Growth and cell survival are unevenly impaired in pixie mutant wing discs

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Summary

It is largely unknown how growth slows and then stops in vivo. Similar to most organs, Drosophila imaginal discs undergo a fast, near-exponential growth phase followed by a slow growth phase before final target size is reached. We have used a genetic approach to study the role of an ABC-E protein, Pixie, in wing disc growth. pixie mutants, like mutants in ribosomal proteins genes (known as Minutes), show severe developmental delay with relatively mild alterations in final body size. Intriguingly, pixie mutant wing imaginal discs show complex regional and temporal defects in growth and cell survival that are compensated to result in near-normal final size. In S2 cells, Pixie, like its yeast homolog RLI1, is required for translation. However, a comparison of the growth of eukaryotic translation initiation factor eIF4A and pixie mutant clones in wing discs suggests that only a subset of translation regulators, including pixie, mediate regional differences in growth and cell survival in wing discs. Interestingly, some of the regional effects on pixie mutant clone growth are enhanced in a Minute background. Our results suggest that the role of Pixie is not merely to allow growth, as might be expected for a translation regulator. Instead, Pixie also behaves as a target of putative constraining signals that slow disc growth during late larval life. We propose a model in which a balance of growth inhibitors and promoters determines tissue growth rates and cell survival. An alteration in this balance slows growth before final disc size is reached.

Key words: Cell growth, Ribosomal proteins, Drosophila
promoting activity of specific signalling pathways during late imaginal disc growth. For example, late in larval development, wing disc cells seem to become refractile to growth-inducing signals from a constitutively activated Dpp receptor (Martín-Castelanos and Edgar, 2002). Thus unknown factors may inhibit the ability of wing disc cells to respond to Dpp after the larval growth period. Two potential inhibitors of late disc growth have been identified. Nitric oxide (NO) inhibits imaginal disc cell proliferation, and NO levels have been shown to increase in late third instar imaginal discs (Kuzin et al., 1996). Wg signalling mediates cell-cycle arrest at the dorsoventral boundary during the third instar as part of the program of disc differentiation (Johnston and Edgar, 1998). More recent work has suggested that this role of Wg signalling in cell cycle arrest may extend to the rest of the wing pouch during the late third instar (Johnston and Sanders, 2003; Giraldez and Cohen, 2003).

Development

Both pixie mutants behave like recessive for translation and that slow growth phase, pixie mutant clones are smallest in regions of the disc where growth is slowest. Together our data are consistent with a model in which pixie function is a target of the growth constraining signals that slow growth during late larval life.

Materials and methods

Fly stocks

The following fly strains were generated or obtained from the Bloomington stock centre, unless otherwise indicated: y w; FRT80B, y w hs-flp122, Ubi-GFP FRT80B, y w; pixL35 FRT80B/TM3 Sb Kr-GFP, w hs-flp122 fneo; mwh FRT80B/TM1 mwh (a gift from A Garcia-Bellido), y w hs-flp122, M(3)66D1 Ubi-GFP FRT80B/TM6B Tb, y w/w; UAS-p35; fRT80B/S6-6 TM6, y w hs-flp122; En-Gal4; Ubi-GFP FRT80B / Sm6-TM6B, y hs-flp122; En-Gal4; FRT80B / SM6-TM6B, y w hs-flp122; Ubi-GFP FRT40A and w; eIF4A1006 40APRT/CyO (a gift from M Galloni) (Galloni and Edgar, 1999).

For most experiments, larvae were collected from short egg lays and reared at defined densities, in order to avoid asynchrony.

dsRNAi

dsRNAi was performed by adding 10 µg dsRNA to 35 mm wells containing 2×10^5 Drosophila Schneider S2 cells as described (Clemens et al., 2000). DNA templates containing 5’ T7 RNA polymerase-binding sites were PCR amplified from plasmid or genomic DNA and transcribed with the Megascript T7 transcription kit (Ambion). Primers contained 5’ T7 RNA polymerase-binding sites preceded by a GAA overhang followed by sense or antisense sequences: pixie sense primer, GCAGAGACACAAACGCGACTG; pixie antisense primer, TATGCAAATGTCAGAAGCGAC; eIF4A sense primer, GCATCTTGGAATCCGGTTGCC; eIF4A antisense primer, GTTCAGAAAGATTCCGACTG.

Translation assays

S2 cells (8×10^6 per point) were incubated for 3 hours in 1 ml Schneider’s Drosophila medium (Gibco), 10% foetal bovine serum, containing 200 µCi Premix [35S] cell labelling mix (1000 Ci/mMol, Amersham Biosciences). For emetine treatment, cells were pre-treated for 30 minutes in 0.1 mM emetine (Sigma), prior to addition of Promix. Cells were lysed in 100 µl per point of lysis buffer [50 mM HEPES (pH 7.5), 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM MnSO4, 50 mM NaF, 10 µg/ml aprotinin, 1 µg/ml leupeptin, 10 µg/ml Pepstatin A, 15 µM TLCK, 1 mM PMSF], and aliquots were removed for protein assay (BioRad DC protein assay kit) and analysis by western blotting. Incorporation of [35S]cysteine and [35S]methionine into total cellular protein was assessed by TCA precipitation. Aliquots (5 µl) of lysate were added to 0.5 ml water and 0.5 ml of 0.5 M NaOH containing 1 mM L-methionine and L-cysteine in glass tubes. Tubes were vortexed, incubated at 30°C for 10 minutes, then 1 ml cold 25% (w/v) TCA was added. Tubes were vortexed and incubated on ice for 5 minutes. Precipitated [35S]-labelled proteins were collected by filtration through Whatman GFC glass fibre filters, washed three times in 5% cold TCA, rinsed in 95% ethanol, dried and quantified by scintillation counting. Each dsRNAi condition was carried out in duplicate, and [35S]cysteine and methionine incorporation into protein was measured in duplicate.

Sucrose sedimentation and western blotting

S2 cells were lysed at a density of 10^6 cells per ml in SDG100 or SDG500 (high salt lysis) buffer (Tyzack et al., 2000) with 0.05 M NaF, 1 mM NaVO4, 0.01% Pepstatin, 0.01% Aprotinin, 0.015 mM TLCK, 0.001% Leupeptin, 1 mM PMSF. Cleared lysates were run through 0.8 M sucrose cushions in the presence of 100 mM or 500 mM (high

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Mitotic recombination and clonal analysis

Mitotic recombination was induced using the FLP/FRT system (Xu and Rubin, 1993). Wild-type or *pix*<sup>MD5413</sup> clones were generated by heat-shocking *y w; FRT80B/ Ubi-GFP FRT80B* or *y w; *pix*<sup>MD5413</sup> FRT80B/ Ubi-GFP FRT80B larvae at 34°C for 30 minutes (clones induced during second instar) or 15 minutes (clones induced during third instar). Similar methods were used to generate *eIF4A1<sup>1006</sup>* clones. To examine clones in adult wings, *hs-flp<sup>U2</sup> bacak, *pix*<sup>MD5413</sup> FRT80B/mmh *P<sup>[f+64C w+]</sup>* FRT80B larvae were heat-shocked for 45-80 minutes and male wings were examined.

Clone median doubling time (MDT) was calculated by dividing the number of hours of the clone induction window by log2( Median clone cell number). As is shown in Fig. 6A, the early clone induction windows overlap by 5 hours with the late 32-hour window, and by 17 hours with the late 44-hour window. Despite this difference in the period of overlap with a faster growth phase, the twin MDT within the two late windows is similar. By contrast, twins generated using the 60 hour clone induction window have a MDT that is intermediate between the fast and slow phases. Because of the lag time between heat-shock and clone generation, the actual overlap of the 32, 37 and 44 hour late windows with the fast phase is likely to be minimal, whereas the 60 hour window spans both fast and slow growth phases (Fig. 6A,D; an intermediate MDT is also observed with other short windows that span the fast and slow phases, data not shown). X-ray induced mitotic clonal analyses revealed exponential growth, with decreases in rate at the larval moults (average MDT=8.5 hours) (Garcia-Bellido and Merriam, 1971). However Johnston and Sanders suggest that MDT increases gradually as development progresses (in the hinge it is 9.5 hours during early second instar to 11.5 hours in the third instar) (Johnston and Sanders, 2003). Our data reveal a fast growth phase followed closely by a slower growth phase, approximating the growth curve demonstrated by Byrant and Levinson (Byrant and Levinson, 1985). Owing to the limitations of the clonal analysis technique, our data do not suggest the rate of deceleration of cell division during late third instar.

To generate clones in a *Minute* background, *y w; FRT80B or y w; *pix*<sup>MD5413</sup> FRT80B/TM3 Sh, *Kr-GFP* males were mated with *y w hs-flp<sup>U2</sup>; *M(3)66D4* Ubi-GFP FRT80B/TM6B *Tb* females and larvae were heat-shocked for 10 minutes. *Tb<sup>+</sup>* larvae were dissected. Poor-growing mutant clones are often observed as fragments around a wild-type twin clone. When clones have a growth advantage over their surrounding tissue, they generally do not fragment, allowing easy recognition of clone boundaries. In the late hinge in the *Minute* background, there is a high frequency of smaller *pix*<sup>MD5413</sup> clones, which contribute to an estimated decrease in hinge MDT when compared with the MDT of these clones in a *Minute<sup>+</sup>* background. In these experiments in a *Minute* background, *Minute<sup>+</sup>* twins survive poorly during the late clone induction windows. In the absence of any associated twins, clone fragments belonging to the same mutant clone may be counted as separate smaller clones. Thus, the estimated MDT is likely to be lower than the actual MDT. In the pouch in a *Minute* background, *pix*<sup>MD5413</sup> clone size and frequency is low, allowing us to be confident of their poor growth when compared with a *Minute<sup>+</sup>* background.

Results

Identification of *pixie*, a novel growth regulator

Mutations in *pixie* were identified in a genetic screen for dominant enhancers of a small-wing phenotype, obtained by expressing a kinase-dead version of *Drosophila* PI3-kinase (KD-Dp110, Fig. 1A-E) (Leevers et al., 1996; Coelho et al., 2005). Additional *pixie* alleles were obtained in a screen for mutations that are lethal in combination with a deficiency that uncovers *pixie* (Dahanukar et al., 1999). Subsequent genetic analyses showed that the mutations obtained in the enhancer of KD-Dp110 screen were dominant-negative alleles (Table 1). Strong *pixie* mutants are homozygous lethal, while weaker hypomorphs are viable but grow slowly, with larval periods up
Development of dominant phenotypes of mutant, M66D1 in wing size relative to thorax size were observed in an $P<0.0000005$, Fig. 1L). Similar, but slight variations in circumference to thorax circumference, when compared with body size, represented by an increase in ratio of wing to body size (Leevers and Hafen, 2004). Thus, the insulin-signalling pathway, which includes Dp110, show decreases in body size (Kispal et al., 2005; Yarunin et al., 2005; Dong et al., 2004), and the phenotypic similarities between pixie and the Minutes, we investigated whether Pixie is also involved in translation. Immunostaining revealed that Pixie is predominantly cytoplasmic in Drosophila S2 Schneider cells and imaginal discs (see Fig. S1 in the supplementary material and Fig. 3), consistent with a role in translation. dsRNAi-mediated depletion of Pixie from Drosophila S2 cells significantly lowers global translation within 2 days (Fig. 2C). Furthermore, sucrose sedimentation experiments suggest that Pixie associates with ribosomes in a salt-sensitive manner, suggesting that the association is peripheral and Pixie is not a part of the core ribosomal complex (Fig. 2D). Further biochemical analyses also suggest that Pixie, like yeast RLI1, is required for normal translation (D. Andersen and S.J.L., unpublished).

**pixie hypomorph wing discs display distinct patterns of elevated cell death**

**pixie** hypomorphs are developmentally delayed and have extended larval periods during which the growth of both whole larvae and imaginal discs is slowed. A higher than normal level of apoptosis is observed in **pixie** mutant wing discs (compare Fig. 4B with A, Fig. 4F-G with C). This increased apoptosis changes from uniformly distributed clusters during the early to mid-third-instar (Fig. 4B) to a distinct pattern in the late third instar. In these late third instar discs (9/12 discs examined a day before wandering and 5/16 examined at wandering), the apoptosis is particularly intense in the wing pouch (Fig. 4F) and there is consistently less apoptosis in the hinge than elsewhere in the disc. Shortly before pupation (in the remaining discs examined a day before and at wandering) apoptosis is less intense and more uniformly distributed (Fig. 4G). Nevertheless, at this stage, it is often elevated at the dorsoventral (DV) boundary and at the edges of the pouch (thin and thick arrow in Fig. 4G). Thus, there is a transient period during late third instar well before pupation, when apoptosis is intense in the wing pouch. Interestingly, the wing discs of two Minutes, $M(3)66D^{+/+}$ and $M(3)95A^{+/+}$ also show a similar pattern of apoptosis during early to mid-third instar.

### Table 1. Phenotypic traits of various pixie allelic combinations

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Viability</th>
<th>Development time</th>
<th>Body size</th>
<th>Wing area (mm²)</th>
<th>Bristle size</th>
</tr>
</thead>
<tbody>
<tr>
<td>yw</td>
<td>Viable</td>
<td>10 days</td>
<td>Normal</td>
<td>1.47±0.05 (n=6)</td>
<td>Normal</td>
</tr>
<tr>
<td>yw; pixL2/+</td>
<td>Viable</td>
<td>10 to 12 days</td>
<td>Reduced</td>
<td>1.37±0.06 (n=7, $P&lt;0.0005^*$)</td>
<td>Reduced</td>
</tr>
<tr>
<td>yw; Df(3L)Scf-R11/+</td>
<td>Viable</td>
<td>10 days</td>
<td>Normal</td>
<td>1.49±0.05 (n=7)</td>
<td>Normal</td>
</tr>
<tr>
<td>yw; pixL3/+</td>
<td>Viable</td>
<td>10 days</td>
<td>Normal</td>
<td>1.5±0.05 (n=12, $P&gt;0.1$)</td>
<td>Normal</td>
</tr>
<tr>
<td>yw; pixL4/4</td>
<td>Viable</td>
<td>10 days</td>
<td>Normal</td>
<td>1.57±0.03 (n=7, $P&lt;0.005^*$)</td>
<td>Normal</td>
</tr>
<tr>
<td>yw; pixL4</td>
<td>Embryonic lethal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>yw; pixL5</td>
<td>Embryonic lethal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>yw; pixL6</td>
<td>Larval lethal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>yw; pixL7</td>
<td>Viable</td>
<td>10 days</td>
<td>Normal</td>
<td>1.45±0.04 (n=6, $P=0.15^*$)</td>
<td>Reduced</td>
</tr>
<tr>
<td>yw; pixL8</td>
<td>Viable</td>
<td>12 to 13.5 days</td>
<td>Reduced</td>
<td>1.38±0.08 (n=11, $P&lt;0.0005^*$)</td>
<td>Reduced</td>
</tr>
<tr>
<td>yw; pixL9</td>
<td>Viable</td>
<td>12 to 15 days</td>
<td>Reduced</td>
<td>1.37±0.08 (n=9, $P&lt;0.0005^*$)</td>
<td>Reduced</td>
</tr>
</tbody>
</table>

YW control and homozygotes are above, homozygotes and transallelic combinations below. Among the heterozygotes, pixL2/+ shows developmental delay and reduced wing area and body size (noted by weighing flies in batches of 10 to 20), pixL3 is a weak allele and homozygotes show no significant difference in wing area compared with yw controls. pixL17 and pixL24 are strong alleles and the transcombinations of pixL24/pixL24 and pixL3/pixL17 show significant but mild decreases in wing area compared with yw heterozygotes (see text).

- *When compared with area of yw; pixL2/+. wings.
- †When compared with area of yw wings.

To double those of wild-type larvae (Table 1). They show mild variation from the normal body size, depending on culture conditions. This variation ranges from a ~10% decrease to a 5-10% increase (Table 1, data not shown) (Coelho et al., 2005). In addition, viable adults display slender and short thoracic macrochaetae (bristles) and occasional eye roughening (Fig. 1F-G, Table 1; data not shown). No additional effects on overall pattern or differentiation are seen. The strong developmental delay, mild variation in body size and presence of slender bristles in **pixie** hypomorphs strongly resemble the dominant phenotypes of **Minutes**, many of which encode ribosomal proteins (see Table 1) (Lambertsson, 1998). Thus, **pixie** behaves as a recessive **Minute**. By contrast, mutants in the insulin-signalling pathway, which includes Dp110, show developmental delay and have correspondingly strong decreases in body size (Leevers and Hafen, 2004). Intriguingly, we observe disproportionate effects on the size of the different parts of the body in **pixie** mutant flies. This disproportion tends towards an increase in wing size relative to body size, represented by an increase in ratio of wing circumference to thorax circumference, when compared with control ($P<0.00000005$, Fig. 1L). Similar, but slight variations in wing size relative to thorax size were observed in an RpL14 mutant, M66D1 ($P=0.00008$, Fig. 1L).

**pixie encodes an ABC-E protein required for translation**

Genetic mapping revealed that **pixie** encodes an ABC-E protein that possesses two N-terminal iron-sulphur binding domains and two C-terminal ABC domains (Coelho et al., 2005) (see Introduction and Fig. 2A,B). DNA sequence analysis of **pixie** alleles identified mis-sense mutations in conserved residues in both the ABC and iron-sulphur domains, confirming that both are essential for Pixie function (Fig. 2B and legend). Diverse cellular functions have been assigned to the human homolog of Pixie, RLI, including inhibiting RNaseL, aiding lentivirus capsid assembly and stabilizing MyoD mRNA (Kerr, 2004; Zimmerman et al., 2002; Doohoo and Lingappa, 2004; Bisbal et al., 2000). Given that the yeast homolog of Pixie is involved in ribosomal biogenesis and translation initiation (Kispal et al.,
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Immunostaining revealed that *Pixie* is expressed uniformly in early and late third instar wing discs (Fig. 3). Thus, the regional differences in cell death in *pixie* mutant discs are unlikely to reflect differing expression levels of the protein in wild-type discs. The adult wings that these discs give rise to, are patterned normally, making it unlikely that the elevated cell death is due to defects in patterning or differentiation (Fig. 1H-K). Furthermore, expression of Vg, a target of Wg signalling is normal in *pixie* mutant and *Minute* discs (data not shown). Although cell death is elevated in the wing discs of *pixie* hypomorphs, the resulting adults have a near normal body size, suggesting that extra cell divisions must compensate for the increased cell death. Indeed, the unusual increase in *pixie* hypomorphic wing size relative to thorax size may be partly due to such compensatory cell division. Expression of the Caspase inhibitor, p35, in the posterior compartment of *pixie* mutant discs increases the ratio of posterior compartment size to anterior compartment size, relative to that in control wings also expressing p35 in the posterior compartment (Fig. 4M-N) (Hay et al., 1994). It has recently been shown that induction of the cell death pathway can generate a growth-promoting signal that increases wing size, when the execution of cell death itself is suppressed by p35. Such a mechanism causes extensive overgrowth in wings in which the death inducer Hid is co-expressed with p35 and involves the expression of Wg in cells in which the death signal is activated (Huh et al., 2004; Perez-Garijo et al., 2004). We do observe that, when p35 is expressed in the posterior compartment of *pixie* mutant discs, Wg is ectopically expressed in that compartment (arrow in Fig. 4N), suggesting that a reported mechanism can operate by which the cell death might be compensated (Huh et al., 2004; Perez-Garijo et al., 2004).

*pixie* is required for balanced growth and cell survival in wing discs

Although mild reductions in *pixie* function can allow an
increase in cell proliferation (to compensate for cell death), stronger reductions in pixie function clearly reduce growth and cell proliferation. When Pixie levels are reduced through RNAi in S2 cells, cell number is reduced and an increased proportion of cells accumulate in G1, indicating that Pixie may be required for G1 to S progression (Fig. 2D, lower panels). Furthermore, wing disc clones of pixL17, a strong hypomorphic lethal allele, are reduced in frequency and clone size compared with their wild-type sister clones (twins, Figs 5, 6). Inhibition of apoptosis in the posterior compartment by expression of p35 significantly rescues the frequency of pixL17 mutant clones (Fig. 5B-D). However, these mutant clones are still smaller than their sister clones, suggesting that pixie is required in the wing disc for cell division and growth as well as cell survival. When pixL17 mutant clones were generated late in larval life, rare clones survived to the adult wing and displayed a strong reduction in cell number but not cell size (see Fig. 5A and its legend). Together, these observations demonstrate that strong apoptosis in the posterior compartment by expression of p35 significantly rescues the frequency of pixL17 mutant clones (Fig. 5B-D). However, these mutant clones are still smaller than their sister clones, suggesting that pixie is required in the wing disc for cell division and growth as well as cell survival. When pixL17 mutant clones were generated late in larval life, rare clones survived to the adult wing and displayed a strong reduction in cell number but not cell size (see Fig. 5A and its legend). Together, these observations demonstrate that strong...
reductions in pixie function reduce both balanced growth and cell survival. Balanced growth is the term used to describe an affect on growth (increase in mass) that is accompanied by a corresponding effect on cell division, thus leaving cell size unaltered (de la Cova et al., 2004).

Changes in the regional requirement for pixie in clonal growth correlate with changes in disc growth rates

During our analysis of pixL17 mutant clones, we noticed that clones generated during late disc growth are more poorly represented in the pouch than the hinge (Fig. 5B-D, Fig. 6 and see below). This regional difference in pixL17 mutant clone growth persisted in the presence of p35 (Fig. 5D) and was observed with other lethal pixie alleles (data not shown). Like the varying pattern of cell death in hypomorphic pixie mutant discs (see above), the impaired growth of pixie mutant clones also varied with developmental stage. Closer examination of the growth curves and patterns of apoptosis in wing discs from M(3)/66D1+/+ larvae (which have a more uniform developmental delay than pixie mutant larvae) revealed that their intense cell death correlates maximally with the slow phase of growth (see Fig. S2 in the supplementary material). We thus investigated whether the strong requirement for pixie in pouch clone growth in late discs also correlated with the slow phase of disc growth. Although pixie is required for cell viability and balanced growth, we shall refer for convenience to reduced clone size and frequency in the following sections as reduced growth.

Direct cell counting methods showed that increase in cell number slows from early-mid third instar onwards (Bryant and Levinson, 1985) (see Fig. S2 in the supplementary material). Johnston and Sanders (Johnston and Sanders, 2003) have shown that wing pouch cells have a longer median doubling time (MDT) than hinge cells. The growth of the twins of pixie mutant clones generated in a pix/+ background is similar to the growth of wild-type clones generated in a wild-type background (compare Fig. 6A,D with Fig. S3 in the supplementary material), enabling an estimation of disc growth rates in our experiments. Early and late clone induction windows were chosen that allowed a similar median number of twin cell divisions (see Fig. 6D). Thus, average mutant clone/twin size is comparable between the early 29-hour and 30-hour windows and the late 44-hour and 37-hour windows (see red numbers in Fig. 6D,G). To analyze an early fast growth phase, clones were examined at the mid-third instar (pink boxes Fig. 6A,D), and to examine the late slow growth phase, clones were examined at larval wandering – the end of the larval growth period (blue boxes Fig. 6A,D). In the experiments in Fig. 6D-F, the average twin MDT during the early fast growth phase is 9.4 hours. During the late slow growth phase, the average twin MDT is 12.8 hours, 27% longer than during the fast growth phase. MDT is on average 13% longer in the pouch than the hinge, during the fast phase and 12% longer during the slow phase (see Fig. 6B,C for regional demarcation). Thus, the pouch and hinge growth rates decrease to a similar extent in going from the fast to slow phase (26 and 28%, respectively; see Materials and methods). Although not quantified, clone sizes in the presumptive thorax (notum) appear similar to those in the hinge.

During the fast growth phase (Fig. 6E) pixL17 mutant clone size and frequency are greatly reduced even within 29 or 30 hours of clone induction, and clones are completely absent within 46 hours. pixL17 clone growth is poorer in the hinge than the pouch. This is consistent with Pixie being required for translation and the fact that the hinge grows faster than the pouch, resulting in stronger competitive pressure on hinge mutant clones (see below). As disc growth slows, pixL17 mutant clones grow better in the hinge than during the fast phase (compare hinge data in Fig. 6E with F). Increasing the length of the clone induction window can normally strengthen the phenotype by decreasing the level of perdurant wild-type Pixie; the late windows (44 hours and 37 hours) are longer than the early windows (30 hours and 29 hours). Therefore, the better growth of pixie mutant clones during the late windows does reflect a lower requirement for pixie in the hinge during slower growth.

By contrast, pixL17 mutant clone growth in the pouch remains poor during part of the slow growth phase (Fig. 6F). This is most clearly seen in the 44-hour window (Fig. 6F). The sudden increase in strength of phenotype as the clone induction window is extended from 37 to 44 hours, suggests that pixie is strongly required in the pouch at the beginning of the slow phase. This strong requirement in the wing pouch for pixie at this stage was seen in two additional independent experiments (the 46 hour late window in Fig. 5D, left panels; and an additional 44 hour late window, data not shown). Furthermore, other strong pixie alleles have a similarly severe clone phenotype in the pouch, compared with the hinge during part of the slow growth phase (data not shown), indicating that this phenotype is not allele specific. Although not quantified, the growth of pixie mutant clones in the notum appears similar to that in the hinge. Thus, the growth defect of pixie mutant clones is unexpectedly stronger in the slower-proliferating pouch area.

![Image](https://example.com/image.png)
in late larval discs. Interestingly, clones that are mutant for the eukaryotic translation initiation factor, eIF4A, show no such regional differences in their ability to grow within the wing disc during both the fast and slow growth phases (Fig. 6G).

Our results reveal a temporal and regional correlation between the growth and survival of strong pixie mutant clones and the pattern of cell death in hypomorphic pixie discs. We detect an interesting change in the regional requirement for Pixie as disc growth rates change. During the fast phase, the reduction in pixie mutant clone growth is stronger in the hinge and during the slow phase it is stronger in the pouch. Importantly, our clonal analysis demonstrates that during the

Fig. 4. pixie mutant and Minute wing imaginal discs show regionally elevated levels of apoptosis. (A-L) Images are projections of confocal z-series taken through TUNEL-labelled wing imaginal discs that have been then inverted to reveal wing disc morphology, disc genotypes are indicated. Discs from (A,B) early to mid-third instar, (F,H) late third instar and (C-E,G,I-L) wandering larvae. Apoptosis is similar to control in discs heterozygous for pixL17 (D) and pixL35 (E), and is enhanced in discs that are transheterozygous for pixL17 and M(3)66D1 (J) and pixL17 and M(3)95A1 (L). Thin arrow in G indicates apoptotic nuclei that lie approximately at the DV boundary; thick arrow indicates those that lie at the pouch borders. (M) Bar charts and wing images showing that expressing p35 in the posterior compartment increases area in pixL17;L35 wings (pix) compared with control (pixL17/+). P/A ratio in pix wings is significantly higher than control, $P=3.8 \times 10^{-10}$. White line in images on the right indicates the AP boundary. (N) Wg protein, detected by immunostaining, is ectopically expressed (arrow) when p35 is expressed in the posterior compartment of pixie mutant discs. Scale bar: 50 μm in A-L.
slow phase, *pixie* function is required more in the slowest growing region of the disc and may suggest that Pixie is a target of the constraining signals that slow growth at this stage (see Discussion).

**The contribution of cell competition to the *pixie* mutant clone phenotype**

The regional differences in the *pixie* growth phenotype may result from regional differences in the intrinsic requirement of the cells for *pixie* function. However, it has been reported that regional differences in mutant clone growth may result from differences in the severity of cell competition across the disc (Simpson, 1979; Moreno et al., 2002; Moreno and Basler, 2004). Cell competition is a process by which the growth of slow-growing clones of mutant cells is further impaired when faster-growing cells surround them (Morata and Ripoll, 1975). Cell competition can be alleviated by making mutant clones in slow-growing *Minute* discs (Morata and Ripoll, 1975).

To study the involvement of cell competition, *pixie* mutant clones were made in the *M(2)66D1/+* background (henceforth referred to as the *Minute* background), using clone induction windows that span the fast and slow growth phases (Fig. 7A, B). When *pixie Minute*+ clones in a *Minute* background are examined at approximately the end of the rapid phase of growth, they are larger than those generated in a non-*Minute* (*Minute+*) background and no longer grow more slowly in the hinge than in the pouch. This is clearly observed by comparing clone size distributions of *pixie* mutant clones in the *Minute* versus *Minute*+ background (compare Fig. 7C with D, and Fig. 7E with F). Thus, *pixie* mutant clones are subject to cell competition; that is, the reduction in mutant clone growth or survival is influenced by the rate of growth of the surrounding cells. These results indicate that the regional differences in *pixie* mutant clone presence during the fast growth phase in normal discs correlates, as a result of cell competition, with the growth rate of the surrounding tissue (see above).

Cell competition has been suggested to be due at least partially to differences in the ability of cells to compete for Dpp (Moreno et al., 2002; Moreno and Basler, 2004). *Minute M(2)C* clones are considered to transduce the Dpp signal inefficiently; thus, these clones ectopically express *brinker* and are eliminated in the wing pouch, a region of high Dpp signalling and low *brinker* expression (Moreno et al., 2002). However, elevated *brinker* expression is not observed in all cases of cell competition (de la Cova et al., 2004). *pixL17* mutant clones in the wing pouch examined during mid third instar and pre-wandering stages do not express *brinker* (see Fig. S5 in the supplementary material), suggesting that *pixie* mutant clones are not eliminated because of an inability to compete for Dpp.

**The *Minute* background enhances the *pixie* mutant clone growth defect during the slow phase**

During the slow growth phase, pouch cells grow more slowly than hinge cells; thus, the poor growth of *pixie* mutant clones sections through the same disc showing that nuclei of surviving *pixL17* clones (white arrow) in the posterior pouch are more basal than their accompanying twins (black arrow). (D) Bar charts representing the number of cells in *pixL17* clones (black), and their twins (grey) in anterior clones (above) and in posterior *p35*-expressing clones (below); pouch clones are on left and hinge clones on right. TUNEL labelling confirmed that *p35* expression blocked apoptosis (data not shown). Although posterior mutant clones are larger, the average mutant/twin clone size in the posterior compartment is still significantly less in the pouch (0.17) than the hinge (0.33), *P*<0.0001.
in the pouch is unlikely to be due to stronger cell competition there. Indeed, our analysis reveals that the representation of *pixie* mutant clones at this stage is not improved in a *Minute* background (compare Fig. 7G with H and Fig. 7I with J). In the hinge, it is difficult to estimate *pixie* mutant clone growth in the *Minute* background because of clone fragmentation (see Materials and methods). However, a growth advantage is not visible. In the pouch, *pixie* mutant clones actually grow more poorly in a *Minute* background than those made in the *Minute* background. It is clear that differences in *pixie* mutant clone growth between the pouch and hinge are maintained, and even exaggerated, in the *Minute* background. An impairment of clone growth in a *Minute* background has not been reported for other mutant clones. We have noted that in the above *Minute* environment, *Minute*/– clones are more rapidly eliminated during the slow phase than during the fast phase (Fig. 7), suggesting that the *Minute* environment during the slow phase constrains growth/cell survival.

To ensure that the *Minute* background used in these experiments effectively reduces cell competition during the slow growth phase, the growth of wild-type (*M*+/+) clones in the same *Minute* background was examined. These *M*+/+ clones do have a growth advantage and are larger than those generated in a *Minute* background, as previously described for other *Minutes* (Simpson and Morata, 1981) (compare Fig. 7K with L). Thus, this *Minute* background reduces cell competition. Intriguingly, although *M*+/+ clones have a growth advantage in this *Minute* background, pouch clones still grow more slowly.
Dynamic growth defects in pixie mutant wing discs

than hinge clones (Fig. 7L). Furthermore, during the fast phase, although the growth of pixie mutant clones is enhanced in a Minute background, these clones also grow more slowly in the pouch than the hinge (Fig. 7D,F). Thus, growth constraints exist in the pouch during most of the third instar. Put together our data suggests that although a Minute background...
background reduces cell competition, it reveals the presence of growth constraints that exist in wing discs. During the slow phase, these constraints are more severe or act more severely on slow growing cells that are mutant for pixie or Rp function. As the pattern of these constraints resemble the regional and temporal differences in wild-type clone growth, they are likely to reflect the constraints that normally exist in disc growth.

**Discussion**

To summarize, we have characterized the role of Pixie, a newly identified *Drosophila* ABC-E protein, in growth control. We have shown that in S2 cells, Pixie is required for normal translation. During wing disc development, its requirement in growth and cell survival varies spatially and temporally. Our clonal analysis suggests that this variation in requirement correlates with growth rate of the surrounding tissue, albeit in an unexpected manner. During the fast growth phase, Pixie is required more in a faster growing region of the disc; during the slow phase, Pixie is required more in a slow growing region.

We have found that reduced Rp and Pixie function, but not eIF4A function, results in regional differences in growth and cell survival. The molecular basis for these differences is not immediately clear and suggests that reducing the function of different components of the translation machinery can have different effects on disc growth and cell survival. Our results imply that the regional differences in growth rates across the wing disc are not accompanied by regional differences in levels of translation that are large enough to result in detectable differences in the requirement for eIF4A (Johnston and Sanders, 2003; Garcia-Bellido and Merriam, 1971) (data shown here). By contrast, the requirement for pixie and rp function does seem to reflect regional differences in other properties that reflect the non-homogenous nature of wing disc cells. It is intriguing that reducing rp or pixie function does not result in randomly distributed clusters of dying cells throughout the wing disc throughout development, instead these clusters are elevated in the wing pouch towards the end of development. However, this non-homogenous nature of wing disc cells is not easily explained by what is known about the activities of signalling pathways that regionally regulate cell survival and proliferation in the wing disc. For example, Wg and Dpp signalling are required for cell survival and balanced growth in the wing pouch (Neumann and Cohen, 1996; Giraldez and Cohen, 2003; Johnston and Sanders, 2003; Martin-Castellanos and Edgar, 2002; Moreno et al., 2002). A temporal analysis of the requirement for Wg signalling revealed that Wg is required more strongly in the wing pouch for cell survival during the fast growth phase (Johnston and Sanders, 2003). This contrasts with the pattern of cell death observed in pixie mutant and Minute discs. Furthermore, clonal analysis suggests that during the fast growth phase, pixie is required more in the hinge than in the wing pouch. Johnston and Sanders also suggest that Wg signalling constrains balanced growth in the wing pouch during the late stages of disc growth. This observation is compatible with the poor growth of pixie mutant clones in the wing pouch during slow phase (see below). However, it is not compatible with a potential role for Wg in compensation of pixie mutant cell death (Huh et al., 2004; Perez-Garijo et al., 2004).

Similar to the elf4A mutant clones, insulin-signalling mutants have not been reported to show regional differences in their effect on wing disc clone growth (S.J.L., unpublished). Besides Minute and pixie mutant clones, clones that express lower levels of Myc also show regional differences in their growth in wing discs (Simpson, 1979; Moreno et al., 2002; Moreno and Basler, 2004). Myc has recently been shown to regulate levels of rRNA and also has the potential to regulate levels of ribosomal proteins (Grewal et al., 2005). Thus far, these regional effects in clone growth of Myc-enderexpressing and Minute clones have been attributed to differences in the severity of cell competition (Moreno et al., 2002; Moreno and Basler, 2004). Our findings however, raise the possibility that some of these regional effects are due to sensitivity to growth constraints. During the slow growth phase, the strong pixie clone phenotype in the pouch is better explained by an increased sensitivity to growth-constraining signals, rather than competition from faster-growing neighbouring cells.

**A model to explain how Pixie might respond to growth constraining signals**

A possible explanation for the dynamic spatial requirement for pixie is that pixie responds to growth promoters that are limiting for growth and also show dynamic spatial expression. However, it is hard to explain why levels of these growth promoters would be limiting in the early hinge and late pouch (where the pixie clone phenotype is stronger), and not the early pouch and late hinge (where the pixie clone phenotype is weaker). Thus, it is unlikely that the varying effects on pixie mutant clone growth can be explained purely on the basis of the differential activity of growth promoters. In addition, we observe that reducing cell competition in Minutes does not remove the regional differences in growth rate of Minute+ clones with a growth advantage – pouch clones still grow more slowly than hinge clones. Our data are more easily explained by a model in which growth results from a balance between the activities of growth promoters and inhibitors. The inhibitors antagonize the activity of the promoters and are at higher levels in the pouch; pixie mutant cells show increased sensitivity to the growth inhibitors. Thus, we propose that during the fast phase, levels of growth inhibitors are low and growth promoters high, and pixie is required more where growth is faster. As inhibitor levels rise during the slow phase and promoter activity drops, pixie is now required more where inhibitor levels are higher. pixie function may be the direct or indirect target of inhibitor activity. Alternatively, pixie function may counteract the activity of the inhibitors.

The model proposed above is based partly on a hypothetical, mathematically simulated model described by Nijhout to explain how disc size is sensed and growth stopped (Fig. 8) (Nijhout, 2003). This model proposes that inhibitor levels are low during the exponential phase of growth, but rise as disc growth slows and remain high during the slow growth phase. Thus, Nijhout’s model proposes differential gene activity (differences in inhibitor levels) between the fast and slow growth phases. Computer simulations using Nijhout’s model show that increases in cell number correlate with changes in activator and inhibitor concentrations over time, and resemble the observed growth curve of imaginal discs. However, our model also requires that the inhibitors are at higher levels in the pouch and thus it is an adaptation of Nijhout’s model (Fig. 8). The ‘gradient of responsiveness’ model, which also
proposes a role for inhibitors in the determination of size, suggests that inhibitors may exist at higher levels in the pouch to counteract the effects of morphogens that are expressed at higher levels there (Serrano and O’Farrell, 1997).

The model presented in Fig. 8 explains broadly the observations made in this paper. However, the hypothesized inhibitors need to be identified. Nijhout’s model presumes that all disc cells synthesize growth promoters and inhibitors (an insulin-like peptide is synthesized by disc cells) (Brogiole et al., 2001). Our adaptation to this model suggests that the inhibitors are synthesized by all disc cells but at higher levels in the disc pouch. Nitric oxide (NO) has been shown to inhibit growth during the late third instar and high levels of NO activity are detected in the wing pouch in late third instar discs (Kuzin et al., 1996). However, the ability of NO to inhibit growth and cell division is only evident shortly before pupation. The pattern of cell death in pixie mutant discs together with the clonal analysis suggests that the proposed sensitivity to growth inhibitor might decrease shortly before pupation (see Fig. 8). Furthermore, we have found that injecting nitric oxide synthase inhibitors into larvae does not reduce cell death in late pixie mutant discs, and in fact occasionally exacerbates it (data not shown). Thus, pixie mutant cells are not dying because of an increased sensitivity to NO. Wg signalling has been proposed to constrain growth in the wing pouch during late larval life (Johnston and Sanders, 2003). However, as discussed above, any interaction between Wg signalling and the pixie mutant phenotype is likely to be complex.

A mild reduction in Pixie function results in an impairment of balanced growth and cell survival that varies regionally and temporally in wing discs. This impairment shows the classical characteristics of compensation known to occur for example in wing discs subjected to X-irradiation (Haynie and Bryant, 1977). Despite intensive cell death, close to normal final disc size is achieved, without disturbing differentiation and pattern. However, the observed ability of pixie mutant and Minute cells to compensate for defects in survival and growth that occur earlier in development is interesting and emphasizes the intrinsic ability of these mutant cells to grow and survive. The varying patterns of cell death with time can be seen as a reflection of the different growth environments that these cells are subjected to as development progresses. These mutant cells succumb to the changing environment but do not fully interfere with the ability of the system to correct the defects that arise due to cell death or even perhaps extra cell proliferation. However, it is clear that this ability of the system to correct itself is compromised to the extent that proportion is not always maintained and mild overgrowth can occur (see Marygold et al., 2005; Coelho et al., 2005). Studying the ability of mutant tissues to respond to growth constraints in vivo should advance our understanding of how the slowing down of growth is triggered.

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Supplementary material
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