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Ro60 and Y RNAs: Structure, Functions and Roles in Autoimmunity

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Abstract

Ro60, also known as SS-A or TROVE2, is an evolutionarily conserved RNA-binding protein that is found in most animal cells, approximately 5% of sequenced prokaryotic genomes and some archaea. Ro60 is present in cells as both a free protein and as a component of a ribonucleoprotein complex, where its best-known partners are members of a class of noncoding RNAs called Y RNAs. Structural and biochemical analyses have revealed that Ro60 is a ring-shaped protein that binds Y RNAs on its outer surface. In addition to Y RNAs, Ro60 binds misfolded and aberrant noncoding RNAs in some animal cell nuclei. Although the fate of these defective Ro60-bound noncoding RNAs in animal cells is not well-defined, a bacterial Ro60 ortholog functions with 3' to 5' exonucleases to assist structured RNA degradation. Studies of Y RNAs have revealed that these RNAs regulate the subcellular localization of Ro60, tether Ro60 to effector proteins and regulate the access of other RNAs to its central cavity. As both mammalian cells and bacteria lacking Ro60 are sensitized to ultraviolet irradiation, Ro60 function may be important during exposure to some environmental stressors. Here we summarize the current knowledge regarding the functions of Ro60 and Y RNAs in animal cells and bacteria. Because the Ro60 RNP is a clinically important target of autoantibodies in patients with rheumatic diseases such as Sjögren's syndrome, systemic lupus erythematosus and neonatal lupus, we also discuss potential roles for Ro60 RNPs in the initiation and pathogenesis of systemic autoimmune rheumatic disease.

Keywords

Ro60; Y RNA; Rsr; autoantigen; noncoding RNA; RNA degradation

Introduction

Ro60 was initially characterized as a soluble antigen targeted by autoantibodies from patients with two systemic autoimmune rheumatic diseases, systemic lupus erythematosus and Sjögren's syndrome. Two independent groups identified the Ro autoantigen using agar-based immunodiffusion assays. One group studied sera from a patient ("Ro") with systemic lupus erythematosus symptoms that did not display reactivity with any known nuclear

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antigen (Clark et al. 1969). A second group identified the antigen while characterizing Sjögren's syndrome patient sera, thus leading to Ro60's other designation as SS-A (Sjögren's syndrome A) (Alsbaugh and Tan 1975). Experiments involving metabolic labeling, biochemical purification and western blotting revealed that two structurally unrelated proteins were recognized by anti-Ro patient sera. One protein, Ro60, was found to be an RNA-binding protein with a molecular weight of 60 kDa (Wolin and Steitz 1984; Yamagata et al. 1984), while the second protein, Ro52/TRIM21, is a 52 kDa ubiquitin E3 ligase that targets cytosolic virus-antibody complexes for degradation (Ben-Chetrit et al. 1988; Mallery et al. 2010). It remains unclear why antibodies to these two seemingly unrelated proteins frequently occur together in patient sera.

Ro60, like many rheumatic disease autoantigens, was found to form a ribonucleoprotein (RNP) complex, consisting primarily of Ro60 and noncoding RNAs (ncRNAs) called Y RNAs (Hendrick et al. 1981; Lerner et al. 1981; Wolin and Steitz 1984; Yamagata et al. 1984). The number of Y RNAs varies between species, but in all studied cases Ro60 has been found complexed with at least one Y RNA. Like other classical ncRNAs found in RNP complexes, Y RNAs have highly conserved structures which are important for RNP assembly and function. The most conserved feature of Y RNAs is a stem generated by base pairing the 5' and 3' portions of the RNA (Wolin and Steitz 1983; Van Horn et al. 1995; Chen et al. 2000; Teunissen et al. 2000; Perreault et al. 2007). Within this stem is a high affinity binding site for Ro60 (Wolin and Steitz 1984; Pruijn et al. 1991; Green CD et al. 1998). The sequence at the top of the stem can take on a variety of configurations depending on the specific Y RNA, but generally forms a number of internal loops and short stems. This portion of the Y RNA can serve as a binding site for other proteins (Fabini et al. 2001; Fouraux et al. 2002; Sim et al. 2012; Chen et al. 2013; Scheckel et al. 2016; Tebaldi et al. 2018), in at least some cases tethering these proteins to Ro60 to generate specialized RNPs.

Since its original identification as a human autoantigen, Ro60 has been found to be evolutionarily conserved. In eukaryotes, Ro60 is present in virtually all animal genomes and most choanoflagellates, single-celled eukaryotes that are the closest relatives to animals (Richter et al. 2018). Although neither Ro60 nor Y RNAs have been identified in higher plants, likely Ro60 orthologs are encoded in the genomes of several green algae (*Chlamydomonas reinhardtii*, *Volvox carteri* and *Tetrahena socialis*) and at least one fungus (*Allomyces macrogynus*). About ~5% of sequenced bacterial genomes contain a Ro60 ortholog (Sim and Wolin 2011), as well as several archaea. Although the identification of Y RNA orthologs has lagged behind that of the Ro60 protein, Ro60 has been found complexed with Y RNAs in all species that have been studied (Hendrick et al. 1981; Reddy et al. 1983; Mamula et al. 1989; O'Brien and Wolin 1994; Van Horn et al. 1995; Chen et al. 2000; Chen et al. 2013; Chen et al. 2014), and bioinformatic approaches have identified potential Y RNAs in more than 275 bacterial and phage genomes.

Consistent with a role in ncRNA quality control, Ro60 is also found bound to variant forms of pre-5S rRNA and U2 small nuclear RNAs (snRNAs) in some animal cell nuclei (O'Brien and Wolin 1994; Shi et al. 1996; Chen et al. 2003). The RNAs bound to Ro60 contain sequence changes that cause them to misfold into incorrect structures (O'Brien and Wolin 1994; Shi et al. 1996; Chen et al. 2003; Fuchs et al. 2006). As biochemical studies revealed

that Ro60 binds RNAs that contain both protein-free helical regions and adjacent protein-free single-stranded 3' ends (Shi et al. 1996; Fuchs et al. 2006), Ro60 may scavenge RNAs that fail to assemble with their specific RNA-binding proteins. Structural studies revealed that Ro60 is doughnut-shaped, and that the single-stranded tail of a misfolded RNA inserts through the central cavity while helical portions interact with the basic outer surface of the Ro60 ring (Stein et al. 2005; Fuchs et al. 2006). Y RNAs bind on the outer face of the Ro60 ring, to a site that partially overlaps with the misfolded RNA binding site (Stein et al. 2005; Fuchs et al. 2006). Although the role of Y RNAs in the putative ncRNA quality control pathway are not well-defined in animal cells, a bacterial Y RNA tethers Ro60 to a ring-shaped nuclease to assist structured RNA degradation and may also act as a gate to regulate access of RNA substrates to the Ro60 central cavity (Chen et al. 2007; Chen et al. 2013).

In both animal cells and bacteria, Ro60 is involved in the cellular response to stress. This role was first identified in bacteria, where Ro60 enhances survival after ultraviolet (UV) irradiation and during prolonged growth in stationary phase (Chen et al. 2000; Wurtmann and Wolin 2010). Additionally, both the bacterial Ro60 and its associated Y RNAs are upregulated following multiple forms of environmental stress (Chen et al. 2000; Tanaka et al. 2004; Chen et al. 2007; Wurtmann and Wolin 2010). In bacteria, Ro60 was found to function with 3' to 5' exoribonucleases to modulate RNA populations in response to stress (Chen et al. 2007; Wurtmann and Wolin 2010). In mammalian cells, Ro60 enhances cellular survival after exposure to UV irradiation (Chen et al. 2003) and accumulates in nuclei after either UV irradiation or oxidative stress (Chen et al. 2003; Sim et al. 2009).

The study of Ro60 RNPs may also be helpful for understanding their potential roles in autoimmunity. Antibodies to Ro60 are, on average, the first antibodies detected in patients that eventually develop systemic lupus erythematosus (Arbuckle et al. 2003; Eriksson et al. 2011). Remarkably, evidence has been presented that lupus autoimmunity may be triggered and sustained by commensal bacteria expressing Ro60 RNPs (Greiling et al. 2018). Additionally, because specific autoimmune disorders such as neonatal lupus (Kephart et al. 1981; Weston et al. 1982), Sjogren's syndrome (Moutsopoulos et al. 1980) and subacute cutaneous lupus erythematosus (Sontheimer et al. 1982) are strongly associated with anti-Ro antibodies, immune complexes containing Ro60 have been proposed to contribute to pathogenesis (Clancy et al. 2010; Green NM and Marshak-Rothstein 2011). We discuss how Ro60 and anti-Ro60 antibodies may participate in these disease syndromes.

Ro60 Structure

Crystal structures of *Xenopus laevis* Ro60 and the Ro60 ortholog (called Rsr for Ro sixty related) from the bacterium *Deinococcus radiodurans* revealed that Ro60 folds to form a monomeric ring (Figure 1) (Stein et al. 2005; Ramesh et al. 2007). The ring is formed by a series of antiparallel α -helical repeats known as HEAT repeats (Andrade and Bork 1995) and is closed by a von Willebrand factor A domain (vWFA). Although vWFA domains are found in a number of intracellular proteins, this domain is best characterized in extracellular matrix and cell adhesion proteins (Whittaker and Hynes 2002). Many vWFA domains, including that of Ro60, contain a divalent cation binding site called a metal ion-dependent adhesion site (MIDAS). In integrins, the MIDAS is a ligand-binding site that also transmits

conformational rearrangements to other regions of the protein upon ligand-binding (Springer 2006). How the MIDAS contributes to Ro60 function is currently unknown.

Only *X. laevis* Ro60 has been crystallized bound to its RNA ligands. An edge on the outer surface of the *X. laevis* Ro60 torus contains a basic patch that mediates the high affinity interaction between Ro60 and Y RNAs (Stein et al. 2005). *In vitro* binding assays and chemical probing revealed that Ro60 binds a bulged helix present in all identified animal cell Y RNAs and predicted that Ro60 contacts conserved bases in the Y RNA major groove (Green CD et al. 1998). This model was confirmed by the co-crystal structure, where Ro60 was found to wedge apart the Y RNA stem (Stein et al. 2005). Surprisingly, although nucleotides on both sides of the helix are conserved, Ro60 primarily interacts with the 5' strand, making both base-specific contacts and interactions with the RNA backbone (Stein et al. 2005).

In addition to binding Y RNAs, Ro60 is found complexed with misfolded ncRNAs in some animal cell nuclei (O'Brien and Wolin 1994; Shi et al. 1996; Chen et al. 2003). Binding assays revealed that Ro60 preferentially binds RNAs with structured regions adjacent to a single stranded 3' end (Fuchs et al. 2006). The *X. laevis* Ro60 torus contains a basic central cavity, ~10–15 Å in diameter. Co-crystallization of Ro60 with a fragment of misfolded 5S rRNA revealed that the minor groove of the 5S rRNA duplex interacts with a basic platform surrounding this cavity, while the adjacent single stranded 3' end inserts through the hole (Fuchs et al. 2006). The interaction between Ro60 and the pre-5S duplex RNA is almost entirely mediated through interactions with the sugar-phosphate backbone, allowing Ro60 to bind a wide variety of structured RNA substrates in a largely non-sequence-specific fashion (Fuchs et al. 2006). Although the crystal structures include only portions of both the Y RNA and misfolded pre-5S rRNA, mutagenesis and biochemical studies support a model in which binding of the remainder of the Y RNA to the basic platform can regulate access of the misfolded pre-5S rRNA, and other RNA substrates, to the Ro60 cavity (Stein et al. 2005). Most *X. laevis* Ro60 residues that are important for Y RNA and misfolded pre-5S rRNA binding are conserved in bacterial Rsr proteins (Stein et al. 2005; Fuchs et al. 2006; Ramesh et al. 2007; Greiling et al. 2018).

One notable difference between the *D. radiodurans* and the *X. laevis* structures is a pronounced shift in the orientation of helices H15-H18 comprising the HEAT repeats (Ramesh et al. 2007). In *D. radiodurans* Rsr, this alteration results in a larger central cavity (Figure 1), which is large enough to accommodate double-stranded RNA (Ramesh et al. 2007). One possible explanation for this difference is the occupancy of the MIDAS in the two structures. For those proteins whose MIDAS ligand is known, an Asp or a Glu from the ligand, together with conserved residues that make up the MIDAS (D-x-S-x-S...T...D) coordinates the divalent cation (Springer 2006). In *X. laevis* Ro60, an acetate from the crystallization buffer completes the coordination of the Mg^{2+} bound at the MIDAS, while in the *D. radiodurans* structure, the MIDAS binds Ca^{2+} and the coordination is completed by a glutamate contributed by an adjacent Rsr in the crystal (Stein et al. 2005; Ramesh et al. 2007). Although both the *X. laevis* and *D. radiodurans* structures have a pseudoligand (acetate or glutamate) completing the MIDAS conformation, it is possible that the presence of the larger and more physiologic glutamate at the Rsr MIDAS results in conformational

changes that widen the cavity. In this case, Ro60 and its bacterial orthologs may be inherently flexible, adopting various conformations when bound to different proteins or RNAs. In support of this idea, the orientation of the HEAT repeats in the RNA-free, Y RNA-bound, and misfolded pre-5S RNA-bound *X. laevis* structures are all slightly different (Stein et al. 2005; Fuchs et al. 2006). Additional structures, together with identification of the putative MIDAS ligand(s), will be required to test this hypothesis.

Y RNAs

Y RNAs are a family of RNA polymerase III transcripts that are defined both by their secondary structure and by their interaction with Ro60. Y RNAs, like many ncRNAs, are modular. One module is composed of a stem, formed by base pairing of the 5' and 3' ends, which contains the conserved high affinity Ro60 binding site (Figure 2). In contrast to the binding of Ro60 to misfolded ncRNAs, which depends on structure rather than sequence, the Ro60 interaction with Y RNAs requires a highly conserved sequence in the Y RNA stem (Figure 2, A-B) (Wolin and Steitz 1984; Puijn et al. 1991; Green CD et al. 1998). The second module of the Y RNA consists of one or more pyrimidine-rich stem loops at the other end of the stem from the 5' and 3' ends (van Gelder et al. 1994; Teunissen et al. 2000). This module contributes to Ro60 function by regulating the subcellular location of Ro60 and its interactions with other proteins and RNAs.

The number of distinct Y RNAs varies between species. Humans have four Y RNAs: hY1, hY3, hY4 and hY5 (Hendrick et al. 1981; Wolin and Steitz 1983, 1984) (Figure 2A). The genes encoding human Y RNAs are single copy and reside within 45 kb on chromosome 7 (Wolin and Steitz 1983; Maraia et al. 1994). Although they are clustered, each Y RNA has its own promoter and is transcribed individually, with Y3, Y4 and Y5 transcribed from the plus strand and Y1 transcribed from the minus strand. Evolutionary analysis suggests that this cluster of 4 Y RNAs represents the ancestral tetrapod Y RNA composition, although both the loss of one or more of these Y RNAs and the acquisition of new Y RNAs has been a common occurrence (Mosig et al. 2007; Perreault et al. 2007). For example, the mouse has only Y1 and Y3 RNA, while *X. laevis* contains Y3, Y4 and Y5 orthologs and an additional Y RNA designated Y α (Hendrick et al. 1981; Wolin and Steitz 1983; O'Brien et al. 1993).

In addition to the canonical Y RNAs, hundreds of variant Y RNA sequences exist throughout the genomes of humans and other primates (Perreault et al. 2005; Perreault et al. 2007). Because most of these sequences lack RNA polymerase III promoters and terminators, they have been assumed to be pseudogenes (Perreault et al. 2005). Consistent with this idea, although Y RNAs require Ro60 binding for their stability (Labbe et al. 1999; Chen et al. 2000; Xue et al. 2003), most of the variant RNAs contain mutations that are predicted to eliminate Ro60 binding (Perreault et al. 2005). Many of the pseudogenes are found within introns, and a majority contain the poly(A) tails and target site duplications that are characteristic of retrotransposition by LINE-1 retrotransposon-encoded proteins (Perreault et al. 2005). Interestingly, Y5 RNA has significantly fewer pseudogenes than the other Y RNAs in all species thus far analyzed (Perreault et al. 2005; Perreault et al. 2007).

In some bacterial Y RNAs, known as YrlA (Y RNA-like A) RNAs (Figure 2C), the second module shares significant structural similarity with tRNAs (Figure 3) (Chen et al. 2014; Wang et al. 2018). A crystal structure of this domain from the YrlA RNA of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) confirmed the presence of tRNA-equivalent regions including an acceptor stem, anticodon stem and D and T stem loops (Wang et al. 2018) (Figure 3, B-C). The crystal structure also revealed that most tertiary interactions that stabilize tRNAs also exist in YrlA (Wang et al. 2018). Despite the overall structural similarities between the YrlA tRNA-like domain and tRNAs, there are notable differences. YrlA lacks an anticodon loop, as the region of YrlA that resembles an anticodon stem is attached to the Ro60 binding module. Additionally, while the acceptor stems of all mature tRNAs ends in 3' CCA, in YrlA RNAs, this feature is replaced by a closed loop. In addition to structural similarities, this class of bacterial Y RNAs behave as tRNA mimetics in cells. They undergo nucleotide modification by TruB and DusA (Chen et al. 2014) (Figure 3A), the tRNA modification enzymes that modify highly conserved uridines in the T and D loops to pseudouridine and dihydrouridine, respectively (Gutgsell et al. 2000; Bishop et al. 2002). How the tRNA-like domain contributes to YrlA RNA function is currently unknown.

Roles of Ro60 and Y RNAs in RNA metabolism

Evidence that Ro60 functions in noncoding RNA quality control

Because Ro60 is bound to aberrant forms of ncRNAs in several vertebrate cell types, it has been proposed to function in ncRNA quality control. This was first described in *X. laevis* oocyte nuclei, where Ro60 was found associated with a large collection of variant pre-5S rRNAs. *X. laevis* contains ~24,000 oocyte-specific 5S rRNA genes, many of which contain nucleotide changes from the consensus “wild-type” sequence (Fedoroff and Brown 1978; Miller et al. 1978). The pre-5S rRNAs bound by Ro60 contain nucleotide changes that cause the RNAs to misfold into incorrect structures as well as additional gene-encoded nucleotides at the 3' end, due to readthrough of the first RNA polymerase III termination signal (O'Brien and Wolin 1994; Shi et al. 1996). Consistent with degradation by a surveillance pathway, the variant pre-5S rRNAs were processed less efficiently than wild-type 5S rRNA, were less stable and were not present in ribosomes (O'Brien and Wolin 1994). Subsequently, Ro60 was found associated with variant U2 snRNAs in mouse embryonic stem cells (Chen et al. 2003). Similar to the findings with *X. laevis* pre-5S rRNAs, the interaction of Ro60 with U2 snRNA variants occurred in nuclei and required that the RNA misfold to form an alternative helix (Chen et al. 2003). In support of the idea that Ro60 binding prevents variant defective noncoding RNA transcripts from assembling into mature RNPs, *C. elegans* lacking Ro60 have increased levels of mutant 5S rRNAs in their ribosomes (Labbe et al. 1999).

Biochemical studies support a model in which Ro60 binds structured ncRNA precursors that fail to assemble with their specific protein partners. Specifically, *in vitro*, Ro60 was found to bind RNAs containing both helices and a single-stranded 3' end of at least 5 nt (Fuchs et al. 2006). However, although Ro60 does not bind wild-type 5S rRNA containing additional 3' nucleotides when oocytes or egg extracts are used as a source of Ro60 protein (O'Brien and Wolin 1994; Shi et al. 1996), the purified Ro60 protein binds the protein-free form of wild-type pre-5S rRNA with an affinity only four-fold lower than that of the misfolded RNA

(Fuchs et al. 2006). Together, these data support a model in which Ro60 normally functions in competition with the specific protein partners intended to interact with that RNA. Consistent with a role as a scavenger, the affinity of Ro60 for its misfolded ncRNA substrates is ~25 to 100 nM (Fuchs et al. 2006), while specific RNA-binding proteins usually bind their targets with affinities ~10 nM (Stefl et al. 2005). As many ncRNAs, such as pre-tRNAs and precursors to snRNAs and small nucleolar RNAs, are transcribed as 3' extended precursors that require post-transcriptional processing to achieve their mature form, the spectrum of Ro60 targets could be extensive.

How does Ro60 affect the fate of its bound ncRNA substrates? One possibility is that Ro60 channels defective RNAs into a decay pathway. Consistent with this role, the fate of the misfolded pre-5S rRNAs bound by *X. laevis* Ro60 is to be degraded (O'Brien and Wolin 1994) and a bacterial Ro60 functions with exoribonucleases to degrade structured RNAs (Chen et al. 2007; Chen et al. 2013). Additionally, Ro60 has been described to bind both sense and antisense transcripts of Alu sequences in human cells (Hung et al. 2015). In support of a role for Ro60 in decay of Alu RNAs, cells deleted for Ro60 were reported to have increased levels of these transcripts (Hung et al. 2015). However, in contrast to bacteria, where a Ro60 ortholog functions with two 3' to 5' exoribonucleases, RNase II and RNase PH, to mature 23S rRNA (Chen et al. 2007), and functions with the 3' to 5' exoribonuclease polynucleotide phosphorylase in rRNA degradation (Wurtmann and Wolin 2010; Chen et al. 2013), an annotated nuclease has not been described to function with the vertebrate Ro60. As the proteins that co-purify with Ro60 from mammalian cells include multi-functional RNA-binding proteins such as Y-box proteins, nucleolin, and the MOV10 RNA helicase (Fouraux et al. 2002; Sim et al. 2012), a role in assisting RNA chaperones or helicases in unfolding misfolded RNAs is also possible.

How might Y RNAs function with Ro60 in ncRNA quality control? Since pre-5S rRNA and Y RNA bind overlapping sites on Ro60 (Stein et al. 2005; Fuchs et al. 2006), and compete for binding to purified Ro60 (Green CD et al. 1998), one role of Y RNAs could be to sterically block misfolded RNAs from accessing the Ro60 cavity (Stein et al. 2005). Evidence that Y RNAs can function in this way has been provided in bacteria, where the Y RNA-free form of the *D. radiodurans* Ro60 ortholog functions in pre-23S rRNA maturation, and Y RNA acts as an inhibitor (Chen et al. 2007). Consistent with a model in which the Y RNA-free form of Ro60 binds misfolded RNAs, approximately half of the Ro60/pre-5S RNA in *X. laevis* oocytes is located within nuclei, while all detectable Y RNAs are cytoplasmic (O'Brien and Wolin 1994). However, in human cells, where Y5 RNA is mostly nuclear (Gendron et al. 2001), Y5 RNA and misfolded pre-5S rRNA co-purified when both components were overexpressed from plasmids, providing evidence that misfolded pre-5S rRNA may access the hY5-bound Ro60 (Hogg and Collins 2007). One possibility is that, as in bacteria (see below), the binding of a partner protein to the Y5 stem-loops prevents this portion of Y5 RNA from sterically blocking the Ro60 substrate-binding surface, allowing pre-5S rRNA to access the Ro60 cavity.

Ro60 and Y RNA function in RNA processing and degradation in bacteria during stress

In bacteria, the Ro60 ortholog Rsr functions with exoribonucleases to alter RNA populations in response to stress (Chen et al. 2007; Wurtmann and Wolin 2010; Chen et al. 2013). Studies of Rsr in the first sequenced bacterial genome with a Ro60 ortholog, *D. radiodurans*, revealed that Rsr is important for survival following UV irradiation (Chen et al. 2000) and enhances survival during prolonged growth in stationary phase (Wurtmann and Wolin 2010). Consistent with a role in stress response, the levels of Rsr and Y RNAs increase following UV irradiation, γ -irradiation, and desiccation (Chen et al. 2000; Tanaka et al. 2004) as well as during heat stress and protracted growth in stationary phase (Chen et al. 2007; Wurtmann and Wolin 2010). Although the targets of Rsr following exposure to DNA damaging agents were not identified, Rsr functions in the maturation of 23S ribosomal RNA during heat stress (Chen et al. 2007). In *D. radiodurans*, the 23S ribosomal RNA is made as a 5' and 3' extended precursor that requires both endonucleolytic cleavage and exonucleolytic trimming to reach the mature form. At the normal growth temperature of 30°C, this process is inefficient, as precursors containing both 5' and 3' extensions accumulate (Chen et al. 2007). At 37°C, which results in heat stress in *D. radiodurans* (Holland et al. 2006), 23S rRNA maturation becomes efficient through a process that requires both Rsr and the 3' to 5' exoribonucleases RNase PH and RNase II (Chen et al. 2007). The incompletely matured 23S transcripts that accumulate in the absence of Rsr end in regions of significant secondary structure (Chen et al. 2007), suggesting that Rsr may function as a processivity factor to facilitate exoribonuclease progression through structured RNA. Y RNA was found to act as an inhibitor of this Rsr function, as cells lacking Y RNA exhibited efficient maturation at both temperatures (Chen et al. 2007). Similarly, overexpression of a mutant Rsr with diminished Y RNA binding capacity resulted in efficient maturation at 30°C (Chen et al. 2007). This observation is consistent with biochemical studies demonstrating that Y RNAs and misfolded pre-5S rRNAs compete for binding to Ro60 *in vitro* (Green CD et al. 1998) and with structural studies that predicted that Y RNA might regulate access of RNA substrates to the central cavity (Stein et al. 2005; Fuchs et al. 2006). These bacterial studies also confirmed that Ro60/Rsr can function as a Y RNA-free protein, as suggested by findings that a pool of Y RNA-free Ro60 occurs in mammalian cell nuclei (Peek et al. 1993), and that Ro60 is found complexed with misfolded pre-5S rRNAs (but not Y RNAs) in *X. laevis* oocyte nuclei (O'Brien and Wolin 1994).

During extended growth in stationary phase, a stress that resembles starvation, *D. radiodurans* Rsr functions with the ring-shaped 3' to 5' exoribonuclease polynucleotide phosphorylase (PNPase) to degrade mature rRNAs (Wurtmann and Wolin 2010). Although bacterial ribosomes are normally very stable, conditions that result in nutrient deficiency can result in rRNA degradation (Deutscher 2009). This allows bacteria to recycle and access nucleotides and amino acids contained within ribosomes and to decrease their energy expenditure by limiting protein production. Using *in vivo* crosslinking followed by immunoprecipitation (CLIP) (Ule et al. 2005), Rsr was found to interact with numerous sites in mature 23S and 16S rRNAs (Wurtmann and Wolin 2010). As the exoribonuclease PNPase, which functions in rRNA quality control and degradation in *E. coli* (Kaplan and Apirion 1975; Cheng and Deutscher 2003) co-purifies with Rsr (Chen et al. 2007), a role for Rsr in facilitating rRNA degradation by PNPase was tested. After extended growth at

stationary phase, Rsr levels increased 30-fold, while the association between Rsr and PNPase increased four-fold (Wurtmann and Wolin 2010). Sucrose gradient sedimentation revealed a 10-fold increase in the amount of PNPase sedimenting with degrading ribosomal subunits at stationary phase. Importantly, in cells lacking Rsr, the increase in PNPase sedimenting with degrading ribosomal subunits was not observed (Wurtmann and Wolin 2010), revealing that Rsr is important for the stable association of PNPase with these targets. Moreover, rRNA levels increased in strains lacking Rsr, Y RNA, or PNPase during extended growth at stationary phase, suggesting that all three components functioned together to degrade rRNA (Wurtmann and Wolin 2010).

Structural and biochemical studies revealed that Rsr, Y RNA and PNPase were complexed to form a double-ringed RNA degradation machine (Figure 4) (Chen et al. 2013). This complex could be both purified from *D. radiodurans* and reconstituted by co-expressing all three components in *E. coli*. Single particle electron microscopy, followed by docking atomic models of Rsr (Ramesh et al. 2007), Ro60 bound to Y RNA (Stein et al. 2005), PNPase (Symmons et al. 2000) and Ro60 bound to misfolded pre-5S rRNA (Fuchs et al. 2006) into the 3D reconstruction, resulted in a model in which the 3' end of a substrate RNA feeds through the Rsr cavity into the PNPase cavity for degradation (Chen et al. 2013).

Biochemical studies revealed that the single-stranded loops at the distal portion of the Y RNA interact with the single-stranded RNA-binding S1/KH domains of PNPase (Chen et al. 2013). When tethered to PNPase, Rsr both enhances the ability of PNPase to degrade structured RNA substrates and reduces its activity on single-stranded RNAs. Thus, by tethering Rsr to PNPase, Y RNA alters the substrate specificity of this enzyme (Chen et al. 2013). This novel RNP machine was termed RYPER (Rsr/Y RNA/PNPase Exoribonuclease RNP). As Rsr, Y RNA and PNPase exhibit genetic interactions that are enhanced during growth at high or low temperatures, during oxidative stress and during recovery from UV irradiation (Chen et al. 2007), the RYPER complex may play a role in a variety of stress responses.

While the use of ncRNA to tether an RNA-binding protein to an exonuclease is a novel way of generating an RNA degradation machine, the RYPER complex shares features of the archaeal and yeast RNA exosomes. Similar to both the archaeal and yeast exosomes (Evguenieva-Hackenberg et al. 2014; Zinder and Lima 2017), RYPER consists of an RNA-binding ring on top of an RNase PH domain-containing ring (Evguenieva-Hackenberg et al. 2014; Zinder and Lima 2017). As in the archaeal exosome (Evguenieva-Hackenberg et al. 2014), phosphorolytic RNA degradation in RYPER takes place within the RNase PH domain-containing ring. In the yeast exosome, RNA is funneled through a catalytically inactive RNase PH-domain ring to reach the hydrolytic 5' to 3' exoribonuclease DIS3 (Zinder and Lima 2017). Similar to the yeast exosome, passage through a catalytically inactive ring (Rsr or Exo1–9) may assist ATP-independent unwinding of structured RNA substrates, facilitating degradation by an active exoribonuclease (Bonneau et al. 2009; Chen et al. 2013).

Y RNAs Regulate the Subcellular Localization of Ro60

Another role of animal cell Y RNAs is to influence the subcellular location of their Ro60 protein partner. This has been best characterized in mouse cells, where a Ro60 mutant that lacks the ability to bind Y RNAs accumulates in nuclei (Sim et al. 2009). Additionally, depletion of either mouse Y3 or both mouse Y RNAs (Y1 and Y3 RNAs) results in nuclear accumulation of Ro60 (Sim et al. 2009). When expressed in mouse cells, *D. radiodurans* Ro60 does not bind mouse Y RNAs but shows only slight nuclear accumulation, suggesting that mouse Ro60, but not the *D. radiodurans* ortholog, contains a nuclear accumulation signal. Analysis of the subcellular localization and Y RNA binding properties of various *D. radiodurans*-mouse chimeric Ro60 proteins suggested that the signal for nuclear accumulation resides in the vicinity of mouse Ro60 HEAT repeat 12. As both the crystal structure of *X. laevis* Ro60 and mutagenesis studies revealed that this region contributes to Y RNA binding (Stein et al. 2005), these data suggest that bound Y RNA masks a nuclear accumulation signal, retaining Ro60 in the cytoplasm (Sim et al. 2009).

In addition to its role in retaining Ro60 in the cytoplasm, Y3 facilitates the nuclear export of Ro60 (Sim et al. 2012). Upon treatment of mouse cells with leptomycin B, a specific inhibitor of the nuclear export receptor CRM1 (Fornerod et al. 1997), Ro60 accumulates in nuclei, indicating that it normally shuttles between the nucleus and cytoplasm (Sim et al. 2012). Although Ro60 does not contain a recognizable CRM1-dependent nuclear export signal (NES), Ro60 is tethered by Y3 RNA to the zipcode binding protein ZBP1/IGF2BP1, an RNA-binding protein whose nuclear export is partially dependent on CRM1 (Nielsen et al. 2003; Oleynikov and Singer 2003; Sim et al. 2012). As depletion of ZBP1 results in nuclear accumulation of Ro60 and Y3 RNA (Sim et al. 2012), these results support a model in which ZBP1 functions as an adaptor for nuclear export of Ro60/Y3 RNPs.

Although most Ro60/Y RNA complexes are localized to the cytoplasm in vertebrate cells, the location of these RNPs can vary in different cell types and can change in response to stress. In human cells, the Ro60/Y5 RNA is primarily nuclear, while the complexes formed by Ro60 with the other three Y RNAs are cytoplasmic (Gendron et al. 2001). Since Y5 is confined to the cytoplasm of *X. laevis* oocytes (O'Brien and Wolin 1994), the nuclear location of the human Y5 could reflect specific protein binding and/or a feature of the human RNA that is lacking in its *Xenopus* ortholog. Consistent with a role for a binding protein, the nuclear RNA-binding protein PUF60 co-purifies with the human Ro60/Y5 RNP (Bouffard et al. 2000; Hogg and Collins 2007). The location of Ro60 RNPs can also change in response to stress, as the mouse Ro60/Y3 RNA complex accumulates in nuclei following UV irradiation of two different cell lines (Chen et al. 2003). As the levels of Ro60 and Y RNAs are unchanged following irradiation, the change in cellular location may reflect a stress-induced rearrangement of the Y3 RNA on the Ro60 surface that exposes the nuclear accumulation sequence.

Y3 RNA is also important for the subcellular location of Ro60 during apoptosis. In early apoptosis, a fraction of Ro60 and other normally intracellular autoantigens traffics to the cell surface and becomes accessible to autoantibodies (LeFeber et al. 1984; Golan et al. 1992; Casciola-Rosen et al. 1994). Although the mechanisms by which these normally cytoplasmic and nuclear RNPs reach the cell surface are unknown, the ability of Ro60 to reach this

destination was found to depend on Y3 RNA (Reed et al. 2013). Specifically, in mouse fibroblasts, a Ro60 that is unable to bind Y RNAs fails to reach the cell surface during apoptosis, and depletion of Y3 RNA also prevents Ro60 surface exposure (Reed et al. 2013). Interestingly, in some cell types, Y RNAs are largely degraded early in apoptosis, leaving only those fragments of Y RNAs which are protected by binding of Ro60 and La, a protein that binds the ends of all nascent polymerase III transcripts (Rutjes et al. 1999). Whether Y3 fragments, as opposed to full length Y3 RNA, are required for surface exposure of Ro60 during apoptosis is not known.

A working model for Ro60 and Y RNA function in RNA metabolism

Although our knowledge of the mechanisms by which Ro60 and Y RNAs affect the fate of Ro60-bound RNA substrates is incomplete, a model can be proposed based on the existing data. Because Ro60 binds the single-stranded 3' ends of structured RNAs via its central cavity (Stein et al. 2005; Fuchs et al. 2006), and binding is not strongly dependent on sequence, Ro60 should be able to bind a variety of structured RNAs, including many ncRNA precursors. Experiments in bacteria reveal that the Ro60 ring can function with 3' to 5' exoribonucleases, either as a free ring or tethered by Y RNA to single-stranded RNA binding domains adjacent to the catalytic portion of the exoribonuclease (Chen et al. 2007; Chen et al. 2013). In either case, Ro60 may function as a processivity factor by assisting ATP-independent unwinding of structured RNAs (Chen et al. 2007; Chen et al. 2013). However, Y RNA-mediated tethering of Ro60 also obstructs the single-stranded binding domain of PNPase and replaces it with the RNA-binding surface of Ro60, thus specializing PNPase for binding structured RNAs and reducing its affinity for single-stranded RNA substrates (Chen et al. 2013). Y RNA-mediated tethering, which places the Rsr ring above the PNPase cavity, may also increase degradation efficiency by allowing single-stranded RNA to thread directly from the Rsr ring into the PNPase cavity (Chen et al. 2013).

Based on these data, we propose that Ro60 and its associated Y RNAs function in conjunction with effector proteins (Figure 5). The Y RNA-mediated tethering of Ro60 to catalytically active partners, such as helicases, RNA chaperones and exoribonucleases, could enhance binding and/or unwinding of structured RNAs, thus increasing the effectiveness of these proteins (Figure 5D). Conversely, as in the case of ZBP1 (Sim et al. 2012), Y RNA-mediated tethering of Ro60 to an effector protein could alter the subcellular location or function of Ro60 (Figure 5E). We envision that the presence of multiple Y RNAs in most species may expand the range of Ro60 functions by allowing Ro60 to be tethered to a larger variety of RNA-binding domains on effector proteins. The ability of Ro60 to function as a Y RNA-free protein may further increase its versatility (Figure 5A), while the ability of Y RNA to block access of RNA substrates to the cavity in the absence of a bound effector protein (Figure 5B) (Green CD et al. 1998; Stein et al. 2005; Chen et al. 2007) provides a mechanism for how Ro60 function can be regulated. Finally, the presence of the Ro60 MIDAS, together with the differences in size and shape of the *X. laevis* Ro60 and *D. radiodurans* Rsr cavities (Stein et al. 2005; Ramesh et al. 2007) suggests that protein-protein interactions at this site may also regulate Ro60 conformation and function.

Other proposed functions for Y RNAs

Both Y RNAs and transcripts derived from their pseudogenes may act as sponges for some RNA-binding proteins, diverting them from their mRNA targets (Figure 5C). The neuron-specific ELAV-like (nELAVL) proteins HuB, HuC and HuD bind U-rich sequences in mRNA 3' UTRs and introns, influencing alternative splicing, mRNA stability and translation (Ince-Dunn et al. 2012; Scheckel et al. 2016). Surprisingly, experiments using *in vivo* crosslinking to identify targets of these proteins in human neuroblastoma cells and brain tissue recovered Y RNA pseudogene transcripts, as well as all four human Y RNAs (Scheckel et al. 2016). As expected, most nELAVL-bound Y RNA sequences contain a U-rich motif (Scheckel et al. 2016). Because overexpression of a wild-type Y3 RNA resulted in decreased association of nELAVL with some intronic targets, it was proposed that Y RNA binding sequesters nELAVL from pre-mRNAs (Scheckel et al. 2016). However, since the majority of Y RNA pseudogenes lack both promoters and terminators (Perreault et al. 2005), it is unclear whether this reflects a role for *bona fide* noncoding Y RNAs, or whether nELAVL binds Y RNA sequences that are embedded within products of pervasive RNA polymerase II transcription and/or transcribed as part of nascent pre-mRNAs.

In contrast to humans, mouse cells contain only two Y RNAs (Y1 and Y3) and few pseudogenes (Hendrick et al. 1981; Perreault et al. 2005). Possibly due to these differences, a recent study of HuD targets in mouse motor neuron cells overexpressing human HuD protein identified only Y3 as a prominent interactor (Tebaldi et al. 2018). Because either overexpressing the human HuD or depleting the endogenous mouse Y3 RNA with siRNAs resulted in enhanced translation of HuD target mRNAs, it was proposed that Y3 acts as a sink for HuD (Tebaldi et al. 2018). Whether these proposed functions of Y RNAs and their pseudogenes involve Ro60 remains to be investigated.

Although Y RNAs are nearly quantitatively bound by Ro60 (Wolin and Steitz 1983; Wolin et al. 2013), and are unstable in cells and tissues lacking Ro60 (Labbe et al. 1999; Chen et al. 2003; Xue et al. 2003), a Ro60-independent role for Y RNAs in DNA replication has been proposed (Christov et al. 2006; Langley et al. 2010). Nuclei isolated from cells arrested late in the G1 phase of the cell cycle can initiate DNA replication in the presence of cytoplasmic extracts prepared from proliferating cells (Krude 2000). Fractionation of the cytosolic extracts and oligonucleotide-directed RNase H cleavage of Y RNAs revealed that extracts lacking Y RNAs were incapable of initiating DNA replication (Christov et al. 2006). Interestingly, removal of the Ro60-bound Y RNA fraction from these extracts did not impede DNA replication, suggesting this function is Ro60 independent (Langley et al. 2010). This observation has been extended to zebrafish, *X. laevis* and *C. elegans*, where inhibition of Y RNA function using antisense morpholinos results in embryonic lethality and arrest of DNA replication (Collart et al. 2011; Kowalski et al. 2015). However, it is difficult to reconcile these findings with the fact that Y RNAs are drastically reduced in both *C. elegans* and mouse cells lacking Ro60, yet no significant effects have been detected on the viability of mice or worms lacking Ro60, or on the viability of cells (Labbe et al. 1999; Chen et al. 2003; Xue et al. 2003). Similarly, no growth defects have been reported when Y RNAs are depleted from mouse cells with either siRNAs or shRNAs (Sim et al. 2009; Reed et al. 2013).

Roles for Ro60 and Y RNAs in the cellular response to stress

Taken together, the existing data from bacterial and animal systems support a function for Ro60 RNPs in the mitigation of cellular stress. In *D. radiodurans*, the bacterium where Ro60 has been best-studied, Ro60 and Y RNAs are upregulated following UV irradiation, starvation, heat stress, γ -irradiation and desiccation (Chen et al. 2000; Tanaka et al. 2004; Chen et al. 2007; Wurtmann and Wolin 2010). Although the exact mechanisms by which Ro60 contributes to bacterial physiology following UV, γ -irradiation and desiccation remain to be defined, the findings that Ro60 contributes to *D. radiodurans* survival after UV irradiation and starvation (Chen et al. 2000; Wurtmann and Wolin 2010) indicate that the upregulation of Ro60 following environmental stress is functionally relevant. Consistent with a general role for Ro60 and Y RNAs in the bacterial stress response, Ro60 and Y RNAs are often encoded within a tightly regulated operon that is implicated in RNA repair because it also encodes RtcB, the RNA ligase that functions to ligate tRNA halves in archaea and animal cells (Chen et al. 2013; Das and Shuman 2013; Burroughs and Aravind 2016). Although the signals that allow transcription of this operon are not well understood, the *S. Typhimurium* operon is transcribed during infection of human cells and upon treatment of *S. Typhimurium* with DNA damaging agents (Westermann et al. 2016; Kurasz et al. 2018). Although the role of animal cell Ro60 RNPs in the response to environmental stress is less studied, Ro60 is important for survival of mouse cells after UV irradiation (Chen et al. 2003) and for formation of dauer larvae, an alternative larval stage formed when worms encounter conditions unfavorable for growth (Labbe et al. 2000).

UV radiation

Ro60 has an evolutionarily conserved role in mitigating stress from UV radiation. In *D. radiodurans*, exposure to UV results in a four-fold increase in Rsr and Y RNA (Chen et al. 2000). As *D. radiodurans* lacking Rsr show decreased survival after exposure to UV (Chen et al. 2000), the upregulation is likely to be a functionally relevant aspect of the response to UV. Although exposure to UV has not been found to increase expression of Ro60 or Y RNAs in animal cells, both mouse embryonic stem cells and mouse fibroblasts show a significant redistribution of Ro60 from the cytoplasm to the nucleus after exposure to UV (Chen et al. 2003; Sim et al. 2009; Sim et al. 2012). Decreased survival after exposure to UV is observed for mouse ES cells lacking Ro60 in culture (Chen et al. 2003) and for keratinocytes irradiated *in vivo* in *RO60*^{-/-} mice (Xue et al. 2003). Specifically, when exposed to UVB light, *RO60*^{-/-} mice have twice as many apoptotic keratinocytes as their wild type littermates. Loss of Ro60 specifically sensitizes cells to UV radiation, as both bacteria and mouse cells lacking Ro60 do not exhibit differences in survival after exposure to ionizing radiation (Chen et al. 2000; Chen et al. 2003).

How Ro60 contributes to survival following UV irradiation remains unknown. Although the ways in which UV irradiation damages DNA and the subsequent effects on cell survival and malignancy are well studied (Ichihashi et al. 2003), the effects of RNA damage are far less well understood. However, there is evidence that RNA can be damaged either directly by UV, or indirectly by reactive oxygen species produced after UV exposure [see (Wurtmann and Wolin 2009) for review]. Interestingly, Ro60 is not the only ring-shaped RNA-binding

protein that increases survival after exposure to UV. Two proteins that are each part of ring-shaped assemblies of Sm-like proteins, bacterial Hfq and *S. cerevisiae* Lsm1, are also important for cell survival following UV irradiation (Tsui et al. 1994; Spicakova et al. 2008). Both proteins form ring-shaped assemblies similar in size to Ro60 and bind single-stranded RNA, although Hfq forms a homohexameric ring and Lsm1 assembles with six other Lsm proteins (Lsm2–7) to form a heterohexamer [reviewed by (Wilusz and Wilusz 2013)]. Although the mechanism by which Hfq increases bacterial survival after UV irradiation is unknown, the Lsm1–7 complex is proposed to assist survival by protecting the 3' ends of mRNAs from degradation (Spicakova et al. 2008). Notably, UVB exposure has been reported to alter the structure of the U1 snRNA *in vivo*, resulting in the generation of RNA fragments (Bernard et al. 2012). Thus, one possibility is that Ro60 functions after UV exposure to target structurally aberrant ncRNAs and their fragments for degradation. A detailed study of RNA populations after UV irradiation of wild-type and *RO60*^{−/−} cells may be helpful for determining how Ro60 contributes to the survival of irradiated cells.

Repressing Alu Elements

Mobile elements make up approximately 45% of the human genome, with the Alu short interspersed elements (SINE) being the most abundant, comprising approximately 10% of the mass of the human genome [see (Batzer and Deininger 2002) for review]. Alu elements are preferentially found in gene-rich regions, and negative effects of their insertion have been linked to human disease [see (Deininger and Batzer 1999) for review]. Alu transcripts are degraded, at least in part, by DICER1 cleavage, and reduced levels of DICER1 result in Alu RNA accumulation (Kaneko et al. 2011). Aberrant accumulation of Alu RNAs in retinal pigmented epithelium, due to reduced DICER1, has been linked to cellular toxicity in age-related macular degeneration (Kaneko et al. 2011). Interestingly, Ro60 was found to bind Alu transcripts by CLIP, and *RO60*^{−/−} cells were reported to have elevated levels of Alu transcripts and interferon-regulated genes (Hung et al. 2015). These results suggest that Ro60 may normally degrade Alu RNAs, preventing them from activating intracellular RNA sensors (Hung et al. 2015). However, in the analyses of this CLIP dataset, individual Alu-derived reads were allowed to map to multiple loci without correcting for the fact that one read can map to multiple sites, a protocol that results in artifactual elevation of repetitive transcripts (Marinov et al. 2015). Additionally, the bioinformatic analyses of the whole-transcriptome sequencing data that revealed increased Alu RNAs in *RO60*^{−/−} cells did not distinguish between reads derived from *bona fide* Alu elements, which are transcribed by RNA polymerase III into ~300 nt polyadenylated RNAs, and the many Alu sequences that are expressed because they are housed within RNA polymerase II-transcribed mRNAs or pre-mRNAs (Hung et al. 2015). Additional studies addressing the roles of Ro60 in mammalian RNA metabolism may clarify these issues.

Resource limitation

Ro60 also plays a role in the response to stress in *C. elegans*. In favorable environmental conditions, *C. elegans* embryos progress through four larval stages to become adult worms. Under stressful conditions, such as high population density, limited food or abnormal temperature, *C. elegans* larvae can enter an alternative stress-resistant state known as dauer [reviewed by (Fielenbach and Antebi 2008)]. Dauer larvae can survive in this state for

months before resuming normal development once environmental conditions have improved. Worms lacking Ro60 have defects in dauer formation, and Ro60 exhibits genetic interactions with a number of genes that modify entry into the dauer state (Labbe et al. 2000).

Ro60 in autoimmunity

Both anti-Ro antibodies and their Ro60 target are subjects of considerable clinical interest. Due to the prevalence of these antibodies in patients with Sjogren's syndrome, the presence of these antibodies is a major criterion for diagnosis (Shiboski et al. 2012). In addition, maternal anti-Ro antibodies are strongly associated with neonatal lupus, a passively acquired autoimmune syndrome that can manifest as permanent cardiac conduction defects and/or sunlight-sensitive rashes. Since the skin lesions resolve when the maternal antibodies clear from the baby's circulation (Lee 2009), the antibodies may play a direct role in pathogenesis. Notably, the skin lesions of neonatal lupus are histologically identical to lesions in adults with subacute cutaneous lupus erythematosus, a highly photosensitive form of skin lupus in which anti-Ro antibodies are nearly always present (Sontheimer 2005; Lee 2009). Lastly, several longitudinal studies have revealed that the earliest detected autoantibodies in systemic lupus erythematosus patients react with Ro60 and can be detected years before onset of clinical symptoms (Arbuckle et al. 2003; McClain et al. 2005; Heinlen et al. 2010; Eriksson et al. 2011). Below, we discuss potential roles for Ro60 in rheumatic diseases as well as the molecular characteristics of Ro60 that may contribute to its involvement in autoimmunity.

Evidence that anti-Ro antibodies may be pathogenic in neonatal lupus

Neonatal lupus is the disease that most clearly links the presence of anti-Ro antibodies to pathology. Neonatal lupus presents as a spectrum of abnormalities that include annular skin lesions and cardiac conduction defects affecting neonates and fetuses of anti-Ro positive mothers (Vanoni et al. 2017). The most common cardiac manifestation of neonatal lupus is congenital heart block (CHB) occurring in the absence of structural abnormalities. CHB, which is classically complete atrioventricular block, is permanent, can be lethal in utero, and often requires pacemaker implantation in surviving infants (Vanoni et al. 2017).

In neonatal lupus, maternal anti-Ro antibodies cross the placental barrier from the mother to the child. Anti-Ro antibodies are detectable in both mother and child at birth but are transient in the child while persisting in the mother (Franco et al. 1981; Scott et al. 1983). The cutaneous manifestations of neonatal lupus resolve spontaneously within the first six months of life, a timeframe that matches the clearance of maternal antibodies from newborns (Lee 2010). The finding that antibodies are deposited in both the skin lesions and heart tissue in these infants supports a direct role for the maternal antibodies in disease pathogenesis (Lee and Weston 1984; Litsey et al. 1985). IgG deposition is accompanied by apoptosis, fibrosis and macrophage infiltration in CHB (Clancy et al. 2004). Neonatal lupus is not a consequence of disease in the mother, as many of the women who give birth to affected children are asymptomatic, although approximately half of them later develop autoimmune disease (Rivera et al. 2009).

The RNA components of Ro60 RNPs are potential TLR activators

While neonatal lupus provides evidence that anti-Ro antibodies can be pathologic, a long-standing question has been how circulating antibodies to an intracellular antigen can cause disease. There is now strong evidence that the nucleoproteins that are targets of the immune response in systemic lupus erythematosus induce inflammation through activation of endosomal toll-like receptors (TLRs) [reviewed in (Marshak-Rothstein and Rifkin 2007; Kawai and Akira 2011; Sharma, Fitzgerald, et al. 2015)]. These endosomal TLRs include the DNA sensor TLR9 and the RNA sensors TLR3, TLR7 and TLR8 (Marshak-Rothstein and Rifkin 2007; Kawai and Akira 2011; Sharma, Fitzgerald, et al. 2015). In the prevailing model, nucleic acid-containing autoantigens, upon release from dying cells, form immune complexes with patient antibodies which are then endocytosed by Fc γ receptor-bearing cells and presented to endosomal toll-like receptors (Marshak-Rothstein and Rifkin 2007; Kawai and Akira 2011; Sharma, Fitzgerald, et al. 2015). Binding of the RNA moieties to TLR7, or the DNAs to TLR9, activates a signaling pathway that results in high levels of IFN production, a common signature of the immune response in autoimmune diseases such as systemic lupus erythematosus, and also triggers cytokine secretion, leading to tissue damage (Kawai and Akira 2011; Crow 2014; Sharma, Campbell, et al. 2015). Support for this model has been provided by studies of mouse models of systemic lupus erythematosus (Sharma, Fitzgerald, et al. 2015) and by the fact that drugs that interfere with endosome acidification, such as hydroxychloroquine, are effective in treating lupus patients (Fava and Petri 2019). As systemic lupus erythematosus patients usually produce many different autoantibodies, it is mostly unclear which autoantigens cause specific symptoms. However, the strong association of anti-Ro antibodies with neonatal lupus, coupled with the fact that Ro60 (but not Ro52) is an RNA-binding protein, supports the hypothesis that Ro60 RNPs, in the form of immune complexes, may activate endosomal RNA-sensing TLRs. Consistent with this hypothesis, Y RNAs can activate autoreactive B cells *in vitro* through a mechanism that involves both TLR7 and TLR3 (Green NM et al. 2012). Additionally, immune complexes consisting of anti-Ro60 antibodies bound to Ro60/Y3 RNA RNPs stimulate macrophages to secrete TNF- α , a proinflammatory cytokine, through a TLR7-dependent mechanism (Clancy et al. 2010).

Mice lacking Ro60 develop a lupus-like syndrome

Remarkably, mice lacking Ro60 develop an autoimmune syndrome resembling systemic lupus erythematosus (Xue et al. 2003). Similar to patients, the mice produce autoantibodies to chromatin and ribosomes and exhibit glomerulonephritis due to immune complex deposition and also sunlight sensitivity (Xue et al. 2003). The mechanism by which loss of Ro60 leads to autoimmunity in these mice has not been elucidated. However, given existing evidence that excess intracellular DNA and RNA can activate cytosolic sensors, resulting in increased Type I interferon production and autoimmunity (Crowl et al. 2017), it is tempting to speculate that the autoimmunity in these mice is caused by aberrant RNA accumulation due to loss of RNA surveillance by Ro60 RNPs. Whatever the exact mechanism, the finding that mice lacking Ro60 develop autoantibodies and immune complex-mediated glomerulonephritis reveals that the normal function of Ro60 is important for preventing autoimmunity.

Evidence that microbial Ro60 orthologs could trigger lupus autoimmunity

Studies of both American and Northern European patients who eventually develop systemic lupus erythematosus have found that autoantibodies precede the onset of clinical symptoms by several years (Arbuckle et al. 2003; Eriksson et al. 2011). Remarkably, the earliest detectable autoantibodies are against Ro60 (Arbuckle et al. 2003; McClain et al. 2005; Heinlen et al. 2010; Eriksson et al. 2011). Epitope mapping experiments revealed that the first detected antibodies to Ro60 are directed against amino acids 169–180 (McClain et al. 2005). Because affinity-purified antibodies against this epitope cross-reacted with a viral protein, the Epstein-Barr virus-encoded EBNA1, it was proposed that the primordial antibodies in lupus patients originate through molecular mimicry between EBNA1 and Ro60 (McClain et al. 2005). However, EBNA1 and Ro60 lack primary sequence similarity, making it mysterious as to how EBNA1 could mimic the Ro60 protein. A subsequent study suggested that molecular mimicry between a Coxsackie virus protein and Ro60 may trigger anti-Ro60 antibodies in Sjogren's syndrome patients (Stathopoulou et al. 2005). More recently, a study in which mouse T cell hybridomas reactive with human Ro60 were found to be stimulated by bacterial peptides resulted in the hypothesis that some commensal bacterial proteins may contain epitopes which mimic portions of human Ro60 and thus act as initiators of an anti-Ro60 autoimmune response (Szymula et al. 2014).

An alternative possibility is that the Ro60 orthologs in bacteria that comprise the human microbiota could initiate the immune response against self Ro60 (Greiling et al. 2018). In contrast to the viral and bacterial proteins evoked as triggers in the above experiments, some commensal Ro60 orthologs show remarkable conservation of amino acids 169–180, the region of human Ro60 implicated in the initiation of lupus autoimmunity (Greiling et al. 2018). Bacteria containing Ro60 orthologs are present in a variety of anatomical sites, including the gut, skin, mouth, the airways, the nasopharyngeal tract, and the urogenital tract (Greiling et al. 2018). Consistent with the idea that commensal Ro60 bacteria could trigger autoimmunity, sera from anti-Ro60-positive lupus patients immunoprecipitate Ro60/Y RNA complexes from lysates of *Pseudopropionibacterium propionicum*, a Ro60-containing commensal that is present in the skin lesions of some patients with subacute cutaneous lupus erythematosus (Greiling et al. 2018). Similarly, cloned T cells from a Ro60-positive lupus patient that were selected for reactivity to either whole *P. propionicum* or the Ro60 ortholog from the gut commensal *Bacteroides thetaiotamicron* were also found to be stimulated by human Ro60 (Greiling et al. 2018). These experiments raise the possibility that anti-Ro60 autoantibodies could arise from an immune response to Ro60 ortholog-containing commensal bacteria. These antibodies might cross-react with human Ro60 and eventually lead to autoimmunity through epitope spreading.

As a test of this model, germ-free mice were monocolonized with the Ro60 ortholog-containing gut commensal *B. thetaiotamicron*. Sera from mice monocolonized with this commensal were positive for anti-human Ro60 antibodies by ELISA, while mice monocolonized with the control (non-Ro60-containing) commensal *E. gallinarum* lacked detectable antibodies against Ro60 (Greiling et al. 2018). Cells collected from the mesenteric lymph nodes and spleens of *B. thetaiotamicron*-colonized mice were also stimulated by exposure to *B. thetaiotamicron* Ro60, suggesting both B and T cell responses

to Ro60 (Greiling et al. 2018). Monocolonization also enhanced Ro60 reactivity in non-obese diabetic (NOD) mice and mice treated with the TLR7 agonist imiquimod, two models of autoimmunity (Greiling et al. 2018). In further support of cross-reactivity between the commensal and mammalian Ro60 proteins, cells collected from the spleens and mesenteric lymph nodes of NOD-monocolonized mice were stimulated by exposure to both human and *B. theta* Ro60 (Greiling et al. 2018).

This model, in which commensal orthologs trigger autoimmunity against human proteins, may be generalizable to other systemic rheumatic autoimmune diseases, such as Sjogren's syndrome, that are characterized by anti-Ro60 antibodies, and may also be applicable beyond the Ro60 autoantigen. A similar process was recently proposed for the induction of autoimmunity in a subset of multiple sclerosis (MS) patients (Planas et al. 2018). GDP-L-fucose synthase is involved in the synthesis of fucose, a common component of oligosaccharides throughout the body, including those on the surface of mucosal intestinal cells. Many bacteria in the gut microbiota synthesize fucose using a highly conserved ortholog of the human GDP-L-fucose synthase. Consistent with initiation of autoimmunity through molecular mimicry with the commensal protein, a subset of MS patients had T cells that responded to both human GDP-L-fucose synthase and peptides derived from the GDP-L-fucose synthase of two gut commensal bacteria (Planas et al. 2018).

Perspectives

Experiments in animal and bacterial cells support a model in which Ro60 and its associated Y RNAs are part of a new mechanism for assisting the remodeling of structured RNAs by effector proteins. In addition to the established role of a bacterial Ro60 and Y RNA in assisting exoribonucleases in degrading structured RNAs (Chen et al. 2007; Chen et al. 2013), we consider it likely that Ro60 and Y RNAs aid other RNA remodeling proteins, such as RNA chaperones or helicases, in unfolding RNA substrates. Moreover, since Ro60 contributes to the survival of both mammalian cells and bacteria following some forms of environmental stress (Chen et al. 2000; Chen et al. 2003; Wurtmann and Wolin 2010), and modulates RNA populations during heat stress and starvation in bacteria (Chen et al. 2007; Wurtmann and Wolin 2010), this role may be particularly important during cell stress.

Although Ro60 can function as either a Y RNA-free protein (Chen et al. 2007) or as a Ro60/Y RNA RNP (Chen et al. 2013), Y RNAs are unstable in the absence of their Ro60 partner. Thus, we predict that most functions of these ncRNAs occur in the context of a Ro60/Y RNA RNP. In support of this hypothesis, Y RNAs tether Ro60 to effector proteins such as the PNPase exoribonuclease (Chen et al. 2013), to ZBP1, an adaptor for Ro60 RNP nuclear export (Sim et al. 2012), and to the RNA-binding protein nucleolin (Fouraux et al. 2002), and also gate access of other RNAs to the Ro60 cavity (Fuchs et al. 2006; Chen et al. 2007). An exciting possibility is that, as proposed (Hogg and Collins 2007), the Y RNA loops may contribute to the recognition of specific RNA substrates, in addition to mediating interactions between effector proteins and Ro60.

Although the existing studies in vertebrate cells and *D. radiodurans* have resulted in a framework for understanding how Ro60 and Y RNAs function, much remains to be learned

regarding the roles of Ro60 RNPs in both animal cells and bacteria. For example, although the most conserved bacterial Y RNA contains a domain that mimics tRNA (Chen et al. 2014; Wang et al. 2018), little is known as to how this domain impacts Ro60 or Y RNA function. In animal cells, the finding that Ro60 is found complexed with misfolded ncRNAs (O'Brien and Wolin 1994; Shi et al. 1996; Chen et al. 2003) hints at role(s) in refolding and/or degrading these RNAs. However, the effector proteins, such as helicases and nucleases, that function with Ro60 in these processes remain to be identified. Since Ro60 and Y RNAs have conserved roles in the response of cells to environmental stress (Chen et al. 2000; Chen et al. 2003; Tanaka et al. 2004; Sim et al. 2009; Wurtmann and Wolin 2010), it is possible that, as in bacteria (Wurtmann and Wolin 2010), the association of animal cell Ro60 RNPs with these or other effector proteins becomes more prominent during specific forms of stress. Additionally, the full repertoire of Ro60 target RNAs remains to be determined. While the binding of Ro60 to structured RNAs containing a protein-free single stranded tail explains how Ro60 recognizes ncRNAs (O'Brien and Wolin 1994; Shi et al. 1996; Chen et al. 2003), it in no way precludes the binding of Ro60 to coding RNAs.

From both a cell biological and a clinical perspective, the mechanism of how the normally cytosolic Ro60 becomes accessible on the cell surface during apoptosis is an important question. Since Y3 RNA is required for Ro60 to accumulate on the cell surface (Reed et al. 2013), it will be interesting to determine if specific protein(s) binding the Y3 loops, or the cleavage of Y RNAs during apoptosis (Rutjes et al. 1999) are a prerequisite. Intriguingly, nucleolin, which binds the large internal loops of Ro60-bound Y1 and Y3 RNAs (Fouraux et al. 2002) is found on the surface of some mammalian cells (Said et al. 2002; Sinclair and O'Brien 2002). From a clinical point of view, because immune complexes containing Ro60 RNPs may drive disease progression in neonatal lupus, disrupting surface exposure of the Ro60 RNP could represent a viable clinical intervention.

It will also be important to better understand the role of Ro60 ortholog-containing commensal bacteria in the initiation and/or maintenance of autoantibodies in systemic lupus erythematosus and related diseases, such as Sjogren's syndrome and subacute cutaneous lupus erythematosus, that are characterized by anti-Ro60 antibodies. The use of whole genome metagenomics, which allows unbiased identification of the entire population of commensals within patient samples (Byrd et al. 2018), should be useful in identifying the full range of Ro60-positive commensals in patient microbiomes, as well as determining whether specific commensals are over-represented. Indeed, if particular commensals play an outsized role in this process, it may be possible to prevent or modify disease pathology by targeting the bacteria involved.

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Figure 1. Crystal structures of Ro60 and its bacterial ortholog Rsr. Structures of (A) *X. laevis* Ro60 (PDB 1YVR) and (B) *D. radiodurans* Rsr (PDB 2NVO), colored from the N-terminus in bright blue to the C-terminus in cyan (see color version of this figure at www.tandfonline.com/ibmg).

Figure 2.

Secondary structures of Y RNAs. (A) The four human Y RNAs. The Ro60-binding module is within a stem formed by pairing the 5' and 3' portions of each Y RNA. Boxed regions indicate sequences that are conserved among all characterized animal cell Y RNAs and are important for Ro60 binding. Structure-probing experiments (Green CD et al. 1998) indicate that this portion of the stem can exist in two conformations (arrow). In the crystal structure of *X. laevis* Ro60 complexed with Y3 RNA (Stein et al. 2005), nucleotides colored magenta are sites of base-specific interactions with Ro60. The effector binding/cavity-gating module

of Y RNAs differs between individual Y RNAs, which may allow for interactions with different effectors. (B) *D. radiodurans* Yrn1 (Y RNA 1) and Yrn2 RNAs. (C) *S. Typhimurium* YrlA and YrlB RNAs. The Rsr binding sites in Yrl RNAs diverge from those of metazoan Y RNAs and bacterial Yrn RNAs and have the consensus sequence GNCGAAN₀₋₁G (green), in which the CGA may correspond to the CGA at the end of the metazoan motif (Sim and Wolin 2018) (see color version of this figure at www.tandfonline.com/ibmg).

Figure 3.

YrIA RNAs are tRNA mimics. (A). *S. Typhimurium* YrIA drawn to resemble tRNA. The positions of the D and T arms are indicated, as are the acceptor stem (AS), variable loop (V) and anticodon stem (AC). The indicated nucleotides were shown to be modified to dihydrouridine (D) and pseudouridine (Ψ) *in vivo* (Chen et al. 2014). The boxed region was crystallized. (B) Crystal structure of the effector-binding domain of YrIA (PDB 6CU1). (C) Structure of *Saccharomyces cerevisiae* tRNA^{Phe} (PDB 4TNA) (see color version of this figure at www.tandfonline.com/ibmg).

Figure 4. Single particle reconstruction of the RYPER complex at 25 Å (EMDB ID: EMD-5389). The docking of atomic models of *S. antibioticus* PNPase (PDB 1E3P) and *X. laevis* Ro complexed with Y RNA (PDB 1YVP) and misfolded RNA (PDB 2I91) into this volume supports a model in which the regions colored in yellow, red and green represent Rsr, Y RNA and PNPase respectively (Chen et al. 2013) (see color version of this figure at www.tandfonline.com/ibmg).

Figure 5.

Model for the ways in which Ro60 and Y RNA can function. (A) Ro60 can function as a Y RNA-free protein. This form of Ro60 binds pre-5S RNAs in *X. laevis* oocyte nuclei and is required for efficient pre-23S rRNA maturation in *D. radiodurans* (O'Brien and Wolin 1994; Chen et al. 2007). (B) A bound Y RNA can block access of other RNAs to Ro60. Y RNA prevents binding of misfolded pre-5S rRNA to purified Ro60 (Green CD et al. 1998) and inhibits pre-23S rRNA maturation in *D. radiodurans* (Chen et al. 2007). (C) Y RNAs can act as sponges to sequester other RNA binding proteins. Y3 RNA interacts with nELAVL proteins, potentially preventing these proteins from binding their intended RNA targets (Scheckel et al. 2016; Tebaldi et al. 2018). Whether Ro60 is part of these complexes is unknown. (D) Y RNAs can tether Ro60 to effector proteins, creating RNPs with altered function. In *D. radiodurans*, Ro60 is tethered to PNPase via Y RNA to form RYPER, which has enhanced activity in degrading structured RNA, compared with PNPase alone (Chen et al. 2013). (E) Y RNAs can tether Ro60 to regulatory proteins. Tethering of Ro60 to ZBP1 by Y3 RNA facilitates nuclear export of the Ro60/Y3 complex (Sim et al. 2012) Although two or more of these states (A-E) can occur within the same cell, the specific state(s) that predominate may differ between cell types and can change in response to stress (see color version of this figure at www.tandfonline.com/ibmg).