Trithorax regulates systemic signaling during Drosophila imaginal disc regeneration

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ABSTRACT
Although tissue regeneration has been studied in a variety of organisms, from Hydra to humans, many of the genes that regulate the ability of each animal to regenerate remain unknown. The larval imaginal discs of the genetically tractable model organism Drosophila melanogaster have complex patterning, well-characterized development and a high regenerative capacity, and are thus an excellent model system for studying mechanisms that regulate regeneration. To identify genes that are important for wound healing and tissue repair, we have carried out a genetic screen for mutations that impair regeneration in the wing imaginal disc. Through this screen we identified the chromatin-modification gene trithorax as a key regeneration gene. Here we show that animals heterozygous for trithorax are unable to maintain activation of a developmental checkpoint that allows regeneration to occur. This defect is likely to be caused by abnormally high expression of puckered, a negative regulator of Jun N-terminal kinase (JNK) signaling, at the wound site. Insufficient JNK signaling leads to insufficient expression of an insulin-like peptide, dILP8, which is required for the developmental checkpoint. Thus, trithorax regulates regeneration signaling and capacity.

KEY WORDS: Chromatin, Regeneration, JNK signaling

INTRODUCTION
Regeneration is a complex process through which an organism replaces damaged or lost tissue. Although planarian flatworms and freshwater Hydra are capable of replacing a complete organism (Elliott and Sánchez Alvarado, 2013; Galliot, 2012) and urodele amphibians and teleost fish can replace whole appendages (Gemberling et al., 2013; McCusker and Gardiner, 2011), the regenerative capacity of mammals is restricted and decreases significantly with maturity and age. Therefore, understanding the regulatory mechanisms that permit and promote regeneration in model organisms is of great importance to the field of regenerative medicine, which seeks to enhance the regenerative capacity of human tissues.

Recent work in different model systems has begun to identify the genes and signal transduction pathways that control regeneration. However, it is not clear how tissue damage leads to the activation of these signals and expression of regeneration genes. Interestingly, specific chromatin modifiers are important for regeneration in several organisms, suggesting that chromatin modification regulates the expression of at least some regeneration genes. For example, pharmacological inhibition of histone deacetylases blocks Xenopus tail regeneration (Tseng et al., 2011), the H3K27me3 demethylase Kdm6b.1 is required for zebrafish fin regeneration (Stewart et al., 2009), the PRC1 component Bmi1 is required for a regenerative response to pancreatitis in mice (Fukuda et al., 2012), several members of the Set1/MLL family of histone methyltransferases are required for the stem cell-based regeneration that occurs in planaria (Hubert et al., 2014), the SWI/SNF component Brg1 (Smarca4 – Mouse Genome Informatics) is essential for mouse epidermal wound repair and hair regeneration (Xiong et al., 2013) and its Drosophila homolog, Brahma, is important for midgut regeneration (Jin et al., 2013). In most of these cases, however, the extent to which tissue damage induces chromatin modification and the genes regulated by these chromatin modifiers during regeneration remain unknown.

Drosophila melanogaster imaginal discs undergo wound repair and regenerative growth, replacing lost tissue and patterning (reviewed by Worley et al., 2012). These tissues are an excellent system for studying regeneration because they are a simple columnar epithelium, but they have complex patterning and fate determination that have been well characterized. Furthermore, the genetic tractability of Drosophila and plethora of available reagents are an advantage over many vertebrate models of regeneration. The recent development of genetic tools that induce tissue ablation and allow regeneration to occur in situ has enabled analysis of the complex signaling and patterning events that occur during imaginal disc repair (Bergantiños et al., 2010; Smith-Bolton et al., 2009). Here, we used these tools to identify the chromatin-modification gene trithorax (trx) (Breen and Harte, 1991; Kuzin et al., 1994) in an unbiased forward genetic screen for genes important for imaginal disc regeneration.

By analyzing regeneration in imaginal discs with reduced levels of Trx, we demonstrate that mutant damaged tissue failed to express sufficient levels of the insulin-like peptide dILP8, which delays entry into metamorphosis to allow time for regeneration to occur (Colombani et al., 2012; Garcell et al., 2012). We propose a model in which Trx is important for ensuring proper levels of Jun N-terminal kinase (JNK) signaling after tissue damage, which in turn induces expression of dIlp8 (Ilp8 – FlyBase). This identification of a mechanism through which one chromatin modifier regulates the local and systemic response to wounding confirms a role for epigenetic regulation of regeneration and opens the door for further identification of regeneration genes.

RESULTS
Tissue heterozygous for trithorax regenerates poorly
To enable large-scale regeneration experiments and forward genetic screens, we use genetic tools to induce tissue damage and regeneration in the developing Drosophila wing imaginal disc (Smith-Bolton et al., 2009). By using rotund (rn)-GAL4 and tubulin-GAL80GT to regulate expression of the pro-apoptotic gene
UAS-reaper, most of the wing pouch (>94%) can be ablated in a spatially and temporally defined manner in hundreds of third instar larvae simultaneously (Smith-Bolton et al., 2009). Briefly, the animals are maintained at 18°C until 7 days after egg laying, which is early third instar. The vials are then placed in a 30°C circulating water bath for 24 h before rapidly cooling to 18°C in an ice-water bath to halt expression of UAS-reaper and returning to an 18°C incubator. The 24 h induction of UAS-reaper is sufficient for almost complete ablation of the re-expressing cells. After ablation, the wing pouch regrows and re-patterns, producing an adult wing upon metamorphosis. Measuring the adult wing size provides a quantification of the extent of regenerative growth (Fig. 1A-F). Measuring a large population of wings determines the average amount of regenerative growth for a specific genetic background.

Using this system, we designed an unbiased forward genetic screen to identify novel and unpredicted regeneration genes that regulate all steps of regeneration, including wound closure, blastema formation, growth, repatterning and the whole-animal developmental arrest that occurs during imaginal disc regeneration.

To identify loci that regulate regeneration, screening conditions were established such that discs in most lines in an isogenic collection of mutations regenerated a moderate amount, producing wings that were smaller than undamaged wings (Fig. 1G; Smith-Bolton et al., 2009). Mutations that led to a consistent increase or decrease in average regenerated wing size could then be isolated.

Two pilot dominant-modifier genetic screens (Smith-Bolton et al., 2009) that used isogenic deficiencies (Ryder et al., 2007) and a collection of growth-control mutants in an isogenic background (Tapon et al., 2002) successfully identified mutations that impaired or promoted tissue regeneration.

These initial screens isolated a deficiency, Df(3R)ED5644, that impaired regeneration as assessed by adult wing size when compared with other isogenic deficiencies (Smith-Bolton et al., 2009; Fig. 1G). Mutations that led to a consistent increase or decrease in average regenerated wing size could then be isolated. Two pilot dominant-modifier genetic screens (Smith-Bolton et al., 2009) that used isogenic deficiencies (Ryder et al., 2007) and a collection of growth-control mutants in an isogenic background (Tapon et al., 2002) successfully identified mutations that impaired or promoted tissue regeneration.

To identify the gene responsible for the Df(3R)ED5644/+ phenotype, we tested smaller deficiencies and mutations in candidate genes within the region, including trx and suppressor of Hairy wing [su(Hw)]. As an additional control in all experiments, we compared regeneration in these mutant lines with regeneration in the commonly used control line w1118 (Hazelrigg et al., 1984), because it was comparable to the isogenic deficiencies that did not impact regeneration in our pilot screen (data not shown). A strong hypomorphic allele of trx, trx2 (Kennison and Tamkun, 1988), consistently regenerated worse than w1118 (Fig. 1H; Fig. S1A).

To confirm our semi-quantitative screen findings, we imaged all wings and calculated the area per wing to quantify the difference in...
size between trxE2/+ and control regenerated wings (Fig. 1I). To
determine whether the difference in size was the result of fewer cells
or smaller cells, we calculated cell density by counting the actin-rich
trichomes that protrude from one vertex of each cell. Cell density
was not different between the control and the mutant (Fig. 1J).

To confirm that the poor regeneration phenotype observed in the
trxE2/+ mutants was indeed the result of a reduction in Trx levels, we
used RNAi to knock down trx in the rn-expressing cells that survived
ablation. We used two independently generated RNAi transgenes
targeting trx: JF01557 from the Transgenic RNAi Project (TRIP)
collection (Ni et al., 2009), which we used in conjunction with UASlicec2 as recommended, and KK108122 from the Vienna Drosophila
Resource Center (VDRC) (Dietzl et al., 2007), which has been confirmed to knock down trx in imaginal wing discs (Mohan et al., 2011). Expression of either RNAi in the
subset of blastema cells that expressed m yellow survived ablation
impaired regeneration (Fig. S1B,C).

Tissue damage does not cause global deregulation of
epigenetically regulated gene expression

Trx acts in the TAC1 complex to methylate Lysine 4 of Histone 3
(H3K4) and is required for the expression of homeotic genes during
development (reviewed by Grimaud et al., 2006). Although Trx has
been linked to H3K4 trimethylation, most H3K4 trimethylation is
carried out by set1, with some contribution by trithorax related (trr)
(Ardehali et al., 2011; Hallson et al., 2012). Loss of trx does not lead
to a visible decrease in H3K4me3 levels in imaginal discs (Kanda et al., 2013). Indeed, recent work has suggested that Trx is a
monomethyltransferase (Tie et al., 2014).

There are two possible explanations for the poor regeneration in
trxE2/+ animals. Regeneration might require a global relaxation of
chromatin-mediated gene silencing, as is thought to underlie the
aberrant cell fate changes called transdifferentiation that can follow
tissue damage (Lee et al., 2005). Alternatively, chromatin changes
might occur only at specific genes that must be induced or silenced
to facilitate regeneration, as can occur during developmental
patternning and growth control (Classen et al., 2009; Oktaba et al.,
2008). Indeed, chromatin remodeling regulates specific regeneration genes after tissue damage, including notch1 and
bmp2 in Xenopus tails, Shh in mouse skin and dlx4a in zebrafish
fins (Stewart et al., 2009; Tseng et al., 2011; Xiong et al., 2013).

To distinguish between these possibilities, we expression of ultrathorax (ubx), which is normally expressed in
the haltere and third thoracic leg discs but is silenced in the wing
disc, except when chromatin-mediated silencing is disrupted
(Fig. S2A,B; Glicksman and Brower, 1988; Wang et al., 2010).
ubx was not expressed in damaged wing discs, indicating that tissue
damage did not relieve silencing at this locus (Fig. S2C-F).
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ubx expression of trx after tissue damage, such as puckered and cabat
(Blanco et al., 2010), or expressed only outside the ablation zone,
such as teashirt (Wu and Cohen, 2002; Fig. S3B-D). Interestingly,
trx itself was not upregulated in the damaged and regenerating wing
discs (Fig. S3E).

Our analysis of Trx in regeneration has been carried out in trxE2/+ tissue, because homozygous trx mutant animals are embryonic
lethal (Kennison and Tamkun, 1988) and mitotic clones of trx
homozygous tissue in imaginal discs fail to grow and are eliminated
through programmed cell death (Kanda et al., 2013). The trxE2 allele
has been used to assess the role of Trx in a variety of processes
(Kanda et al., 2013; Klymenko and Müller, 2004; Mauerung
and Paro, 2002). Our genetic analysis, comparing this allele with a
weaker trx allele (trxve) and a chromosomal deficiency, suggests that it
is a very strong hypomorph (Fig. S4). Thus, we sought to identify
how regeneration was impaired in the trxE2/+ mutant tissue.

Early regeneration is normal in trxE2/+ tissue

We examined trxE2/+ regenerating wing discs for a regeneration
blasta, which is the zone of proliferating cells that forms by R24
(Kiehle and Schubiger, 1985; Smith-Bolton et al., 2009).
The signaling molecule Wingless (Wg) is expressed in the regeneration
blasta when damage is caused by a cut or by tissue ablation
(Gibson and Schubiger, 1999; Schubiger et al., 2010; Smith-Bolton
et al., 2009). Wg was expressed in the trxE2/+ regenerating
tissue (Fig. 2A-C). Furthermore, marking cells in S phase using
EdU incorporation demonstrated that a blastema formed in the
trxE2/+ damaged wing discs (Fig. 2D-F). The intensity of 5-ethyl-2-"deoxyuridine (EdU) immunostaining was not different between
control and trxE2/+ regenerating discs at R24 (Fig. 2G). We also
used phospho-histone H3 to mark mitotic cells (Hendzel et al.,
1990; Fig. S5H-M). Thus, the mutant tissue formed a blastema that
appeared to proliferate appropriately.

To confirm that the wing primordium was regrowing at the same
rate in control and trxE2/+ regenerating discs, tissue size was
compared by measuring the area of the wing disc that expressed the
wing primordium marker nubbin (Ng et al., 1995). Average wing
primordium size was not different between control and trxE2/+ regenerating discs at R24 (Fig. 2L-N) or R48 (Fig. 2O-Q).

Trx is important for the developmental checkpoint that
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Given that the wing primordia in trxE2/+ and control regenerating
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Analysis of the role of Trithorax during regeneration

To quantify gene expression during regeneration, we identified two
genes, gapdh2 (Tso et al., 1985) and CG12703, used as controls in
previous studies of imaginal disc growth (Classen et al., 2009), that
did not change relative expression after tissue damage (Fig. S3A).

Using these reference controls and mRNA from whole wing
imaginal discs, we detected elevated relative expression of genes
that are upregulated after tissue damage, such as puckered and cabat
(Blanco et al., 2010), or expressed only outside the ablation zone,
such as teashirt (Wu and Cohen, 2002; Fig. S3B-D). Interestingly,
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Given that the wing primordia in trxE2/+ and control regenerating
discs at R48 were the same size, the deficit in regeneration in the
trx^{E2/+} tissue presumably occurred after this time. Immunostaining for cleaved Caspase 3 at R48 stained only pockets of debris, marked by absence of Nubbin and whole nuclei, and did not show any regenerated tissue undergoing apoptosis in trx^{+/} discs (Fig. 3A-H). Therefore, the trx^{E2/+} regenerating tissue was not lost via apoptosis.

Tissue damage in imaginal discs activates a developmental checkpoint, which induces a delay in pupariation. This developmental delay requires retinoid synthesis and the insulin-like peptide dILP8 (Colombani et al., 2012; Garelli et al., 2012; Halme et al., 2010). To determine whether trx^{E2/+} regenerating animals activated the developmental checkpoint, we quantified pupariation rates in undamaged and regenerating animals. Although normally developing trx^{E2/+} and control animals pupariated at the same rate, trx^{E2/+} animals with damaged wing discs appeared to pupariate approximately 1 day before control animals with damaged wing discs when new pupae were counted once a day (Fig. 3I). We confirmed this premature pupariation by quantifying the number of animals that had pupariated every 12 h (Fig. 3J). Therefore,
although these two genotypes had regenerated to a similar extent at R48, the \( \text{trxE}^{E2/}+ \) animals entered pupariation, while the control animals continued to regenerate for approximately 1 day more. Furthermore, use of RNAi to reduce Trx levels also led to premature pupariation (Fig. S6A,B).

These differences in timing of pupariation were due to differences in timing of Ecdysone signaling, as measured by expression of the Ecdysone-responsive gene \( E74 \) (Fig. 3K; Burtis et al., 1990). Levels of \( E74 \) expression remained low at R24 in control and \( \text{trxE}^{E2/}+ \) larvae (Fig. 3K). However, \( E74 \) expression had peaked at R48 in \( \text{trxE}^{E2/}+ \)
control animals (Fig. 3N). Thus, extending the time to pupariation largely rescued the regeneration defect of trxE2+/+ wing discs. Other, unidentified factors might account for the small remaining difference between trxE2+/+ and control regeneration.

dilp8 expression is reduced in trx/+ mutants

Given that dILP8 is expressed in damaged imaginal discs and is required for induction of the delay in pupariation (Colombani et al., 2012; Garelli et al., 2012), we examined dilp8 expression. Interestingly, dilp8 expression in trxE2+/+ regenerating discs was reduced at R24 compared with control regenerating discs (Fig. 4A).

The reduced dilp8 expression could account for the shortened time for regeneration and smaller wings in trxE2+/+ animals. Indeed, animals heterozygous for the hypomorphic allele dilp8MI00727 (Colombani et al., 2012) showed premature pupariation and reduced adult wings after damage (Fig. 4B,C).

Because dilp8 had significantly reduced expression in trxE2+/+ damaged tissue, it is possible that Trx is required to modify chromatin at the dilp8 locus after wounding to alleviate silencing. However, the region around dilp8 is characterized as open chromatin in S2 cells and lacks H3K4me3 and H3K27me3 in embryos, whole larvae and adults according to data deposited in the modENCODE database (Celniker et al., 2009; Kharchenko et al., 2011). Given that H3K4 becomes methylated at the start site of many transcriptionally active genes, including dilp8 in regenerating tissue (data not shown), it is possible that Trx acts directly on the dilp8 locus after tissue damage. However, it is also possible that Trx controls expression of a regulator of dilp8, either in the disc itself or in another tissue that impacts disc growth. Changing Ecdysone levels, by feeding the larvae either 20-hydroxyecdysone or erg2Δ yeast, did not affect dilp8 expression (Fig. S7C,D). Therefore, we sought to identify a regulator of dilp8 expression in the imaginal disc that is misregulated in the trxE2+/+ regenerating tissue.

JNK signaling is reduced in trx/+ mutants

Previous reports have suggested that JNK signaling regulates dilp8 expression (Colombani et al., 2012; Katsuyama et al., 2015). Indeed, examination of the dilp8 locus in GenomeSurveyor (Kazemian et al., 2011) shows conservation of a predicted AP-1 binding site (Perkins et al., 1988) about 4 kb upstream of the dilp8 (CG14059) start site. If Trx acts through JNK signaling to regulate DILP8 expression, modification of JNK signaling might replicate or rescue the trxE2+/+ phenotype. Indeed, wing discs heterozygous for the gene encoding JNK, basket (bsk) (Sluss et al., 1996), regenerated poorly as assessed by adult wing size, similar to wing discs heterozygous for trxE2+ (Fig. 5A). In addition, increasing Puc levels via rn-Gal4 and UAS-puc (Bischof et al., 2013) eliminated the normal damage-induced developmental delay and any regenerative response (Fig. 5B,C). Given the central role that JNK signaling plays in wound closure, regeneration and the damage-induced developmental delay, this complete abrogation of regeneration was not surprising.

To determine whether JNK signaling was reduced in trxE2+/+ regenerating wing discs, we assessed levels of phosphorylated JNK, expression of a transgenic reporter of JNK signaling (Chatterjee and Bohmann, 2012), and expression of the JNK signaling target gene puckered (puc) (Martin-Blanco et al., 1998). To assess levels of

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Fig. 3. trx heterozygous animals had reduced delay of pupariation. (A-H) Control (A-D) and trxE2+/+ (E-H) regenerating wing discs at R48. Cleaved Caspase 3 (A,E; red in merge), DNA (TO-PRO3; B,F; blue in merge), Nubbin (C,G; green in merge) and merge (D,H). The cleaved Caspase 3 observed was in the debris that remained adjacent to the healed and regenerating epithelium. (I) Pupariation rates (in hours after egg lay), with pupae counted every 24 h. Undamaged discs: three independent experiments, control n=100, trxE2+/+ n=62. Damaged discs: seven independent experiments, control n=362, trxE2+/+ n=225. (J) Pupariation rates (in hours after egg lay), with pupae counted every 12 h. Six independent experiments, control n=199, trxE2+/+ n=117. **P<0.01 at 228 and 240 h. (K) Relative expression of E74 quantified by qRT-PCR using whole larvae. Between three and six independent samples per time point. (L) Control and trxE2+/+ animals had adult wings of similar size after regeneration when grown on soft erg2 yeast food to delay pupariation. Eight independent experiments, control n=188 wings, trxE2+/+ n=92 wings. (M) trxE2+/+ regenerating animals grown on firm erg2 yeast food had adult wings similar in size to those of control regenerating animals grown on standard food. Control regenerating animals grown on erg2 yeast food delayed longer and had larger adult wings. Seven independent experiments, control animals fed standard food n=267 wings, trxE2+/+ animals fed standard food n=267 wings, control animals fed erg2 yeast food n=338 wings, trxE2+/+ animals fed erg2 yeast food n=144 wings. Percentages of animals with fully regenerated wings were not significantly different between control animals fed standard food and trxE2+/+ animals fed erg2 yeast food, P=0.96. (N) Two representative individual experiments in which animals grown on firm erg2 yeast food had adult wings similar in size to those of control regenerating animals grown on standard food. Control regenerating animals with the prematurer pupariation phenotype in the trxE2+/+ background had (Hal02254; Colombo et al., 2012) showed premature pupariation and reduced adult wings after damage (Fig. 4B,C). Importantly, overexpressing DILP8 using a UAS-dilp8 transgene (Garelli et al., 2012) expressed via rn-Gal4 during the thermal shift in the rn-expressing cells that survived ablation restored proper dilp8 expression levels and rescued the premature pupariation phenotype in the trxE2+/+ regenerating animals, as well as overall wing size (Fig. 4D-F; Fig. S7A,B).
activated JNK, we immunostained regenerating discs using an anti-phospho-JNK antibody. In two independent experiments, phospho-JNK was significantly reduced in the control regenerating tissue compared with control regenerating tissue at day 10 (Fig. 5D). In a third experiment, expression was highly variable and not statistically different between the genotypes (data not shown). Given that dilp8 expression, phospho-JNK levels and the TRE-red reporter were all reduced in the mutant regeneration blastema, we concluded that JNK signaling was reduced.

### Misregulation of puc represses JNK signaling in trx/+ mutant regenerating tissue

As a third assessment of transcription downstream of JNK signaling, we assessed expression of the target gene puc, which encodes a phosphatase that dephosphorylates and negatively regulates JNK (Martin-Blanco et al., 1998). To our surprise, puc expression levels were significantly elevated in the trx/+ regenerating tissue compared with control regenerating tissue at R24, as measured by both an enhancer trap (Ring and Martinez Arias, 1993) and qRT-PCR of puc mRNA (Fig. 6A-C). This result raised the possibility that Trx negatively regulates puc and that the elevated puc caused the reduction of JNK signaling and dilp8 expression in the trx/+ mutant (Fig. 6D). Importantly, RNAi against trx also caused an increase in puc expression and a reduction in dilp8 expression (Fig. S8).

To confirm that elevated puc expression was responsible for the decrease in dilp8 expression, we quantified dilp8 expression in regenerating discs that were heterozygous mutant for both trx and puc. In these doubly heterozygous mutant animals, puc expression...
Fig. 5. JNK signaling is reduced in trxEx2/+ mutant regenerating tissue. (A) Extent of regeneration as measured by adult wing size. Note that the trxEx2/+; bsk1/+ and bsk1/+; trxEx2/+ animals all consistently regenerated poorly relative to control animals, although the differences among the mutants were not statistically significant at each wing size and were highly variable across experiments, possibly owing in part to high lethality in the bsk1/+ and bsk1/+; trxEx2/+ regenerating animals. Three independent experiments, control n=70 wings, trxEx2/+ n=54 wings, bsk1/+ n=122 wings, bsk1/+; trxEx2/+ n=162 wings. (B) Pupariation timing of regenerating animals (in hours after egg lay). UAS-puc was expressed under the control of m-GAL4. The difference in pupariation at 216 h between control and m>puc was significant, **P<0.01. Three independent experiments, control n=158 pupae, trxEx2/+ n=117 pupae, m>puc n=64 pupae. (C) Extent of regeneration as measured by adult wing size. The distributions of adult wing sizes between control and m>puc were significantly different, χ² test P<0.01. Four independent experiments, control n=325 wings, trxEx2/+ n=270 wings, m>puc n=114 wings. (D,E) Phosph-JNK immunostaining in control (D) and trxEx2/+ (E) R24 discs. (F) Quantification of phospho-JNK staining in the blastema as defined by Nubbin expression. Control and trxEx2/+ n=9 discs each. **P<0.01. (G-J) Expression of the puc-lacZ (G,I) and TRE-Red (H,J) reporters in undamaged (G,H) and damaged (I,J) wing discs. (K) Quantification of dsRed average pixel intensity in the wing pouch or regeneration blastema in undamaged wing discs (n=4), as well as regenerating discs at R0 (n=14), R24 (n=8) and R48 (n=7). **P<0.01 compared with undamaged discs. (L-T) Wing imaginal discs showing anti-Wg (L,O,R), dsRED (M,P,S) and the merge of Wg (green), dsRED (red) and DAPI (blue) (N,Q,T). Images are of a mock-ablated wing disc (L-N), a control disc at R24 (O-Q) and a trxEx2/+ disc at R24 (R-T). Arrows mark cellular debris, which was retained in disc folds at similar frequencies in all ablated genotypes. (U) Quantification of AP-1 reporter expression in the Wg-expressing blastema in two independent experiments. Pockets of debris occurred randomly in all genotypes and were excluded from the analysis. Examples are marked by arrows in P and confirmed by absence of nuclear DAPI staining. **P=3×10⁻⁶. Control n=7 discs, trxEx2/+ n=4 discs. *P=0.02. Control n=10 discs, trxEx2/+ n=11 discs. Scale bars: 100 µm. All error bars are s.e.m. R, number of hours after tissue damage.
was similar to expression in control regenerating tissue (Fig. 6E). Importantly, dilp8 expression levels were similar to expression levels in control regenerating discs (Fig. 6F). These animals pupariated at the same time as control regenerating animals and regenerated to the same extent as control animals (Fig. 6G,H). Thus, reduction of puc in the trxE2/+ regenerating tissue restored systemic signaling, developmental timing and regenerative capacity.

Although the genetic interaction experiments alone can only suggest a regulatory relationship, the changes in puc expression, phospho-JNK levels and the AP1-dsRED reporter in the trxE2/+ regenerating tissue, as well as the restoration of proper puc and dilp8 expression levels in the pucE69, trxE2/+ regenerating tissue, indicate that puc expression and JNK activity are downstream of trx. As Trx is generally thought to promote rather than repress gene expression, Trx is not likely to act directly on the puc locus in the damaged tissue. Therefore, we propose that Trx regulates expression of an unknown factor, which in turn regulates puc expression.

**DISCUSSION**

This work sought to identify the role that the chromatin modifier Trx plays in regulating regeneration. Our results demonstrate that the primary problem in trx heterozygous regenerating animals was insufficient time for regeneration. We propose a model in which reduced Trx levels lead to abnormally high expression of puc, which suppresses JNK signaling, which leads to insufficient dilp8 expression (Bosch et al., 2008, 2005; Colombani et al., 2012). Although JNK signaling was reduced in the trx heterozygous tissue, it was still sufficient to promote wound healing, blastema formation,

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**Fig. 6. Increased puc expression limits dilp8 expression and regenerative capacity.** (A) Relative expression levels of puc quantified by qRT-PCR in undamaged and regenerating wing discs. n was between five and ten independent samples per genotype and time point. *P<0.02, **P<0.0005, ***P<5×10⁻⁵. (B,C) Anti-β-galactosidase immunostaining of discs containing the puc-lacZ enhancer trap. Scale bars: 100 µm. (B) Control disc at R24. (C) trxE2/+ disc at R24. Similar results were obtained in three independent experiments. (D) Model showing trx negatively regulating puc expression, which regulates JNK signaling and dilp8 expression. Regulation of dilp8 by trx independently of JNK is also possible. Arrows do not imply direct interaction. (E) Relative expression levels of puc determined by qRT-PCR, in regenerating wing discs. P=0.66, indicating no significant difference (n.s.), control n=4, trxE2/+ n=5, pucE69, trxE2/+ n=5 independent biological replicates. (F) Relative expression levels of dilp8 determined by qRT-PCR, in regenerating wings discs. P=0.82, indicating no significant difference (n.s.), control n=5, trxE2/+ n=4, pucE69, trxE2/+ n=5 independent biological replicates. (G) Pupariation timing of regenerating animals (in hours after egg lay). The differences between control and pucE69, trxE2/+ were not significant. Four independent experiments. Control n=97, pucE69/+ n=80, trxE2/+ n=36, pucE69, trxE2/+ n=128. (H) Extent of regeneration as measured by adult wing size. All results are significantly different from each other, χ² P<0.01. Four independent experiments, control n=263 wings, pucE69/+ n=246 wings, trxE2/+ n=130 wings, pucE69, trxE2/+ n=377 wings. Scale bars: 100 µm. All error bars are s.e.m. R, number of hours after tissue damage.
and regenerative growth. Thus, dilp8 expression seems more sensitive to changes in JNK signaling than other transcriptional targets of this pathway or it might require additional input from a Trx-dependent but JNK-independent mechanism (Fig. 6D). It is likely that further reduction in Trx levels or activity would impair other JNK-dependent aspects of regeneration. Thus, we have identified a Trx-dependent mechanism that controls the scope and magnitude of regeneration signaling.

It is possible that Trx regulates JNK signaling in a similar manner in other contexts. Indeed, puc expression levels appeared to be slightly increased in undamaged trx<sup>E74</sup>/ discs compared with controls (Fig. 6A), and phospho-JNK levels were expected to be reduced outside the regeneration blastema in trx<sup>E74</sup>/ discs (Fig. 5D,E). Interestingly, when a trx RNAi construct was expressed in the developing notum, adult flies had a weak notum malformation phenotype, whereas a bsk RNAi construct induced a moderate notum malformation phenotype, suggesting that Trx can affect the JNK-dependent process of notum fusion (Munnery-Widmer et al., 2009). Furthermore, animals homozygous for one mutant allele, <i>trx<sup>00427</sup></i>, are reported to have a groove in the adult notum suggestive of incomplete notum fusion (‘Insertion alleles’, communication to Flybase by Berkeley Drosophila Genome Project, 1993). To our knowledge, however, no report has implicated Trx in regulation of the JNK-dependent process of embryonic dorsal closure or described a dorsal closure phenotype for any <i>trx</i> allele.

We do not yet know how Trx regulates <i>puc</i> expression after tissue damage. The <i>puc</i> region contains histone modifications and binds chromatin modulators, including members of the NURF complex and Polycomb, according to the modENCODE database (Celniker et al., 2009). Although these modENCODE data are not from isolated imaginal discs, these findings suggest that regulatory mechanisms other than AP-1 are likely to contribute to the control of <i>puc</i> expression throughout development. Our results caution against using <i>puc</i> expression as the sole indicator of JNK signaling activity. As Trx is generally thought to promote rather than repress gene expression, Trx might regulate a repressor of <i>puc</i> expression. Indeed, GenomeSurveyor (Kazemian et al., 2011) predicts binding sites for many transcription factors in the <i>cis</i>-regulatory modules defined by the AP-1 binding sites in the <i>puc</i> locus. Several of these predicted binding factors can act as repressors of transcription and are in turn located in genomic regions that, according to modENCODE data, contain highly modified chromatin, and thus are candidates for mediating the influence of Trx on <i>puc</i>. We tested mutants of the five genes that we identified as fitting these criteria: <i>sloppy-paired 1</i>, <i>tramtrack</i>, <i>caupolican</i>, <i>traffic jam</i> and <i>earnuff</i> (Gómez-Skarmeta et al., 1996; Grossniklaus et al., 1992; Harrison and Travers, 1990; Kawashima et al., 2003; Weng et al., 2010). None of the mutants had impaired regeneration; therefore, none is likely to be the factor that regulates <i>puc</i> downstream of <i>trx</i> (Fig. S9A-E), which remains to be identified.

We had initially predicted broad changes in gene expression in the <i>trx</i> mutant regenerating tissue, resulting in multiple defects throughout the regeneration process. However, expression of many of the regeneration genes we tested was not impaired (Fig. S9F,G), possibly because the <i>trx</i> heterozygote reduces the gene dosage only by half. Strong changes in expression were detected in only a few specific genes, such as <i>cycE</i>, <i>puc</i> and <i>dilp8</i>. However, it remains possible and even likely that additional genes are regulated by Trx after tissue damage that were not detectable in the heterozygous mutant. Indeed, chromatin modification could rapidly and efficiently alter the developmental program in damaged tissue to enable regeneration. Importantly, RNAi knockdown of the planarian homolog of <i>trx</i>, <i>Smed-mll1/2</i>, did not prevent formation of a regeneration blastema but did impair regeneration of particular cells (Hubert et al., 2014), suggesting that Trx and its homologs play specific roles in regeneration across species.

In summary, our unbiased genetic screen identified a chromatin-modification gene, <i>trx</i>, as a key regulator of regeneration. Reducing levels of Trx in damaged tissue led to reduced JNK signaling and limited dilp8 expression, resulting in a failure to complete regenerative growth before the onset of pupariation and metamorphosis. Thus, we have proposed a model in which Trx is important for regulating the expression of the phosphatase Puckered, which modulates JNK activity. We have not ruled out the possibility that Trx also regulates <i>dilp8</i> directly or that it regulates additional regulators of <i>dilp8</i> expression. This work has demonstrated that chromatin modification after wounding can regulate specific signaling events and expression of key genes. A genome-wide examination of changes in histone modification and chromatin state in regenerating tissue will identify more genes that are regulated epigenetically upon wounding, including novel regeneration genes that will contribute to our understanding of wound repair. Our findings also illustrate the importance of fine-tuned regulation of regeneration signaling, because changes in the magnitude or duration of feedback inhibition can significantly alter the regenerative outcome.

**MATERIALS AND METHODS**

**Tissue ablation and genetic screen**

Ablation was induced as previously described (Smith-Bolton et al., 2009), using expression of <i>UAS-reaper</i> and a 24 h thermal shift to 30°C to induce cell death. The genetic screen was carried out as previously described (Smith-Bolton et al., 2009). For all experiments, ablation was induced in the early third instar, which is 7 days after egg laying at 18°C. Mock-ablated controls experienced the shift to 30°C for 24 h alongside the ablating animals, but lacked the ablation-inducing transgenes.

**Fly lines and genetics**

Flies were maintained on standard molasses-based food. Regeneration experiments were carried out on modified Bloomington standard media containing malt and 0.3% tegosept (Apex). Fly lines were obtained from the Bloomington <i>Drosophila</i> Stock Center, FlyORF, the Vienna <i>Drosophila</i> Resource Center or colleagues (see supplementary materials and methods for details).

**Imaging adult wings**

Adult wings were mounted in Gary’s Magic Mount [Canada balsam (Sigma) dissolved in methyl salicylate (Sigma)]. Images were taken on an Olympus SZX10 microscope using CellSens Dimension software with the Extended Focal Image feature. Wing area was measured in ImageJ. Samples included both females and males. Undamaged control wings were averaged to give a standard area. The area of each experimental wing was then calculated as a fraction of the standard. Trichomes were counted in ImageJ within a 50×50 pixel box.

**Immunohistochemistry**

Immunostaining was carried out as previously described (Smith-Bolton et al., 2009). Wing discs were imaged on a Zeiss LSM 510 or a Zeiss LSM 700 confocal microscope. Images were processed using ZEN lite (Zeiss), ImageJ (NIH) and Photoshop (Adobe). Details for antibodies, labeling and image analysis can be found in supplementary materials and methods.

**Molecular biology**

qRT-PCR was carried out as previously described (Classen et al., 2009). Wing disc tissue was used for all experiments except the E74 qRT-PCR, in which whole larvae were used. For qRT-PCR of whole larvae, mRNA was
levels were normalized to undamaged discs were extremely low. fold changes for undamaged discs are shown. For all primer sequences, sources for primer 20-hydroxyecdysone were in tubes that contained vehicle (ethanol) only fed to the larvae at a concentration of 0.6 mg/ml of food in a microfuge tube windows were transferred to fresh vials. 20-Hydroxyecdysone (Sigma) was in animals that pupariated at the same time, pupae formed within 24 h contained 6.5% agar. Firm food contained 7.5% agar. Eggs were laid on prepared as previously described (Katsuyama and Paro, 2011). Soft food consisting of 15 wing discs or five whole larvae. Power SYBR Green Master Mix (ABI) was used and reactions were run on an ABI Step One Plus Real-Time PCR System. Analysis was done by the ΔΔCt method and expression levels were normalized to gapdh2. Fold changes relative to control undamaged discs are shown. For all primer sequences, sources for primer sequences or sources for primers, see Table S1. The variation observed in fold changes for dilp8 at R24 was probably because expression levels in undamaged discs were extremely low.

Pupariation quantification and Ecdysone manipulation
Pupariation rates were quantified by counting newly formed pupae every 12 or 24 h. Pupariation was delayed by raising larvae on food made of erg2 mutants, strain 4027088 (ATCC, Manassas, VA, USA). Food was prepared as previously described (Katsuyama and Paro, 2011). Soft food contained 6.5% agar. Firm food contained 7.5% agar. Eggs were laid on grape plates, from which larvae were picked on day 2 after egg laying, and transferred to erg2 yeast food, 50 larvae per vial. To compare regeneration in animals that pupariated at the same time, pupae formed within 24 h windows were transferred to fresh vials. 20-Hydroxyecdysone (Sigma) was fed to the larvae at a concentration of 0.6 mg/ml of food in a microfuge tube as previously described (Halme et al., 2010). Controls that were not fed 20-hydroxyecdysone were in tubes that contained vehicle (ethanol) only mixed with the food.

Statistical analysis
All statistical analyses were performed using Student’s t-test in Excel except where noted that χ² tests were used. Results were considered statistically significant with P<0.02.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
R.K.S.-B. and A.S. designed the approach, R.K.S.-B., A.S. and S.J.K. designed, carried out and interpreted the experiments, and R.K.S.-B., A.S. and S.J.K. designed, prepared the manuscript.

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