Cap-n-collar promotes tissue regeneration by regulating ROS and JNK signaling in the *Drosophila* wing imaginal disc

Amanda R. Brock, Mabel Seto, and Rachel K. Smith-Bolton

Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801
Running title: Cnc regulates tissue regeneration

Key words:

Cap-n-collar

Nrf2

*Drosophila*

imaginal disc

regeneration

reactive oxygen species

Corresponding author:

Rachel Smith-Bolton

601 S. Goodwin Ave

Urbana, IL 61801-3761

217-244-4183

rsbolton@illinois.edu
ABSTRACT

Regeneration is a complex process that requires an organism to recognize and repair tissue damage, as well as grow and pattern new tissue. Here we describe a genetic screen to identify novel regulators of regeneration. We ablated the *Drosophila melanogaster* larval wing primordium by inducing apoptosis in a spatially and temporally controlled manner and allowed the tissue to regenerate and repattern. To identify genes that regulate regeneration, we carried out a dominant modifier screen by assessing the amount and quality of regeneration in adult wings heterozygous for isogenic deficiencies. We have identified 31 regions on the right arm of the third chromosome that modify the regenerative response. Interestingly, we observed several distinct phenotypes: mutants that regenerated poorly, mutants that regenerated faster or better than wild type, and mutants that regenerated imperfectly and had patterning defects. We mapped one deficiency region to *cap-n-collar (cnc)*, the *Drosophila* Nrf2 ortholog, which is required for regeneration. Cnc regulates reactive oxygen species levels in the regenerating epithelium, and affects JNK signaling, growth, debris localization, and pupariation timing. Here we present the results of our screen and propose a model wherein Cnc regulates regeneration by maintaining an optimal level of reactive oxygen species to promote JNK signaling.
Regeneration has long captured the interest of scientists, who seek to understand the complex pathways that enable some animals to replace missing structures after injury. Model organisms across animal phyla have been used to identify genetic regulators of regrowth and repatterning, demonstrating considerable evolutionary conservation in the factors that are used during regeneration, including Wnt, JNK, Hippo, and reactive oxygen species (ROS) signaling. For example, Wnt signaling plays a vital role in reestablishing axis polarity in the invertebrate flatworm planarians (PETERSEN and REDDIEN 2008, 2009), and Wnts are upregulated during head regeneration in the invertebrate cnidarian hydra (HOBMAYER et al. 2000). Furthermore, Wnt signaling is required for regeneration in vertebrate models, including the zebrafish caudal fin (WEHNER et al. 2014), the axolotl limb (KAWAKAMI et al. 2006), and the murine liver, where it plays an important role in cellular differentiation (WILLIAMS et al. 2010). JNK signaling is also required for regeneration of planarians (TASAKI et al. 2011) and murine liver regeneration (BEHRENS et al. 2002). Yorkie/YAP, the transcription factor and effector of the Hippo pathway, is required for proper planarian regeneration (LIN and PEARSON 2014), for cricket leg regeneration (BANDO et al. 2009), and is activated during mammalian liver regeneration (GRIJALVA et al. 2014).
ROS are also important for tissue repair across phyla. ROS are required in planarians for efficient head and tail regeneration (PIROTTE et al. 2015) and in Drosophila for imaginal disc regeneration (SANTABÁRBARA-RUIZ et al. 2015), compensatory proliferation (FOGARTY et al. 2016) and dorsal closure, a model for wound healing (MULIYIL and NARASIMHA 2014). ROS signals are also required in vertebrates during wound healing (SCHÄFER and WERNER 2008; NIETHAMMER et al. 2010; YOO et al. 2012) and regeneration (BEYER et al. 2008; GAURON et al. 2013; LOVE et al. 2013). However, the complex relationship between ROS and other signaling pathways during regeneration remains unclear, as does the mechanism by which ROS are constrained to appropriate levels.

The requirement for the same signaling pathways during regeneration across species strongly suggests that conserved mechanisms regulate many aspects of regeneration and that much can be learned about regeneration by studying model organisms. However, many regeneration models have technical limitations, including lack of a sequenced genome, no ability to make transgenic animals, a significantly long regeneration time, or labor-intensive methods of causing tissue damage. Thus, work in these models would be complemented by an approach in a more genetically tractable model system.

The wing imaginal disc of the fruit fly, Drosophila melanogaster, is capable of substantial regeneration, enabling us to ask questions about the regulation of
regeneration using this genetically tractable model system. Experiments in the mid-twentieth century by Ernst Hadorn and colleagues discovered that fragmented imaginal discs could regenerate upon transplantation into a young larva or an adult fly abdomen (reviewed in WORLEY et al. 2012). Imaginal discs regenerate by forming a zone of proliferating cells called a blastema at the site of tissue damage, enabling regrowth (HADORN et al. 1949; HADORN and BUCK 1962; reviewed in WORLEY et al. 2012). These studies were invaluable for understanding consequences of damage to imaginal discs. However, the laborious nature of these experiments made genetic screens challenging.

A few genetic screens have been carried out in imaginal discs to identify regeneration genes. First, a screen of lacZ-containing P-element insertion lines, using a temperature-sensitive cell-lethal mutation to induce damage, identified loci that induced expression of the enhancer-trap lacZ after tissue damage (BROOK et al. 1993). Second, the Schubiger lab performed a dominant-modifier genetic screen to identify mutations that modify the frequency of transdetermination by exploiting the fact that ubiquitous expression of wg in the leg disc recapitulates the leg-to-wing fate changes occasionally seen after tissue damage. They also screened for expression changes of P element-insertion reporter lines in the regeneration blastema (MCCLURE and SCHUBIGER 2008). However, technical limitations did not allow either group to screen for inability to regenerate. Additionally, multiple labs have screened for regulators of
compensatory proliferation, a similar but distinct process by which scattered apoptotic cell death can induce proliferation in neighboring cells (Gerhold et al. 2011; Fan et al. 2014; Meserve and Duronio 2015). While many signaling pathways might be involved in both regeneration and compensatory proliferation, these screens were not designed to identify genes that are required to replace a tissue after catastrophic damage.

Instead of manual fragmentation of imaginal discs, overexpression of wg, or induction of scattered cell death, we use genetic tools to induce extensive tissue ablation in the wing primordium (Smith-Bolton et al. 2009). This ablation system enables sophisticated genetic experiments because it provides temporal and spatial control of tissue damage and subsequent regeneration. This system also enables large-scale, unbiased screening for genes that are necessary for the process of regeneration. Indeed, studies using genetically induced tissue damage have already confirmed roles in imaginal disc regeneration for conserved signaling pathways, including Wnt signaling (Smith-Bolton et al. 2009), JNK signaling (Bergantinos et al. 2010), Hpo/Yki signaling (Grusche et al. 2011; Sun and Irvine 2011), and ROS (Santabarbara-Ruiz et al. 2015; Fogarty et al. 2016).

Two pilot forward genetic screens using an EMS-induced mutant collection and a small portion of a deficiency collection demonstrated that genetically induced
imaginal wing disc ablation can be used to identify genes that are required for regeneration and that restrict regeneration (SMITH-BOLTON et al. 2009). Genes identified in these pilot screens included the chromatin modifier Trithorax, which regulates regeneration signaling (SKINNER et al. 2015), and Taranis, which is a novel regeneration-specific regulator of posterior wing cell fate (SCHUSTER and SMITH-BOLTON 2015). Thus, genetic screens using this regeneration model successfully identified novel genetic regulators of regeneration that are likely to be conserved in vertebrates.

Here we present the results of a dominant-modifier deficiency screen covering 20% of the Drosophila genome. We identified 17 regions of chromosome 3R that contain one or more genes that are required for regeneration, and 14 regions that, when hemizygous, cause enhanced regeneration. We have shown that the specific gene within one of the regions that is required for regeneration is cap-n-collar (cnc), which encodes the Drosophila homolog of vertebrate Nrf2. Furthermore, we demonstrate that Cnc coordinates the response to injury-produced ROS, by constraining the ROS signal to appropriate levels and thereby regulating the appropriate signaling response to tissue damage and thus, the cellular and developmental processes downstream of those early signals.
MATERIALS AND METHODS

Fly Stocks

Flies were reared on standard molasses medium at 25° unless otherwise noted. The line w^{1118};;;m-GAL4, UAS-reaper, tubGAL80^{TM6B}, tubGAL80 (BRAND and PERRIMON 1996; MCGUIRE et al. 2003; SMITH-BOLTON et al. 2009; SCHUSTER and SMITH-BOLTON 2015) was used to induce ablation in developing wing discs, referred to later as the “ablation chromosome.” Deficiency lines from the DrosDel collection (RYDER 2004), the Exelixis collection (PARKS et al. 2004; THIBAULT et al. 2004), and the Bloomington Stock Center collection (COOK et al. 2012) were used to conduct the screen. All deficiency lines were ordered from the Bloomington Stock Center, with the exception of Df(3R)ED10951, which came from the Drosophila Genomic Resources Center in Kyoto, Japan. A list of deficiency lines used can be found in Supplemental Table 1. w^{1118} was used as a control. cnc^{03921} (PERRIMON et al. 1996), cnc^{EY08884} (BELLEN et al. 2004), Irk1^{M108404} and Irk1^{MB08423} (VENKEN et al. 2011), nub^{M105126} (nub-GFP) (NAGARKAR-JAISWAL et al. 2015; KHAN et al. 2016), UAS-eGFP (HALFON et al. 2002), cnc^{HMS02021} (Ni et al. 2011), and P{CaryP}attP40 (MARKSTEIN et al. 2008) were from the Bloomington Stock Center. RNAi lines targeting Cnc (VDRC ID #101235 and #108127) were from the Vienna Drosophila Resource Center (DIETZL et al. 2007). TRE-red (CHATTERJEE and BOHLMANN 2012) and UAS-cncC (SYKIOTIS and BOHLMANN 2008), were gifts from Dirk Bohmann.
Genetic Screen

The screen is outlined in Figure 1. Briefly, deficiency lines were crossed to the ablation chromosome. Embryos were collected on a grape juice plate for 4-5 hours at room temperature in dark conditions. The plates were moved to 18°. Larvae were staged and selected two days after egg lay to synchronize development and then moved from the grape juice plate to standard Bloomington cornmeal media that was churned and had yeast paste added. For each genotype, three vials of 40-50 larvae per vial were selected. The vials were then placed at 18° until day seven after egg lay. On the morning of day seven, when the larvae were in the early third instar as determined by counting mouth hooks, the vials were placed in a 30° water bath for 24 hours, during which ablation of the wing pouch occurred. After 24 hours they were placed in an ice-water bath for one minute to stop ablation and returned to 18°. For screening experiments, the vials remained at 18° until after eclosion, at which point their wing size was scored and they were discarded. Wings from the same genotype and date of egg lay were pooled and scored by relative size compared to a control undamaged wing (Figure 1C). This process was repeated three times to have three independent replicates of the experiment. The average regeneration index was determined by calculating the sum of the products of percentage wing size and the percentage of the population at each size. To determine an absolute wing size, flies of the correct genotype and ablation status were frozen, wings were
mounted in Gary’s Magic Mount (Canada Balsam dissolved in methyl salicylate, [Sigma]), and imaged on an Olympus SZX10 microscope with an Olympus DP21 camera with CellSens Dimensions software. The area was measured in ImageJ (SCHNEIDER et al. 2012).

**Pupariation timing experiments**

Flies underwent the same treatment as above with some minor differences. Six sets of 40-50 larvae were selected and moved to standard Bloomington cornmeal media in vials. Three vials underwent the thermal shift as above while three vials remained at 18° to serve as the non-ablated control. The ablated and non-ablated experiments were performed at the same time to control for environmental variables. Starting at day 9 and continuing to day 15, new pupal cases were counted on the side of each vial every 24 hours. The vials from the same genotype and ablation status were pooled and graphed to show the percentage of the population that had pupariated at each day after egg lay. For Table 1, the time at which 50% of the animals had entered pupariation was interpolated from the graph.

**Immunostaining**

Immunostaining was performed as previously described (SCHUSTER and SMITH-BOLTON 2015; SKINNER et al. 2015). The Nubbin antibody was a gift from S. Cohen and was used at a 1:200 dilution (NG et al. 1996). The PH3 antibody came from Millipore and was used at a 1:500 dilution. Anti-dMyc came from
Santa Cruz Biotechnology (sc-28207) and was used at a 1:500 dilution. Anti-Wg came from the Developmental Studies Hybridoma Bank and was used at a 1:100 dilution (BROOK and COHEN 1996). The Developmental Studies Hybridoma Bank (DSHB) was created by the NICHD of the NIH and is maintained at the University of Iowa, Department of Biology, Iowa City, IA 52242. Discs were mounted in Vectashield (VectorLabs) and imaged on a Zeiss LSM 700 Confocal microscope.

**ROS Detection**

This protocol was adapted from a previously published protocol (OWUSU-ANSAH et al. 2008). Briefly, larvae were dissected and collected in Schneider’s Insect Medium (Sigma; S0146). The dihydroethidium (Thermo Fisher Scientific; D11347) was reconstituted in DMSO, then 1μL of DHE was dissolved in 1mL of Schieder’s Medium. The tissue was incubated in DHE in the dark for 5 minutes, washed 3X in Schneider’s Medium, fixed for 7 minutes in 7% formaldehyde in 1X PBS, washed once in 1X PBS, then immediately mounted in Vectashield (VectorLabs), and imaged on a Zeiss LSM 700 Confocal.

**ROS Feeding**

This protocol was adapted from previous published protocols (GROVER et al. 2009; SANTABÁRBARA-RUIZ et al. 2015). To increase ROS levels, food was supplemented with a solution of 1% Sucrose and either 0% (control) or 0.5% H₂O₂. A circle of Whatman paper was placed on top of the food, and 1 mL of solution was added to the Whatman paper at the end of the 24-hour temperature
shift, such that regenerating larvae were consuming excess ROS throughout regeneration.

**Image Analysis**

All image analysis was done in ImageJ (NIH). To measure the wing pouch size, the area that immunostained with anti-Nubbin was measured by outlining and measuring in ImageJ. For PH3 measurements, the wing primordium was identified by outlining the Nubbin-positive region, and mitoses were counted using the ImageJ cell-counter tool. Orthogonal images of the imaginal discs were generated using the ImageJ reslice tool on a Z-stack of images. For DHE, eGFP, and TRE-dsRed intensity analysis in orthogonal slices, three equal-sized rectangles were placed in each region (apical, epithelium, basal), and the average fluorescence intensity was measured. Three measurements were taken per region, per image.

**Reagent and data availability**

All relevant data are included in the paper. *Drosophila* lines are available upon request. Raw data from primary and secondary screens available upon request.
RESULTS

Wing disc ablation system and screening methods

To facilitate genetic screening for regeneration genes, we used a method of genetically ablating the wing disc using the GAL4/UAS/GAL80ts system that has been previously described (Figure 1A,B; see methods)(SMITH-BOLTON et al. 2009). Briefly, apoptosis was induced in the wing-blade primordium by driving expression of the pro-apoptotic transgene UAS-reaper with rotund-GAL4, which is expressed in the majority of the wing primordium. This ablation was turned on and off with a temperature-sensitive GAL80 (GAL80ts), which represses GAL4 when active. At 18°, GAL4 was inhibited by GAL80ts, preventing apoptosis; shifting to 30° relieved the inhibition and allowed tissue ablation. All three components are on a single chromosome for ease of genetic manipulation, hereafter referred to as the ablation chromosome. The amount of regeneration expected in controls was regulated by adjusting the developmental stage of the larvae at the beginning of ablation and the amount of time that ablation was allowed to continue. The wing primordium was ablated over the course of 24 hours starting at the early third instar (day 7 after egg laying) (Figure 1B). After the temperature shift back to 18° the wing discs regenerated, and the resulting adult wings were qualitatively scored by assessing the size of each adult wing and counting the numbers of wings that were approximately <25%, 25%, 50%,
75% or 100% the size of a normal adult wing (Figure 1C). This semi-quantitative evaluation enabled the screening of approximately 900 wings or 6-10 genotypes per week.

We used this ablation system to conduct a dominant-modifier genetic screen using chromosomal deficiencies. We adjusted the timing of the ablation such that a control population, generated by crossing the ablation chromosome to \( w^{1118} \), had a majority of its wings regenerate to 50% of normal wing size. This moderate amount of regeneration in the control wings enabled us to identify both enhancers and suppressors of regeneration similar to the previously reported pilot screens (Smith-Bolton et al. 2009)(Figure 1D). Each line was screened three times to ensure reproducible results. We screened three collections of isogenic deficiency lines to maximize coverage of the right arm of the third chromosome: the Bloomington Stock Center and Exelixis Deficiency collections (Parks et al. 2004; Cook et al. 2012), which were generated in the same genetic background and were screened together, as well as the DrosDel deficiencies (Ryder 2004), which we screened separately to control for differences in genetic background (Supplemental Table 1). We found that the proportion of lines that had poor, normal, and enhanced regeneration was similar among the three collections (Figures S1 and S2), indicating that such separate treatment was likely unnecessary. We calculated an average regeneration index number from the semi-quantitative scores. Variations in regeneration occurred between
experiments due to food batch differences and ambient environmental factors outside of our control (SKINNER et al. 2015; VONESCH et al. 2016). Therefore, assessment of regenerative capacity for each deficiency line was determined by its score in comparison to the other lines screened at the same time, not in comparison to the complete set of screened lines, resulting in the intermixing of normal and altered regenerative ability observed in Figures S1 and S2. Seventeen deficiency regions were identified that displayed reduced regenerative capacity (Table 1). Thirty-five deficiency regions were identified that displayed enhanced regenerative capacity (Table 2). Of these thirty-five, two also displayed consistent defects in patterning of the wing.

Secondary screen for disruption of developmental timing

Regenerative capacity is linked to the developmental age of the tissue, and in turn, damaged tissue can regulate developmental progression. Damaged imaginal discs are capable of extending the third larval instar, delaying metamorphosis to allow sufficient time for regeneration (HALME et al. 2010). These damaged discs secrete an insulin-like peptide, dILP8, that regulates the production in the ring gland of ecdysone, the hormone that regulates the developmental transition between the larval and pupal stages (COLOMBANI et al. 2012; GARELLI et al. 2012). Despite this ability to stall development, there is a limited developmental window during which imaginal disc regeneration is possible. If tissue is damaged after a particular point in development,
metamorphosis will continue without adequate time for regeneration (Smith-Bolton et al. 2009). This restriction is likely due to a spike in ecdysone levels that occurs before pupariation, as larvae with a reduced ability to synthesize ecdysone have a prolonged window in which to regenerate (Katsuyama and Paro 2013). Epigenetic silencing prevents at least one regeneration-specific enhancer from responding to damage at this stage of development (Harris et al. 2016), suggesting that broader epigenetic silencing of regeneration genes may prevent full regeneration if damage occurs just before metamorphosis.

Given these connections between developmental progression and regenerative capacity, it was important to determine whether deficiency lines with a regeneration phenotype also had defects in developmental or pupariation timing. Note that direct comparisons between regenerating larvae, which spend 24 hours at 30° (Fig. 1A), and developing larvae, which remain at 18°, cannot be made due to the effects of temperature on development (Fig. 2A). Therefore, we compared the pupariation timing of each deficiency line during regeneration (Fig. 2 B, C, and D) or during development (Fig. 2 B’, C’, and D’) to a w1118 control that experienced the same temperature and ablation treatments.

There are three different ways a mutation can alter apparent regenerative capacity by altering developmental timing. First, poor regeneration can occur if the deficiency contains one or more genes that are responsible for signaling the
need for a delay in progression to metamorphosis, resulting in a shortened time for regeneration. Second, enhanced regeneration can occur if the deficiency contains one or more genes that are required for normal development such that the mutant animal develops slowly even without tissue damage, resulting in ablation of a smaller amount of tissue and/or a longer time to regenerate (Figure 2B and B’). Finally, enhanced regeneration can occur if the deficiency contains one or more genes that restrict the delay in pupariation after tissue damage, resulting in a longer time for regeneration in the mutant (Figure 2C and C’). By contrast, a mutation that specifically affects regenerative growth would do so without affecting either normal developmental timing or the delay of entry into metamorphosis after tissue damage (Figure 2D and D’).

We performed a secondary screen of the 51 deficiency regions that scored in our primary screen (Tables 1 and 2). Based on these results, the animals heterozygous for the deficiencies fell into 3 categories. In Category 1, mutant populations took longer than controls for 50% of the animals to pupariate in the absence of tissue damage, indicating an overall developmental delay. These lines with slow development also had enhanced delays in entering metamorphosis after tissue damage (Figure 2B and B’). Only two deficiencies that caused poor regeneration (16%) fell into Category 1 (Figure 2D). These mutants likely experienced extremely slow growth during normal development and regeneration. Twenty-one deficiencies that caused enhanced regeneration
(60%) fell into Category 1 (Figure 2E). These mutant animals likely had imaginal discs that were less mature at the time of ablation and had more time to regenerate, accounting for the apparent increase in regenerative capacity. No mutations in Category 1 were selected for further analysis.

In Category 2, mutant populations developed at the same rate as controls, but had a regeneration-specific delay in metamorphosis (Figure 2C and C’). Two deficiencies that caused poor regeneration (16%) fell into Category 2 (Figure 2E), indicating that loss of a gene or genes within these regions significantly impaired regeneration such that adult wings were smaller than controls even with additional time for regrowth. Eleven deficiencies that caused enhanced regeneration (31%) fell into Category 2 (Figure 2E). These regions may contain genes that affect the expression of dILP8, or the systemic response to this signal that regulates entry into metamorphosis. The additional time for regeneration likely accounts for the apparent increase in regenerative capacity.

Surprisingly, none of the poorly regenerating deficiency lines had a reduction in time to pupariation. However, our lab has previously shown that animals heterozygous mutant for the gene trithorax exhibit poor regeneration because of a reduction in dilp8 expression and time before pupariation (SKINNER et al. 2015).
In Category 3, mutant populations developed normally and delayed metamorphosis at the same rate as controls. These deficiencies were most likely to contain one or more genes that regulate regeneration itself (Figure 2D and D'). Twelve of the poorly regenerating deficiency lines (75%) had no changes in developmental timing relative to the control line (Figure 2E). Only three of the deficiencies that enhanced regeneration (8.5%) did so without affecting developmental timing relative to the control line (Figure 2E). Below we describe the identification and characterization of one gene within a deficiency in Category 3.

**Identification of cap-n-collar as a *Drosophila* regeneration gene**

To identify the genes that are responsible for regulating regeneration, we mapped the regeneration phenotypes to smaller regions and then to individual genes. We first mapped the phenotype of *Df(3R)ED6103* because it displayed a strong phenotype. We tested three overlapping deficiency lines for a regeneration phenotype (Figure S3A,B), and identified a smaller region, which contained two genes, that was likely responsible for the phenotype. We tested mutants of the two genes, the transcription factor *cap-n-collar* (*cnc*) (Mohler et al. 1991), a homolog of vertebrate Nrf2, and *Inwardly Rectifying Potassium Channel-1* (*Irk1*) (MacLean et al. 2002) for altered regenerative capacity. The majority of the population of *cnc*^{03921/+} wings only regenerated to 25% of normal size (Figure 3A), whereas the *irk1/+* mutants regenerated comparably to controls, indicating
that cnc is most likely the gene responsible for the phenotype. Similar results were obtained for a second, independently derived allele, cnc\textsuperscript{EY08884} (SFigure 3C). Measurement of absolute wing size showed that heterozygous mutants did not regenerate to the extent controls did, despite not having a size difference in the absence of tissue damage (Figure 3B), indicating that cnc is required for regeneration.

Approximately 5% of rn-GAL4 expressing cells survive ablation and contribute to the regenerating tissue (SMITH-BOLTON \textit{et al.} 2009; SKINNER \textit{et al.} 2015), enabling expression of transgenic constructs in our regeneration system. Larvae with cnc levels reduced in the wing pouch via RNAi also regenerated poorly (SFigure 3D). Interestingly, the severity of the phenotype was comparable between the heterozygous mutant and the RNAi knockdown. By contrast, overexpression of Cnc via \textit{UAS-cncC}, the isoform known to respond to stress (SYKIOTIS and BOHMANN 2008), led to better regeneration than in controls as assessed by our semi-quantitative assay (Figure 3C), further supporting a role for Cnc in regulating the regeneration of the wing primordium. To confirm that ablation was not reduced in flies expressing UAS-Cnc, we measured the size of the wing pouch at the end of the temperature shift. At R0, the average wing pouch size of \textit{UAS-Cnc} animals was the same as controls, indicating they experienced equivalent amounts of cell death (SFigure 4). The enhanced regeneration
induced by overexpression of Cnc was confirmed by measurement of absolute wing size (Figure 3D).

The basic leucine-zipper transcription factor Cnc is a well-characterized stress-response gene that is activated in the presence of reactive oxygen species (ROS) and xenotoxic compounds. It subsequently activates transcription of antioxidant enzymes and detoxifying proteins (reviewed in SYKIOTIS and BOHMANN 2010). There are three protein isoforms, although CncA has no known function. The CncB isoform is required for head development (GELLON et al. 1997; McGINNIS et al. 1998). CncC regulates intestinal stem cell proliferation (HOCHMUTH et al. 2011), the 26S proteasome (GRIMBERG et al. 2011), aging (RAHMAN et al. 2013; TSAKIRI et al. 2013), and occupies the early-ecdysone puffs during metamorphosis (DENG and KERPPOLA 2013). Nrf2, the vertebrate ortholog of Cnc, is also required for epidermal wound healing (BEYER et al. 2007a) and murine liver regeneration (BEYER et al. 2007b). Interestingly, hyperactivation of Nrf2 also negatively impacts murine liver regeneration (KÖHLER et al. 2014), indicating that further insight into how Cnc/Nrf2 regulates regeneration is necessary. We characterized the cnc\textsuperscript{3921/+} regenerating tissue to clarify how this transcription factor regulates regeneration in Drosophila.

Cnc is required for the initial growth response to tissue damage
One of the early steps of regeneration is organization of a zone of proliferation at the damage site called a blastema. To test if \textit{cnc}^{03921+/+} animals fail to regenerate due to a defect in proliferation, we measured the differences in blastema growth between \textit{cnc}^{03921+/+} and control regenerating wing discs. We measured the size of the developing wing primordium during regeneration as marked by expression of the wing primordium gene \textit{nubbin}. We did not observe a significant difference in the size of the primordium between \textit{cnc}^{03921+/+} and control animals at the beginning of or after 24 hours of regeneration (R0 and R24) (Figure 4 A,B). We next measured the number of mitotic nuclei in the blastema by immunostaining with anti-phosphohistone H3 (PH3). At R0, there were significantly fewer PH3+ nuclei in the \textit{cnc}^{03921+/+} wing primordia than in controls (Figure 4 A'). Calculating the mitotic rate showed that \textit{cnc}^{03921+/+} discs had 20% fewer mitotic nuclei per 100 \(\mu\text{m}^2\) of blastema (Figure 4 A''), suggesting that the \textit{cnc}^{03921+/+} discs were slower to begin proliferation in response to the massive tissue loss.

Such a subtle difference in proliferation rate may require several rounds of cell division before it affects the overall size of the blastema. Indeed, while the average regenerating \textit{cnc}^{03921+/+} wing pouch size was the same as controls at R24, it began lagging behind controls at R48 and continued to be smaller than controls at R60 (Figure 4 B). Interestingly, the mitotic rate in the \textit{cnc}^{03921+/+} wing pouch was equal to that of controls at R24 and R48, indicating that the \textit{cnc}^{03921+/+} blastema was able to proliferate appropriately after the early stages of
regeneration (Figure 4 C). The mitotic rate was actually higher in the \( \text{cnc}^{03921/+} \)
regenerating wing pouch at R60, which is shortly before the animals enter metamorphosis. These late rounds of proliferation were insufficient to make up for the slow initial regenerative growth. Thus, the \( \text{cnc}^{03921/+} \) discs grew slowly during the early stages of regeneration, likely because of a defect in activating proliferation due to insufficient expression of Cnc targets.

**Cnc is required for proper timing of pupariation after tissue damage**

To determine the extent to which Cnc regulates the systemic signaling that delays metamorphosis, another process required for regeneration after tissue damage, we quantified the rate of pupariation in control, \( \text{cnc}^{03921/+} \), and \( \text{UAS-cnc} \) animals with regenerating wing discs. \( \text{cnc}^{03921/+} \) and \( \text{UAS-CncC} \) animals developed at the same rate as controls when undamaged (Figure 4 D). However, \( \text{cnc}^{03921/+} \) did not delay pupariation as long as controls after the tissue had been damaged, while \( \text{UAS-CncC} \) delayed pupariation longer than controls (Figure 4 E). The difference in time of entry to metamorphosis was approximately 12 hours early for \( \text{cnc}^{03921/+} \). Interestingly, the original Df that scored in our genetic screen did not cause a reduction in time to pupariation. However, this large Df removes many genes, and may remove additional loci that impact pupariation timing. By contrast, the \( \text{UAS-CncC} \) regenerating wing pouches had an extra 12 hours before pupariation. These animals were not different in size from controls at R60 (SFigure 4), suggesting that this difference in pupariation timing may account for
most or all of the differences in the final adult wing size and thus regenerative capacity induced by overexpression of Cnc. Because pupariation delay after tissue damage is regulated by dilp8, which is regulated by JNK signaling (COLOMBANI et al. 2012; KATSUYAMA et al. 2015), the differences in pupariation timing in cnc<sup>03921/+</sup> and UAS-cncC could be due to misregulation of JNK signaling in Cnc mutants. Thus, the cnc<sup>03921/+</sup> mutants regenerate poorly due to a combination of reduced growth early in regeneration, and reduced time for regeneration caused by premature pupariation, both of which are consistent with defects in JNK signaling.

**Cnc regulates debris localization after tissue ablation.**

Another early step in regeneration is reestablishing tissue continuity and clearing cellular debris. We noticed that there was a difference in the distribution of cellular debris among the different genotypes. Because the cellular debris may indicate differences in wound closure and debris clearance, and the debris itself may signal to the regenerating epithelium and impact regeneration signaling, we quantified the distribution of cellular debris following ablation. To mark the debris, we expressed UAS-eGFP with the ablation chromosome, such that eGFP was transiently expressed in the same cells that expressed reaper. Thus, GFP was found in dead and dying cells, as well as any m-GAL4 expressing cells that survived the ablation (Fig. 5A-B).
To obtain a more complete view of the debris location, we examined orthogonal slices of the wing primordium during regeneration, noting the location of the GFP-containing debris. We measured fluorescence intensity within the region apical to the epithelial sheet and below the peripodium, within the epithelium as marked by the Nubbin antibody, and within the region basal to the epithelial sheet (Figure 5 C,D). The amount of GFP in the epithelium was not significantly different between the genotypes at R24 (Figure 5 E), indicating that the number of cells surviving ablation was similar. However, the GFP levels in the \( cnc^{03921/+} \) mutant epithelium at R48 were significantly higher than in the \( w^{1118} \) epithelium. Given that Cnc/Nrf2 can regulate proteasomal and lysosomal degradation in other contexts (GRIMBERG et al. 2011; NAGY et al. 2013; PICKERING et al. 2013), the persistent GFP in the epithelium suggests that the \( cnc^{03921/+} \) mutants may have been unable to degrade the GFP, or, if the epithelial cells ingest debris, they were less able to degrade the debris they were clearing.

In the ablation system used here, the large field of debris was extruded apically (Figure 5 C,F), which is contrary to most single-cell extrusion from epithelial sheets and may occur because such a large number of cells die simultaneously. Interestingly, in \( cnc^{03921/+} \) ablated discs there was significantly more debris in the region basal to the epithelium (Figure 5 D,G), suggesting that these regenerating discs may have an impaired ability to move dying cells and debris to the apical surface, or that there is a difference in wound closure, resulting in an atypical
distribution of debris. This failure to remove debris directionally and efficiently may affect regeneration by imposing physical constraints or by impairing or changing the location of any pro-regeneration signals emitted by dying cells.

**ROS levels are higher in regenerating tissue with reduced Cnc**

Cnc/Nrf2 is closely associated with changes in expression of antioxidant enzymes in response to ROS (ITOH et al. 2004; SYKIOTIS and BOHMANN 2008). Damage to imaginal discs leads to ROS production by the resulting cellular debris and by the healing epithelium (SANTABÁRBARA-RUIZ et al. 2015; FOGARTY et al. 2016; reviewed in SERRAS 2016). To determine the extent to which Cnc affects levels of ROS in the regeneration blastema, we used dihydroethidium (DHE) staining (OWUSU-ANSAH et al. 2008) to observe ROS levels in control and cnc<sup>03921/+</sup> regenerating discs after 24 hours of regeneration. ROS levels were high in the cellular debris, but also present in the epithelium, which is the area of interest (Figure 6 A). Undamaged samples had very low levels of ROS (Figure 6 B’). Damaged samples had higher levels of ROS in the regenerating epithelium compared to an undamaged disc (Figure 6 C’), as previously reported (SANTABÁRBARA-RUIZ et al. 2015). However, cnc<sup>03921/+</sup> mutants had significantly higher levels of ROS in the regenerating epithelium compared to controls (Figure 6 C-G), indicating that Cnc is required to control ROS levels in the blastema during regeneration. The elevated ROS in the cnc<sup>03921/+</sup> regenerating epithelium could have several deleterious effects, including increasing damage and cell
death in the blastema and interfering with regeneration signaling, contributing to
the poor regeneration observed in the cnc03921/+ tissue.

**Cnc regulates regeneration signaling**

ROS presence in damaged tissue induces wound healing and activation of JNK
signaling (SANTABÁRBARA-RUIZ et al. 2015; FOGARTY et al. 2016). Additionally, the
cnc/+ mutants showed defects in wound healing, blastema formation, growth,
and pupariation timing, processes that are regulated by JNK signaling (BOSCH et
GARELLI et al. 2012). Thus, reduction of Cnc levels might affect JNK signaling,
either directly through misregulation of its transcriptional targets or indirectly
through misregulation of ROS levels. To examine JNK signaling, we quantified
expression of a TRE-red transcriptional reporter (CHATTERJEE and BOHMANN
2012). While expression levels were high in the cellular debris, our region of
interest was the regenerating epithelium (Figure 7 A). This reporter was higher in
the epithelium of control discs immediately after tissue ablation (R0) than in
cnc03921/+ regenerating discs at R0 (Figure 7 B-D). Thus, cnc03921/+ damaged
tissue did not have the same initial levels of JNK signaling as control damaged
tissue. Interestingly, after 48 hours of regeneration, levels of JNK signaling were
comparable in cnc03921/+ regenerating discs and controls, further suggesting that
Cnc activity is more important early in regeneration (SFigure 5 A-C).
These data suggest that Cnc regulates JNK signaling during the early stages of regeneration. There are two possible mechanisms through which Cnc could be required for JNK signaling, which are not mutually exclusive. First, direct genetic targets of Cnc may promote JNK signaling. Second, there may be an optimal level of ROS to activate JNK signaling, and excessive amounts of ROS might decrease JNK activation. To determine whether elevated ROS can inhibit JNK signaling, we exposed regenerating tissue to ectopic ROS and assessed the effects on JNK activation. We supplemented the food with 0.5% H₂O₂ and stained with DHE to confirm that consumption of H₂O₂ by the larvae increased the amount of ROS in regenerating discs (Figure 7 E, F). We also observed that increased ROS in undamaged discs did activate JNK expression, as expected (SANTABÁRBARA-RUIZ et al. 2015) (Figure 7 G-I). Interestingly, ectopically increasing ROS for the first 24 hours of regeneration lowered JNK activation (Figure 7 J-M), supporting the idea that excess ROS can dampen the JNK signal. Thus, Cnc ensures proper levels of JNK signaling at least in part through maintaining optimal ROS levels, although additional Cnc-dependent mechanisms of regulating JNK have not been ruled out.

To assess the influence of Cnc on regeneration signaling and gene expression downstream of JNK, we assessed Wg and Myc expression levels in cnc⁰３⁸₂₁/⁺ regenerating discs via immunostaining. Quantification of staining intensity showed statistically significant reduction in levels of both Wg and Myc in
Cnc03921/+ regenerating discs at R24 compared to controls (SFigure 5D-I). These small yet significant reductions in Wg and Myc may be a result of the reduction in JNK signaling. Expression of Myc was comparable to controls by R48 (SFigure 5F). The Cnc03921/+ regenerating discs resolved Wg expression to normal third instar patterning by R48.

All together, these data led us to a model wherein ROS is necessary to activate JNK signaling after tissue damage, but too much ROS reduces JNK signaling, and Cnc is required to maintain ROS at the appropriate level (Figure 8A). When Cnc levels are lowered, ROS levels increase such that JNK signaling is lowered, leading to regeneration defects (Figure 8B). Our data do not rule out a role for Cnc in activating the JNK pathway independently of ROS levels; indeed, it is possible that Cnc regulates JNK through both mechanisms. Furthermore, we have not ruled out the possibility that Cnc affects regeneration independently of JNK.

DISCUSSION

The first regeneration gene mapped and identified from our screen of chromosome arm 3R was cap-n-collar. In contrast to other regulators identified through our pilot genetic screen (Smith-Bolton et al. 2009; Schuster and Smith-Bolton 2015; Skinner et al. 2015), reduction of Cnc levels affected multiple
factors during regeneration. Our data demonstrate that Cnc has an important role in maintaining proper ROS levels during regeneration, presumably through regulating expression of antioxidant genes (reviewed in PITONIAK and BOHMANN 2015). ROS are one of the earliest signals that tissue damage has occurred (NIETHAMMER et al. 2010; YOO et al. 2012). However, ROS themselves are also agents of tissue damage (FRIDOVICH 1978). Thus, there is likely an ideal level of ROS for stimulating regeneration, and too much or too little ROS could impair regenerative capacity (Figure 8A). Recent work has shown that ROS activates JNK signaling during regeneration (SANTABÁRBARA-RUIZ et al. 2015). However, we show here that the reduction of Cnc results in increased ROS (Figure 6), but decreased JNK signaling (Figure 7). Thus, there is not a direct linear relationship between ROS levels and amount of JNK activation, and there is a point at which excess ROS dampens JNK activation (Figure 8B). Interestingly, our data also show that Cnc affects many regenerative processes that are regulated by JNK signaling, including wound closure and debris localization, early blastema proliferation, and stalling entry to pupariation. We propose a model where cap-n-collar has important regulatory roles during regeneration. First, it regulates the response to the presence of reactive oxygen species (ROS) and keeps ROS at the appropriate levels for efficient regeneration. Second, it regulates JNK signaling, via ROS levels and possibly via additional transcriptional targets (Figure 8B). Third, it may regulate regenerative processes independently of JNK (Fig. 8A and B)
While work to date in *Drosophila* has focused on the role of Cnc in the cellular response to stress and to xenobiotic factors (DENG and KERPPOLA 2013, 2014), as well as oxidative stress related to aging and aging-related neurological disorders (SYKIOTIS and BOHMANN 2008, 2010; BARONE *et al.* 2011; RAHMAN *et al.* 2013), work in vertebrates has identified Nrf2 as an important regulator of epidermal wound healing (BEYER *et al.* 2007a; SCHÄFER and WERNER 2008) and liver regeneration (BEYER *et al.* 2007b; BEYER and WERNER 2008; WAKABAYASHI *et al.* 2010; DAYOUB *et al.* 2013; ZOU *et al.* 2014). Thus, by establishing a complementary model to explore the role of Cnc in imaginal disc repair and regeneration, we will be able to gain deeper understanding of the role of this transcription factor in both invertebrate and vertebrate tissue repair. For example, overactivated Nrf2 negatively impacts liver regeneration (KÖHLER *et al.* 2014). Given the similarities in the imaginal disc, where both too much and too little ROS can result in insufficient JNK signaling for regeneration, further elucidation of the mechanism relating ROS, Cnc/Nrf2, and JNK signaling in response to damage or other stressors may offer insight into the protective mechanisms at work in vertebrate organisms. Systematic identification of the transcriptional targets of Cnc during wound healing and regeneration, which is beyond the scope of this work, will be tremendously informative concerning regulation of regeneration in this and other systems.
In addition to the chromosomal deficiency that removed cnc, the genetic screen described here has isolated chromosomal deficiencies that remove a variety of important regeneration genes, likely including wound response genes, growth regulators, genes required for patterning, systemic signaling factors, and novel genes that have heretofore unknown roles in regeneration. The secondary screen we carried out provides some clues to the different processes regulated by each mutant. For example, the genes identified by mapping the phenotypes within the deficiencies that affect the timing of metamorphosis will further characterize the mechanism of systemic signaling that coordinates growth regulation and metamorphosis after tissue damage (Colombani et al. 2012; Garelli et al. 2012; Jaszcak et al. 2015). The secondary screen also narrowed down the number of deficiencies that likely contain negative regulators of regeneration to three. These negative regulators may act as a brake on cellular growth, but their reduction did not result in obvious overgrowth. They regenerated faster but still respected endogenous organ size control mechanisms. Such factors are of particular interest because inhibition of their vertebrate orthologs might have the potential to enhance regeneration without inducing tumor formation.

We have only begun to characterize the genes identified in this screen. As the number of mapped deficiency regions increases and we identify and characterize novel regeneration genes, this screen will prove to be a powerful tool in our quest
to characterize regeneration. Furthermore, we expect that by expanding this screen to other chromosome arms, we will generate a more complete understanding of the regulation of regeneration in the *Drosophila* imaginal wing disc.

**FIGURE LEGENDS**

**Figure 1. Outline of the deficiency screen.** A) The genotype of the ablation chromosome and the genetic cross used in the screen. B) A diagram of the tissue ablation system. Briefly, at 18° GAL80\textsuperscript{TS} inhibits GAL4 and *UAS-reaper* is not expressed. Shifting to 30° at day 7 after egg lay (AEL) relieves the inhibition and *reaper* expression induces apoptosis. On day 8 AEL, stocks are shifted back to 18° and regeneration occurs. Starting at approximately day 20 AEL, adult flies eclose, enabling scoring of wing size. C) A semi-quantitative scoring scale for the adult wings that resulted from regenerated wing primordia. D) Tissue ablation was timed so that the majority of a population of control wings regenerate to 50% of normal size, so that screening could identify populations of wings that were larger or smaller than controls.

**Table 1. Deficiency regions that reduced regenerative capacity**
The deficiency regions that, when heterozygous, caused wing discs to regenerate poorly. The developmental timing of pupariation and regeneration-specific timing of pupariation is given in the number of days before or after controls ($w^{1118}$) that 50% of the animals had entered pupariation (see methods).

N/A* This deficiency is lethal over the TM6B-$Tb$ balancer, which is necessary for the experiment.

Table 2. Deficiency regions that enhanced regenerative capacity

The deficiency regions that, when heterozygous, caused wing discs to regenerate better than controls. The developmental timing of pupariation and regeneration-specific timing of pupariation is given in the number of days before or after controls ($w^{1118}$) that 50% of the animals had entered pupariation (see methods).

Figure 2. Secondary screen for defects in developmental and regeneration timing

Categorization of mutant lines according to the rates at which the mutant populations reached pupariation during normal development and after tissue damage. A) The rate at which larvae at 18° reach pupariation compared to that of larvae experiencing the 24-hr, 30° temperature shift without ablation. B) $Df(3R)ED5518/+ \text{ pupariated four days later than controls following tissue damage, 3 independent experiments, } w^{1118} n=157, Df(3R)ED5518/+ n=64.; B')$
the same line also delayed pupariation during normal development, 3
independent experiments, \(w^{1118}\) n=184 wings, \(Df(3R)ED5518/+,\) n=64 wings. C) \(Df(3R)BSC320/+\) delayed pupariation one day longer than controls following
tissue damage, 3 independent experiments, \(w^{1118}\) n=165, \(Df(3R)BSC320/+,\) n=96. C’) the same line developed at the same rate as controls when undamaged, 3
independent experiments, \(w^{1118}\) n=173, \(Df(3R)BSC320/+,\) n=96; D) \(Df(3R)BSC140/+\) pupariated at the same rate as controls following tissue
damage, 3 independent experiments, \(w^{1118}\) n=128, \(Df(3R)BSC140/+,\) n=69; D’) the same line also developed at the same rate as controls when undamaged, 3
independent experiments, \(w^{1118}\) n=129, \(Df(3R)BSC140/+,\) n=52; E) The
secondary screening results for lines that regenerated poorly compared to
controls: 2 lines developed slower than controls, 2 lines developed normally but
had an increased recovery period following tissue damage, and 12 lines had no
discernible pupariation delay after tissue damage. Data for individual lines are in
Table 1. The secondary screening results for lines that regenerated better than
controls: 21 lines developed slower than controls, 11 lines developed normally
but had an increased recovery time following tissue damage compared to
controls, and 3 lines had enhanced regeneration with no discernible change in
pupariation timing relative to controls. Data for individual lines are in Table 2.

Figure 3. Cnc is required for regeneration
A) Comparison of populations of adult wings after regeneration for control (w^{1118}), cnc^{03921}/+, Irk1^{M08404}/+, and Irk1^{MB08423}/+ animals. Error bars display standard error of the mean (S.E.M.). Three independent experiments, w^{1118} n=228 wings, cnc^{03921}/+ n=122 wings, Irk1^{M08404}/+ n=160 wings, and Irk1^{MB08423}/+ n=116 wings.

B) Measurement of adult wing sizes resulting from undamaged or ablated imaginal discs, in square millimeters. cnc^{03921}/+ (undamaged male n=56 wings, undamaged female n=84 wings, regenerated male n=53 wings, regenerated female n=82 wings) and w^{1118} (undamaged male n=103 wings, undamaged female n=165 wings, regenerated male n=105 wings, regenerated female n=154 wings). Male and female wings were separated because of sexually dimorphic adult wing size. There was no significant difference between undamaged cnc and w^{1118} wing sizes (p>0.5), however there was a significant difference in wing size following tissue damage in both males and females using the Students T-test. *p<0.05, **p<0.005. Error bars represent S.E.M.

C) Comparison of populations of adult wings after imaginal disc ablation with the genotypes w^{1118}, cnc^{03921}/+, and UAS-cncC. Error bars represent S.E.M. Three independent experiments, w^{1118} n=233 wings, cnc^{03921}/+ n=206 wings, and UAS-cncC n=176 wings.

D) Measurement of w^{1118} and UAS-cncC adult wing sizes resulting from undamaged or regenerated imaginal discs in square millimeters. w^{1118} male n=70 wings, female n=131 wings, UAS-cncC male n=155 wings, female n=204 wings. Error bars represent S.E.M. * p<0.05, **p<0.005, using the Students T-test.
Figure 4. Cnc is required for early blastema proliferation and regulates entry to metamorphosis.

A) Average wing pouch size in control and cnc^{03921/} regenerating discs at R0, as marked by anti-Nubbin immunostaining. n= between 11 and 15 discs for each sample, from at least 2 independent experiments. *p< 0.05. A’) Average number of mitoses, as marked by anti-phospho Histone H3 (PH3), per wing pouch, as marked by anti-Nubbin, in the same discs as A. A’’) The average number of mitoses per 100 μm², calculated from the same discs. Error bars represent S.E.M.

B) Average wing pouch size in control and cnc^{03921/+} regenerating discs at R24, R48, and R60, as marked by anti-Nubbin immunostaining. n= between 12 and 22 discs for each sample, from at least 2 independent experiments. *p< 0.05, Students T-test.

C) Average number of mitoses per 100 μm², calculated from the same discs. Error bars represent S.E.M.

D) The percentage of normally developing animals that had formed pupae on the side of the vial each day after egg lay. These animals contained the ablation chromosome but were reared at 18 degrees and so did not induce ablation. Three independent experiments, total n for w^{1118} = 205, UAS-cncC = 207, cnc^{03921/+} = 103.

E) The percentage of animals with damaged imaginal discs that had formed pupae on the side of the vial each day after egg lay. The thermal shift to 30 degrees accelerated development such that the timing in D cannot be compared to the timing in E. Three independent experiments, total n for w^{1118} = 177, UAS-cncC = 154, cnc^{03921/+} = 95. Error bars are SEM.
Figure 5. Cnc prevents persistence of basally localized cellular debris

Distribution of cellular debris marked by UAS-eGFP expression in regenerating discs. A) Typical view of a wing imaginal disc in the XY-plane. B) Schematic of wing discs in the YZ-plane, which is the orientation of all confocal images shown (orthogonal slices). UAS-eGFP is expressed during the 24 hour ablation period; 24 hours after ablation has ended, the visible GFP is in the debris in the lumen between the peripodium and disc epithelium. C,D) Orthogonal slices showing the debris fields (eGFP, green) and regenerating epithelia (anti-Nubbin, red; DAPI, blue) at R24. In w1118, the debris is apical to the disc epithelium (C). In cnc03921/+ mutants, the debris is located both apical and basal to the disc epithelium (D). The scale bar in C is equal to 20 µm. E-G) Quantification of fluorescence intensity in the epithelium (E), and regions apical (F), and basal (G) to the epithelium of the two genotypes at R24 and R48. Error bars are SEM. *p<0.05, Student’s T-test.

Figure 6. ROS levels are higher in the blastema when Cnc levels are reduced

A) Schematic of a disc in the XY plane. A nubbin-GFP enhancer trap labels the debris and the regenerating epithelium. Dihydroethidium (DHE) fluorescence marking ROS is present in high levels in the cellular debris and lower levels in the disc epithelium. B-D) Wing discs presented in the YZ-plane expressing a nubbin-
GFP reporter to mark the wing primordium and debris (B,C,D) with DHE staining to indicate ROS levels (B',C',D'). Discs are \( w^{1118} \) undamaged (B,B'), \( w^{1118} \) R24 (C,C'), or \( cnc^{03921/+} \) R24 (D, D'). Yellow dotted lines outline the diffuse nub-GFP expression in the regenerating wing pouch, and exclude the bright GFP with puncta in the debris. Scale bar equals 20 \( \mu m \). E) DHE levels were quantified by measuring fluorescence intensity in three equal-sized boxes in the epithelial layer of each disc. Undamaged, \( n=13 \), \( w^{1118} \), \( n=15 \), \( cnc^{03921} \), \( n=15 \). Error bars are SEM. *p<0.05, Students T-test.

Figure 7. JNK signaling is reduced during regeneration by exposure to excessive ROS

A) Schematic of a wing disc in the YZ plane. A reporter for JNK signaling, TRE-red, is present at high levels in the debris and at lower levels in the disc epithelium, which is identified by Nubbin antibody (green). B,C) Wing discs at the end of the ablation period (R0) with TRE-red (red) and anti-Nubbin (green) of the genotypes \( w^{1118} \) (B) and \( cnc^{03921/+} \) (C). Scale bar in B equals 20 \( \mu m \). D) Quantification of TRE-red fluorescence levels in the disc epithelium ( \( w^{1118} n=20, \) \( cnc^{03921/+} n=16 \) ) (E,F) DHE staining of wing imaginal discs from larvae fed 0% and 0.5% \( H_2O_2 \). Note that feeding 0.5% \( H_2O_2 \) to the larvae results in higher ROS levels in the wing discs. G,H) Undamaged \( w^{1118} \) wing discs from larvae fed 0% \( H_2O_2 \), or 0.5% \( H_2O_2 \) (H,H') with anti-Nubbin (green), TRE-dsRed (red), and DAPI (blue). G',H') The TRE-dsRed alone, adjusted for brightness and contrast to
enhance visibility. I) Quantification of TRE-dsRed performed on unadjusted images (see methods): 0% H$_2$O$_2$,$^{11}$w$^{11}$ n= 14, 0.5% H$_2$O$_2$, $w^{11}$ n=20. J-M) TRE-dsRed (red) and anti-Nubbin (green) in wing discs after 24 hours of regeneration. J,J') $w^{11}$, fed 0% H$_2$O$_2$; K,K') $cnc^{03921}$/+, fed 0% H$_2$O$_2$; L,L') $w^{11}$, fed 0.5% H$_2$O$_2$. M) Quantification of J-L. (0% H$_2$O$_2$,$w^{11}$ n= 31, $cnc^{03921}$/+ n=25; R24 0.5% H$_2$O$_2$, $w^{11}$ n=30). Note that TRE-red levels in $cnc^{03921}$/+discs are equal to those in $w^{11}$ discs with ectopic 0.5% H$_2$O$_2$. Error bars are SEM. *p<0.05 ** p<0.005, Student’s T-test.

**Figure 8. Model for Cnc activity during regeneration**

A) Proposed relationship between ROS levels, Cnc, JNK signaling, and regenerative capacity. Injury induces ROS, which in turn activate JNK and Cnc activity. Cnc transcriptional targets constrain ROS levels and may also regulate JNK activity. Thus, there is an ideal range of ROS levels at which regeneration is most efficient. B) Model where Cnc levels are reduced. Injury induces ROS, which in turn activate JNK and Cnc. Less Cnc activity results in high ROS levels and low JNK activation, due to an inhibitory effect of high ROS and possibly due to reduction of other important Cnc targets.

**Acknowledgments**

The authors would like to thanks R. Roberts-Galbraith and S.J. Khan for their
critical reading of the manuscript and helpful comments; D. Bohmann and S. Cohen for reagents; the Bloomington Stock Center (NIH P40OD018537), the Vienna Drosophila Resource Center and the Kyoto Stock Center for *Drosophila* lines; and the Developmental Studies Hybridoma Bank (NICHD, University of Iowa) for antibodies. This work was funded by a Young Investigator Award from the Roy J. Carver Charitable Trust (#12-4041) and a grant from the NIH (NIGMS R01GM107140).

**REFERENCES CITED**


BEYER T. a, WERNER S., 2008 The cytoprotective Nrf2 transcription factor controls insulin receptor signalling in the regenerating liver. Cell Cycle 7: 874–879.


BROOK W. J., OSTAFICHUK L. M., PIORECKY J., WILKINSON M. D., HODGETTS D. J.,
RUSSELL M. a, 1993 Gene expression during imaginal disc regeneration
detected using enhancer-sensitive P-elements. Development 117: 1287–
1297.

BROOK W. J., COHEN S. M., 1996 Antagonistic Interactions Between Wingless
and Decapentaplegic Responsible for Dorsal-Ventral Pattern in the

CHATTERJEE N., BOHMANN D., 2012 A Versatile \( \Phi \)C31 Based Reporter System for
Measuring AP-1 and Nrf2 Signaling in Drosophila and in Tissue Culture (B

COLOMBANI J., ANDERSEN D. S., LEOPOLD P., 2012 Secreted Peptide Dilp8

J. M., KAUFMAN T. C., COOK K. R., 2012 The generation of chromosomal
deletions to provide extensive coverage and subdivision of the Drosophila

DAYOUB R., VOGEL A., SCHUETT J., LUPKE M., SPIEKER S. M., KETTERN N., HILDT
E., MELTER M., WEISS T. S., 2013 Nrf2 activates augmenter of liver
regeneration (ALR) via antioxidant response element and links oxidative

DENG H., KERPPOLA T. K., 2013 Regulation of Drosophila Metamorphosis by


GARELLI a., GONTIO a. M., MIGUELA V., CAPARROS E., DOMINGUEZ M., 2012 Imaginal Discs Secrete Insulin-Like Peptide 8 to Mediate Plasticity of Growth


HOCHMUTH C. E., BITEAU B., BOHMANN D., JASPER H., 2011 Redox regulation by keap1 and Nrf2 controls intestinal stem cell proliferation in drosophila. Cell Stem Cell 8: 188–199.


in mice by activation of genes involved in cell-cycle control and apoptosis. 

Hepatology 60: 670–678.


Meserve J. H., Duronio R. J., 2015 Scalloped and Yorkie are required for cell cycle re-entry of quiescent cells after tissue damage. Development 142: 2740–2751.


OWUSU-ANSAH E., YAVARI A., BANERJEE U., 2008 A protocol for in vivo detection of reactive oxygen species. Protoc. Exch. doi:10.103...


PITONIAK A., BOHMANN D., 2015 Mechanisms and Functions of Nrf2 Signaling in


Supplemental Information

**Figure S1. Regeneration indices for BSC and Exelixis Deficiencies.** Average regeneration index for each line screened, calculated from the regeneration
indices of three independent experiments. Regeneration index = approximate wing size multiplied by percentage of wings at that size. Blue, no effect on regeneration; purple, reduced regeneration; green, enhanced regeneration.

**Figure S2. Regeneration indices for DrosDel Deficienacies.** Average regeneration index for each line screened, calculated from the individual regeneration indices of three independent experiments. Regeneration index = approximate wing size multiplied by percentage of wings at that size. Blue, no effect on regeneration; purple, reduced regeneration; green, enhanced regeneration. Star indicates the Df that removed cnc.

**Figure S3. Identification of Cnc as a regeneration gene.** A) Schematic of the genomic region of Df(3R)ED6103, which caused poor regeneration in the genetic screen. The relative positions of overlapping chromosomal deficiencies and their impact on wing disc regeneration are noted, as well as the location of cnc and Irk2. The yellow box indicates the region deduced to contain the relevant gene based on the regeneration phenotypes of the chromosomal deficiencies. B) Relative regenerative capacities of wing discs heterozygous for the indicated overlapping chromosomal deficiencies as assessed by adult wing size. Three independent experiments, w^{1118} n = 270 wings, ED6103/ + n = 168 wings, BSC527/ + n = 90 wings, Exel6274/ + n = 182 wings, Exel6280/ + n = 156 wings. C) Adult wing size of cnc^{EY08884}/ + wings (male n = 86, female n = 144) compared to
\textit{w^{1118}} controls (male n=82, female n= 106). ** p<0.005.

**Figure S4. Overexpression of CncC does not increase regenerative growth.**

Average regenerating wing pouch sizes of control and \textit{UAS-cncC} discs at R0, R24, R48, and R60 do not show consistent increases in the growth of the CnC overexpressing discs. The apparent increase at R48 was lost by R60. *p<0.5. Error bars are SEM. \textit{w^{1118}} n=11, 11, 28, 32 and \textit{cnc^{03921}} n=9,11,22,24 (R0, R24, R48, R60, respectively).

**Supplemental Figure 5. Regeneration Signaling is reduced in the beginning of regeneration in \textit{cnc/+} mutants**

A,B) Orthogonal slices of regeneration blastemas after 48 hours of regeneration. The epithelial layer is marked by Nubbin (green), JNK signaling is marked by \textit{TRE-red}, and the nuclei are labeled by DAPI (blue). A) \textit{w^{1118}}, B) \textit{cnc^{03921}/+}. C) Quantification of \textit{TRE-red} levels in the regenerating epithelium. (D, E, G, H) R24 discs were co-stained with α-Myc and α-Wg antibodies in \textit{w^{1118}/+} (D,G) and \textit{cnc^{03921}/+} (E,H). Fluorescence intensity was quantified at both R24 and R48 for Myc (F) and at R24 for Wg (I). For \textit{w^{1118}}, R24 n=16 discs, R48 n=19. For \textit{cnc^{03921}/+}, R24 n=17, R48 n=9. The scale bar in H equals 50 μm. *p<0.05, Students T-test.

**Table S1**
The deficiency regions screened, with chromosomal breakpoints, a note about their regeneration phenotype, and their provenance.
A

$\text{w^{118}; +; rn-GAL4, UAS-reaper, tubGAL80^{ts}}$

\[\text{TM6B, tubGAL80}\]

$\times$

$\text{Dl(3R)}$

\[\text{TM6B or TM6C}\]

B

wing pouch

days after egg lay

temperature

\[18^\circ \rightarrow 30^\circ\]

C

Percentage of Population

D

Enhanced

Reduced

Control

Enhanced

wing size

0% 25% 50% 75% 100%
**Table One: Deficiency regions that reduce regenerative capacity**

<table>
<thead>
<tr>
<th>Deficiency Line</th>
<th>Developmental Delay (Relative to Control)</th>
<th>Expanded Recovery Period (Relative to Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(3R)BSC678</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Df(3R)ED6361</td>
<td>1 day</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)ED6265</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Df(3R)ED6280</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Df(3R)ED6103</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Df(3R)BSC677</td>
<td>None</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)ED5331</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Df(3R)ED5156</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Df(3R)Exel6270</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Df(3R)BSC467</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Df(3R)BSC516</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Df(3R)ED5942</td>
<td>N/A*</td>
<td>N/A*</td>
</tr>
<tr>
<td>Df(3R)BSC321</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Df(3R)BSC620</td>
<td>None</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)BSC489</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Df(3R)BSC498</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Df(3R)Exel9013</td>
<td>1 day</td>
<td>1 day</td>
</tr>
</tbody>
</table>
## Table Two: Deficiency regions that have enhanced regenerative capacity

<table>
<thead>
<tr>
<th>Deficiency Line</th>
<th>Developmental Delay (Relative to Control)</th>
<th>Expanded Recovery Period (Relative to Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(3R)BSC140</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Df(3R)ED10257</td>
<td>1 day</td>
<td>2 days</td>
</tr>
<tr>
<td>Df(3R)BSC138</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Df(3R)BSC819</td>
<td>None</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)ED6232</td>
<td>None</td>
<td>2 days</td>
</tr>
<tr>
<td>Df(3R)ED5177</td>
<td>None</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)Exel8269</td>
<td>1 day</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)Exel7327</td>
<td>1 day</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)BSC748</td>
<td>None</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)Exel6169</td>
<td>None</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)Exel7320</td>
<td>1 day</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)ED5642</td>
<td>1 day</td>
<td>2 day</td>
</tr>
<tr>
<td>Df(3R)Exel6195</td>
<td>None</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)ED5911</td>
<td>1 day</td>
<td>2 days</td>
</tr>
<tr>
<td>Df(3R)Exel6214</td>
<td>None</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)BSC320</td>
<td>None</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)ED5780</td>
<td>1 day</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)ED6220</td>
<td>1 day</td>
<td>2 days</td>
</tr>
<tr>
<td>Df(3R)ED6025</td>
<td>None</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)BSC549</td>
<td>1 day</td>
<td>2 days</td>
</tr>
<tr>
<td>Df(3R)BSC874</td>
<td>None</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)ED5454</td>
<td>3 days</td>
<td>3 days</td>
</tr>
<tr>
<td>Df(3R)BSC681</td>
<td>1 day</td>
<td>1 day</td>
</tr>
<tr>
<td>Df(3R)ED5612</td>
<td>1 day</td>
<td>2 days</td>
</tr>
<tr>
<td>Df(3R)ED5664</td>
<td>1 day</td>
<td>2 days</td>
</tr>
<tr>
<td>Df(3R)BSC793</td>
<td>1 day</td>
<td>2 days</td>
</tr>
<tr>
<td>Df(3R)BSC749</td>
<td>2 days</td>
<td>2 days</td>
</tr>
<tr>
<td>Df(3R)ED5092</td>
<td>2 days</td>
<td>3 days</td>
</tr>
<tr>
<td>Df(3R)ED5518</td>
<td>3 days</td>
<td>3 days</td>
</tr>
<tr>
<td>Df(3R)ED5428</td>
<td>3 days</td>
<td>3 days</td>
</tr>
<tr>
<td>Df(3R)ED6187</td>
<td>2 days</td>
<td>3 days</td>
</tr>
<tr>
<td>Df(3R)ED6058</td>
<td>None</td>
<td>2 days</td>
</tr>
<tr>
<td>Df(3R)ED5100</td>
<td>2 days</td>
<td>3 days</td>
</tr>
<tr>
<td>Df(3R)ED5514</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Df(3R)ED10951</td>
<td>4 days</td>
<td>4 days</td>
</tr>
</tbody>
</table>
**A**

![Graph A](https://via.placeholder.com/150)

**B**

![Graph B](https://via.placeholder.com/150)

**E**

Deficiencies with reduced regeneration

- 2
- 12

Deficiencies with enhanced regeneration

- 3
- 21

**C**

![Graph C](https://via.placeholder.com/150)

**D**

![Graph D](https://via.placeholder.com/150)

**B’**

![Graph B’](https://via.placeholder.com/150)

**C’**

![Graph C’](https://via.placeholder.com/150)

**D’**

![Graph D’](https://via.placeholder.com/150)

**Not regenerating with temp. shift**

**Regenerating Developing (Undamaged)**

- Df(3R)ED5518/+  
  - Blue line: Not regenerating with temp. shift
  - Red line: No temp. shift

- Df(3R)BSC320/+  
  - Blue line: Df(3R)BSC320/+  
  - Red line: w1118

- Df(3R)BSC140/+  
  - Blue line: Df(3R)BSC140/+  
  - Red line: w1118

Legend:

- Develops slower than WT (False Positives)
- Longer damage recovery period than WT
- Same developmental and recovery timing as WT
A. Graph showing the percentage of population with undamaged and regenerating wing sizes.

B. Graph comparing wing size (mm²) between UAS-CncC and various genotypes.

C. Graph showing the percentage of population with different wing sizes.

D. Graph comparing wing size (mm²) between regenerating genotypes.
Figure 1. (A–C) Quantification of mitotic activity in different conditions. Average pouch size, average number of PH3-positive cells, and number of mitoses/100 μm² are shown for different conditions: W^{1118} cnc/+, w1118 cnc/+, and UAS-CncC cnc03821. Significant differences are indicated by an asterisk (*).

Figure 2. (D–E) Percentage of population pupariated over time. The graphs show the percentage of population pupariated for undamaged and regenerating conditions, with different genotypes: W^{1118} cnc/+, cnc03821, and UAS-CncC.
A. Nubbin-GFP labelled debris (high ROS)
Disc Epithelium (moderate ROS)

B. nubbin-GFP
C. w1118
D. cnc09321/+

E. Average Fluorescence Intensity

B'. DHE
C'.
D'.

No Damage

**A**

TRE-red (debris)

TRE-red and α-Nubbin (epithelium)

**B**

w1118

TRE-red

**B’**

w1118, Undamaged

DHE

**C**

cnc03921/+ 

TRE-red

**C’**

cnc03921/+ 

TRE-red

**E**

R24

DHE

**F**

R24

DHE

**G**

w1118, Undamaged

0% H₂O₂

**H**

w1118, Undamaged

0% H₂O₂

**I**

Undamaged

0% H₂O₂

0.5% H₂O₂

**J**

TRE-red

w1118

**J’**

0% H₂O₂

**K**

TRE-red

cnc03921/+ 

**K’**

0% H₂O₂

**L**

TRE-red

w1118

**L’**

0.5% H₂O₂

**D**

Avg. Fluorescence Intensity (TRE-red)

**M**

Avg. Fluorescence Intensity (TRE-red)
A

Damaged Tissue → Reactive Oxygen Species → Cnc activation → JNK Signalinng → Pupariation timing, wound closure, proliferation

B

Damaged Tissue → Reactive Oxygen Species → Cnc activation → JNK signaling → Defects in: Pupariation timing, wound closure, proliferation