The miRNA machinery targets Mei-P26 and regulates Myc protein levels in the *Drosophila* wing

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MicroRNAs (miRNAs) have been implicated in cell-cycle regulation and in some cases shown to have a role in tissue growth control. Depletion of miRNAs was found to have an effect on tissue growth rates in the wing primordium of *Drosophila*, a highly proliferative epithelium. Dicer-1 (Dcr-1) is a double-stranded RNaseIII essential for miRNA biogenesis. Adult cells lacking *dcr-1*, or with reduced *dcr-1* activity, were smaller than normal cells and gave rise to smaller wings. *dcr-1* mutant cells showed evidence of being susceptible to competition by faster growing cells *in vivo* and the miRNA machinery was shown to promote G₁–S transition. We present evidence that Dcr-1 acts by regulating the TRIM-NHL protein Mei-P26, which in turn regulates dMyc protein levels. Mei-P26 is a direct target of miRNAs, including the growth-promoting *bantam* miRNA. Thus, regulation of tissue growth by the miRNA pathway involves a double repression mechanism to control dMyc protein levels in a highly proliferative and growing epithelium.

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Introduction

Regulation of gene expression at the transcriptional level has a central role in development and physiology; however, the relevance of post-transcriptional gene regulation is increasingly recognized. MicroRNAs (miRNAs), endogenous small non-coding RNAs, 22 nucleotides long, that repress target transcripts (Flynt and Lai, 2008), confer a novel layer of post-transcriptional regulation. Dicer-1 (Dcr-1) is a crucial element for miRNA biogenesis (Lee *et al*, 2003). Therefore, impairing Dcr-1 activity provides a means to assess the role of the miRNA pathway in a given biological process. Loss of *dcr-1* produces defects in *Drosophila* and vertebrate stem cell

maintenance and causes a delay in G₁–S transition in these cells (Hatfield *et al*, 2005; Jin and Xie, 2007; Wang *et al*, 2007). In the developing mouse limb, loss of *dcr-1* leads to growth defects (Harfe *et al*, 2005). The main effectors mediating the activity of Dcr-1 in these processes have not been identified. The wing imaginal disc of *Drosophila* is a very suitable model system to analyse at a cellular level the role of miRNAs in a highly proliferative epithelium and to identify such effectors.

The fly wing primordium arises as a group of 30–40 cells in the embryonic ectoderm that proliferates during 5 days to reach a final size of around 50 000 cells and gives rise after metamorphosis to the adult wing (García-Bellido and Merriam, 1971; Madhavan and Schneiderman, 1977). Here we have analysed the role of Dcr-1 in growth control in the developing wing. Dcr-1 is required for cell and tissue growth, promotes G₁–S transition and *dcr-1* mutant cells are eliminated by a process of cell competition. We present evidence that the *dMyc* proto-oncogene (Johnston *et al*, 1999) contributes to the role of the miRNA pathway in these processes. TRIM32, the mouse orthologue of *Drosophila* Mei-P26 (Page *et al*, 2000), has been shown to show ubiquitin ligase activity, bind to c-Myc and target it for degradation (Schwamborn *et al*, 2009). We present evidence that the miRNA machinery, acting through Mei-P26, regulates dMyc activity, and as a consequence regulates cell and tissue growth rates and E2F activity. Mei-P26 is a direct target of the growth-promoting *bantam* miRNA. This study identifies the elements of an integrated regulatory network involving miRNAs, regulators of cell growth and of cell proliferation.

Results and discussion

Reduced growth in the absence of Dcr-1

We first used the Gal4/UAS technique to express an RNAi construct of *dcr-1* (*dcr-1^{RNAi}*) to reduce Dcr-1 activity in specific territories within the developing wing. To assess the efficacy of the *dcr-1^{RNAi}* transgene, we measured the levels of 11 miRNAs known to be expressed in wing imaginal discs by quantitative PCR. Nine of these miRNAs were reduced (Figure 1A). We also analysed the capacity of *dcr-1^{RNAi}* to reduce the processing of the *bantam* miRNA in the wing cells. Expression of *dcr-1^{RNAi}* reduced *bantam* activity as visualized by increased levels of a *bantam* activity sensor (Brennecke *et al*, 2003) in the wing imaginal disc (Supplementary Figure S1). The magnitude of this effect was less than that observed in clones of cells mutant for a null allele of *dcr-1* (*dcr-1^{Q1147X}*) (Lee *et al*, 2004), Supplementary Figure S1). Expression of a sensor lacking the *bantam* target sites (Brennecke *et al*, 2003) was not affected upon *dcr-1^{RNAi}* expression (Supplementary Figure S1). Thus, *dcr-1^{RNAi}* expression leads to an intermediate condition where Dcr-1 activity and miRNA levels are reduced

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Figure 1 Reduced cell growth in the absence of Dcr-1 activity. (A) Quantitative PCR experiment comparing the relative level of mature miRNAs between the anterior (a, white bars) and posterior (p, blue bars) compartments of *en> dcr-1^{RNAi}* late third instar wing discs. The reference gene *U27* was used for data normalization for RNAs from different compartments. The relative levels of miRNAs in the a compartment were set to '1' (white bars). *en> dcr-1^{RNAi}* drives expression of *dicer-1^{RNAi}* and GFP in the p compartment. The y-axis shows the percentages of miRNA expression level relative to the a compartment. A wing disc is depicted to visualize a (white) and p (blue) compartments of a mature wing disc. (B) Cells in an *en> dcr-1^{RNAi}* adult wing. The red line indicates the boundary between the anterior (a) *dcr-1^{RNAi}*-non-expressing and posterior (p) *dcr-1^{RNAi}*-expressing cells. Note the reduced cell size of the posterior cells. (C, D) Cuticle preparations of adult wings expressing GFP, *dcr-1^{RNAi}* and/or *dcr-1* in the *patched* (*ptc*, C) or *engrailed* (*en*, D) domains (labelled blue) in different genetic backgrounds. (E–G) Histograms plotting the size (E, F) and cell density values (G), normalized as a percent of the control GFP-expressing wing values, of the *ptc* and *en* domains expressing different transgenes. The error bars indicate the standard deviation. Only adult wing males were analysed. (E) In *ptc> dcr-1^{RNAi}*, a significant decrease in the size of the *ptc* domain was observed when compared with *ptc> GFP* wings ($P < 10^{-5}$). (F, G) In *en> dcr-1^{RNAi}*, a significant decrease in the size of the *en* domain was observed when compared with *en> GFP* wings ($P < 10^{-7}$), and a significant increase in the cell density of the *en* domain was observed when compared with *en> GFP* wings ($P < 10^{-5}$). Coexpression of *dcr-1*-rescued tissue ($P < 10^{-10}$) and cell size ($P < 10^{-2}$) defects. Tissue size values: *ptc> GFP* = 100 ± 8.4 (n of wings = 10); *ptc> dcr-1^{RNAi}* = 75 ± 5.1 (n = 12); *en> GFP* = 100 ± 5.7 (n = 10); *en> dcr-1^{RNAi}* = 75 ± 5.1 (n = 10); *en> dcr-1^{RNAi} > dcr-1* = 110 ± 5.8 (n = 12). Cell density values: *en> GFP* = 100 ± 7.6 (n = 10); *en> dcr-1^{RNAi}* = 123 ± 9.4 (n = 10); *en> dcr-1^{RNAi} > dcr-1* = 95 ± 2.6 (n = 10). (H, I) Adult wings with clones of cells lacking *dcr-1* activity. The mutant tissue in adult wings (genotype: *forked^{36a} hs-FLP; FRT 82 P(forked +) M(3)95A2/ FRT82 dcr-1^{Q1147X}*) was marked by absence of the *P(forked⁺)* rescue construct (see Materials and methods). In panel H, the red bar indicates the mutant bristles and blue arrows indicate the *wild-type* bristles. The red line in panel I indicates the boundary between *wild-type* and mutant tissue. *Wild-type* and *M(3)95A2/+* adult wings show a similar cell size (Morata and Ripoll, 1975).

but not eliminated. Expression of *dcr-1^{RNAi}* caused an autonomous reduction in cell size in the adult wing (Figure 1B and G; see also Supplementary Table S1). As a consequence, the size of the tissue that expressed *dcr-1^{RNAi}* was also reduced (Figure 1C–F). These effects were rescued by co-expression of a *wild-type* form of *dcr-1* (Figure 1D, F and G). Decreasing the amount of *dcr-1* by 50% enhanced the size defects caused by *dcr-1^{RNAi}* expression (Figure 1D) and the viability of these

flies was drastically reduced (data not shown). Similar adult cell size defects were observed in clones of cells mutant for a null allele of *dcr-1* (*dcr-1^{Q1147X}*, Figure 1H and I).

To further study the requirement of Dcr-1 in tissue growth, we induced clones of *dcr-1^{Q1147X}* mutant cells in the wing primordium and compared their size with their *wild-type* twin clones resulting from the same mitotic recombination event. In clones analysed 72 h after induction, activity of Dcr-1 was

Figure 2 Dcr-1 and cell competition. (A, B) Wing discs with clones of cells lacking *dcr-1* activity marked by absence of GFP (white). Clones were induced 72 h before dissection. Note the reduced size of the mutant clones (in black) when compared with the control *wild-type* twins (in white). (C) Graphs showing the relative sizes (clone areas, in arbitrary units) of individual pairs of *dcr-1*^{-/-} clones (black bars) and *dcr-1*^{+/+} twins (grey bars). Two different alleles of *dcr-1* were used in panels A–C. Genotypes: *hs-FLP; FRT 82 Ubi-GFP/FRT82 dcr-1*^{Q1147X} and *hs-FLP; FRT 82 Ubi-GFP/FRT82 dcr-1*^{d102}. Only those wing discs with low frequency of clones and twins were scored to facilitate the quantification and reduce the possibility of fusion of neighbouring clones or twins. (D–F) Wing discs with clones of cells lacking *dcr-1* activity marked by absence of GFP (white in panels D and E, green in panel F) and induced 72 h (D, F) or 96 h (E) before dissection. Note that mutant clones tend to break (red arrowheads in panel D) and enter apoptosis (labelled by TUNEL staining, red, F) 72 h after induction, and they are frequently lost from the epithelium 96 h after induction (E). (G) Wing discs with clones of cells lacking *dcr-1* activity (right panels) or *wild-type* for *dcr-1* (left panels) and generated by the *Minute* technique to give clone cells a growth advantage. Clones were labelled by absence of GFP expression (white) and induced 96 h before dissection. The genotypes were: *hs-FLP; FRT 82 Ubi-GFP M(3)95A2/FRT82 dcr-1*^{Q1147X} and *hs-FLP; FRT 82 Ubi-GFP M(3)95A2/FRT82*. (H) Histogram plotting the size of *dcr-1* (*n* clones = 21) and *wild-type Minute*(+) clones (*n* clones = 18) induced 96 h before dissection. Clone size was normalized as a percent of the *wild-type Minute*(+) clone size. The error bars indicate the standard deviation. The difference between both genotypes was statistically significant ($P < 10^{-8}$).

already reduced, monitored by the reduced activity of the *bantam* miRNA (Supplementary Figure S1). Clones of *dcr-1*^{Q1147X} mutant cells were on average smaller than their corresponding *wild-type* twin clones (Figure 2B and C; see also references Friggi-Grelin *et al*, 2008; Martin *et al*, 2009). Clones of cells mutant for *dcr-1*^{d102}, another allele of *dcr-1*, gave similar results (Figure 2A and C). We noticed that these clones were frequently fragmented (Figure 2D) and that many clones were eliminated from the wing disc by 96 h after induction (Figure 2E). A total of 31.5% of the *wild-type* twin clones induced 96 h before dissection were lacking their corresponding *dcr-1* mutant clones (*n* of twin clones/wing disc = 4.1; *n* of *dcr-1* clones/wing disc = 2.4; *n* of discs = 10). Most *dcr-1* mutant clones were positive for TUNEL staining, a marker of apoptotic cells (Figure 2F). These observations suggest that *dcr-1* mutant

cells are eliminated through cell competition, a process by which slower growing cells are detected and removed through apoptosis (Morata and Ripoll, 1975; Moreno *et al*, 2002; de la Cova *et al*, 2004; Moreno and Basler, 2004; Li and Baker, 2007). To test this we gave the mutant cells a relative growth advantage using the *Minute* technique to impair growth of the other cells (see section Materials and methods). In a *Minute*/+ background, *dcr-1* mutant clones were recovered at the same frequency as *wild-type* control clones induced 96 h before dissection (*[dcr-1 M(+)]* = 3.2 clones/wing disc, *n* = 32 clones; *[M(+)]* = 3 clones/wing disc, *n* = 30 clones). However, *dcr-1* mutant clones were much smaller than the *wild-type* control clones (Figure 2G and H). Similar results were obtained with clones of cells mutant for *Argonaute-1*, a component of the miRISC complex. *Argonaute-1*

Figure 3 Dcr-1 regulates G₁-S transition. (A–J) Wing discs with clones of cells lacking *dcr-1* activity (A, D, E, G, I; genotype: *hs-FLP; FRT 82 lacZ M(3)95A2/FRT82 dcr-1^{Q1147X}*) marked by absence of β -gal (green), or expressing *dcr-1^{RNAi}* (B, C, F, H, J) in the *patched* (*ptc*) domain (red arrowheads) and labelled to visualize in pink or white cyclin-B (CycB; A, B), cyclin-E (CycE; C, D), dE2F activity (an E2F1 responsive reporter *ORC1-GFP* was used, antibody to GFP; E, F), PCNA (G, H) and Dap (I, J) protein expression. Clones were induced 72 h before dissection. (K) Cuticle preparations of adult wings expressing *dcr-1^{RNAi}* in the *engrailed* (*en*) domain in several genetic backgrounds. (L, M) Histograms plotting the size (L) and cell density (M) of the *en* domain in adult wings expressing *dcr-1^{RNAi}* in different genetic backgrounds. Tissue size and cell density values were normalized as a percent of the values of control GFP-expressing wings. The error bars indicate the standard deviation. Adult wing males and females were analysed. In *en>dcr-1^{RNAi}*, a significant decrease in the size of the *en* domain was observed when compared with *en>GFP* wings ($P < 10^{-7}$ in males and $P < 10^{-3}$ in females). Halving the dose of *dap* or coexpression of *CycE* significantly rescued this phenotype ($P(\text{dap}^4) < 10^{-5}$, $P(\text{Rbf}^{\text{slis5}}) < 10^{-5}$, $P(\text{CycE}) < 10^{-8}$). In *en>dcr-1^{RNAi}* males, a significant increase in the cell density of the *en* domain was observed when compared with *en>GFP* wings ($P < 10^{-3}$). Halving the dose of *dap* or coexpression of *CycE* significantly rescued the cell size defects ($P(\text{dap}^4) < 10^{-3}$, $P(\text{CycE}) < 10^{-3}$). Tissue size values (males): *en>GFP* = 100 ± 5.7 ($n = 12$); *en>dcr-1^{RNAi}* = 75 ± 5.1 ($n = 10$); *en>dcr-1^{RNAi}; dap⁴/+* = 122 ± 7.3 ($n = 7$); *en>dcr-1^{RNAi}>CycE* = 111 ± 4.5 ($n = 12$). Tissue size values (females): *en>GFP* = 100 ± 7.4 ($n = 12$); *en>dcr-1^{RNAi}* = 66 ± 1.7 ($n = 12$); *Rbf^{slis5}/+* = 80 ± 3.1 ($n = 12$); *en>dcr-1^{RNAi}>CycE* = 106 ± 4.5 ($n = 10$). Cell density values (males): *en>GFP* = 100 ± 7.6 ($n = 10$); *en>dcr-1^{RNAi}* = 123 ± 9.4 ($n = 10$); *en>dcr-1^{RNAi}; dap⁴/+* = 105 ± 4.5 ($n = 10$); *en>dcr-1^{RNAi}>CycE* = 106 ± 4.5 ($n = 10$).

mutant clones were smaller than their *wild-type* twin clones, were lost from the epithelium and their recovery rate was increased when given a growth advantage with the *Minute* technique (Supplementary Figure S1, and data not shown). Taken together, these results suggest that the miRNA machinery is required for cell and tissue growth.

Dcr-1 regulates E2F activity and p21/Dap protein levels

Loss of *dcr-1* has been reported to produce a delay in G₁-S transition in *Drosophila* and vertebrate stem cells (Hatfield et al, 2005; Jin and Xie, 2007; Wang et al, 2007). We analysed the distribution of the cell-cycle stages in *dcr-1* mutant wing disc cells using antibodies against several cell-cycle markers (Figure 3A–D and Supplementary Figure S2). In cells mutant for *dcr-1* or expressing *dcr-1^{RNAi}*, expression of Cyclin-B (CycB) protein, which accumulates during G₂ (Whitfield et al, 1990), was reduced (Figure 3A and B), whereas that of Cyclin-E (CycE) protein, which accumulates during G₁ (Knoblich et al, 1994), was increased (Figure 3C and D). *Wild-type* control clones generated in a *Minute* heterozygous

background did not show any change in CycB or CycE protein levels (Supplementary Figure S2). These data suggest that loss of *dcr-1* leads to a G₁-S delay.

The G₁-S transition is negatively regulated by the CDK inhibitor Dap (the *Drosophila* p21/p27 orthologue), which traps the CycE-CDK2 complex in a stable but inactive form (de Nooij et al, 1996; Lane et al, 1996), and by retinoblastoma family proteins Rb1 and Rb2, which interact with and negatively regulate E2F (van den Heuvel and Dyson, 2008). In cells with reduced Dcr-1 activity, Dap protein expression was increased (Figure 3I and J) and E2F activity was reduced (Figure 3E–H), visualized using the dE2F1-responsive reporter *ORC1-GFP* (Asano and Wharton, 1999; labelled dE2F in the figures) and PCNA, a target of dE2F. *Wild-type* control clones generated in a *Minute* background did not show any change in PCNA protein levels (Supplementary Figure S2). Altogether, these results suggest that miRNAs normally promote cell division by limiting the expression of Dap and by increasing the activity of E2F.

Figure 4 Dcr-1 regulates dMyc protein levels. (A–F) Wild-type wing discs (E, F) or wing discs with reduced *dcr-1* activity (A–D) labelled to visualize dMyc protein (red or white; A, B, D, E) or mRNA (purple; C, F) expression. In panel A, clones of cells were generated (genotype: *hs-FLP; FRT 82 Ubi-GFP M(3)95A2/FRT82 dcr-1^{Q1147X}*) and marked by absence of GFP (green). In panels B–D, *dcr-1^{RNAi}* was expressed in the *patched* (*ptc*, red arrowheads in panels B and C) or *engrailed* (*en*, red brackets in panel D) domains. The *en* domain was also labelled in panel D by expression of GFP. (G–I) Wing discs overexpressing *mei-P26* (G, G', H), dMyc and *mei-P26* (I, J) or dMyc and GFP (K, L) in the *engrailed* (*en*; brackets in panels G, G', H, I) domain and labelled to visualize dMyc protein (red or white; G, G', I, K), Mei-P26 protein (green; I), GFP protein (green; K) or dMyc mRNA (purple; H, J, L). In panel G', wing discs were cultured in the presence of MG132, a proteasome inhibitor, for 3 h. (M–O) Wing discs with reduced *dcr-1* activity labelled to visualize Mei-P26 protein (red or white) or *mei-P26* mRNA (purple) expression. In panels M and M', clones of *dcr-1* mutant cells were generated (genotype: (M) *hs-FLP; FRT 82 lacZ M(3)95A2/FRT82 dcr-1^{Q1147X}* and (M') *hs-FLP; FRT 82 lacZ/FRT82 dcr-1^{Q1147X}*) and marked by absence of β -gal (green). In panels N and O, *dcr-1^{RNAi}* was expressed in the *patched* (*ptc*; red arrowheads) domains. In panel O, sense and antisense RNA probes were used. (P, Q) Wing discs expressing *dcr-1^{RNAi}* and *mei-P26^{RNAi}* (ID number: 7553) in the *patched* (*ptc*, red arrowheads) domain and labelled to visualize dMyc (green or white) and Mei-P26 (red or white) protein expression in panel P, and dMyc mRNA (purple) expression in panel Q. (R, S) Wing discs with clones of *AGO1* mutant cells marked by absence of GFP and labelled to visualize Mei-P26 (R) and dMyc (S) protein expression (red or white). Genotype: *hs-FLP; FRT G13 Ubi-GFP/FRT13 AGO1¹⁴* (R) and *hs-FLP; FRT G13 Ubi-GFP M(2)12/FRTG13 AGO1¹⁴* (S).

Cell-cycle regulators like Dap, Rbf or CycE have been reported to not exert a direct effect on tissue growth in mature wing discs and adult wings (Neufeld *et al*, 1998; see also Supplementary Table S1). However, their expression is altered under conditions where reduced miRNA pathway activity affects tissue growth. In this context we asked whether manipulating CycE, Dap or Rbf levels would influence the growth-reducing effects of miRNA depletion. Increased CycE or reduced Dap or Rbf protein levels were able to overcome the effects of *dcr-1* depletion. Under these circumstances, cell size was rescued (Figure 3M) and reduction in the size of the wing territory was compensated (Figure 3K and L). These results imply that the influence of cell-cycle regulators on tissue growth is not normally limiting, but that it can be shown under conditions where requirement for their activity is sensitized. Consistent with this view, increased CycE was shown to overcome the defects on tissue growth caused by depletion of Notch signalling in early wing and eye primordia (Kenyon *et al*, 2003; Rafel and Milan, 2008).

Regulation of dMyc levels by the activity of Dcr-1

The proto-oncogene *dMyc* regulates cell growth, tissue growth and G₁–S transition, and differences in dMyc levels induce cell competition in *Drosophila* tissues (Johnston *et al*, 1999; de la Cova *et al*, 2004; Moreno and Basler, 2004). Reduced dMyc expression leads to cellular and clonal pheno-

types resembling some aspects of reduced Dcr-1 activity. Intriguingly, we found that dMyc protein and mRNA were expressed at lower than normal levels in cells depleted of *dcr-1* (Figure 4A–F). We also monitored the effects of Dcr-1 depletion on the activity of the PI3K and *hippo* pathways, two pathways involved in growth control in *Drosophila* tissues (Neufeld, 2003; Pan, 2007), but found no changes (Supplementary Figure S3). These results suggest a specific role of the miRNA pathway in regulating dMyc protein levels.

We next determined the mechanism used by the miRNA machinery to regulate dMyc protein levels. The murine TRIM-NHL protein TRIM32 has been reported to bind to, ubiquitinate and degrade c-Myc (Schwamborn *et al*, 2009). We tested whether the *Drosophila* TRIM32 orthologue Mei-P26, known to regulate cell growth and proliferation in stem cells (Neumuller *et al*, 2008), had a similar role in wing disc cells. Overexpression of Mei-P26 induced a strong reduction in dMyc protein levels and a milder reduction in dMyc mRNA levels (Figure 4G and H). To investigate whether regulation of dMyc protein levels by Mei-P26 is a consequence of reduced dMyc mRNA levels, we analysed the capacity of Mei-P26 to downregulate exogenously expressed dMyc. For this purpose, we compared dMyc protein and mRNA levels in wing discs overexpressing dMyc and GFP (in *en-gal4; UAS-GFP, UAS-dMyc* larvae; Figure 4K and L) or dMyc and Mei-P26 (in *en-gal4; UAS-Mei-P26, UAS-dMyc* larvae; Figure 4I and J). Mei-P26 reduced

exogenously induced dMyc protein with no obvious effect on dMyc mRNA levels.

To determine whether dMyc protein might be degraded by Mei-P26 in a proteasome-dependent manner, we incubated wing discs overexpressing Mei-P26 with the proteasome inhibitor MG-132. In drug-treated wing discs, dMyc protein degradation was prevented (Figure 4G'). In control solvent-treated discs, overexpression of Mei-P26 induced a strong reduction in dMyc protein levels (data not shown). In *wild-type* wing discs, expression of dMyc is reduced along the dorsal-ventral (DV) compartment boundary by the activity of Notch (Herranz *et al*, 2008). In drug-treated wing discs overexpressing Mei-P26, we noticed that cells located at the DV boundary and expressing Mei-P26 expressed high levels of dMyc, suggesting that high levels of Mei-P26 override the effect of Notch on dMyc levels. Consistent with this view, the Notch-regulated gene *wingless* was also repressed upon Mei-P26 overexpression (Supplementary Figure S4).

We next analysed dMyc protein levels in a situation of reduced Mei-P26 activity. As the available *mei-P26* alleles did not lead to a visible reduction in Mei-P26 protein levels in the clones of cells in the wing disc (data not shown), we used the Gal4/UAS technique to express three independent RNAi transgenes that target *mei-P26* (*mei-P26^{RNAi-7553}*, *mei-P26^{RNAi-106754}* and *mei-P26^{RNAi-11429}*). All three were able to visibly reduce Mei-P26 protein levels when expressed in specific territories in the wing disc (Figure 4P; Supplementary Figure S4, and data not shown). Expression of these constructs did not induce a significant change in dMyc protein levels in the developing wing (Figure 4P and Supplementary Figure S4). These data suggest that 'normal' levels of Mei-P26 activity are not limiting for dMyc protein levels. However as shown above, elevated Mei-P26 levels can lead to reduction of dMyc. This raises the possibility that regulation of Mei-P26 activity could provide a means to control dMyc levels.

Consistent with this view, *mei-P26* is predicted to be a target for regulation by miRNAs (Stark *et al*, 2005) and cells mutant for *dcr-1* or with reduced *dcr-1* activity showed increased levels of Mei-P26 protein but not *mei-P26* mRNA (Figure 4M–O). Cells depleted of Argonaute-1, a component of the miRISC complex, also expressed higher levels of Mei-P26 and showed reduced levels of dMyc (Figure 4R and S). Archipelago, the F-box component of an SCF-ubiquitin ligase, is known to negatively regulate the levels and activity of dMyc protein *in vivo* (Moberg *et al*, 2004). Archipelago protein levels were not affected in cells mutant for *dcr-1* (Supplementary Figure S3). To test whether the increase in Mei-P26 protein levels was responsible for the reduction in dMyc expression in *dcr-1* mutant cells, we analysed dMyc protein and mRNA levels when both Dcr-1 and Mei-P26 were depleted. Expression of *mei-P26^{RNAi}* together with *dcr-1^{RNAi}* was able to prevent the increase of Mei-P26 and thereby prevent the reduction of dMyc protein and mRNA levels that was seen when Dcr-1 was depleted alone (Figure 4P and Q, and Supplementary Figure S4). These results indicate that the miRNA machinery regulates dMyc by repressing Mei-P26 protein production.

Contribution of dMyc to the growth and E2F activity defects caused by Dcr-1 depletion

The above results indicate that the miRNA machinery regulates dMyc levels through the activity of Mei-P26 (Figure 4).

As reduced Dcr-1 activity led to cellular and clonal phenotypes resembling the consequences of reduced dMyc activity (Johnston *et al*, 1999; de la Cova *et al*, 2004; Moreno and Basler, 2004), we examined the contribution of dMyc to the defects caused by depletion of Dcr-1. For this purpose, we restored dMyc expression and analysed tissue and cell size in *dcr-1^{RNAi}*-expressing adult wings. The Gal4/UAS technique was used to express high levels of dMyc in the same domain as *dcr-1^{RNAi}*. As well, two different heterologous promoters (*heat-shock*, *hs*, and *tubulin*, *tub*) were used to drive dMyc expression throughout the wing disc (*hs-dMyc* (Johnston *et al*, 1999) and *tub-dMyc* (Moreno and Basler, 2004)). The tissue and cell size defects caused by *dcr-1^{RNAi}* in adult wings were largely rescued in all three combinations (Figure 5A). Notably, *hs-dMyc* does not express dMyc at levels that cause significant tissue overgrowth on its own under the conditions used (Supplementary Table S1). Therefore it is unlikely that suppression of the Dcr-1 depletion phenotypes by *hs-dMyc* reflects the independent action of Dcr-1 depletion and dMyc overexpression. This possibility cannot be excluded for the *tub-dMyc* and *UAS-dMyc* combinations, which express higher levels of dMyc. To further investigate this issue, we made use of a different means to restore dMyc activity that does not involve dMyc overexpression. Expression of two independent *mei-P26^{RNAi}* transgenes to deplete Mei-P26 together with *dcr-1^{RNAi}* restores dMyc protein to near-endogenous levels (Figure 4P and Supplementary Figure S4). This combination effectively suppressed the Dcr-1 depletion phenotypes without dMyc overexpression (Figure 5A). Taken together these observations are consistent with the hypothesis that the low level of dMyc is a principal contributor to the cell and tissue growth phenotypes caused by Dcr-1 depletion.

As shown above, *dcr-1* mutant clones contained fewer cells than *wild-type* control clones and were eliminated from the wing disc by 96 h after induction (see also Figure 5B, C and E). Expression of dMyc in these clones completely suppressed their loss (Figure 5D and E), but clone size was not completely recovered (Figure 5E). Thus, the reduced level of dMyc explains many, but not all, of the effects of Dcr-1 on cell-cycle regulators. To explore the effects of restoring dMyc expression in more depth, we examined the expression of cell-cycle regulators in dMyc-rescued *dcr-1* mutant or *dcr-1^{RNAi}*-expressing cells. Again, the three different experimental settings described above were used to restore dMyc expression. CycB and CycE proteins were restored to *wild-type* levels (compare Figure 5F, H, K, M, O and Q with Figure 3A–D). E2F activity was completely rescued (Figure 5G, L and P). However, Dap protein levels remained high in dMyc-rescued *dcr-1* mutant or *dcr-1^{RNAi}*-expressing cells (Figure 5I, J, N and R). The fact that these cells showed increased Dap but normal CycE protein levels suggests that regulation of CycE levels can be uncoupled from Dap in this situation.

A role of the bantam miRNA in reducing Mei-P26 protein levels

bantam was identified as a growth-promoting miRNA and as an inhibitor of apoptosis in *Drosophila* tissues (Brennecke *et al*, 2003). *bantam* inhibits apoptosis by targeting the proapoptotic gene *hid* (Brennecke *et al*, 2003), but the target genes that mediate the role of *bantam* in promoting tissue growth have not been identified so far. Interestingly, *mei-P26* is predicted to be a target for regulation by *bantam* (Stark

Figure 5 *dMyc* contributes to the activity of Dcr-1 in regulating cell and tissue growth and E2F activity. (A) Histograms plotting the size and cell density values of the *en* domains expressing different transgenes. Tissue size and density values were normalized as a percent of the values of control GFP-expressing wings. The error bars indicate the standard deviation. Only adult wing males were analysed. In *en>dcr-1^{RNAi}*, a significant decrease in the size of the *en* domain was observed when compared with *en>GFP* wings ($P < 10^{-7}$). Coexpression of *dMyc* or *mei-P26^{RNAi}* significantly rescued this phenotype ($P(\text{dMyc}) < 10^{-9}$ and $P(\text{mei-P26^{RNAi}}) < 10^{-4}$). Mild ubiquitous expression of *dMyc* was also able to rescue this phenotype ($P(\text{hs-dMyc}) < 10^{-5}$ and $P(\text{tub-dMyc}) < 10^{-9}$). In *en>dcr-1^{RNAi}*, a significant increase in the cell density of the *en* domain was observed when compared with *en>GFP* wings ($P < 10^{-3}$). Coexpression of *dMyc* or *mei-P26^{RNAi}* significantly rescued the cell size defects ($P(\text{dMyc}) < 10^{-5}$ and $P(\text{mei-P26^{RNAi}}) < 10^{-4}$). Mild ubiquitous expression of *dMyc* was also able to rescue this phenotype ($P(\text{hs-dMyc}) < 10^{-8}$ and $P(\text{tub-dMyc}) < 0.08$). Tissue size values: *en>GFP* = 100 ± 5.7 ($n = 12$); *en>dcr-1^{RNAi}* = 75 ± 5.1 ($n = 10$); *en>dcr-1^{RNAi}>dMyc* = 118 ± 9.5 ($n = 12$); *en>dcr-1^{RNAi}; hs-dMyc* = 99 ± 13.3 ($n = 10$); *en>dcr-1^{RNAi}; tub-dMyc* = 108 ± 5.8 ($n = 10$); *en>dcr-1^{RNAi}; mei-P26^{RNAi} 106754* = 86 ± 11.2 ($n = 10$). Cell density values: *en>GFP* = 100 ± 7.6 ($n = 10$); *en>dcr-1^{RNAi}* = 123 ± 9.4 ($n = 10$); *en>dcr-1^{RNAi}>dMyc* = 105 ± 2.98 ($n = 10$); *en>dcr-1^{RNAi}; hs-dMyc* = 89 ± 3.5 ($n = 10$); *en>dcr-1^{RNAi}; tub-dMyc* = 111 ± 17 ($n = 10$); *en>dcr-1^{RNAi}; mei-P26^{RNAi} 106754* = 105 ± 3 ($n = 10$). (B–D) Wing discs with clones of wild-type cells (B), mutant cells for *dcr-1* (C) and mutant cells for *dcr-1* and expressing *dMyc* (D) (genotypes: A: *hs-FLP tub-Gal4 UAS-GFP; FRT82 arm-lacZ/FRT82 Gal80*; B: *hs-FLP tub-Gal4 UAS-GFP; FRT82 dcr^{Q1147X}/FRT82 Gal80*; C: *hs-FLP tub-Gal4 UAS-GFP; UAS-dMyc/+; FRT82 dcr^{Q1147X}/FRT82 Gal80*). Clones were labelled by presence of GFP and induced 96 h before dissection. In panel B, twin clones were labelled by absence of β -gal expression (in red). Wing discs in panels C and D were labelled to visualize *dMyc* protein expression in red. (E) Frequency and size (in number of cells) of clones shown in panels B–D. To minimize the effects of fusion of clones, only those wild-type clones with one twin were quantified and wing discs with obvious fusions were discarded. (F–R) Wing discs labelled to visualize in red or white cyclin-B (CycB; F, K, O), PCNA (G, L, P), cyclin-E (CycE; H, M, Q) and Dap (Dap; I, J, N, R) protein expression, and in green GFP protein expression. In panels F–I, clones of cells mutant for *dcr-1* and expressing GFP and *dMyc* were generated (genotype: *hs-FLP tub-Gal4 UAS-GFP; UAS-dMyc/+; FRT82 dcr^{Q1147X}/FRT82 Gal80*). Clones were induced 72 h before dissection. In panels J–R, the following transgenes were expressed in the *patched* (*ptc*) domain: *dcr-1^{RNAi}*, *dMyc* and GFP (J), *dcr-1^{RNAi}* and GFP (K–N) and *dcr-1^{RNAi}* and *mei-P26^{RNAi}* (ID number: 106754, O–R). In panels K–N, *dMyc* was expressed at lower levels using the *tub-dMyc* construct.

et al, 2005 and Supplementary Figure S5). We then tested the capacity of *bantam* to target MeiP-26 in wing disc cells and to restore the growth defects caused by depletion of Dcr-1. Restoring *bantam* expression was able to rescue the tissue and cell size defects caused by *dcr-1^{RNAi}* in adult wings (Figure 6A) and restored Mei-P26 to normal levels (Figure 6B) in wing disc cells expressing *dcr-1^{RNAi}* (compare with Figure 4N). *dMyc* protein levels were also rescued (Figure 6C), most likely as a consequence of normalization of Mei-P26 levels. *bantam* overexpression on its own was found to reduce endogenous Mei-P26 to lower than normal levels *in vivo* (Figure 6D).

To assess the function of the predicted *bantam* target sites in the 3'UTR of *mei-P26*, we expressed an *mei-P26* 3'UTR luciferase reporter in S2 cells and assessed the capacity of *bantam* to reduce its activity. *bantam* reduced luciferase

activity in a manner that depended on the presence of the predicted *bantam* target site (Figure 6F and G). These data indicate that Mei-P26 is a direct target of the *bantam* miRNA. We observed, however, that clones of *bantam* mutant cells did not show increased Mei-P26 protein levels in the wing disc (Figure 6E). To test the possibility that other miRNAs with sites in the *mei-P26* 3'UTR (Supplementary Figure S5) might also contribute to Mei-P26 regulation, we expressed candidate miRNAs in S2 cells and asked whether they could regulate the *mei-P26* reporter (Supplementary Figure S5). *miR-137* was found to reduce the expression of an *mei-P26* 3'UTR luciferase reporter in S2 cells in a manner that depended on the presence of the predicted miRNA target sites (Figure 6H and I). These results suggest that multiple miRNAs might contribute to regulation of Mei-P26 *in vivo*.

Figure 6 The role of *bantam* in mediating the activity of Dcr-1 in regulating Dap and Mei-P26 protein levels. **(A)** Histograms plotting the size and cell density values of the *en* domains expressing GFP or *dcrl-1^{RNAi}*. Tissue size and density values were normalized as a percent of the values of control GFP-expressing wings. The error bars indicate the standard deviation. Only adult wing males were analysed. In *en>dcrl-1^{RNAi}*, a significant decrease in the size of the *en* domain and a significant increase in the cell density was observed when compared with *en>GFP* wings ($P < 10^{-7}$ and $P < 10^{-3}$, respectively). Mild ubiquitous expression of *bantam* (with the *hs-bantam* transgene) was able to rescue tissue size ($P < 10^{-9}$) and cell density ($P < 10^{-9}$). Tissue size values: *en>GFP* = 100 ± 5.7 ($n = 12$); *en>dcrl-1^{RNAi}* = 75 ± 5.1 ($n = 10$); *en>dcrl-1^{RNAi}; hs-bantam* = 119 ± 6.3 ($n = 12$). Cell density values: *en>GFP* = 100 ± 7.6 ($n = 10$); *en>dcrl-1^{RNAi}* = 123 ± 9.4 ($n = 10$); *en>dcrl-1^{RNAi}; hs-bantam* = 94 ± 4.2 ($n = 10$). **(B–E)** Wing discs labelled to visualize Mei-P26 (red or white; **B**, **D**, **E**) or dMyc (red or white; **C**), and GFP protein expression in *ptc-Gal4; UAS-dcrl-1^{RNAi}*, *UAS-bantam-GFP* (**B**, **C**) and *ptc-Gal4; UAS-bantam-GFP* (**D**) larvae, and in clones of cells mutant for *bantam* (genotype: *hs-FLP; M(3L) ubi-GFP FRT80B/ban^{Δ1}FRT80B*) and marked by the absence of GFP (green) (**E**). The *patched* (*ptc*) domain is indicated by a red arrowhead. **(F)** Gal4-directed expression of *UAS-bantam* inhibited luciferase activity from a reporter containing the *mei-P26* 3'UTR (see Materials and methods), and in a much weaker manner the mutant 3'UTR in which the *bantam* 5' seed region was deleted. The error bars indicate the standard deviation. The difference between intact and mutated UTR reporters was statistically significant ($P < 0.05$). **(G)** Cartoon depicting the different Firefly-luciferase constructs containing wild-type or mutated *mei-P26* 3'UTR used in panel **F**. **(H)** Expression of mir-137 genomic fragments inhibited luciferase activity from a reporter containing the *mei-P26* 3'UTR (see Materials and methods), and in a much weaker manner the mutant 3'UTRs (mut1 and mut2) in which the mir-137 target sites were mutated. The error bars indicate the standard deviation. The difference between intact and mutated UTR reporters was statistically significant ($P < 0.05$). **(I)** Cartoon depicting the different Firefly-luciferase constructs containing wild-type or mutated forms of the *mei-P26* 3'UTR used in panel **H**. The seed region is marked in red and the mutation are marked in blue and underlined.

Concluding remarks

Here we have analysed the role of the miRNA pathway in the *Drosophila* wing, a highly proliferative epithelium. In the absence of Dcr-1, cell and tissue size was reduced, and G₁–S transition was compromised. When confronted with wild-type cells, *dcrl-1* mutant cells were eliminated by cell

competition. We have provided evidence that regulation of dMyc contributes to the roles of Dcr-1 in these processes. dMyc levels were reduced in Dcr-1-depleted cells. Restoring dMyc expression rescued the defects in cell and tissue size, E2F activity and cell competition caused by absence of Dcr-1.

Figure 7 The miRNA machinery regulates dMyc protein levels by targeting Mei-P26. An illustration describing the regulatory network described in this work by which the miRNA machinery maintains dMyc levels by targeting Mei-P26.

The murine Mei-P26 homologue TRIM32 has been reported to bind to, ubiquitinate and degrade cMyc (Schwamborn *et al*, 2009). We present evidence that Mei-P26 overexpression reduces dMyc protein levels in a proteasome-dependent manner and also causes a reduction in *dMyc* mRNA levels. Whether Mei-P26 has a direct and independent role in modulating *dMyc* mRNA levels or this is rather an indirect consequence caused by reduction in dMyc activity is unclear. Finally, our data indicate that the miRNA pathway contributes to regulating dMyc levels by reducing the levels of Mei-P26 *in vivo*. We have identified *bantam* and *mir-137* as two miRNAs that target the *mei-P26*-3'UTR. These results define a frame by which the miRNA pathway is required in a growing epithelium to maintain sufficient levels of dMyc expression for tissue growth (illustrated in Figure 7).

In cells mutant for *dcr-1* we did not detect any major change in the activity of the growth-promoting *PI3K* and *hippo* pathways nor in the activity of the main signalling pathways involved in organizing growth and patterning of the developing wing (eg Wingless, Notch, Dpp and Hedgehog; Supplementary Figure S3 and S6). These observations reinforce the principal contribution of reduced dMyc to the growth defects observed in Dcr-1-depleted cells. Although the failure to observe an effect upon knockout of an miRNA-processing factor does not indicate the lack of crucial roles of single miRNAs in these pathways, our results suggest that the general effect of the miRNA machinery in these pathways is most probably neutralized by the opposite effects of single miRNAs targeting positive and negative modulators.

Deregulated expression of the proto-oncogene *Myc* occurs frequently in human malignancies including cancer (Meyer and Penn, 2008). Similarly, alterations in the expression of *miRNA* genes contribute to the pathogenesis of many human diseases and several miRNAs function as oncogenes when overexpressed (Croce, 2009). The gene-regulatory relationship described in this work and mediated by the activity of certain miRNAs targeting Mei-P26 and regulating dMyc protein levels opens the possibility of a tight causal relationship between deregulated miRNAs and increased Myc expression in certain types of cancer.

Finally, it is interesting to note that *Drosophila* Mei-P26 and its murine and *Caenorhabditis elegans* homologues TRIM32 and *nhl-2* have been shown to bind to components of the miRISC complex and regulate the activity of the miRNA machinery (Neumuller *et al*, 2008; Hammell *et al*, 2009; Schwamborn *et al*, 2009). These results together with our observations on the negative regulation of *mei-P26* by miRNAs raise the possibility that a feedback loop might be used by the miRNA machinery to regulate its own activity levels.

Materials and methods

Drosophila strains

dcr-1^{Q1147X} and *dcr-1^{d102}* (Hatfield *et al*, 2005); E2F1-responsive reporter (Asano and Wharton, 1999); *hs-dMyc* and *UAS-dMyc* (Johnston *et al*, 1999); *tub-dMyc* (Moreno and Basler, 2004); *bantam^{Δ1}*, *UAS-bantam-GFP* (Brennecke *et al*, 2003); *UAS-CycE* (Knoblich *et al*, 1994); *UAS-me-P26^{RNAi}* (ID numbers: 7553 and 106754, Vienna Drosophila RNAi Center, Austria; ID number: 11429, National Institute of Genetics, Japan); *mei-P26^{mfsl}*, *mei-P26^{fs2}* (Page *et al*, 2000); *hs-bantam* (Neumuller *et al*, 2008); *AGO1¹⁴* (Yang *et al*, 2007). Other stocks are described in Flybase.

Antibodies

Guinea pig anti-dMyc (Herranz *et al*, 2008); rabbit anti-Mei-P26 (Neumuller *et al*, 2008); mouse anti-Dap (de Nooij *et al*, 1996); rabbit anti-CycE (see Supplementary Figure S2 for control stainings), rabbit anti-CycB and mouse anti-PCNA (Santa Cruz Biotechnology); mouse anti-CycB (Developmental Studies Hybridoma Bank); rabbit anti-β-gal (Cappel); sense and antisense digoxigenin-labelled RNA probes of *dMyc* and *mei-P26*, *in situ* hybridization and TUNEL staining were performed as described by Herranz *et al* (2008).

Mosaic analysis

Minute clones. The FLP/FRT system was combined with the Minute technique (Minute mutations are defective in ribosomal proteins, and in heterozygous conditions induce a delay in growth rates; Morata and Ripoll, 1975) to induce clones covering large areas of the wing. These clones were *wild type* for the Minute mutation and had a growth advantage over the neighbouring Minute/+ heterozygous wing cells.

Clones in the adult wing were generated in the following genotype:

forked^{36Δ}hs-FLP; FRT 82 P(forked+) M(3)95A2/FRT82 dcr-1^{Q1147X}.

Mutant cells were marked by absence of the *P(forked+)* rescue construct.

Clones in the wing discs were generated in the following genotypes:

hs-FLP/+; FRT82 dcr-1^{Q1147X}/FRT82 M(3)95A2 Ubi-GFP
hs-FLP/+; FRT82 dcr-1^{Q1147X}/FRT82 M(3)95A2 arm-lacZ
hs-FLP/+; FRT82 dcr-1^{d102}/FRT82 M(3)95A2 Ubi-GFP
hs-FLP/+; FRT82/FRT82 M(3)95A2 Ubi-GFP
hs-FLP/+; FRT82/FRT82 M(3)95A2 arm-lacZ
hs-FLP; M(3L) ubi-GFP FRT80B/ban^{Δ1}FRT80B
mei-P26^{mfsl}FRT18/ubi-GFP FRT18; hs-FLP/+
mei-P26^{fs2}FRT18/ubi-GFP FRT18; hs-FLP/+
hs-FLP; FRT G13 Ubi-GFP /FRT13 AGO1¹⁴
hs-FLP; FRT G13 Ubi-GFP M(2)J2/FRTG13 AGO1¹⁴
 Mutant cells were marked by absence of GFP or β-gal.

Twin/clone analysis. The following genotypes were used to generate loss-of-function clones by the classic FLP/FRT system:

hs-FLP/+; FRT82 dcr-1^{Q1147X}/FRT82 Ubi-GFP
hs-FLP/+; FRT82 dcr-1^{d102}/FRT82 Ubi-GFP

Mutant cells were marked by the absence of GFP expression and their twins by the expression of two copies of GFP.

MARCM clones. The following genotypes were used to generate loss-of-function clones by the MARCM (mosaic analysis with a repressible cell marker) technique to simultaneously express diverse transgenes in the clones (Lee and Luo, 2001):

hs-FLP tub-Gal4 UAS-GFP; UAS-dMyc/+; FRT82 dcr-1^{Q1147X}/FRT82 tub-Gal80;
hs-FLP tub-Gal4 UAS-GFP;; FRT82 dcr-1^{Q1147X}/FRT82 tub-Gal80;
 Mutant cells were marked by the presence of GFP
hs-FLP tub-Gal4 UAS-GFP/+;; FRT82 arm-LacZ/FRT82 tub-Gal80.
 Clones were marked by the presence of GFP and their twins by the absence of β -gal.

Quantification of miRNA expression levels

Fifty wing discs from *en-gal4; UAS-GFP; UAS-dcr-1^{RNAi}* larvae were dissected and the GFP-expressing and non-expressing domains isolated using a GFP Leica dissecting-scope. Total RNA from the GFP-expressing and non-expressing cells was extracted using the TRIzol Reagent (Invitrogen). The same procedure was repeated twice. Primer sets designed to amplify mature microRNAs (and U27 as a reference gene for data normalization of RNAs from different compartments) were obtained from Applied Biosystems. Products were amplified from 20 ng total RNA samples from the respective genotypes by TaqMan microRNA assay using a quantitative PCR machine (Applied Biosystems).

Luciferase-reporter assays

S2 cells were co-transfected with a plasmid that expresses the yeast transcription factor Gal4 under the control of the *Drosophila* actin promoter (actin-Gal4), a control construct expressing *Renilla* luciferase and a luciferase reporter. Dual luciferase assays were performed 72 h after transfection following the manufacturer's instructions (Promega). The luciferase reporters contained the following: the mei-P26 3'UTR, a mutated version of the mei-P26 3'UTR lacking the predicted bantam target site, or two mutated versions of the mei-P26 3'UTR with one nucleotide mutated in the predicted mir-137 target sites. To investigate the role of bantam in targeting the mei-P26-3'UTR, the transfection mix included a Gal4-regulated construct expressing a genomic fragment with or without the bantam hairpin (genomic rescue constructs described previously; Brennecke et al, 2003), referred as UAS-bantam and UAS-bantam- Δ hairpin, respectively. Normalized luciferase activity of cells transfected with UAS-bantam was compared with transfections with UAS-bantam- Δ hairpin. To investigate the role of other miRNAs in targeting the mei-P26-3'UTR, genomic fragments containing miRNA hairpins were cloned under the tubulin promoter. Luciferase activity of each UTR construct was measured and normalized to *Renilla* control and compared with the normalized luciferase activity of an empty vector. All transfections were performed in triplicate each time and data were obtained from at least three independent experiments.

Imaginal disc culture

en-gal4; UAS-me-P26 third instar wing discs were incubated in cl-8 cell medium for 3 h before fixation and staining. For treatment with MG-132, discs were cultured in medium containing 40 μ M MG132 (experimental discs) or an equal volume of DMSO (control discs).

Quantification of cell and tissue growth parameters in adult wings and wing discs

dcr-1^{RNAi} expression. *en-gal4* or *ptc-gal4* virgins were crossed with males carrying different UAS or heat-shock transgenes in different genetic backgrounds and allowed to lay eggs for 24 h at 25°C. The resulting larvae developed at 29°C until adulthood. At least 10 adult flies per genotype were mounted in Faure medium. Size of the *engrailed* and *patched* domains was measured using the Image J Software (NIH, USA). Cell density in these domains and in neighbouring ones were measured as the number of hairs (each wing cell differentiates a hair) per defined area. The following areas were used to measure cell densities: (1) two conserved regions

between veins L4 and L5 (*engrailed* domain) and veins L3 and L4 (neighbouring tissue) in the *engrailed-gal4* experiments and (2) two conserved regions between veins L3 and L4 (*patched* domain) and veins L4 and L5 (neighbouring tissue) in the *ptc-gal4* experiments. Final area and cell density values were normalized as a percent of the control *en-gal4, UAS-GFP* or *ptc-gal4, UAS-GFP* values (Supplementary Table S1).

dcr-1 twin/clone size analysis. *hs-FLP/+; FRT82 dcr-1^{Q1147X}/FR82 Ubi-GFP* and *hs-FLP/+; FRT82 dcr-1^{d102}/FR82 Ubi-GFP* larvae were grown at 25°C, treated for 45 min at 37°C during early second instar and dissected 72 and 96 h later. Clone and twin size was measured using the Image J Software (NIH, USA) and the values were presented in arbitrary units (Figure 2C).

dcr-1M(+) clone size analysis. *hs-FLP/+; FRT82 dcr-1^{Q1147X}/FR82M(3R)^{w124}Ubi-GFP* and *hs-FLP/+; FRT82/FR82M(3R)^{w124}Ubi-GFP* larvae were grown at 25°C, treated for 3 min at 37°C during first instar and dissected 96 h later. Clone size was measured using the Image J Software (NIH, USA) and the values were normalized as a percent of the size of the *wild-type Minute(+)* clones (Figure 2H). The heat-shock treatment was short to reduce the frequency of clones per wing and avoid fusion of clones.

dcr-1M(+) clone frequency analysis. *hs-FLP/+; FRT82 dcr-1^{Q1147X}/FR82M(3R)^{w124}Ubi-GFP* and *hs-FLP/+; FRT82/FR82M(3R)^{w124}Ubi-GFP* larvae were grown at 25°C, treated for 3 min at 37°C during first instar and dissected 96 h later. Clone frequency was measured. The heat-shock treatment was short to reduce the frequency of clones per wing and avoid fusion of clones.

dcr-1 clones expressing dMyc. Larvae of the following genotypes were grown at 25°C, treated for 45 min at 37°C during first instar and dissected 96 h later: (1) *hs-FLP tub-Gal4 UAS-GFP;; FRT82 arm-LacZ/FRT82 tub-Gal80*; (2) *hs-FLP tub-Gal4 UAS-GFP; UAS-dMyc/+; FRT82 dcr-1^{Q1147X}/FRT82 tub-Gal80*; and (3) *hs-FLP tub-Gal4 UAS-GFP;; FRT82 dcr-1^{Q1147X}/FRT82 tub-Gal80*. The frequency of clones and size of the clones (in number of cells) in the wing pouch region were measured (Figure 5E).

Using Microsoft Excel, average values and the corresponding standard deviations were calculated and *t*-test analysis was performed.

Supplementary data

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

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Conflict of interest

The authors declare that they have no conflict of interest.

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