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A screen for genes that function in leg disc regeneration in *Drosophila melanogaster*

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Abstract

Many diverse animal species regenerate parts of an organ or tissue after injury. However, the molecules responsible for the regenerative growth remain largely unknown. The screen reported here aimed to identify genes that function in regeneration and the transdetermination events closely associated with imaginal disc regeneration using *Drosophila melanogaster*. We screened a collection of 97 recessive lethal *P-lacZ* enhancer trap lines for two primary criteria: first, the ability to dominantly modify *wg*-induced leg-to-wing transdetermination and second, for the activation or repression of the *lacZ* reporter gene in the blastema during disc regeneration. Of the 97 *P-lacZ* lines, we identified six genes (*Krüppelhomolog- 1*, *rpd3*, *jing*, *combgap*, *Aly* and *S6 kinase*) that met both criteria. Five of these genes suppress, while one enhances, leg-to-wing transdetermination and therefore affects disc regeneration. Two of the genes, *jing* and *rpd3*, function in concert with chromatin remodeling proteins of the Polycomb Group (PcG) and trithorax Group (trxG) genes during Drosophila development, thus linking chromatin remodeling with the process of regeneration.

Keywords

Drosophila; Imaginal discs; Regeneration; Transdetermination; Wingless; Blastema; Chromatin remodeling; Enhancer trap; *P*-elements; Genetic screen

1. Introduction

There are three different mechanisms that organisms use to re-grow and replace lost or damaged body parts, and often, more than one mechanism can function within different tissues of the same organism. Muscle and bone, for example, repair themselves by activating a resident stem cell population, while the liver regenerates by compensatory proliferation of normally quiescent differentiated cells (Fausto, 2006; Shi and Garry, 2006). Appendage/fin regeneration in lower vertebrates occurs by a process termed epimorphic regeneration (Akimenko et al., 2003; Morgan, 1901; Poss et al., 2003; Stoick-Cooper et al., 2007), which proceeds in three distinct stages: (1) wound healing and migration of the surrounding epithelial cells to form the wound epidermis, (2) formation of the regeneration blastema – a mass of undifferentiated and proliferating cells of mesenchymal origin and (3) regenerative outgrowth and pattern re-formation. Whether these diverse modes of regeneration share a common molecular and genetic basis is not known.

Regeneration in the Drosophila imaginal discs, the primordia of the adult fly appendages, closely parallels epimorphic limb/fin regeneration in lower vertebrates. Cells in the imaginal discs are rigidly determined to form specific adult structures (e.g., legs and wings) by the third

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larval instar. If the discs are fragmented at this time and cultured *in vivo*, they will regenerate (Karlsson, 1980; Schubiger and Hadorn, 1968; Tiong et al., 1977). Disc regeneration begins 12 h after wounding, when transient heterotypic contacts are made between peripodial (squamous epithelium) and columnar cells (disc proper) near the cut edges of the wound (Bosch et al., 2005; Reinhardt and Bryant, 1981; Reinhardt et al., 1977). These initial contacts involve microvilli-like extensions and provide temporary wound closure. Then, approximately 24 h after wounding, homotypic cell contacts (between columnar or between squamous cells) are made involving the close apposition of cell membranes and cellular bridges, which eventually (48 h after wounding) restore the physical continuity of the disc (Reinhardt and Bryant, 1981; Reinhardt et al., 1977). Before and during wound healing, cell division is randomly distributed throughout the disc (Graves and Schubiger, 1982). However, once completed (36–48 h after wounding), division is only observed in cells near the wound site (Kiehle and Schubiger, 1985). These cells are known as the regeneration blastema. Thus, like appendage regeneration in lower vertebrates, disc regeneration involves wound healing followed by blastema formation.

Blastema cells are responsible for the regeneration and repatterning of the entire missing disc fragment (Abbott et al., 1981; Kiehle and Schubiger, 1985). Thus, these cells exhibit remarkable developmental plasticity. For example, in anterior- only leg disc fragments, some blastema cells will switch to posterior identity and establish a novel posterior compartment in the regenerate (Abbott et al., 1981). Gibson and Schubiger (1999) found that this anterior/posterior conversion occurs during heterotypic wound healing, when *hedgehog (hh)*-expressing peripodial cells induce ectopic *engrailed* (*en*) expression in the apposing anterior columnar cells. In addition, the disc blastema, like its vertebrate counterpart, is able to form a normal regenerate (complete leg disc and adult leg) when isolated from the remaining disc fragment(Karpen and Schubiger, 1981; Brockes and Kumar, 2005). Regenerative plasticity is also observed when a few blastema cells switch fate to that of another disc type (e.g., leg-to-wing), in a phenomenon known as transdetermination (Gehring, 1966; Gehring et al., 1968; Hadorn, 1963;Wildermuth, 1968). Transdetermination events are closely associated with regenerative disc growth. Clonal analysis, for example, has shown that blastema cells first regenerate the missing disc structures, and only then, are they competent to transdetermine (Gehring, 1967;Wildermuth, 1968).

Little is known about how the regeneration blastema forms in the fragmented leg disc, although ectopic Wingless (Wg/Wnt1) expression is detected along the cut site, both prior to and during blastema formation (Gibson and Schubiger, 1999) (unpublished observations). Wg is a developmental signal in many different tissues and animals; in flies Wg patterns all of the imaginal discs, functioning as both a morphogen and mitogen to regulate disc cell fate and growth (Johnston and Sanders, 2003; Strigini and Cohen, 1999). In lower vertebrates, Wnt ligands are key regulators of blastema formation during epimorphic regeneration (Kawakami et al., 2006; Stoick-Cooper et al., 2007). Thus, activation of Wg within the disc blastema is potentially important for regeneration. This idea is consistent with the observation that ubiquitous expression of *wg* during the second or third larval instars, in unfragmented leg discs, is sufficient to induce a regeneration blastema in the proximodorsal region of the disc, known as the weak point (Johnston and Schubiger, 1996; Sustar and Schubiger, 2005). Moreover, ubiquitous expression of *wg* mimics the pattern deviations associated with leg disc fragmentation and subsequent regeneration, including the duplication of ventral with concomitant loss of dorsal pattern elements and leg-to-wing transdetermination events (Johnston and Schubiger, 1996; Maves and Schubiger, 1995). Thus, leg disc regeneration can be examined using two experimental protocols: fragmentation or ubiquitous *wg* expression. However, it is important to point out that only fragmentation-induced regeneration involves wound healing.

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Precisely which molecules and signaling pathways are required for the process of regeneration remain poorly understood, partly because the organisms historically used to study regeneration (e.g., newts and salamanders) have been refractory to genetics and molecular manipulations. Recently, however, the use of new genetic techniques together with 'regeneration' model systems – such as planarians, hydra and zebrafish have given researchers the opportunity to examine the mechanisms of regeneration and to identify the genes, proteins and signaling pathways that regulate different regenerative processes (Broun et al., 2005, 1999; Cebria et al., 2002; Nechiporuk and Keating, 2002; Reddien et al., 2005; Schummer et al., 1992; Technau and Bode, 1999; ten Dijke and Hill, 2004; Thummel et al., 2006). For example, a large scale RNAi-based screen was performed to survey gene function in planarian tissue homeostasis and regeneration (Reddien et al., 2005). Out of ~1000 genes examined, RNAi knock-down of 240 displayed regeneration-related phenotypes, including defects in wound healing, blastema formation and blastema cell differentiation (Reddien et al., 2005). Despite these studies, however, it remains unclear whether regeneration requires only the modulation of genes expressed at the time of injury, the reactivation of earlier developmental genes and/or signaling pathways, or the activation of novel genes specific to the process of regeneration. Thus, a major interest in the field of regenerative biology is the identification of gene products that regulate blastema formation, blastema growth and regenerative cellular plasticity. We have performed a genetic screen aimed at identifying genes that regulate cellular plasticity and regeneration using Drosophila prothoracic leg discs. We screened a collection of 97 recessive lethal *P*-element *lacZ* (*PZ*) insertion lines (Russell et al., 1998) and identified six genes that function in *wg*-induced leg disc regeneration, including genes with functional ties to Wg signaling as well as chromatin remodeling proteins.

2. Results

2.1. PZ insertions that modify wg-induced regeneration and transdetermination

We have focused on the regeneration of prothoracic leg discs because the process of regeneration in these discs has been well-characterized both at the descriptive and molecular level (Johnston and Schubiger, 1996; Maves and Schubiger, 1995; Maves and Schubiger, 1998; Sustar and Schubiger, 2005). Leg disc regeneration can be induced either by disc fragmentation and *in vivo* culture or by ubiquitous *wg* expression in mid-second or early third instar larvae (Johnston and Schubiger, 1996; Sustar and Schubiger, 2005). Regeneration induced by both experimental protocols causes leg-to-wing transdetermination in the proximodorsal region of the leg disc, known as the weak point (Maves and Schubiger 1995; Johnston and Schubiger 1996; Sustar and Schubiger 2005). Transdetermination events, therefore, are closely associated with regenerative disc growth and proliferation (Tobler, 1966; Schubiger, 1973; Sustar and Schubiger, 2005). For example, when regeneration is augmented by additional disc injury both the frequency and area of transdetermination increase (Tobler, 1966). Conversely, when regenerative proliferation and growth are inhibited, transdetermination is not observed (Schubiger, 1973; Sustar and Schubiger, 2005). These observations indicate that we can screen mutations in genes for their function in leg disc regeneration by examining the frequency of transdetermination. Here we focus specifically on leg-to-wing transdetermination because it involves the ectopic activation of the wing selector gene *vestigial* (*vg*) in leg disc cells (Maves and Schubiger, 1995). Furthermore, activation of *vg* in leg-to-wing transdetermination has been molecularly characterized and is known to require the *vg-Boundary Enhancer* (*vgBE*) (Kim et al., 1996; Maves and Schubiger, 1998; Williams et al., 1991). Since both fragmentation and ubiquitous *wg* expression induce regeneration and transdetermination, we conducted our screen by overexpressing *wg* – a much simpler procedure than fragmentation and *in vivo* culture.

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Previously, 470 recessive lethal *P-lacZ* enhancer trap lines were screened for ectopic expression after imaginal disc injury and regeneration (Russell et al., 1998). In this study, Russell et al. (1998) induced disc regeneration *in vivo* by random cell death using two different methods: (1) a temperature sensitive cell–lethal mutation in *suppressor of forked* [$su(f)^{12}$] and (2) a short exposure to gamma radiation. Out of the 470 *PZ* insertions, a total of 118 were identified with ectopic *lacZ* expression after gamma radiation, and 47 of those were also activated after *su(f)*-induced cell death (Russell et al., 1998). The design of this screen identified genes that potentially function in radiation repair and/or apoptosis, as well as disc regeneration. Therefore, we re-examined the 118 *PZ* insertion lines, of which only 97 remain as viable fly stocks, for their role in *wg*-induced leg disc regeneration.

Ubiquitous expression of *wg* (*Act5C>wg*) during the third larval instar induces leg-to-wing transdetermination with a high frequency (>90% of leg discs analyzed) (Maves and Schubiger, 1998; Sustar and Schubiger, 2005). This protocol was modified in order to identify *PZ* insertions that both enhance and suppress *wg*-induced leg-to-wing transdetermination and disc regeneration. A protocol of ubiquitously expressing *wg* (*Act5C>wg*) during the mid-second larval instar (60 h after egg deposition, AED) was selected because leg-to-wing transdetermination was observed at a moderate frequency of 32% (n = 60 discs) (Fig. 1A, B and Table 1). Hereafter, we refer to this frequency as the *wg*-induced transdetermination control. Leg-to-wing transdetermination was detected by the ectopic expression of Vg in leg disc cells (Maves and Schubiger, 1995) (Fig. 1B). To identify genes that function in regeneration, we screened for *PZ* insertions that when heterozygous mutant (*PZ/+*) would dominantly enhance or suppress the frequency of the *wg*-induced transdetermination control. Fig. 1 outlines the genetic details of the screen. Out of an initial collection of 97 lethal *PZ* insertions, we tested 90 for their ability to modify leg disc regeneration. Seven *PZ* insertions were excluded from the screen because the fly stocks were no longer recessive lethal (see Supplementary Table 1). Each *PZ* line was examined in three replicate experiments to ensure a consistent interaction (Supplementary Table 1). Of the 90 lines, 17 significantly suppressed and 2 enhanced the *wg*-induced transdetermination control (Table 1). To rule out possible interactions due to genetic background and to further confirm a functional interaction with *wg*, we tested additional mutant alleles of 10 genes that were identified as modifiers (*taiman*, *Secβ61*, *polo*, *Krüppel homolog-1*, *ken and barbie*, *cyclinA*, *jing*, *combgap*, *schnurri* and *Nup154*), and found that 9 similarly modified *wg*-induced leg-to-wing transdetermination (Supplementary Table 1). *schnurri* (*shn*) was excluded from the screen because an amorphic allele of *shn* (shn^1) did not significantly modify the frequency of *wg*-induced transdetermination (data not shown).

To verify that ectopic Vg expression in the leg discs reflects actual leg-to-wing transdetermination events, we allowed animals overexpressing *wg* and heterozygous for the *PZ* insertion (*Act>wg/PZ*, *Act>wg; PZ/+*), to develop through metamorphosis and examined the presence of wing cuticle in the adult legs (Fig. 1C and D). As shown in Table 1, nearly all of the *PZ* insertions (15 out of 17) that suppressed Vg expression in the leg discs, showed a similar and corresponding reduction of leg-to-wing transdetermination in the adult leg cuticle. In four *PZ* insertions [*l(3)05203*, *rpd3*, *S6k* and *l(3)01344*], however, the frequency of ectopic Vg expression in the leg discs did not follow the frequency of leg-to-wing transdetermination in the cuticle. For example, the *rpd3* insertion decreased the frequency of Vg expression in the leg discs, yet the fraction of adult legs with wing cuticle increased, although not significantly (Table 1). This difference may be caused by the development of wing cuticular structures which do not originate from *vg*-expressing leg disc cells; ventral wing hinge structures, such as the yellow club, for example, do not arise from *vg*-positive cells in the wing disc (Fig. 1C and D). Additionally, the two *PZ* insertions that enhanced Vg frequency in the leg discs, *l(3)01344* and *S6k*, did not similarly increase the frequency of leg-to-wing transdetermination in the adult cuticle (Table 1). The reasons for this discrepancy could be related to the differences in

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resolution between the two analyses: we can detect as few as 2–3 Vg-expressing cells in the leg disc through antibody staining, yet identifying small wing cuticular structures, such as a single wing bristle, within the adult leg cuticle is much more difficult to recognize. Nevertheless, we find that the expression of Vg in leg disc cells is a reliable indicator of transdetermination to wing identity and subsequent terminal differentiation of adult wing structures.

2.2. Expression of PZ insertions during leg disc regeneration

Having identified 19 genes that dominantly modify *wg*-induced leg-to-wing transdetermination, we defined the *lacZ* expression of these genes to establish possible correlations with disc regeneration. Specifically, we tested for changes of candidate gene expression in the *wg*-induced regeneration blastema (proximodorsal cells of the leg disc). For this, first thoracic leg discs were harvested from wandering third instar *PZ/+* control larvae and *PZ/Act>wg* or *Act>wg/+; PZ/+* experimental larvae 2.5 days (120 h AED) after the induction of *wg* during the mid-second instar (60 h after egg deposition, AED). Both control and experimental leg discs were stained for β-gal. Among the 19 PZ insertion lines, we observed four different classes of *lacZ* expression changes: (1) no change from the endogenous *lacZ* expression pattern, (2) complete or partial loss of *lacZ* expression, (3) ubiquitous expression throughout the leg disc and (4) blastema-specific expression. Representative examples of the four classes are shown in Fig. 2. Among the entire *PZ* collection (90 insertions), the most common expression pattern following ubiquitous *wg* expression was that of no change from the endogenous *lacZ* expression pattern (Fig. 2A). In contrast, among the 19 *PZ* insertions that modified *wg*-induced leg-to-wing transdetermination eight displayed expression limited to the regeneration blastema (Fig. 2B). These findings indicate that the screen found an enrichment for genes activated in the regeneration blastema following ubiquitous *wg* expression (Fig. 2A). Three of the 19 *PZ* insertions, *ken and barbie* (*ken*), *cyclin A* (*cycA*) and *CG30947*, showed minor or undetectable expression changes upon *wg*-induced regeneration (data not shown). *ken* and *cycA* were ubiquitously expressed, while *CG30947* displayed low levels of expression during regeneration (Fig. 2C, Class 1; data not shown). One of the 19 *PZ* insertions, *oho23B*, showed complete loss of the endogenous *lacZ* expression pattern upon *wg*-induced regeneration, including loss of expression in the presumptive tarsal segments and chordotonal organ (Fig. 2C, Class 2). In seven of the 19 *PZ* insertions, *Krüppel-homolog-1 (Kr-h1)*, *Nup154*, *rpd3*, *S6 kinase* (*S6k*), *Secβ61*, *l(2)00248*, *Syx13*, expression was low or absent in prothoracic leg discs of third instar larvae, but increased significantly and ubiquitously in the discs during leg disc regeneration (Fig. 2C, Class 3 and Fig. S1). Finally, a total of eight lines, *jing*, *taiman* (*tai*), *combgap* (*cg*), *polo*, *l(3)05203*, *l(3)01629*, *l(3)01344*, *Aly*, exhibited *lacZ* expression limited to the *wg*-induced regeneration blastema (Fig. 2C, Class 4 and Fig. S2). The blastema-specific expression of these genes during *wg*-induced regeneration, together with their ability to modify leg-to-wing transdetermination, suggests that they function in disc regeneration as well as regulate disc cell plasticity.

2.3. mRNA expression of PZ lines that modify wg-induced regeneration

A major criterion of our screen was to identify genes activated specifically in the blastema during leg disc regeneration. Because *P*-elements can insert into chromosomes in either the 5′ or 3′ direction, or detect only a subset of enhancers and possibly even disrupt regulation by insertion, it is necessary to establish that the *lacZ* reporter expression represents the actual expression of the adjacent gene. For many of the *PZ* insertions identified, both the insertion site and adjacent gene were known. To verify the *lacZ* expression from the enhancer trap lines, we made cDNA probes to the genes that modified transdetermination, and performed *in situ* hybridization in wild-type and *wg*-overexpressing leg discs. Five *PZ* lines [*l(3)01629*, *tai*, *Syx13*, *l(2)00248* and *l(3)05203*] were not examined, either because the *P*-element insertions were near uncharacterized genes or full length cDNAs were not available (Table 1). Of the 14

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genes examined by *in situ* hybridization, 11 (*ken*, *cycA*, *CG30947*, *oho23B*, *Secβ61*, *Nup154*, *rpd3*, *S6k*, *jing*, *Aly* and *cg*) reliably reproduced the enhancer trap expression pattern from the *PZ* insertion lines (Fig. 3A–K, Table 1). Expression of the *ken*, *cycA* and *CG30947* genes did not alter significantly after ubiquitous *wg* expression (Fig. 3A–C, Class 1), while expression of *oho23B* in the distal primordia of the leg disc (data not shown) was lost after *wg*-induced regeneration (Fig. 3D, Class 2). Transcripts from *Secβ61*, *Nup154*, *rpd3*, and *S6k* were ubiquitously expressed following *wg* overexpression (Fig. 3E–H, Class 3). In most leg discs (>50%), *rpd3* and *S6k* expression were found at significantly higher levels in the regeneration blastema ($n = 26$ and 32 discs, respectively; Fig. 3G and H, black arrowheads). Consistent with these observations, we found that the enhancer trap expression of these two genes, following ubiquitous *wg* expression, was noticeably higher in *vg*-expressing leg cells that had transdetermined to wing identity (Fig. 4A–A‴ and data not shown). Thus, upon *wg*-induced regeneration these two genes are expressed throughout the entire leg disc, but at noticeably higher levels in the blastema (Fig. 3G, H and Table 1). In contrast, in situ hybridization of the *jing*, *Aly* and *cg* genes detected blastema-specific expression in the majority of leg discs analyzed (>60%, $n = 19$, 24 and 27 discs, respectively; (Fig. 3I–K, Class 4). *jing-lacZ* expression was consistently adjacent to, but never overlapping with, leg disc cells expressing ectopic Vg (Fig. 4B–B‴; data not shown), while the enhancer trap expression of *cg* and *Aly* were coincident with *vg*-expressing leg disc cells (Fig. 4C–C‴; data not shown). Of the remaining three genes, we identified two, *l(3)01344* and *Kr-h1*, whose expression by *in situ* hybridization differed from the enhancer trap expression pattern (Fig. 5A–B). In situ hybridization of *l(3)01344* revealed uniform and low levels of expression in prothoracic leg discs from third instar larvae (data not shown) which was reduced to undetectable levels upon *wg*-induced regeneration (Fig. 5A, Class 2). This differed significantly from the *l(3)01344-lacZ* expression pattern which was found in the *wg*-induced regeneration blastema (Fig. 5A′). Additionally, *Kr-h1-lacZ* expression was ubiquitous after *wg* induction (Fig. 5B′), yet by *in situ* hybridization *Kr-h1* transcripts were detected specifically in the regeneration blastema (70%, $n = 23$; Fig. 5B, Class 4). Finally, we could not reliably detect expression of the *polo* gene by *in situ* hybridization. Thus, the enhancer trap expression analysis together with *in situ* hybridization experiments, has allowed us to identify six genes (*jing*, *Aly*, *cg*, *Kr-h1*, *rpd3* and *S6k*) that are activated in the regeneration blastema upon ubiquitous *wg* expression. Heterozygous mutations in the *jing*, *Aly*, *cg*, *Kr-h1* and *rpd3* genes dominantly suppressed, while *S6k* enhanced *wg*-induced leg-to-wing transdetermination (Table 1), supporting the conclusion that they function in regeneration.

2.4. Modifications of transdetermination in other discs

Ubiquitous *wg* expression induces not only leg-to-wing transdetermination, but other transdetermination events to wing in the eye, antenna, maxillary palp and genital discs (Table 2) (Johnston and Schubiger, 1996). To determine whether transdetermination, in general, is altered by our modifier genes we examined the frequencies of *wg*-induced transdeterminations in these other discs. Of the six blastema-specific genes that modified *wg*-induced leg-to-wing transdetermination, heterozygous mutations in two (*jing* and *cg*) significantly suppressed all *wg*-induced transdeterminations in the other discs (Table 2). These findings reveal that these genes generally promote regeneration and cell fate plasticity. Interestingly, the remaining four genes (*Kr-h1*, *Aly*, *rpd3* and *S6k*) modified *wg*-induced transdeterminations in a context-dependent manner. For example, animals that were heterozygous mutant for *Kr-h1* suppressed transdetermination events in all of the discs except the genital disc (Table 2). This suggests that some genes can modify cell fate plasticity, in general, while others act in a more disc-specific manner.

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2.5. Blastema formation is altered in genes that modify leg-to-wing transdetermination

To investigate how these genes function in regeneration we examined blastema formation in animals heterozygous mutant for the *PZ* insertions. Ubiquitous expression of *wg* during the third larval instar (72 h AED) induces a regeneration blastema within 48 h (82%, *n* = 22 discs; Fig. 6A) (Sustar and Schubiger, 2005). As the blastema is formed, the surrounding cells exit the cell cycle (Fig. 6A′) (Sustar and Schubiger, 2005). In four of the six *PZ* insertions, we observed significant changes in blastema formation and thus regeneration. Leg discs from *jing/+* animals rarely formed a blastema, instead, cell replication was randomly distributed throughout the leg discs both 48 and 72 h after *wg* induction (62%, *n* = 21 discs; 84.6% *n* = 26 discs, respectively) (Fig. 6B and B′). *Aly/+* and *cg/+* discs, in contrast, were able to form a blastema, yet oneday later compared to the *wg*-expressing controls (Fig. 6A, A′, C, C′, D and D′). Instead of cell replication limited to the blastema at 48 h, *Aly/+* and *cg/+* leg discs showed a random distribution of cell replication (54.5%, *n* = 22; 53%, *n* = 18, respectively) (Fig. 6C and D). However, after 72 h of *wg* expression a blastema formed in *Aly/+* (81%, *n* = 22) and *cg/+* (84%, *n* = 19) leg discs with a frequency similar to the *wg*-expressing controls (74%, *n* = 23) (Fig. 6C′ and D′). Like *Aly/+* and *cg/+* animals, *rpd3/+* animals were able to form a blastema, but at a reduced frequency when compared to the *wg*-expressing controls (55%, *n* = 21). Leg discs that failed to form a blastema showed random cell replication (30%) or no replication (15%). Similar patterns of replication were observed in *rpd3/+* leg discs after 72 h of *wg* expression (data not shown). Blastema formation in *S6k/+* and *Kr-h1/+* animals was not significantly different from the *wg*-expressing control animals (data not shown). Taken together, these results provide evidence that two wild-type copies of *jing*, *Aly*, *cg* and *rpd3* are required for the initiation of regeneration and/or the timing of regenerative proliferation, growth and cellular plasticity.

3. Discussion

3.1. wg-inducible genes involved in leg disc regeneration

Extensive studies have focused on the proliferation and pattern regulation of regenerating imaginal discs (Abbott et al., 1981; Adler, 1981; Bryant et al., 1977; Dale and Bownes, 1985; French et al., 1976; Gibson and Schubiger, 1999; Johnston and Schubiger, 1996; Kiehle and Schubiger, 1985; Mattila et al., 2004; Maves and Schubiger, 1998; Sustar and Schubiger, 2005). In contrast, relatively few studies have investigated the molecular basis for regenerative growth in the discs, including the question of how the regeneration blastema forms and what gene products contribute to its proliferation and remarkable cellular plasticity (Mattila et al., 2005). Here we describe an enhancer trap screen designed to identify genes with changed gene expression during leg disc regeneration as well as required for regenerative proliferation and growth.

This screen identified 19 genes that when heterozygous mutant (*PZ/+*), dominantly modify *wg*-induced leg-to-wing transdetermination, which serves as a functional assay for disc regeneration (Table 1). Of the 19 genes, 37% are transcription factors or involved in transcriptional regulation (*tai*, *Krh1*, *ken*, *jing*, *cg*, *rpd3* and *Aly*), 21% function in cell cycle regulation and growth (*oho23B*, *S6k*, *polo* and *cycA*), 10.5% play a role in protein secretion (*Secβ61* and *Syx13*), and 31% are of other or unknown function [*l(3)01629*, *CG30947*, *l(2)00248*, *l(3)05203*, *l(3)01344*, *Nup154*]. The identification of transcription factors as the most frequent class of genes that modify *wg*-induced leg disc regeneration was similarly observed in a DNA microarray screen designed to identify genes enriched in leg disc cells that transdetermine to wing (Klebes et al., 2005). Together, these findings strongly suggest that transcription factors and their downstream targets play a prominent role in disc cell plasticity.

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Using *lacZ* expression analyses, together with whole mount *in situ* hybridization experiments, we verified the expression patterns of the 19 genes that modified *wg*-induced leg-to-wing transdetermination (Figs. 2–5). This analysis identified several different expression patterns upon *wg*-induced regeneration, including a loss of gene expression, ubiquitous expression and genes with expression limited to the regeneration blastema (Figs. 2–5). Such observations indicate that a complex change of gene expression, both negative and positive, mediates the process of epimorphic regeneration. Six (*jing, Alyi cg, rpd3, Kr-h1* and *S6k*) of the 19 modifiers displayed expression limited to the regeneration blastema (Table 3), indicating that we have identified novel markers of regeneration and transdetermination. The blastema-specific expression patterns of *jing*, *Aly*, *cg*, *Kr-h1*, *rpd3* and *S6k* raised the intriguing possibility that these genes may be functionally involved in the formation, cell proliferation or maintenance of the blastema during disc regeneration (Table 3). Indeed, upon ubiquitous *wg* expression *jing*/+ animals rarely formed a regeneration blastema, indicating that two wild-type copies of *jing* are required for the initiation of the regenerative process. In contrast, *Aly*/+ and *cg*/+ animals formed a normal blastema, but only after a one-day delay (Fig. 6). Therefore, two wild-type copies of the *Aly* and *cg* genes are required for the proper timing of regeneration. In addition, we found that the frequency of blastema formation was reduced in *rpd3*/+ animals, implicating this gene in the process of regeneration. Interestingly, heterozygous mutations in all four of these genes (*jing*, *Aly*, *cg* and *rpd3*) strongly suppress *wg*-induced leg-to-wing transdetermination (Table 3). We speculate that the transdetermination frequency declines in these mutant animals because the initiation and/or timing of blastema formation is delayed. This idea is consistent with all previous work which has shown that blastema cells are only competent to transdetermine after they have regenerated the missing disc structures (Gehring, 1967;Wildermuth, 1968). Heterozygous mutations in *Kr-h1* and *S6k* did not significantly alter the formation of the *wg*-induced regeneration blastema, however, these genes did affect regeneration-induced transdetermination (Table 3). Such results suggest that *Kr-h1* and *S6k* specifically function to modulate the cell fate changes that occur as a consequence of regeneration.

3.2. Chromatin remodeling genes and regenerative cellular plasticity

We call attention to two genes identified in our screen: *jing* and *rpd3*. Our analysis showed that upon regeneration, the expression of *jing* and *rpd3* is blastema-specific and functional assays revealed that both genes function in blastema formation and disc cell plasticity during regeneration. Jing is a Zn-finger transcriptional repressor required for Drosophila wing development and proximodistal (P/D) axis formation in the developing leg (Culi et al., 2006). Drosophila Rpd3 is a histone deacetylase (HDAC) that alters local chromatin architecture by deacetylating histone tails, leading to chromatin compaction and transcriptional silencing (De Rubertis et al., 1996). Both *jing* and *rpd3* function in concert with the Polycomb Group (PcG) and trithorax Group (trxG) proteins during Drosophila development (Collins and Treisman, 2000; Culi et al., 2006). This is significant because two previous studies have reported the differential regulation of several PcG and trxG proteins in regenerating leg disc cells (Klebes et al., 2005; Lee et al., 2005). These studies have also shown that the *wg*-induced transdetermination frequency is altered in PcG and trxG heterozygous mutant flies. PcG and trxG proteins sustain lineage-specific transcriptional programs, and thus cell identities, over multiple cell divisions through epigenetic modification of chromatin structure, notably through covalent histone modifications (Ringrose and Paro, 2004). Our findings of *jing* and *rpd3* activation in the blastema during disc regeneration and their mutations altering blastema formation supports the idea that regenerative proliferation, plasticity and transdetermination events are regulated or driven by changes in chromatin remodeling (Klebes et al., 2005; Lee et al., 2005).

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3.3. Regeneration genes and the process of transdetermination

Investigations into the molecular basis of transdetermination have shown that inputs from the Wg, Decapentapelagic (Dpp) and Hedgehog (Hh) signaling pathways activate key selector genes out of their normal developmental context, such as ectopic Vg activation in the leg disc, which then drives cell-fate switches (Maves and Schubiger, 2003). Several of the genes identified in our screen have functional ties to Wg, Dpp and Hh signaling pathways. For example, Cg is a zinc-finger transcription factor that is required for proper transcriptional regulation of the Hh signaling effector gene *Cubitus interruptus* (*Ci*) (Campbell and Tomlinson, 2000). In *cg* mutant wing and leg discs, *Ci* expression is lowered in the anterior compartment, resulting in the ectopic activation of *wg* and *dpp* and significant disc overgrowth (Campbell and Tomlinson, 2000). Another gene identified in our screen–*ken*, functions in concert with Dpp to direct the development of the Drosophila terminalia (Lukacsovich et al., 2003). Further characterizations of whether these genes and other modifiers of transdetermination and regeneration affect Wg, Dpp and Hh expression and/or signaling may shed light on the regulation of regeneration and regeneration-induced proliferation and cell fate plasticity.

4. Conclusion

So far, only one previous study has examined the molecular basis of blastema formation in the Drosophila imaginal discs after injury. Mattila et al. (2005) reported that the Jun-N terminal Kinase (JNK) signaling pathway recruits cells into the regenerative cell cycles. Thus, our screen contributes significantly to the identification of genes functionally involved in imaginal disc regeneration. Further characterization of the genes identified in this screen will provide important insights into the mechanism of epimorphic regeneration, not only in the Drosophila imaginal discs, but also in appendage regeneration in lower vertebrates, and ultimately aid in the development of therapies to replace or re-grow tissues lost to disease or injury.

5. Experimental procedures

5.1. Fly stocks and genetics

Flies were reared at 25 °C and on standard Drosophila media containing cornmeal and molasses. The lethal *P-lacZ* insertion lines used in the screen (see Supplementary Table 1), and the different mutant alleles of the *PZ* insertions (*tai*^{K15101}, *Secβ61*^{K02307}, *polo*^{KG03033}, *Kr-h1*^{K04411}, *ken*^{1}, *cycA*^{EY11746}, *jing*^{3404}, *cg*^{1}, *Nup154*^{EY13350}, *shn*^{1}) tested for function in *wg*-induced regeneration, were received through the Bloomington Stock Center. Each second chromosome *PZ* line was balanced over a *CyO-GFP* chromosome and each third chromosome *PZ* line was balanced over a *TM6B, Tb* chromosome in a *y w hs-flp*^{122} background. Females of the balanced *PZ* lines were then crossed to *y w; Actin5c>y*$^{+}$*>wg* males (K. Basler) (Fig. 1). Egg collections were taken for 1–2 h after a 1 h pre-collection. To overexpress *wg* and induce regeneration, the progeny were heat-shocked 60 h after egg deposition (AED) for 75 min at 37 °C (Fig. 1). Using this protocol *wg* is overexpressed in >90% of the disc cells after heat shock (Maves and Schubiger 1995). Animals heterozygous for the *PZ* insertion and *Actin5c>y+>wg* were identified by the absence of *GFP* (2nd chromosome *PZ* lines) or *Tb* marker (3rd chromosome *PZ* lines), and either dissected 2.5 days (120 h AED) after *wg* induction or allowed to develop through metamorphosis. For all experiments, the *y w hs-flp*^{122}; *Actin5c>y*$^{+}$*>wg* animals were used as the control. To examine the formation of the regeneration blastema, ubiquitous *wg* expression was induced at 72h AED in animals heterozygous mutant for the following *PZ* insertions: *jing*^{01094}, *Aly*^{02267}, *cg*^{07659}, *rpd3*^{04556}, *Krh1*^{10642} and *S6k*^{07084}.

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5.2. Immunocytochemistry and in situ hybridization

Discs were fixed in 4% formaldehyde in PBS. The following primary antibodies were used in overnight incubations at 4 °C in PBNT: rabbit anti-Vestigial (1:200, S Carroll), rabbit anti-β-galactosidase (1:1000, Cappel). Confocal images were collected with Bio-Rad MRC 600.

Imaginal discs were hybridized with digoxigenin-labeled anti-sense and sense RNA probes as previously described (O'Neill and Bier, 1994). Probes were made from full-length cDNA containing plasmids (provided by BDGP).

For blastema analysis, BrdU (10 μg/ml) incorporation was performed at both 48 and 72 h after *wg* expression (induced at 72 h AED) for 20 min before a 30 min fixation.

5.3. Cuticle analysis

Transdetermination is only observed in pharate adults (adult flies unable to eclose after differentiation). Such animals were boiled in 5 N KOH for 15 min, washed two to three times with H_2O and mounted in Faure's mounting media (Ashburner, 1989).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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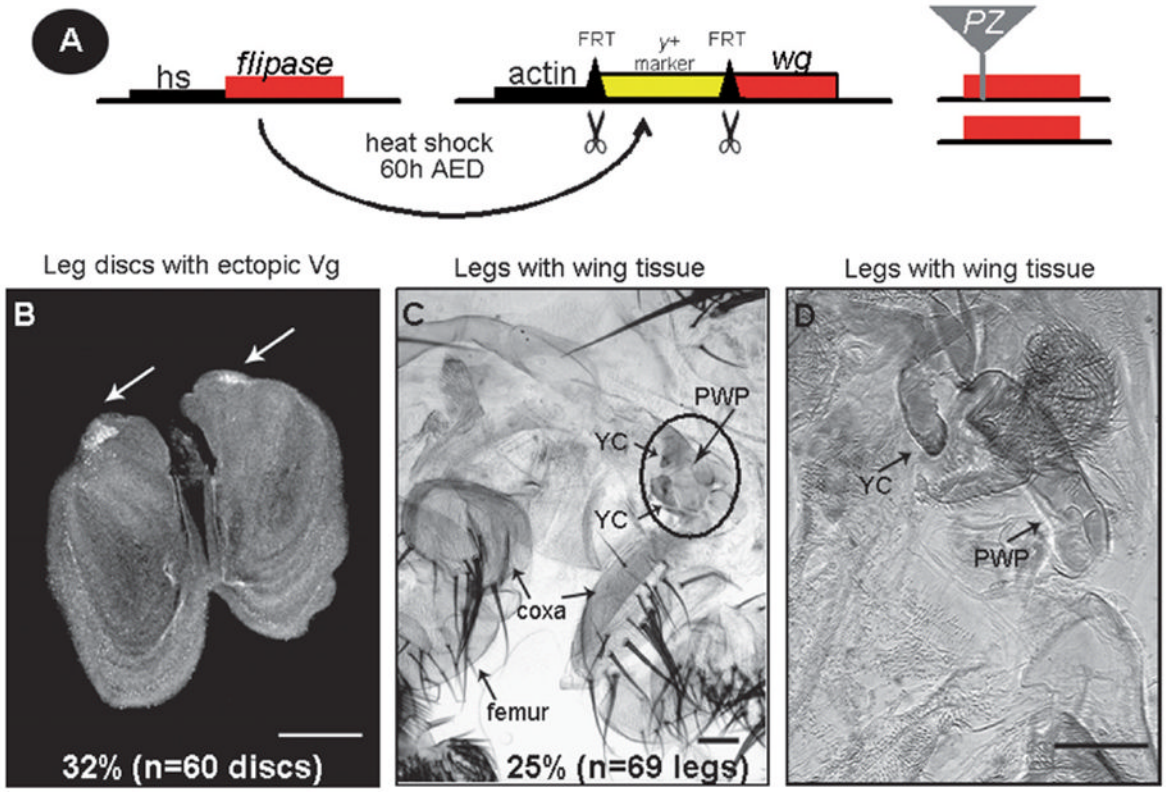

Fig. 1.

wg-induced regeneration and transdetermination. (A) Leg-to-wing transdetermination was induced in each heterozygous *PZ* insertion line by ubiquitously expressing *wg* in mid-second instar larvae (60 h after egg deposition, AED) using the FLP/FRT system(for details see Section 5). (B) Using this protocol, *wg* overexpression alone (*Actin5C>wg*, referred to as the *wg*-induced transdetermination control) induces ectopic Vg expression (white arrows) in leg discs with a moderate frequency of 32% (n = 60 discs).White scale bar, 50 μm. (C and D) Leg cuticle from*Actin5C>wg* pharate adults (differentiated but not enclosed animals) contain wing tissue with a frequency of 25% (n = 69 legs). (C) Black circle within the leg cuticle highlights ventral wing hinge structures including the yellow club (YC) and pleural wing process (PWP). The proximal leg segments, coxa and femur, are labeled. (D) Leg cuticle with transdetermined wing structures (YC and PWP) at a higher magnification than in (C). Black scale bars, 100 μm.

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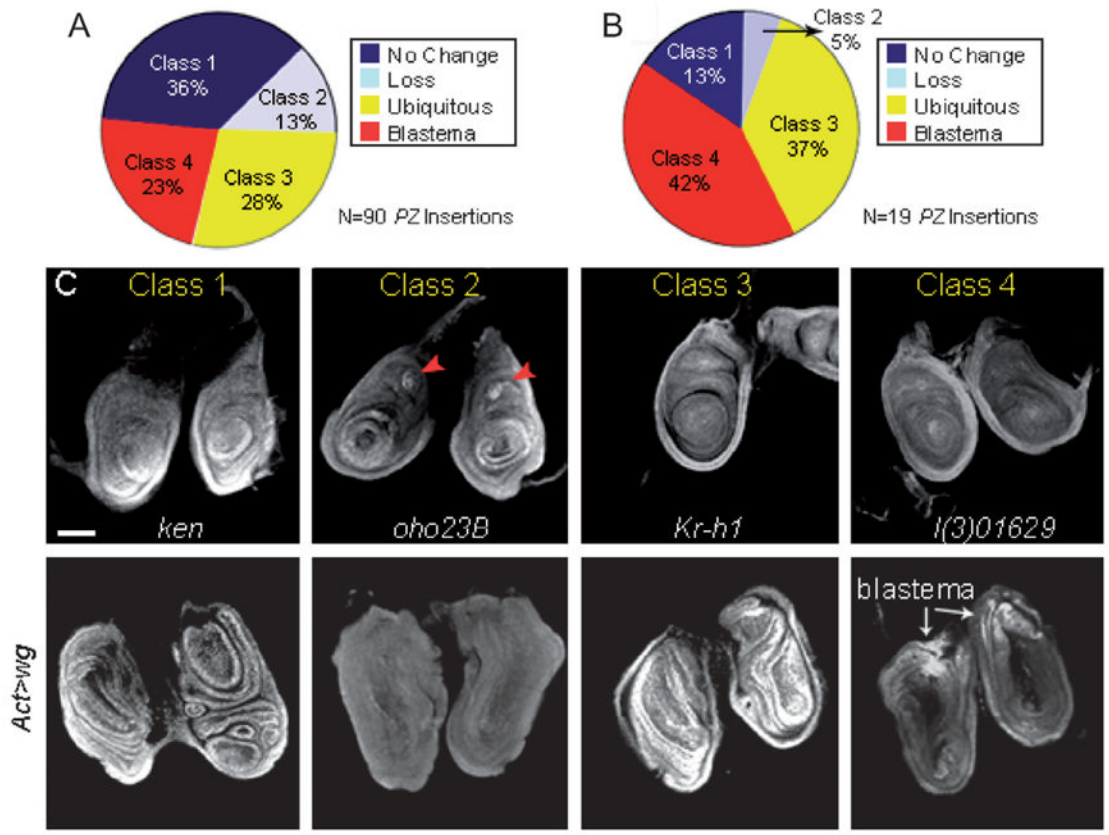

Fig. 2.

LacZ expression of enhancer trap lines that modify leg disc regeneration. *PZ* insertions lines were examined for *lacZ* expression in the regeneration blastema (proximodorsal region) following ubiquitous *wg* expression. (A) Pie-chart shows the distribution of the 90 *PZ* insertions into four different classes of *lacZ* expression: Class 1 lines show no expression change upon ubiquitous *wg* expression (36%, blue), Class 2 lines show partial or complete loss of expression (13%, light blue), Class 3 lines exhibit ubiquitous expression (28%; yellow) and Class 4 lines display expression limited to the regeneration blastema (23%; red). (B) Pie-chart shows the *lacZ* expression patterns of the 19 *PZ* insertions identified to modify *wg*-induced leg-to-wing transdetermination. Note that there is an enrichment for genes that exhibit blastema-specific expression following ubiquitous *wg* expression (Class 4, 42%; red). (C) Representative examples of the four classes of *lacZ* expression patterns. Upper panels show endogenous expression in prothoracic leg disc pairs from four *PZ* lines [*ken* and *barbie* (*ken*), *oho23B*, *Krüppel-homolog-1* (*Kr-h1*), and *l(3)01629*]. The leg discs were dissected from wandering third instar larvae. Lower panels show *lacZ* expression of *ken*, *oho23B*, *Kr-h1*, and *l(3)01629* after ubiquitously expressing *wg (Act > wg)* during the mid-second instar. Leg disc pairs were dissected 2.5 days (120 h AED) after *wg* induction. Red arrowheads mark *lacZ* expression in the chordotonal organ. Scale bar, 50 μm.

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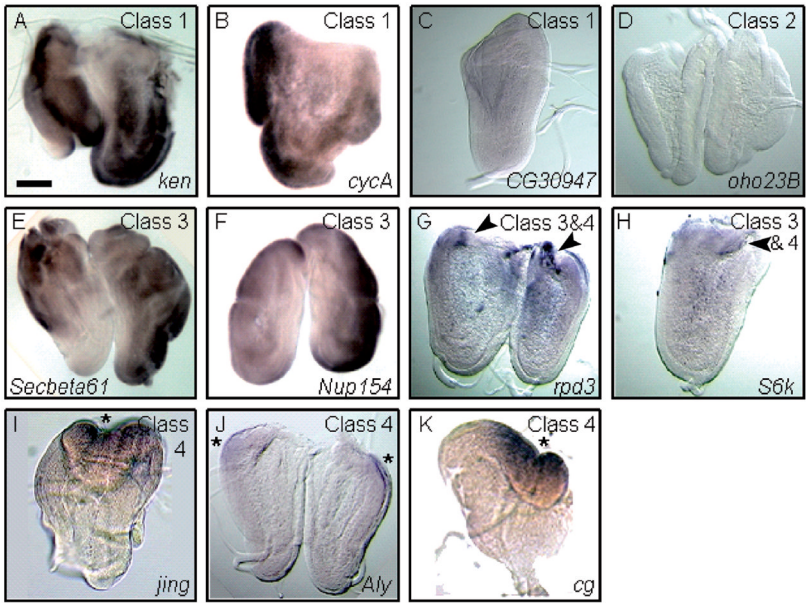

Fig. 3.

Whole mount *in situ* hybridization to *wg*-overexpressing leg discs. (A–K) *wg*-overexpressing leg discs were hybridized with probes from genes that modify *wg*-induced leg-to-wing transdetermination. Expression of the *ken* (A), *cycA* (B), *CG30947* (C), *oho23B* (D), *Secβ61* (E), *Nup154* (F), *rpd3* (G), *S6k* (H), *jing* (I), *Aly* (J) and *cg* (K) genes by *in situ* hybridization reproduced the enhancer trap expression pattern. The ubiquitous expression of the *ken* and *cycA* genes and low level expression of *CG30947* did not significantly alter upon *wg* misexpression (Class 1). Expression of *oho23B* was not detected upon *wg*-induced leg disc regeneration (Class 2), while the *Nup154*, *S6k*, *rpd3* and *Secβ61* genes were ubiquitous or broadly expressed in the dorsal region of *wg*-overexpressing leg discs (Class 3). Note that *rpd3* and *S6k* expression is considerably higher in the *wg*-induced regeneration blastema (black arrowheads in G and H; Class 3 and 4). The *jing*, *Aly*, and *cg* genes exhibit blastema-specific expression following ubiquitous *wg* expression (Class 4). Asterisks mark the proximodorsal regeneration blastema. Scale bar, 100 μm.

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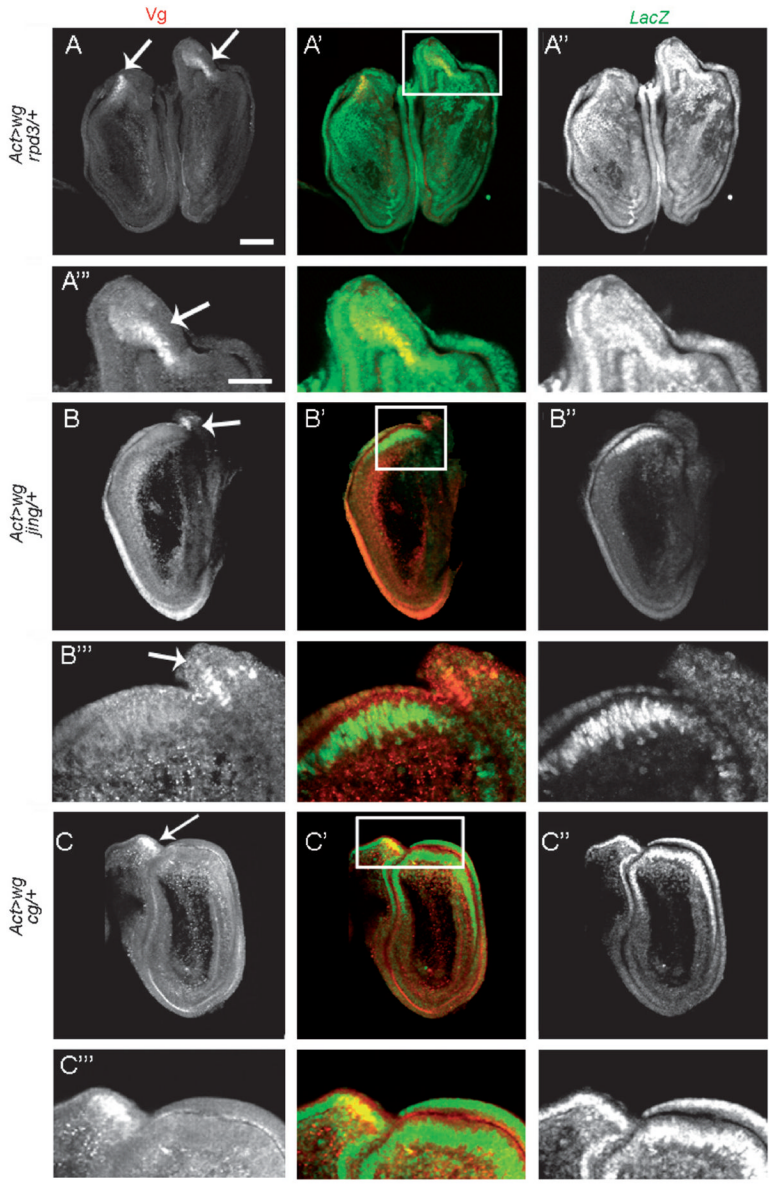

Fig. 4.

Expression of *PZ* lines in transdetermined leg disc cells. (A–A″) *Actin5C>wg; rpd3/+* prothoracic leg disc pair; (B–B″) *Actin5C>wg, jing/+* prothoracic leg disc; (C–C″) *Actin5C>wg, cg/+* prothoracic leg disc. (A‴–C‴) are enlargements of the boxed regions in A′–C′, respectively. The single channels are shown in first (Vg expression) and last (*LacZ* expression) columns. In the merged images Vg expression is in red and *LacZ* expression is in green. White arrows mark Vg-expressing leg disc cells. (A′–A‴) *LacZ* expression from *PZ* insertion *rpd3* is ubiquitous after *wg* overexpression, yet higher expression levels are detected in transdetermined leg disc cells. (B′–B‴) After *wg* overexpression, *jing-lacZ* expression is localized to the regeneration blastema and adjacent to, but not overlapping with, *vg*-expressing leg disc cells. (C′–C‴) *LacZ* expression from *PZ* insertion *cg* is restricted to the regeneration blastema after *wg* induction, and generally co-localizes with *vg*-expressing leg disc cells (4/4 discs). White scale bars, 50 μm.

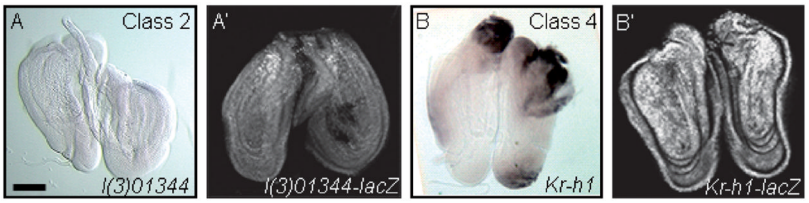

Fig. 5.

In situ hybridization of the *l(3)01344* and *Kr-h1* genes reveals different expression patterns from the enhancer trap lines. (A and B) *wg*-overexpressing leg discs were hybridized with probes to the *l(3)01344* and *Kr-h1* genes. In situ hybridization of *l(3)01344* (A) and *Kr-h1* (B) does not reproduce the *lacZ* expression pattern from the enhancer trap lines which are shown in (A′) and (B′), respectively. (A) The low level of *l(3)01344* expression in wild-type leg discs (data not shown) is down-regulated upon *wg*-induced leg disc regeneration (Class 2). (B) In situ hybridization of the *Kr-h1* gene detects blastema-specific expression following ubiquitous *wg* expression (Class 4). Scale bar, 100 μm.

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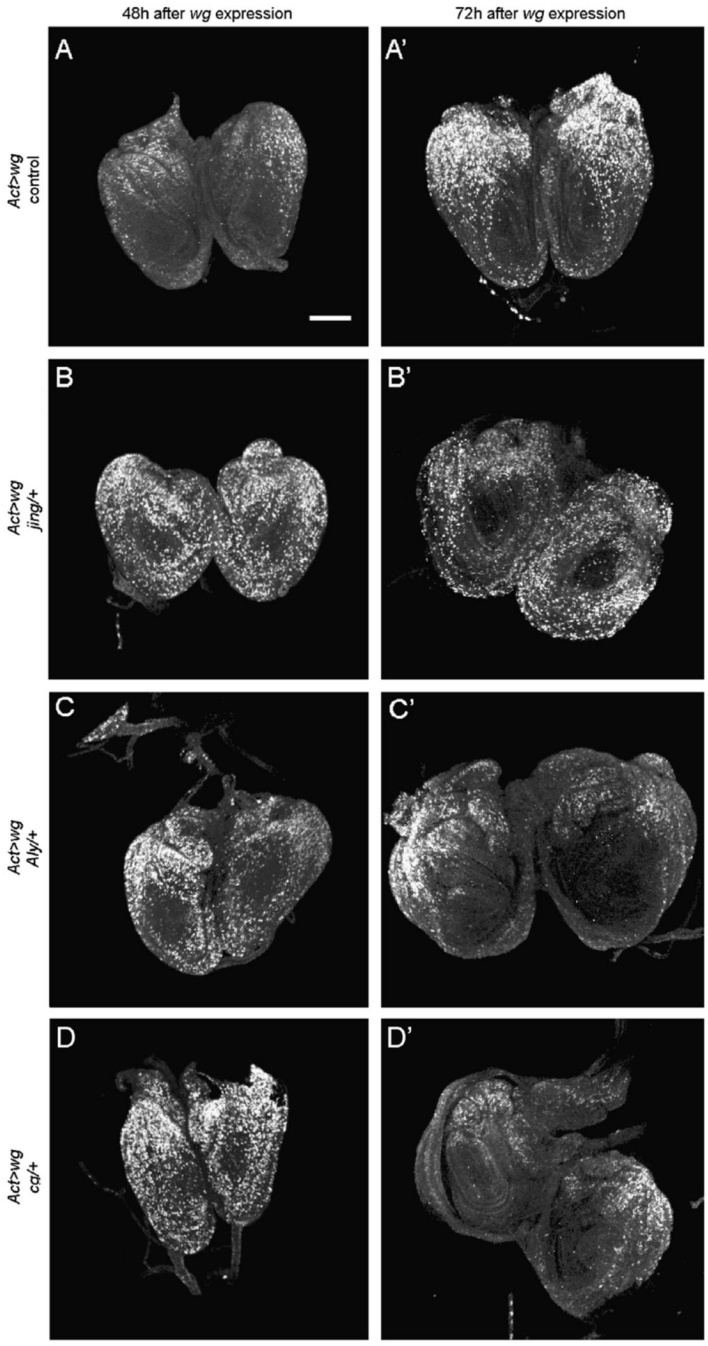

Fig. 6.

Blastema formation is altered by heterozygousmutations of *jing*, *Aly*and *cg*. BrdUlabeling showscell division in leg discs 48 and 72 h after ubiquitous *wg* expression (induced at 72 h AED) in control animals expressing *wg* alone (A and A′), *jing*/+ animals (B and B′), *Aly*/+ animals (C and C′) and *cg*/+ animals (D and D′). (A) At 48 h, cell replication is maintained in the proximodorsal leg cells, the regeneration blastema, and arrested in ventral non-blastema cells. This phenotype is observed in 82% of leg discs analyzed (n = 22). (A′) At 72 h, the blastema grows in size and expands ventrally. (B and B′) In *jing*/+ leg discs a blastema rarely forms, instead, random cell replication is observed at 48 h and again at 72 h after *wg* induction. (C, C′ and D, D′) *Aly*/+ and *cg*/+ leg discs form a blastema, yet with a one-day delay compared

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to the *wg*-expressing control animals. (C and D) At 48 h, the majority of *Aly/+* and *cg/+* leg discs exhibit randomcell replication, yet one day later, after 72 h of *wg* expression, (C′ and D ′) a regeneration blastema is observed. Note that in all cases the cell cycle is arrested in the center of the discs, the primordia of tarsal segments 2–5. Scale bar, 50 μm.

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Table 1

PZ lines that suppress or enhance wg-induced leg-to-wing transdetermination

Genotype	*LacZ* expression	*In situ* expression	Frequency (%) of Vg expression in leg discs (*n* = discs)	Frequency (%) of legs with wing tissue (*n* = discs)
Act>wg			32 (60)	25 (69)
Suppressors				
l(3)01629	Class 4	N/A	4 (113)*	11(19)
taiman (*tai*^{01351})	Class 4	N/A	5 (97)*	8 (24)
Secβ61^{07214}	Class 3	Class 3	6 (107)*	6 (46)**
polo^{01673}	Class 4	N/A	7 (53)**	15 (34)
Krüppel-homolog-1 (*Kr-h1*^{10642})	Class 3	Class 4#	8 (128)*	10 (30)
Syx13^{01470}	Class 3	N/A	8 (97)*	6 (31)**
ken and barbie (*ken*^{02970})	Class 1	Class 1	9 (114)*	3 (40)**
oho23B^{03575}	Class 2	Class 2	9 (88)*	13 (23)
cyclin A (*cycA*^{03946})	Class 1	Class 1	10 (119)*	19 (54)
l(2)00248	Class 3	N/A	10 (96)*	12 (30)
CG30947^{1483a}	Class 1	Class 1	10 (80)**	15 (20)
l(3)05203	Class 4	N/A	11 (74)**	23 (40)
rpd3^{04556}	Class 3	Class 3/4	12 (122)**	35 (20)
jing^{01094}	Class 4	Class 4	13 (113)**	3 (32)**
combgap (*cg*^{07659})	Class 4	Class 4	13 (100)**	10 (20)
Nup154^{01501}	Class 3	Class 3	13 (68)**	8 (26)
Aly^{02267}	Class 4	Class 4	15 (117)**	5 (40)**
Enhancers				
l(3)01344	Class 4	Class 2#	59 (92)**	25 (24)
S6 kinase (*S6k*^{07084})	Class 3	Class 3/4	48 (82)**	19 (48)

Data shown are from prothoracic leg discs and adult legs. 90 *PZ* insertion lines (see Supplementary Table 1) were examined for their ability when heterozygous mutant to dominantly modify wg-induced regeneration and transdetermination. Transdetermination frequency in leg discs and adult cuticle were generated from animals heat shocked at 60 h AED (37 °C) to induce ubiquitous *wg* expression.

* Asterisk(s) indicate *p*-values <0.001;

** indicate *p*-values within 0.01–0.05. The *lacZ* and *in situ* expression patterns of each *PZ* insertion falls into 4 different classes after ubiquitous *wg* expression (see Fig. 2, for details). The number sign (#) indicates that the *lacZ* expression pattern differed significantly from the *in situ* expression pattern (Fig. 5). N/A, *in situ* expression is not available.

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Table 2

Effects of modifier genes on transdetermination to wing in eye-antennal and genital discs

Genotype	Eye-to-wing (%) (*n*)	Antenna-to-wing (%) (*n*)	Maxillary palp-to-wing (%) (*n*)	Genital-to-wing (%) (*n*)
Act>wg (control)	25 (53)	28 (71)	20 (71)	29 (24)
rpd3^{04556}	0 (28)**	0 (28)*	11 (28)	14 (28)
jing^{01094}	0 (27)**	0 (27)*	4 (27)**	0 (15)**
cg^{07659}	0 (29)**	0 (29)*	0 (29)**	5 (22)**
Kr-h1^{10642}	0 (20)**	0 (20)**	5 (20)**	46 (22)
Aly^{02267}	4 (24)**	4 (24)	15 (20)	20 (20)
S6k^{07084}	22 (32)	0 (32)*	22 (32)	13 (23)

Data shown are from eye-antennal (which includes the maxillary palp) and genital discs. The number of discs with ectopic Vg expression per total number of discs examined is given in percentages. Transdetermination frequency in eye-antennal, maxillary palp and genital discs were generated from animals heat shocked at 60 h AED (37 C) to induce ubiquitous *wg* expression. Asterisk(s) (*) indicate *p*-values <0.001; (**) indicate *p*-values within 0.001–0.05.

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Table 3

Summary of genes that function in leg disc regeneration

Gene	Expression during regeneration	Blastema formation	*wg*-induced transdeterminations				
			Leg-to-wing	Eye-to-wing	Antenna-to-wing	Maxillary palp-to-wing	Genital-to-wing
Kr-h1^	Blastema-specific	NC	S	S	S	S	NC
rpd3	Ubiquitous/blastema	Reduced	S	S	S	NC	NC
jing^	Blastema-specific	Reduced	S	S	S	S	S
cg^	Blastema-specific	Delayed	S	S	S	S	S
Aly	Blastema-specific	Delayed	S	S	S	NC	NC
S6k	Ubiquitous/blastema	NC	E	NC	S	NC	NC

Six genes were identified that met both criteria of our screen, that is, they displayed expression in the regeneration blastema during leg disc regeneration and significantly modified regeneration-induced leg-to-wing transdetermination. Expression patterns are based on whole-mount *in situ* hybridization in the leg disc. S, suppresses; E, enhances; NC, no significant change.

^Indicates that more than one allele was tested and found to modify *wg*-induced leg-to-wing transdetermination. All of the genes when heterozygous mutant dominantly modify at least one other (besides leg-to-wing) *wg*-induced transdetermination event.