

# Guide RNA acrobatics: the one-for-two shuffle

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**RNA modifications are crucial for the proper function of the RNAs. The sites of pseudouridines are often specified by dual hairpin guide RNAs, with one or both hairpins identifying a target uridine. In this issue of *Genes & Development*, Jády and colleagues (pp. 70–83) identify a novel mechanism by which a single guide RNA hairpin can specify two uridines adjacent to each other or separated by 1 nt; i.e., one for two or guide RNA acrobatics.**

Pseudouridine ( $\Psi$ ) is so abundant that 65 years ago it was identified in RNA as a “fifth” nucleotide (Davis and Allen 1957). Now, it has become a household name through the COVID-19 pandemic, as its N1-methyl derivative forms an important basis of the current mRNA vaccines. Perhaps lesser known is that even the SARS-CoV-2 RNA itself contains  $\Psi$ s (Fleming et al. 2021).  $\Psi$  is most famous for its presence in the highly abundant tRNAs, ribosomal RNAs (rRNAs), and spliceosomal small nuclear RNAs (snRNAs).  $\Psi$  is critical for fine-tuning the function of these stable RNAs. The ~130  $\Psi$ s in human snRNAs and rRNAs are formed by H/ACA RNA protein complexes (H/ACA RNPs) in a process called pseudouridylation (Fig. 1A). These RNPs consist of one H/ACA guide RNA with a 5' and 3' hairpin (Fig. 1B), each stabilized by the same four core proteins: the pseudouridine synthase NAP57 (also known as dyskerin), NOP10, NHP2, and GAR1 (for review, see Kiss et al. 2010). The hairpins harbor a central pseudouridylation pocket that identifies a target uridine (U) in rRNA or snRNA by site-directed base pairing (Fig. 1B, blue). Hybridization to either side of the substrate U and an unpaired nucleotide (N) positions them underneath the distal stem of the hairpin (Fig. 1B, N $\Psi$  and shaded gray, respectively). Arranged in this manner, the enzyme NAP57 of that hairpin isomerizes the substrate U to  $\Psi$  by rotating its base 180° and reattaching it as 5-ribosyluracil (Fig. 1A). Normally, a single pseudouridylation pocket guides the formation of a single U to  $\Psi$ . In this issue of *Genes & Development*, Jády et al.

(2022) document for the first time the capacity for pseudouridylation of two consecutive Us (or removed by 1 nt) by a single pocket. This is made possible through contortions of the guide loop and distal stem of one of the H/ACA RNA hairpins, here termed “guide RNA acrobatics” (Fig. 1C).

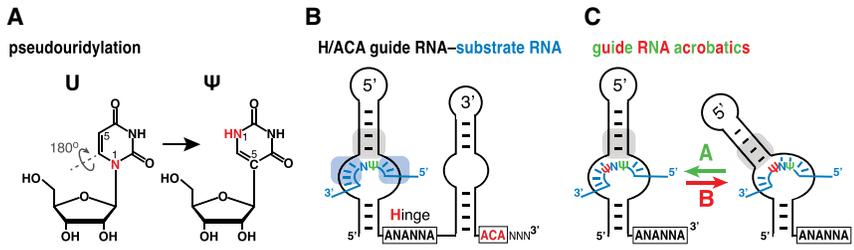
The investigators begin with the orphan SNORA53 that had not been assigned to guide RNA modification or to any other function. By manipulating the base-pairing registers of the distal stem (Fig. 1B, shaded gray) between the guide and substrate RNA (Fig. 1B, shaded blue), the 5' hairpin of SNORA53 could be folded into two configurations, A and B (Fig. 1C). This shuffle could position both  $\Psi$ 3747 and  $\Psi$ 3749 of 28S rRNA for potential pseudouridylation by SNORA53, thus serving as guide RNA for the two unassigned  $\Psi$ s (Fig. 1C). Indeed, knockout of SNORA53 using CRISPR/Cas 9 technology resulted in the loss of pseudouridylation at positions U3747 and U3749 of 28S rRNA. Importantly, restoring SNORA53 expression in the knockout cells rescued pseudouridylation at those positions, cementing the role of SNORA53 in this activity. However, the question remained of whether the 5' hairpin was solely responsible for both modifications or whether perhaps the 3' hairpin was also involved. Analysis of the originally identified configurations A and B of the 5' hairpin (Fig. 1C) made it evident that the deletion or mutation of 3 or 2 nt at the bottom of the distal stem would lock the hairpin into configuration A or B, respectively (Fig. 1C, shaded gray). Remarkably, by constructing the two mutant versions of the snoRNA and expressing one or the other in the SNORA53 knockout cells, the investigators succeeded in rescuing the  $\Psi$ 3747 or  $\Psi$ 3749 modification individually, as predicted by the construct validating the one-for-two role of the 5' hairpin. To leave no stone unturned, the investigators next turned from manipulating the guide RNA sequence to that of the substrate RNA. For that purpose, they expressed mutated 28S rRNA target sequence from a minigene. They removed a normally bulged U in the upstream 28S rRNA-SNORA53 hybrid region (Fig. 1B, left, shaded blue), thereby

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**Figure 1.** (A) Pseudouridylation is the isomerization of uridine (U) to pseudouridine ( $\Psi$ ). It involves breaking of the N-glycosidic bond, rotation of the base by 180°, and reattachment via a carbon–carbon glycosidic bond. (B) Schematic of an H/ACA guide RNA hybridized with one substrate RNA (blue) in its 5' hairpin pseudouridylation pocket. Either or both hairpins can guide pseudouridylation. An unpaired nucleotide (N) and the target U/ $\Psi$  (green) are positioned

underneath the distal stem of the hairpin (shaded gray). The 5' and 3' antisense elements hybridized to the substrate RNA are highlighted (shaded blue). The two hairpins are separated by the conserved sequence of the namesake hinge region and end in the ACA sequence exactly 3 nt from the 3' end. (C) In the novel mechanism described by Jády et al. (2022), a single RNA hairpin guides the pseudouridylation of two adjacent uridines (or separated by one N) by assuming two conformations, A and B, through guide RNA acrobatics.

stabilizing this double-stranded region and preventing the RNA acrobatics. As predicted, this restricted pseudouridylation to U3747. With this and additional controls, the investigators confirmed their original prediction of single H/ACA RNA pseudouridylation loops with dual guide capacity.

They go on to inspect all  $\Psi$ s in rRNA and snRNA that are next to each other or separated by 1 nt. Although many of these are selected by dedicated pseudouridylation loops (one for one), three pairs in addition to 28S rRNA  $\Psi$ 3747/3749 were eventually identified as targeted by single loops (one for two): U2 snRNA  $\Psi$ 43/44 and  $\Psi$ 89/91, and 18S rRNA  $\Psi$ 1045/1046. These were specified by the 3' hairpins of SCARNA8, SCARNA1, and SNORA57, respectively. Many of those  $\Psi$ s had not been assigned guide RNAs yet, but knockout and rescue experiments with wild-type and mutant guide RNAs confirmed that the guide RNA acrobatics results were similar to those described for SNORA53. Overall, 6% of all pseudouridines in rRNAs and snRNAs are generated by this novel, single-hairpin mechanism.

The most remarkable case is perhaps that of human U2 snRNA  $\Psi$ 43 and  $\Psi$ 44. These  $\Psi$ s follow three  $\Psi$ s at positions 37, 39, and 41 (for review, see Morais et al. 2021). Pseudouridylation of these five  $\Psi$ s is guided by three different H/ACA RNAs using separate mechanisms: one  $\Psi$  by an individual hairpin (one for one; SCARNA15, 3'), two by both hairpins of the same guide RNA (two for two; SCARNA4), and two by a single hairpin (3') using the newly identified guide RNA acrobatics (one for two; SCARNA8).

Using extensive mutational analysis, the investigators document that the base pairings of the guide RNA with the substrate RNA (i.e., the H/ACA antisense sequences) (Fig. 1B, shaded blue) are the major determinants of the dual guide activity. However, in the case of SNORA53, manipulation of the lower half of the distal stem was sufficient for locking it into configuration A or B (Fig. 1C, shaded gray). Importantly, these determinants of the 5' hairpin of SNORA53 and those of the 3' hairpins of SNORA57, SCARNA1, and SCARNA8 are evolutionarily remarkably conserved from humans to fish, underlining the important functional property of the one-for-two mechanism.

The molecular mechanism underlying guide RNA acrobatics will likely take some time to dissect. As suggested by the investigators, the H/ACA RNP core proteins, in particular GAR1, may play a role in RNA rearrangement. Structural analysis of the H/ACA RNP shows GAR1 bound to the guide RNA-associated pseudouridine synthase NAP57, albeit at a distance from NAP57's catalytic aspartate (Li and Ye 2006; Ghanim et al. 2021). Nonetheless, the observation that in an active H/ACA RNP GAR1 is cross-linked to the substrate U puts this protein at the center of catalysis (Wang and Meier 2004). Obviously, many interesting questions remain to be answered regarding the mechanism of guide RNA acrobatics. Is there a specific order to the modification of the two Us? Does it occur in one or two interactions? What regulates the 100% modification level at both sites? Is there a control mechanism to assess pseudouridylation? While we are awaiting answers, guide RNA acrobatics add an exciting novel mechanism to RNA flexibility, structure, and function.

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