRY-associated (PRY) region that extends the domain to form a PRY/SPRY fusion, also known as the B30.2 or RFP-like domain (D'Cruz et al., 2013). Proteins lacking the PRY region also contain N-terminal extensions which, analogous to the PRY motif, form an integral part of the SPRY domain architecture (D'Cruz et al., 2013). The PRY/SPRY and related folds are classically known for mediating protein-protein interactions, having various roles in innate immune responses (see TRIM25 below) (D'Cruz et al., 2013). Often associated with the PRY/SPRY domain are the C-terminal subgroup One Signature (COS) domain, which associates with microtubules, and the fibronectin type III (FN3) domain, found in many extracellular matrix proteins (Ozato et al., 2008).

A smaller number of TRIM proteins contain the *NCL-1*, *H2A*, *LIN-41* (NHL) domain, found in many proteins in eukaryotes and prokaryotes, and serving as binding interfaces for various interactions (Slack and Ruvkun, 1998). This domain folds into a β -propeller structure resembling the well-studied tryptophan-aspartic acid WD40 domain (Edwards et al., 2003). Studies on BRAT demonstrated association of its NHL domain with single-stranded RNA, and currently all members of the TRIM-NHL subfamily are thought to bind RNA (see below for details) (Loedige et al., 2013, 2014). Often associated with the NHL domain is the filamin (FIL) domain, which may localise the protein to the cytoskeleton through interactions with actin (Ozato et al., 2008). In a few members of the TRIM family there are also zinc-binding PHD finger and bromodomains known for their interaction with modified chromatin-associated histones during gene regulation (Ragvin et al., 2004). In addition, a few members contain the meprin and TRAF

homology (MATH) and ADP ribosylation factor (ARF) domains, among others. Thus, the C-terminal domains mediate substrate specificity and confer TRIM proteins with a rich diversity of biological functions.

Several salient reviews on TRIM proteins have highlighted their role in immunity during viral infection (Nisole et al., 2005; Gack et al., 2007; Ozato et al., 2008; Sparrer and Gack, 2018; van Gent et al., 2018) and in disease processes (Hatakeyama, 2011; Tocchini and Ciosk, 2015; Hatakeyama, 2017; Watanabe and Hatakeyama, 2017; Crawford et al., 2018). For more focussed syntheses of TRIM protein function as ubiquitin E3 ligases, the reader is referred to overviews by Ikeda and Inoue (2012) and Ebner et al. (2017). In this review, we will focus on TRIM family members for which there exists clear evidence of RNA binding such as those containing the PRY/SPRY and NHL domains, and discuss potential functional and structural links between their ligase function and association with RNA.

TRIM-NHL proteins

The TRIM-NHL family is a relatively small subfamily which is defined by the presence of an NHL domain at the C-terminus (see Figure 4A). This family includes five members in humans (TRIM2, TRIM3, TRIM32, TRIM56 and TRIM71). Almost all of these proteins have six NHL repeat regions which together form an NHL domain.

The NHL domain is a β -propeller containing six blades each made up of four anti-parallel β -sheets named β -a to β -d (Figure 4A). Each blade is made up of three β -strands from one NHL repeat plus one β -strand from the previous NHL repeat, with the exception of the first blade which brings the N-terminal and C-terminal amino acids of the domain into contact. Blade I is made up of one β -strand from the N-terminus and three β -strands from the C-terminus. In the case of TRIM32, the fourth NHL repeat region is replaced with a sequence which, although compatible with β -strand formation, is not an NHL repeat (Slack and Ruvkun, 1998). However, it is not clear what effect this has on the structure of the domain.

The canonical NHL domain has a 'top' defined as the side on which the β -b to β -c loop is located (Edwards et al., 2003). So far, all known interactions of the NHL domain with RNA have been mediated by this 'top' surface (Loedige et al., 2015; Kumari et al., 2018). However, protein ligands can bind either the 'top' (Lee et al., 2006) or 'bottom' surface (Cho et al., 2006).

It has been noted that all TRIM-NHLs possess a positively charged 'top' surface, suggesting that RNA binding

Figure 4: Structure of the NHL domain and its RNA-binding site. (A) Structure of the BRAT NHL domain from Edwards et al. (2003) with NHL repeats coloured according to their order from red to purple with a 'top' view on the left and a side view on the right. Blades and β -strands are also numbered according to convention. (B) Structure of UUGUUG bound BRAT-NHL from Loedige et al. (2015) (PDB:4ZLR). (C) Structure of TRIM71-NHL bound to a 13mer from the mab-10 3'UTR from Kumari et al. (2018) (PDB:6FQL).

is a conserved feature of this protein family (Loedige et al., 2014). However, it has been proposed that the RNA targets and binding modes may vary widely between NHL domains (Kumari et al., 2018) as there are large differences in charge distributions and a general lack of conservation between most TRIM-NHL paralogs present in a given organism.

It has previously been noted that NHL domains bear a marked similarity to WD40 domains (Edwards et al., 2003). These were found to be enriched within the mESC mRNA interactome (Kwon et al., 2013) and display remarkable versatility in their interactions, including RNA binding (Lau et al., 2009; Stirnimann et al., 2010). It may therefore be helpful to consider the NHL domains within the broader context of RNA binding β -propellers.

RNA sequence preferences of TRIM-NHL proteins

The best understood TRIM-NHL protein, BRAT, was initially thought to bind RNA indirectly through its interaction with Pumilio (Pum) (Arama et al., 2000; Sonoda and Wharton, 2001) but has since been demonstrated to

bind RNA directly (Loedige et al., 2014). NHL domains are now seen as crucial for RNA binding; for example, it was shown that swapping the NHL domains of TRIM71 and TRIM32 swapped their mRNA targets (Loedige et al., 2013).

RNAcompete (Ray et al., 2009) experiments identified UU[G/A]UU[G/A] and UUUACA as the RNA motifs for BRAT and its paralog Mei-P26 (both *D. melanogaster* proteins), respectively, (Loedige et al., 2015) while Davis et al. (2018) found that the *C. elegans* ortholog of Mei-P26, NHL-2, preferentially binds poly-uridine.

Binding motifs for TRIM71, LIN-41 and Wech (human, *C. elegans* and *D. melanogaster* orthologs, respectively) deviate substantially from those of the BRAT orthologues and are characterised by a single highly conserved adenine (Loedige et al., 2015). The lack of clear binding motifs found for TRIM71/LIN41 in this study may be explained by (Kumari et al., 2018)'s observation that binding to TRIM71 is dependent on the formation of a stem loop. Based on data from Loedige et al. (2015) and Laver et al. (2015), they demonstrated that while most NHL domains specify an RNA sequence motif, the NHL domain of LIN41/TRIM71 has a mixed motif dependent both on structure and sequence. Binding necessitates a stem-loop containing a three nucleotide loop with a strong preference for those containing a U-A base pair at the end of the stem and a purine at the third position in the loop. The importance of the interactions was confirmed by the subsequent finding that mutations in residues that interact with the adenine and the purine are linked to congenital hydrocephalus (Furey et al., 2018).

The two structurally characterised NHL domains BRAT and TRIM71 show very different RNA binding modes: the 'top' surface of BRAT-NHL has a sequence of grooves and clefts that accommodate specific bases lying flat along the NHL domain (six bases length total), whereas TRIM71 binds the trinucleotide loop of the stem loop end-on through a positively charged shallow central cavity (Loedige et al., 2015; Kumari et al., 2018) (Figure 4B and C). Thus, as for many other RBDs, for example, RRM (Daubner et al., 2013), the adaptability of this domain architecture allows for a wide variety of RNA binding specificities and makes it currently impossible to predict this selectivity based on protein sequence or structure alone.

The NHL domain mediates both protein-protein and protein-RNA interactions. RNA binding to the NHL may be regulated by protein binding at nearby or overlapping sites. For example, the binding site for the protein Miranda on BRAT-NHL overlaps with that of RNA (Lee et al., 2006; Loedige et al., 2015). Miranda binding was shown to be inhibitory of RNA binding and is thought to be important in regulating mRNA transcriptional downregulation by BRAT.

Despite the sequence and structure specificity of NHL domains, binding can be relatively weak, with affinities in the lower micromolar range (Davis et al., 2018; Kumari et al., 2018). This is similar to RNA affinities of many other RBDs in isolation, like RRM or CSDs, which also bind RNA in the low micromolar range (Maris et al., 2005; Hennig et al., 2014a) but increase affinity up to 1000-fold in the context of neighbouring domains or multi-RBP-RNA complexes due to cooperative binding (Hennig et al., 2014b). Similarly, for TRIM proteins the coiled-coil domain mediates dimerisation and thus each complex features two NHL domains. These could, for instance, reach across long stretches of RNA to bind to their preferred motifs on a single mRNA, which would in turn increase binding affinity of TRIM proteins to their respective RNA targets. This effect could be even further increased if, as suggested in (Koliopoulos et al., 2016), further RING-B-box mediated oligomerisation occurred (Figure 5). The functional consequence of TRIM-NHL mediated RNA binding will be reviewed and discussed in the next section.

RNA-dependent functions of TRIM-NHL proteins

TRIM-NHL proteins carry out a variety of roles, many of them relating to the acquisition and maintenance of differentiated cellular identity as well as translation regulation (Tocchini and Ciosk, 2015). We will focus on roles that relate to RNA binding and regulation, as well as some of the functional parallels between paralogous and orthologous TRIM-NHL proteins.

Figure 5: Diagram showing how dimerisation and potentially oligomerisation as discussed in Koliopoulos et al. (2016) can drive higher affinity and selectivity in TRIM-NHL protein binding to RNA.

BRAT, Mei-P26 and their orthologs

One of the best understood TRIM-NHL proteins is the *D. melanogaster* BRAT, which notably lacks an N-terminal RING domain and is involved in a variety of translational repression roles (Arvola et al., 2017). BRAT interference causes an increase in the levels of active Notch (NICD, Notch intra-cellular domain) and leads to cells with a more proliferative phenotype (Mukherjee et al., 2016).

BRAT segregates asymmetrically between *D. melanogaster* larval neuronal stem cells, leading to post-transcriptional dMyc inhibition through direct RNA binding and differentiation into a ganglion mother cell (Betschinger et al., 2006; Lee et al., 2006; Laver et al., 2015). During differentiation of ovarian germline stem cells into cytotoblasts, BRAT interacts with Pum to repress Mad and cMyc (Harris et al., 2011). BRAT also drives the differentiation from neuroblasts to intermediate neural precursors through binding of *deadpan* and *zelda* mRNA at their 3'UTR and exploits the different affinities of these 3'UTRs to achieve different levels of repression (Reichardt et al., 2018). Furthermore, BRAT is involved in axon maintenance through translational repression of *src64B*, possibly by direct binding, and regulates neuromuscular synapse size and density by repressing Mad translation (Shi et al., 2013; Marchetti et al., 2014).

The earliest known function was discovered in *Drosophila* embryos, where BRAT controls body patterning by generating a gradient of the protein morphogen Hunchback. The fly embryo contains a uniform distribution of *hunchback* mRNA (Tautz, 1988) but has an anterior-posterior gradient in Nanos (Wang and Lehmann, 1991). BRAT interacts with Nanos and Pum to repress *hunchback* translation (Sonoda and Wharton, 2001; Arvola et al., 2017). The complex forms on the 3'UTR of *hunchback* mRNA through binding of Nanos response elements, themselves composed of two conserved boxes that bind BRAT and Pum-Nanos (Wharton and Struhl, 1991; Murata and Wharton, 1995; Loedige et al., 2014; Weidmann et al., 2016; Arvola et al., 2017).

The BRAT orthologs in humans are TRIM2 and TRIM3, two closely related proteins (67% identity). Although they are only distantly related to BRAT and have kept their RING domain, they play similar roles including regulating neuron polarisation, switching cells to asymmetric division and regulating NICD and cMyc [see Figure 2; Balastik et al. (2008); Khazaei et al. (2011); Chen et al. (2014); Tocchini and Ciosk (2015); Mukherjee et al. (2016); Kumari et al. (2018)].

Although little is known about the role of RNA binding in the function of TRIM2 and TRIM3, both are abundant

constituents of mRNPs. They bind myosin V, which transports mRNPs to the synapse (Ohkawa et al., 2001; Kanai et al., 2004), but do not ubiquitinate myosin and are not essential for mRNP trafficking (Balastik et al., 2008; Schreiber et al., 2015). Both TRIM2 and TRIM3 have been linked to the regulation of long-term potentiation (Ohkawa et al., 2001; Cheung et al., 2010; Schreiber et al., 2015). The rapid changes in protein expression and stability involved in this synaptic plasticity could implicate both post-transcriptional and ubiquitination mediated regulation.

More closely related to BRAT but absent in vertebrates are Mei-P26 (*D. melanogaster*) and NHL-2 (*C. elegans*). Mei-P26 is a cofactor of the microRNA induced silencing complex (miRISC) where it interacts with Argonaute-1 to inhibit a variety of miRNAs, in particular *bantam*, a regulator of proliferation and apoptosis. Mei-P26 drives differentiation, suppresses mitotic proliferation and, like BRAT, suppresses dMyc expression (Neumuller et al., 2008). NHL-2 is also a cofactor of the miRISC (Hammell et al., 2009) but can also localise to peri-nuclear granules (P-granules) present in germline cells involved in mRNA regulation and potentially in totipotency maintenance (Wang and Seydoux, 2014; Davis et al., 2018), where it interacts with 22G-RNAs involved in the CSR and WAGO pathways, possibly determining the fate of a subset of 22Gs (Davis et al., 2018).

TRIM71, LIN41 and Wech

TRIM71 and its *C. elegans* ortholog LIN41 are enriched in processing bodies (P-bodies). There, they form part of the RISC complex and ubiquitinate Argonaute-2 as well as interacting with Dicer and regulating AGO-1 (Rybak et al., 2009; Zou et al., 2013). These interactions are decreased upon RNase treatment indicating that it is at least partially mediated by RNA binding (Loedige et al., 2013). Once RNA binding has occurred, TRIM71 acts as a translational repressor through its CC-Filamin domain; this repression can occur independently of the RISC complex (Loedige et al., 2013). Similarly, LIN41 participates in both transcript degradation and translational repression. Interestingly, positioning of the LIN41 binding site at either the 5'UTR or 3'UTR was shown to regulate whether translational repression or transcript degradation, respectively, occurred (Aeschmann et al., 2017).

TRIM71/LIN41 acts as a 'roadblock' to differentiation. This activity is exercised, similarly to other TRIM-NHL proteins, in neuronal and germline cells (Tocchini et al., 2014; Mitschka et al., 2015). It represses various differentiation markers such as the transcription factor EGR1 and *ccnd2*

in neurons, in many cases through interactions with the 3'UTR, but does not appear to drive stem cell marker maintenance (Slack et al., 2000; Loedige et al., 2013; Worringer et al., 2014; Mitschka et al., 2015).

In adult neurons, LIN-41 promotes axon regeneration after injury and was shown to be involved in a negative regulation loop with its well established repressor *let-7* through its regulation of the argonaute protein Alg-1 (Zou et al., 2013).

The TRIM71 *D. melanogaster* ortholog, Wech, has mostly been studied in relation to a potential role as a scaffolding protein at muscle attachment sites (Loer and Hoch, 2008; Loer et al., 2008) but, interestingly, has been shown to be protective against CAG-repeat based RNA toxicity (Shieh and Bonini, 2011).

TRIM32, NHL-1 and Abba

In mouse neurons, TRIM32 plays a crucial role in neuronal differentiation by becoming polarised between progenitor and daughter cells and determining cell differentiation fate by down-regulating cMyc and binding AGO1 to activate *let-7a* (Schwamborn et al., 2009).

Similar to TRIM2 and TRIM3, TRIM32 binds myosin through its NHL domain as part of its role in muscle remodelling (Kudryashova et al., 2005). TRIM32 orthologs are relatively poorly studied: Abba (*D. melanogaster*) is involved in sarcomere organisation (LaBeau-DiMenna et al., 2012; Domsch et al., 2013) while NHL-1 (*C. elegans*) is involved in stress responses in neuronal cells (Volovik et al., 2014).

TRIM56

Finally, TRIM56, which is often not included in discussions of NHL-TRIM proteins but is closely related and possesses NHL-like repeats (Liu et al., 2016), has been identified to be part of the human RNA interactome (Kwon et al., 2013). It is a key component of the Toll like receptor 3 (dsRNA sensing) pathway and inhibits the replication of influenza viruses, however this activity is dependent only on a 63 amino acid-long segment of the C-terminus, not on a fully formed NHL domain (Shen et al., 2012; Liu et al., 2016). However, in the case of bovine diarrhoea virus, the entire C-terminus as well as the E3 ubiquitin ligase activity were necessary to restrict viral RNA replication (Wang et al., 2011). A review by Garcia-Moreno et al. (2018) drew parallels between the RNA binding dependence of E3 ligase activity observed in TRIM25 by Choudhury et al. (2017) and the potential for a similar mechanism for TRIM56.

In summary, TRIM-NHL proteins play crucial roles during cell differentiation, especially in neuronal and germline cells. Their ability to act both post-transcriptionally and post-translationally is ideally suited to these steps during which cellular states must undergo rapid and dramatic changes. All the described functions are directly or at least indirectly related to RNA binding. A more in-depth study of these proteins can shed light on networks and checkpoints involved (Mitschka et al., 2015) as well as how they came to evolve. A detailed understanding of the interplay between these proteins' ubiquitination and RNA binding activities will most likely be crucial to a more complete understanding of their biological roles.

Although most direct TRIM-RNA interactions described to date are mediated by the NHL domain, new work is starting to uncover a role for non-NHL mediated TRIM protein-RNA interactions, particularly the so far unique case of RNA binding by TRIM25 through its PRY/SPRY domain (Kwon et al., 2013; Choudhury et al., 2017).

RNA-binding of the PRY/SPRY containing TRIM25

So far RNA-binding has only been shown for a single member of the TRIM-SPRY family, TRIM25, which exhibits diverse functions in innate immunity, morphogenesis and cell proliferation (Orimo et al., 1999; Gack et al., 2007; Castanier et al., 2012; Qin et al., 2015; Takayama et al., 2018). TRIM25 was identified by RNA interactome capture studies from mESCs (Kwon et al., 2013). Meanwhile, it has been shown that TRIM25 interacts with a wide variety of RNAs, including the 3'-UTRs and exons of mRNAs (Kwon et al., 2013; Choudhury et al., 2017), lincRNAs (Choudhury et al., 2017), miRNAs (Choudhury et al., 2014), viral RNAs and their corresponding RNPs (Manokaran et al., 2015; Meyerson et al., 2017). No clear consensus motif for RNA-binding has been identified, although G- and C-rich sequences are overrepresented in CLIP-data (Choudhury et al., 2017). *In vitro* assays found no clear preference for single or double stranded RNA (Sanchez et al., 2018).

This broad range of RNA targets in part reflects TRIM25's broad and often poorly understood range of functions. The case of precursor-of-microRNA let-7 (pre-let-7a-1) might give insights into the general principle behind these interactions: TRIM25 recruits the pluripotency promoting factor Lin28 and the terminal uridylyltransferase 4 (TUT4). TUT4, possibly after activation through ubiquitination by TRIM25, poly-uridylylates pre-let-7 and marks it for RNA degradation (Choudhury et al., 2014).

Efforts have been made to identify the regions responsible for RNA binding. Kwon et al. (2013) originally mapped the RNA-binding of TRIM25 to the CC region based on co-purification experiments. Later work identified an amino acid stretch in the PRY motif as crosslinking to RNA (Castello et al., 2016). Deletion of this peptide abolished RNA-binding of the full-length protein in electrophoretic mobility shift assays (EMSAs) (Choudhury et al., 2017).

Neither the isolated PRY/SPRY domain nor TRIM25 Δ CC bound in these assays, indicating a critical role of the CC, either through direct interaction or by mediating dimerisation. In addition, the linker connecting the PRY/SPRY and CC domains (referred to as L2 linker) contains a short lysine-rich motif that contributes to RNA-binding (Sanchez et al., 2018).

Together, these results suggest that although several regions in the protein are likely involved in RNA-binding, the PRY/SPRY domain plays a critical role. This would be the first example of RNA-binding by a PRY/SPRY domain. Most PRY/SPRY domains are protein-protein interaction domains, with a few examples adapted to binding of large supramolecular assemblies like viral capsids or antibodies (Woo et al., 2006; Keeble et al., 2008; Biris et al., 2012). Although the fold of the core domain is highly conserved, the varying length and amino acid composition of the four flexible regions v1–v4 allow for binding of this remarkably broad variety of substrates (Song et al., 2005) (Figure 6A). It is therefore not surprising that several of the mutants described by Sanchez et al. (2018) as affecting RNA-binding cluster in the v1 and v2 region, and the proposed RNA-binding region from Choudhury et al. (2017) contains the complete v1 region (Figure 6B and C).

The TRIM25 PRY/SPRY domain is necessary and sufficient for the interaction with substrates such as Retinoic Acid Inducible Gene I (RIG-I) or Zinc finger antiviral protein (ZAP), two well-known RBPs involved in innate immunity (Gack et al., 2007; Li et al., 2017).

The link between RNA binding and ubiquitination

An influence of RNA-binding on ubiquitination was first reported by Choudhury et al. (2017). RNase I treatment strongly decreased auto-ubiquitination of TRIM25 as well as TRIM25-mediated ubiquitination of ZAP *in vitro*. Deletion of its proposed RNA-binding region showed a similar phenotype, in addition to abolishing RNA-binding. However, parts of this deletion were shown to be involved in the interaction between the PRY/SPRY and CC domains, which proved to be crucial for ubiquitination of

Figure 6: Charge distribution and conservation of TRIM-PRY/SPRY domains and the likely RNA-binding site.

(A) Sequence alignment of several human TRIM-SPRY domains reveals variable regions v1-v4 (numbering corresponds to TRIM25).

(B) Structural alignment of all published structures of primate TRIM-SPRY domains [TRIM5 α (PDB:2LM3), TRIM20 (PDB:4CG4), TRIM21 (PDB:2IWG), TRIM25 (PDB:6FLM), TRIM72 (PDB:3KB5)]. The variable regions are highlighted in the same colour scheme as in A.

(C) Comparison with the postulated RNA-binding region (Choudhury et al., 2017) in yellow and mutants described to affect the RNA-binding of TRIM25 (Sanchez et al., 2018) in red shows that they cluster in the variable region, possibly indicating that RNA-binding is not a conserved feature of TRIM-PRY/SPRY domains. (D) The PRY/SPRY domain of TRIM25 (PDB:6FLN) interacts weakly with the CC and the two domains form a shared, strongly positively charged surface (in blue), that may allow for cooperative RNA-binding.

RIG-I *in vivo* (Koliopoulos et al., 2018) and a similar role in ZAP ubiquitination appears likely, possibly explaining the observed phenotype in an RNA-independent way.

The RNA dependence of TRIM25's catalytic activity was, however, also reported elsewhere (Sanchez et al., 2018). Mutation of a lysine-rich stretch in the L2 linker region reduced not only RNA-binding, but also ubiquitination of RIG-I *in vivo* and therefore suppression of viral replication.

RNA-binding might enhance ubiquitination activity by at least two different mechanisms: either by facilitating substrate recruitment through binding to the same RNA or by directly increasing catalytic activity through allosteric changes of TRIM25 (Choudhury et al., 2017). In support of the first mechanism, both ZAP and RIG-I are well-known RNA-binding molecules and co-localisation of TRIM25 and RIG-I in stress granules is RNA-dependent (Sanchez et al., 2018). Although TRIM25 and RIG-I co-purify when either of them is immuno-precipitated from cells (Gack et al., 2007), there is no clear evidence for a direct interaction *in vitro*, suggesting that additional factors like RNA might be needed for the interaction. There is no indication of preferential binding of TRIM25 to RIG-I bound RNA over free RNA (Sanchez et al., 2018).

An allosteric effect of RNA-binding on ubiquitin E3 ligase activity would most likely be accomplished by facilitating interactions between the substrate which recognises the PRY/SPRY domain and the catalytic RING finger. Sanchez et al. (2018) propose that this is achieved through rearrangement of the disordered L2 linker upon RNA-binding. Alternatively, RNA might stabilise the weak CC:PRY/SPRY interaction, either through direct interactions with both domains or by allosterically modulating binding of the PRY/SPRY to the CC. Interestingly, the crystal structure of a CC-PRY/SPRY construct of TRIM25 shows the PRY/SPRY domain interacting with the CC region (Koliopoulos et al., 2018). However, in solution this interaction between both domains is rather weak (Koliopoulos et al., 2018). The PRY/SPRY domain being dissociated from the CC with the long flexible linker being distal to the E2-Ub bound RING would make ubiquitination of substrates difficult. Intriguingly, both domains together form a larger positively charged surface (Figure 6D), which could accommodate RNA. This RNA could decrease the average distance of the substrate to the E2-Ub conjugate significantly. Allosteric effects could also directly enhance interactions with the substrate.

RIG-I binds double-stranded RNA with a 5'-triphosphate (5' ppp) moiety through its helicase and C-terminal domains (Hornung et al., 2006). It is likely that TRIM25 and RIG-I will bind the same RNA molecule, thereby facilitating the interaction between E3 ligase and substrate. RNA-binding could not only increase the ubiquitination

activity of TRIM25 by facilitating CC:PRY/SPRY interactions, but will also lead to a conformational change of RIG-I that makes the N-terminal CARD domains, that are otherwise bound to the helicase domain in an auto-inhibited state, accessible for ubiquitination by TRIM25 (Kolakovsky et al., 2012) (Figure 7). Poly-ubiquitination of the CARD domains and release of unanchored K63-linked ubiquitin chains triggers filament formation of the mitochondrial antiviral signalling protein (MAVS) and causes IFN expression (Hou et al., 2011). A recent report identifies the long non-coding RNA Lnczc3h7a as binding both the TRIM25 PRY/SPRY and RIG-I helicase domain, thereby facilitating their interaction and TRIM25-dependent ubiquitination of RIG-I. Interestingly, Lnczc3h7a does not activate RIG-I and might bind the helicase domain via a novel interaction site independent of the known binding site for double-stranded RNAs (Lin et al., 2019).

Despite the clear evidence for an activating function of RNA on TRIM25 *in vitro*, the example of the Dengue virus subgenomic RNA (sfRNA) shows that the situation is less clear *in vivo*. Mutations in the sfRNA of the Dengue virus not only increased affinity of this RNA to TRIM25, but also decreased IFN- β expression, possibly indicating reduced RIG-I signalling (Manokaran et al., 2015). Immunoprecipitation of RIG-I from sfRNA-transfected cells still co-purified TRIM25, indicating that the interaction with RIG-I is not impaired, but showed a higher proportion of ubiquitinated TRIM25. This may indicate that sustained activation of TRIM25 E3 ligase activity by sfRNA facilitates auto-ubiquitination and increased proteasomal degradation of TRIM25, thereby decreasing RIG-I signalling.

RNA binding in other PRY/SPRY TRIM proteins

The role of the PRY/SPRY domain in RNA-binding of TRIM25 has led to the suggestion that RNA-binding might be a conserved feature of TRIM-PRY/SPRY domains and that other members of the PRY/SPRY-carrying TRIM family might bind RNA. So far evidence for this is sparse. Replacement of the proposed RNA-binding peptide in the TRIM25 PRY/SPRY domain with homologous sequences from other TRIM proteins (TRIM5 α , TRIM21, TRIM27 and TRIM65) preserves both RNA binding and auto-ubiquitination activity (Choudhury et al., 2017). However, the interpretation that RNA binding is found in more PRY/SPRY domains is controversial, as the results can also be explained by preserved interactions between PRY/SPRY and CC domains. Given the broad spectrum of interaction partners of PRY/SPRY domains and the fact that published mutants affecting

Figure 7: One potential, albeit speculative mechanism of RNA-assisted RIG-I ubiquitination in accordance with experimental evidence.

(A) In the absence of RNA the substrate-binding PRY/SPRY domain interacts only transiently with the CC (Koliopoulos et al., 2018). (B) RNA-binding induces a conformational change of TRIM25 through remodeling of the linker connecting the CC and PRY/SPRY domain (Sanchez et al., 2018) or direct interaction with both domains (Kwon et al., 2013; Choudhury et al., 2017), leading to its activation. (C) RIG-I binds the same RNA-strand via its helicase and C-terminal domain (CTD), leading to opening of the auto-inhibited state (Kolakofsky et al., 2012). (D) TRIM25 poly-ubiquitinates the RIG-I CARD domains, triggering RIG-I signalling. This mechanism is, of course, speculative and other mechanisms are possible, for example, other factors might be included to mediate RIG-I-CARD domain interactions with the TRIM25 PRY/SPRY domain. An example for such a factor might be the long non-coding RNA *Lnczc3h7a*, that binds both TRIM25 and RIG-I, but does not remove auto-inhibition of RIG-I (Lin et al., 2019).

RNA binding are mostly located in the poorly conserved loop regions, it is unlikely that RNA binding is a broadly conserved feature of PRY/SPRY domains.

Nevertheless, TRIM65, a close relative of TRIM25, was found in the interactome of miRNAs, although it is likely that interaction with the known RNA-binding TNRC6 proteins accounts for this observation rather than direct protein-RNA interactions (Li et al., 2014). Another recently described RNA-binding TRIM is TRIM26, which seems to interact specifically with the microRNA miR-18b (Treiber et al., 2017; Trendel et al., 2019). Unlike TRIM25 and TRIM65, which are ancient proteins with homologs in all vertebrates, TRIM26 is part of a younger, mammalian specific and more rapidly evolving subgroup of TRIM proteins (Sardiello et al., 2008). The large evolutionary distance and the absence of reported RNA-binding for other PRY/SPRY domains suggest, that RNA-binding in PRY/SPRY domains might have evolved more than once.

Co-localisation of substrates on the RNA and phase separation

The case of TRIM25 suggests that RNA-binding in TRIM proteins might generally play a role in substrate recruitment, with binding of both substrate and E3 ligase to the same RNA facilitating their interaction. While other TRIM proteins have been less studied in this context, TRIM25 and TRIM71 give insights into the links between RNA binding, RNP formation and ubiquitination.

In extension of this idea, RNA-induced biological condensation might lead to enrichment of TRIM proteins and their substrates in ‘membrane-less organelles’. This phase separation is usually strongly coupled to multivalency (Li

et al., 2012) and RNA-binding in addition often promotes macromolecular condensation (Lin et al., 2015). Given that TRIM proteins with their diverse and redundant interaction sites distributed over several domains are archetypal multivalent proteins, it is not surprising that phase separation is a common theme in TRIM proteins with TRIM19 (PML) condensation into nuclear bodies being the prime example (Dyck et al., 1994). TRIM proteins also feature large intrinsically disordered regions (Uversky, 2014) that have been implicated in phase-separation (Malinowska et al., 2013). Formation of TRIM containing cell compartments in response to overexpression was shown for most TRIM proteins (Reymond et al., 2001). Among those proteins are also RNA-binding TRIM proteins and, at least in the case of TRIM25, an RNA-dependence for the formation of these stress granules was shown (Sanchez et al., 2018). Redundant TRIM binding sites are found in several RNA targets, possibly supporting RNA-induced phase-separation (Kumari et al., 2018). Macromolecular condensates remain liquid and as such allow for free diffusion of moderately large molecules, including proteins, and exchange with the surrounding liquid while at the same time featuring very high protein concentrations and frequent encounters between proteins (Banani et al., 2017). The catalytic activity of TRIM proteins opens up the possibility of a fine-tuned regulation of macromolecular condensation, as post-translational modifications (PTMs) often act as switches to induce or repress phase separation and ubiquitination regulates the formation of some membrane-less organelles (Mittag et al., 2017; Dao et al., 2018; Herhaus and Dikic, 2018). Although so far, no TRIM protein was shown to directly regulate phase separation by ubiquitination, TRIM19 auto-sumoylation facilitates nuclear body formation (Shen et al., 2006). While auto-ubiquitination of TRIM proteins in the cytosol promotes proteasomal degradation, resulting in short life-times (Diaz-Griffero et al., 2006; Versteeg et al., 2013), poly-ubiquitinated proteins are likely protected from proteasomal degradation in phase-separated droplets. Phase-separation in addition to facilitating substrate recruitment by increasing local concentration might therefore also regulate stability. TRIM proteins are also subject to other PTMs that might have an influence on phase-separation (Stacey et al., 2012; Lee et al., 2018). This might explain why the RNA-binding of some TRIM proteins' changes upon stress (Garcia-Moreno et al., 2019; Trendel et al., 2019). Macromolecular condensation into membrane-less organelles provides a powerful and elegant hypothesis to explain localisation of low-abundance proteins with their substrates and enable another layer of post-translational regulation, but it should not be forgotten that most proteins will phase-separate given the right condition (Dumetz et al., 2008). It is therefore not always clear if results from

over-expressed proteins or *in vitro* reconstituted systems reflect the situation at much lower physiological concentrations. While phase separation is a relatively new concept in biology (Li et al., 2012), in the context of TRIM proteins it can be seen as the newest evolution of the idea of functional multimerisation. The clearest example of functional multimerisation in TRIM proteins are the highly ordered hexagonal lattices that TRIM5 α forms on retroviral capsids (Ganser-Pornillos et al., 2011) and even spontaneously (Li et al., 2016). Trimeric BB structures form the junctions of these networks, connected by the CC dimers (Li and Sodroski, 2008). The PRY/SPRY domains are found on one side of the network, where they are prearranged for interaction with the viral capsid, while the RING domains form trimers on the other side of the network (Biris et al., 2013; Fletcher et al., 2018). While it was a popular hypothesis that similar forms of oligomerisation might be a common feature of other TRIM proteins, the highly ordered two-dimensional (2D) crystals formed by TRIM5 α through well-defined interactions between B-boxes are structurally and functionally different from liquid-like macromolecular condensates held together by numerous, weak, often unspecific, interactions. To the best of our knowledge, TRIM5 α remains the only member of the TRIM family to form these kind of well-ordered oligomers and generalisations from TRIM5 α to other TRIM proteins are therefore difficult.

miRNA regulation

The most obvious case in which TRIM proteins are involved in phase separation is the role they play within the miRISC complex which is segregated into P-bodies. It was established relatively early on that miRNA regulation was central to the role of many TRIM proteins (Loedige and Filipowicz, 2009; Wulczyn et al., 2011). This is most heavily studied in the case of TRIM71, itself a target of the miRNA *let-7*, which also localises to P-bodies where it ubiquitinates AGO2 and leads to reduced silencing efficiency (Rybak et al., 2009). TRIM71 also negatively regulates *let-7* levels through its binding and regulation of the argonaute protein ALG-1 (Zou et al., 2013) and was demonstrated to bind pri-miR-29a through its apical loop, driving its downstream processing and repression of proteins TET2 and TET3 (Treiber et al., 2017). TRIM25 has also been postulated to regulate *pre-let-7* as described above (Choudhury et al., 2014).

The homologs Mei-P26, and NHL-2 are also members of the miRISC complex as discussed above (Neumuller et al., 2008; Hammell et al., 2009; Davis et al., 2018). BRAT, Mei-P26 and TRIM32 all bind AGO1 (Neumuller et al., 2008; Schwamborn et al., 2009). While NHL-2 binds ALG-1 and

upregulates *let-7* and *lsy-6* activity (Hammell et al., 2009) and TRIM32 upregulates *let-7*, Mei-P26 was reported to globally decrease miRNA levels (Neumuller et al., 2008; Schwamborn et al., 2009).

A common observation in most of these studies is that the regulation exercised by TRIM-NHL proteins is highly specific to particular miRNAs, presumably achieved via direct TRIM-RNA interactions, suggesting they may play a crucial role in regulating specificity of miRNA network changes.

Comparison with other RNA-binding E3-ligases

The TRIM proteins discussed above are a significant addition to the list of known proteins that combine RNA-binding and E3-ubiquitin ligase activity. Previously, only around thirty out of the approximately 600 human Ubiquitin E3 ligases were known to bind RNA (Cano et al., 2010). As in the case of TRIM25, several of these have ubiquitination activity regulated by RNA-binding (Cano et al., 2012; Zhang et al., 2015a). A particularly interesting case is Roquin, which promotes mRNA decay (Leppek et al., 2013) by binding stem-loop motifs known as constitutive decay elements (CDEs) (Schlundt et al., 2014). Roquin-mediated RNA-binding can either up- or down-regulate the E3 ligase activity dependent on the E2 bound, allowing for regulation of the linkage type of poly-ubiquitin chains (Zhang et al., 2015a). This is achieved by a bipartite architecture with two rigid modules connected by flexible linkers. Both modules contain RNA-binding sites and RNA-binding causes major domain rearrangements also involving the catalytic RING domain (Zhang et al., 2015a). This situation might resemble the architecture of TRIM proteins with the rigid tripartite motif and the additional C-terminal domains.

Concluding remarks

In this review we hope to have demonstrated that RNA interaction is crucial to understanding the role of many TRIM proteins. Current knowledge prompts us to postulate two hypotheses about TRIM-RNA interaction. (i) TRIM proteins bind RNAs to regulate their fate, whether it will be RNA decay, translation regulation or other functions around the central dogma. (ii) Regulatory RNAs and RNA pathogens bind TRIM proteins to regulate their ubiquitination efficiency. These aspects of the field have

opened up many exciting lines of enquiry: how are the RNA binding functions of these proteins coupled to their more classical ubiquitination roles? Can RNA binding sites act as TRIM-specific degrons? How does the inherently dimeric and potentially multimeric nature of these proteins impact how they recognise and recruit their RNA targets? How can we reconcile the variability observed in the domain architecture of certain orthologous TRIMs with their relatively well-conserved biological roles? How can we develop a solid understanding of the specific cellular environments such as membraneless organelles in which these proteins operate?

Many of these questions could not be tackled without the recent developments in RNA specific methods, 'omics' techniques and structural biology methods capable of addressing large and flexible complexes. These developments make RNA-TRIM interactions a challenging yet manageable field of study and, given the biologically central role played by many of these proteins, exciting developments are to be expected.

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