RNA ligases and RNA repair

Overview of RNA repair – There is an emerging appreciation of the existence of "RNA repair" pathways that rely on RNA ligases to maintain or manipulate RNA structure in response to purposeful RNA breakage events. RNA breaks destined for repair are inflicted by sequence-specific or structure-specific endoribonucleases during physiological RNA processing (e.g. tRNA splicing; kinetoplast mRNA editing) and under conditions of cellular stress (e.g., virus infection, unfolded protein response). RNA cleavage can occur either: (i) by a transesterification mechanism that yields 2',3'-cyclic-PO_4 and 5'-OH ends; or (ii) via a hydrolytic mechanism that leaves 3'-OH and 5'-PO_4 ends. RNA repair enzymes capable of sealing 2',3'-cyclic-PO_4/5'-OH breaks or 3'-OH/5'-PO_4 breaks are present in diverse taxa in all phylogenetic domains of life.

ATP-dependent RNA ligases join 3'-OH and 5'-PO_4 RNA termini via three nucleotidyl transfer steps similar to those of DNA ligases: (Step 1) ligase reacts with ATP to form a covalent ligase-(lysyl-Nz)–AMP intermediate plus pyrophosphate; (Step 2) AMP is transferred from ligase-adenylate to the 5'-PO_4 RNA end to form an RNA–adenylate intermediate (AppRNA); and (Step 3) ligase catalyzes attack by an RNA 3'-OH on the RNA–adenylate to seal the two ends via a phosphodiester bond and release AMP. In order for these RNA ligases to repair breaks formed by transesterification, the 2',3'-cyclic-PO_4 and 5'-OH ends must first be "healed". End-healing entails hydrolysis of the 2',3'-cyclic phosphate (by a phosphoesterase enzyme) to form a 3'-OH and phosphorylation of the 5'-OH (by a polynucleotide kinase enzyme) to form a 5'-PO_4.

Phage T4 polynucleotide kinase/phosphatase (Pnkp) and RNA ligase 1 (Rnl1) are prototypal RNA healing and sealing enzymes. They function *in vivo* to repair a break in *E. coli* $tRNA^{Lys}$ triggered by phage-activation of a latent host-encoded anticodon nuclease PrrC. PrrC specifically incises $tRNA^{Lys}$ at a single

site in the anticodon loop, 5' of the wobble uridine, to generate a 2',3'-cyclic-PO_4//5'-OH break. If Pnkp and Rnl1 are not present, depletion of the pool of functional $tRNA^{Lys}$ by PrrC blocks phage protein synthesis and arrests the infection before it can spread. However, Pnkp and Rnl1 repair the broken tRNAs and thereby thwart the host defense mechanism. Pnkp remodels the ends of the broken tRNA as follows: (i) it converts the 2',3'-cyclic-PO_4 to a 3'-PO_4 and then hydrolyzes the 3'-PO_4 to a 3'-OH; and (ii) it transfers the gamma phosphate from ATP to the 5'-OH end to form a 5'-PO_4. Rnl1 then joins the 3'-OH and 5'-PO_4 RNA ends. We have probed in depth the structural basis for catalysis by T4 Pnkp and Rnl1 and the features of the PrrC anticodon nuclease that are required for "ribotoxicity" *in vivo*.

Fungal tRNA Splicing as a Paradigm of RNA Repair – Intron-containing tRNAs are widespread in eukarya the intron is usually located in the anticodon loop of the pre-tRNA and must be removed precisely for the tRNA to function in protein synthesis. tRNA splicing occurs in two stages: (i) intron excision and (ii) joining of the broken tRNA halves. A tRNA splicing endonuclease catalyzes dual incisions of the pre-tRNA at the exon-intron borders to yield 2',3'-cyclic-PO_4 and 5'-OH termini. The joining phase of tRNA splicing has been characterized best in fungi and plants, where a single multifunctional tRNA ligase enzyme (Trl1 in yeast; AtRNL in *Arabidopsis*) repairs the ends of the broken tRNA half-molecules. The healing and sealing steps of yeast/plant tRNA splicing involve distinctive intermediates and an additional repair step not seen in the phage T4 tRNA repair pathway. Trl1 and AtRNL perform three reactions: (i) the 2',3'-cyclic-PO_4 of the proximal tRNA half-molecule is hydrolyzed to a 3'-OH,2'-PO_4 by a cyclic phosphodiesterase (CPD); (ii) the 5'-OH of the distal half-molecule is phosphorylated by a kinase; and (iii) the 3'-OH,2'-PO_4 and 5'-PO_4 ends are sealed by an ATP-dependent RNA ligase to form a spliced tRNA with an unconventional 2'-PO_4, 3'-5' phosphodiester at the splice junction. The final step of removing of the 2'-PO_4 is performed by a separate enzyme, Tpt1.

Trl1 (827-aa) and AtRNL (1104-aa) are homologous proteins composed of separable healing and sealing enzyme modules. The C-terminal CPD module belongs to the "2H" phosphoesterase superfamily, named for the pair of HxT peptides that comprise the CPD active site. The central kinase module is a member of the P-loop phosphotransferase superfamily, named for a signature GxGK(S/T) motif that binds the NTP phosphate donor. The N-terminal ATP-dependent ligase domain belongs to the covalent nucleotidyltransferase enzyme superfamily that includes classic RNA/DNA ligases and mRNA capping enzymes. We've shown that: (i) each component catalytic module of tRNA ligase is essential for yeast cell growth; (ii) mutations in the active sites of the CPD, kinase, and ligase domains are lethal *in vivo*; and (iii) phage and plant tRNA repair systems are "portable" and can complement growth of a yeast *trl1*Δ null mutant. These findings empowered us to develop yeast *trl1* mutants as genetic models for studies of phage, plant, and fungal tRNA repair systems and the identification and characterization of novel tRNA repair activities from eukarya, archaea, and bacteria.

During the last several years we made major strides in (i) elucidating the unique biochemical requirement

for a 2'-PO_4 in the ligation step of fungal/plant tRNA ligase; (ii) developing a kinetic mechanism for the healing and sealing steps; (iii) highlighting distinctive kinetic properties and NTP specificities of the kinase components of fungal *versus* plant tRNA ligase; and (iv) solving the crystal structure of the kinase domains of *Candida* tRNA ligase.

tRNA Ligase as Antifungal Drug Target – Fungal/plant tRNA ligases are essential agents of informational and stress response pathways involving the repair of tRNA breaks (during tRNA splicing) and mRNA breaks (during the unfolded protein response). The biochemical outcomes and end-specificities of the healing and sealing reactions they catalyze are unique compared to the tRNA repair systems elaborated by viruses, bacteria, and metazoans. Indeed, metazoan proteomes have no discernable homologs of the sealing domain of fungal/plant tRNA ligase. Metazoa rely for end-joining during tRNA splicing and the unfolded protein response on RtcB enzymes, a novel clade of RNA ligases co-discovered by my lab. RtcB is a GTP-dependent RNA ligase with no structural similarity to the classic ATP-dependent ligase superfamily. We've shown that RtcB splices 3'-PO_4 and 5'-OH ends via a unique chemical mechanism entailing the formation of covalent RtcB-(histidinyl)-GMP and $RNA_{3'}pp_{5'}G$ intermediates. There is no 5' kinase end-healing step in the RtcB pathway of RNA repair. RtcB is conspicuously absent from the proteomes of pathogenic fungi. This scenario highlights tRNA ligase as a target for the discovery of new antifungals.

To fortify the case for fungal tRNA ligase as a drug target, we need to understand the properties of Trl1 enzymes produced by fungi that cause human disease. To that end, we have characterized Trl1 from the human fungal pathogens *Aspergillus fumigatus*, *Coccidioides immitis*, and *Candida albicans*. *A. fumigatus* causes invasive pulmonary disease in immunocompromised individuals, especially those with hematological malignancies or who have undergone bone marrow or solid organ transplantation. *C. immitis* is the agent of San Joaquin Valley Fever, a disease prevalent in the US desert Southwest region. *C. albicans* causes a spectrum of illnesses ranging from local infections of oral and genital mucosa to invasive systemic infections with significant morbidity and mortality in immunocompromised hosts. The *Aspergillus*, *Coccidioides*, and *Candida* Trl1 enzymes all strongly prefer GTP as the NTP phosphate donor for their 5' kinase reactions.

We recently solved a 2.2 Å crystal structure of the *Candida* Trl1 kinase domain in complex with GDP and Mg^{2+} in the active site. The P-loop phosphotransferase fold of the kinase is embellished by a unique "G-loop" element that accounts for guanine nucleotide specificity. Mutations of amino acids that contact the guanine nucleobase efface kinase activity in vitro and Trl1 function in vivo.

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