A Wingless and Notch double-repression mechanism regulates G1–S transition in the *Drosophila* wing

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The control of tissue growth and patterning is orchestrated in various multicellular tissues by the coordinated activity of the signalling molecules Wnt/Wingless (Wg) and Notch, and mutations in these pathways can cause cancer. The role of these molecules in the control of cell proliferation and the crosstalk between their corresponding pathways remain poorly understood. Crosstalk between Notch and Wg has been proposed to organize pattern and growth in the *Drosophila* wing primordium. Here we report that Wg and Notch act in a surprisingly linear pathway to control G1–S progression. We present evidence that these molecules exert their function by regulating the expression of the *dmec* proto-oncogene and the *bantam* micro-RNA, which positively modulated the activity of the E2F transcription factor. Our results demonstrate that Notch acts in this cellular context as a repressor of cell-cycle progression and Wg has a permissive role in alleviating Notch-mediated repression of G1–S progression in wing cells.

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Introduction

During development of multicellular organisms, Wnt/Wingless (Wg)- and Notch-signalling pathways are involved in the determination of a variety of cell fates and in the control of tissue growth (Bray, 2006; Clevers, 2006). In many cellular contexts, genetic manipulations that change the activities of these two proteins result in cancer (Bienz and Clevers, 2000; Radtke and Raj, 2003). Thus, tight regulation of Notch and Wnt/Wg is crucial for proper development and survival of multicellular organisms.

The wing primordium of *Drosophila* is a very suitable model system to define, at a genetic and cellular level, the role of these pathways in the development of highly proliferative tissues. Cell-fate specification by Notch and Wg is a

well-known and defined process; by contrast, little is known about their role in the control of cell proliferation. The wing primordium arises as a group of 30–40 cells in the embryonic ectoderm that proliferates during the three larval stages to reach a final size of around 50 000 cells (García-Bellido and Merriam, 1971; Madhavan and Schneiderman, 1977). Early in development, the wing becomes subdivided into a dorsal (D) and a ventral (V) cell population, or compartment, by the activity of the LIM-Homedomain transcription factor Apterous in D cells (Diaz-Benjumea and Cohen, 1993; Blair *et al.*, 1994), and cell interactions between D and V cells lead to activation of Notch at the compartment boundary (Diaz-Benjumea and Cohen, 1995; de Celis *et al.*, 1996). During the third instar larval stage, Notch activity induces Wg expression at the dorsal–ventral (DV) boundary (Diaz-Benjumea and Cohen, 1995; de Celis *et al.*, 1996), and another set of cell interactions between boundary and nearby non-boundary cells takes place to maintain Notch activity and Wg expression at the DV boundary (Figure 1). The DV boundary has a role in organizing growth of the whole-wing primordium (Diaz-Benjumea and Cohen, 1993). Notch and Wg have been postulated as being responsible for this organizing activity, although data are controversial (Giraldez and Cohen, 2003; Johnston and Sanders, 2003). Late in development, cells at the DV boundary are characterized by cell-cycle arrest and define the so-called Zone of Non-proliferating Cells (ZNC; O'Brochta and Bryant, 1985; Phillips and Whittle, 1993). Wg has been reported to induce this cell-cycle arrest (Johnston and Edgar, 1998; Duman-Scheel *et al.*, 2004).

Here we have revised the role of Wg and Notch in the control of cell proliferation and present evidence that a Wg and Notch double-repression mechanism controls G1–S transition in the wing primordium. Our results indicate that these signalling molecules exert their function through regulation of the *bantam* micro-RNA (Brennecke *et al.*, 2003) and the proto-oncogene *dmec* (Johnston *et al.*, 1999). Our work clarifies and simplifies the role of Notch and Wg in cell-cycle control in the *Drosophila* wing and provides a very suitable model by which to analyse the function of Notch- and Wg-signalling pathways in the regulation of the cell-cycle machinery.

Results and discussion

The ZNC is defined by Notch activity

Stable activation of Notch and expression of Wg along the DV boundary relies on a positive feedback loop between boundary and non-boundary cells (Figure 1). Boundary cells activate the receptor Notch and express Wg, whereas non-boundary cells respond to Wg and signal back by expressing the Notch ligands Serrate and Delta. Boundary and non-boundary cells are characterized late in development by their cell-cycle arrest and constitute the ZNC (O'Brochta

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Figure 1 Wg- and Notch-mediated cell interactions involved in DV boundary formation. (A) Late third instar wing imaginal disc labelled to visualize Wg (green) and Senseless (red) protein expression in boundary and non-boundary cells, respectively. (B) Illustration describing the cell interactions that take place between boundary (white) and non-boundary (black) cells that lead to activation of Notch (N) and expression of Wg and Cut along the DV boundary of the *Drosophila* wing primordium. N, Notch; Dl, Delta; Ser, Serrate.

and Bryant, 1985). Anterior non-boundary cells are arrested in G2 by the activity of Wg (Johnston and Edgar, 1998; Figure 8A). Wg exerts its function through its target genes *achaete* and *scute*, which inhibit the expression of *cdc25/string*, the universal eukaryotic regulator of the G2/M transition. Posterior cells and anterior boundary cells are arrested in G1 (Johnston and Edgar, 1998). This arrest has also been postulated to be defined by the activity of Wg (Johnston and Edgar, 1998; Duman-Scheel *et al*, 2004). Two observations suggest that Notch, and not Wg, is responsible for the cell-cycle arrest in G1. First, the Wg-signalling pathway is blocked in boundary cells by activity of Notch, as recently reported (Buceta *et al*, 2007; Figure 1B). Second, some low-threshold target genes of Notch, like *crumbs*, are expressed in a broader domain that includes both boundary and non-boundary cells and corresponds to the ZNC (Figure 2A and B; Herranz *et al*, 2006). Here we have readdressed the role of Wg and Notch in this process.

The ZNC is characterized by its failure to incorporate bromodeoxyuridine (BrdU) (O'Brochta and Bryant, 1985) or by the reduced activity levels of the E2F1 transcription factor (Johnston and Edgar, 1998; Figure 2A and B). E2F proteins, such as *Drosophila* E2F1, regulate the expression of a number of genes required for S-phase, and their activity is inhibited by the Retinoblastoma (Rb) proteins like *Drosophila* Rbf (Rb-family protein, reviewed by Dyson, 1998). The Rb pathway is a key regulator of the G1-S transition and *Drosophila* Rbf is required for G1 arrest in the ZNC (Duman-Scheel *et al*, 2004). Activity of E2F1, labelled as dE2F in the Figures, can

be visualized by observing the expression of the E2F1-responsive reporter *ORC1-GFP* (Asano and Wharton, 1999). It consists of the *ORC1* promoter bearing functional E2F-binding sites driving the expression of an unstable Ftz-GFP-Myc fusion protein. As the half-life of this protein is short, this reporter monitors newly synthesized protein and permits examination of cell-cycle-regulated transcription *in vivo*.

We first analysed the role of Notch in defining the ZNC. In a temperature-sensitive *Notch* loss-of-function background (*N^{ts2}*) reared for 48 h at restrictive (29°C) temperature, cells at the ZNC incorporated BrdU and expressed *ORC1-GFP* (Figure 2C and D). Blocking Notch activation by expression of a dominant-negative form of the Notch ligand Delta or the nuclear Notch effector Mastermind (Giraldez *et al*, 2002) caused similar effects (Figure 2E and F). Clones of cells located at the ZNC and mutant for the Suppressor of Hairless (*Su(H)*) transcription factor, the nuclear mediator of Notch signalling (Bray, 2006), incorporated BrdU and expressed the E2F1-responsive reporter (Figure 2G and H; see also Supplementary Figure S1). We used a fluorescence-associated cell sorter (FACS) to collect data on the cellular DNA content of dissociated, GFP-sorted, ZNC wing disc cells, and to confirm the role of Notch in defining the G1 block. Blocking Notch signalling in these cells led to a decrease in the fraction of cells in G1 (Supplementary Figure S1). Altogether, these results imply that the activity of the Notch-signalling pathway is required for the cell-cycle arrest in G1 that takes place at the ZNC.

Wg has been reported to be required for the G1 arrest at the ZNC, as blocking Wg signalling in all wing margin cells causes cells to enter into S-phase (in *C96; UAS-TCF^{DN}* larvae; Figure 2N and O; and Johnston and Edgar, 1998; Duman-Scheel *et al*, 2004; see also Figure 2R for the pattern of expression of the *C96-Gal4* driver). We further analysed the requirement of the Wg pathway in this process. Unexpectedly, clones of cells mutant for *arrow*, the Wg coreceptor (Wehrli *et al*, 2000), and located at the ZNC, did not incorporate BrdU (Figure 2I; Supplementary Figure S1; 74 out of 84 clones located at the ZNC did not incorporate BrdU; *n* (scored discs) = 33). Similarly, expression in a subset of ZNC cells of a dominant-negative form of the LEF-1/TCF transcription factor (*TCF^{DN}*), the nuclear mediator of canonical Wg signalling (Logan and Nusse, 2004), did not increase BrdU nor E2F1 activity levels either (Figure 2J; Supplementary Figure S1). Altogether, these results indicate that Wg signalling is not required for the cell-cycle arrest in G1 that occurs at the ZNC. The effects reported on G1 progression after blocking Wg signalling in all wing-margin cells (Johnston and Edgar, 1998; Duman-Scheel *et al*, 2004) can be explained by the Notch- and Wg-dependent positive feedback loop that operates at the DV boundary (Figure 1B), which may also compromise the activity of Notch in these conditions. Indeed, expression of the Notch-regulated genes *cut* and *wg* was reduced (Figure 2K and L; and data not shown), and increasing Notch activity levels, by means of expression of an activated form of the Notch receptor (*N^{INTRA}*), counteracted the effects of *TCF^{DN}* expression and restored *cut* expression (Figure 2M), reduced E2F activity (Figure 2Q) and BrdU incorporation (Figure 2P). Blocking Wg signalling only in a subset of wing-margin cells did not compromise Notch activity, as reported by expression of *cut* and *wingless*

Figure 2 The ZNC is defined by the activity of Notch. Late third instar wing discs labelled to visualize cells in S-phase by BrdU incorporation (antibody to BrdU in red or white (**A, C, E, G, I, N, O, P**)) or expressing the E2F1-responsive reporter *ORC1-GFP* (antibody to GFP in pink or white (**B, D, F, H, J, Q**)). (A, B) Wild-type wing discs labelled to visualize *crumbs-lacZ* expression (antibody to β -gal, green). Note failure to incorporate BrdU (A) or low levels of E2F1 activity (B) in those cells expressing high levels of the Notch-regulated gene *crumbs*, which corresponds to the ZNC. The DV compartment boundary is marked in this and other panels (d, dorsal; v, ventral). (C, D) *Notch^{ts2}* wing discs reared at the restrictive temperature and labelled to visualize Wg protein expression (green). (E, F) *ptc-Gal4; UAS-Delta^{DN}* (E) and *sal-Gal4; UAS-mam^{DN}* (F) wing discs labelled to visualize Senseless (Sens, green in E) or Wg (green in F) protein expression. Sens expression was used to label the DV boundary. Red lines label the domain of *Delta^{DN}* or *mam^{DN}* expression. (G, H) Wing discs with clones of cells lacking *Su(H)* activity (*Su(H)⁸*), marked by the absence of GFP (G) or β -gal (H) in green. The ZNC is marked by horizontal lines in G. (I) Wing disc with clones of cells lacking *arrow* activity (*arrow²*) marked by the absence of GFP. The ZNC is marked by horizontal lines. (J) *dpp-Gal4 Gal80ts; UAS-TCF^{DN}* wing disc reared at 29°C during 30 h and labelled to visualize Wg (green) protein expression. The *dpp-Gal4* domain is marked by arrowheads. (K–R) Wild-type (K, N), *C96-Gal4; UAS-TCF^{DN}* (L, O), *C96-Gal4; UAS-TCF^{DN} UAS-N^{inttra}* (M, P, Q) and *C96-Gal4; UAS-GFP* (R) wing discs labelled to visualize Cut (green, K–M) or GFP (green, R) protein expression, BrdU incorporation (red, N–P) or activity of the E2F1 responsive reporter *ORC1-GFP* (antibody to GFP in pink, Q). The ZNC is marked by horizontal dashed lines.

Figure 3 Regulation of E2F activity by Notch and Wg. (A–H) *Abruptex*^{M1} (A, B), *dpp-Gal4;UAS-N^{intra}* (C, C', D, D'), *ap-Gal4 Gal80ts, UAS-GFP; UAS-TCF^{DN}* wing (E, F), *ap-Gal4 Gal80ts, UAS-GFP; UAS-Axin* (G) and *dpp-Gal4 Gal80ts; UAS-TCF^{DN}* (H) late third instar wing discs labelled to visualize cells in S-phase by BrdU incorporation (antibody to BrdU in red or white (A, C, C', E, G)) or expressing the E2F1-responsive reporter *OCT1-GFP* (antibody to GFP in pink or white (B, D, D', F, H)). *crb-lacZ* (antibody to β -Gal in green (A, B)), Wingless (Wg, green (C, C', D, D', H)) and GFP (green (E, G)) protein expression is shown. Panels C' and D' show higher magnifications of the discs in panels C and D. The anterior–posterior (ap) boundary is labelled to show the cell-autonomous block in G1 imposed by the activity of N. *N^{intra}* activity is labelled by the expression of Wg (red line) and the G1 block by reduced levels of BrdU and dE2F activity. (I, J, K) *ap-Gal4, Gal80ts, UAS-GFP; UAS-TCF^{DN}* (I), *wild-type* (J) and *ap-Gal4 Gal80ts, UAS-GFP; UAS-N^{intra}* (K) early third instar wing discs labelled to visualize BrdU incorporation (red or white) and GFP (green) expression. The DV compartment boundary is marked in all panels (d, dorsal; v, ventral). Wing discs shown in panels E–I were reared at 29°C during 24 h.

(Figures 3H, 4F and F'). This might be caused by the fact that the Notch- and Wg-dependent positive feedback loop that operates at the DV boundary is not affected in these conditions. Note also that TCF^{DN} expression in these experiments was temporally controlled by the Gal4/Gal80ts system (see Materials and methods for details) and induced during the third instar stage (30 h before dissection) to circumvent the requirement of Wg signalling in this feedback loop.

A G1–S checkpoint controlled by a Wg and Notch double-repression mechanism

Notch activity is required in ZNC cells to induce cell-cycle arrest in G1, most probably by a reduction in E2F1 activity levels (Duman-Scheel *et al*, 2004). We then examined whether ectopic activation of Notch could reduce E2F1 activity levels and cause cell-cycle arrest in the rest of the wing cells, the so-called wing-blade cells (Figure 1A). For this

Figure 4 A Wg and Notch double-repression mechanism regulates G1 progression. (A–D) *dpp-Gal4*, *UAS-GFP*; *UAS-N^{intra}**UAS-E2F* (A), *ap-Gal4*, *Gal80ts*, *UAS-GFP*; *UAS-TCF^{DN}*, *UAS-E2F* (B), *ptc-Gal4*, *Gal80ts*, *UAS-GFP*; *UAS-N^{intra}*, *UAS-CycE* (C) and *ap-Gal4*, *Gal80ts*, *UAS-GFP*; *UAS-TCF^{DN}*, *UAS-CycE* (D) late third instar wing discs labelled to visualize cells in S-phase by BrdU incorporation (antibody to BrdU in red or white) and GFP (green) protein expression. (E–G) *ap-Gal4*, *Gal80ts*, *UAS-GFP*; *UAS-TCF^{DN}* (E) and *dpp-Gal4*, *Gal80ts*, *UAS-GFP*; *UAS-TCF^{DN}* (F, F', G) late third instar wing discs labelled to visualize Wingless protein (Wg, red or white, E, G), *wg* mRNA (F), *wg-lacZ* expression (antibody to β -gal, F') and GFP (green, E) protein expression. Arrowheads in panels F, F' and G indicate the *dpp-gal4* expression stripe. (H) *ap-Gal4*, *Gal80ts*, *UAS-GFP*; *UAS-TCF^{DN}*, *UAS-mam^{DN}* late third instar wing disc labelled to visualize cells in S-phase by BrdU incorporation (antibody to BrdU in red or white) and GFP (green) protein expression. (I) Wing disc with clones of cells lacking arrow activity (arrow²), marked by the absence of GFP in green, and labelled to visualize Wg expression (red or white). Note ectopic expression of Wg in the clones (red arrows). (J) *ap-Gal4*, *Gal80ts*, *UAS-GFP*; *UAS-TCF^{DN}*, *UAS-mam^{DN}* late third instar wing discs labelled to visualize cells expressing the E2F1-responsive reporter *OCT1-GFP* (antibody to GFP in pink or white) and Wg (green) protein expression. Wing discs shown in panels B and D–J were reared at 29°C during 30 h. The DV compartment boundary is marked in all panels (d, dorsal; v, ventral).

purpose, we monitored these parameters in wing discs mutant for the *Notch* gain-of-function allele *Abruptex^{M1}* or expressing an activated form of the Notch receptor (*N^{intra}*). In both cases, the ZNC was expanded (Figure 3A–D), as revealed by the reduced activity levels of E2F1 and the failure to incorporate BrdU. Notch induced this cell-cycle arrest in all

cells located in the presumptive wing blade and at any time during the third instar larval stage (Figure 3C and K). Reduction in the activity of E2F1 mediated this cell-cycle arrest, as overexpression of E2F (known to bypass the negative effects of Rbf protein) restored cell-cycle progression, as monitored by BrdU incorporation (Figure 4A).

To confirm that these cells were arrested in G1, we coexpressed N^{INTRA} together with cyclin E, the key regulator and rate-limiting factor controlling G1–S transition in wing cells (Knoblich *et al*, 1994; Neufeld *et al*, 1998). Interestingly, these cells incorporated BrdU (Figure 4C, compare with Supplementary Figure S2). Coexpression of *cdc25/string*, the rate-limiting factor of G2–M transition, was not able to rescue the failure to incorporate BrdU (Supplementary Figure S2). Altogether, these results indicate that high levels of Notch induce a G1 block by reducing the activity of E2F1. We noted that high levels of Notch activity were able to induce an increase in BrdU incorporation and E2F activity in the proximal part of the wing (wing hinge, data not shown), and this increase might be the cause of the overgrowth phenotype observed in *Abruptex*^{M1} wing discs.

Notch activation along the DV boundary induces Wg expression. Wg protein is secreted and forms a long-range protein gradient that reaches every wing-blade cell (Zecca *et al*, 1996; Neumann and Cohen, 1997; Strigini and Cohen, 2000). We then analysed the role of Wg in G1–S progression in these cells. For this purpose, we blocked the Wg pathway by expressing the dominant-negative form of TCF (TCF^{DN}) or axin, and monitored BrdU incorporation and E2F1 activity levels. TCF^{DN} or axin expression was temporally controlled by the Gal4/Gal80ts system (see Materials and methods for details) and induced during the third instar stage (30 h before dissection) to circumvent the requirement of Wg signalling in cell survival and wing-fate specification (Giraldez and Cohen, 2003; Johnston and Sanders, 2003). We chose to use strong Gal4 drivers to completely block the Wg pathway. Interestingly, high levels of expression of TCF^{DN} or axin caused cell-cycle arrest in wing-blade cells. Those cells unable to transduce the Wg signal did not incorporate BrdU (Figure 3E and G; Supplementary Figure S1), and in these cells the expression levels of the E2F1-responsive reporter *ORC1-GFP* were reduced (Figure 3F and H). All cells located in the presumptive wing blade and at any time during the third instar larval stage required activation of the Wg pathway to pass through S-phase (Figure 3E, G and I; compare with Figures 2A and 3J). This cell-cycle arrest was due to a reduction in E2F1 activity levels, as coexpression of E2F rescued the cell proliferation defects (Figure 4B). Expression of *cyclin E*, and not *cdc25/stg*, was able to restore cell-cycle progression in the presence of TCF^{DN} (Figure 4D; Supplementary Figure S2). Taken together, these results indicate that Wg signalling is required in wing blade cells to reach the appropriate levels of E2F1 activity to trigger G1 progression.

We noticed that reduced Wg signalling was also able to compromise the mitotic activity of wing blade cells (Supplementary Figure S2). We then analysed the possible regulation of the G2–M transition in these conditions. In wing cells expressing TCF^{DN}, coexpression of *cdc25/string*, the rate-limiting factor of G2–M transition, was not able to rescue mitotic activity and BrdU incorporation (Supplementary Figure S2). In contrast, expression of *cyclin E* was able to rescue both of them (Supplementary Figure S2; Figure 4D). Thus, the cell-cycle arrest imposed by lack of Wg signalling takes place only in G1.

Notch- and Wg-signalling pathways exert opposite effects on G1–S transition. Notch imposes G1 arrest whereas Wg is required for G1–S progression. Interestingly, Notch is known

to be repressed by the activity of Wg at different levels of its pathway during wing development (Axelrod *et al*, 1996; Rulifson *et al*, 1996; Micchelli *et al*, 1997; de Celis and Bray, 1997; Neumann and Cohen, 1998). Consistent with this, blocking the Wg pathway in the dorsal compartment or in a stripe along the anterior–posterior compartment boundary induced ectopic Notch activation, as monitored by the expression of Wg protein, *wg* mRNA and *wg-lacZ* (Figure 4E–G), and clones of cells mutant for *arrow*, the Wg co-receptor, and located far away from the DV boundary express Wg (Figure 4I). These observations suggest that the opposite effects of Notch and Wg in the G1–S transition could be explained by a Wg-mediated repression of the Notch pathway and a consequent alleviation of the G1 block. To test this, we blocked the Notch pathway in cells unable to transduce the Wg signal and analysed their capacity to progress through G1. Interestingly, wing cells expressing the dominant-negative form of the nuclear Notch effector Mastermind (Mam^{DN}), together with TCF^{DN} in the dorsal compartment, incorporated BrdU and increased E2F1 activity levels (Figure 4H and J, compare with Figure 3E and F). Note that the expression pattern of Wg is restored (compare Figure 4E and J). These results imply that a Wg and Notch double-repression mechanism controls G1–S transition in wing cells.

***dMyc* and bantam micro-RNA mediate regulation of G1–S by Wg and Notch**

The proto-oncogene *dMyc* promotes G1–S progression in *Drosophila* cells (Johnston *et al*, 1999) and the cell-cycle arrest in G1 that occurs at the ZNC is defined by the absence of *dMyc* expression (Johnston *et al*, 1999; Duman-Scheel *et al*, 2004; Figure 5A and B). We then analysed the regulation of *dMyc* expression by the activities of Notch and Wg. Interestingly, and consistent with the above results, down-regulation of *dMyc* expression at the ZNC is defined by the activity of Notch, and not Wg (Johnston *et al*, 1999; Duman-Scheel *et al*, 2004). Blocking Notch activation by a dominant-negative form of Mastermind (Giraldez *et al*, 2002), or by using a *Notch*^{ts2} loss-of-function background raised at the restrictive temperature during 48 h, led to expression of *dMyc* at the ZNC (Figure 5C–E). The effects reported on *dMyc* expression after blocking Wg signalling in all ZNC cells (Johnston *et al*, 1999; Duman-Scheel *et al*, 2004; see also Figure 5F) can be explained again by compromised Notch activity, due to the Wg and Notch positive feedback loop that operates at the DV boundary. Increasing Notch activity levels was able to counteract the effects of TCF^{DN} expression (Figure 5G).

Expression of activated forms of the receptor Notch (N^{INTRA}), or the transcription factor Suppressor of Hairless (Su(H)-VP16), led to a reduction in *dMyc* expression levels in wing-blade cells (Figure 5J and K; and Supplementary Figure S3). Ectopic expression of Wg in these cells did not cause such a reduction (Figure 5M). The cell-cycle arrest in G1 that takes place in cells expressing N^{INTRA} was at least in part due to a reduction in *dMyc* expression levels, as coexpression of *dMyc* induced cells to enter into the S-phase, as shown by BrdU incorporation (Figure 5Q). This observation correlates with the upregulation of E2F1 activity levels in these cells (Figure 5R). We then examined the contribution of reduced levels of *dMyc* in the G1 block that occurs at the ZNC. To address this, we analysed the ability of reduced *dMyc*

Figure 5 Regulation of dMyc expression by Notch and Wg. (A–N, P) *Wild type* (A, B), *Notch^{ts2}* reared at the restrictive temperature for 48 h (C), *ap-Gal4*, *UAS-GFP*; *UAS-mam^{DN}* (D), *C96-Gal4*; *UAS-mam^{DN}* (E, H), *C96-Gal4*; *UAS-TCF^{DN}* (F), *C96-Gal4*; *UAS-TCF^{DN}UAS-N^{intra}* (G), *C96-Gal4*; *UAS-mam^{DN} UAS-dMyc^{dsRNA}* (I), *dpp-Gal4*; *UAS-N^{intra}* (J, K), *ap-Gal4*, *Gal80ts*, *UAS-GFP*; *UAS-TCF^{DN}* (L, N) *dpp-Gal4*; *UAS-Wg* (M), and *ap-Gal4*, *Gal80ts*, *UAS-GFP*; *UAS-TCF^{DN}*, *UAS-mam^{DN}* (P) late third instar wing discs labelled to visualize dMyc protein (blue or white (A, D, E–J, M, N, P)) or dMyc mRNA (B, C, K, L) expression, and Wingless (Wg, red (A, E, J, M)), Cut (red (F, G)), Distalless (Dil, red (N, P)) or GFP (green (D, N, P)) protein expression. The DV compartment boundary in panels A and B is indicated by a dashed line. Note dMyc expression in GFP-expressing cells abutting the DV compartment boundary in D (compare with A) as well as in ZNC cells lacking Notch activity (C, E, H). (O, Q, R) *ap-Gal4*, *Gal80ts*, *UAS-GFP*; *UAS-TCF^{DN}*, *UAS-dMyc* (O), *dpp-Gal4*; *UAS-N^{intra}*, *UAS-dMyc* (Q, R) late third instar wing discs labelled to visualize cells in S-phase by BrdU incorporation (antibody to BrdU in red or white (O, Q)), or expressing the E2F1-responsive reporter OCT1–GFP (pink (R)). GFP (O) and dMyc (R) protein expression (green) is also shown. Arrowheads in panels Q and R indicate the *dpp-gal4* expression stripe. Wing discs shown in panels L and N–P were reared at 29°C during 30 h. (S) Wild-type wing disc labelled to visualize dMyc (blue) and Distalless (Dil, red) protein expression.

expression to impose a G1 block at the ZNC in a situation of reduced Notch activity. In wing discs expressing a dominant-negative form of Mastermind along the DV boundary, dMyc expression and BrdU incorporation were increased in ZNC cells (Figure 5H). Decreasing dMyc protein levels in these cells, by means of a dMyc RNA interference construct, restored the ZNC, as revealed by the absence of BrdU incorporation (Figure 5I). These results indicate that reduced dMyc levels caused by the activity of Notch contribute to define the G1 block at the ZNC.

As reported above, a Wg and Notch double-repression mechanism induces G1–S transition in wing cells not located at the ZNC. This mechanism correlates with the presence of dMyc expression, as wing cells unable to transduce the Wg signal (expressing TCF^{DN}) showed reduced levels of dMyc (Figure 5L and N) and coexpression of Mam^{DN}, together with TCF^{DN}-rescued dMyc expression levels (Figure 5P). More interestingly, regulation of G1–S transition by this double-repression mechanism was mediated by the presence of dMyc, as coexpression of dMyc together with TCF^{DN} rescued G1–S progression, as visualized by BrdU incorporation (Figure 5O). We noticed that the Wg and Notch double-repression mechanism did not regulate the expression of other Wg-regulated genes like *Distalless*, as wing cells unable to transduce the Wg signal lost expression of *Distalless* independently of Mam^{DN} coexpression (Figure 5N and P, compare with Figure 5S).

Altogether, these results indicate that dMyc mediates the activities of Notch and Wg in the regulation of G1–S transition in the wing disc, defining the G1 cell-cycle arrest at the ZNC and facilitating G1 progression in the rest of cells. Interestingly, there was a striking correlation between dMyc expression and the activity of the *bantam* micro-RNA, which controls cell-cycle progression in the *Drosophila* wing (Figures 6A and 7A; Brennecke *et al*, 2003). For this reason, we then analysed the role of *bantam* in this process. To monitor *bantam* activity, we used a green fluorescent protein (GFP) *bantam* sensor that expresses GFP under the control of a ubiquitously active tubulin promoter and has two perfect *bantam* target sites in its 3' untranslated region (Brennecke *et al*, 2003). When present, the *bantam* micro-RNA reduces GFP expression through its RNA interference (RNAi) effect. The expression pattern of GFP is thus a negative image of the activity pattern of *bantam* micro-RNA. Brennecke *et al* (2003) showed that the ZNC is characterized by higher levels of the *bantam* sensor (Figure 6A), and ectopic expression of *bantam* induces ZNC cells to enter into S-phase, as shown by BrdU incorporation. We also monitored E2F activity levels in these cells and verified that levels of E2F activity rose upon *bantam* expression in ZNC cells (Figure 6M), thereby indicating that absence of *bantam* contributes to inducing G1 cell-cycle arrest through regulation of E2F1 activity.

We then analysed the regulation of *bantam* activity by Notch and Wg. Blocking Notch activation by means of a dominant-negative form of Mastermind (Giraldez *et al*, 2002) reduced the expression of the *bantam* sensor at the ZNC (Figure 6B), and expression of an activated form of the Notch receptor (N^{INTRA}) led to an increase in its expression levels in wing-blade cells (Figure 6C and C'). The activity of Notch is not mediated by Wg, as Wg ectopic expression did not cause reduction of *bantam* activity levels (Figure 6D), and Wg signalling was not required for repression of *bantam*

activity in ZNC cells, either. Again, the reported effects of Wg signalling on *bantam* activity (Brennecke *et al*, 2003) can be explained by compromised Notch activity, as increased Notch activity levels were able to counteract the effects of TCF^{DN} expression on *bantam* activity levels (Figure 6E–G). The cell-cycle arrest in G1 that occurs in cells expressing N^{INTRA} was at least, in part, due to a reduction in *bantam* expression levels, as coexpression of *bantam* induced cells to enter into the S-phase, as shown by BrdU incorporation (Figure 6L). We then examined the contribution of reduced *bantam* activity in defining the G1 block that takes place at the ZNC. In wing discs with reduced Notch signalling along the DV boundary, BrdU incorporation was increased in ZNC cells (Figure 5H). We noted that Notch activity levels were not completely reduced in these conditions, as Wg and Cut were still expressed in some cells at the DV boundary (Figures 5E and 6H). Thus, this background might be able to respond to changes in the levels of *bantam* activity. Indeed, halving the dose of *bantam* restored the ZNC, as revealed by the absence of BrdU incorporation (Figure 6H). These results indicate that reduced *bantam* activity contributes to define the G1 block at the ZNC.

In the rest of the wing cells, a Wg and Notch double-repression mechanism controls G1–S transition. There is again a striking correlation between the activity levels of *bantam* and this double-repression mechanism. Indeed, wing cells unable to transduce the Wg signal (expressing TCF^{DN}) showed increased expression of the *bantam* sensor (Figure 6I), and coexpression of Mam^{DN}, together with TCF^{DN}, restored the expression levels of the *bantam* sensor back to the endogenous ones (Figure 6J). More interestingly, regulation of the G1–S transition by this double-repression mechanism was mediated by the presence of *bantam*, as coexpression of *bantam*, together with TCF^{DN}, rescued G1 cell-cycle progression, as visualized by BrdU incorporation (Figure 6K).

dMyc and *bantam* micro-RNA are two independent effectors of Notch and Wg

Notch imposed a G1 arrest at the ZNC by reducing the activity of *bantam* and the expression of dMyc. Wg repressed Notch and facilitated G1 progression in the rest of wing cells through dMyc and *bantam*. Expression of either of these genes was sufficient to induce G1 progression and bypass the effects of Notch or absence of Wg. We then questioned whether these effectors are independently regulated by Notch and Wg, or, alternatively, whether their expression depends on each other.

Overexpression of *bantam* induced an increase in dMyc protein expression in wing-blade cells and at the ZNC (Figure 7B, compare with Figure 7A), and overexpression of dMyc caused an increase in the activity levels of *bantam* (Figure 7E). By contrast, clones of cells mutant for *bantam* did not show reduction in dMyc protein levels (Figure 7C and D), and reduced levels of dMyc did not considerably affect *bantam* activity levels (Figure 7G). Altogether, these results indicate that dMyc and *bantam* are able, when overexpressed, to modulate each other's expression or activity levels, but are independently regulated by Notch and Wg.

Are dMyc and *bantam* two independent effectors regulating G1 progression? The evidence for dMyc is clear, as in the presence of Notch or absence of Wg signalling, dMyc

Figure 6 Regulation of *bantam* activity by Notch and Wg. (A–G; I, J) Wild type (A, A', E), *ap-Gal4; UAS-mam^{DN}* (B), *dpp-Gal4; UAS-N^{intra}* (C, C'), *dpp-Gal4; UAS-Wg* (D), *C96-Gal4; UAS-TCF^{DN}* (F), *C96-Gal4; UAS-TCF^{DN} UAS-N^{INTRA}* (G), *dpp-Gal4; UAS-TCF^{DN}* (I) and *dpp-Gal4; UAS-TCF^{DN}, UAS-mam^{DN}* (J) late third instar wing discs labelled to visualize expression of the *bantam* sensor (blue and white), and BrdU incorporation (red (A)), Cut (red (A')), Gal4 (red (B)), Wg (red (C, C', I)) or Sens (red (D–G, J)) protein expression. Note symmetric expression of the *bantam* sensor in dorsal and ventral cells abutting the DV compartment boundary (dashed line) in panels A and A', and lack of expression of the *bantam* sensor in dorsal cells abutting the DV compartment boundary (dashed line) in panel B (see also magnification). Note elevated levels of expression of the *bantam* sensor in the stripe of *dpp-Gal4* expression (red arrowheads) in panels C and I, when compared with panels A, B, D and J. (H, K–M) *C96-Gal4; UAS-TCF^{DN}/bantam^{Δ1}* (H), *dpp-Gal4; UAS-TCF^{DN}, UAS-ban-GFP* (K), *dpp-Gal4; UAS-N^{intra}, UAS-ban-GFP* (L) and *dpp-Gal4; UAS-ban* (M) late third instar wing discs labelled to visualize BrdU incorporation (red and white H, K, L) or E2F activity (red or white, M). Cut (green, H), GFP (green, K, L), Sens (blue, K) and Wg (blue, L, M) protein expression is also shown. Wing discs shown in panels I–K were reared at 29°C during 30 h.

Figure 7 Crosstalk between dMyc and bantam. (A) Wild-type wing disc labelled to visualize the *bantam* sensor (blue) and dMyc protein expression (red and white). (B) *ptc-Gal4; UAS-bantamGFP* wing disc showing dMyc expression (red and white) and GFP (green). (C, D) Clones of cells lacking *bantam* activity (*ban^{Δ1}*) marked by the absence of GFP. dMyc protein expression is shown in red or white. Note dMyc expression in the clones (red arrows). (E–G) *sal-Gal4; UAS-dMyc* (E), *dpp-Gal4; UAS-N^{intra}, UAS-dMyc* (F), and *sal-Gal4; UAS-dMyc^{dsRNA}* (G) wing discs labelled to visualize expression of the *bantam* sensor (blue) and dMyc protein (red in E, red or white in G). The DV compartment boundary is marked in panels E and F (d, dorsal; v, ventral). (H) *ptc-Gal4; UAS-dMyc^{dsRNA}, UAS-bantamGFP* late third instar wing disc labelled to visualize BrdU incorporation (red), GFP (green) and dMyc (blue or white) protein expression. (I) *C96-Gal4; UAS-bantam* late third instar wing disc, grown at 18°C and labelled to visualize expression of the *bantam* sensor (blue), BrdU incorporation (red) and dMyc protein expression (white).

expression had the capacity to rescue G1 progression (Figure 5M, O and P), and this happened in the presence of reduced levels of *bantam* activity. Note that dMyc overexpression was able to increase *bantam* activity levels only very mildly at the ZNC where Notch activity is high (Figure 7E), and was completely unable to restore the low activity levels of *bantam* imposed by Notch activation (compare Figures 6C and 7F). By contrast, *bantam* might rescue G1 progression through regulation of dMyc (Figure 7B). Note that in this case *bantam* is able to bypass the effect of Notch at the ZNC and induce an increase in dMyc expression (Figure 7B). We then analysed the capacity of *bantam* to induce G1 progression after reducing dMyc expression levels. Expression of *bantam*, together with a *dmyc* double-stranded RNA (dsRNA) construct, induced G1–S progression in wing cells (Figure 7H), and this happened in the presence of very low levels of dMyc (Figure 7H). Altogether, these results imply that *bantam* and dMyc are two independent effectors of Wg and Notch that regulate G1 progression.

These effectors are able to substitute each other when overexpressed in situations of reduced Wg signalling or high levels of Notch activity. However, their endogenous levels do not appear to do so, as loss of *dMyc* or *bantam* activity in clones of cells compromises cell-cycle progression (Johnston *et al*, 1999; Brennecke *et al*, 2003), and reduced levels of

either *dMyc* or *bantam*, were able to define the ZNC (Figures 5 and 6). This suggests that the endogenous levels of both effectors are rate-limiting. Interestingly, coexpression of both effectors at endogenous, wing-blade levels was able to drive G1–S progression in ZNC cells. As shown in Figure 7I, ZNC cells with wing-blade levels of *bantam* activity and dMyc protein were able to enter S-phase, as monitored by BrdU incorporation. Thus, wing cells require the activity of both *bantam* and dMyc to drive G1–S transition.

Concluding remarks

Here we have analysed the role of Notch and Wg in the regulation of G1–S progression in the *Drosophila* wing and have provided evidence that a surprisingly linear pathway, based on a Wg and Notch double-repression mechanism, controls this transition (Figure 8B). Notch activity autonomously blocks cells in G1 and defines the ZNC at the DV boundary of the *Drosophila* wing. Wg activity is required in wing-blade cells to repress Notch signalling and allow cells to enter the S-phase. These signalling molecules exert their function in a linear pathway through the *bantam* microRNA and the *dmyc* proto-oncogene, two independent effectors controlling the activity of the E2F1 transcription factor and regulate G1-to-S progression. Notch is known to be repressed by the activity of Wg at different levels of its

Figure 8 Different effects of Notch and Wg in cell-cycle control in the *Drosophila* wing. **(A)** Illustration describing the control of the G2-to-M transition in the anterior (a) compartment by the activity of Wg through its target genes *achaete* and *scute* (*ac-sc*). *ac-sc* are known to repress the expression of *Drosophila cdc25 string* (*stg*) in non-boundary cells. Note Wg does not exert any function in this process in posterior (p) cells. Notch represses *ac-sc* expression in boundary cells, this facilitating G2 progression in these cells. **(B)** Illustration describing the control of the G1-S transition by the activities of Notch (N) and Wingless (Wg) at the ZNC and in wing-blade cells, through the downstream effectors *bantam* and *dMyc*.

pathway (Axelrod *et al*, 1996; Rulifson *et al*, 1996; Michelli *et al*, 1997; de Celis and Bray, 1997; Neumann and Cohen, 1998), but the level at which Wg represses Notch activity to facilitate *dMyc* expression, *bantam* activity and G1-S progression remains to be elucidated.

There is evidence in mammals that Notch can act as a tumour-suppressor gene or as an oncogene depending on the cellular context (reviewed by Radtke and Raj, 2003). The cellular context should then modulate the differential response of the cells to changes in Notch activity, in some cells leading to hyper-proliferation, and in others to quiescence. How this context and the differential response

are defined at the molecular level remains unclear. *Drosophila*, again, provides a very suitable model system to address this issue. Although our results highlight that Notch is involved in the inhibition of G1-S progression in wing discs, in other developmental contexts Notch exerts a proliferative function (Baonza and Freeman, 2005; Firth and Baker, 2005). Interestingly, in both cases, Notch exerts an effect through Rbf and E2F to positively or negatively control the G1-S transition. We, therefore, speculate that the molecular context is then defined by the effectors available, like *dMyc* and *bantam*, or by the presence of nuclear factors that act as a switch in the ultimate activation/inactivation of E2F through Rbf.

It is interesting to note that there are three consensus sequences for binding of Su(H) to DNA in the *dMyc* gene (data not shown), and in the particular case of human T-cell acute lymphoblastic leukaemia/lymphoma, *c-myc* proto-oncogene acts as a direct downstream target of Notch1 (Sharma *et al*, 2006; Weng *et al*, 2006). These results raise the possibility that *dMyc* might be a direct target of Notch in the *Drosophila* wing. However, the fact that *dMyc* is negatively regulated by Notch and an activated form of Su(H) (Su(H)-VP16) is able to repress *dMyc* expression, suggests that Notch regulates *dMyc* through a Notch target gene that may act as a repressor.

The DV boundary behaves as an organizer of growth in the developing wing, as ectopic DV boundaries induce non-autonomous proliferation of the surrounding tissue, and loss of the DV boundary leads to absence of wing tissue (Diaz-Benjumea and Cohen, 1993). Notch has been shown to mediate this organizing activity (Irvine and Wieschaus, 1994; de Celis *et al*, 1996), and previous reports showed that Wg is not sufficient to promote growth in the absence of an endogenous DV boundary (Klein and Arias, 1998; Baena-Lopez and Garcia-Bellido, 2003; Zecca and Struhl, 2007). These results are consistent with the data obtained in this work and suggest that the organizing activity of the DV boundary should not be attributed to the activity of Wg. Our results indicate that the organizing activity of Notch is strictly non-autonomous, as Notch induces cell-cycle arrest in a cell-autonomous manner. In this context, Wg signalling alleviates Notch-mediated repression of G1-S progression in wing cells. The role of Wg can be defined as permissive rather than instructive, as wing cells unable to respond to Notch and Wg do proliferate. Altogether, these results indicate that unknown signalling molecules regulated by Notch might exert the organizing activity of the wing DV boundary.

Materials and methods

Drosophila strains

crb^{MH. M2} (*crb-lacZ* in the text), *UAS-Ser^{DN}* and *UAS-D^{DN}* (Herranz *et al*, 2006); E2F1-responsive reporter (Asano and Wharton, 1999), *UAS-dE2F1 UAS-DP* (*UAS-E2F* in the text; Neufeld *et al*, 1998, a gift from B Edgar), *UAS-dMyc* (Johnston *et al*, 1999, a gift from B Edgar), *ban^{Δ1}*, *ban-sensor-GFP* and *UAS-bantam-GFP* (Brennecke *et al*, 2003, a gift from S Cohen), and *UAS-dMyc^{RNAi}* (Vienna *Drosophila* RNAi Center), *UAS-mam^{DN}* (Helms *et al*, 1999). Other stocks are described in Flybase.

Antibodies

Guinea pig anti-Senseless (Nolo *et al*, 2000, a gift from H Bellen), mouse anti-Wg and mouse anti-BrdU are described in the Developmental Studies Hybridoma Bank; rabbit anti-β-gal (Cappel)

was also used. Anti-dMyc antibody was raised in guinea pig against recombinant full-length dMyc protein. The specificity of the anti-dMyc antibody was tested in wing discs overexpressing dMyc and in wing discs in which dMyc expression is reduced by means of a dsRNA construct of *dmyc* (see Supplementary Figure S4). BrdU staining, antisense digoxigenin-labelled RNA probes of *dMyc* and *wg* mRNAs and *in situ* hybridization was performed as described by Milan *et al* (1996).

Spatiotemporal gene expression targeting in *Drosophila*

We made use of the TARGET system developed by McGuire *et al* (2004). Adult flies carrying the Gal4 drivers *ptc-Gal4*, *dpp-Gal4* or *ap-Gal4* and the thermosensitive version of the Gal4 repressor Gal80 (Gal80^{ts}) under the control of the *tubulin* promoter (*tubulin-Gal80ts*) were crossed with transgenic flies bearing one or more UAS transgenes. Flies were allowed to lay eggs over a period of 24 h at 18°C. The progeny was then grown at 18°C to maintain the Gal4/UAS system in a switched-off condition and transferred to 29°C for a range of periods during larval development to induce Gal4/UAS-dependent expression.

Larval genotypes

The TARGET system:

tub-Gal80^{ts}/UAS-dTcf^{DN}; *dpp-Gal4/E2F-sensor*
tub-Gal80^{ts}/UAS-dTcf^{DN}; *UAS-mam^{DN}*; *dpp-Gal4*,
E2F-sensor/UAS-mam^{DN}
tub-Gal80^{ts}/+; *dpp-Gal4*, *UAS-GFP/UAS-N^{inttra}*, *UAS-E2F*,
UAS-Dp
tub-Gal80^{ts}/+; *dpp-Gal4*, *UAS-GFP/UAS-N^{inttra}*, *UAS-CycE*
tub-Gal80^{ts}/+; *dpp-Gal4*, *UAS-GFP/UAS-N^{inttra}*, *UAS-dMyc*
ap-Gal4, *UAS-GFP/UAS-dTcf^{DN}*; *tub-Gal80^{ts}/+*

ap-Gal4, *UAS-GFP/UAS-dTcf^{DN}*; *UAS-E2F*, *UAS-Dp/tub-Gal80^{ts}*
ap-Gal4, *UAS-GFP/UAS-dTcf^{DN}*, *UAS-mam^{DN}*; *tub-Gal80^{ts}/+*
ap-Gal4/UAS-dTcf^{DN}, *UAS-mam^{DN}*; *tub-Gal80^{ts}/UAS-mam^{DN}*
ap-Gal4/UAS-Tcf^{DN}, *UAS-GFP*; *tub-Gal80^{ts}/UAS-dMyc*
ptc-Gal4, *UAS-GFP/UAS-dTcf^{DN}*; *UAS-CycE/tub-Gal80^{ts}*
ptc-Gal4, *UAS-GFP/UAS-Tcf^{DN}*; *UAS-BantamD/tub-Gal80^{ts}*
ptc-Gal4, *UAS-UAS-BantamD/UAS-Myc^{dsRNA}*; *tub-Gal80^{ts}/+*
Clonal analysis:

hs-FLP/+; *Su(H)⁸FRT40A/ubi-GFP FRT40A*
hs-FLP/+; *Su(H)⁸FRT40A/ubi-GFP FRT40A*; *E2F-sensor/+*
hs-FLP/+; *FRT42D ubi-GFP/FRT42D pwn arr²*
hs-FLP/+; *M(3L) ubi-GFP FRT80B/bantam^{A1}FRT80B*

Supplementary data

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

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References

- Asano M, Wharton RP (1999) E2F mediates developmental and cell cycle regulation of ORC1 in *Drosophila*. *EMBO J* **18**: 2435–2448
- Axelrod JD, Matsuno K, Artavanis-Tsakonas S, Perrimon N (1996) Interaction between wingless and Notch signaling pathways mediated by Dishevelled. *Science* **271**: 1826–1832
- Baena-Lopez LA, Garcia-Bellido A (2003) Genetic requirements of vestigial in the regulation of *Drosophila* wing development. *Development* **130**: 197–208
- Baonza A, Freeman M (2005) Control of cell proliferation in the *Drosophila* eye by Notch signaling. *Dev Cell* **8**: 529–539
- Bienz M, Clevers H (2000) Linking colorectal cancer to Wnt signaling. *Cell* **103**: 311–320
- Blair SS, Brower DL, Thomas JB, Zavortink M (1994) The role of *apterous* in the control of dorsoventral compartmentalization and PS integrin gene expression in the developing wing of *Drosophila*. *Development* **120**: 1805–1815
- Bray SJ (2006) Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* **7**: 678–689
- Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM (2003) bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene *hid* in *Drosophila*. *Cell* **113**: 25–36
- Buceta J, Herranz H, Canela-Xandri O, Reigada R, Sagues F, Milan M (2007) Robustness and stability of the gene regulatory network involved in DV boundary formation in the *Drosophila* wing. *PLoS ONE* **2**: e602
- Clevers H (2006) Wnt/beta-catenin signaling in development and disease. *Cell* **127**: 469–480
- de Celis JF, Bray S (1997) Feedback mechanisms affecting Notch activation at the dorsoventral boundary in the *Drosophila* wing. *Development* **124**: 3241–3251
- de Celis JF, Garcia-Bellido A, Bray SJ (1996) Activation and function of *Notch* at the dorsal-ventral boundary of the wing imaginal disc. *Development* **122**: 359–369
- Diaz-Benjumea FJ, Cohen SM (1993) Interaction between dorsal and ventral cells in the imaginal disc directs wing development in *Drosophila*. *Cell* **75**: 741–752
- Diaz-Benjumea FJ, Cohen SM (1995) Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the *Drosophila* wing. *Development* **121**: 4215–4225
- Duman-Scheel M, Johnston LA, Du W (2004) Repression of dMyc expression by Wingless promotes Rbf-induced G1 arrest in the presumptive *Drosophila* wing margin. *Proc Natl Acad Sci USA* **101**: 3857–3862
- Dyson N (1998) The regulation of E2F by pRB-family proteins. *Genes Dev* **12**: 2245–2262
- Firth LC, Baker NE (2005) Extracellular signals responsible for spatially regulated proliferation in the differentiating *Drosophila* eye. *Dev Cell* **8**: 541–551
- García-Bellido A, Merriam JR (1971) Parameters of the wing imaginal disc development of *Drosophila melanogaster*. *Dev Biol* **24**: 61–87
- Giraldez AJ, Cohen SM (2003) Wingless and Notch signaling provide cell survival cues and control cell proliferation during wing development. *Development* **130**: 6533–6543
- Giraldez AJ, Perez L, Cohen SM (2002) A naturally occurring alternative product of the mastermind locus that represses notch signalling. *Mech Dev* **115**: 101–105
- Helms W, Lee H, Ammerman M, Parks AL, Muskavitch MA, Yedvobnick B (1999) Engineered truncations in the *Drosophila* mastermind protein disrupt Notch pathway function. *Dev Biol* **215**: 358–374
- Herranz H, Stamatakis E, Feiguin F, Milan M (2006) Self-refinement of Notch activity through the transmembrane protein Crumbs: modulation of gamma-secretase activity. *EMBO Rep* **7**: 297–302
- Irvine K, Wieschaus E (1994) *fringe*, a boundary specific signalling molecule, mediates interactions between dorsal and ventral cells during *Drosophila* wing development. *Cell* **79**: 595–606
- Johnston LA, Edgar BA (1998) Wingless and Notch regulate cell-cycle arrest in the developing *Drosophila* wing. *Nature* **394**: 82–84
- Johnston LA, Prober DA, Edgar BA, Eisenman RN, Gallant P (1999) *Drosophila* myc regulates cellular growth during development. *Cell* **98**: 779–790
- Johnston LA, Sanders AL (2003) Wingless promotes cell survival but constrains growth during *Drosophila* wing development. *Nat Cell Biol* **5**: 827–833
- Klein T, Arias AM (1998) Different spatial and temporal interactions between Notch, wingless, and vestigial specify proximal and distal pattern elements of the wing in *Drosophila*. *Dev Biol* **194**: 196–212

- Knoblich JA, Sauer K, Jones L, Richardson H, Saint R, Lehner CF (1994) Cyclin E controls S phase progression and its downregulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. *Cell* **77**: 107–120
- Logan CY, Nusse R (2004) The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* **20**: 781–810
- Madhavan MM, Schneiderman HA (1977) Histological analysis of the dynamics of growth of imaginal discs and histoblast nests during the larval development of *Drosophila melanogaster*. *Roux Arch Dev Biol* **183**: 269–305
- McGuire SE, Mao Z, Davis RL (2004) Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in *Drosophila*. *Sci STKE* **2004**: pl6
- Micchelli CA, Rulifson EJ, Blair SS (1997) The function and regulation of cut expression on the wing margin of *Drosophila*: Notch, Wingless and a dominant negative role for Delta and Serrate. *Development* **124**: 1485–1495
- Milan M, Campuzano S, Garcia-Bellido A (1996) Cell-cycling and patterned cell proliferation in the wing primordium of *Drosophila*. *Proc Natl Acad Sci USA* **93**: 640–645
- Neufeld TP, de la Cruz AF, Johnston LA, Edgar BA (1998) Coordination of growth and cell division in the *Drosophila* wing. *Cell* **93**: 1183–1193
- Neumann CJ, Cohen SM (1997) Long-range action of Wingless organizes the dorsal–ventral axis of the *Drosophila* wing. *Development* **124**: 871–880
- Neumann CJ, Cohen SM (1998) Boundary formation in the *Drosophila* wing: the POU-domain protein Nubbin attenuates Notch activity. *Science* **281**: 409–413
- Nolo R, Abbott LA, Bellen HJ (2000) Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in *Drosophila*. *Cell* **102**: 349–362
- O'Brochta DA, Bryant PJ (1985) A zone of non-proliferating cells at a lineage restriction boundary in *Drosophila*. *Nature* **313**: 138–141
- Phillips R, Whittle JRS (1993) *wingless* expression mediates determination of peripheral nervous system elements in late stages of *Drosophila* wing disc development. *Development* **118**: 427–438
- Radtke F, Raj K (2003) The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat Rev Cancer* **3**: 756–767
- Rulifson EJ, Micchelli CA, Axelrod JD, Perrimon N, Blair SS (1996) *wingless* refines its own expression domain on the *Drosophila* wing margin. *Nature* **384**: 72–74
- Sharma VM, Calvo JA, Draheim KM, Cunningham LA, Hermance N, Beverly L, Krishnamoorthy V, Bhasin M, Capobianco AJ, Kelliher MA (2006) Notch1 contributes to mouse T-cell leukemia by directly inducing the expression of c-myc. *Mol Cell Biol* **26**: 8022–8031
- Strigini M, Cohen SM (2000) Wingless gradient formation in the *Drosophila* wing. *Curr Biol* **10**: 293–300
- Wehrli M, Dougan ST, Caldwell K, O'Keefe L, Schwartz S, Vaizel-Ohayon D, Schejter E, Tomlinson A, DiNardo S (2000) arrow encodes an LDL-receptor-related protein essential for Wingless signalling. *Nature* **407**: 527–530
- Weng AP, Millholland JM, Yashiro-Ohtani Y, Arcangeli ML, Lau A, Wai C, Del Bianco C, Rodriguez CG, Sai H, Tobias J, Li Y, Wolfe MS, Shachaf C, Felsher D, Blacklow SC, Pear WS, Aster JC (2006) c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev* **20**: 2096–2109
- Zecca M, Basler K, Struhl G (1996) Direct and long-range action of a Wingless morphogen gradient. *Cell* **87**: 833–844
- Zecca M, Struhl G (2007) Recruitment of cells into the *Drosophila* wing primordium by a feed-forward circuit of vestigial autoregulation. *Development* **134**: 3001–3010