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Report

MicroRNAs Act as Cofactors in Bicoid-Mediated Translational Repression

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Summary

Noncoding RNAs have recently emerged as important regulators of mRNA translation and turnover [1, 2]. Nevertheless, we largely ignore how their function integrates with protein-mediated translational regulation. We focus on Bicoid, a key patterning molecule in *Drosophila*, which inhibits the translation of *caudal* in the anterior part of the embryo [3, 4]. Previous work showed that Bicoid recruits the cap-binding protein d4EHP on the *caudal* mRNA to repress translation [5]. Here we show that *miR-2* family microRNAs are essential cofactors in the repression of *caudal*. Using an in vivo sensor, we demonstrate that Bicoid acts through a 63 nt response element in the *caudal 3′ UTR* that includes a single *miR-2* target site. Mutating that site abolishes Bicoid-mediated repression, and this effect can be partly reversed by expressing a microRNA with compensatory changes that restore binding to the mutated target. Four predicted Bicoid splice isoforms are capable of *caudal* repression, including two that lack the d4EHP interaction domain; all four isoforms require the microRNA target for repression. The synergy between Bicoid and microRNAs appears to have evolved recently in the context of the drosophilid *caudal BRE*. The discovery that microRNAs play an essential role in Bicoid-mediated translational repression opens up new perspectives on Bicoid's function and evolution.

Results and Discussion

Bicoid (Bcd) is a key regulator that functions as a morphogen to define the anterior-posterior axis of *Drosophila* embryos [6, 7]. It fulfils this role by acting both as a transcriptional activator and as a translational repressor of different target genes in early blastoderm embryos [3, 4, 8–10]. Bicoid evolved recently, within cyclorrhaphan flies, from a homeobox-containing gene of the Hox family [11–14], by acquiring a suite of new properties that include its anterior localization in early embryos, a major change in its DNA binding specificity, and the ability to bind RNA and to regulate translation [15]. Bicoid thus serves as an excellent paradigm for the evolution of gene functions.

The only known translational target of Bicoid is the posterior patterning gene *caudal*, whose maternally transcribed messenger RNAs (mRNAs) are ubiquitously distributed in early embryos [3, 4]. Bicoid-mediated translational repression generates an inverse (posterior) gradient of Caudal (Cad) protein (Figure 1A). Previous work showed that *caudal* repression involves direct binding of the Bicoid homeodomain to the 3′ untranslated region (UTR) of *caudal* mRNA and recruitment of the cap-binding protein d4EHP [3–5, 16, 17]. This mechanism has served as a new paradigm for translational repression via competitive cap binding [18]. Here we report that Bicoid-mediated repression is more complex than previously thought and involves an unsuspected contribution from microRNAs.

In Vivo Sensor for Bicoid-Mediated Translational Repression

To monitor Bicoid's ability to regulate translation in vivo, we established a fluorescent sensor. It consists of a transgene expressing nuclear-localized EGFP followed by the *caudal 3′ UTR*, under the constitutive *tub-α1* promoter (Figure 1B). A control sensor contains the SV40 early polyadenylation sequence instead of the *caudal 3′ UTR*. We inserted both constructs at the same location in the *Drosophila* genome using phiC31-mediated integration (see the Supplemental Experimental Procedures available online). Flies carrying these constructs expressed high levels of EGFP protein in their ovaries, which perdured to embryonic stages. This ubiquitous maternal EGFP prevented us from observing Bicoid-mediated translational repression in early embryos. However, by expressing Bicoid protein uniformly in oocytes and early embryos, using *nanos-GAL4* and a *UAS-Bcd* construct carrying the *fs(1)K10 3′ UTR* instead of the *bicoid 3′ UTR*, we observed that Bicoid exerted a strong repression on the *caudal 3′ UTR* sensor (Figure 1B). The control sensor, carrying the SV40 polyA, was insensitive to Bicoid (Figure 1B). Thus, we developed a sensor that recapitulates Bicoid-mediated repression on the *caudal 3′ UTR* during oogenesis.

Using a modified sensor, expressing an unstable EGFP-Cad fusion protein with the *caudal 3′ UTR*, we were able to detect the graded activity of Bicoid in early embryos (see Figure S1A). However, the weaker and graded fluorescence obtained with that sensor was less reliable for quantitative measurements.

Bicoid Isoforms Lacking the d4EHP-Interaction Domain Are Still Capable of Repression

The five known splice isoforms of *bicoid* are predicted to encode different protein isoforms (http://flybase.org; Figure 1C), but potential functional differences of these isoforms had not been tested. We examined the functional properties of each Bicoid isoform by generating UAS lines for each isoform and expressing them uniformly in the oocyte and early embryo using the *nanos-GAL4* driver (see the Supplemental Experimental Procedures).

The transcriptional capability of each isoform was tested by examining the expression of a known transcriptional target of Bicoid, *hunchback*, in embryos. We found that isoforms D to G are capable of driving ectopic *hunchback* expression, whereas isoform A, which lacks the homeodomain, is not (see Figure S1B). Next we examined the translational capability of each isoform using the *caudal 3′ UTR* sensor described earlier. Isoforms D to G repressed the sensor, whereas isoform A had no effect (Figure 1D). Consistent results were obtained using different *UAS-Bcd* insertions. Repression by isoforms D to G

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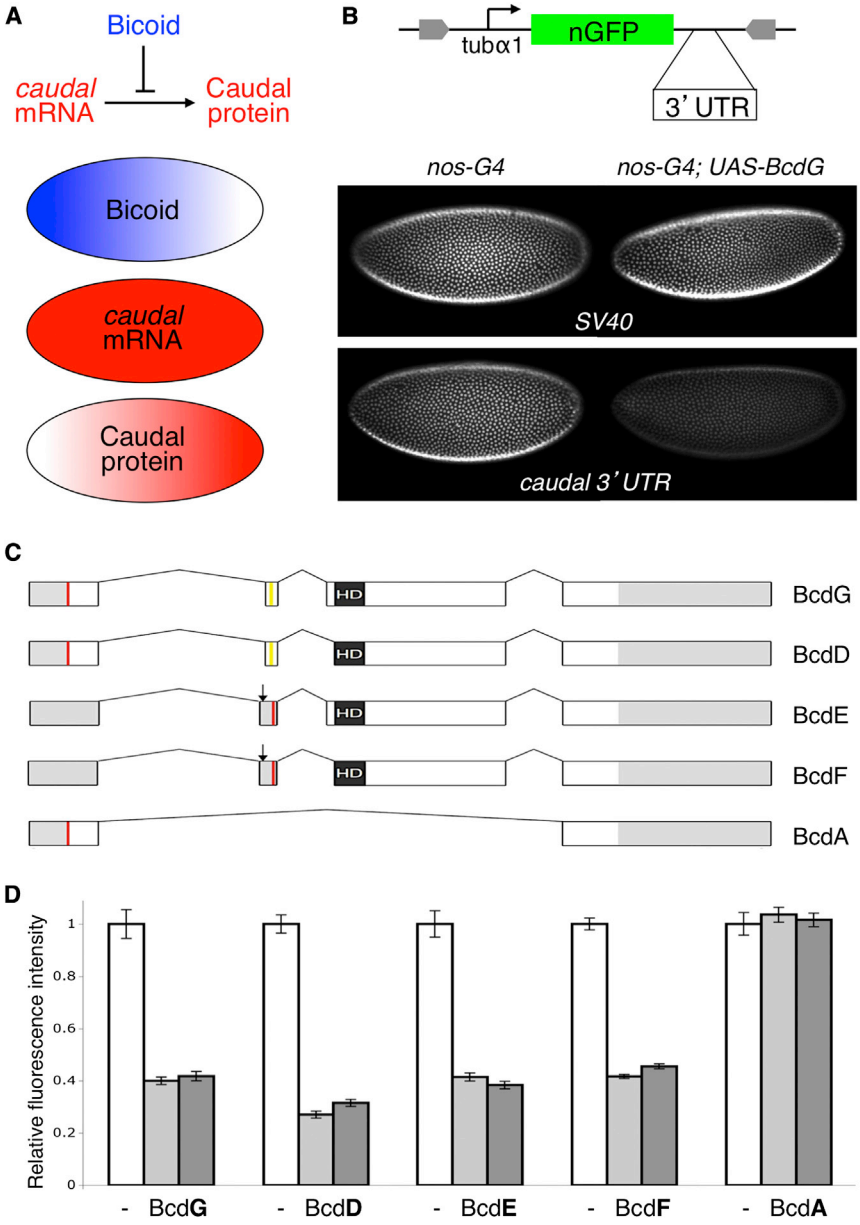

Figure 1. In Vivo Sensor Reveals Translational Regulation by Bcd Isoforms

(A) Schematic representation of Bicoid and Caudal distributions in early *Drosophila* embryos. Maternal *caudal* mRNA is uniformly distributed. The anterior gradient of Bcd represses *caudal* translation, resulting in an opposite gradient of Caudal protein [3, 4].

(B) The in vivo sensor of Bcd-mediated translation consists of a maternally active promoter (*tub-α1*) driving expression of nuclear-localized EGFP followed by 3′ UTR sequences whose activity we are testing. Using the sensor carrying the *SV40 3′ UTR*, we observe high levels of GFP fluorescence in blastoderm embryos, both in the presence and absence of maternally expressed Bcd (*nos-GAL4* versus *nos-GAL4; UAS-BcdG*). In contrast, we observe that Bcd is able to exert a strong repression on the sensor carrying the *caudal 3′ UTR*. Fluorescence images for each sensor were captured using identical settings. The *SV40 3′ UTR* sensor is expressed at higher levels than the *caudal 3′ UTR* sensor, so we used shorter exposure times to image those embryos.

(C) Representation of the five splice isoforms of Bcd (see http://flybase.org). The longest protein isoform is BcdG. Isoforms D and F utilize an alternative splice acceptor in exon 3, generating proteins that lack a short sequence just upstream of the homeodomain (HD). Isoforms E and F utilize an alternative splice acceptor in exon 2, which results in the introduction of a stop codon (arrow) in frame with the first AUG; an alternative in-frame AUG in exon 2 may be used to initiate translation in these mRNAs (AUG start codons marked in red). Isoform A lacks exons 2 and 3, generating a protein that lacks both the homeodomain and the d4EHP-interaction domain. Putative coding sequences are shown in white, 5′ and 3′ UTRs in gray, the d4EHP interaction domain [5] is marked in yellow and the homeodomain (HD) in black.

(D) Assaying translational repression ability of each Bcd isoform using the *caudal 3′ UTR* sensor. Fluorescence was quantified on cycle 11 blastoderm embryos laid by females carrying single insertions of the sensor, *nanos-GAL4* and *UAS-Bcd*; two lines, carrying different insertions of the *UAS-Bcd* transgene, were tested for each isoform (gray bars). The mean fluorescence intensity of each set was quantified in relation to control embryos from females carrying the sensor and *nos-GAL4* (white bars). Note that isoforms E and F, which lack the characterized d4EHP interaction domain, are still capable of robust repression. Error bars represent one SE.

See also Figures S1 and S2.

was also seen with Caudal antibody stainings in early embryos (Figure S1C). Sensor mRNA levels are not significantly affected by the presence of Bicoid isoforms D to G (Figure S2A), suggesting that these isoforms exert their effects primarily by translational repression. These results show that isoforms D to G are all potentially contributing to the transcriptional and translational activities of Bicoid, whereas isoform A is unlikely to do so.

Strikingly, isoforms E and F lack the d4EHP-binding domain, which is thought to be necessary for translational repression via d4EHP recruitment and competitive inhibition at the cap [5]. The fact that these isoforms are equally capable of repressing *caudal* suggests the existence of alternative mechanisms of Bicoid-mediated translational repression.

The *caudal 3′ UTR* Contains a Bicoid-Response Element with a Putative MicroRNA Target Site

To examine how Bicoid exerts its repression, we identified a short fragment of the *caudal 3′ UTR* that can mediate Bicoid-dependent repression in vivo. Previous work had defined a 350 nt Bicoid response element (BRE) [3]. Guided by sequence conservation, we narrowed that activity down to a 63 nt fragment, *BRE(257–319)*, that encompasses nucleotides 257–319 of the *caudal 3′ UTR* (Figure 2). When incorporated into the control sensor construct, this fragment recapitulates Bicoid-dependent repression (Figure 2E). Bicoid does not significantly reduce *BRE(257–319)* sensor mRNA levels (Figure S2B), suggesting that the effect is largely due to translational repression.

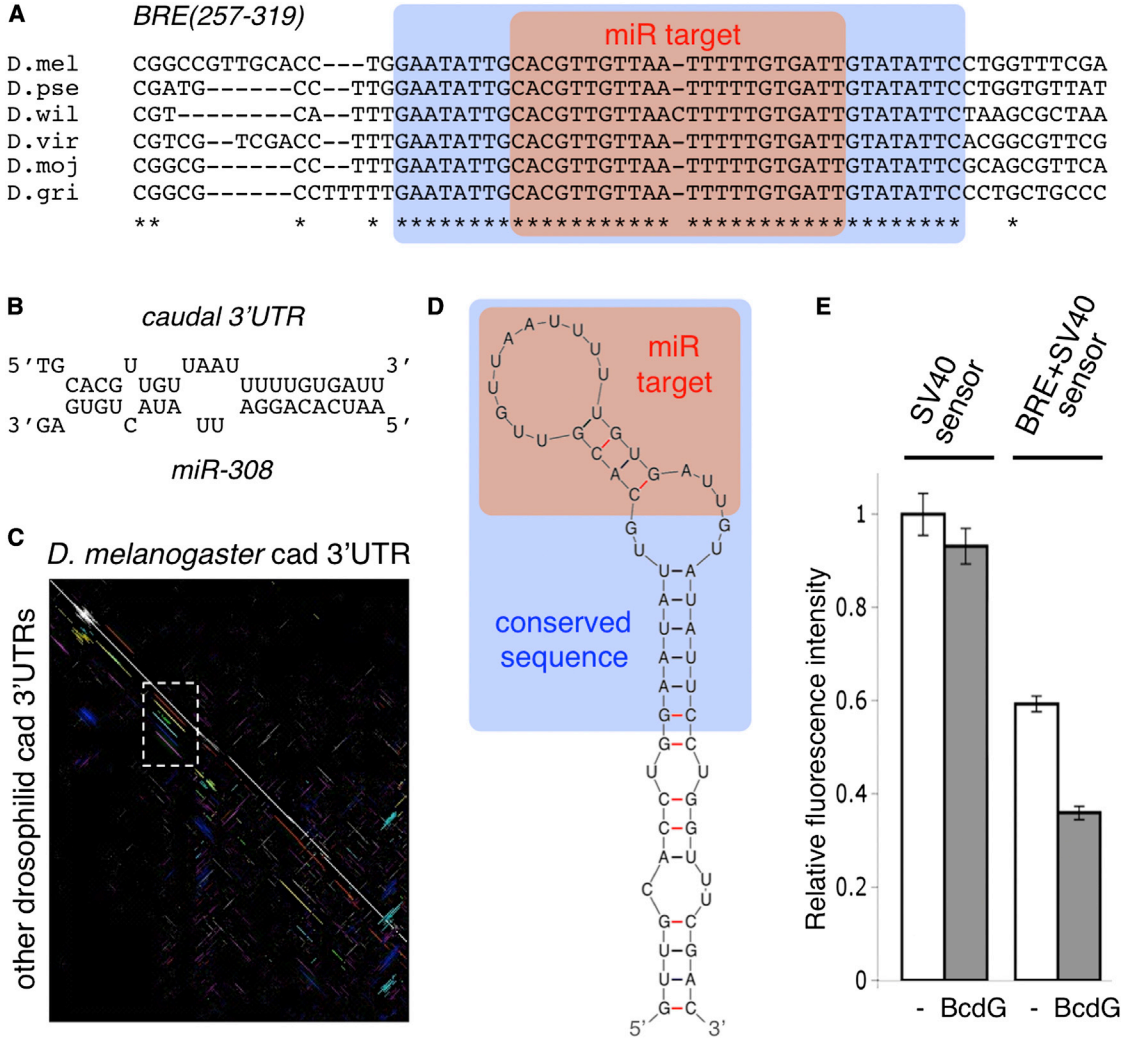

Figure 2. The Bicoid Response Element in the *Caudal 3′ UTR* Contains a Putative MicroRNA Binding Site

(A) Sequence alignment of *BRE(257–319)* among drosophilid species. The conserved sequence and putative microRNA target site are highlighted in blue and red, respectively.

(B) Predicted interaction of putative microRNA target site in the *caudal 3′ UTR* with *Drosophila miR-308*; similar interactions are predicted with other microRNAs that share the *miR-2* seed sequence (*miR-2*, *miR-6*, *miR-11*, *miR-13*, and *miR-308* [19, 20]).

(C) Dot plot of *Drosophila melanogaster caudal 3′ UTR* sequence compared to the homologous 3′ UTRs of other drosophilid species. Different pairwise comparisons are shown in separate colors. The only region where all drosophilid sequences show significant conservation is indicated in the box.

(D) Putative RNA secondary structure of *BRE(257–319)*; the conserved portion of the sequence and the putative microRNA target site are highlighted.

(E) *BRE(257–319)* is sufficient to mediate Bcd-dependent repression in vivo. We compare the activities of a control sensor, carrying the *SV40 3′ UTR*, and a sensor carrying *BRE(257–319)* just upstream of the *SV40 3′ UTR*. Fluorescence was quantified in cycle 11 blastoderm embryos laid by females carrying single insertions of each sensor and *nanos-GAL4*, in the presence or absence of *UAS-BcdG* (white and gray bars, respectively). The mean fluorescence intensity of each set was quantified in relation to the control sensor in the absence of Bcd. The presence of *BRE(257–319)* confers Bcd sensitivity to the *SV40 3′ UTR*. Lower overall fluorescence levels in the *BRE(257–319)* sensor could be mediated by the microRNA target site. Error bars represent one SE. We note that the *BRE(257–319)* sensor is expressed at much higher levels than the *caudal 3′ UTR* sensor, in the absence of Bicoid, suggesting that additional elements outside of *BRE(257–319)* contribute to *caudal mRNA* repression independently of Bicoid.

See also Figures S2 and S3.

As we describe below, certain mutations within *BRE(257–319)* abolish responsiveness to Bicoid. Thus, elements contained within *BRE(257–319)* are both necessary and sufficient to mediate responsiveness to Bicoid.

BRE(257–319) displays a number of interesting features. First, within the *caudal 3′ UTR*, it is the region with the highest degree of sequence conservation among drosophilids (Figures 2A and 2C). Second, RNA secondary structure predictions suggest that the fragment may fold into a stable hairpin structure (Figure 2D). Third, the distal part of that hairpin harbors a putative target site for microRNAs of the *miR-2* family, including microRNAs *miR-2*, *miR-6*, *miR-11*, *miR-13*, and *miR-308*, which share the same seed sequence (Figure 2B). The putative microRNA target lies at the center of the conserved region. The presence of a conserved microRNA target site within the Bicoid-responsive element suggests that the translational regulation of *caudal* could involve an interaction between Bicoid and microRNAs.

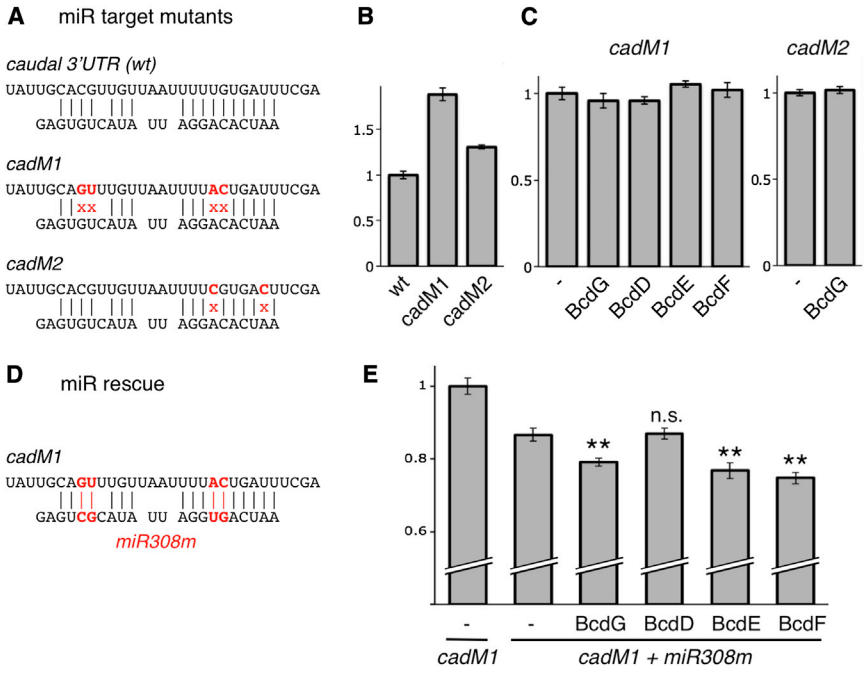

Figure 3. The MicroRNA Target Site in *BRE(257–319)* Is Essential for Bcd-Mediated Repression

(A) To test the role of microRNA binding, we generated two sets of mutations in the *caudal 3′ UTR*, named *cadM1* and *cadM2*, which are predicted to completely disrupt binding of *miR2* family microRNAs. The mutations (highlighted in red) disrupt binding at the microRNA seed sequence [21, 22] but preserve the secondary structure of the 3′ UTR (shown in Figure 2D).

(B) The fluorescence intensity of the *cadM1* and *cadM2 in vivo* sensors was quantified in cycle 11 blastoderm embryos, relative to the intensity of the wild-type *caudal 3′ UTR* sensor ("wt"), in the absence of Bcd. Both mutant sensors displayed significantly higher fluorescence levels than the wild-type.

(C) The fluorescence intensity of the *cadM1* and *cadM2* sensors was not affected by the expression of Bcd (embryos laid by *nos-GAL4*; *UAS-Bcd* females).

(D) To rigorously test the requirement of microRNA binding for Bcd-mediated repression, we asked whether sensitivity to Bicoid can be recovered by providing a mutated microRNA (*miR308m*) that restores binding to *cadM1*.

(E) The fluorescence intensity of the *cadM1* sensor is reduced in the presence of *miR308m*, and sensitivity to Bcd is partially restored. The strength of Bcd-mediated repression is limited, but highly significant with Bcd isoforms G, E, and F. We do not detect an effect with Bcd isoform D, which may point to additional isoform-specific requirements.

All error bars represent one SE (two asterisks indicate 99% statistical confidence; n.s. indicates no statistical significant difference, using a t test).

Mutations in the MicroRNA Target Site Abolish Bicoid-Mediated Repression

To directly test whether the putative microRNA target site is necessary for Bicoid-mediated repression, we generated two sets of mutations (*cadM1* and *cadM2*) that disrupt microRNA binding while preserving the putative secondary structure of the *caudal 3′ UTR* (Figure 3A). These mutations were introduced into our sensor constructs in the context of the entire *caudal 3′ UTR* and were tested in vivo, in the presence or absence of Bicoid.

In the absence of Bicoid, both mutant sensors gave higher readings than the unmutated *caudal 3′ UTR* sensor (Figure 3B), consistent with the expected repressive effect of microRNA binding. Remarkably, adding Bicoid produced no significant change in expression levels of either *cadM1* or *cadM2* (Figure 3C); all the Bicoid isoforms tested (isoforms D to G) were incapable of repression. These results show that the predicted microRNA target site contains essential elements for Bicoid-mediated repression.

Providing a MicroRNA with Compensatory Changes Restores Bicoid-Mediated Repression

The mutant sensor results are consistent with the idea that microRNAs could be involved in Bicoid-mediated repression. This is plausible, as *miR-2* family microRNAs are expressed during oogenesis and deposited in the early embryo [19, 23]. However, an alternative explanation could be that the same point mutations inadvertently disrupt Bicoid binding in a microRNA-independent manner. To address this issue, we asked whether Bicoid responsiveness in a mutant sensor could be rescued by providing a microRNA carrying compensatory changes that restore binding to the sensor. Such a rescue would demonstrate that microRNAs have a direct role in Bcd-mediated repression.

To conduct this experiment, we took the precursor hairpin for a *miR-2* family member, *miR-308*, and introduced point mutations that restore binding to the *cadM1* sensor (Figure 3D). We also introduced mutations to preserve the secondary structure of the precursor hairpin, in order to allow normal processing of the microRNA [24, 25]. We placed this mutant microRNA, called *miR-308m*, under the constitutive *tub-α1* promoter and inserted it into the *Drosophila* genome, on the same chromosome as *cadM1* (see the Supplemental Experimental Procedures).

The presence of *mir-308m* lowered the expression of the mutant sensor (Figure 3E), partially restoring the levels that we observe with the unmutated *caudal 3′ UTR*. This result suggests that *mir-308m* interacts with *cadM1* in vivo. The repressive effect of *mir-308m* is lower than that of endogenous microRNAs on the wild-type *caudal 3′ UTR* (compare first two columns on Figure 3B with Figure 3E), which could be due to lower expression or inefficient processing of *miR-308m*, or due to lower efficiency of the microRNA-target interaction.

Adding Bicoid further repressed *cadM1* expression in the presence of *mir-308m* (Figure 3E), demonstrating that by restoring the microRNA-target interaction we can partially rescue Bicoid-dependent repression. The extent of Bicoid repression is small but reproducible for Bicoid isoforms G, E, and F (compare with Figure 1D). This result demonstrates that microRNA binding to the *caudal 3′ UTR* has a direct role in Bicoid-dependent repression. The fact that the restoration of Bcd repression is partial may reflect a problem in the biogenesis of the mutant microRNA, or effects of the *cadM1* mutation that are independent of microRNA binding (e.g., unanticipated effects on secondary structure, effects on recruitment of other cofactors).

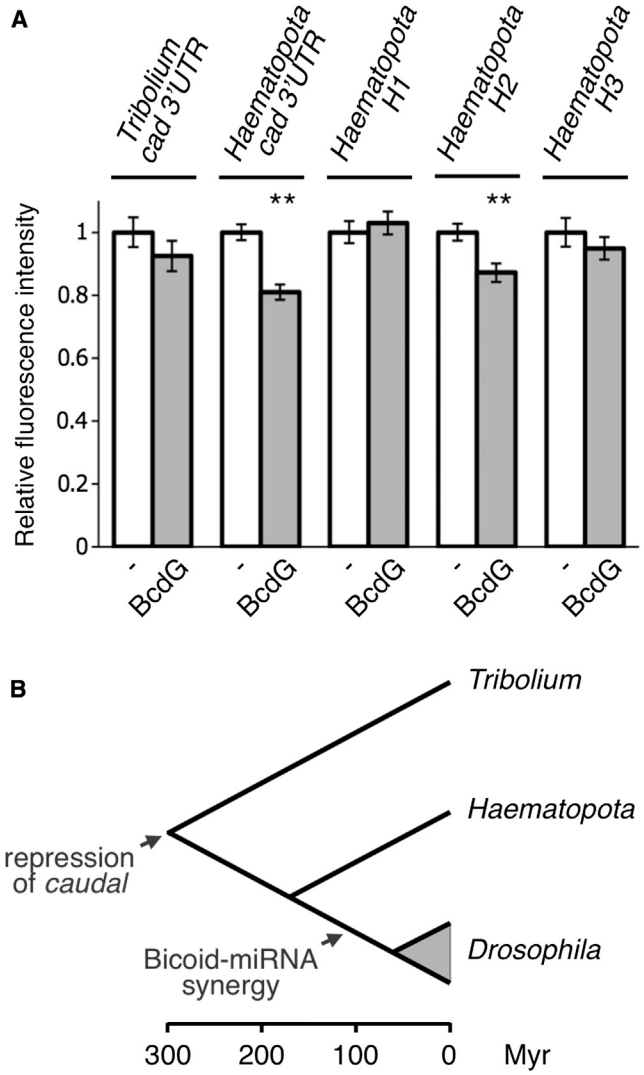

Figure 4. Evolution of Synergy between MicroRNAs and Bcd

(A) Sensors carrying the *Tribolium castaneum* and *Haematopota caudal 3′ UTRs* were assayed for their ability to mediate Bcd-dependent repression. The *Tribolium caudal 3′ UTR* sensor showed no significant difference in the presence or absence of Bcd. In contrast, the *Haematopota caudal 3′ UTR* sensor was significantly repressed by Bcd. To dissect the Bcd-responsive element in *Haematopota caudal 3′ UTR*, we subdivided that sequence into three fragments (*H1*, *H2*, and *H3*) predicted to form distinct stem-loop structures. Sensor constructs show that only one of these fragments, the 42 nt long *H2*, can mediate Bcd-dependent repression. Error bars represent one SE; two asterisks indicate 99% statistical confidence using a t test.

(B) Previous studies have indicated that translational repression of maternal *caudal* mRNA is likely to be an ancient feature that predates the evolution of Bicoid [26, 27]. Although *caudal mRNAs* from *Tribolium*, *Haematopota*, and *Drosophila* may share some *cis*-regulatory signals that can mediate repression by Bcd ([26] and present work), our results suggest that the synergy between microRNAs and Bcd probably arose after the divergence of these species, concomitant with the functional specialization of Bcd in cyclorrhaphan flies [11–15].

See also Figure S4.

MicroRNA-Bicoid Synergy Evolved Recently and Is Context Specific

Translational repression of *caudal* at the anterior pole of the embryo may be an ancient feature that predates the evolution of Bicoid: *caudal* appears to be translationally repressed in the beetle *Tribolium castaneum*, which does not possess a distinct Bicoid homolog [26, 27]. Strikingly, Wolff et al. showed that *Tribolium caudal* mRNA could be repressed in a Bicoid-dependent manner when expressed in *Drosophila* embryos [26], suggesting that Bicoid recognizes the same *cis*-regulatory signal on *caudal* mRNA as the ancestral (Bicoid-independent) mechanism of repression. Could the microRNA target site represent that ancestral conserved signal?

To address this question, we examined the functional properties of *caudal 3′ UTRs* from the beetle *Tribolium castaneum* and the noncyclorrhaphan dipteran *Haematopota pluvialis*, two insects that do not possess Bicoid [11, 13]. A sensor carrying the entire *Tribolium caudal 3′ UTR* showed no significant Bcd-dependent repression in *Drosophila* (Figure 4A), suggesting that the result of Wolff et al. is not mediated through the 3′ UTR. However, we found that a sensor carrying the *Haematopota caudal 3′ UTR* sequence is able to mediate a moderate Bicoid-dependent repression in *Drosophila* (Figure 4A). We mapped this activity to a 42 nt fragment of the *3′ UTR*, that we name *H2*, which is sufficient for Bicoid-dependent repression (Figure 4A). *H2* is predicted to form a hairpin structure and it is capable of weakly binding Bicoid in a gel shift assay in vitro (see Figure S4). Importantly, *H2* lacks any predicted microRNA target sites (including targets for *Drosophila* microRNAs), which suggests that Bicoid acts on this fragment in the absence of microRNA binding.

Thus, we find no evidence to suggest that the role of microRNAs in anterior *caudal* repression predates the evolution of Bicoid (Figure 4B). The synergy of Bicoid with microRNAs appears to have evolved in the context of a specific *caudal BRE* and may be absent outside of the cyclorrhaphan fly lineage.

Conclusions

Our results show that Bicoid cooperates with microRNAs to repress *Drosophila caudal* mRNA. The interaction occurs within a 63 nt region of the *caudal 3′ UTR* and is essential for repression of that specific target. However, microRNAs do not appear to be necessary for Bicoid repression on all targets (see results on the *Haematopota 3′ UTR*), which suggests that the target mRNA plays an important role in determining which components are involved in the repression. Target mRNAs with different sequence or structural motifs may assemble different repressive complexes, involving different sets of proteins and regulatory RNAs.

Bicoid and microRNAs could cooperate in a number of different ways to achieve *caudal* repression. One possible mechanism could involve cooperative binding of Bicoid and microRNA/RISC complexes on the BRE. Although previous work indicated that Bicoid can bind specifically to the *caudal 3′ UTR* in vitro [4, 16, 17], our gel shift experiments suggest that this interaction is weak and not highly specific (see Figure S3). Cooperative binding of microRNAs and Bicoid could enhance the strength and the specificity of this interaction. Bicoid and microRNAs might also cooperate in establishing a translational repression complex that involves several components, including d4EHP and Ago [5, 28]. Thus, the synergy between Bicoid and microRNAs may occur at the level of both mRNA binding and translational repression.

These results point to a previously unappreciated level of complexity in *caudal* repression, involving both proteins and microRNAs, echoing some recent findings in other systems [2, 29, 30]. Bicoid remains the factor that provides the spatial specificity in *caudal* repression, which serves to transmit

positional information in the developing embryo. However, the synergy with microRNAs provides an additional layer of regulation and opportunities for regulatory evolution.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.06.041.

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