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Nuclease integrated Kinase Super Assemblies (NiKs) and Their Role in RNA Processing

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Abstract

Here we highlight the Grc3/Las1 complex, an essential RNA processing machine that is well conserved across eukaryotes and required for processing the pre-ribosomal RNA (pre-rRNA). Las1 is an endoribonuclease that cleaves the pre-rRNA while Grc3 is a polynucleotide kinase that phosphorylates the Las1-cleaved RNA product. Recently we showed that Grc3 and Las1 assemble into a higher-order complex composed of a dimer of Grc3/Las1 heterodimers that is required for nuclease and kinase activity. Unexpectedly, we found that the Grc3/Las1 complex draws numerous parallels with two other eukaryotic nucleases, Ire1 and RNase L. In this perspective we explore the similarities and differences between this family of Nuclease integrated Kinase super assemblies (NiKs) and their distinct roles in RNA cleavage.

Keywords

Grc3; Las1; Ribosome Biogenesis; IreI; RNase L; HEPN Domain

RNA Processing and the HEPN Endoribonuclease Family

More than 50 years after the discovery of ‘giant’ pre-rRNA and its processing pathway (Scherrer, et al. 1963), the RNA community is still unraveling the intricate and coordinated regulation of this essential cellular process. A growing number of endo- and exo-nucleases have been identified to support the foundation for pre-rRNA processing and deregulation of many of these nucleases have been linked to disease underscoring their importance (Butterfield, et al. 2014, Martin and Li 2007, Mattijssen, et al. 2010, Tafforeau, et al. 2013). The poly-cistronic pre-rRNA (35S in yeast/47S in mammals) must undergo extensive RNA processing in eukaryotic cells to remove two external transcribed spacers (5´ ETS and 3´ ETS) and two internal transcribed spacers (ITS1 and ITS2), thereby generating the mature 18S, 5.8S, and 25S/28S rRNA (Fernandez-Pevida, et al. 2015, Henras, et al. 2015, Tomecki, et al. 2017). Removal of the ITS2 is an important mid-to-late stage processing event that is initiated by a single endonucleolytic cleavage step at the C2 site (site 4 in mammals) (Konikkat and Woolford 2017). Despite uncovering the C2 cleavage site almost four decades ago (Veldman, et al. 1981), the responsible endoribonuclease, Las1, was only recently

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identified through a comprehensive bioinformatics analysis and confirmed with *in vivo* studies (Anantharaman, et al. 2013, Gasse, et al. 2015).

Las1 belongs to the HEPN (higher eukaryote and prokaryote nucleotide binding) Superfamily of ribonucleases, which represents a group of metal-independent endoribonucleases involved in many types of RNA processing. Endoribonucleases which contain the small α-helical HEPN domain harbor a canonical RΦxxxH motif (where Φ is commonly N, D, or H and x is any residue) responsible for RNase function (Figure 1A) (Anantharaman, et al. 2013, Aravind and Koonin 1999, Grynberg, et al. 2003). Aside from the catalytic motif there is little sequence similarity among HEPN family members (Anatharaman, 2013). HEPN domains work in concert by either homo- or hetero-dimerization to form a composite and functional nuclease active site (Davidov and Kaufmann 2008, Han, et al. 2014, Huang, et al. 2014, Lee, et al. 2008, Liu, et al. 2017a, Liu, et al. 2017b, Niewoehner and Jinek 2016, Pillon, et al. 2017). Their metal-independent catalysis produces a 2′,3′-cyclic phosphate and 5′-hydroxyl group at the site of incision (Figure 1B). Bacteria contain several examples of HEPN nucleases many of which are involved in host RNA defense systems such as the CRISPR effectors C2c2 and Cas13 (Anantharaman, et al. 2013, Liu, et al. 2017b, Shmakov, et al. 2015). Furthermore, many eukaryotic RNases have been established as members of the expanded HEPN family, including Ire1, RNase L, and Las1 (Figure 1A) (Anantharaman, et al. 2013).

We recently characterized the nuclease activity of Las1 and unexpectedly found that it is reliant on the polynucleotide kinase Grc3 for higher-order assembly and HEPN nuclease activation (Pillon, et al. 2017). Together Las1 and Grc3 assemble into a complex resembling the activated forms of Ire1 and RNase L, which are both dependent upon kinase domains for coordinating higher-order assembly and activation of their HEPN domains (Table 1). This similarity was unexpected because the nuclease domain from Las1 and the kinase domain from Grc3 are from two separate proteins, while the kinase and nuclease domains from Ire1 and RNase L are encoded within the same polypeptide chain (Figure 2A). Here we discuss the molecular mechanisms regulating this important group of Nuclease integrated Kinase super assemblies (NiKs) that play crucial cellular roles through the nicking of their RNA targets.

The ITS2 Processing Machinery – Grc3/Las1

Despite its short history as an endoribonuclease, previous studies established that Las1 is essential for cell viability. Las1 (lethal in the absence of Ssd1) was originally identified as an essential nuclear protein in *Saccharomyces cerevisiae* with roles in cell morphogenesis and cell growth (Doseff and Arndt 1995). Subsequent studies in yeast and mammalian cells linked Las1 (Las1L in mammals) to the maturation of the large ribosomal subunit (Castle, et al. 2012, Castle, et al. 2010, Castle, et al. 2013, Kitano, et al. 2011, Schillewaert, et al. 2012). In addition to being required for ribosome production, Las1L has also been associated with human disease. *LAS1L* is an X-linked gene, and mutations within *LAS1L* have been identified in several families with X-linked intellectual disability (Hackmann, et al. 2016, Hu, et al. 2016). Studies have also shown that contextual fear induces the alternative splicing of *LAS1L* suggesting it may play a role in learning (Poplawski, et al.

2016). Moreover, a missense mutation in *LAS1L* (Human S477N) was uncovered in a patient with spinal muscular atrophy with respiratory distress (SMARD) further underscoring the critical importance of Las1 (Butterfield, et al. 2014).

Las1 contains a well conserved N-terminal HEPN domain (Anantharaman, et al. 2013, Castle, et al. 2010). The Las1 HEPN harbors the consensus RΦxxxH motif (^{129}RHWGTH in *S. cerevisiae*) and disruption of the arginine and histidine residues (RΦxxxH) confirmed Las1 is indeed the endoribonuclease responsible for C2 cleavage *in vivo* (Figure 1A) (Gasse, et al. 2015). Analogous to other HEPN nucleases, Las1 cleavage generates a terminal 2´,3´ cyclic phosphate on the 7S pre-rRNA and a 5´-OH on the 26S pre-rRNA (Figure 1B) (Gasse, et al. 2015). The 5´-OH of the 26S pre-rRNA is subsequently phosphorylated by the essential polynucleotide kinase Grc3, which marks the pre-rRNA for processing by the 5´-exonuclease Rat1 and its co-factor Rai1 to remove the remaining ITS2 spacer (Gasse, et al. 2015).

Grc3 (Nol9 in mammals) is a well conserved member of the RNA specific Clp1/Grc3 polynucleotide kinase (PNK) subfamily, which utilizes ATP to catalyze the transfer of the γ-phosphate to the 5´-end of RNA (Braglia, et al. 2010, Heindl and Martinez 2010). A high-throughput RNA processing screen originally identified Grc3 as an essential protein required for removal of the ITS2 (Peng, et al. 2003) and several studies identified associations between Grc3 and Las1 homologues (Castle, et al. 2012, Castle, et al. 2013, Kitano, et al. 2011, Sydorskyy, et al. 2003). Moreover, Grc3 and Las1 are dependent upon one another for protein stability (Castle, et al. 2013) and the Grc3/Las1 complex was shown to withstand glycerol gradients and high salt concentrations (Castle, et al. 2012, Gasse, et al. 2015, McCann, et al. 2015). Collectively, these studies led us to hypothesize that Grc3 and Las1 form a stable obligate complex essential for coordinating pre-rRNA processing.

To ascertain whether the interaction between Grc3 and Las1 mediates enzymatic regulation of this RNA processing machinery, we characterized *S. cerevisiae* Grc3/Las1 oligomerization and enzymatic function using a combination of biochemical assays and *in vivo* studies. On its own, Las1 displayed weak nuclease activity that was robustly enhanced in the presence of Grc3 (Pillon, et al. 2017). Correspondingly, we found that efficient Grc3 kinase activity was dependent upon the presence of the Las1 nuclease domain (Pillon, et al. 2017). We discovered that the Grc3/Las1 complex assembles into a super-dimer, composed of a dimer of Grc3/Las1 heterodimers (Figure 2B). The Las1 HEPN nuclease domain drives this higher-ordered assembly which is further stabilized by the Grc3 PNK domain (Pillon, et al. 2017). Intriguingly, enzymatic studies revealed molecular cross-talk between the Grc3 kinase and the Las1 nuclease domains. Mutation of key active site residues in either the nuclease or kinase domain had significant effects on both kinase and nuclease activity. For example, mutation of the arginine and histidine residues (^{129}RHWGTH) within the Las1 HEPN motif abolished nuclease activity and significantly reduced kinase activity *in vitro* while having no impact on the ability to form the super-dimer (Pillon, et al. 2017). Taken together, our results reveal that Las1 and Grc3 organize into a super assembly to facilitate molecular cross-talk between the nuclease and kinase domains, which is critical to ensure efficient and accurate processing of the C2 site. Interestingly, this is not unique to Grc3/Las1 since similar activation requirements have been reported for Ire1 and RNase L, thus

suggesting a common underlying regulatory theme for these specialized RNA processing machines.

The Ire1/RNase L Ribonuclease Family

Ire1 Kinase/Nuclease

Ire1 (inositol-requiring enzyme 1) is a well-studied protein that plays a fundamental role in the unfolded protein response (Coelho and Domingos 2014, Korennykh and Walter 2012, Maurel, et al. 2014, Sano and Reed 2013). Ire1 is a type I transmembrane protein composed of an N-terminal endoplasmic reticulum (ER) luminal domain, a transmembrane helix, and a large cytoplasmic C-terminal region which includes a protein kinase domain followed by an HEPN domain, also referred to as a kinase extension nuclease (KEN) domain (Figure 2A) (Anantharaman, et al. 2013, Lee, et al. 2008). Elevated levels of unfolded proteins in the ER are sensed by the Ire1 luminal domain leading to the oligomerization of Ire1 and the activation of the unfolded protein response (Aragon, et al. 2009, Credle, et al. 2005, Gardner and Walter 2011, Korennykh, et al. 2009). The luminal, kinase, and HEPN domains as well as, a short linker connecting the kinase domain to the transmembrane segment, all participate in Ire1 higher-ordered assembly (Figure 2B) (Korennykh, et al. 2009, Lee, et al. 2008). Ire1 activates the unfolded protein response through the nonconventional splicing of *HAC1* mRNA in yeast or *XBP1* mRNA in metazoan cells (Gardner, et al. 2013). Removal of introns from the transcription factors Hac1 or Xbp1 leads to the activation of genes involved in ER stress adaptation (Korennykh and Walter 2012). During extended ER stress, Ire1 from metazoan cells also degrades a cohort of mRNAs resulting in gene repression, through the RIDD (Regulated IRE1-Dependent Decay) pathway (Bhattacharyya 2014, Maurel, et al. 2014).

RNase L Pseudo-kinase/Nuclease

RNase L (ribonuclease latent) is a tightly controlled cytoplasmic endoribonuclease that is activated by an interferon response in higher eukaryotes (Table 1) (Chakrabarti, et al. 2011, Gusho, et al. 2016). RNase L includes an N-terminal ankyrin repeat domain followed by a pseudo-protein kinase domain and HEPN domain (Huang, et al. 2014). The pseudo-kinase and HEPN domains of RNase L share sequence and structural homology with the kinase-HEPN domains from Ire1 (Han, et al. 2014, Huang, et al. 2014, Lee, et al. 2008). Under normal cellular conditions RNase L is in an inactive monomeric conformation. Activation of an interferon response by pathogen or damage associated double stranded RNA leads to the production of the second messenger 2-5A by oligoadenylate synthetases (Kristiansen, et al. 2011). RNase L is the only known target of 2-5A, which binds to the ankyrin repeat domain and activates RNase L through dimerization (Figure 2B) (Dong and Silverman 1995, Gusho, et al. 2016). Higher-order assembly of RNase L leads to the cleavage of its RNA targets, such as rRNA, and viral and cellular mRNAs (Andersen, et al. 2009, Chakrabarti, et al. 2011, Cooper, et al. 2015, Gusho, et al. 2016, Rath, et al. 2015). RNase L efficiently cleaves single stranded regions of RNA to effectively degrade the RNA targets. RNA degradation by RNase L has been linked with numerous downstream events such as inhibition of translation, removal of viral genomes, and the activation of cell signaling pathways, highlighting the importance of RNase L in coordinating cellular responses (Gusho, et al. 2016).

Higher-order Assembly Mediates Nuclease-Kinase Cooperativity

Despite their diverse cellular activities, Ire1, RNase L, and Grc3/Las1 all undergo an oligomerization-driven activation mechanism. All three NiKs rely on the self-association of their respective HEPN and kinase domains (Table 1). Ire1 and RNase L super assembly is triggered by their respective ligand binding domains in response to cellular stressors (Figure 2B). In contrast, Grc3/Las1 does not have a known ligand binding domain and remains constitutively assembled as a super-dimer, to support ribosome assembly (Table 1). Grc3/Las1 also encodes three additional domains (Grc3 N- and C- terminal domains and Las1 coiled coil domain) of unknown function that may modulate the activities of this RNA processing machinery (Figure 2A-B).

Ire1, RNase L and Grc3/Las1 share similar mechanisms of nuclease catalysis. All three NiKs harbor the canonical HEPN motif (RΦxxxH) and rely on the integrity of their kinase domains for RNase activity (Table 1). Super assembly is required to form a composite HEPN active site that is competent for RNA cleavage. This is supported by the observation that disrupting the conserved histidine residue within both HEPN motifs (RΦxxxH) of the Ire1 and RNase L dimer abolishes nuclease activity; whereas trans-complementation assays forming chimeric Ire1 and RNase L encoding a single histidine residue per HEPN dimer was sufficient for nuclease activity *in vitro* (Han, et al. 2014, Korennykh, et al. 2011). Other HEPN nucleases including C2c2, Cas13, and Csm6 have similarly revealed a composite active site suggesting this may be a common theme among the HEPN endoribonuclease family (Liu, et al. 2017a, Liu, et al. 2017b, Niewoehner and Jinek 2016). Despite lacking an HEPN domain, the bacterial RNA degradosome also draws similar parallels where the catalytic domain relies on higher-order assembly for RNase activity, as well as, provides a critical scaffold to license the ATPase activity of its binding partner (Callaghan, et al. 2005, Carpousis 2007, Chandran, et al. 2007, Hughes 2016).

While the kinase domains from Ire1, RNase L, and Grc3/Las1 are distinct, they all work cooperatively with their HEPN domains. Ire1 contains a Ser/Thr protein kinase domain, whose only known target is Ire1 itself. Auto-phosphorylation of Ire1 is not required for nuclease function *in vitro*, but it appears to play a regulatory role *in vivo* (Gardner, et al. 2013, Prischi, et al. 2014). In contrast to Ire1, the pseudo-kinase domain within RNase L is thought to be inactive and contains a number of nonconventional residues within the kinase C-lobe which likely accounts for its lack of kinase function (Huang, et al. 2014). Despite this difference in kinase activity, ATP binding to the Ire1 and RNase L kinase domains is required to activate the HEPN domains (Dong and Silverman 1999, Dong, et al. 1994, Huang, et al. 2014, Sidrauski and Walter 1997, Wreschner, et al. 1982). Unlike Ire1 and RNase L, Grc3/Las1 is a polynucleotide kinase and the nucleotide-bound state of the Grc3 PNK is not required to support RNA cleavage by Las1 *in vitro* (Huang, et al. 2014, Lee, et al. 2008, Pillon, et al. 2017, Sidrauski and Walter 1997). Despite this distinction, disruption of the Grc3 Walker B motif, which prevents the transfer of the γ-phosphate from an ATP donor onto RNA, resulted in RNA cleavage deficiency (Pillon, et al. 2017). Furthermore, rather than simply playing a regulatory role, the PNK domain from Grc3/Las1 also directly participates in RNA processing by phosphorylating the Grc3/Las1 cleavage product (Gasse, et al. 2015). Taken together, this high cooperativity between the kinase and endoribonuclease

domains likely contributes to the precise targeting of these nucleases to their RNA substrates.

RNA Substrate Recognition

NiKs share a common RNA cleavage mechanism that targets discreet sites in diverse cellular RNAs. This is in stark contrast to several characterized bacterial HEPN nucleases that exhibit non-specific RNA cleavage activity (Liu, et al. 2017a, Liu, et al. 2017b, Niewoehner and Jinek 2016). The Grc3/Las1 complex has exquisite endoribonuclease specificity for the C2 site encoded within the ITS2 of pre-rRNA, where changes to the local RNA sequence and/or structure negates cleavage (Figure 2B) (Pillon, et al. 2017, Poothong, et al. 2017, van Nues, et al. 1995a, van Nues, et al. 1995b). Ire1 also shows precise splicing specificity within tandem stem loops of *HAC1*/*XBP1* mRNA (Figure 2B) (Gonzalez, et al. 1999, Poothong, et al. 2017, Sidrauski and Walter 1997). Meanwhile, RNase L directs cleavage to AU-rich mRNAs, where incision typically follows the dinucleotides UU or UA (Figure 2B) (Floyd-Smith, et al. 1981, Han, et al. 2014, Wreschner, et al. 1981). Atomic structures of Ire1 and RNase L reveal variable HEPN secondary structure, known as α-helix/loop elements (HLE). While important for RNA cleavage efficiency, these variable elements are dispensable for RNA targeting (Han, et al. 2014, Korennykh, et al. 2011). Thus, the distinguishing features contributing to RNA specificity among NiKs remains elusive.

While a consensus Grc3/Las1 RNA cleavage motif remains undefined, intriguing parallels can be draw from RNase L. Grc3/Las1 nicks the pre-rRNA following an adenine nucleotide at the C2 site within the ITS2 of *S. cerevisiae*. Interestingly, the nucleotides immediately surrounding the C2 site (UAˆG; where ˆ denotes the cleavage site) mimic the universal RNase L cleavage motif (UNˆN; where N is any nucleotide) where studies have shown a preference for UAˆN and UUˆN (Figure 2B) (Geerlings, et al. 2000, Han, et al. 2014, Pillon, et al. 2017). Corresponding with the notion that Grc3/Las1 may recognize a similar UNˆN motif for RNA cleavage, mutations to the adjacent uracil (UAˆG) and adenine (UAˆG) abrogates C2 cleavage by Grc3/Las1 *in vitro* (Pillon, et al. 2017).

The C2 site within pre-rRNA is the only known Grc3/Las1 target; however, the wide breadth of known RNA targets of other NiKs members suggests the possibility for additional unidentified substrates. For example, Ire1 from metazoans not only targets *XBP1* mRNA for cleavage, but also degrades a number of other RNAs in the RIDD pathway (Bhattacharyya 2014, Maurel, et al. 2014). The universal RNase L motif (UNˆN) is also an abundant sequence encoded in eukaryotic mRNAs suggesting RNase L may be capable of degrading most mRNAs it encounters in its super assembly state. Moreover, new developments in high-throughput sequencing have revealed the presence of numerous RNAs with 2′,3′-cyclic phosphate and 5′-hydroxyl ends, which could be uncharacterized NiKs family cleavage products (Peach, NAR 2015 and Schutz RNA, 2010). Taken together, uncovering new RNA targets will be a critical step in expanding our understanding of the Grc3/Las1 molecular repertoire in nucleolar RNA processing.

Concluding Remarks

The constitutive Grc3/Las1 super-dimer is a distinct feature from stress-activated Ire1 and RNase L; thus, raising the question for how this nuclease-kinase super assembly is regulated. A recently emerging theme amongst the HEPN nuclease family is that dimerization is required, but not sufficient for activation. To form a competent nuclease active site, HEPN domains must bind a ligand or engage a specific RNA substrate to properly orient its catalytic residues. For example, similar to RNase L, the newly discovered CRISPR-associated Csm6 nuclease is activated in the presence of a cyclic oligoadenylate substrate (Kazlauskiene, et al. 2017, Niewoehner, et al. 2017). Likewise, the two HEPN domains from Cas13a, a type VI-A CRISPR-Cas RNA-guided ribonuclease, undergo a structural rearrangement upon binding target CRISPR RNA to activate the HEPN active site (Liu, et al. 2017a). We propose that the Grc3/Las1 HEPN domains are activated through a similar mechanism, where by recognition of specific residues surrounding the C2 cleavage site most likely licenses the Grc3/Las1 machinery for RNA cleavage.

In conclusion, we have established Grc3/Las1 as a defining member of the NiKs family. The NiKs family represents a specialized class of endoribonucleases with unique mechanisms of assembly and activation, that participate in diverse cellular roles through the cleavage of their RNA targets. While the parallels between Ire1 and RNase L have been apparent for many years, the similarities with Grc3/Las1 were unexpected and suggest that there may be more examples of nuclease integrated kinase super assemblies that have yet to be discovered.

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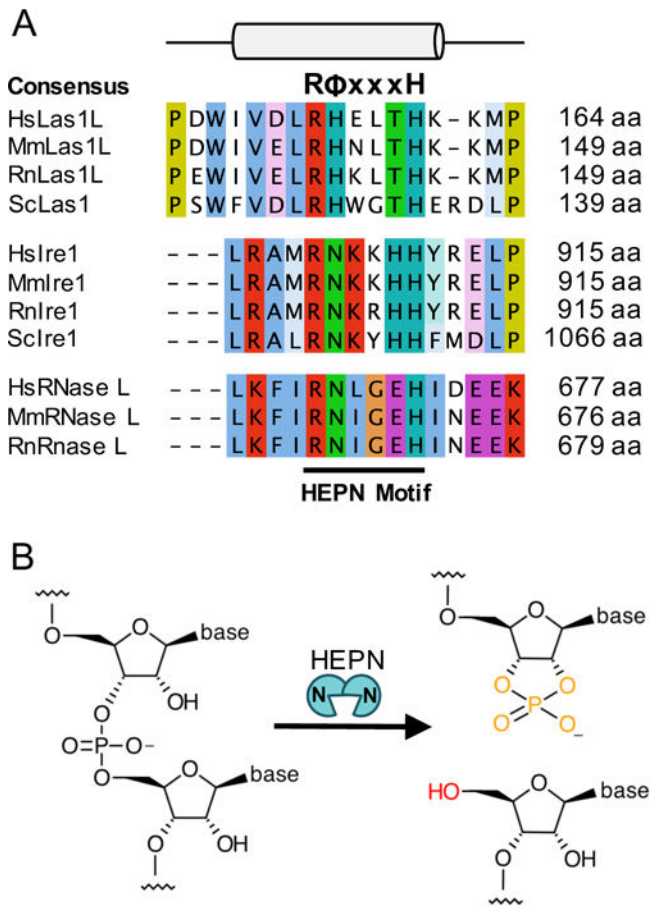

Figure 1.

Las1, Ire1, and RNase L are HEPN Endoribonucleases. (A) Secondary structure guided sequence alignment (Pei, et al. 2008) of the conserved HEPN motif (RΦxxxH) from *Homo sapiens* (Hs), *Mus musculus* (Mm), *Rattus norvegicus* (Rn), and *S. cerevisiae* (Sc) Las1, Ire1, and RNase L. The predicted α-helix is shown as a cylinder and the HEPN motif is marked by a black bar. (B) Cartoon schematic of RNA cleavage by metal-independent HEPN nucleases (N; cyan). The resulting 2′,3′-cyclic phosphate and 5′-hydroxyl are colored in orange and red, respectively.

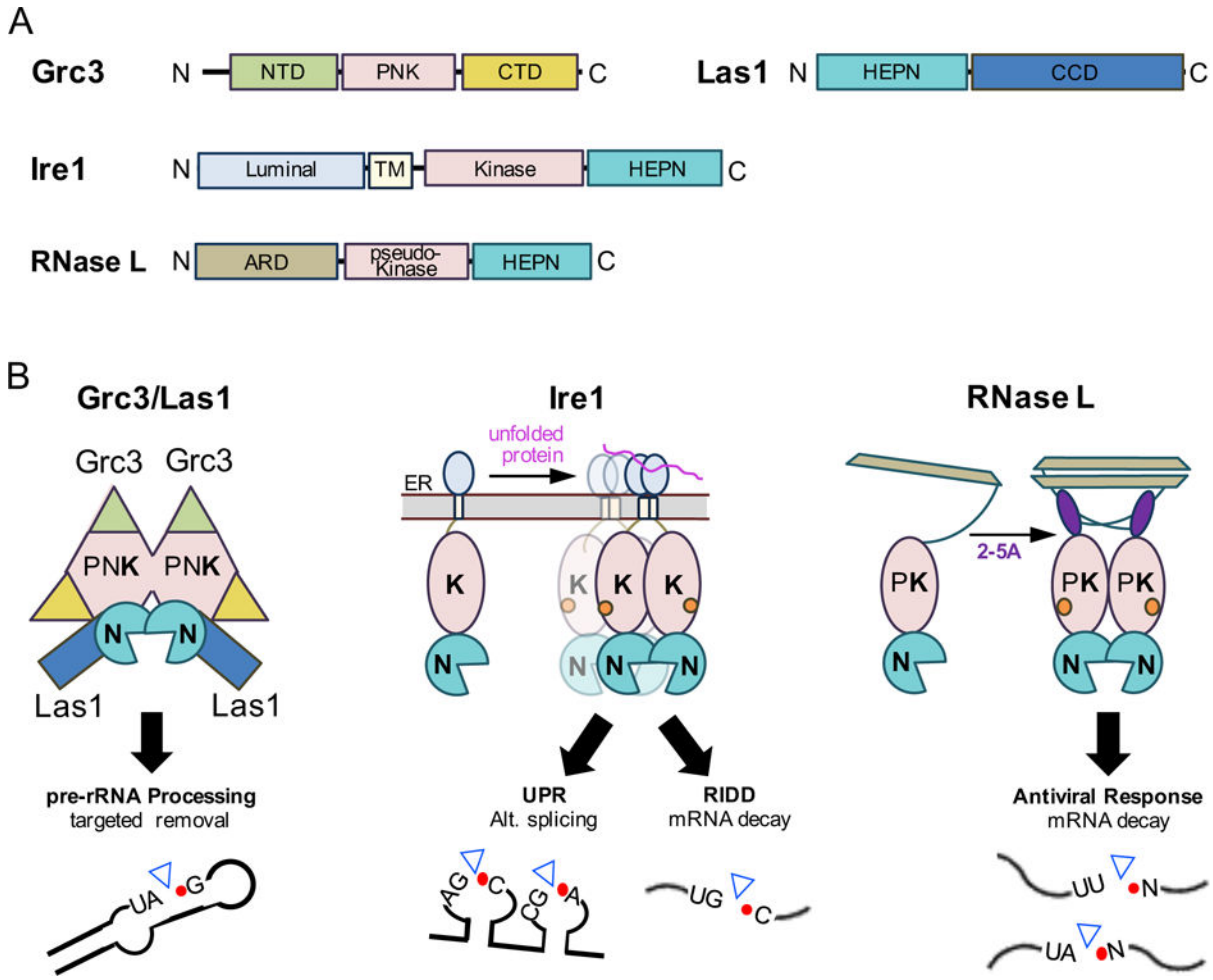

Figure 2.

Oligomerization-driven Activation of Grc3/Las1, Ire1, and RNase L. (A) Domain architecture of Grc3, Las1, Ire1, and RNase L. NTD and CTD denote the N- and C-terminal domains while CCD is the coiled coil domain. TM marks the transmembrane domain and ARD represents the ankyrin repeat domain. (B) The Grc3 polynucleotide kinase (PN**K**; beige) is constitutively assembled into a super-dimer with the Las1 HEPN (N; cyan) forming the active ITS2 RNA processing machinery that targets the C2 site (UAˆG in *S. cerevisiae*) for RNA cleavage. The Grc3 NTD and CTD are shown in green and yellow, respectively, whereas the Las1 CCD is dark blue. The Ire1 luminal domain (light blue) binds unfolded protein (pink) in the ER to trigger self-association through the luminal, protein kinase (K; beige) and HEPN (N; cyan) domains. Nucleotide (orange) is also required to activate the Ire1 nuclease leading to alternative splicing of *HAC1* mRNA in yeast (AGˆC and CGˆA), as well as, mRNA decay (UGˆC) by the RIDD pathway in metazoan cells. RNase L binds the interferon response second messenger 2-5A (purple) through its ankyrin repeat domain (brown). This triggers dimerization through its ankyrin repeat, pseudo-kinase (P**K**; beige) and HEPN (N; cyan) domains. Like Ire1, the nucleotide bound state of the pseudo-kinase activates the RNase L nuclease for mRNA decay at sites encoding the universal UNˆN motif,

where N represents any nucleotide. Blue arrowheads mark the RNA incision site and the red dots indicate the resulting 5´-hydroxyl end.

Table 1

Functional Features of NiKs.

	Grc3/Las1	Ire1	RNase L
Similarities			
Dual Enzymatic Function	PNK/RNase	protein kinase/RNase	pseudo-kinase/RNase
Active State	super-assembly	super-assembly	super-assembly
RΦxxxH Motif	RHxxTH	RNKxHH	RNxGEH
RNase Metal Dependency	independent	independent	independent
RNase Active Site	composite	composite	composite
Nuclease Activation	kinase-dependent	kinase-dependent	kinase-dependent
Differences			
Conservation	eukaryotes	eukaryotes	higher eukaryotes
Subcellular Localization	nucleolus	ER	cytoplasm
Higher-order Assembly	constitutive	UPR-induced	2-5A-induced
Bifunctional Kinase/RNase	trans-acting	cis-acting	cis-acting
Kinase Target	polynucleotide	protein	N/A
Nuclease Target	pre-rRNA	mRNA	rRNA/mRNA