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A characterization of the effects of Dpp signaling on cell growth and proliferation in the *Drosophila* wing

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SUMMARY

Cell proliferation and patterning must be coordinated for the development of properly proportioned organs. If the same molecules were to control both processes, such coordination would be ensured. Here we address this possibility in the *Drosophila* wing using the Dpp signaling pathway. Previous studies have shown that Dpp forms a gradient along the AP axis that patterns the wing, that Dpp receptors are autonomously required for wing cell proliferation, and that ectopic expression of either Dpp or an activated Dpp receptor, Tkv^{Q253D}, causes overgrowth. We extend these findings with a detailed analysis of the effects of Dpp signaling on wing cell growth and proliferation. Increasing Dpp signaling by expressing Tkv^{Q253D} accelerated wing cell growth and cell cycle progression in a coordinate and cell-autonomous manner. Conversely, autonomously inhibiting Dpp signaling using a pathway specific inhibitor, Dad, or a mutation in *tkv*, slowed wing cell growth and division, also in a coordinate fashion. Stimulation of cell cycle progression by Tkv^{Q253D} was blocked by the cell cycle inhibitor RBF, and required normal activity of the growth effector, PI3K. Among the known Dpp targets, *vestigial* was the only one tested that was required for Tkv^{Q253D}-induced growth. The growth response to altering Dpp signaling varied regionally and temporally in the wing disc, indicating that other patterned factors modify the response.

Key words: *Drosophila*, Tkv, Proliferation, Growth, E2F, PI3K, Vestigial

INTRODUCTION

Development in multicellular organisms requires the coordination of growth and patterning. In the case of *Drosophila*, the developing adult epidermal tissues, partitioned into imaginal discs, grow at the same time that localized secreted signals effect patterning. The secreted factors Hedgehog (Hh), Wingless (Wg) and Decapentaplegic (Dpp) form gradients that confer positional identity to cells along the different axes (Lawrence and Struhl, 1996). In the wing imaginal disc, a narrow strip of cells just anterior to the anteroposterior (AP) compartment boundary expresses Dpp, a BMP2/4 homolog. Type I serine/threonine kinase receptors transduce the signal after ligand (Dpp) binding to type II serine/threonine receptors and heterotetramerization (Massagué, 1998). To date, Smad proteins are the only BMP receptor substrates known to propagate Dpp signaling intracellularly. Phosphorylated Smads move to the nucleus where they act as transcriptional co-repressors or co-activators (Massagué and Chen, 2000). Two type I BMP receptors have been described in *Drosophila*. Thick veins (Tkv) is the principle Dpp receptor and the Saxophone receptor (Sax) can synergize with Tkv signaling mainly via another BMP family member, GGB-60A, in areas of low Dpp concentration (Singer et al., 1997; Haerry et al., 1998). Cells at different distances from the Dpp source are exposed to different levels of Dpp and, as a consequence, different sets of target genes are activated (Nellen et al., 1996; Lecuit et al., 1996). For example, *spalt* is expressed only in cells close to the AP boundary because its activation requires high levels of Dpp, whereas *optomotor-blind* (*omb*) is expressed in cells extending further from the AP border, because its activation requires less Dpp. Thus, the Dpp gradient divides the wing disc into different regions, each expressing a different combination of Dpp target genes.

Imaginal wing cells divide exponentially during larval development and in 3.5 days the number of cells increases 1000-fold (Bryant and Simpson, 1984). The cells divide with an 8-14 hour cell cycle that resembles that of vertebrate cells in having both G1 and G2 gap phases. Cyclin E is the limiting factor for the G1/S progression, and the Cdc25 homolog String is the limiting factor for G2/M transition (Milán et al., 1996; Neufeld et al., 1998). Clonal analysis has shown that cells in all regions of the wing disc divide during the larval period (González-Gaitán et al., 1994**)**. Although the wing cells do divide at different rates according to position (Garcia-Bellido and Merriam, 1970), obvious patterns of cell division are not observed until the late third larval instar when cells in the 'zone of non-proliferating cells' (ZNC) transiently arrest their division (O'Brochta and Bryant, 1985; Johnston and Edgar, 1998).

An early and still influential model that addressed the coordination between patterning and growth in imaginal discs

is the polar-coordinate model (French et al., 1976; Bryant and Simpson, 1984). This model is partially based on the observation that surgical removal of a fragment of an imaginal disc triggers regenerative growth, such that both the missing tissue and missing positional information are restored. The model supposes that discontinuities in positional information at the edges of a surgical cut stimulate cell proliferation, and cell intercalation, in order to recover missing positional values. Regeneration stops when cells are once again surrounded by other cells with similar positional information, as in the unperturbed situation. When applied to normal disc growth, this idea suggests that cell proliferation may be a consequence of steep slopes in the gradients of morphogens that confer cell identities, such as the one established by Dpp (Lawrence and Struhl, 1996). In this case, cell proliferation would be expected to subside when the slopes of these morphogen gradients were reduced as a consequence of growth of the tissue (Serrano and O'Farrell, 1997; Day and Lawrence, 2000).

According to this view, non-autonomous effects on cell proliferation should be observed whenever discontinuities in a morphogen gradient are generated experimentally. Yet, except in the case of regenerating disc fragments, this has not been reported. Genetic manipulations in vivo have provided little if any direct evidence that a gradient of Dpp is required for tissue growth during normal wing disc development. On the contrary, overexpression of Dpp in patterns expected to abolish the gradient fails to block growth of the disc, and instead results in overgrowth (Nellen et al., 1996; Haerry et al., 1998). Nevertheless, there is ample evidence that Dpp signaling regulates cell growth in the wing. For example, clones of cells that lack the Dpp receptors Thick veins (Tkv) or Punt, or downstream transducers such as Mad and Schnurri, are smaller than their sister clones and are eventually eliminated from the wing cell population (Burke and Basler, 1996; Kim et al., 1997). Additionally, ectopic expression of Dpp or an activated version of Tkv (Tkv^{Q235D}) results in massive over-proliferation of cells, often forming wing outgrowths or duplications (Capdevila and Guerrero, 1994; Zecca et al., 1995; Burke and Basler, 1996; Lecuit et al., 1996; Nellen et al., 1996). Taken together, these data suggest that Dpp might promote cell growth and/or proliferation directly, in a cell autonomous manner. We have aimed to clarify the relationship between patterning and growth by characterizing, in detail, the cell autonomous effects of activating or inhibiting Dpp signaling in the wing disc.

MATERIALS AND METHODS

Fly stocks

y w hs-flp^{122}
y w hs-flp^{122}; UAS-tkv^{Q253D} UAS-CD2 (III) (Nellen et al., 1996)
y w hs-flp^{122}; UAS-RBF/SM6-TM6B (II) (Neufeld et al., 1998)
y w hs-flp^{122}; UAS-RBF; UAS-tkv^{Q253D} UAS-CD2/ SM6-TM6B
y w hs-flp^{122}; UAS-Δp60 (II) (Weinkove et al., 1999)
y w hs-flp^{122}; UAS-Δp60; UAS-tkv^{Q253D} UAS-CD2
y w hs-flp^{122}; UAS-dad (II) (Tsuneizumi et al., 1997)
w; Act5c>CD2>Gal4 UAS-GFP (III) (Neufeld et al., 1998)
w; UAS-p35; Act5c>CD2>Gal4 UAS-GFP (Neufeld et al., 1998)
y w hs-flp^{122}; M(2)32^{A1} π-myc 52A Ub-GFP FRT40A (52A Ub-GFP) (W. Bender)
w; *tkv*^{7} FRT40A/SM6-TM6B (Penton et al., 1994)
w; FRT 40A
A9 Gal4 (X) (Haerry et al., 1998; Khalsa et al., 1998)
l(1)omb^{3198}/FM6y; Act5c>CD2>Gal4 UAS-GFP (*l(1)omb*^{3198}) (Grimm and Pflugfelder, 1996)
y w; 70 FLP3A/CyO P[y^{+}]; UAS-tkv^{Q253D} UAS-CD2 (70FLP3A) (Golic et al., 1997)
y w hs-flp^{122}; *vg*^{83b27R}/CyO Act5c-GFP; UAS-tkv^{Q253D} UAS-CD2/TM6B (*vg*^{83b27R}) (Williams et al., 1990) CyO Act5c-GFP: Bloomington Center)
w; *vg*^{83b27R}/CyO Act5c-GFP; Act5c>CD2>Gal4 UAS-GFP
y w hs-flp^{122}; *cdk4*^{3}; UAS-tkv^{Q253D} UAS-CD2 (*cdk4*^{3}) (Meyer et al., 2000)
y w; *cdk4*^{3}/CyO P[y^{+}]; Act5c>CD2>Gal4 UAS-GFP

Cell doubling time, clone area measurements and induction of transgenes

Cell doubling time calculation and clone area measurement were as described previously (Prober and Edgar, 2000). Flp/Gal4 clones (Struhl and Basler, 1993; Pignoni and Zipursky, 1997; Neufeld et al., 1998) were generated by crossing females y w hs-flp^{122} or y w hs-flp^{122}; UAS-X to males w; UAS-p35; Act5c>CD2>Gal4 UAS-GFP. Larvae were heat shocked 72 hours after egg deposition (AED) for 20 minutes at 37°C, dissected and fixed at 99 hours AED. To measure clone area, females y w hs-flp^{122} or y w hs-flp^{122}; UAS-tkv^{Q253D} UAS-CD2 were crossed with males w; Act5c>CD2>Gal4 UAS-GFP or w; UAS-p35; Act5c>CD2>Gal4 UAS-GFP. Larvae were heat shocked at 48 hours AED for 20 minutes at 37°C, and dissected and fixed at 120 hours AED. Clones were scored in medial or lateral areas, or in the entire wing pouch. For the BrdU experiment shown in Fig. 2D, clones were induced at 48 hours with a 30-minute heat shock at 37°C and analyzed at 96 hours AED. Clones in experiments shown in Fig. 1A, Fig. 5 and Fig. 7B were induced with a 30-minute heat shock at 37°C and analyzed at 120 hours AED. Clones in Fig. 6 correspond to the same experiment shown in the FACS profile. For the experiment shown in Fig. 7A, larvae were heat shocked at 72 hours AED for 1.5 hours at 37°C, and wing discs dissected at 120 hours AED. For the experiment shown in Fig. 3B, females y w hs-flp^{122} or y w hs-flp^{122}; UAS-X were crossed with males A9-Gal4 and imaginal discs were dissected from female larvae at 120 hours AED. Cell death was visualized with Acridine Orange as described (Neufeld et al., 1998).

Mitotic recombination

Mitotic recombination was induced using the FLP/FRT method (Xu and Rubin, 1993). Females y w hs-flp^{122}; M(2)32^{A1} π-myc 52A Ub-GFP FRT40A were crossed with males w; *tkv*^{7} FRT40A/SM6-TM6B or w; FRT 40A. Larvae were heat shocked at 37°C for 2 hours at 72 hours AED. Tb^{+} animals were dissected at 168 hours AED (late third instar, delay is due to the Minute genetic background).

Flow cytometry

Females y w hs-flp^{122}; UAS-X or y w hs-flp^{122} were crossed with males w; Act5c>CD2>Gal4 UAS-GFP. Larvae were heat shocked at 48 hours (Figs 5, 6) or 72 hours AED (Fig. 1B) at 37°C for 1.5-2 hours to obtain about 50% of experimental cells. For the experiment in Fig. 6, larvae were heat shocked for only 50 minutes. Tb^{+} larvae were selected for dissection at 120 hours AED. Approximately 20 discs per genotype were dissociated for experiments in Fig. 1B, Fig. 4, and 50 for experiments in Figs 5, 6. FACS analysis was performed as described (Neufeld et al., 1998).

Histology

Larvae were dissected in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde/PBS for 20-30 minutes. For the experiment with *vg*^{83b27R} tissue were fixed in 8% paraformaldehyde for 1 hour, as the normal fixation protocol proved insufficient. Hoechst 33258 (Acros, 1 μg/ml) was used to label nuclei. Wing imaginal discs were

mounted in Fluoroguard (BioRad). BrdU (Sigma, 100μg/ml) incorporation was carried out in culture for 30 minutes before tissue fixation (Johnston and Schubiger, 1996). For immunostaining, discs were blocked for 1 hour at room temperature with 5% normal goal serum in PBS 0.1% Tween 20 (PBT). The following antibodies were used in overnight incubations at 4°C in PBT: mouse anti-BrdU (Becton Dickinson, 1:100), rabbit anti-Stg (AP RBRFB 1:10), guinea pig anti-CycE (1:400, T. Orr-Weaver), rabbit anti-phospho histone H3 (D. C. Allis, 1:2000), rabbit anti-phospho Mad (SP1 1:10000, C.-H. Heldin), rabbit anti-Spalt (AP 1:30, R. Schuh), mouse anti-CycD (DCDII 1:20, W. Du) and rabbit anti-Vg (AP 1:20, S. B. Carroll). Incubations with secondary antibodies were done at room temperature for 3-4 hours in PBT. Anti-mouse or anti-rabbit Cy3 (Jackson ImmunoResearch, 1:500) was used except for the CycE antibody, in which case signal was detected with Alexa Fluor 568 anti-guinea pig (Molecular Probes, 1/1500). Fluorescent images were collected on a Leica TCSSP confocal microscope for Fig. 2D (20×), Fig. 3A and Fig. 4 (10×1.21 zoom), and Figs 5, 6 (40× magnification); on a Deltavision S/A30 microscope for Fig. 3B (20×); or on a Leitz DMRD epifluorescence microscope with a RT SLIDER SPOT Digital Camera (Diagnostic Instruments, Inc.) for Fig. 1A, Fig. 7 (10× or 20× magnification). For Deltavision images in Fig. 3B, 'stitch' function was used.

RESULTS

Tkv^{Q253D} accelerates cell proliferation

To address the cell autonomous effects of the Dpp signaling pathway, we have used the Flp/Gal4 method to activate or suppress Dpp signaling in clones of cells marked with GFP (Struhl and Basler, 1993; Pignoni and Zipursky, 1997; Neufeld et al., 1998). First, we expressed a mutant version of the Dpp type I receptor Thick veins, Tkv^{Q253D}, containing a point mutation in the glycine/serine rich domain (GS). This mutation mimics the receptor phosphorylation that occurs upon ligand binding, and therefore renders the receptor constitutively active and ligand independent (Nellen et al., 1996; Lecuit et al., 1996). Tkv^{Q253D} expression strongly activates the Dpp signaling pathway, inducing high levels of the phospho-Mad transducer and expression of two Dpp targets, *omb* and *spalt* (Nellen et al., 1996; Lecuit et al., 1996; Jazwinska et al., 1999; Tanimoto et al., 2000). First we induced clones of cells that expressed Tkv^{Q253D} in early second instar larvae (at 48 hours AED) and allowed the cells to proliferate until the end of larval development (120 hours AED). As noted in previous studies (Nellen et al., 1996; Burke and Basler, 1996), wing cell clones expressing Tkv^{Q253D} showed smooth borders compared with control clones, which showed jagged borders, and were also larger than control clones (Fig. 1A). This phenotype was stronger in lateral areas of the disc, far from the endogenous Dpp source. Approximately half of the lateral clones were completely round and bulged out of the disc epithelium, generating extra folds around them (not shown). This phenotype was not seen when Tkv^{Q253D} was expressed throughout the disc (see Fig. 3B, Fig. 7A), indicating that the round bulging clonal phenotype is a consequence of abnormal heterotypic interactions between Tkv^{Q253D}-expressing cells and wild-type cells.

Induction of clones by heat shock allowed us to control the age of the clones, and to infer cell proliferation rates from the number of cells per clone. As cell death was observed by Acridine Orange staining in Tkv^{Q253D}-expressing clones (not shown), we co-expressed the apoptotic inhibitor p35 to block cell death (Hay et al., 1994). This is necessary to obtain accurate proliferation rate measurements, which are

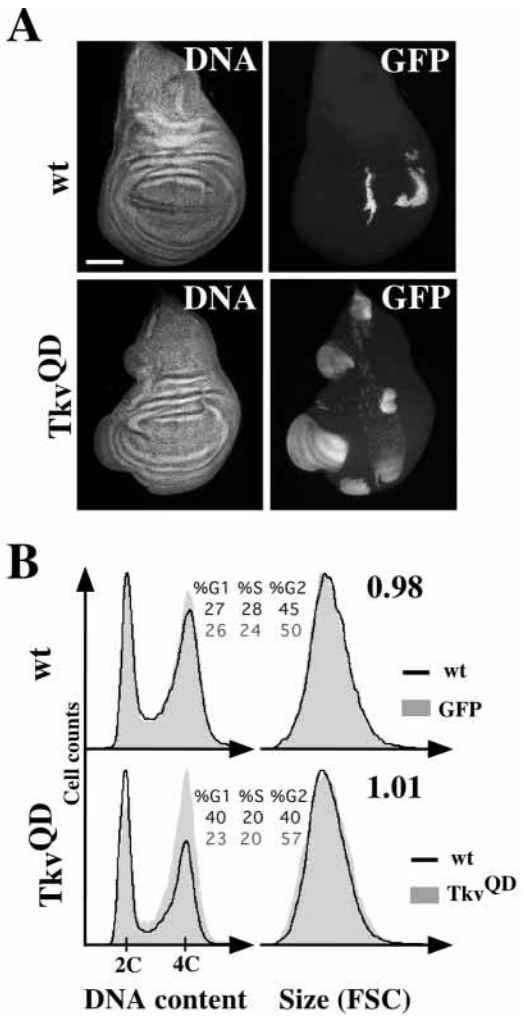

Fig. 1. (A) Activation of Dpp signaling using the activated receptor Tkv^{Q253D} produces large clones with smooth borders that minimize contact with neighboring wild-type cells. Left panels show digital camera images of the disc morphology by Hoechst 33258 DNA stain. Right panels show Flip-out/Gal4 clones in the same tissue marked with GFP. Tkv^{QD} clones are round (GFP), compare Tkv^{QD} clones (bottom right) with wild-type clones (upper right). Tkv^{QD} clones seem also larger than control clones. This is most obvious in areas of the tissue far from the AP boundary. For all the imaginal disc images, dorsal is upwards and anterior is leftwards. Scale bar: 100 μm. (B) Tkv^{Q253D} overexpression alters cell cycle phasing but not cell size. A parallel experiment, in which only GFP was expressed, was used as an external control (wt, top panels). Cells in the same tissue not expressing GFP (and therefore no transgene) were used as an internal control. Black traces correspond to the internal controls and gray filled traces correspond to the GFP-expressing experimental populations. Numbers in the top right corner of the size histograms show the ratio of the mean size of GFP^+ cells (experimental population) versus the mean size of GFP^- cells (control population) from the forward scatter data (FSC). Expression of the activated receptor Tkv^{QD} causes a decrease in the G1 population and an increase in G2 population. Tkv^{QD} does not dramatically affect cell size. Tkv^{QD} has the same effects in notum and wing regions (data not shown).

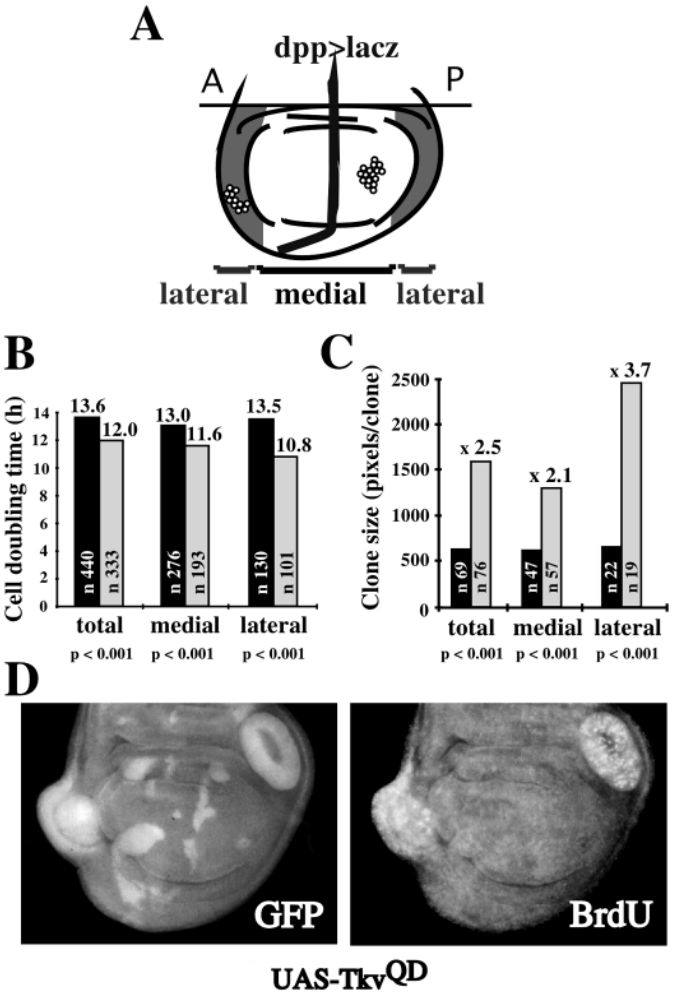

Fig. 2. Tkv^{Q253D}-expressing cells proliferate and grow faster than do wild-type cells. (A) Area definition for doubling time and clone area measurement experiments. *dpp* expression is indicated by the black line down the center of the disc. For wing definition, the longest fold along the AP axis was used as a border. For mediolateral definition, the most lateral folds were used. Clones exterior to them were considered lateral clones, and clones interior to them were considered medial clones. (B) Cell doubling times of Tkv^{QD}-expressing cells and control cells expressing GFP alone. The caspase inhibitor p35 was co-expressed in both experiments. Clones were scored in the entire wing (total), and in medial and lateral areas. Numbers of clones counted in each area are noted inside the bars. Black bars represent the control experiment and gray bars the Tkv^{QD} samples. Numbers on top of each bar correspond to the cell doubling time. *P* values are noted below each category. Tkv^{QD} cell doubling time is significantly reduced and lateral cells are dividing with a 20% shorter cell cycle (2.7 hours shorter, from 13.5 to 10.8 hours doubling time). (C) Clone size of Tkv^{QD} and wild-type Flip-out/Gal4 clones shown as pixels per clone. Nomenclature and color code as in B. Numbers on top of the gray bars correspond to the increase in Tkv^{QD} clone size compared with control clones. Tkv^{QD} clones are significantly larger than control clones and lateral clones are more than 3.5 times larger. (D) S-phase cells visualized by BrdU incorporation in Tkv^{QD} Flip-out/Gal4 clones. Left panel shows clone position by GFP signal and right panel shows BrdU incorporation. Lateral clones show a strong BrdU incorporation.

confounded by cell death. We induced clones and let them proliferate for a short time in the period of larval development when imaginal wing cells proliferate exponentially. Because of the regional phenotype described above, we counted the number of cells per clone in lateral and medial areas, as well as in the entire presumptive wing region (see Fig. 2A for area definition). Cells over-expressing the activated Dpp receptor proliferated faster than control cells (Fig. 2B, Table 1). This phenotype was stronger in lateral areas, where Tkv^{Q253D}-expressing cells proliferated 20% faster than controls. Tkv^{Q253D}-expressing cells proliferated 10% faster than controls in the medial region. This regional phenotype reflects the graded activity of endogenous Dpp signaling (Tanimoto et al., 2000; Teleman and Cohen, 2000); lateral areas normally low in Dpp are more sensitive to signaling activation.

To further analyze the cellular phenotype, we performed flow cytometry (FACS) using co-expressed GFP to identify Tkv^{Q253D}-expressing cells. The GFP-negative cell population from the same discs was used as an internal control (Neufeld et al., 1998). GFP expression alone did not cause a significant change in the cell cycle profile. Tkv^{Q253D} overexpression shifted the distribution of cells in the different phases of the cell cycle. A smaller proportion of the Tkv^{Q253D}-expressing cells were in the G1 phase and greater proportion in G2 (Fig. 1B, DNA content histogram, Table 1). These data, together with the shorter doubling time of these cells, suggests that Tkv^{Q253D} preferentially promotes G1/S progression. This cell cycle phenotype was more severe if the activated receptor was expressed for a longer period of time (Figs 5, 6).

To address more carefully the autonomy of the effects of Tkv^{Q253D}, we analyzed the expression patterns of String and Cyclin E protein in discs containing Tkv^{Q253D}-expressing clones. String and Cyclin E limit progression of the imaginal disc cell cycle through G2/M and G1/S transitions, respectively (Milán et al., 1996; Neufeld et al., 1998). We also assessed S-phase progression in Tkv^{Q253D}-expressing clones using BrdU incorporation, and mitosis by phospho-Histone H3 detection. The BrdU incorporation assay yielded a result consistent with increased proliferation within Tkv^{Q253D}-expressing clones in lateral regions of the discs: these clones showed a uniform increase in BrdU uptake (Fig. 2D). Increased BrdU incorporation was limited to within the Tkv^{Q253D}-expressing clones, and no non-autonomous effects were detected. This

Table 1. Effects in response to Dpp signaling manipulation

Manipulation	Cell cycle	Cell size	Proliferation rate	Growth rate
UAS-Tkv^{Q253D}	↓G1 ↑S/G2*	⩾*	↑†	↑‡,§,¶
tkv^{7} mutant	↑G1 ↓S/G2*	=**	n.a.	↓**
UAS-Dad	n.a.	n.a.	↓††	↓‡‡

n.a., not addressed; ↑, increase; ↓, decrease; ⩾, slightly larger or similar; =, similar.
*Figs 1B, 5, 6.
†Fig. 2B.
‡Figs 2C, 3B, 5, 6.
§Nellen et al., 1996.
¶Haerry et al., 1998.
**Fig. 4.
††Fig. 3A.
‡‡Fig. 3B.

result implies that Tkv^{Q253D} stimulates cell proliferation cell-autonomously. We did not detect any changes in Cyclin E, String or phospho-Histone H3 expression levels in Tkv^{Q253D}-expressing clones or surrounding cells (data not shown).

Although raising the levels of Dpp signaling increased rates of cell proliferation, it did not appear to bypass the developmentally programmed proliferation arrest that occurs at the end of larval development (Schubiger and Palka, 1987). Tkv^{Q253D}-expressing clones induced late in larval development (96 hours AED) and analyzed 1 day after proliferation normally ceases (pupae at 168 hours AED) contained the same number of cells as control clones (data not shown). The same result was obtained when p35 was co-expressed. In addition, Tkv^{Q253D}-expressing clones induced early (48 hours AED) and analyzed in pupae (168 hours AED) did not contain mitotic cells, as visualized by anti-phospho Histone H3 staining (data not shown). This suggests that a dominant, developmentally programmed signal prevents Tkv^{Q253D}-expressing cells from continuing to divide beyond the normal proliferation stage.

Tkv^{Q253D} autonomously promotes cell growth

Induction of cell proliferation does not necessarily indicate increased growth (Neufeld et al., 1998). To more directly assess the ability of Tkv^{Q253D} to induce growth, we measured areas of the disc epithelium encompassed by Tkv^{Q253D}-expressing clones. Clones were induced early in larval development and analyzed at the end of the larval period. The average area of Tkv^{Q253D}-expressing clones was 2.5 times larger than that of control clones (Fig. 2C, Table 1), indicating that Tkv^{Q253D}-expressing cells grew faster than wild-type cells. This phenotype depended on the position of the clone in the anterior-posterior axis. Clones in the lateral areas, far from the source of endogenous Dpp, showed the strongest phenotype. Fifty percent of these lateral clones were larger than the largest control clone. On average, lateral clones expressing Tkv^{Q253D} were 3.7 times larger than lateral control clones (Fig. 2B). This effect on clone size was observed even without p35 co-expression (data not shown).

The cellular growth effects of Tkv^{Q253D} were further assessed using FACS analysis to measure cell size. We used the ratio of the mean forward light scatter (FSC) of GFP^+ cells versus GFP^- cells as a cell size indicator. GFP expression did not cause a significant change in cell size. Tkv^{Q253D}-expressing cells analyzed by FACS generally showed a size that was not significantly different from wild-type cells (Fig. 1B, size histogram; Table 1). In some experiments, however, these cells were slightly larger than controls (Fig. 6). The fact that Tkv^{Q253D}-expressing clones were much larger than controls, but consisted of cells of roughly normal size, confirms that Tkv^{Q253D} accelerated cell cycle progression. Taking our in situ and FACS analysis together, we conclude that activation of Dpp signaling coordinately increases both rates of cell proliferation and cell growth.

Inhibition of Dpp signaling suppresses cell cycle progression and cell growth

To complement these experiments, we analyzed the effects of autonomously inhibiting Dpp signaling by overexpressing the pathway-specific inhibitor Dad, or by generating cell clones mutant for *tkv*. Dad is an inhibitory Smad protein that, when overexpressed, blocks *omb* expression and the adult wing

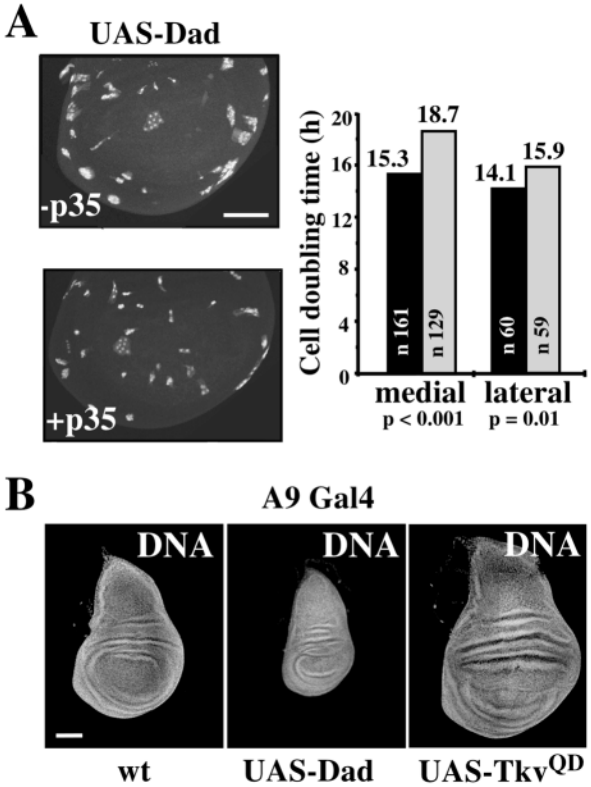

Fig. 3. Inhibition of Dpp signaling pathway by overexpression of Dad impairs cell proliferation and tissue growth. (A) Cell doubling times of Dad-expressing cells and control cells. Left panels correspond to confocal images showing GFP marked cells. Clones in the medial area of the tissue were recovered only when the apoptotic inhibitor p35 was co-expressed. The big cell clone in the -p35 experiment corresponds to a clone in the peripodial membrane and not to a clone in the columnar epithelium. Histogram nomenclature and color code as in Fig. 2B. Dad-expressing cells (gray bars) divide more slowly than do wild-type cells (black bars). Medial cells divided with a 22% longer cell cycle (3.4 hours longer, from 15.3 to 18.7 hours doubling time). (B) A9 Gal4 driver was used to express Dad or Tkv^{QD} throughout most of the wing disc. Cells were visualized by Hoechst 33258 DNA stain. Dad expression causes a reduction of the disc size (58% of wild-type size, n=7; Dad n=10, P<0.001), mainly along the AP axis. Conversely, Tkv^{QD} expression promotes enlargement of the disc (154% compared with wild-type size, n=7; Tkv^{QD} n=5, P<0.001). Deltavision images were acquired at the same magnification. Scale bars: 100 μm.

phenotypes induced by ectopic Dpp signaling (Tsuneizumi et al., 1997). It is normally activated by Dpp signaling and expressed in a broad domain centered on the AP axis. Smad inhibitory proteins are induced by the activation of similar pathways in other systems, and inhibit signaling in a negative regulatory loop by blocking Smad phosphorylation or translocation to the nucleus (Inoue et al., 1998; Massagué and Chen, 2000). When Dad was overexpressed using the Flp/Gal4 method, clones were not recovered in the dorsomedial area of wing blade (Fig. 3A). However, Dad-expressing clones were recovered in medial areas when the apoptotic inhibitor p35 was co-expressed (Fig. 3A). These clones contained fewer cells than controls, indicating that Dad overexpression impairs proliferation of cells at medial positions (Table 1). The cell doubling time of Dad overexpressing medial cells was more

than 3 hours (22%) longer than the control doubling time (Fig. 3A). Slow-growing cells are eliminated by a mechanism known as cell competition when normal growing cells surround them (Morata and Ripoll, 1975; Simpson and Morata, 1981). Because Dad overexpressing cells proliferate slowly, this may explain why they are not recovered unless the apoptotic inhibitor p35 is co-expressed.

To better understand the basis of this proliferative defect, we generated *tkv*$^{-}$ clones by mitotic recombination (Burke and Basler, 1996; Singer et al., 1997). We used a recessive lethal allele, *tkv*^{7}, that carries a point mutation in a conserved glutamate residue in the kinase domain and results in loss of expression of Dpp targets (Penton et al., 1994; Campbell and Tomlinson, 1999). In the medial wing pouch, *tkv*^{7} clones survived for 36 hours but were lost within 48 hours of induction (in the 72-120 hours AED interval). In lateral areas, *tkv*^{7} mutant clone survival was greater, however mutant clones were still small compared with wild-type twin spots, and showed round morphology (data not shown). This lateral-medial survival phenotype has been previously reported using different *tkv* alleles, and reflects the lower requirement for Dpp signaling in lateral areas of the wing imaginal disc (Burke and Basler, 1996). However, no cellular phenotypes were described in that study.

We next used flow cytometry to analyze *tkv*^{7} cells. To counteract cell competition and enrich the population of mutant cells, we used a cell lethal *Minute* mutation, M(2)32^{A1}, that carries a lesion in ribosomal protein S13, and slows growth when heterozygous (Saeboe-Larssen and Lambertsson, 1996). As M$^{-/-}$ cells are not viable, only M$^{+/+}$ cells were recovered after mitotic recombination. These M$^{+/+}$ cells were *tkv*^{7} homozygous. In the Minute background, *tkv*^{7} cells survived at least 4 days and colonized more tissue than in a wild-type background. However, they were still growth impaired relative to wild-type cells growing in the same Minute$^{+/-}$ background, and they still appeared mainly in lateral areas (Fig. 4, arrows). Approximately 30% of the *tkv*^{7} discs showed an aberrant morphology (Fig. 4), probably caused by abnormal adhesive interactions between mutant and wild-type cells. *tkv*^{7} cells showed a cell cycle profile consistent with a proliferation defect; the S phase fraction was extremely reduced and the G1 fraction was increased (Fig. 4, DNA content histogram; Table 1). This phenotype was opposite to that of cells overexpressing Tkv^{Q253D}, which had a shortened G1 (Fig. 1B, Fig. 5, Fig. 6). FACS analysis also showed that *tkv*^{7} cells were not detectably different in size from control cells (Fig. 4, size histograms; Table 1). Previous studies indicate that when cell cycle progression is specifically delayed, cell size increases as cells continue to grow at the normal rates (Nasmyth and Nurse, 1981; Weigmann et al., 1997; Neufeld et al., 1998). As *tkv*^{7} cells proliferated very slowly while maintaining a normal cell size, evidently they were impaired for growth as well as cell cycle progression.

Interestingly, M(2)32^{A1}/+ cells were larger than wild-type cells (Fig. 4, size histogram in wild-type experiment). This suggests that these cells divided more slowly than they grow, and thus that the growth defect caused by the Minute mutation affects cell cycle progression preferentially. In fact, in both budding and fission yeast cell cycle control genes are sensitive to translational conditions (Polymenis and Schmidt, 1997; Daga and Jiménez, 1999; Grallert et al., 2000). Our studies using another Minute mutation that encodes a ribosomal protein, M(3)95A, detected no size alteration in M/+ cells, and thus this effect may be gene specific (Neufeld et al., 1998) (T. Reis and B. A. E., unpublished).

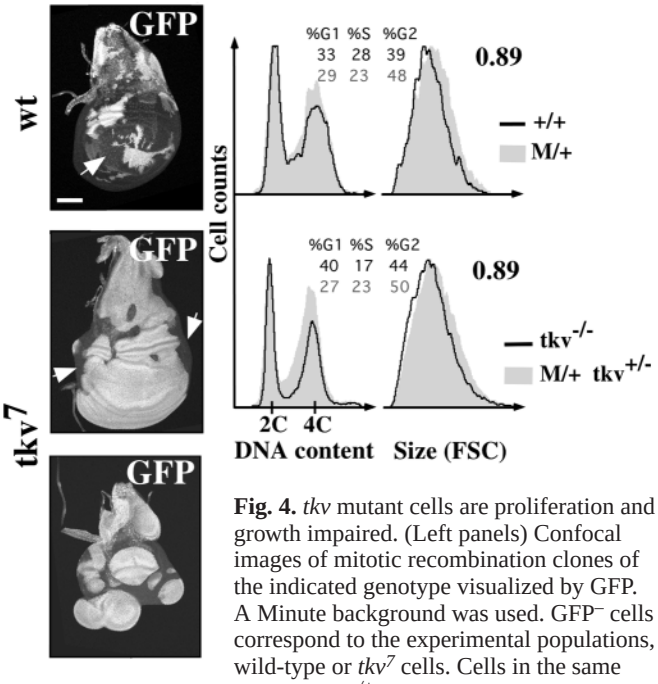

Fig. 4. *tkv* mutant cells are proliferation and growth impaired. (Left panels) Confocal images of mitotic recombination clones of the indicated genotype visualized by GFP. A Minute background was used. GFP^{-} cells correspond to the experimental populations, wild-type or *tkv*^{7} cells. Cells in the same disc expressing GFP and therefore *M*$^{-/+}$ (wild-type experiment) or *M*$^{-/+}$ *tkv*$^{7/+}$ (*tkv*^{7} experiment) were used as an internal controls. *tkv*^{7} cells colonize much smaller an area than do wild-type cells and they are found mainly in the lateral areas (arrows, compare GFP^{-} population in wt and *tkv*^{7} experiments). Some *tkv*^{7} discs show an abnormal morphology (lower panel in *tkv*^{7} experiment). Scale bar: 100μm. (Right panels) FACS analysis of the same experiment as in left panels. Gray filled traces correspond to the internal GFP^{+} control cells, and black traces correspond to the GFP^{-} experimental populations. Numbers in the top right-hand corner in the size histograms show the ratio of the mean size of GFP^{-} cells (experimental population) versus the mean size of GFP^{+} cells (control population) from FSC data. *tkv*^{7} cells show a severe reduction in the S phase and G2 populations, and an increase in G1 population (DNA content histogram). Cell size is not affected. *tkv*^{7} has the same effects in notum and wing regions (data not shown).

Using a third approach to avoid the effects of cell competition, we induced Dad ubiquitously throughout the wing disc using the A9-Gal4 driver (Haerry et al., 1998; Khalsa et al., 1998). This caused a reduction of disc size (Fig. 3B; Table 1). This size reduction was especially pronounced along the AP axis and thus was opposite to the phenotype resulting from Tkv^{Q253D} expression using the same driver, which enlarged the wing disc preferentially along the AP axis (Fig. 3B, Table 1) (Haerry et al., 1998). These results are consistent with those described above in showing that inhibition of Dpp signaling reduces growth and impairs proliferation, whereas activation of Dpp signaling increases growth and accelerates proliferation.

Tkv^{Q253D} induced proliferation is suppressed by the *retinoblastoma* homolog RBF

The results presented so far show that Dpp signaling can regulate both cell growth and cell proliferation. Next, we

addressed the relationship between these two effects. Growth and proliferation could be two independent effects of the activation of Dpp signaling or, alternatively, the induction of proliferation might be a secondary consequence of increased cell growth. To distinguish between these possibilities, we simultaneously activated Dpp signaling and inhibited cell cycle progression by co-expressing Tkv^{Q253D} and RBF, a *Drosophila retinoblastoma* homolog. RBF negatively regulates E2F activity and specifically suppresses cell cycle progression when overexpressed (Du et al., 1996; Neufeld et al., 1998; Datar et al., 2000). Clones of cells co-expressing RBF and Tkv^{Q253D} contained as few cells as RBF-expressing clones (Fig. 5). These cells showed a less pronounced increase in S and G2 populations than cells expressing Tkv^{Q253D} alone, indicating that RBF blocked the proliferative effect of Tkv^{Q253D}. Interestingly, FACS analysis indicated that cells co-expressing RBF and Tkv^{Q253D} were significantly larger than cells overexpressing RBF alone (see FSC ratios in Fig. 5). This suggests that RBF-dependent inhibition of proliferation did not entirely block the ability of Tkv^{Q253D} to drive cell growth.

Clonal expression of Tkv^{Q253D} frequently caused lethality during pupal stages, and those animals that did eclose (28%, with a mild 30-minute heat shock at 48 hours AED) showed a variety of wing phenotypes, including extra vein tissue, vein loss and notches in the wing. Seven percent had outgrowths in proximal regions of the wing (not shown). Interestingly, co-expression of RBF with Tkv^{Q253D} suppressed these phenotypes. Animals co-expressing Tkv^{Q253D} and RBF eclosed at control rates and showed, at most, very mild morphological defects (not shown). We attribute this to the small size of these clones, which would contribute very little tissue to the adult even if they were not eliminated by cell competition (their likely fate). This rescue and the data presented above indicate that cell proliferation induced by Dpp signaling can be blocked by co-expressed RBF. Therefore, Dpp signaling probably requires E2F activity to stimulate cell proliferation.

Tkv^{Q253D} induced proliferation requires PI3K signaling

Next, we activated the Dpp pathway in growth-impaired cells. If growth and cell cycle progression were independently regulated by Tkv, we would expect to detect the proliferative effect of Tkv^{Q253D} even in growth-impaired cells. Alternatively, if Tkv^{Q253D} were to promote cell cycle progression indirectly via stimulating cellular growth, the proliferative effect of Tkv^{Q253D} should be inhibited when cell growth is impaired.

To suppress cell growth we overexpressed a truncated version of p60, Δp60. This is an adaptor molecule for the class I PhosphoInositide 3-Kinase (PI3K/Dp110 in *Drosophila*). Dp110 signaling has been characterized in *Drosophila* as a potent growth inducer (Weinkove et al., 1999; Böhni et al., 1999; Verdu et al., 1999; Brogiolo et al., 2001). Adaptor molecules, such as p60, bind to the Dp110 kinase and recruit it to the Insulin Receptor, allowing full activation of the enzyme (Wymann and Pirola, 1998). Δp60 binds the Insulin Receptor but cannot bind Dp110, and thus inhibits Dp110 signaling in a dominant-negative manner (Weinkove et al., 1999). When expressed in wing cells, Δp60 reduces cell size and strongly delays G1 progression (Weinkove et al., 1999) (Fig. 6). Flp/Gal4 clones expressing Δp60 contained very few

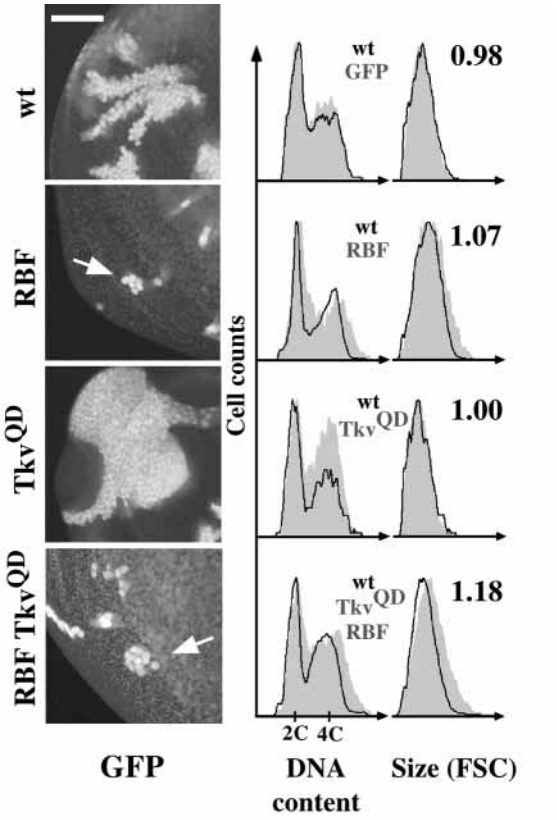

Fig. 5. RBF expression blocks Tkv^{Q253D} induced proliferation. (Left panels) Confocal images of Flip-out/Gal4 lateral clones of the indicated genotype visualized by GFP signal. (Right panel) FACS analysis of a similar timing experiment. Black traces show the GFP^- internal control population, and gray filled traces show the GFP^+ experimental population. Numbers in the top right-hand corner in the size histograms show the ratio of the mean FSC of GFP^+ cells (experimental population) versus the mean FSC of GFP^- cells (control population). Clones expressing RBF and Tkv^{QD} contain as few cells as RBF-expressing clones (arrows). These cells do not show the characteristic cell cycle profile of Tkv^{QD}-expressing cells (DNA content histogram) and are bigger than cells expressing RBF alone (size histogram). Scale bar: 50 μm.

cells compared with controls (Fig. 6). Overexpressed Δp60 also dominantly blocked the growth and proliferation effects of Tkv^{Q253D}. Clones of cells that co-expressed Δp60 and Tkv^{Q253D} contained as few cells as those expressing Δp60 alone, and these cells were just slightly larger than those expressing Δp60 alone (Fig. 6). Thus, loss of growth resulting from loss of PI3K activity cannot be rescued by hyperactivating Dpp signaling, and cell proliferation induced by Dpp probably requires Dp110 activity (Table 2). These results are consistent with the model in which Dpp-driven cell growth indirectly promotes cell cycle progression.

Although clonal growth was blocked by co-expressing Δp60 and Tkv^{Q253D}, cells that co-express Δp60 and Tkv^{Q253D} did not show the G1 delay characteristic of cells expressing Δp60 alone. Thus, Tkv^{Q253D} appeared to be able to promote G1/S progression even in the presence of Δp60 (Fig. 6). This suggests that some aspects of cell cycle progression induced by Tkv^{Q253D} may be Dp110 independent. However, the slight increase in size observed in cells co-expressing Δp60 and

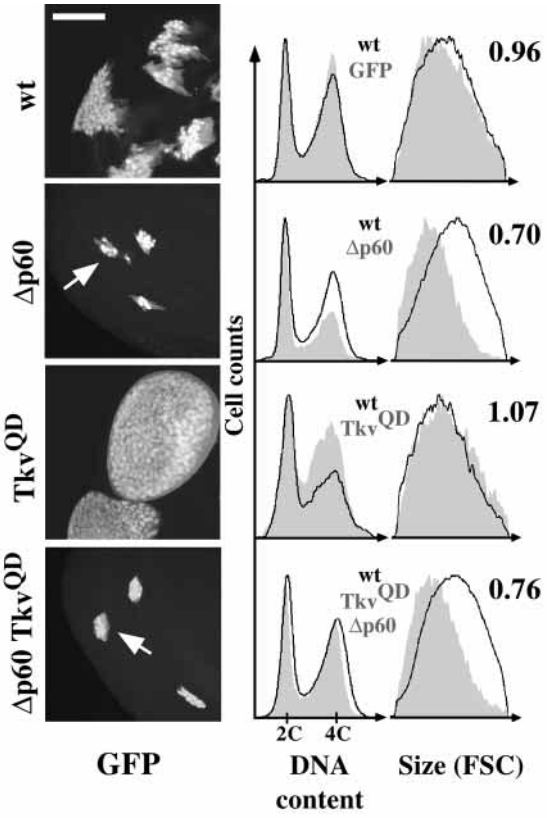

Fig. 6. PI3K/Dp110-dependent and -independent effects of Tkv^{Q253D} overexpression. (Left panels) Confocal images of Flip-out/Gal4 lateral clones of the indicated genotype visualized by GFP signal. (Right panel) FACS analysis of the same experiment. Nomenclature and color code as in Fig. 5. Clones co-expressing Δp60 and Tkv^{QD} contain as few cells as Δp60-expressing clones (arrows). The same phenotype was observed when cell death was blocked by co-expressing p35 (data not shown). These cells are slightly larger than Δp60 cells (size histogram) but show a wild-type cell cycle profile (DNA content histogram). Scale bar: 50 μm.

Tkv^{Q253D} makes us unable to rule out the possibility that this effect on G1/S progression also occurred indirectly, as a consequence of increased growth.

Vestigial is required for Tkv^{Q253D} induced growth

In the wing imaginal disc, *omb*, *spalt* and *vestigial* (*vg*) have been reported to respond to Dpp signaling (Grimm and Pflugfelder, 1996; de Celis et al., 1996; Kim et al., 1996). We wanted to know which if any of these genes was involved in controlling tissue growth effected by Tkv^{Q253D}. *spalt* is probably not required, as Spalt protein is not induced by Tkv^{Q253D} expression in the lateral areas of the wing disc, where we see the strongest overgrowth effects (Nellen et al., 1996; Lecuit et al., 1996; Jazwinska et al., 1999) (data not shown) (Table 2). In the case of *omb* and *vg*, we used null alleles as a genetic background in which the expression of the activated Dpp receptor was induced. We used a strong heat shock late in larval development to induce Tkv^{Q253D} expression in most of the cells, as in Fig. 3B. Expression of Tkv^{Q253D} in a null $l(1)omb^{3198}$ background promoted tissue overgrowth, just as in a wild-type background, indicating that Tkv^{Q253D} can promote growth in the absence of Omb (data not shown; Table 2).

Table 2. Complex responses to Dpp signaling

Genes	Required for Tkv^{Q253D}-induced growth	Induced by Tkv^{Q253D}
sal	n.a.	Only in wing pouch*,†,‡,§
omb	–*	+†,‡
vg	+¶	–**
cycD/cdk4	–*	–*
PI3K/Dp110	+††	n.a.

n.a., not addressed; +, required/induced; –, not required/not induced.
*Data not shown.
†Nellen et al., 1996.
‡Lecuit et al., 1996.
§Jazwinska et al., 1999.
¶Fig. 7A.
**Fig. 7B.
††Fig. 6.

By contrast, Tkv^{Q253D} was not able to promote tissue growth in a null vg^{83b27R} background (Fig. 7A, Table 2). This result points to Vg as a possible effector of growth induced by Dpp signaling. Consistently, ectopic Vg expression induces wing-like outgrowths in imaginal discs (Kim et al., 1996). However, we were surprised to find that clones expressing Tkv^{Q253D} did not show increased levels of Vg protein, regardless of their position in the disc (Fig. 7B, Table 2). Some lateral clones did express Vg, but these most probably originated in the Vg expression domain. In fact, clones in lateral positions where Vg is expressed over-grew better than in other regions. These results suggest that activation of Dpp signaling is not sufficient to induce Vg expression, but that Tkv^{Q253D} and Vg might synergize to effect tissue growth.

Dpp signaling does not effect growth via Cyclin D/Cdk4

In our effort to identify effectors of Tkv^{Q253D}-induced growth, we also tested whether Dpp signaling was able to induce Cyclin D (CycD). Overexpressed *Drosophila* CycD/Cdk4 promotes cell growth and cell proliferation in a coordinate manner, much as does Tkv^{Q253D} (Datar et al., 2000). The coordinate induction of growth and cell proliferation by CycD/Cdk4 has been attributed to its dual effects on the cell cycle regulator RBF, and on other unidentified growth regulatory targets. We detected no effect on CycD protein expression when Tkv^{Q253D} was expressed in clones (data not shown; Table 2). We also tested whether Tkv^{Q253D} could induce tissue growth in a *cdk4* null background (Meyer et al., 2000). Tkv^{Q253D} induced overgrowing clones in $cdk4^{-/-}$ wing discs just as it did in wild-type discs (data not shown). These results indicate that Cyc/Cdk4 is neither induced by nor required for tissue growth effected by Dpp signaling (Table 2). This is consistent with the results reported in Fig. 5, showing that Tkv^{Q253D}, unlike CycD/Cdk4, is incapable of counteracting the effects of the cell cycle inhibitor RBF.

DISCUSSION

Dpp signaling stimulates cell growth and proliferation

Using assays that distinguish growth-specific and cell cycle-

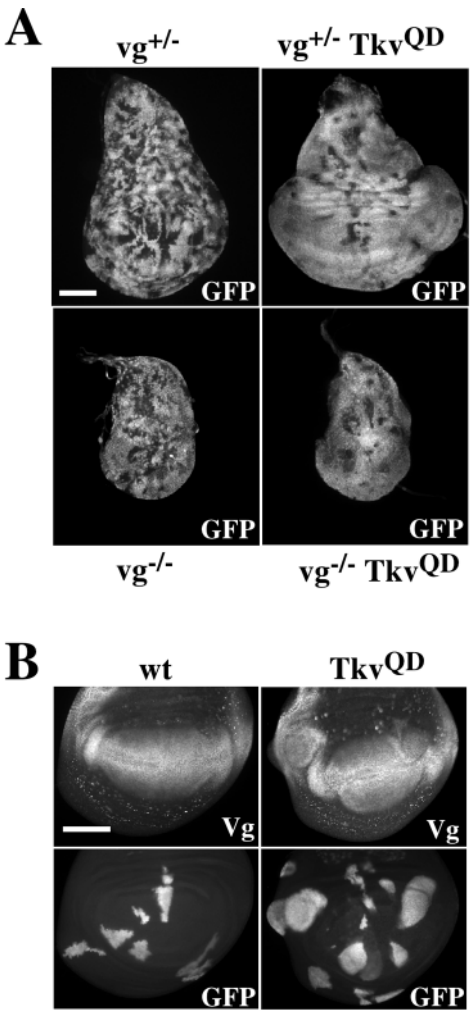

Fig. 7. Tkv^{Q253D} requires Vg to promote tissue growth but does not induce Vg expression. (A) Digital camera images of imaginal wing disc overexpressing GFP alone or with Tkv^{QD} in a *vg* mutant background. vg^{83b27R} heterozygous animals develop normally and wing discs reach normal size (upper left panel). Tkv^{QD} induces wing disc over-growth in a vg^{83b27R} heterozygous background, just as in a wild-type background (top right panel). The growth effect of Tkv^{QD} is blocked in a vg^{83b27R} homozygous animal (lower right panel); the size of the wing discs is similar to the size of vg^{83b27R} homozygous discs (lower left panel). (B) Digital camera images of Flip-out/Gal4 clones expressing GFP alone (lower left) or with Tkv^{QD} (lower right). Normal Vg expression pattern is shown in the upper left panel. Tkv^{QD} expression does not induce Vg protein upregulation, regardless of clone position (top right panel). Scale bars: 100 μm.

specific effects, we found that cell autonomous activation of Dpp signaling induces both cell growth (mass accumulation) and cell cycle progression. Cells expressing the activated Dpp receptor, Tkv^{Q253D}, outgrew controls, and exhibited a 'balanced' mode of growth in which cell cycle progression and growth were accelerated to the same degree, and cell size was not appreciably altered (Fig. 1B, Fig. 2, Table 1). We obtained opposite complementary effects by inhibiting Dpp signaling cell autonomously using a hypomorphic allele of the receptor, tkv^{7}, or the Dpp pathway-specific inhibitor, Dad. In these cases, cells had a very slow division cycle but no size defect (Fig. 3, Fig. 4, Table 1). Thus as in the gain-of-function experiments, cell growth and cell cycle progression were coordinately affected. Consistent with the idea that Dpp signaling affects cell growth directly, these results were not substantially altered when cell death was blocked by the caspase inhibitor, p35. These findings extend earlier studies that indicated a role for Dpp signaling in tissue growth (Capdevila and Guerrero, 1994; Zecca et al., 1995; Burke and Basler, 1996; Nellen et al., 1996; Lecuit et al., 1996), but did not distinguish between cell cycle, cell growth and cell viability effects.

The 'balanced' effects on cell growth and cell proliferation caused by Tkv^{Q253D} differ markedly from results obtained when other growth stimulatory factors were manipulated in the developing wing. Ras, Myc and PI3K have all recently been shown to autonomously stimulate wing cell growth (Prober and Edgar, 2000; Johnston et al., 1999; Weinkove et al., 1999). Growth mediated by ectopic expression of these factors leads to a truncated G1 phase, which in the case of Ras and Myc has been attributed to post-transcriptional upregulation of the G1/S regulator Cyclin E (Prober and Edgar, 2000). However, hyperactivation of Ras, Myc or PI3K signaling did not increase overall rates of wing cell proliferation, apparently because of a failure to stimulate G2/M progression. Consequently, these factors drove 'unbalanced' growth characterized by substantial increases in cell size. By contrast, ectopic Tkv^{Q253D} causes an increase in overall rates of cell division. Thus, Tkv^{Q253D} must induce G2/M as well as G1/S progression. Although we have not detected any changes in Cyclin E or String levels by immunofluorescence, it is possible that small differences not detectable by antibody staining are responsible for G1/S and G2/M promotion.

Elusive growth effectors and region-specific responses to Dpp

Although early studies of wing development suggested that gradients of signaling might be the driving force that promotes cell growth in the wing, recent work has suggesting that Dpp signaling need not be employed in a gradient to stimulate growth (Lecuit et al., 1996; Nellen et al., 1996; Burke and Basler, 1996; Serrano and O'Farrell, 1997; Day and Lawrence, 2000). We found that Dpp signaling in Tkv^{Q253D}-expressing clones was intense and homogenous, as assayed by anti-phospho-Mad staining (Tanimoto et al., 2000), even in lateral areas (data not shown). This suggests that gradients of Dpp signaling within these clones had been obliterated. Nevertheless, a variety of assays indicated that cell proliferation was promoted uniformly and autonomously throughout the clones, rather than at their edges, where sharp differentials of signaling intensity occur (Fig. 1, Fig. 2D). Gradient models also predict non-autonomous effects on growth in regions bordering Tkv^{Q253D}-expressing clones. Although we did not directly analyze cell growth rates in these regions, our inspection of markers for cell cycle progression did not detect major non-autonomous effects on cell proliferation. Thus, all of our observations suggest that absolute intracellular levels of Dpp signaling, rather than gradients, are important for growth.

As previously shown (Burke and Basler, 1996) survival of tkv^{-}cells was better in regions of the wing that experience low level Dpp signaling. However, even in lateral regions far from the Dpp source, tkv^{-} cells have a growth and proliferation

defect (Fig. 4). This suggests that all cells in the wing disc, including lateral cells, receive and require at least low levels of a Tkv ligand for normal growth. This leads us to suggest that some of the Dpp targets that mediate its growth effects might not have regionalized, nested expression patterns like two well-characterized Dpp targets, *spalt* and *omb* (which appear not to be mediators of Tkv^{Q253D}-induced growth; Table 2) (Grimm and Pflugfelder, 1996; de Celis et al., 1996). Instead, it seems plausible that some of the Dpp targets that mediate cell growth and proliferation are more uniformly expressed in regions where Dpp is required.

How might Dpp, expressed in a gradient, drive expression of growth regulatory targets more uniformly? It has been proposed that induction of target genes in cells receiving low levels of Dpp must overcome the activity of the transcriptional repressor, Brinker (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999; Sivasankaran et al., 2000). *brinker* mutant clones in lateral areas of the wing disc exhibit a round morphology and over-growth phenotypes that are similar to Tkv^{Q253D}-expressing clones (Jazwinska et al., 1999). *brinker* mutant discs also exhibit a dramatic over-growth phenotype along the AP axis similar to discs that overexpress Tkv^{Q253D} ubiquitously (as in Fig. 3B), or discs that overexpress Dpp in its own domain (Nellen et al., 1996; Lecuit et al., 1996; Campbell and Tomlinson, 1999). Thus, it seems plausible that all wing cells require a threshold level of Dpp activity to grow, and that in lateral regions this threshold is equal to the amount of signaling activity needed to overcome repression of Dpp growth targets by Brinker. When Brinker is lost or Tkv^{Q253D} is expressed in lateral regions, this threshold level of signaling may be greatly surpassed, causing increased expression of growth regulators and acceleration of cell growth rates beyond normal levels.

As suggested by earlier studies, we found that the growth response of a cell to altered Dpp signaling varied according to its location in the disc. Ectopic Tkv^{Q253D} caused the strongest over-growth phenotypes in lateral regions, far from the source of endogenous Dpp (Fig. 1A, Fig. 2, Fig. 7B), whereas inhibition of Dpp signaling had the strongest phenotypes in medial areas of the disc, where Dpp levels are normally high (Burke and Basler, 1996) (Fig. 3A, Fig. 4). Similar region-specific responses have been observed in experiments in which Notch or Wingless signaling was activated ectopically using cell autonomous effectors, or ligands (Go et al., 1998) (L. Johnston, personal communication). What is the significance of these region-specific responses? Without knowing the pertinent growth regulatory targets of these signaling systems, we can only speculate. Perhaps the differential responses reflect cooperation between several regionally expressed signals that affect tissue growth, both positively and negatively, in a combinatorial fashion. Our observations relating to *vg* seem consistent with this possibility. *vg* is required by Tkv^{Q253D} to promote tissue growth, yet Vg protein is not up-regulated by ectopic Tkv^{Q253D}, and Tkv^{Q253D} is capable of promoting overgrowth in wing regions where Vg is not detectable (Figs 1, 7; Table 2). The complex growth responses of cells to Dpp signaling, summarized in Table 2, illustrate how much is unknown about mechanisms of growth control. New, more global, approaches to studies of growth modulation will be required before we can understand its regulation by patterning signals. Important tasks for future studies include identifying the Dpp targets that stimulate cellular metabolism to effect growth, and determining how these targets integrate input from other patterning signals such as Wingless, Notch, Hedgehog and the EGFR ligands.

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