Smaug

An unexpected journey into the mechanisms of post-transcriptional regulation

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Drosophila Smaug is a sequence-specific RNA-binding protein that can repress the translation and induce the degradation of target mRNAs in the early *Drosophila* embryo. Our recent work has uncovered a new mechanism of Smaug-mediated translational repression whereby it interacts with and recruits the Argonaute 1 (Ago1) protein to an mRNA. Argonaute proteins are typically recruited to mRNAs through an associated small RNA, such as a microRNA (miRNA). Surprisingly, we found that Smaug is able to recruit Ago1 to an mRNA in a miRNA-independent manner. This work suggests that other RNA-binding proteins are likely to employ a similar mechanism of miRNA-independent Ago recruitment to control mRNA expression. Our work also adds yet another mechanism to the list that Smaug can use to regulate its targets and here we discuss some of the issues that are raised by Smaug's multi-functional nature.

Post-transcriptional regulation of gene expression can be mediated by *cis*-acting elements within mRNAs that can be recognized by RNA-binding proteins and their accessory factors. One such protein, Smaug, is a highly-conserved, sequence-specific RNA-binding protein that plays a prominent role in posttranscriptional regulation in the early *Drosophila* embryo through its ability to repress translation and induce mRNA decay.¹⁻⁴ Indeed, Smaug is required to destabilize up to two-thirds of the unstable mRNAs in the early *Drosophila* embryo.⁵ Smaug and its homologs regulate target mRNAs through

a conserved Sterile Alpha Motif domain that binds to well-defined stem-loop structures, known as Smaug Recognition Elements (SREs).^{1-4,6-8}

In addition to RNA-binding proteins, mRNAs can also be regulated by *cis* elements that represent binding sites for non-coding RNAs where recognition is mediated by base pairing. For instance, microRNAs (miRNAs) are major post-transcriptional regulators of gene expression.⁹ Traditionally miRNA-mediated regulation occurs when a miRNA, bound to an Argonaute (Ago) protein, recruits Ago to an mRNA to trigger Ago-dependent translational repression and/or decay (Fig. 1A).

An Unexpected Mechanism of Smaug-Mediated Translational Repression

In Pinder and Smibert¹⁰ we uncovered a novel miRNA-independent mechanism of Ago1 recruitment through the Smaug RNA-binding protein. We initially found that Ago1 coimmunoprecipitates with Smaug. To test the significance of the Ago1/Smaug interaction in Smaug function we assessed the role of Ago1 in the regulation of Smaug's archetypal target mRNA, *nanos* (*nos*). Smaug binds to *nos* mRNA through two SREs in the *nos* mRNA's 3' untranslated region (UTR), thereby repressing *nos* translation.^{1-3,11} Using an Ago1 mutant we showed that Ago1 is required to repress *nos* translation as well. We also showed that Ago1 co-purifies with *nos* mRNA and that this interaction remarkably requires Smaug but does not require a targeting miRNA.

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Figure 1. Mechanisms of recruitment of the Ago1/Smaug complex. **(A)** The standard mechanism of Ago1 recruitment to an mRNA via the complementarity between a bound miRNA and the target. **(B)** The model for the Smaug-dependent miRNA-independent recruitment of Ago1 to mRNAs such as *nos* through Smaug's interaction with an SRE stem/loop. Note that, as discussed in more detail in the text, Ago1 in this complex may contain an associated miRNA which, while not base paired with the target mRNA and therefore not involved in Ago1 recruitment, could be important for translational repression. **(C)** The Ago1/Smaug complex could be recruited to some mRNAs through base pairing between a miRNA and the transcript. **(D)** The Ago1/Smaug complex could be recruited to other mRNAs through the combined interaction of Smaug with an SRE and the complementarity of a miRNA to the transcript.

Taken together, our work indicates that Smaug, bound to the *nos* 3'UTR, can bypass the need for a targeting miRNA to recruit Ago1 to *nos* and repress its translation (Fig. 1B).

While a miRNA is not required for the interaction between Ago1 and *nos* mRNA it is possible that a miRNA that is not annealed to *nos* mRNA is necessary for steps downstream of recruitment. For instance, incorporation of a miRNA may induce allosteric changes in Ago1 that facilitate binding to accessory proteins that are necessary for translational repression. Indeed, Djuranovic et al.¹² suggest that miRNA-dependent allosteric changes are important for Ago-mediated repression, while Elkayam et al.¹³ have demonstrated that miRNA binding stabilizes Ago proteins.

Our observation that an RNA-binding protein can recruit Ago1 to a target mRNA in the absence of a targeting miRNA represents a new mechanism of post-transcriptional control that is unlikely to be limited to Smaug. Indeed, comparison of mRNAs that co-purify with Ago from normal cells vs. cells that lack miRNAs suggests that Ago proteins can co-purify with specific mRNAs in the absence of miRNA-targeting.^{14,15} Furthermore, Friend et al.¹⁶ have shown that a reporter

RNA that is not expected to contain a miRNA-targetable *cis* element is regulated by the Pumilio RNA-binding protein in complex with Ago in vitro.

While our data indicate that Smaug recruits Ago1 to *nos* mRNA through a miRNA-independent mechanism, the Ago1/Smaug complex could bind to other mRNAs through alternative mechanisms. For example, Ago1 could recruit Smaug to an mRNA through a miRNA binding site in the absence of an SRE (Fig. 1C). Alternatively, an mRNA that carries both an SRE and a miRNA-binding site could be bound by the complex through both *cis* elements (Fig. 1D). In this case the interaction with both *cis* elements simultaneously could significantly increase the stability of the Smaug/Ago1/mRNA complex. If these alternative mechanisms of recruitment do indeed exist it would be interesting to assess what effect the mode of recruitment has on mechanisms that regulate the target mRNA.

Smaug Employs Multiple Mechanisms to Regulate Its Target mRNAs

The first indication that Smaug is multifunctional came from our work that suggested Smaug could both repress

translation and induce transcript degradation and the fact that translational repression preceded transcript decay.¹⁷ Subsequently, we showed that Smaug interacts with the Cup protein which in turn interacts with the cap binding protein eIF4E, and both in vitro and in vivo evidence indicated that the Smaug/Cup/eIF4E complex blocks 40S ribosome subunit recruitment, thereby repressing translation initiation.¹⁸ While other groups have generated data consistent with a role for Cup in Smaug-mediated translational repression,^{19,20} recent work by Igreja et al.²¹ suggests that the interaction between Cup and eIF4E is not always necessary for Cup-dependent translational repression. This conclusion was drawn from experiments where Cup was targeted to a reporter mRNA by fusing it to a heterologous RNA-binding domain which may have had unanticipated effects on Cup function. Consistent with this possibility, the authors found that when Cup is recruited to an mRNA via its interaction with the RNA-binding protein Bruno the Cup/eIF4E interaction is required for full repression. Taken together these data suggest that the mechanism of Cup recruitment to a target mRNA likely influences the mechanism involved in Cup-mediated regulation.

In addition to its interaction with Cup, Smaug also regulates target mRNAs through its interaction with the Ccr4/Not deadenylase complex, and Smaug-mediated Ccr4/Not recruitment results in removal of a transcript's poly(A) tail.²²⁻²⁵ Consistent with the role of the poly(A) tail in stabilizing an mRNA,²⁶ Smaug-mediated deadenylation induces transcript decay. Smaug-mediated deadenylation and degradation of *nos* mRNA has been proposed to involve a complex that contains Smaug, Aubergine (a piwi-type Ago), and the Ccr4/Not deadenylase.²⁷ Recruitment of this complex is thought to involve both Smaug binding to the *nos* SREs and the interaction of a piwiRNA, bound to Aubergine, that is complementary to sequences in the *nos* 3' UTR.

The poly(A) tail also plays a role in translation initiation and in principle Smaug could also repress translation through deadenylase recruitment.²⁶ However, whether deadenylation inhibits translation beyond simply decreasing mRNA levels has only been rigorously tested for one Smaug target mRNA, *Hsp83*, and in this case deadenylation does not repress *Hsp83* translation.²²

A final mechanism of Smaug function is indicated by work using in vitro translation extracts which indicate that Smaug can repress translation at a step after recruitment of the 40S ribosome.²⁰ This work also uncovered a slow step in Smaug-mediated translational repression that is ATP-dependent and that results in the formation of a very stable repressed mRNA/protein complex. While the mechanisms that underlie these aspects of Smaug function are currently not known, it is intriguing that Ago1-mediated translational repression in *Drosophila* embryo in vitro translation extracts also requires ATP.²⁸ The results of future work to test if Ago1 is involved in the ATP-dependence of Smaug repression will be of great interest.

Unanswered Questions

A number of questions are raised by Smaug's ability to employ multiple mechanisms to regulate its target mRNAs. For instance, how are Smaug's translational repression and transcript destabilization functions related? Current data

suggest that Smaug-mediated translational repression and transcript decay are separate processes. For example, in early embryos, components of the Ccr4/Not deadenylase complex do not co-immunoprecipitate with either Cup or eIF4E suggesting that the Smaug/Cup/eIF4E complex and Smaug/CCR4/Not deadenylase complex are distinct.²² In addition, Smaug-mediated translational repression functions in early embryos prior to the onset of Smaug-mediated transcript decay.¹⁷ Taken together these data suggest that different Smaug functions might be important at different stages of development.

Another important question is why Smaug is capable of employing multiple mechanisms to repress translation? One possibility is that redundancy provides a buffering system that ensures protein expression is prevented in the event that a single mechanism of repression is insufficient. For example, if the block to translation initiation imposed by the Smaug/Cup complex is inefficient then repression mechanisms that function at a step downstream of 40S ribosomal subunit recruitment could come into play. Alternatively, since the ATP-dependent mechanism of Smaug-mediated repression, which leads to a very stable repressed mRNA/protein complex, is slow, the Smaug/Cup interaction might be required to function prior to establishing the stably repressed mRNA. In a related model the various mechanisms of Smaug-mediated translational repression could cooperate together, perhaps in a step-wise fashion, which ultimately incorporates repressed mRNAs into stable mRNA/protein complexes.

A final question is what determines which of Smaug's repressive mechanisms is used to regulate particular Smaug targets? Smaug's multi-functionality could permit Smaug to regulate different mRNAs through different mechanisms, and studies of Smaug's two best-characterized target mRNAs, *nos* and *Hsp83*, suggest that this is indeed the case. While Smaug represses *nos* translation, it plays only a modest role in regulating *nos* mRNA stability.^{1-3,22,29} In contrast, Smaug plays a major role in regulating *Hsp83* mRNA stability while it does

not regulate *Hsp83* translation.^{22,25} The molecular mechanisms that underlie these differences are not understood, but SRE location could play a role as the *Hsp83* SREs are in the transcript's open reading frame, while the SREs in *nos* are in the 3'UTR. An alternative explanation is that additional *cis* elements within Smaug target mRNAs and the *trans*-acting factors that bind them influence the mechanisms of Smaug function. For example, the *Hsp83* mRNA contains a translational enhancer in its 3'UTR raising the possibility that this element is able to override Smaug's ability to repress *Hsp83* translation.³⁰

Future Prospects

While considerable progress has been made in understanding Smaug function, many questions remain unanswered. Genome-wide experiments where Smaug or Ago1 are immunoprecipitated and copurifying mRNAs are identified by microarray or deep sequencing will identify transcripts that are associated with these proteins. Similar experiments using mutations in Smaug that block RNA-binding and in Ago1 that block miRNA binding will identify other transcripts that are bound by Ago1/Smaug through the same mechanism as *nos* and indicate whether other mRNAs are bound by Ago1/Smaug through the alternative mechanisms outlined in Figure 1.

Uncovering the molecular mechanisms that underlie repression mediated by the Ago1/Smaug complex and the ATP-dependent mechanism of Smaug-mediated translational repression will also be important next steps. Identification of mutations in Smaug that specifically disrupt its interactions with various binding partners will allow for an assessment of the contribution that each of these interactions make to Smaug function. These mutants will also allow for investigations of how these different mechanisms might function with one another to regulate target mRNAs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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