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The nuclear export receptor XPO-1 supports primary miRNA processing in *C. elegans* and *Drosophila*

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MicroRNA (miRNA) biogenesis proceeds from a primary transcript (pri-miRNA) through the pre-miRNA into the mature miRNA. Here, we identify a role of the *Caenorhabditis elegans* nuclear export receptor XPO-1 and the cap-binding proteins CBP-20/NCBP-2 and CBP-80/NCBP-1 in this process. The RNA-mediated interference of any of these genes causes retarded heterochronic phenotypes similar to those observed for animals with mutations in the *let-7* miRNA or core miRNA machinery genes. Moreover, pre- and mature miRNAs become depleted, whereas primary miRNA transcripts accumulate. An involvement of XPO-1 in miRNA biogenesis is conserved in *Drosophila*, in which knockdown of Embargoed/XPO-1 or its chemical inhibition through leptomycin B causes pri-miRNA accumulation. Our findings demonstrate that XPO-1/Emb promotes the pri-miRNA-to-pre-miRNA processing and we propose that this function involves intranuclear transport and/or nuclear export of primary miRNAs.

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Introduction

According to the current model of miRNA biogenesis, miRNAs are transcribed by RNA polymerase II as capped and polyadenylated primary miRNAs (pri-miRNA) of several hundred or thousands of nucleotides in length (Bracht *et al*, 2004; Cai *et al*, 2004; Lee *et al*, 2004). The microprocessor complex, composed of Drosha and DGCR8 (DRSH-1 and PASH-1, respectively, in *Caenorhabditis elegans*), cleaves the pri-miRNAs in the nucleus to generate pre-miRNAs, characterized by their hairpin structures and size of ~70 nt (Denli *et al*, 2004; Gregory *et al*, 2004; Han *et al*, 2004; Landthaler *et al*, 2004). Subsequently, cleavage of the pre-miRNA by the cytoplasmic RNase Dicer (DCR-1) releases the mature miRNA (Grishok *et al*, 2001; Hutvágner *et al*, 2001; Ketting *et al*, 2001), which is loaded into a functional miRNA-induced silencing complex (miRISC) containing an Argonaute (AGO; ALG-1 and ALG-2 in *C. elegans*) protein (Grishok *et al*, 2001; Hutvágner *et al*, 2004) and a GW182 protein (AIN-1 and AIN-2; Ding *et al*, 2005; Liu *et al*, 2005; Rehwinkel *et al*, 2005; Zhang *et al*, 2007) at its core.

In vertebrates and flies, Exportin-5 (Exp5) connects the two nucleolytic processing steps by exporting the nuclear pre-miRNA into the cytoplasm for further cleavage by Dicer (Yi *et al*, 2003; Bohnsack *et al*, 2004; Lund *et al*, 2004). However, although the miRNA biogenesis machinery is generally conserved in *C. elegans*, the nematode genome contains no orthologue of Exp5 (Supplementary Figure S1 and see, Bohnsack *et al*, 2004; Murphy *et al*, 2008).

The depletion of several components of the miRNA core machinery in *C. elegans* results in developmental phenotypes that resemble those seen upon the loss of the *let-7* miRNA, such that these phenotypes provided the first indication for a function of DCR-1, ALG-1/2, and AIN-1/2 in the miRNA pathway (Grishok *et al*, 2001; Ketting *et al*, 2001; Ding *et al*, 2005; Zhang *et al*, 2007). These so-called heterochronic phenotypes are particularly apparent in a subset of skin cells, the seam cells. In wild-type animals, these cells exit the cell cycles at the larval-to-adult (L/A) transition, fuse into a syncytium, and contribute to the formation of a specific cuticular structure, the adult alae. In *let-7* mutant and miRNA pathway mutant animals, cell cycle exit and/or cell differentiation fail, resulting in extra seam cell divisions, delay, or lack of formation of the seam cell syncytium and/or the alae. Moreover, on more complete loss of *let-7* or general miRNA activity, animals die by vulval bursting at the L/A transition.

In this study, we show that depletion of the nuclear export receptor XPO-1 or either subunit of the nuclear cap-binding complex (CBC), NCBP-1/CBP-80 and NCBP-2/CBP-20, causes vulval bursting and heterochronic phenotypes in *C. elegans*. This is caused by a defect in the miRNA biogenesis at the level of primary miRNAs, and a similar function in miRNA biogenesis is also observed for the *Drosophila* XPO-1 orthologue Embargoed. We propose that XPO-1, possibly in conjunction with the CBC, mediates the intranuclear transport and/or nuclear export of primary miRNAs.

Results

xpo-1 is a heterochronic gene in C. elegans

Exp5 is a member of the importin β-superfamily that mediates the nuclear export of pre-miRNAs in flies and

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mammals. As *C. elegans* lacks an Exp5 orthologue (Supplementary Figure S1 and see, Bohnsack *et al*, 2004; Murphy *et al*, 2008), we were interested in testing whether other nuclear export receptors support miRNA biogenesis in *C. elegans*. The CSE1L/CAS orthologue, XPO-2, has previously been identified as a suppressor of the *let-7(n2853)* mutation (Ding *et al*, 2008), indicating a negative role—by genetic criteria—in miRNA function, and thus arguing against a miRNA biogenesis-promoting activity. We therefore investigated the other two *C. elegans* exportins, XPO-1 and XPO-3. The exportin XPO-1 is the orthologue of yeast and human CRM1/XPO1, which mediates nuclear export of the spliceosomal U snRNAs (Hutten and Kehlenbach, 2007), whereas XPO-3 is the orthologue of human Exportin-t and yeast Los1p, which mediates tRNA nuclear export (Großhans *et al*, 2000). *xpo-1* has also previously been identified as one among >200 genes, depletion of which enhanced vulval bursting for a weak *let-7* allele in an RNAi-sensitized, *eri-1* mutant background, although a function in miRNA biogenesis remained elusive (Parry *et al*, 2007).

To obtain evidence for a possible function of either transport receptor in miRNA biogenesis or function, we exposed wild-type animals to RNAi by feeding against *xpo-1*, *xpo-3* or a control plasmid and scored animal survival and alae defects in young adults. To avoid sterility or embryonic lethality phenotypes, we initiated RNAi on synchronized first larval (L1) stage animals. Animals treated with mock RNAi exhibited wild-type vulvae and alae (Figures 1A, D and 2A, F). By contrast, depletion of the *C. elegans* Argonaute, *alg-1*, caused both vulval bursting and alae defects (Figures 1B, D and 2B, F).

Animals exposed to *xpo-3(RNAi*) appeared wild type (Figures 1D and 2F), although RT–PCR confirmed efficient mRNA depletion (data not shown). This finding suggests that under our experimental conditions sufficient XPO-3 protein might still be available to promote tRNA nuclear export. Alternatively, as in yeast and *Drosophila*, in which Exp-t orthologues are non-essential or not encoded in the genome, respectively (Supplementary Figure S1 and see, Großhans *et al*, 2000; Shibata *et al*, 2006), partially redundant tRNA nuclear export pathways might compensate for the loss of XPO-3 in larvae.

By contrast, *xpo-1(RNAi)* caused the characteristic vulval bursting and alae break phenotypes (Figures 1C, D and 2C, F), previously observed for depletion of other core components of the *C. elegans* miRNA pathway (Grishok *et al*, 2001; Ketting *et al*, 2001; Denli *et al*, 2004), including *alg-1* (Figures 1B, D and 2B, F). Surviving animals were sterile for reasons that we have not investigated.

A more detailed analysis confirmed that *xpo-1(RNAi)* caused true heterochronic phenotypes. Thus, *xpo-1(RNAi)* animals displayed unfused seam cells at the young adult stage, when seam cells in wild-type animals would be fused (Supplementary Figure S2). Moreover, the number of seam cells in young adult *xpo-1(RNAi)* animals was increased relative to mock RNAi animals (Supplementary Figure S3), and this was due to extra seam cell division in the young adult stage and not cell-fate transformations or extra cell divisions during larval stages (Supplementary Figure S3).

In summary, we have shown that *xpo-1(RNAi)* phenocopies multiple aspects of the *let-7* heterochronic phenotype, including lethality and defects in seam cell differentiation and proliferation control, establishing *xpo-1* as a *bona fide* heterochronic gene.

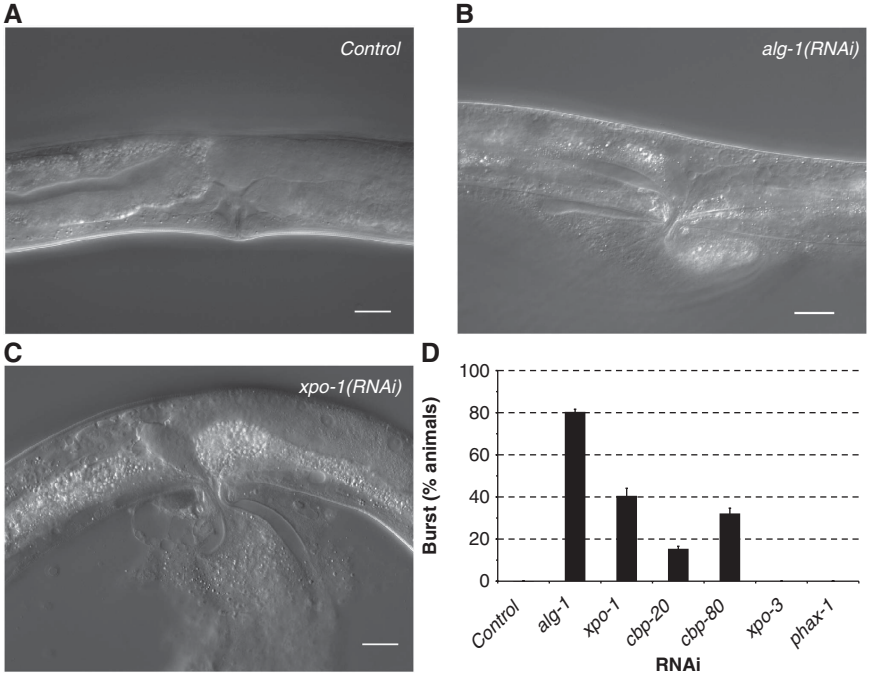

Figure 1 RNAi against *xpo-1*, *ncbp-1/cbp-80*, or *ncbp-2/cbp-20* causes animals to die by vulval bursting. Unlike (**A**) the healthy control animals, (**B**) *alg-1(RNAi)* and (**C**) *xpo-1(RNAi)* adults have protruding vulvae and often die by bursting through the vulva. (**D**) This phenotype is also penetrant on depletion of *cbp-20* or *cbp-80*, whereas RNAi against *xpo-3* or *phax-1* has no effect (independent experiments $n \geqslant 2$, each $n \geqslant 165$ animals). 'Control' in this and subsequent figures denotes animals that were fed bacteria carrying the insertless L4440 parental RNAi vector. Error bars = s.e.m. Scale bars are 20 μm.

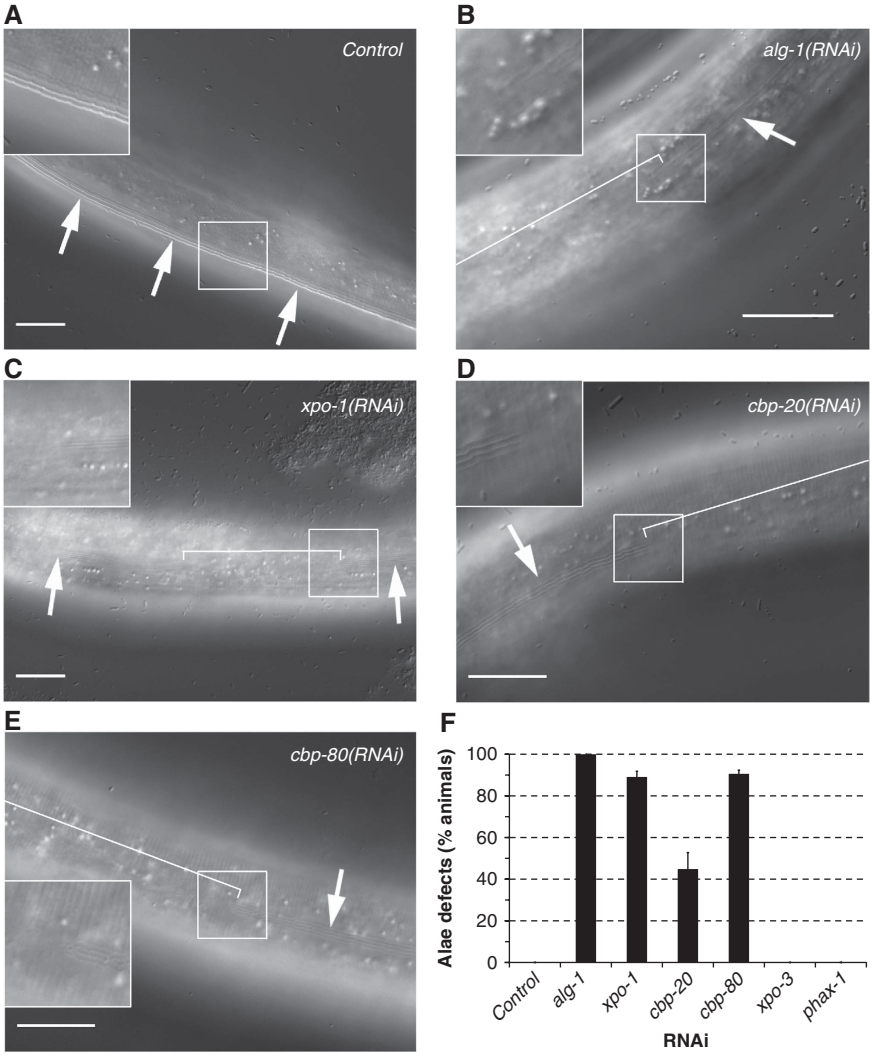

Figure 2 xpo-1(RNAi), ncbp-1/cbp-80(RNAi), and ncbp-2/cbp-20(RNAi) cause alae defects. (**A**) Control animals display strong and complete alae (arrows), whereas (**B**–**F**) alae in animals treated with RNAi as indicated are broken or absent altogether (brackets indicate alae breaks; for (**F**), independent experiments $n \geqslant 3$, each $n \geqslant 22$ animals for control, *xpo-1(RNAi)*, *cbp-20(RNAi)* and *cbp-80(RNAi)*; for *alg-1(RNAi)* one experiment with 34 animals). Residual alae in the mutant animals (arrows) are much weaker than in the control. Error bars = s.e.m. Scale bars are 20 μm.

XPO-1 is required for normal let-7 accumulation

The extensive resemblance of *xpo-1(RNAi)* and *let-7* mutant phenotypes is consistent with a function of XPO-1 in *let-7* biogenesis. In accord with this idea, we also observed that *xpo-1(RNAi)*-induced vulval bursting was largely suppressed by a loss-of-function mutation in the *let-7* target *lin-41*, which also suppresses vulval bursting of *let-7* mutant animals (data not shown). However, Parry *et al* (2007) had previously examined whether depletion of *xpo-1* affected mature and/or pre-*let-7* levels and failed to find any evidence to support this idea. We wished to re-examine this issue in the light of the stronger vulval bursting phenotypes that we observed in comparison to Parry *et al* (2007), who required *xpo-1* depletion in the *eri-1(mg366)*; *let-7(mg279)* background to observe significant bursting. Indeed, when we examined the abundance of mature *let-7* in *xpo-1(RNAi)* animals, we observed a ~50% decrease relative to control RNAi animals (Figure 3A). This finding supports a possible function of XPO-1 in *let-7* biogenesis.

The cap-binding complex is a potential co-factor of XPO-1 in let-7 biogenesis

To mediate nuclear export of U snRNAs, vertebrate XPO1/CRM1 functions with three adaptor proteins—the cap-binding complex (CBC) comprising CBP20 and CBP80, and the PHAX protein (Izaurralde *et al*, 1995; Ohno *et al*, 2000). More recently, CBC was shown to be required for efficient miRNA accumulation in plants and to affect pre- and pri-miRNA levels in flies and mammals, respectively (Gregory *et al*, 2008; Kim *et al*, 2008; Laubinger *et al*, 2008; Gruber *et al*, 2009; Sabin *et al*, 2009). Finally, both *ncbp-2/cbp-20* (*F26A3.2*) and *ncbp-1/cpb-80* (*F37E3.1*) caused vulval bursting when depleted in *eri-1(mg366)*; *let-7(mg279)* animals (Parry *et al*, 2007). Thus, to test whether PHAX and CBC also function in *C. elegans* miRNA biogenesis, we depleted them by RNAi.

Animals exposed to RNAi against *phax-1* (*Y71H2B.2*) displayed neither vulval bursting nor alae defects (Figures 1D and 2F) although *phax-1* mRNA was efficiently (~70%)

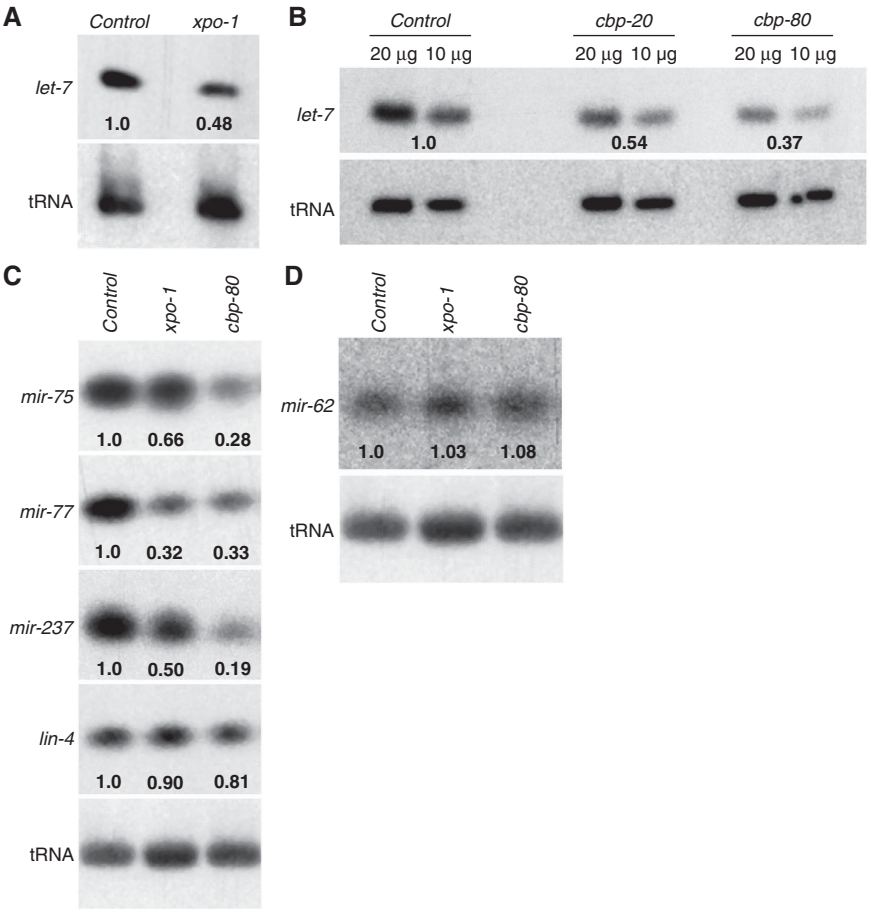

Figure 3 Depletion of *xpo-1*, *cbp-20*, or *cbp-80* causes a widespread decrease in mature miRNA, but not in mature mirtron levels. (**A**–**D**) Northern blots using total RNA from synchronized late L4 stage animals exposed to RNAi as indicated. Oligonucleotides complementary to the indicated mature miRNAs or $tRNA^{Gly(TCC)}$ were used. To facilitate a comparison, two different amounts of total RNA were loaded in (**B**) as indicated. (**D**) The accumulation of the mirtron *mir-62* is not affected by the depletion of *xpo-1* and *cbp-80* (same membrane as in (**C**), re-probed and tRNA shown again for comparison). Numbers represent the quantification by phosphoimager, normalized to $tRNA^{Gly}$ levels.

depleted as determined by RT–PCR (data not shown). Although it remains possible that residual PHAX-1 suffices for function, these data argue against a major role of PHAX-1 in *let-7* biogenesis. In contrast, and similar to *xpo-1(RNAi)*, depletion of *cbp-20* or *cbp-80* by RNAi caused penetrant alae defects and vulval bursting (Figures 1D and 2D–F); the latter phenotype being suppressed by the *lin-41(ma104)* mutation (data not shown). Finally, the levels of the mature *let-7* miRNA were significantly reduced on *CBC* depletion (Figure 3B), supporting the idea that CBC, like XPO-1 might be involved in miRNA biogenesis. As a parsimonious explanation, we propose that CBP-20 and CBP-80 function together with XPO-1 in the nuclear export of *let-7*.

XPO-1 and the CBC are widely required for miRNA accumulation

Mutations in the core miRNA machinery cause *let-7*-like phenotypes even for factors generally required for miRNA function (Figures 1B, D and 2B, F; Grishok *et al*, 2001; Denli *et al*, 2004). Therefore, we tested the possibility that XPO-1 and CBC are required for the accumulation of other miRNAs. As depletion of *cbp-20* caused less penetrant developmental phenotypes than RNAi against *cbp-80*, we focused our analysis on *xpo-1* and *cbp-80*. We noted that these differences in *cbp-20* and *cbp-80* depleted animals occurred although relative depletion efficiency was comparable for both mRNAs (Supplementary Figure S4A), possibly indicating that differences in protein stability or differences in protein abundance already before depletion might render CBP-20 more refractory to efficient depletion by RNAi.

We examined the abundance of *lin-4*, *mir-75*, *mir-77*, and *mir-237*, four larvally expressed miRNAs (Lim *et al*, 2003), and found that their levels were decreased on *xpo-1* and *cbp-80* knockdown (Figure 3C), although the effect on *lin-4* was modest (but see below), presumably due to its early expression in L1. We conclude that XPO-1/CRM1 and CBC are required for the biogenesis of many *C. elegans* miRNAs, including *let-7*, providing a molecular explanation for the developmental phenotypes.

XPO-1 and CBC act upstream of mature miRNA

If XPO-1 and CBC act directly and jointly in miRNA biogenesis, the involvement of CBC might suggest a function linked to a capped miRNA precursor, that is, pri-miRNA, rather than the uncapped pre- or mature miRNAs. To test this possibility, we examined the abundance of *let-7* biogenesis intermediates. Although low pre-*let-7* levels in wild-type animals preclude efficient detection, depletion of *dcr-1* yields a readily detectable accumulation of pre-*let-7* (Grishok *et al*, 2001; Ketting *et al*, 2001). Our failure to detect pre-*let-7* on *xpo-1*

or *CBC* depletion thus ruled out a significant accumulation (data not shown). Indeed, when we overexpressed *let-7* ~five-fold from an integrated DNA array (Weidhaas *et al*, 2007 and Supplementary Figure S5A), depletion of *xpo-1* or *cbp-80* decreased pre-*let-7* levels relative to control RNAi (Figure 4A), indicating that *xpo-1* and *cbp-80* function upstream of Dicer-mediated pre-*let-7* processing.

We used RT–qPCR to examine the accumulation of pri-*let-7* and *C. elegans*-specific SL1-pri-*let-7*, which is derived from the pri-miRNA by *trans*-splicing (Figure 4B; Bracht *et al*, 2004). Unlike for pre-*let-7* and mature *let-7*, we observed that levels of these potential export substrates did not decline in *xpo-1*-, *cbp-20*-, or *cbp-80*-depleted animals but instead increased (data not shown). However, the extent of accumulation varied substantially among different experiments. As pri-*let-7* expression is dynamic during the L4 stage (A Pasquinelli, personal communication), we addressed the possibility that slightly divergent staging of the animals might account for this variability among the different trials. We performed a time-course analysis using *lin-42*, an mRNA expression of which peaks once during each larval stage (Jeon *et al*, 1999), as a reference (Supplementary Figure S5B; Materials and methods section). Both pri-*let-7* and SL1-pri-*let-7* were dynamically expressed during the L4 stage, starting from low levels, peaking around the time of maximum *lin-42* levels, and subsequently declining (Figure 4C and D). This dynamic was unchanged on *xpo-1*, *cbp-20*, or *cbp-80* depletion, but the levels of both transcripts were consistently increased at all time points relative to the control animals (Figure 4C–E). Although *xpo-1(RNAi)* enhanced the accumulation of SL1-*let-7* particularly strongly, *cbp-20/-80 (RNAi)* preferentially affected pri-*let-7* accumulation.

Next, we extended our study to the primary transcripts of *lin-4*, *mir-237*, *mir-48*, and *mir-77*, none of which has been reported to undergo *trans*-splicing, and for none of which we could amplify a *trans*-spliced product using RT–PCR with an SL1-specific primer and a pri-miRNA specific primer (data not shown). We observed that all four pri-miRNAs accumulated on *xpo-1(RNAi)*, *cbp-20(RNAi)*, and *cbp-80(RNAi)* relative to the control (Figure 4E and data not shown),

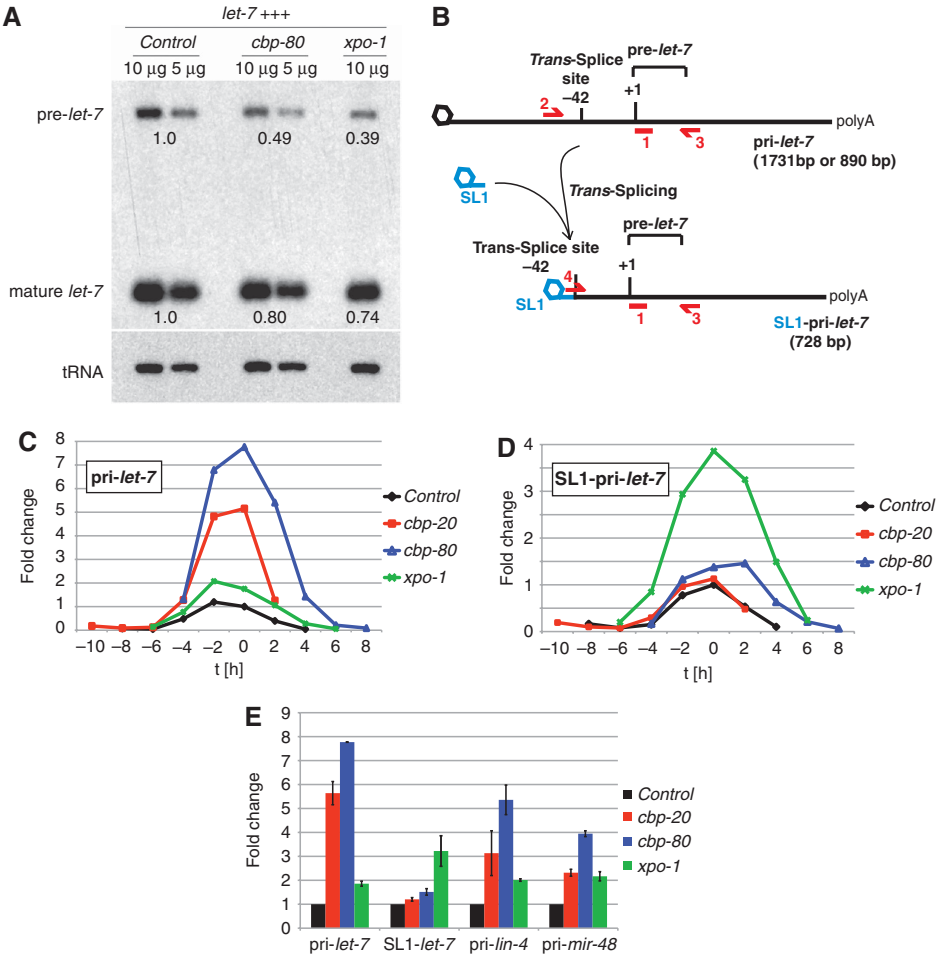

Figure 4 Depletion of XPO-1 or CBC reduces pre-*let-7* levels and increases pri-miRNA accumulation. (**A**) Pre-*let-7* levels are reduced in *let-7*-overexpressing animals (*let-7*$^{+++}$) exposed to *xpo-1(RNAi)* or *cbp-80(RNAi)*. Total amounts of RNA were loaded as indicated. (**B**) Schematic representation (not to scale) of the primary (pri-*let-7*) and *trans*-spliced SL1-pri-*let-7* transcripts. The positions of oligonucleotides used for RT–qPCR and Northern blot are highlighted in red. Mature and pre-*let-7* are detected by probe (1); pri-*let-7* by primers (2) and (3); SL1-pri-*let-7* by primers (3) and (4). (**C**) The levels of pri–*let-7* and (**D**) SL1-pri-*let-7* change dynamically during the L4 stage and are elevated in the *xpo-1(RNAi)*, *cbp-80(RNAi)*, and *cbp-20(RNAi)* animals. Time (*x*-axis) is relative to the peak of *lin-42* mRNA levels in L4, which we defined as $t = 0$ h (see Supplementary data). Pri- and SL1-pri-*let-7*-levels were arbitrarily set to 1 in the control RNAi strain at $t = 0$ h. The experiment was performed in biological duplicate, a representative example is shown. (**E**) Pri-*let-7*, SL1-pri-*let-7*, pri-*lin-4*, and pri-*mir-48* levels were determined in biological duplicates for the time points of peak *lin-42* expression. The average fold change, compared with the RNAi control, is shown. Error bars indicate actual measurements.

suggesting that XPO-1 and the CBC act on the primary transcripts, and confirming that XPO-1 and CBC are widely required for miRNA biogenesis.

A mirtron miRNA is not affected by depletion of xpo-1 or CBC

To further test the idea that XPO-1 and CBC act on pri-miRNAs, we examined accumulation of *mir-62*. *mir-62* belongs to the mirtron subclass of miRNAs, which reside in short introns of host mRNA genes, from which the—uncapped—pre-miRNA is released through nuclear mRNA splicing, bypassing processing by Drosha (Okamura *et al*, 2007; Ruby *et al*, 2007). We expected that reduced levels of XPO-1 and CBP-80 would not affect the accumulation of this mature miRNA if these factors acted on primary miRNAs. Indeed, depletion of *xpo-1* or *cbp-80* failed to decrease the levels of the mature *mir-62*, as predicted by our model (Figure 3D). In addition, this result indicates that splicing activity is not appreciably impaired in the *xpo-1(RNAi)* and *cbp-80(RNAi)* animals, as mature mirtron accumulation requires splicing.

Expression of miRNA pathway components is not affected by XPO-1 or CBC depletion

The fact that a mirtron miRNA accumulates normally in the presence of reduced XPO-1 or CBC levels suggests that not only splicing but also dicing and Argonaute binding are not adversely affected. However, to directly examine whether XPO-1 or CBC might affect the levels of miRNA pathway components, we examined the level of Dicer (DCR-1) using an antibody against the endogenous protein (Duchaine *et al*, 2006). We also examined its mRNA levels and those of Drosha (*drsh-1*), Pasha (*pash-1*), the miRNA Argonautes (*alg-1* and *alg-2*), and the GW182 orthologues (*ain-1* and *ain-2*) by RT–qPCR. Our experiments revealed that none of these factors was depleted by RNAi against *xpo-1* or *CBC* (Supplementary Figure S4B and C). Notably, we saw some elevation of Dicer protein and Drosha, Argonaute, and GW182 mRNAs consistent with the suggestion of widespread autoregulation of miRNA pathway components by miRNAs (Zisoulis *et al*, 2010). Regardless of the cause of this effect, these results argue against an impairment of miRNA activity through depletion of core miRNA pathway genes on *xpo-1* or *CBC* knockdown. These data thus further support the idea that XPO-1 and CBC have a direct role in supporting miRNA biogenesis at the level of pri-miRNA.

Emb, the Drosophila XPO-1 orthologue, also regulates pri-miRNA processing

The miRNA biogenesis pathway is well conserved in diverse organisms and recent data show a requirement of the CBC for efficient miRNA processing and/or activity in plants, mammals, and flies (Gregory *et al*, 2008; Kim *et al*, 2008; Laubinger *et al*, 2008; Gruber *et al*, 2009; Sabin *et al*, 2009) (JSY and ECL, unpublished data). In contrast, similar data are not available for XPO-1.

To elucidate if Embargoed (Emb), the *Drosophila* XPO1 orthologue, has a function in the biogenesis of miRNAs, we depleted it in S2 cells by soaking of *Emb* dsRNA. As in *C. elegans*, we observed an accumulation of several pri-miRNAs when Emb was depleted (Figure 5A, Supplementary Figure S6A). We confirmed the specificity of

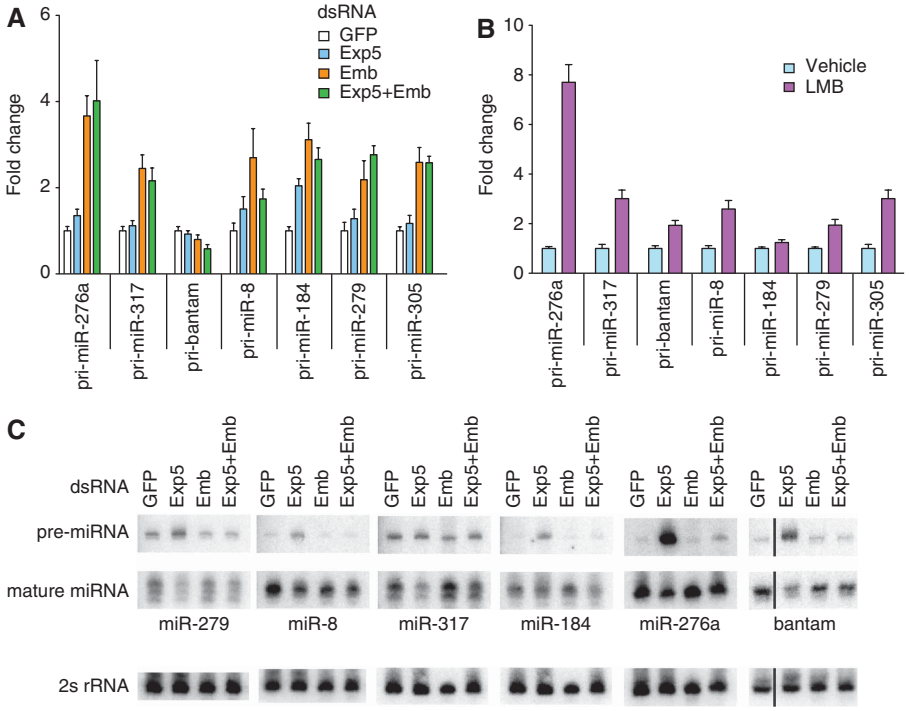

Figure 5 Accumulation of pri-miRNAs on depletion of Emb/Crm1 activity in *Drosophila*. (**A**) Depletion of Emb by RNAi increases the accumulation of several pri-miRNAs. Fold changes are relative to the cells soaked with control dsRNA (GFP) and normalized against pre-*rp49*. Knockdown efficiencies are depicted in Supplementary Figure S6. (**B**) Inhibition of Emb activity by LMB (25 ng/ml) confirms the increased accumulation of pri-miRNAs. Abundance of pri-miRNAs was analysed after 2-h treatment. (**C**) Northern blots using total RNA from cells exposed to dsRNA as indicated. The accumulation of pre-miRNAs on depletion of Exp5 is suppressed when Emb is depleted simultaneously. For *bantam*, non-adjacent lanes of a single autoradiograph are shown.

this effect by obtaining comparable results when blocking Emb activity with Leptomycin B (LMB; Figure 5B). Leptomycin B specifically inhibits XPO1/CRM1 by binding covalently to a conserved cysteine residue (Kudo *et al*, 1999), and this modification prevents substrate binding by occupying the substrate-binding site (Dong *et al*, 2009). Within as little as 2 h, LMB treatment caused an accumulation (at least two-fold) of several pri-miRNAs relative to the vehicle-treated control (Figure 5B). Thus, acute and chronic depletion of Emb activity causes pri-miRNA accumulation, with the rapidity of the effect arguing for a direct involvement of Emb in miRNA biogenesis.

Although some mature miRNAs seemed to be moderately changed on Emb depletion, the effect was generally weak (Figure 5C), as previously observed on CBC depletion in flies (Sabin *et al*, 2009). Thus, it seems that compensatory effects downstream in miRNA biogenesis or turnover can compensate for the decreased pri-miRNA processing. Alternatively, incomplete Emb depletion might sustain sufficient export capacity in cells exposed to RNAi against Emb. Finally, Emb might only have a minor or partially redundant role in miRNA biogenesis in *Drosophila*.

To test whether a redundant function was performed by the pre-miRNA export receptor, Exportin-5, we co-depleted Emb and Exp5. Surprisingly, we found that the double depletion did not decrease mature miRNA levels beyond what was seen with Exp5 single depleted cells. Nonetheless more surprisingly, RNAi against Emb suppressed the accumulation of the pre-miRNA that occurs in an Exp5 single knockdown (Figure 5C). Although these experiments involve partial knockdown and not null mutations, precluding strong statements about epistasis, these findings suggest that in *Drosophila* Emb acts upstream of, rather than in parallel to, Exportin-5.

Discussion

We have shown in this study that *C. elegans xpo-1*, *cbp-20*, and *cbp-80* are heterochronic genes that are required for proper execution of the L/A switch mediated by *let-7*. We have further observed that all three factors are important for the accumulation of miRNAs, including *let-7*, providing a molecular explanation for the developmental phenotypes. We note that a previous study failed to observe a significant decrease in *let-7* on *xpo-1* depletion (Parry *et al*, 2007). However, as *xpo-1(RNAi)*-induced vulval bursting in that study required the sensitized *eri-1(mg366); let-7(mg279)* background, less efficient *xpo-1* depletion than under our experimental conditions seems a likely cause of the discrepancy (Gregory *et al*, 2008).

The fact that depletion of *xpo-1* and *CBC* both decreased mature and pre-miRNA levels, but increased pri-miRNA levels, points to their function in miRNA biogenesis at a step upstream of the pre-miRNA, that is, at the level of pri-miRNAs. Formally, we cannot rule out that these functions might differ for XPO-1 and CBC. However, as XPO-1, CBP-20, and CBP-80 complexes are known in vertebrates (Ohno *et al*, 2000), the shared molecular and developmental phenotypes seen in the *C. elegans* RNAi mutants suggests that they also function as a complex in the *C. elegans* miRNA biogenesis pathway. As *C. elegans* lacks the canonical pre-miRNA export receptor, Exp5, a function in miRNA nuclear export is a strong possibility.

Pri-miRNA nuclear export would require cytoplasmic processing of the pri-miRNA (generally considered a nuclear event), and it is therefore of particular interest that CBC and Drosha have recently been shown to co-immunoprecipitate in flies and humans (Gruber *et al*, 2009; Sabin *et al*, 2009), suggesting the possibility of a large shuttling complex that contains the pri-miRNA processing activity. Processing of pri-miRNAs in *C. elegans* might then occur at, or during transit through, the nuclear pore. Nonetheless, as Drosha localization in *C. elegans* is currently unknown, and localization using various GFP-tagged Drosha transgenes has yielded inconsistent results (IB and HG, unpublished data), alternative explanations remain possible.

Previous studies on fly and human CBC reported a function in miRNA biogenesis that involved an interaction with the serrate homologous protein ARS2 (Gruber *et al*, 2009; Sabin *et al*, 2009). However, ARS2 is only present in proliferating cells, and impairs the accumulation of a specific subset of miRNAs (Gruber *et al*, 2009). If CBC functioned in miRNA biogenesis exclusively through its interaction with ARS2, one would predict a similarly specific function. As the effect of CBC depletion on mature miRNA levels has not been reported for humans and only for one miRNA in flies—*bantam*, the levels of which remained unchanged (Sabin *et al*, 2009)—this possibility remains to be addressed. However, the fact that depletion of E01A2.2, the *C. elegans* ARS2 homologue, does not result either in vulval bursting or in alae defects (IB and HG, unpublished data), and that all miRNAs that we had investigated were affected by the depletion of CBC, suggests that in *C. elegans* some, or possibly all, CBC functions in miRNA biogenesis are independent of ARS2. Consistent with a difference in CBC function between *C. elegans* and humans or flies, Gruber *et al*, 2009 and Sabin *et al* (2009) also observed a reduction of pri-miRNA levels on depletion of CBC, whereas we observed that *C. elegans* pri-miRNAs accumulate in this situation.

It thus seems possible that CBC has a conserved yet diverging function in miRNA biogenesis in different organisms, and this also seems to be true for XPO-1: our studies of *Drosophila* Emb reveal that this XPO-1 orthologue also regulates the miRNA biogenesis at the step of pri-miRNA processing, although *Drosophila* does harbour a miRNA export receptor, Exp5. However, the fact that Emb depletion does not enhance Exp5 phenotypes at the level of mature miRNA accumulation, but does suppress pre-miRNA accumulation, suggests that Emb functions upstream of, rather than in parallel to, Exp5. One possible function could be intranuclear transport of the pri-miRNA, as previously demonstrated for U3 snoRNA in human cells (Boulon *et al*, 2004).

While this paper was under preparation, CRM1/XPO-1 was reported to regulate the nuclear–cytoplasmic localization of mature miRNAs in cultured mammalian cells, suggesting that this nuclear export receptor might additionally modulate miRNA activity after miRNA biogenesis has been completed (Castanotto *et al*, 2009). It is unclear whether this function would be conserved in *C. elegans*. However, if it were, it would be insufficient to explain several of our observations, that is, the accumulation of pri-miRNA, the depletion of pre-miRNA, and the lack of an effect on the levels of the *mir-62*

mirtron. Nonetheless, we cannot rule out that beyond the functions in pri-miRNA biogenesis that we describe here, XPO-1 would additionally affect mature miRNA localization.

When considering the possibility of conserved, yet differing functions in miRNA biogenesis, one striking feature of the *C. elegans* miRNA pathway is that many or all of its canonical miRNAs are expressed from their own promoters (Supplementary data and Martinez *et al*, 2008), whereas a large fraction of vertebrate miRNAs are 'intronic' such that nuclear Drosha processing releases them from their host genes (Kim and Kim, 2007). It is tempting to speculate that a varying dependence on CBC for miRNA biogenesis in *C. elegans* and in humans might explain these divergent gene organization patterns, with a more general requirement in *C. elegans* necessitating the production of capped transcripts, from 'intergenic' miRNA loci.

Materials and methods

C. elegans strains

C. elegans strains used were: wild-type N2; MT7626: *let-7(n2853)* (Reinhart *et al*, 2000); CT19: N2;*zaIs3[let-7(+) myo-3*::*gfp]* (Weidhaas *et al*, 2007); *him-5;[ajm-1*::*gfp, rol-6]*; JR672: N2;*wIs54 [scm*::*gfp]* (Koh and Rothman, 2001); and GR1434: *wIs54 [scm*::*gfp]*V;*let-7(n2853)* (Hayes *et al*, 2006).

The *C. elegans* cap-binding complex and PHAX-1

Using reciprocal BLAST search, we identified F26A3.2 (*ncbp-2*) and F37E3.1 (*ncbp-1*) as the closest CBP20 and CBP80 homologues, respectively, in *C. elegans* (data not shown). The genes encoding these proteins are named *ncbp-1* (CBP80) and *ncbp-2* (CBP20), following the human nomenclature. For clarity, we used *cbp-20/-80* and CBP-20/-80 throughout the text when referring to gene and protein, respectively. A PHAX homologue has already been identified previously (Ohno *et al*, 2000). We verified that this gene, *Y71H2B.2*, was indeed the closest PHAX homologue in *C. elegans* (data not shown) and named it *phax-1*.

RNAi and RNAi constructs

The RNA-mediated interference was performed by feeding, starting with synchronized L1 larvae. 'Control' in all RNAi experiments denotes animals that were fed bacteria carrying the insertless L4440 parental RNAi vector. Appropriate developmental stages of worms were verified by vulval and gonad development using DIC optics. The RNAi constructs targeting *ncbp-1/cbp-80* and *ncbp-2/cbp-20* are from an RNAi library (Kamath *et al*, 2003). *xpo-1(RNAi)*, *xpo-3 (RNAi)*, and *phax-1(RNAi)* were constructed as described in the Supplementary data.

RNA isolation and northern blot

Worms were mixed with Trizol (Invitrogen) and either ground in liquid nitrogen or freeze-thawed as described previously (Bethke *et al*, 2009). The RNA was extracted according to the manufacturer's instructions. Total RNA was separated on 10 or 15% PAGE–urea gels and transferred on to a membrane (Zeta-Probe GT, BioRad for UV cross-linking and Hybond-Nx, Amersham for chemical cross-linking) by wet or semidry blotting. Cross-linking was carried out either by UV irradiation plus baking or by chemical cross-linking as described previously (Pall *et al*, 2007). Single-stranded DNA oligonucleotides complementary to the sequence of interest were used except for *let-7* and *mir-62*, in which an LNA-modified oligonucleotide (Exiqon) was used to facilitate detection. Probes were 5′ end-labelled with ATP-γ-[^{32}P] and polynucleotide kinase according to standard protocols. Hybridization was carried out overnight in 4 × SSPE (0.6 M NaCl, 40 mM NaH_2PO_4, 4 mM EDTA), 7% SDS, 25% formamide at 37°C for the DNA oligonucleotides and in 4 × SSPE, 6% SDS, 50% formamide at 60°C (*mir-62* LNA) or 65°C (*let-7* LNA).

let-7 LNA (*hsa-let-7a*): 5′-AACTATACAACCTACTACCTCA-3′;
Cel-lin-4: 5′-TCACACTTGAGGTCTCAGGGA-3′;
Cel-mir-75: 5′-AAGCCGGTTGGTAGCTTTAA-3′;
Cel-mir-77: 5′-TGGACAGCTATGGCCTGATGAA-3′;
Cel-mir-237: 5′-AAGCTGTTCGAGAATTCTCAGGGA-3′;
Cel-mir-62 LNA: 5′-CTGTAAGCTAGATTACATATCA-3′;
Cel-tRNA (tGly): 5′-GCTTGGAAGGCATCCATGCTGACCATT-3′.

For *Drosophila* cell culture experiments, endogenous total RNAs were isolated from dsRNA- or drug-treated S2R$^+$ cells by Trizol (Life Technologies). Northern blot analyses were performed to analyse the pre- and mature miRNA levels: 15–20 μg total RNA per lane were separated by 12% polyacrylamide gels, transferred onto GeneScreen plus-charged nylon membranes (PerkinElmer), and probed with γ-^{32}P-labelled LNA oligonucleotides (pre-designed by Exiqon) antisense to *miR-8*, *miR-276a*, *miR-279*, and *miR-317* or DNA oligonucleotides (IDT) antisense to *bantam* (5′-AATCAGCTTT CAAAATGATCTCA-3′), *miR-184* (5′-GCCCTTATCAGTTCTCCGTCCA -3′), and 2S rRNA (5′-TACAACCCTCAACCATATGTAGTCCAAGCA-3′).

RT–qPCR

The RT–qPCR analysis was performed to examine the abundance of primary miRNAs. Total RNA was diluted to 500 ng/μl and treated with DNaseI (Ambion; DNA-free) according to the manufacturer's protocol. The cDNA synthesis was performed with the ImProm-II reverse transcription system (Promega) using oligo-dT primers following the manufacturer's protocol. The resulting cDNA was used for real-time PCR with the Absolute qPCR SYBR green ROX mix (ABgene), gene-specific oligonucleotides, and an ABI Prism 7000 machine. Detailed description of normalization, time adjustments by expression of *lin-42* and sequences of gene-specific oligonucleotides can be found in the Supplementary data.

The primer sets for primary transcripts of *bantam*, *miR-8*, *miR-276a*, *miR-279*, *miR-305*, and *miR-317* were designed as previously described (Martin *et al*, 2009), and the primer sets for *pri-miR-184*, Exp5, Emb, H2B, and *pre-rp49* can be found in the Supplementary data. To analyse gene expression, pri-miRNA levels were normalized to *pre-rp49*, and means and s.e.m. values of technical triplicates were plotted. Two additional biological replicates are shown in Supplementary Figure S6.

Knockdown of endogenous gene expression in Drosophila

To investigate the effect of knockdowns in miRNA biogenesis, we performed dsRNA soaking in S2R$^+$ cells. The *GFP* dsRNA sequence was obtained from a published template (Förstemann *et al*, 2005). Approximately 500-bp fragments of other target genes were amplified from *D. melanogaster w*$^-$ genomic DNA using the primers listed below:

Dme-Exp5-dsRNA_F_XhoI: 5′-AGAGCTCGAGCTGGAGGATCAG CTCAATCG-3′
Dme-Exp5-dsRNA_R_XbaI: 5′-AGAGGTCTAGAGACGGAGCAG CTCGTAGAAC-3′
Dme-emb-dsRNA_F_XhoI: 5′-CCGCTCGAGACTGGGAGACATT CATCAG-3′
Dme-emb-dsRNA_R_XbaI: 5′-GCTCTAGAGAACCATGCTTAAA CACATG-3′

The PCR-amplified fragments were cloned into the *Xho*I/*Xba*I sites of pLitmus (NEB), which contains opposing T7 promoters flanking the cloning site. The dsRNAs were synthesized from pLitmus using MEGAscript T7 Kit (Ambion).

To knock down the expression of endogenous genes, 2.5×10^6 S2R$^+$ cells were soaked with 15 μg dsRNA in a 6-well plate for 4 days and transferred into another 6-well plate and soaked with 15 μg dsRNA for another 4 days.

Inhibition of Emb activity by leptomycin B treatment

To analyse the effect of direct inhibition of Emb protein activity on pri-miRNA level, we treated 8×10^5 S2R$^+$ cells with 25 ng/ml LMB (Sigma) or vehicle control (70% methanol) in 12-well plates for 2 h. The treatment with a higher dosage of LMB (50 ng/ml) resulted in a similar accumulation of pri-miRNAs. In a time-course experiment, 8×10^5 S2R$^+$ cells in 12-well plate were treated with 75 ng/ml LMB or vehicle control for 0, 1, 2, and 4 h. As pri-mir-317 level progressively increased upon treatment (data not shown), we selected the 2 h as a representative mid-level time point.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contribution. HG conceived the project; IB and HG designed and analysed *C. elegans* experiments, which IB performed; JSY and ECL designed and analyzed *Drosophila* experiments, which JSY performed. The authors jointly wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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