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Wingless Promotes Proliferative Growth in a Gradient-Independent Manner

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Abstract

Morphogens form concentration gradients that organize patterns of cells and control growth. It has been suggested that, rather than the intensity of morphogen signalling, it is its gradation that is the relevant modulator of cell proliferation. According to this view, the ability of morphogens to regulate growth during development depends on their graded distributions. Here, we describe an experimental test of this model for Wingless, one of the key organizers of wing development in *Drosophila*. Maximal Wingless signalling suppresses cellular proliferation. In contrast, we found that moderate and uniform amounts of exogenous Wingless, even in the absence of endogenous Wingless, stimulated proliferative growth. Beyond a few cell diameters from the source, Wingless was relatively constant in abundance and thus provided a homogeneous growth-promoting signal. Although morphogen signalling may act in combination with as yet uncharacterized graded growth-promoting pathways, we suggest that the graded nature of morphogen signalling is not required for proliferation, at least in the developing *Drosophila* wing, during the main period of growth.

INTRODUCTION

Morphogens are signalling molecules that contribute to pattern formation in tissues by forming a concentration gradient and controlling target gene expression in a dose-dependent manner (1, 2). Morphogens have also been implicated in the control of tissue growth, although this activity is still poorly understood. Most of the evidence for this role arises from studies of developing appendages of *Drosophila*, especially the imaginal discs of the wing. These epithelial sacs originate from an embryonic primordium of about 50 cells that reaches a final size of 30,000 to 50,000 cells before formation of the wing proper (3).

Two morphogens can be considered the cardinal organizers of wing development: Decapentaplegic (Dpp), which patterns the anterior-posterior (AP) axis, and Wingless (Wg), which spreads from the dorsoventral (DV) boundary (2). It is widely thought that the cells of the prospective wing, the so-called pouch region of the wing imaginal disc, integrate the information from these two signals to differentiate appropriately. In addition, these two signals are thought to modulate cell survival and overall wing growth (4-7). Indeed, removing the activity of either Dpp or Wg after territorial segregation of the wing and the

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thorax leads to a strong reduction in wing size (8, 9). How the contributions of Dpp and Wg are summed and coordinated with other growth-regulating mechanisms is largely unknown. Indeed, even though these two signals are homologous to known vertebrate growth factors, little is known about the downstream events relevant to growth that they trigger in wing imaginal discs.

Much effort has been devoted to understanding the role of Dpp in the growth of wing imaginal discs. Intriguingly, in this tissue, the rate of cell proliferation is not directly related to the local concentration of Dpp, because the pattern of growth and proliferation is relatively uniform (10) whereas the distribution of Dpp is graded. One elegant model suggests that it is the slope of the Dpp gradient that may be the key determinant of growth (7). This possibility has received experimental support from the observation that artificial on-off steps in the pattern of Dpp activity trigger excess proliferation along the central region of the AP axis (11). Subsequent work by the same authors has led to the notion that the Dpp gradient could contribute to the graded activity of Fat-Hippo signalling, which itself would be the central regulator of growth (12). This is an attractive model because it might explain, at least in part, how Fat-Hippo signalling becomes graded even though it itself does not appear to involve a diffusible molecule (13); however, another study suggests that wing growth can proceed in the presence of uniform Dpp signalling (14). Therefore, it is an open question whether graded signalling by Dpp, or any other morphogen, is required for tissue growth. Here, we addressed this question for another wing morphogen, Wg.

Wingless is the main member of the Wnt family of secreted glycolipoproteins (<http://www.stanford.edu/~rnusse/wntwindow.html>) in *Drosophila*. Wnt signalling triggers proliferation in many situations, and indeed excess Wnt signalling is associated with numerous cancers (15, 16). Nevertheless, evidence suggests that in the imaginal discs of the wing, during the third instar (a time when extensive growth takes place), enhanced Wingless signalling induces a premature stop in proliferation (4). At this stage, near the DV boundary, where Wg signalling is most intense, the expression of genes that encode several regulators of the cell cycle, such as E2F-1 or String (the *Drosophila* homolog of CDC25) is decreased, and a zone of nonproliferating cells forms (17, 18).

Despite the apparent anti-proliferative effect of high amounts of Wg, there is evidence that Wg provides a positive input to proliferation in regions of the discs located outside of the DV boundary area (9, 19, 20). Therefore, the role of Wingless in controlling the proliferation of prospective wing cells remains ambiguous. Another important function attributed to Wingless in imaginal discs, especially in regions where signalling is least intense, is that of survival factor (4, 19). Clones of cells that cannot transduce the Wingless signal activate apoptosis and are quickly eliminated, even during the third instar (4, 19). However, it appears that large patches of tissue that do not receive a Wg signal are able to survive and grow (21, 22). This observation, as well as the development of tools that enable fine experimental control of Wg signalling, provides an opportunity to revisit the role of Wg in the proliferation of prospective wing cells and also to test directly whether its graded distribution is relevant to growth control.

RESULTS

Mild ectopic Wingless signalling leads to enlargement of the prospective wing

A temperature-sensitive inhibitor of Gal4, Gal80^{ts} (produced from a Tubulin-G80^{ts} transgene), can be used to modulate Gal4-dependent gene expression in *Drosophila* (23). This was combined with the *nubbin-Gal4* (*nubG4*) driver, which is active throughout the prospective wing (fig. S1A). Thus, Arm^{S10}, an activated form of Armadillo (Arm, the *Drosophila* β -catenin), could be expressed at various concentrations in the prospective

imaginal discs. Although one cannot be sure that Arm^{S10} recapitulates all of the effects of Wingless, it does trigger known downstream events, including the activation of genes that encode wing margin markers, such as *senseless (sens)*(4), and repression of the expression of *string* (24).

Adult flies of the genotype *nubG4* Tubulin-G80^{ts} UAS-Arm^{S10} raised at 29°C (when Gal80^{ts} is inactive) had stunted wings with most cells seemingly adopting a margin fate (Fig. 1, A to C), which is specified by maximal signalling (21). Therefore, Arm^{S10} triggers premature cell cycle arrest and differentiation throughout the wing primordium. As expected, the areas of expression of *sens* and *heat-shock protein 23 (hsp23)*, two genes that respond to enhanced signalling, were widened in discs expressing Arm^{S10} (Fig. 1, D and E). Conversely, repression of the expression of *dally-like (dlp)*, normally a characteristic of the DV boundary area, extends throughout the pouch (Fig. 1, D and E).

When only moderate ectopic expression of Arm^{S10} was induced (as, for example, in *nubbin-Gal4*, Tubulin-G80^{ts}, UAS-Arm^{S10} flies raised at 25°C or 17°C), only a small number of ectopic bristles appeared (Fig. 1, F to K) and a few ectopic *sens*-expressing cells were detected (fig. S1). Therefore, mild ectopic signalling caused only subtle changes in cell fate; however, it had a substantial effect on wing size. To appreciate this effect, it is important to consider that because the Gal80^{ts} system relies on temperature modulation to control ectopic signalling, it is crucial that discs from these flies are compared to discs from control, wild-type flies raised at the same temperature.

This is especially true because wing size is highly temperature dependent in the wild type (25, 26). At either 25°C or 17°C, wings that expressed Arm^{S10} were enlarged compared to those of the corresponding controls (compare the adult wings in Fig. 1, A to J, and see the graph in L). This result suggests that moderate Wingless signalling triggers extra wing growth.

To confirm and characterize the growth-promoting effect of moderate Wingless signalling in the prospective wing, we turned to two attenuated forms of the ligand. The first carries a change of Ser²³⁹, which normally bears a palmitoleic acid moiety, to alanine (*wg*^{S239A})(27). This mutant protein, which is tagged and thus can be detected with antibodies against Hemagglutinin (HA) or Wingless, is present at a reduced abundance in the extracellular space compared to that of wild-type Wingless, either because of its reduced stability there or because of a defect in its secretion (27). As a result, its ability to trigger signal transduction is strongly reduced (27). Production of Wingless^{S239A} in the embryonic epidermis with *en-Gal4* (*enG4*) or *hedgehog-Gal4* (*hhG4*) had no noticeable effect on embryonic patterning (as judged by the pattern of denticle hairs that normally form on the ventral epidermis). By contrast, production of wild-type Wingless with the same Gal4 drivers leads to stereotypical pattern defects and embryonic lethality with 100% penetrance (28). Therefore, by comparison to the wild-type protein, Wingless^{S239A} must be poorly active. Nevertheless, the increased abundance of this protein in the wing pouch, under the control of *nubG4*, caused substantial enlargement of the wing (Fig. 2, A and B, and fig. S2A) without noticeable changes in cell size (fig. S2B). A similar effect was seen after ectopic expression of a temperature-sensitive form of Wingless, Wingless^{C104S} (Wingless^{ts}), at a semipermissive temperature (22°C), under the control of *nubGal4* (Fig. 2, C and D, and fig. S2A). These results are consistent with the suggestion that moderate or mild ectopic Wingless signalling leads to increased cell proliferation.

Mild ectopic Wingless signalling promotes growth by stimulating proliferation

What is the cellular basis of the Wingless^{S239A}-dependent enlargement of the wing? To address this question, Wingless^{S239A} was expressed in the posterior compartment and its

effect there was compared with that in the anterior compartment. Expression of UAS-Wingless^{S239A} with either *hhG4* (Fig. 2, E to G) or *enG4* (fig. S2, C and D) led to a marked increase in the size of the posterior compartment (Fig. 2, E to G). Note that, by comparison to the situation in control wings, the size of the anterior compartment was also enlarged, although less markedly than that of the posterior compartment (Fig. 2, E to G). This is probably due to the ability of Wingless^{S239A} to spread across the boundary between the two compartments; Wingless^{S239A} may even have increased mobility compared to that of wild-type Wingless because of the absence of the palmitoleic acid moiety.

We next confirmed the ability of Wingless^{S239A} to affect the rate of cell proliferation. Cells were randomly marked by Flp-mediated recombination in *hhG4* UAS-Wingless^{S239A} wing discs. They were then allowed to proliferate for 48 hours, and the surface area of the territory colonized by their progeny was assessed. Visual inspection indicated that, on average, clones were larger in the posterior compartment (where Wingless^{S239A} was expressed) than in the control anterior compartment (Fig. 2H), which was confirmed by quantification (Fig. 2I; Mann-Whitney test, $Z < 0.0001$). The data on clone size were converted into cell-doubling times, which appeared to be reduced by about 22% on average in the Wingless^{S239A}-containing anterior compartment (Fig. 2J) compared to those in control posterior compartments. In accordance with this finding, we detected a relative increase in cell proliferation, as assayed by staining with an antibody against phosphohistone H3 (PH3) (Fig. 2K and fig. S2E) or incorporation of 5-bromo-2'-deoxyuridine (fig. S2F), in Wingless^{S239A}-containing territories. A similar increase in the abundance of these two markers of proliferation was also seen earlier during larval development [84 hours after egg laying (AEL); fig. S2E]. It must be noted that no clear-cut increase in PH3 immunoreactivity was seen in clones containing Wingless^{S239A} (as opposed to marked clones induced in a posterior compartment that uniformly contained Wingless^{S239A}), perhaps because such clones produced insufficient amounts of Wingless^{S239A} for a substantial effect on the normally spotty distribution of PH3 to be detectable (fig. S3).

Altogether, our results suggest that a mild and sustained increase in Wingless signalling stimulates cell proliferation in prospective wing cells. It is likely that the extent of Wingless signalling required to stimulate extra growth is relatively low because Wingless^{S239A} does not trigger the formation of ectopic margin bristles, nor does it substantially disrupt the patterning of veins (Fig. 2, A to F). To further estimate the extent of signalling required for growth stimulation, the effect of Wingless^{S239A} on the expression of various known target genes was assessed. As expected from the wing phenotype, no effect on the expression of *sens* could be detected (fig. S2G). Nevertheless, Wingless^{S239A} did activate signalling because it caused a moderate increase in the expression of Vestigial (Vg) (fig. S2H). Moreover, the expression of Dlp, which is repressed by Wingless signalling, appeared to be slightly inhibited in the Wingless^{S239A}-expressing territory (fig. S2I). Therefore, we conclude that a moderate increase in Wingless signalling, to an extent that had little or no effect on patterning, stimulated proliferation.

The growth-stimulating activity of mild Wingless signalling is gradient independent

So far, our data have shown that mild Wingless signalling stimulated proliferative growth; however, because of the presence of a gradient of endogenous Wingless, these experiments have not directly addressed the need for graded signalling in the proliferative response. Therefore, we sought to uniformly activate mild Wingless signalling in a Wingless-deficient background. Because Wingless is required for embryogenesis, homozygous wingless mutant cells were specifically generated in imaginal disc precursors by Flp-mediated mitotic recombination (29). Widespread recombination in the posterior compartment was achieved with a combination of *hhG4* and UAS-Flipase (Flp) as a source of Flp recombinase. Moreover, homozygous mutant cells were given a growth advantage with the Minute

technique (30). As previously described (22), this genetic combination enables sufficient early Wingless signalling for the specification of wing fate and leads to the subsequent elimination of all Wingless-expressing cells in the posterior compartment (Fig. 3A).

Only occasional Wingless-expressing cells were detected at the beginning of the third instar stage (72 hours AEL, fig. S4A). Wingless-deficient posterior compartments were reduced in size compared to control wild-type posterior compartments, and this was correlated with the loss of a substantial portion of the posterior tissue in adult wings (Fig. 3B) and wing discs (Fig. 3, C and D; see quantification in I and further details in fig. S5). Moreover, expression of P35, a viral inhibitor of apoptosis, did not substantially restore normal wing size in the absence of Wingless (fig. S6 and quantification in Fig. 3I, compare gray bar and purple), suggesting that loss of tissue in the absence of Wingless is primarily caused by reduced cell proliferation. Thus, Wingless signalling would be required to sustain the rate of proliferation that normally takes place during disc growth. However, growth and proliferation were not completely abolished in third instar posterior compartments that lacked a source of Wingless (Fig. 3, D and J). It is unlikely that residual growth relies on the spreading of Wingless from the anterior compartment because posterior compartments composed entirely of Frizzled and Frizzled2 (*fz fz2*) mutant cells, which cannot respond to Wingless, also grew, albeit to a lesser extent than did Wingless-deficient compartments (see Materials and Methods) (22). Therefore, Wingless signalling appears to be required, but not absolutely essential, for sustained proliferation in wing imaginal discs.

The ability to generate posterior compartments devoid of Wingless during the third instar enabled the direct testing of whether graded Wingless signalling was required for the growth-stimulatory activity of Wingless during this period. Uniform, low-intensity Wingless signalling was introduced into the Wingless-deficient posterior compartments by expressing the gene encoding Wingless^{S239A} under the control of *hhG4* (Fig. 3E and fig. S4B). This led to the restoration of posterior compartment size (Fig. 3, E to G, quantified in I) and of the rate of proliferation in this compartment (Fig. 3H, quantified in J). Note that the total amount of Wingless signalling in rescued wings was not sufficient for overgrowth. One must note also that although the rescued wings were surprisingly well proportioned, they lacked a proper margin in the posterior compartment (detail in Fig. 3F). Such tissue loss is likely to reduce the overall growth that can be achieved. The lack of margin bristles, which require maximal Wingless signalling for their formation, confirms that the signalling activity of Wingless^{S239A} was rather limited. Indeed, expression of *Sens*, a late target of enhanced Wingless signalling, was not activated by Wingless^{S239A} in Wingless-deficient posterior compartments of third instar wing imaginal discs (fig. S4C).

Intermediate and low signalling activity of Wingless triggers proliferation

Next, we sought to further define the amount of Wingless that was required to trigger proliferation. As mentioned above, the primary defect of Wingless^{S239A} is its relative scarcity in the extracellular space. To directly compare the amount of extracellular Wingless^{S239A} to that of extracellular wild-type Wingless, Wingless^{S239A} was produced in Wingless-deficient posterior compartments of larvae cultured at 25°C, and the discs were incubated with an antibody specific for Wingless according to a protocol designed to detect solely extracellular antigens (31). As expected, the signal was uniform in the posterior compartment (the *hhG4* expression region), whereas the normal gradient was detected in the anterior compartment (Fig. 4A). Thus, staining intensity in the two compartments could be compared (Fig. 4B). Fluorescence intensity scans revealed that, under these conditions, Wingless^{S239A} was present at an intermediate abundance at the cell surface: immunoreactivity was below the normal peak of the gradient but higher than in much of the tail end of the gradient (Fig. 4B). This was consistent with the behaviour of the target genes. When Wingless^{S239A} was the only source of signalling activity, *Sens* was not expressed (fig.

S2F), but the expression of Vg was increased to an extent higher than that seen at the tail end of the gradient in the control anterior compartment (Fig. 3H).

As shown above, this intermediate signalling triggered extra proliferation in the presence of endogenous Wingless (Fig. 2) without a noticeable change in the expression of Sens (fig. S4). Measurements of fluorescence intensity showed that the endogenous Wingless gradient was steep (Fig. 4, B and D) (32). Superimposition of the Wingless gradient onto the pattern of expression of sens and dlp (Fig. 4C) showed that the activation of Sens and the repression of Dlp corresponded to the presence of a high quantity of Wingless at the surface (Fig. 4, C and D). Further away from the site of Wingless production, around the area where Sens was no longer expressed, the quantity of Wingless dropped precipitously and remained nearly unchanged in the rest of the pouch (Fig. 4D). Therefore, in wild-type discs, it appears that most cells were exposed to a relatively low amount of Wingless.

DISCUSSION

In this study, we found that an attenuated form of Wingless, Wingless^{S239A}, which cannot activate the expression of so-called high target genes, increased the proliferation of prospective wing cells in *Drosophila*. Because high Wingless signalling is associated with a premature stop in proliferation, it appears that Wingless has a bimodal effect on proliferation in this tissue, inhibiting proliferation when at a high abundance and stimulating proliferation when at an intermediate abundance. The positive effect on proliferation is consistent with the observation that wing size is strongly reduced in the absence of Wingless and suggests that, in wild-type wings, Wingless might contribute positively to proliferation, except very near the source, where it would be inhibitory.

The availability of an attenuated form of Wingless enabled us to devise a direct test of whether graded Wingless signalling was required in the pro-proliferative region. Wingless^{S239A} was added uniformly to tissue lacking endogenous Wingless, thus creating a situation in which the only source of Wingless, although attenuated, was uniformly distributed. In such a condition, the proliferation deficit caused by the absence of Wingless was fully rescued by the uniform presence of Wingless^{S239A}. This result provides strong evidence that, during the main period of wing growth (72 to 120 hours AEL), a graded distribution of Wingless is not required for it to stimulate cell proliferation. A role for graded Wingless signalling before this stage, although unlikely, cannot be excluded.

Sharp differences in signalling by Dpp, another important wing morphogen, trigger ectopic proliferation (11), suggesting that, in contrast to Wingless, graded Dpp could be used as a proliferative cue. However, another study shows that proliferation proceeds under conditions of uniform Dpp signalling (14). Excess proliferation at sharp Dpp signalling interfaces (11) could conceivably be a response to pattern discontinuity and does not necessarily demonstrate the need for graded Dpp signalling during normal growth. Therefore, spatially graded morphogen signalling is unlikely to be a key determinant of proliferation during normal development of the wing imaginal disc. However, our data do not exclude the possibility that either another graded activity, for example, that of Fat-Hippo signalling (13) or an autonomously maintained gradient of Vg, controls tissue growth (33-35). Nevertheless, it is unlikely that such a growth-promoting gradient would be continuously sustained by graded morphogen signalling. Perhaps Wingless or Dpp can be seen as permissive signals that modulate or amplify the action of instructive growth regulators? It is worth noting that proliferation continues (albeit in a much reduced manner) in the absence of Wingless. One possibility is that Dpp and Wg have an additive role in controlling wing growth; that is, Dpp may be able to contribute to residual growth in the absence of Wingless.

Alternatively, as mentioned above, additional pro-proliferative cues, either acting at a short range (13, 36-38) or relying on uncharacterized long-range signals (37), could be at work.

How does Wingless promote uniform growth in the wing disc outside of the DV boundary? Fluorescence intensity measurements showed that the Wingless gradient was very steep, dropping off rapidly away from the source. Therefore, there is a relatively narrow region in which Wingless signalling is sufficient to cause premature arrest of proliferation and activation of the expression of *sens* during the third instar stage (Fig. 4E). In much of the rest of the pouch, the prospective wing is exposed to relatively low amounts of Wingless (Fig. 4E). Such a low abundance of Wingless is required to increase proliferation because, in the absence of Wingless, proliferation is much reduced (although not completely eliminated). Interestingly, in this signalling range, there is scope for increased proliferation in response to higher amounts of Wingless (Fig. 4E). One would therefore expect that in the wild-type fly, proliferation would increase where Wingless in the gradient increases from its low abundance while still remaining below the amount required for the expression of *sens*. Because of the steepness of the gradient of Wingless, however, the area where this range of Wingless signalling occurs is very narrow, and this probably explains why a zone of increased proliferation cannot be detected in wild-type discs (Fig. 4E). Nevertheless, our results show that much of the wing imaginal disc has an inbuilt potential for Wingless-dependent extra growth (Fig. 4E) and that such potential remains untapped under normal circumstances. It is difficult to assess whether this feature is functionally important, but it is conceivable that it could enable extra growth in some aberrant developmental situations (39). It could also be a relevant target for microevolution and size-scaling of organs in response to external factors, because it would provide a mechanism to modulate wing size without causing profound changes in pattern formation.

MATERIALS AND METHODS

Fly strains

All of the fly strains used in this study are described at <http://flybase.org>, unless otherwise indicated. UAS-Wingless^{S239A} was described by Franch-Marro et al.(27). All experiments were carried out at 25°C, unless otherwise indicated.

Measurements of adult wings

The surface areas of wings or wing compartments were measured in >10 adult wings with ImageJ. To obtain an estimate of cell density in adult wings, the density of trichomes was used as a proxy because each cell makes one trichome (40).

Mosaic analysis, cell-doubling time, and measurement of clone size

Marked clones were generated either in normal discs or in discs expressing UAS-Wingless^{S239A} under the control of *hhG4*. For the latter, the genotype was *ywf 36a hsp70-flp; FRT40A Ubi-GFP/FRT40A ck1 f+; hhG4/UAS-Wingless^{S239A}*. Clones were induced at 48 to 72 hours AEL by a 25-min heat shock at 37°C, and the larvae were fixed 48 hours after the heat shock. Details on clone size measurement are as follows. Clone areas were measured in ImageJ in projections obtained from at least 20 confocal sections. Clone areas were then normalized to the area of the pouch (defined as the area within the first fold; see detail in fig. S3). In this experiment, the presence of nuclear green fluorescent protein (GFP) enabled us to count the number of cells in the clones. This was used to estimate the cell doubling time as described previously (4). Clones expressing UAS-Wingless^{S239A} (induced at 48 to 72 hours) were generated in the following genotypes:

yw hs-flp1.22; actin FRT y + FRTGal4,UAS-GFP; UAS-Wingless^{S239A} (actin Flp-out clones);

yw hsp70-flp; tubulin FRT stop FRT Gal4,UAS-lacZ; UAS-Wingless^{S239A} (tubulin Flp-out clones).

These clones were analyzed in larvae 120 hours AEL by staining with an antibody specific for PH3. To generate posterior compartments depleted of Wingless-expressing cells, larvae of the following genotypes were used:

FRT40A Cyc-E UbiGFP/FRT40A wgCX4; *hhG4* UAS-Flp/+;

FRT40A M arm-lacZ/FRT40A wgCX4; *hhG4* UAS-Flp/+. Removal of the essential signalling receptors, Fz and Fz2, is a more efficient means of abrogating Wingless signalling (22). However, because rescue by Wingless^{S239A} cannot be assessed in an *fz fz2* background, all of the rescue experiments described here were performed in a wingless mutant background. To generate a Wingless-deficient posterior compartment that also expressed either UAS-Wingless^{S239A} or UAS-P35, the following genotypes were used:

FRT40A Cyc-E UbiGFP/FRT40A wgCX4; *hhG4* UAS-Flp/UAS-Wingless^{S239A};

FRT40A M arm-lacZ/FRT40A wgCX4; *hhG4* UAS-Flp/UAS-Wingless^{S239A};

FRT40A Cyc-E UbiGFP/FRT40A wgCX4; *hhG4* UAS-Flp/UAS-P35;

FRT40A M arm-lacZ/FRT40A wgCX4; *hhG4* UAS-Flp/UAS-P35.

In discs of the above genotypes, the relative number of proliferating cells (detected with an antibody against PH3) in the posterior compartment was measured at 96 to 110 hours AEL with ImageJ. These numbers were divided by the relevant surface area to obtain estimates of the density of the cells. The profile of the Wingless gradient and the expression of target genes were obtained by measuring fluorescence intensity with ImageJ.

Histology

BrdU labeling and immunostaining of imaginal discs were performed according to standard protocols. The following primary antibodies were used: mouse or rabbit antibody against HA, mouse antibody against BrdU, and mouse antibody against Dlp (Hybridoma Bank), rabbit antibody against P35 (Stratagene), rabbit antibody against Hsp23 (a gift of J. F. Santarén), rabbit (Upstate Biotechnology) or rat (Abcam) antibody against PH3, antibody against Vg (a gift of S. Carroll), antibody against Sens (a gift of H. Bellen), and chicken antibody against b-galactosidase (Abcam). Secondary antibodies labeled with Alexa 488, Alexa 555, or Cy5 were obtained from Molecular Probes. Extracellular staining was performed as described by Strigini and Cohen (31). Imaginal discs were mounted in Vectashield (Vector Laboratories). Micrographs were acquired with a Leica SP5 confocal microscope. Images were processed with Photo-shop CS3 (Adobe).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Wingless signalling has a bimodal effect on cell proliferation. (A) *nubG4; TubG80^{ts}* wing (control) at 29°C. (B) *nubG4/UAS-Arm^{S10}; TubG80^{ts}* wing at 29°C. (C) Detail from (B). The red arrowhead points to an ectopic bristle. Scale bars represent 0.25 mm. (D) The presence of Hsp23, Sens, and Dlp, detected by immunofluorescence in a *nubG4; TubG80^{ts}* disc cultured at 29°C (control). (E) Expression of the same proteins in a *nubG4/UAS-Arm^{S10}; TubG80^{ts}* disc cultured at 29°C. Note the expansion of the areas in which Hsp23 and Sens (markers of the wing margin) are produced and the reduction of the area in which Dlp (a marker of wing blade) is found. The effects were weaker in the central domain of the wing where *nubG4* was less abundantly expressed [red arrowhead in (E); see also fig. S1A]. (F to H) Wings from flies of the genotype *nubG4; TubG80^{ts}* [(F); control] or *nubG4/UAS-Arm^{S10}; TubG80^{ts}* [(G) and detail in (H)] raised at 25°C. Red arrowheads point to ectopic bristles (H). (I to K) Wings from flies of the genotype *nubG4; TubG80^{ts}* [(I); control] and *nubG4/UAS-Arm^{S10}; TubG80^{ts}* [(J) and detail in (K)] that were raised at 17°C. (L) Surface areas of wings from flies of genotype *nubG4; TubG80^{ts}* or *nubG4/UAS-Arm^{S10}; TubG80^{ts}* cultured at the indicated temperatures. The areas measured correspond to those shaded in green in (A). Error bars indicate SD, and statistical significance was assessed with a Student's t test. **P < 0.01, n > 10. All of the images shown are representative of more than 10 samples examined.

Fig. 2.

Moderate Wingless signalling stimulates cell proliferation. (A) *nubG4* wing from a fly raised at 25°C. (B) *nubG4/UAS-Wingless^{S239A}* (*UASwg[S239A]*) wing from a fly raised at 25°C. (C) *nubG4* wing from a fly raised at 22°C. (D) *nubG4/UAS-Wingless^{ts}* (*UASwg[ts]*) wing from a fly raised at 22°C. (E) *hhG4* wing from a fly raised at 25°C. Shading marks the A (blue) and P (green) compartments (E). (F) *hhG4 UAS-Wingless^{S239A}* wing at 25°C. (G) Mean surface area of whole wings (A+P), anterior compartments (A), or posterior compartments (P) in *hhG4* (black bars; control) or *hhG4 UAS-Wingless^{S239A}* (white bars). Statistical significance was assessed with the Student's t test. **P* < 0.05, ***P* < 0.01; *n* > 10. Error bars indicate the SD. (H) Clonal growth of "equivalent" twin spots [one sister is marked by the absence of GFP (absence of white staining on the right-hand side) whereas the other is marked with 2· GFP, intense white staining on the right-hand side]. These twin spots were generated in an *hhG4/UAS-Wingless^{S239A}* background. (I) Histogram showing the surface area of 2· GFP clones, located in the anterior (A, control, black bars) or posterior (P, expressing *Wingless^{S239A}*, green bars) compartments (*P* < 0.0001, Mann-Whitney test). (J) Nuclear counts in the clone were used to deduce the cell-doubling time (in hours) in the anterior (black bars) and posterior compartments (white bars) of *hhG4* (control, left) and *hhG4/UAS-Wingless^{S239A}* (right) discs. (K) Cell proliferation (as assayed with an antibody specific for PH3, labeled in purple) was relatively increased in the *Wingless^{S239A}*-expressing posterior compartment. Three representative examples of (K) were recorded with *n* > 10 for all other panels. Scale bars represent 0.5 mm.

Fig. 3.

Wingless signalling promotes cell proliferation in a gradient-independent manner. (A to H) Imaginal discs and wings comprising a posterior compartment composed entirely of *wg*CX4 homozygous cells (recognized by the absence of GFP). In (E) to (H), all of the cells of the posterior compartment expressed Wingless^{S239A} (*hhG4* UAS-Wingless^{S239A}) in addition to being devoid of endogenous Wingless. Wg (A and E), Vg (C and G), and PH3 (D and H) stainings are shown in purple. (I) Histogram showing the ratio between the surface areas of the anterior and posterior compartments in various genetic backgrounds as described below. In the wild type, the A/P ratio was around 1.1. This ratio was slightly changed by the presence of P35 in the posterior compartment (black bar). Lack of Wingless in the posterior compartment [as in (A) to (D)] caused a reduction in surface area, hence an increased A/P ratio (gray bar). This reduction was rescued by adding Wingless^{S239A} [white bar, genotype as in (E) to (H)]. By contrast, P35 had only a mild rescuing effect (purple bar; see also fig. S2, C and D). (J) The surface density of PH3 staining was significantly rescued by the addition of Wingless^{S239A}. A student's t test was used to assess significance (***P* < 0.01). Error bars indicate the SD. See the Materials and Methods for more details about the genotypes used. Scale bars represent 50 μ m for confocal images and 0.5 mm for adult wings. More than 10 samples were analyzed for all panels except (F), which is representative of 8 samples. Wg^{S239A}-expressing flies had to be removed manually from the pupal case; they could not close naturally because of leg defects.

Fig. 4.

The abundance of extracellular Wingless determines its effect on growth and the expression of target genes. (A) The amount of extracellular Wingless produced by the UAS-Wingless^{S239A} transgene. The posterior compartment was devoid of endogenous Wingless and expressed UAS-Wingless^{S239A} under the control of *hhG4* (right half of the disc). White and blue dashes mark the lines used to generate fluorescence intensity scans. (B) Fluorescent intensity showing the relative amounts of extracellular Wingless in the anterior (A, black line) and posterior (P, blue line) compartments. (C) A disc from a wild-type fly stained at the end of third instar for extracellular Wingless (Extracell. Wg, green), Dally-like (Dlp, red), and Senseless (Sens, blue). White dashes define the line used for the fluorescence intensity measurements shown in (D), which shows the intensity profile for extracellular Wingless (blue line) and Dlp (red line). (E) Diagram correlating the presence of extracellular Wingless with the expression of target genes and the proliferative response. The bracket in (D) and (E) indicates the prospective wing margin. All of the panels are representative of more than 10 samples examined.