Coordinate post-transcriptional repression of Dpp-dependent transcription factors attenuates signal range during development

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ABSTRACT
Precise control of the range of signalling molecule action is crucial for correct cell fate patterning during development. For example, Drosophila ovarian germline stem cells (GSCs) are maintained by exquisitely short-range BMP signalling from the niche. In the absence of BMP signalling, one GSC daughter differentiates into a cystoblast (CB) and this fate is stabilised by Brain tumour (Brat) and Pumilio (Pum)-mediated post-transcriptional repression of mRNAs, including that encoding the Dpp transducer, Mad. However, the identity of other repressed mRNAs and the mechanism of post-transcriptional repression are currently unknown. Here, we identify the Medea and schnurri mRNAs, which encode transcriptional regulators required for activation and/or repression of Dpp target genes, as additional Pum-Brat targets, suggesting that tripartite repression of the transducers is deployed to desensitise the CB to Dpp. In addition, we show that repression by Pum-Brat requires recruitment of the CCR4 and Pop2 deadenylases, with knockdown of deadenylases in vivo giving rise to ectopic GSCs. Consistent with this, Pum-Brat repression leads to poly(A) tail shortening and mRNA degradation in tissue culture cells, and we detect a reduced number of Mad and shn transcripts in the CB relative to the GSC based on single molecule mRNA quantitation. Finally, we show generality of the mechanism by demonstrating that Brat also attenuates pMad and Dpp signalling range in the early embryo. Together our data serve as a platform for understanding how post-transcriptional repression restricts interpretation of BMPs and other cell signals in order to allow robust cell fate patterning during development.

KEY WORDS: BMP, Brat, Post-transcriptional repression, Pum, Differentiation, Mad, Medea, Schnurri

INTRODUCTION
The bone morphogenetic proteins (BMPs) constitute a major conserved family of signalling proteins that regulate a range of cellular processes during development and homeostasis, including cell fate determination (Ramel and Hill, 2012). For example, in Drosophila the BMP signalling molecule Dpp patterns fates over a range of cell diameters in different developmental contexts (Affolter and Basler, 2007), with ovarian germline stem cells (GSCs) maintained in response to an exquisitely short-range Dpp signal. Each Drosophila ovary consists of 16-20 ovarioles and at the tip of each ovariole is the germarium (Fig. 2A), a structure containing two or three GSCs that reside within a surrounding somatic cell niche (Harris and Ashe, 2011; Eliazer and Buszczak, 2011). Dpp released from the niche is highly restricted in range through sequestration by extracellular collagen IV (Wang et al., 2008) and the requirement for the niche-associated glypicans Dally for Dpp stability (Guo and Wang, 2009; Liu et al., 2010). The extracellular Dpp signal is transduced within GSCs through the activation of the Mothers against dpp (Mad) and Medea (Med) transcription factors. Phosphorylated Mad (pMad) forms a complex with Med that activates Dpp target genes, whereas recruitment of the Schnurri (Shn) co-repressor to the pMad-Med complex confers transcriptional repression (Hamantoglu et al., 2014). In GSCs, the pMad-Med-Shn complex directly represses transcription of bag of marbles (bam), which encodes a key differentiation factor (Chen and McKearin, 2003a,b; Pyrowolakis et al., 2004), as well as fused, encoding a kinase that phosphorylates the Dpp receptor, targeting it for proteasomal degradation (Xia et al., 2010, 2012). When a GSC divides, one daughter remains within the niche while the other, the cystoblast (CB) is displaced posteriorly and consequently receives a lower level of Dpp signal. This results in de-repression of Bam and subsequent differentiation (Kirilly and Xie, 2007; Harris and Ashe, 2011).

Translational controls also play important roles in regulating the balance between GSC self-renewal and differentiation. Like other stem cells, Drosophila GSCs are maintained by two major translational repressors: the Puf proteins Pumilio (Pum) and Nanos (Nos) (Gilboa and Lehmann, 2004; Wang and Lin, 2004). One target of Pum-Nos repression within GSCs is brain tumor (brat) mRNA, encoding a translational repressor that acts as a differentiation factor in both the female germ line and in neural stem cells (Betschinger et al., 2006; Lee et al., 2006; Harris et al., 2011). Recently it has been shown that Pum-Nos also repress mei-P26 mRNA in GSCs by recruiting the CCR4-NOT deadenylation complex (Joly et al., 2013). Mei-P26 is required at low levels in GSCs and plays a role in self-renewal through repressing translation of Brat as part of a Pum-Nos-Mei-P26 complex (Li et al., 2012). In contrast, Mei-P26 is expressed at high levels in CBs and differentiating cysts where it promotes differentiation and restricts proliferation through inhibition of the miRNA pathway (Neumüller et al., 2008).

Translational repression also plays a significant role in maintaining CB fate and promoting differentiation. Together with the RNA helicase Bgcn, and potentially also Sex-lethal and Mei-P26 (Chau et al., 2009; Li et al., 2013), Bam represses translation of Nos in the CB (Li et al., 2009) thereby relieving repression of differentiation factors such as Brat. Brat itself is able to form a complex with Pum (Sonoda and Wharton, 2001) and post-transcriptionally repress mRNAs encoding both Mad and the growth factor Myc in CBs (Harris et al., 2011). As CBs are born relatively close to the niche, repression of Mad mRNA might be important to reduce Mad protein levels in CBs, thereby ensuring that bam is transcribed even in the presence of low levels of Dpp signal. In addition to Pum-Brat repression, other mechanisms restrict Dpp signal transduction in CBs,
including Fused and Smurf-dependent targeting of the Dpp receptor Thickveins to the proteasome (Xia et al., 2010, 2012) and depletion of the mRNA encoding the BMP receptor Saxophone by miRNA repression (Iovino et al., 2009). However, despite the importance of Pum-Brat repression in this system, relatively little is known about the precise mechanism of repression.

In this work we present evidence that, in addition to Mad, Pum-Brat also post-transcriptionally repress other BMP signal transducers. In addition, we provide mechanistic detail underpinning Pum-Brat repression of target mRNAs by showing a requirement for deadenylases. Furthermore, we show that Brat represses Mad expression in the embryo, demonstrating that antagonism between Brat repression and BMP signalling also exists in other developmental contexts.

RESULTS

Pum and Brat post-transcriptionally regulate the Med and shn mRNAs

Dpp-dependent transcriptional activation is mediated by a complex of transcription factors pMad and Med, whereas a pMad-Med-Shn complex mediates transcriptional repression (Hamaratoglu et al., 2014). As we have previously provided evidence that Pum-Brat post-transcriptionally represses Mad mRNA via its 3′ UTR (Harris et al., 2011) we wished to determine whether similar regulation also exists for Med and shn mRNAs. Initially, we analysed the Med and shn 3′ UTR sequences for matches to the Pum binding consensus (Gerber et al., 2006) and the recently identified Brat binding site (Laver et al., 2014; Laver et al., 2015). This revealed multiple matches to both in each 3′ UTR (Fig. 1A), suggesting that these might be potential regulatory targets.

We next tested whether these 3′ UTRs confer post-transcriptional regulation in a tissue culture assay. A tagged construct bearing the Med coding sequence and both the 5′ and 3′ UTRs (Med-med3′) was transfected into Drosophila S2 cells, in the absence or presence of Pum and/Brat. S2 cells express Pum, Nos and Brat (modENCODE RNAseq data, Graveley et al., 2011). However, as Med mRNA regulation by Pum-Brat occurs in the absence of Nos in the CB, we mimicked this situation by reducing Nos levels in S2 cells using nos RNAi, as described previously (Harris et al., 2011). Co-transfection of Pum and Brat significantly reduced expression compared to Med-med3′ alone (Fig. 1B, B′) and no repression was observed in the absence of the Med 3′ UTR (Fig. 1C, C′). This suggests that Med mRNA might be post-transcriptionally regulated by Pum and Brat, in a manner similar to that we have described for Mad mRNA (Harris et al., 2011). We next tested whether shn expression is also repressed post-transcriptionally by expressing a GFP construct bearing the shn 3′ UTR (GFP-shn3′) in S2 cells in the presence of nos RNAi. GFP-shn3′ expression was repressed when co-transfected with either Pum or Brat (Fig. 1D, D′), in contrast to the GFP control that showed no repression by Pum and Brat (data not shown; Harris et al., 2011).

To determine the contribution to this regulation of the identified Pum or Brat sites in the Med and shn 3′ UTRs (Fig. 1A), we carried out site directed mutagenesis of two Pum and three Brat sites in each 3′ UTR. In the case of the shn 3′ UTR, the three Brat sites chosen included one matching the consensus described by Laver et al. (2014) (GUUUGU) and two matching the consensus identified by Laver et al. (2015) by both RIP-chip and RNAcompete (UGUUA), whereas the three remaining sites match the consensus identified by RIP-chip only. Wild-type and mutated UTRs were cloned into a dual luciferase reporter vector and transfected into S2 cells. With the Med 3′ UTR, the relative Renilla expression decreases upon Pum and Brat addition (Fig. 1E), mirroring the results obtained with the Med-med3′ reporter (Fig. 1B). The degree of repression is significantly reduced when the Pum and Brat sites are mutated (Fig. 1E), but not abolished, which might reflect incomplete disruption of binding when Pum and Brat are overexpressed, the presence of additional cryptic sites or that the 3′ UTR can be regulated through another mechanism. When testing the regulation of the shn 3′ UTR, we found that addition of Pum-Brat to the wild-type 3′ UTR repressed expression as expected. However, compared to the wild type, the mutant 3′ UTR was significantly de-repressed in both the absence and presence of Pum-Brat, comparable to the ~2 fold effect recently reported in a similarly designed repression assay in which Brat binding sites were also mutated (Laver et al., 2015). Taking into account this baseline de-repression, we detected no difference in Pum-Brat repression of the mutant 3′ UTR versus the wild type, which we speculate is because of the presence of additional sites that we have not mutated (Fig. 1A). Unlike the wild-type shn 3′ UTR, we do not observe de-repression of the wild-type Med 3′ UTR in the absence of added Pum and Brat when the Pum-Brat sites are mutated, which we attribute to the endogenous levels of Brat being insufficient to repress the Med 3′ UTR. Consistent with this, the effect on the Med-med3′ reporter when Pum alone is added is weaker and less significant than that observed when only Pum is added to the GFP-shn3′ reporter (compare Fig. 1B′ and D′).

Brat repression of Med and Shn in vivo

To test whether post-transcriptional repression of Med and Shn by Brat is relevant in vivo, we visualised Med protein in germlaria from wild-type flies and those with brat mutant germ lines (generated by tissue-specific flipase mediated mitotic recombination). In wild-type germlaria Med is present at higher levels in GSCs than CBs (Fig. 2B), consistent with a requirement for active Dpp signalling in these cells. In contrast, there is an expanded pattern of Med expression in a germline mutant for the bratnull null allele (Fig. 2B) and Med-GFP staining when Brat expression was knocked down in the CB using bam-Gal4VP16 to drive a brat shRNA (Fig. 2C), consistent with Brat acting as a repressor of Med expression in the ovary. We also visualised Shn protein in the germlaria using a Shn-GFP fusion, which is present at higher levels in GSCs than CBs in wild-type ovaries (Fig. 2D). However, when Brat expression was knocked down using a nos-Gal4VP16 driven brat shRNA transgene, we observed expanded Shn-GFP expression in cells bearing round spectosome organelles (Fig. 2D), providing in vivo support for shn mRNA also being post-transcriptionally repressed by Brat.

A conserved tryptophan residue within Pum is important for repression of Mad mRNA 3′ UTR

To determine the mechanism of Pum-Brat post-transcriptional repression, we focused on regulation of Mad mRNA. In terms of a potential effect of Pum on translation initiation, studies in Xenopus have shown that Pum2 represses translation by competing with eIF4E for binding to the 5′ 7-methyl guanosine cap (5′ m7G), thereby blocking translation initiation. In addition, the ability of Pum2 to out-compete eIF4E requires a conserved Trp residue (W344 in Xenopus) (Cao et al., 2010). As this Trp residue is also conserved in Drosophila (W783, Fig. 3A) we investigated whether mutation of W783 to Gly affected the ability of Pum to repress translation.

A GFP-reporter construct bearing the Mad 3′ UTR (GFP-mad3′), which we have used previously as a reporter of Pum-Brat repression (Harris et al., 2011), was transfected into Drosophila S2 cells along with Pum, Brat or mutant Pum (PumW783G) expression plasmids, in the presence of nos RNAi. Western blot analysis revealed repression of GFP-mad3′ by Pum. However, we observed a significant reduction
in this repression when the PumW783G construct was transfected (Fig. 3B,C), even though the wild-type and mutant Pum proteins accumulate to similar levels (Fig. 3B), suggesting that this residue is important for full repression. Transfection of Brat alone resulted in a similar level of repression to Pum-Brat co-transfection, suggesting that there might be sufficient endogenous Pum in S2 cells to allow repression with the addition of Brat, masking the effect of the transfected Pum. Consistent with this, transfection of PumW783G together with Brat only relieves repression moderately, with the repression observed potentially as a result of the activity of endogenous wild-type Pum and Brat. However, together these data suggest that the W783 residue is required for Pum to fully repress translation, and that Drosophila Pum might act in a similar manner to vertebrate Pum2, blocking translation through preventing eIF4E from binding to the 5′ 7 mG cap.

Repression by Pum-Brat requires recruitment of deadenylases

In addition to effects on initiation, repression of protein levels can often correlate with the deadenylation of mRNAs (Wiederhold and Passmore, 2010). In the Drosophila embryo and GSCs, there is evidence that the inhibition of translation by Pum and Nos involves
recruitment of the CCR4-NOT deadenylase complex (Kadyrova et al., 2007; Joly et al., 2013). Therefore, we investigated whether CCR4-NOT complex deadenylases play a role in Pum-Brat post-transcriptional repression. In Drosophila the CCR4-NOT complex is composed of seven proteins including NOT1-NOT4 and two deadenylases Pop2 (also known as CAF1) and CCR4 (Barckmann and Simonelig, 2013; Temme et al., 2010). Initially we used RNAi to knock down CCR4 and Pop2 expression in S2 cells, followed by transfection of GFP-mad3′ with and without Pum and Brat. Successful reduction in CCR4 and Pop2 protein levels by RNAi was verified by western blot (Fig. 4A). Visualisation of GFP levels revealed that knockdown of Pop2 caused significant loss of Pum-Brat repression of GFP-mad3′, whereas knockdown of CCR4 had no effect (Fig. 4B,B′). Although Joly et al. (2013) observed that CCR4 is the crucial deadenylase interacting with Pum in GSCs, our results here are consistent with Pop2 playing a more dominant role in the CCR4-NOT complex in S2 cells (Temme et al., 2010). It is interesting that only partial loss of repression was observed, however this might be a result of incomplete knockdown of Pop2 (Fig. 4A). One explanation for the requirement for Pop2 is that Pum-Brat could recruit the deadenylase complex to the target mRNA to promote its degradation through removal of the poly(A) tail.

To further investigate the role of the deadenylases, we tested whether there was a requirement for either CCR4 or Pop2 for CB differentiation in vivo. shRNA transgenes for either CCR4 (also known as twin) or Pop2 were used to knock down expression of these deadenylases in CBs by driving shRNA expression with bam-Gal4VP16. Knockdown of either deadenylase resulted in an increased number of cells with round spectrosome organelles in the germarium (Fig. 4C-F). Wild type germarium have on average four cells bearing round spectrosomes in each germarium, whereas we observed an average of 6.0±0.93 cells per germarium when CCR4 was knocked down (n=20) and 6.15±0.80 cells per germarium when Pop2 was knocked down (n=15). The additional cells bearing round spectrosomes in CCR4 and Pop2 knockdown germarium were pmad positive and Bam negative and therefore resemble undifferentiated GSC-like cells (Fig. 4C,D). As loss of either deadenylase in the complex is sufficient to cause the additional GSC-like cell phenotype it would appear that CCR4 and Pop2 do not function redundantly and it is possible that neither deadenylase is expressed at sufficiently high level to compensate for loss of the other.

The increase in pmad levels might reflect higher Med mRNA levels, as would be predicted if Pum-Brat repression involves recruitment of the CCR4-NOT complex. Consistent with this, we also observed ectopic expression of both Med-GFP and Shn-GFP in the additional GSC-like cells when CCR4 and Pop2 were knocked down (Fig. 4E,F). The up-regulation of Mad, Med and Shn protein levels provides a molecular explanation for the ectopic GSC-like cells observed, as a result of continued repression of bam transcription outside of the niche. Taking these results together with our observations in S2 cells, we hypothesise that in the absence of deadenylase activity, Mad, Med and shn transcripts persist in germline cells outside of the niche, allowing the persistence of Dpp signalling that results in delayed onset of Bam expression, and subsequent differentiation. Deadenylation might therefore be required to destabilise Mad transcripts hastening their degradation and removal from CBs.

**Pum-Brat targeted transcripts undergo poly(A) shortening and degradation**

Whereas some evidence exists for deadenylation-independent roles of the CCR4-NOT complex in translational repression (Chekulaeva et al., 2011; Cooke et al., 2010; Weidmann et al., 2014), more generally it has been proposed that this complex reduces translation and destabilises mRNA in a process reliant upon poly(A) tail removal (Temme et al., 2004; Chicoine et al., 2007). In order to assess whether repression by Pum-Brat leads to a loss of mRNA transcripts, we initially measured GFP-mad3′ transcript levels in S2 cells, with and without Pum-Brat co-transfection. The level of the GFP-mad3′ transcript in samples derived from cells co-transfected with Pum and Brat was reduced by 80% compared to controls (Fig. 5A). This decrease in mRNA levels implies that Pum-Brat mediated repression involves mRNA degradation. Furthermore, PAT-assays reveal that the poly(A) tail length of transcripts bearing
the Mad 3' UTR decreases in the presence of Pum-Brat (Fig. 5B). Although the majority of transcripts detected in both cases have a tail length of less than 50A because of preferential PCR amplification of shorter transcripts, the maximum tail length detected in unpressed samples was 200A whereas in Pum-Brat repressed samples this reduces to around 70A. Poly(A) tail length also increased to around 350A when endogenous Brat was knocked down by RNAi prior to transfection (Fig. 5B). Knockdown of either CCR4 or Pop2 by RNAi resulted in a similar increase in poly(A) tail length and knockdown of Pop2 in particular resulted in a greater number of transcripts retaining longer tails in the presence of Pum and Brat (Fig. 5B). In contrast we found no difference in poly(A) tail length of tubulin transcripts in the same samples (Fig. 5C). These data suggest that Pum-Brat recruitment to the Mad 3’ UTR leads to a shortening of poly(A) tail length and transcript instability and this process requires the CCR4-NOT complex deadenylases.

To determine whether Mad transcripts are also degraded in CBs we used Stellaris fluorescence in situ hybridisation to label and quantify Mad transcripts within GSCs and CBs in wild type ovaries. GSCs were identified from their position within the niche and CBs identified as cells expressing Bam (Fig. 5D). GSCs had on average 50.8±13.8 Mad transcripts per cell whereas the mean number of transcripts in CBs was 33.0±8.0. The number of Mad transcripts detected in CBs ranged from 50% to about 80% of the number detected in GSCs in the same germarium (P<0.01, n=13; Fig. 5D,G). Similarly, we found that the mean number of shn transcripts decreased from 42.3±7.2 in GSCs to 28.2±6.9 in CBs with, on average, a 32% reduction in the number of transcripts detected in CBs versus GSCs in the same germarium (P<0.01, n=12; Fig. 5E,G). It is possible that the variability observed relates to the age of the CBs when the samples are fixed. The transcript number would be expected to be similar in GSCs to 28.2±6.9 in CBs with, on average, a 32% reduction in the number of transcripts detected in CBs versus GSCs in the same germarium (

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Dpp target genes in brat11 GLC embryos, we also observed a significant increase in the number of amnioserosa cells from 133.6±6.5 in wild-type to 181.3±11.5 in brat11 GLCs (Fig. 6D,D′). We also counted amnioserosa cell number to investigate a genetic interaction between the brat11 and Med14 loss-of-function alleles. To this end, we analysed progeny from females that were either Med14+/+ or brat11+/+. Med14+/+ or brat11+/+ crossed to Med14 heterozygous males. When the females were Med14+/+, embryos showed variable reductions in amnioserosa number, consistent with a reduced Dpp signalling response. However, we found that the phenotype could be partially rescued when the females were also heterozygous for the brat11 mutation, indicated by a greater proportion of embryos with a higher number of amnioserosa cells (Fig. 6E), consistent with antagonism between Brat and the Dpp pathway. Taken together, these results show that Brat has a negative regulatory effect on Dpp signalling in the early embryo, consistent with a general role for Brat as a post-transcriptional
repressor of the Dpp signal transduction pathway in different developmental contexts.

**DISCUSSION**

In this study we provide evidence that Pum-Brat represses Mad, Med and shn mRNAs in CBs, preventing transduction of the Dpp self-renewal signal. Mechanistically we show that lower levels of the Pop2 deadenylase are associated with weaker Pum-Brat repression in a tissue culture assay. Consistent with this, knockdown of the Pop2 or CCR4 deadenylases in the germarium gives rise to a similar phenotype of extra GSC-like cells as we have previously reported for the brat mutant (Harris Fig. 5. Pum-Brat repression leads to deadenylation and degradation of target RNAs. (A) Quantification of GFP-mad3' mRNAs in S2 cell extracts by qPCR. Graph shows the normalised mean of three biological repeats relative to Rp49, there is a 78% reduction in GFP-mad3' mRNA level following Pum-Brat (PB) repression (error bar represents s.d., ***P<0.001 by t-test). (B) RACE-PAT assay shows poly(A) tail length of GFP-mad3' mRNAs following Pum-Brat repression and/or treatment with brat, CCR4 or Pop2 RNAi in S2 cells. (C) There is no reduction in poly(A) tail length of beta-tubulin mRNAs extracted from the same transfected S2 cells. (D-F) Mad (D), shn (E) and Tbp (F) mRNAs detected by Stellaris fluorescence in situ hybridisation (red) in representative bam-GFP; vasa-GFP germlia co-stained with anti-GFP (green). Dotted lines indicate GSCs (white arrows) and CBs (Bam-positive); scale bar: 5 μm. (G) Ratio (GSC:CB) of the mean number of Mad, shn and Tbp transcripts per cell in each germarium, normalised to the number in GSCs (n=13). Transcripts were quantified in GSCs and CBs using Imaris software. There is a significant reduction in Mad and shn, but not Tbp, transcript number in CBs versus GSCs (**P<0.01, ***P<0.001 by t-test, error bar represents s.d.).
et al., 2011). We show that these extra GSC-like cells are a result of repression of bam by higher pMad, Med and Shn levels in cells outwith the niche, suggesting that these deadenylases are required in CBs to initiate timely differentiation. We present evidence that the repressed Mad mRNA has a shorter poly(A) tail, and we detect fewer Mad and shn mRNA molecules in CBs relative to GSCs. The simplest interpretation of these data is a model whereby Pum-Brat repression of the target mRNAs involves recruitment of the CCR4-NOT complex, resulting in poly(A) tail shortening and degradation of target transcripts, reducing Dpp signal transduction (Fig. 7). Finally, we detect expanded pMad and Dpp target gene expression in brat mutant embryos, suggesting that this repression also occurs at other developmental stages.
Although knockdown of CCR4 and Pop2 results in extra GSC-like cells, these cells are able to differentiate eventually as observed previously in the brat mutant (Harris et al., 2011). Presumably these cells are pushed further from the niche with each GSC division, so receive progressively lower levels of Dpp, which acts at short range (Harris and Ashe, 2011). Therefore, even if Mad protein continues to accumulate in CCR4/Pop2 knockdown germaria it is unlikely to be activated far from the niche because of a lack of Dpp, allowing bam de-repression and differentiation. Other Pum-Brat-independent mechanisms promote differentiation in CBs by attenuating the Dpp signal, including Fused/Smurf-dependent proteolysis of Thickveins (Xia et al., 2010; Casanueva and Ferguson, 2004). In addition, Dpp-independent mechanisms, such as repression of the miRNA pathway (Neumüller et al., 2008) and lower levels of translation overall (Zhang et al., 2014), also promote differentiation. As these mechanisms respond to either a reduction in Dpp signalling or are downstream of Bam (Xia et al., 2012; Neumüller et al., 2008; Zhang et al., 2014), they would be inactive in the ectopic CCR4/Pop2 knockdown GSCs. Therefore, the number of ectopic GSCs we detect should act as a readout of the Dpp signal range in the germarium, which would be equivalent to around three cell diameters.

CCR4 has also been shown to be a Pum-Nos co-factor in GSCs and is essential for GSC self-renewal (Joly et al., 2013). In CCR4 mutants GSCs are lost to differentiation over time, even though they do not upregulate bam expression, eventually resulting in empty ovarioles. This has been attributed to loss of translational repression of Mei-P26 and possibly other differentiation factors by Pum-Nos (Joly et al., 2013). In contrast, we observed that knockdown of CCR4 in CBs blocked Bam de-repression and differentiation which we interpret as being as a result of higher Mad, Med and Shn levels following loss of Pum-Brat repression of the target mRNAs. We suggest that as CCR4 depletion in the CB does not affect GSC maintenance, GSCs would divide as normal. However, the daughter with reduced CCR4 activity does not upregulate Bam. CCR4 bam
double mutants give rise to a mix of the bam mutant phenotype of
tumorous undifferentiated cells with the germline loss phenotype
characteristic of the CCR4 mutant, with the latter prevailing over
time (Joly et al., 2013). Therefore, although CCR4 bam double
mutant germline cells can differentiate, the process appears to be
slower than normal, which could explain why we capture extra
GSC-like cells when we knock down CCR4 in the CB.

As discussed above, our data suggest a model whereby Pum-Brat
recruit deadenylases resulting in a shorter poly(A) tail and
subsequent destabilisation of target transcripts. However, we also
find that complete Pum-Brat repression depends on a conserved Trp
residue W783, which allows Pum to interact with the 5′ cap
and hence block translation initiation in other species (Cao et al.,
2010). Therefore, we cannot rule out that the Pum-Brat repression
mechanism is primarily through effects on translation initiation, via
an interaction of Pum and the 5′ cap which prevents elf4E
recruitment (Fig. 7), and that subsequent CCR4-NOT complex
recruitment and deadenylation is potentially a secondary effect.
This scenario would be similar to Pum-Brat-Nos repression of
hh mRNA, as although there is evidence for deadenylation of
hh mRNA (Wreden et al., 1997), this appears to be secondary to the
repression mechanism (Chagnovich and Lehmann, 2001). To add to
this complexity, Brat itself has recently been shown to mediate both
translation repression and degradation of mRNAs, including
maternal mRNAs, in the embryo (Laver et al., 2015). We note that
a complex relationship also exists between translational repression and mRNA deadenylation/degradation with respect to
miRNA regulation (Wileczynska and Bushell, 2015). Controversy
exists over whether translation repression precedes deadenylation
(Meijer et al., 2013) or deadenylation is dominant (Eichhorn et al.,
2014), with the added complexity of context-dependent effects
(Subtelny et al., 2014). Overall, these studies highlight the
difficulties that have been encountered in defining the order and
primary target of post-transcriptional control mechanisms, with
multiple effects usually observed.

Another possibility for the function of CCR4 in CBs is that it is
independent of deadenylation, given the precedents for this in other
contexts. CCR4-NOT is recruited to miRNA regulated mRNAs via
GW182 (also known as Gawky) proteins (Fabian et al., 2009; Ilnek
et al., 2010), and can repress poly(A)− as well as poly(A)− RNAs,
suggesting that CCR4-NOT can inhibit translation independently of
deadenylation (Chekulaeva et al., 2011). Similarly, the Pop2
homologue CAF1 has been shown to repress poly(A)− mRNAs in
Xenopus oocytes and Pop2 lacking deadenylase activity retains
some ability to repress cap-dependent translation initiation (Cooke
et al., 2010). It is clear that further studies are required to precisely
define the roles of CCR4 and Pop2 in regulating post-transcriptional
repression in the CB.

We observed expanded expression of pMad and Dpp target genes,
with associated increased numbers of amnioserosa cells, in brat
mutant embryos, suggesting that Brat is required to limit the range
of Dpp signal transduction by post-transcriptionally repressing
Mad mRNA and, by extrapolation, Med and shn mRNAs. As Mad
protein accumulates in a wild-type embryo when Brat is present, this
suggests that repression is normally incomplete. This might relate to
relative levels of the maternally expressed target mRNAs and that of
the Brat protein and/or other members of the repression complex.
Alternatively, it is possible that a feature of post-transcriptional
repression during development is that it is used for fine-tuning
protein levels rather than for a complete shutdown of protein
synthesis. This is the case for Pum-Nos repression of mei-P26
mRNA in GSCs, with a particular level of Mei-P26 protein
associated with GSC self-renewal (Li et al., 2012), whereas high
Mei-P26 promotes differentiation (Neumüller et al., 2008).

We suggest that repression of Mad mRNA in the embryo also
involves Pum, so that the repression mechanism would be the same
as we observed in S2 cells and in the ovary, which both implicate
Pum (Harris et al., 2011). However, it has been suggested that the
Mad 3′ UTR can be repressed by Brat alone in S2 cells,
independently of Pum (Loedige et al., 2014), and Med mRNA has recently been identified as a Brat but not Pum target in the
embryo based on RIP-seq analysis (Laver et al., 2015). The reason
for this discrepancy is unclear but it is possible that Brat could
inhibit Mad or Med mRNA translation in the absence of Pum in
some circumstances, for example in the embryo where we
hypothesise partial repression occurs as described above, although
optimal repression might require both Pum and Brat. Consistent
with this, Brat has recently been shown to function largely
independently of Pum in the embryo, although some mRNAs are
co-regulated by both (Laver et al., 2015). Brat has also been
implicated in repression of Mad and hence modulation of BMP
signalling at neuromuscular junction (NMJ) synapses (Shi et al.,
2013). Given the differences in the NMJ phenotypes associated with
the pum and brat mutants (Shi et al., 2013; Menon et al., 2004), the
NMJ synapse might represent one context where Brat repression
of Mad is Pum-independent. However, the same study also suggested
co-repression of Mad by Brat and Pum in presynaptic neurons (Shi
et al., 2013), identifying another situation where Pum and Brat
potentially cooperate to repress Mad.

A major way in which a cell responds to changes in its
environment, including chemical signals, is through the coordinated
regulation of sets of genes at the transcriptional level. This varies
in complexity from the co-regulation of genes arranged in
centromeres within the genomes of prokaryotes, C. elegans and plants
(Ishihama, 2010; Blumenthal, 2004; Boycheva et al., 2014) to the
more complex coordination of whole gene expression programmes
by particular transcription factors (Bonn and Furlong, 2008). At
the level of translation, there is recent evidence for
colocalisation of mRNAs encoding components of protein complexes or pathways
within particular cytoplasmic granules that might allow coordinated
translation (Lui et al., 2014; Gao et al., 2015). Therefore, a logical
extension of these findings would be the coordinated post-
transcriptional repression of mRNAs encoding proteins within
specific complexes or pathways to limit their time of action. Our
data, identifying the mRNAs encoding all three components of the
Mad-Med-Shn complex as targets of Pum-Brat post-transcriptional
repression, support this idea. This tripartite regulation might
represent an important fail-safe mechanism to ensure that Dpp
signal transduction is blocked efficiently in the CB, permitting
differentiation despite proximity of this cell to the niche. In contrast,
as discussed above, in the early embryo it appears that partial
repression modulates interpretation of the Dpp signal. In this way,
post-transcriptional repression of the Dpp signal transducers can act
as a ‘volume’ control, offering great flexibility in regulating the
strength of the signal response either across a field of cells or more
specifically within individual cells depending on the levels of Pum-
Brat. We predict that post-transcriptional regulation of specific
components of other signalling pathways will be widely used as a
mechanism to refine the cellular response during development.

MATERIALS AND METHODS

Fly stocks
Fly stocks used were: y w6723 (used as wild type in all experiments); bam-
GFP (Chen and McKearin, 2003b); Pop2, P[TRIP.HMJ21614] and twin,
**In situ hybridisation and immunofluorescence**

Embryos were collected from yeast/apple juice plates and fixed and stained using standard techniques. Ovaries were dissected from adults after maturing on yeast/apple juice plates and fixed according to standard techniques. Primary antibodies used were: anti-pSmad3 (1:1000, EMD Millipore), rabbit anti-GFP (1:200, Torrey Pines Biolabs TP401); mouse anti-spectrin (1:20, DSHB Hybridoma Product 1952, deposited to the DSHB by R. R. Williams); rabbit anti-Mad (1:100, Sutherland Stowers Institute, USA). Secondary antibodies used were donkey anti-mouse and donkey anti-rabbit Alexa594 (Life Technologies A21207) and goat anti-mouse-AP (Promega 1140). Secondary antibodies were used at 1:10,000 dilution.

**Tissue culture and western blot**

S2 cells were cultured in Schneider’s Modified Drosophila Media (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin. DNA constructs for transfection were generated using standard techniques and the PumW783G mutation and mutations in Pum and Brat binding sites were generated by site-directed mutagenesis (Invitrogen) in Drosophila S2 cells. For RNAi, S2 cells were transfected with pTRIP.HMS00493 shRNA (Harvard Medical School TRIP project, Bloomington Stock Center); bam-Gal4 (Chen and McKearin, 2003b); Med1/TMS, PBac[Med-GFP.FLAG] and PBac[Shn-GFP.FLAG] (Bloomington Stock Center). brat11 clonal germ lines were generated by FLP/FRT recombination as described previously (Harris et al., 2011). brat11 germine clone embryos were generated using the FLP-DFS system: larvae of genotype hs-FLP; brat11-FRT/Pvop3-FRT were heat shocked for 3 h on two consecutive days, adult females were then crossed to brat11-FRT/CyO males and the resulting embryos were collected.

**Statistical analysis**

Western blot IR signals were quantified using Li-Cor Odyssey software and qPCR signals were quantified using Opticon Monitor3 software. Error bars represent the standard deviation (s.d.) of at least three biological repeats with Student’s t-tests used for significance.

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**Author contributions**

This paper was written by F.G.N. and H.L.A. Medea western blots, Medea immunostaining and Dpp target gene in situ hybridisations were carried out by R.E.H. and luciferase assays were carried out by C.S. All other experiments were carried out by F.G.N. with technical assistance from C.S.

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**Competing interests**

The authors declare no competing or financial interests.
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