PERSPECTIVE

RNAi in *Xenopus*: look before you leap

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RNAi has revolutionized reverse genetics; however, RNAi is not necessarily ubiquitous or constitutive. Lund and colleagues (pp. 1121–1131) show that microRNA (miRNA) effector Argonautes (Ago) are limiting and easily saturated during early *Xenopus* embryogenesis. Moreover, this stage is devoid of slicing capacity. Supplementation of Ago proteins rescued endogenous miRNA activity in the presence of exogenous siRNAs, and, excitingly, ectopic Ago2 could now support RNAi in *Xenopus*. These observations may potentially facilitate RNAi in other recalcitrant model organisms.

'It slices! It dices! And so much more!'

So exclaimed an enthused Ron Popeil, as his saccharine grin illuminated the set of his quintessential 1960s infomercial for the Ronco Veg-o-Matic. This catchphrase is now firmly entrenched in the American pop culture psyche, but, to modern biologists, "It slices! It dices!" immediately evokes the core enzymatic activities of RNAi. Akin to Mr. Popeil's slick gadget, the cutting actions of RNAi provide a powerful shortcut to assess gene loss of function. Unfortunately, precision in its usage can sometimes be elusive, and returns are more easily made with kitchen contraptions than with precious research effort and resources.

Many biologists now take the ability to apply RNAi for granted, but it is instructive to recall that it was not always so. The initial description of RNAi involved injection of long dsRNA triggers into *Caenorhabditis elegans* (Fire et al. 1998). However, in mammalian cells, dsRNA induces the antiviral interferon response via PKR, leading to global translational suppression independent of trigger sequence (Clemens 1997). A mechanistic understanding of RNAi eventually led to a solution. The key observation was that input dsRNA is not the active species per se; rather, it is processed into functional 21-nucleotide (nt) siRNAs that mediate target suppression (Zamore et al. 2000). Consequently, direct introduction of siRNA duplexes proved to bypass PKR, paving the way for exploitation of RNAi in mammalian cells (Elbashir et al. 2001).

Other mechanistic knowledge has improved the efficacy and specificity of RNAi. For example, the elucidation

of asymmetric duplex strand selection helped to minimize off-target regulatory activities of siRNA passenger strands (Khvorova et al. 2003; Schwarz et al. 2003). Synthetic shRNAs designed to mimic endogenous microRNAs (miRNAs) mediate effective transgenic silencing (Paddison et al. 2002), but their overly robust expression can saturate the endogenous miRNA export machinery (Grimm et al. 2006). Coexpression of Exportin5, the miRNA export protein, can alleviate this effect (Yi et al. 2005). Knowledge of miRNA:target interactions also revealed undesired regulatory effects of guide strands on off-target transcripts bearing seed matches (Jackson et al. 2006). Reciprocally, meta-analysis of transcriptome profiling experiments following siRNA treatment revealed competition or saturation of miRNA pathways, resulting in broad up-regulation of endogenous miRNA targets (Khan et al. 2009). A possible solution to these problems may come with the selection of ultrapotent shRNAs that are highly active at low levels, even as single-copy insertions (Fellmann et al. 2011).

Limiting Ago proteins and lack of slicing activity in early *Xenopus* embryos

With all of this progress, though, several popular model organisms, including fish and frogs, remain stubbornly recalcitrant to RNAi. In this issue of *Genes & Development*, Dahlberg and colleagues (Lund et al. 2011) explore the side effects of injecting siRNAs into *Xenopus* zygotes. This induces a range of morphological defects suggesting broad developmental perturbation, and these effects are independent of siRNA sequence (Lund et al. 2011). Upon investigating miRNA biogenesis following siRNA injection, Lund et al. (2011) found that the conversion of mature miRNAs from precursor hairpins was inhibited. In early *Xenopus* embryos, the predominant miRNA is miR-427, which participates in turnover of maternal messages (Lund et al. 2009). This function is analogous to that of zebrafish miR-430, a dominantly expressed early miRNA that degrades the bulk of maternal transcripts during the maternal-zygotic transition (Giraldez et al. 2006). The accumulation of mature miR-427 was significantly reduced in siRNA-injected embryos, and miR-427 reporters were no longer deadenylated (Lund et al. 2011), providing a potential explanation for the developmental phenotypes induced by generic siRNAs.

The accumulation of pre-miRNAs upon siRNA injection might suggest a defect in Dicer activity; however,

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overexpression of the Dicer cofactor TRBP (HIV-1-transactivating response RNA-binding protein), did not rescue generic siRNA-induced phenotypes. Instead, overexpression of multiple exogenous human Ago proteins could alleviate the nonspecific effects of siRNA injection (Lund et al. 2011). Although the precise mechanism of modulation of Dicer activity by the availability of Ago proteins remains unclear, similar accumulation of certain pre-miRNAs was observed in Ago-deficient mutants in worms and mice (Grishok et al. 2001; O'Carroll et al. 2007). Transfer of small RNA products from the Dicer complex to Ago proteins might facilitate turnover of the dicing cycle (Chendrimada et al. 2005; Gregory et al. 2005; Maniataki and Mourelatos 2005; MacRae et al. 2008). Western analysis showed that bulk Ago proteins are present at very low levels in oocytes and early embryos prior to mid-blastula transition, although their levels increase progressively over the next 2 d of development. This is in contrast to Dicer and Exportin5, whose protein levels remain constant across these stages. Thus, Ago proteins are particularly prone to saturation in the early frog embryo.

On the basis of these data, Lund et al. (2011) postulated that saturation of Ago proteins by injected siRNAs interferes with dicing of *pre-mir-427*, whose impaired maturation results in inappropriate stability of maternal messages, leading to developmental abnormalities. It would be interesting to see whether this conjecture can be confirmed by microarray analysis, to see whether miR-427 targets are nonspecifically up-regulated by diverse siRNAs, similar to the accumulation of miR-430 target transcripts in *Dicer*-mutant fish embryos (Giraldez et al. 2006). In any case, the saturability of endogenous Ago pools mirrors observations in other systems where elevation of Ago proteins can increase the accumulation of miRNAs or endo-siRNAs (Yigit et al. 2006; Diederichs and Haber 2007) and enhance on-target suppression (Diederichs et al. 2008; Grimm et al. 2010).

Any of three human Ago proteins tested (hAgo1/3/4) could alleviate the cohort of miRNA processing and developmental defects caused by siRNA injection (Lund et al. 2011). However, the capacity of early frog oocytes and embryos to cleave target messages was observed only upon expression of hAgo2, the sole vertebrate Slicer Argonaute. Therefore, endogenous *Xenopus* Ago2 protein is either not present or not functional at this stage, despite the fact that *ago2* transcripts can be detected (Armisen et al. 2009; Lund et al. 2011). Consistent with this, siRNA passenger strands (which are normally cleaved by Ago2 in mammalian cells) are stable in *Xenopus* oocytes, but not in hAgo2-supplemented embryos. This may reflect occlusion of endogenous Ago proteins with siRNA duplexes, which could further contribute to the potent inhibition of miRNA biogenesis and activity by generic siRNAs.

Finally, the Dicer-independent, Ago2-mediated biogenesis of vertebrate miR-451 provided another convenient genetic assay for slicing capacity (Yang and Lai 2010). The 42-nt *pre-mir-451* hairpin is too short to be cleaved by Dicer and is instead loaded directly into Ago2, which cleaves its 3' arm to generate a 30-nt species whose 3' end

is resected to yield mature 23-nt miR-451 (Cheloufi et al. 2010; Cifuentes et al. 2010; Yang et al. 2010). Injected *pre-mir-451* hairpins were stable in *Xenopus* oocytes and early embryos, further indicating the absence of endogenous Slicer activity. However, coinjection with human Ago2, but not catalytically inactive Ago2, now resulted in successful maturation of miR-451 (Lund et al. 2011).

Evolutionary dichotomy in the usage of endo-siRNAs in different vertebrate oocytes

The tests of Lund et al. (2011) clearly document the low level of Ago proteins and absence of slicer activity in *Xenopus* oocytes and early embryos. Curiously, these features do not seem to be shared in mammals. As mentioned, most mammalian cells lack the capacity to use long dsRNA as RNAi triggers, due to activation of the interferon pathway. Nevertheless, it has been known for some time that mouse oocytes are amenable to the RNAi technique (Svoboda et al. 2000). In fact, mouse oocytes and early embryos happen to be specific settings where endogenous dsRNA generates abundant siRNAs. These derive from antisense-transcribed pseudogenes hybridized to their sense counterparts, as well as from specific transposon classes and some long inverted repeats, and these endo-siRNAs detectably mediate target regulation via Ago2 (Tam et al. 2008; Watanabe et al. 2008).

A surprising twist is that, while miRNAs are also expressed in mouse oocytes, miRNA-mediated regulation appears to be globally suppressed at this stage (Ma et al. 2010; Suh et al. 2010). This is evidenced most convincingly by the fact that *ago2* and *dicer* mutants exhibit common defects at the earliest zygotic divisions, whereas loss of the Drosha partner DGCR8 causes neither mutant phenotypes nor substantial changes across the transcriptome at this stage. These differences exist despite the fact that mature miRNAs are depleted in *dgcr8* mutant early embryos, similar to *dicer* mutants. Based on these findings, it is inferred that, among Dicer-dependent small RNAs, early mammalian development may rely exclusively on endo-siRNAs and not on miRNAs.

In light of this, it is not clear why *Xenopus* does not supply active Ago2 protein maternally. Overexpression of Ago2 in oocytes did not cause obvious developmental defects that might otherwise have indicated some need to restrict slicing at this stage (Lund et al. 2011). Perhaps some clues to the underlying biology may become apparent through molecular phenotypes in transcriptome analysis. The seemingly opposite requirements for miRNAs and endo-siRNAs in the early development of fish and frogs (Giraldez et al. 2006; Lund et al. 2009) compared with mice (Ma et al. 2010; Suh et al. 2010) reminds us that, even as we seek to apply previously gained knowledge to new biological settings, it is prudent to perform research with an open mind.

Prospects for applying RNAi in frogs and fish

Even if we do not yet understand why *Xenopus* oocytes do not exhibit endogenous Slicer activity, the observation

that seemingly few defects occur after injection of hAgo2 provides a tantalizing prospect for developing effective RNAi in *Xenopus*. Currently, reverse genetics in model systems such as frogs and fish is mainly accomplished using morpholinos. These modified antisense oligonucleotides are usually designed to hybridize with translational initiation or splice sites to block protein production (Hulstrand et al. 2010). While this strategy has been developed as a viable technique, it is not without caveats. For example, the dilution of injected oligonucleotides during cell division limits their capacity for inhibition to early development. In addition, the injection approach does not afford cell or tissue selectivity. Perhaps most importantly, morpholinos can cause general toxicity, so phenotypic specificity must be carefully evaluated using titration series.

The mechanistic work of Dahlberg and colleagues (Lund et al. 2011) suggests that on-target RNAi may be possible when supplementing *Xenopus* with Ago2, broadening the palette of gene suppression tools in frogs. Curiously, coinjection of synthetic miR-427 siRNA duplex with hAgo2 did not promote precocious target deadenylation as efficiently as in their earlier experiments with *pre-mir-427*, which did not use exogenous Ago2 (Lund et al. 2009). This suggests that the generation of fully functional miR-427–RNA-induced silencing complexes (RISC) is potentiated by dicing, as proposed in mammalian systems (Gregory et al. 2005; Kim et al. 2005; Maniataki and Mourelatos 2005). Therefore, injection of shRNA-type hairpins may prove efficacious. It is worth noting that the miR-451 system may provide some conceptual benefits to conventional shRNAs, since miR-451-class substrates mature exclusively in Ago2/Slicer and lack star strands (Yang and Lai 2010). Indeed, the *mir-451* backbone is amenable to functional reprogramming in zebrafish and mammalian cells (Cheloufi et al. 2010; Cifuentes et al. 2010; Yang et al. 2010), and the *mir-451* system was shown to work in frogs as well (Lund et al. 2011).

Even in systems such as *Drosophila* and mammalian cells, which have benefited from more than 10 years of effort on optimizing RNAi specificity and efficacy, studies from even the past couple of months continue to document substantial technical improvements (Fellmann et al. 2011; Ni et al. 2011). The stage is now set for in-depth studies toward optimization of RNAi in *Xenopus* using the Ago2 trick. If this strategy can be shown to be broadly effective, it will be a great boon to amphibian research. Perhaps more tantalizing is the suggestion that a similar strategy may help to achieve on-target RNAi in zebrafish, where RNAi invariably induces nonspecific effects (Zhao et al. 2008; Fjose and Zhao 2010), conceivably due to inhibition of miR-430-mediated regulation (Giraldez et al. 2006). The outcome of such tests is eagerly awaited.

Overall, the studies of Lund et al. (2011) highlight that RNAi should always be interpreted with caution, especially in untested cell types or tissues. Who would have guessed that *Xenopus* oocytes and early embryos can dice, but cannot slice? Beyond testing of multiple triggers and appropriate negative controls in RNAi experiments,

the possibility of nonspecific effects due to titration of endogenous miRNAs should also be considered (Grimm et al. 2006; Zhao et al. 2008; Khan et al. 2009; Fjose and Zhao 2010; Lund et al. 2011). On the other hand, these studies strongly affirm the value of basic mechanistic studies in aiding the exploitation of gene regulatory mechanisms for designed purposes. Clearly, if RNAi technology is to become a clinical tool, the intended cells must be able to support ectopic small RNAs without affecting endogenous miRNA-mediated regulation, and with a minimum of unintended regulatory consequences. Hopefully, clever strategies can be devised to circumvent such problems.

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