**Regenerative growth is constrained by *brain tumor* to ensure proper patterning in
*Drosophila***

Syeda Nayab Fatima Abidi¹ and Rachel K. Smith-Bolton^{1,*}

¹ Department of Cell and Developmental Biology, University of Illinois Urbana-Champaign, Urbana, IL 61801, USA

* rsbolton@illinois.edu

Abstract

Some animals respond to injury by inducing new growth to regenerate the lost structures. This regenerative growth must be carefully controlled and constrained to prevent overgrowth and to allow correct organization of the regenerating tissue. However, the factors that restrict regenerative growth have not been identified. Using a genetic ablation system in the *Drosophila* wing imaginal disc, we have identified one mechanism that constrains regenerative growth, impairment of which leads to erroneous patterning of the final appendage. Regenerating discs with reduced levels of the RNA-regulator Brain tumor (Brat) exhibit enhanced regeneration, but produce adult wings with disrupted margins that are missing extensive tracts of sensory bristles. In these mutants, aberrantly high expression of the pro-growth factor Myc and its downstream targets leads to loss of cell-fate specification. Thus, Brat ensures that the regenerating tissue forms the proper final structure by constraining expression of pro-regeneration genes.

Introduction

Regeneration is the remarkable process by which some organisms replace tissues and organs after damage such that both morphology and function are restored. Complete regeneration requires several steps to occur correctly including wound healing, cell proliferation, and proper patterning and cell-fate specification in the newly formed tissue. The degree of regenerative capacity varies among different species, ranging from whole-body regeneration in hydra and planaria to limited tissue regeneration in

mammals. Work in several model organisms has identified signaling pathways and molecular mechanisms that are important for initiating and executing regenerative growth after tissue damage, including JNK signaling (Bergantiños et al., 2010; Bosch et al., 2010, 2005; Martín et al., 2017; Tasaki et al., 2011), JAK/STAT signaling (Bando et al., 2013; Katsuyama et al., 2015; Verghese and Su, 2017), EGFR signaling (Fan et al., 2014; Jiang et al., 2011; Jin et al., 2015; Nakamura et al., 2007), Hippo signaling (Bando et al., 2009; Grijalva et al., 2014; Grusche et al., 2011; Hayashi et al., 2014; Sun and Irvine, 2011), Wnt signaling (Hanovice et al., 2019; Hobmayer et al., 2000; Kawakami et al., 2006; McClure and Schubiger, 2008; Schubiger et al., 2010; Smith-Bolton et al., 2009; Wehner et al., 2014), and Myc (Harris et al., 2016; Smith-Bolton et al., 2009). Many of these mechanisms are also important during normal development, and the process of regeneration was traditionally thought to be a redeployment of earlier developmental steps (Bosch et al., 2010; Gupta et al., 2013; Harris et al., 2016; Mader and Cameron, 2004; Muneoka and Bryant, 1982; Nakamura et al., 2007; Roensch et al., 2013). However, recent evidence suggests that regeneration is not a simple reiteration of development but can employ regeneration-specific regulatory mechanisms (Bosch et al., 2010; Harris et al., 2016; Luttrell et al., 2016; McCusker and Gardiner, 2013; Myohara, 2004; Schuster and Smith-Bolton, 2015; Vizcaya-Molina et al., 2018). Indeed, faithful regeneration likely requires additional mechanisms, since regrowth happens in the presence of wound-response signaling and in a developed juvenile or adult organism. Additionally, pro-growth pathways that are used during normal development are often activated in new ways and at higher strengths in the regenerating tissue (Bergantiños et al., 2010; Grusche et al., 2011; Katsuyama et al.,

2015; Smith-Bolton et al., 2009). These augmented pro-growth signals must be constrained as regeneration progresses to prevent overgrowth and to enable re-establishment of pattern and cell-fate specification. Thus, growth suppressors and additional patterning factors are likely used to terminate regeneration and allow differentiation (Sun and Irvine, 2014). However, despite our understanding of the pro-growth signals needed for regeneration, we do not yet know what factors exist in different model organisms to restrain growth and promote re-patterning of regenerating tissue.

Drosophila melanogaster imaginal discs, precursors of adult fly appendages, are simple columnar epithelia that have well-characterized, complex expression of patterning genes that determine cell-fate specification. Imaginal discs undergo regeneration after damage (Hariharan and Serras, 2017), and we have previously used a genetic ablation system to study patterning in the regenerating tissue (Schuster and Smith-Bolton, 2015; Smith-Bolton et al., 2009). Here we identify the RNA-regulator Brain tumor (Brat) as a key constraint on regenerative growth that ensures proper formation of the regenerated structure. Brat is a member of the TRIM- (tripartite motif containing)-NHL (NCL-1, HT2A, and LIN-41) family of proteins and functions as a translational repressor by binding to its target RNAs either independently or in a complex with Pumilio and Nanos (Loedige et al., 2015, 2014; Sonoda and Wharton, 2001). It acts as a potent differentiation factor and tumor suppressor in neural and ovarian germline stem cell lineages (Arama et al., 2000; Betschinger et al., 2006; Harris et al., 2011; Lee et al., 2006). Human and mouse orthologs of Brat, TRIM3 and TRIM32 respectively, also possess tumor-suppressor

activity in glioblastomas and are required for neuronal and muscle differentiation (Chen et al., 2014; Kudryashova et al., 2012; Nicklas et al., 2012; Schwamborn et al., 2009).

We show that regenerating wing imaginal discs with reduced levels of Brat regenerate better than controls, but the resulting adult wings have a disrupted margin. The margin loses some of the characteristic sensory bristles and veins, demonstrating an error in cell-fate specification. Importantly, these phenotypes are regeneration-specific, as they are not observed in the mutant animals after normal development. The enhanced regeneration is due to increased expression of the growth regulators Myc and Wingless as well as upregulation of *ilp8*, which delays metamorphosis and allows the damaged tissue more time to regenerate. Intriguingly, this seemingly beneficial elevated Myc expression is also responsible for the aberrant cell-fate specification at the wing margin, through misregulation of downstream target genes, including Chronologically inappropriate morphogenesis (Chinmo). Hence, Brat acts as an important growth regulator and protective factor by constraining Myc and Chinmo levels during regeneration to prevent errors in patterning, cell-fate specification, and differentiation in the regenerating tissue.

Results

Brat suppresses regenerative growth and is required for wing margin cell-fate specification during regeneration

To identify genes important for regenerative growth and re-patterning, we performed a candidate screen, using our wing imaginal disc ablation system (Smith-Bolton et al., 2009). The primordial wing was targeted for ablation at the early third-instar larval stage by using *rotund-GAL4* to drive the expression of the proapoptotic gene *reaper* for 24 hours (Figure 1A). Our ability to restrict damage to 24 hours was provided by *tubulin-GAL80^{ts}*, which can inhibit GAL4 activity at 18°C, but allows GAL4-driven cell death at 30°C in the 24-hour window. The extent of wing imaginal disc regeneration in the larvae was reflected in the adult wing size. Hence, the resulting adult wings were scored based on size and patterning features to identify mutants that affect genes that are involved in regulating regenerative growth and establishment of cell fates. There is inherent variability in this system because of its sensitivity to environmental conditions such as temperature, humidity, and food quality, causing the results of different experiments to vary slightly (Brock et al., 2017; Khan et al., 2017; Skinner et al., 2015; Sun and Irvine, 2011; Vonesch et al., 2016). Animals with the same genotype within an experiment also showed some variation, due to stochastic differences in the time each animal takes to eclose, with animals that take longer to eclose having larger wings (Khan et al., 2017; Smith-Bolton et al., 2009). However, differences between control and mutant animals using this system are reproducible, consistent, and have identified key regeneration genes (Brock et al., 2017; Khan et al., 2017; Schuster and Smith-Bolton, 2015; Skinner et al., 2015).

Using this genetic ablation system, we identified the gene *brain tumor (brat)* as an important regulator of regenerative growth. *brat^{1/+}* mutants that did not experience

damage during development had adult wings that were not significantly different in size from controls (Figure S1A). However, after ablation and regeneration were induced, *brat*^{1/+} mutants showed enhanced regeneration and had adult wings that were, on average, much larger than controls that had also undergone regeneration (Figure 1B and 1C). We confirmed this enhanced regeneration phenotype in heterozygotes for three other *brat* mutant alleles: *brat*¹⁹², *brat*¹⁵⁰ (Luschnig et al., 2004) and *brat*¹¹ (Wright et al., 1981)(Figure S1B).

Interestingly, we also discovered a role for *brat* in cell-fate specification during regeneration. After normal development, *brat*^{1/+} mutants had adult wings that were patterned normally (Figure 1D-E and Figure S1C). To confirm that loss of *brat* does not cause patterning errors during normal development, we knocked down Brat levels in the entire wing pouch using *brat* RNAi, which resulted in adult wings that were patterned normally (Figure S1D and S1E). A previous study in which Brat levels were reduced in the anterior and posterior compartments of the wing also did not report any patterning defects (Ferreira et al., 2014). However, when discs were ablated and allowed to regenerate, *brat* heterozygous mutant wings showed aberrant patterning such that the wing margin lost sensory bristles and vein material (Figure 1F and 1G). By contrast, control regenerated wings lost margin tissue at a lower frequency (Figure 1H and 1I). Furthermore, the extent of margin tissue lost was not as severe in control regenerated wings as compared to *brat*^{1/+} regenerated wings (Figure 1H and 1I). Similar to the enhanced regeneration seen in *brat* mutants, we confirmed the loss-of-margin defect in heterozygotes for the additional three mutant alleles (Figure S1F).

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137 ***brat* regulates entry into metamorphosis**

138 Tissue damage in imaginal discs can induce a systemic response in the larvae, which
 139 extends the larval phase of development and delays pupariation (Halme et al., 2010;
 140 Smith-Bolton et al., 2009). This delay in pupariation is due to expression of the relaxin-
 141 like peptide *ilp8* in damaged discs (Colombani et al., 2012; Garelli et al., 2012). To
 142 determine whether *brat* mutants regenerated better due to an enhanced delay in
 143 pupariation, we measured rates of pupariation in control and mutant animals. We found
 144 that during normal development, control and *brat*^{1/+} animals pupariated at the same
 145 time, indicating that the two genotypes develop at similar rates (Figure S2A). After disc
 146 damage, *brat* mutants delayed pupariation an additional day compared to controls in
 147 which discs were also damaged (Figure 2A and Figure S2B). Note that direct
 148 comparisons cannot be made between regenerating larvae that spent 24 hours at 30°C
 149 (Figure 2A, Figure S2B) and normally developing larvae that remain at 18°C (Figure
 150 S2A), due to the effects of temperature on development. Our data show that *brat*^{1/+}
 151 mutants are able to stay in the larval stage even longer than controls, giving them more
 152 time to regenerate.

153

154 To determine why discs with reduced Brat had an increased delay in pupariation, we
 155 measured *ilp8* transcript levels through qPCR. Undamaged control animals express
 156 very low *ilp8* levels. However, after regeneration was induced, we saw an 80-fold
 157 increase in *ilp8* levels in controls, while the *brat*^{1/+} animals showed a 140-fold increase

(Figure 2B). Thus, *brat* suppresses *ilp8* during regeneration, regulating the timing of pupariation.

***brat* restricts growth and proliferation during regeneration**

Regenerative growth occurs through localized cell proliferation at the wound site (Abbott et al., 1981; Smith-Bolton et al., 2009). The proliferating cells, known as the blastema, give rise to the regenerated tissue. The blastema and the subsequent regenerated wing pouch can be labeled with the wing primordium marker Nubbin (Nub) (Ng et al., 1995). To determine whether *brat*^{1/+} discs regenerated better due to increased growth rates in the wing pouch, we measured the area of the Nub-expressing cells in control and *brat*^{1/+} regenerating discs. In the initial stages of regeneration, the control and mutant had similar Nub-expressing areas, indicating equal ablation and equal early regrowth. However, by 48 hours after tissue damage (recovery time 48, or R48), *brat*^{1/+} wing discs had a significantly bigger Nub-expressing pouch than the control (Figure 2D-F), indicating that *brat*^{1/+} mutants were regenerating faster than controls. To assess whether this difference in growth rates was due to differences in proliferation, we counted cells going through mitosis by quantifying Phospho-histone H3 (PH3)-positive nuclei in the regenerating blastema. Reduction of *brat* resulted in a significantly higher number of PH3-positive nuclei per area at R0, but this increased proliferation had subsided to normal levels by R24 (Figure 2G-I). To confirm that the increased number of PH3-positive nuclei were not simply due to an increased number of total cells in the blastema, we also counted total Nubbin-positive nuclei in the regenerating blastema to

calculate a ratio of proliferating cells to blastema cells (Figure S2C). These data confirm increased proliferation in *brat*^{1/+} regenerating discs at R0. Differences in proliferation early in regeneration often become evident later when measuring wing pouch area (Brock et al., 2017). Therefore, reduction of *brat* gives the regenerating tissue a growth advantage early in regeneration, resulting in a measurable difference in tissue area by R48.

Wingless (Wg) and Myc are regulators of regenerative growth and are upregulated at the wound site after damage (McClure and Schubiger, 2008; Schubiger et al., 2010; Smith-Bolton et al., 2009). Interestingly, Brat regulates stem cell differentiation in the brain by suppressing self-renewal signaling pathways such as Wnt signaling, and acting as a post-transcriptional inhibitor of Myc, to enable specification of progenitor cell fate (Betschinger et al., 2006; Komori et al., 2014). Additionally, Brat overexpression can suppress Myc at the post-transcriptional level in wing disc epithelial cells, although loss of *brat* does not lead to elevated Myc protein in wing discs during normal development (Ferreira et al., 2014). To determine whether these regulators of regenerative growth are upregulated in *brat*^{1/+} regenerating discs, we examined the expression of Wg and Myc. Wg is normally expressed along the Dorso-ventral (DV) boundary and in two concentric circles at the inner and outer edge of the wing pouch (Couso et al., 1993) (Figure 3A), and Myc is expressed in the wing pouch, but is repressed in the cells at the DV boundary as they undergo cell cycle and growth arrest (Wu and Johnston, 2010) (Figure 3B). Both Wg and Myc expression were comparable to controls in undamaged *brat*^{1/+} discs (Figure S3A-E). When damage is induced, Wg is upregulated throughout

the blastema by R0 (Smith-Bolton et al., 2009) (Figure 3C). Reduction of *brat* expression resulted in significantly higher levels of Wg expression at R0 (Figure 3D and 3E) but not at R24 (Figure 3F). After ablation, Myc expression is elevated in the regenerating tissue (Smith-Bolton et al., 2009) (Figure 3G and 3H). *brat*^{1/+} discs showed significantly higher levels of Myc at R0, which were sustained through R24 (Figure 3I-K). Thus, loss of *brat* caused an increase in the levels of both Wg and Myc early in regeneration. The elevated expression of these growth regulators likely explains the high proliferation seen in *brat*^{1/+} discs at R0, and the larger wing pouch at R48.

***brat* is required for margin cell-fate specification during regeneration**

Reduction of *brat* during regeneration caused patterning defects specifically at the wing margin, resulting in the loss of vein at the margin and loss of sensory bristles (Figure 1G). Thus, *brat* is required for correct cell-fate specification at the DV boundary during regeneration. The wing imaginal disc is divided into the dorsal and the ventral compartments, with expression of the LIM-homeodomain protein Apterous (Ap) in dorsal cells. The juxtaposition of the dorsal and ventral cells forms the DV boundary, which develops into the adult wing margin (Diaz-Benjumea and Cohen, 1993) (Figure 4A). Notch (N) and Wg signaling at the DV boundary are crucial for the correct organization and cell-fate specification at the boundary (Micchelli et al., 1997). *cut* (*ct*) and *achaete* (*ac*) are margin-specific genes that are expressed downstream of N and Wg signaling. *ct* is required for the specification of the wing margin, and *ac* specifies the

pro-neural sensory organ precursors (Becam and Milán, 2008; Micchelli et al., 1997) (Figure 4A).

To investigate whether the errors in fate specification seen in *brat*^{1/+} discs were due to a compromised compartment boundary, we examined the expression of Ap using the *ap-lacZ* reporter. *ap-lacZ* expression showed a clear DV boundary in the undamaged control discs (Figure 4B). The DV boundary remained intact after ablation in control and *brat*^{1/+} discs (Figure 4C-D, Figure S4A and S4B). *ap-lacZ* expression was also seen in the debris found in the damaged wing imaginal disc, due to the perdurance of β -gal. Wg expression was restored to its normal DV expression by R48 in both control and *brat*^{1/+} discs (Figure S4C and S4D). Therefore, the patterning defects were not caused by disruptions in the DV boundary or changes in Wg expression.

Next, we examined N signaling in *brat*^{1/+} discs due to its critical role in specifying fates at the DV boundary. We used a N signaling reporter, which uses *Notch Response Elements* (NREs) that bind to the Notch co-receptor Suppressor of Hairless, to drive the expression of GFP (Saj et al., 2010). No difference was detected in the expression of the N reporter for undamaged control and *brat*^{1/+} discs (Figure 4E and 4F). N signaling at the DV boundary was restored by R24 in controls and continued at R48 (Figure 4G and 4H). Note that the reporter signal can also be seen in cellular debris in the regenerating discs due to the perdurance of GFP. Interestingly, *brat*^{1/+} discs showed highly elevated levels of the N signaling reporter at both these time points (Figure 4I and

4J). This result is consistent with recent evidence demonstrating Brat's ability to attenuate N nuclear transport in the brain (Mukherjee et al., 2016). We wondered whether this elevated N signaling could also disrupt margin fates. However, overexpressing the N-intracellular domain in the wing pouch during the 24-hour ablation period (Figure S4E and S4F) resulted in adult wings that were patterned remarkably well, with significantly fewer wings showing any margin defects when compared to the control (Figure S4G). Thus, increased N activity during regeneration suppresses margin defects. Additionally, decreasing N signaling activity in *brat*^{1/+} regenerating discs by using a mutation in the *anterior pharynx defective 1 (aph-1)* gene was unable to rescue the loss of *brat* phenotype. *aph-1*^{D35/+} discs showed significantly reduced N signaling during normal development (Figure S4H-J) and at R24 during regeneration (Figure S4K-M), but could not rescue the loss of margin phenotype in the *brat* mutant (Figure S4N). Thus, while Brat constrains N signaling during regeneration, the elevated N signaling in *brat*^{1/+} mutants does not cause the margin cell-fate specification defects.

***brat* specifies margin fate by controlling the expression of Cut and Achaete**

To understand how patterning was disrupted in *brat*^{1/+} regenerating discs, we examined expression of margin cell-fate genes. Cut (Ct) expression was present along the DV boundary in both undamaged control and *brat*^{1/+} discs (Figure 4K and 4L), consistent with our results showing that adult undamaged *brat*^{1/+} wings do not have margin defects (Figure S1D). In control regenerating discs, Ct expression was detected at the DV boundary at R72, which is when regeneration and repatterning are largely complete

(Figure 4M). By contrast, Ct expression was either not observed in *brat*^{1/+} discs or was still missing in segments of the DV boundary at R72 (Figure 4N and 4O). These results indicate a specific error in cell-fate specification, as the DV boundary was intact at R72 (Figure S4A and S4B). Undamaged control and *brat*^{1/+} discs also showed appropriate Ac expression in two stripes of cell directly flanking the DV boundary in the anterior half of the disc (Figure 4P and 4Q). Ac expression was also detected in control regenerating discs at R72 (Figure 4R). While Ac-expressing cells appeared in *brat*^{1/+} discs, they were not clearly separated across the DV boundary (Figure 4S). This finding is consistent with previous reports showing that Ct suppresses Ac at the margin, and mutations in *ct* lead to aberrant expression of Ac at the DV boundary, followed by degeneration of the wing margin through cell death (Couso et al., 1994; Jack et al., 1991).

High Myc expression perturbs margin cell-fate specification during regeneration

Our results show that Brat both restricts regenerative growth and ensures correct cell-fate specification at the wing margin. Interestingly, JNK signaling in regenerating tissue can cause aberrant posterior-to-anterior cell-fate changes, which can be suppressed by a regeneration-specific protective factor, Taranis, to ensure correct patterning of the regenerating tissue (Schuster and Smith-Bolton, 2015). Therefore, we wondered whether unconstrained regenerative growth, or unconstrained expression of growth drivers, could also have deleterious side effects such as loss of margin cell fates. As Wg expression is normal during late regeneration and we have ruled out elevated N

signaling as the causative factor for the cell-fate errors that occurred in *brat*^{1/+} regenerating discs, we wondered whether high Myc levels caused by reduced Brat could cause the margin defects.

Brat overexpression can suppress Myc in wing imaginal disc cells (Ferreira et al., 2014), and in undamaged wing discs Brat protein levels were elevated at the DV boundary where Myc was reduced (Figure 5A and 5A'), suggesting that Brat may regulate Myc at the DV boundary. Furthermore, our results showed that regenerating *brat*^{1/+} mutant discs experienced elevated Myc levels compared to controls (Figure 3G-K). Previous studies demonstrated that Brat regulates Myc at the post-transcriptional level (Ferreira et al., 2014). To confirm that Brat is also regulating *myc* post-transcriptionally during regeneration, we measured *myc* transcript levels through qPCR. Regenerating discs showed significantly increased transcription of *myc* compared to undamaged controls. However, there was no significant difference in *myc* transcript levels between regenerating control and *brat*^{1/+} discs at R0 and R24 (Figure 5B and 5C), indicating that Brat's regulation of Myc must be post-transcriptional.

To test whether the elevated Myc protein levels could cause margin defects during regeneration and phenocopy the *brat* mutation, we overexpressed Myc in the wing pouch during the 24-hour ablation period. Myc was highly upregulated at R0 (Figure 5D-F), but Myc levels had returned to normal by R24 (Figure 5F). Overexpression of Myc also resulted in a significantly higher number of proliferating nuclei in the regenerating

tissue at R0, similar to *brat*^{1/+} discs (Figure 5G-I). Remarkably, we observed that adult wings resulting from Myc-overexpressing regenerating discs also showed margin defects similar to the *brat*^{1/+} wings (Figure 5J and 5K). Moreover, the frequency of margin defects in the adult wings resulting from Myc-overexpressing regenerating discs was even higher than in adult wings resulting from *brat*^{1/+} regenerating discs (Figure 5L), demonstrating that elevated levels of Myc alone can cause errors in margin cell-fate specification. Similar to *brat*^{1/+} discs, *ap-lacZ* expression showed that the compartment boundary was not compromised in Myc-overexpressing regenerating discs (Figure 5M and 5N). Likewise, Ct expression was missing in segments at the DV boundary as in the *brat*^{1/+} discs (Figure 5O and 5P).

Overexpressing Myc for a 24-hour window during normal development resulted in 3 adult wings out of 730 that showed any margin defects (Figure S5A). Even in these wings, only one segment of the margin was affected. To rule out the possibility that transient overexpression of Myc during development may not be sufficient to perturb patterning, we overexpressed Myc continuously after the animals entered the third instar larval stage. Continuous Myc overexpression proved to be lethal for many animals. While the flies that survived had significantly smaller wings, the margins showed no defect (Figure 5Q and 5R). 31 hours of Myc overexpression during normal development showed significantly higher Myc protein levels (Figure 5S-U) but did not interfere with Ct expression (Figure 5V and 5W). These data indicate that high Myc levels do not cause cell-fate specification errors during normal development, and the

extensive loss of wing margin induced by high Myc expression is a regeneration-specific phenotype.

We hypothesized that if the *brat* phenotype was due to elevated Myc levels, we would be able to rescue the phenotype by reducing Myc levels in the *brat* mutant. For this purpose, we used *dm⁴*, which is a null allele of Myc (Pierce et al., 2004). Surprisingly, we observed that the *dm⁴/+* mutants alone showed margin defects in the regenerated wings at a frequency similar to *brat¹/+*, even though the *dm⁴/+; brat¹/+* double mutant showed slightly reduced frequency of margin defects (Figure S5B). To confirm that Myc levels were reduced in the *dm⁴/+* mutants, we quantified Myc protein through immunostaining. We observed that there was no significant difference in Myc expression levels between the *dm⁴/+* mutant and control, both during development and regeneration (Figure S5C and S5D). Indeed, Myc levels were trending higher in the *dm⁴/+* discs during regeneration. The failure of the *dm⁴* mutation to reduce Myc levels could be due to compensatory expression of the functional copy of the Myc locus. We next tried reducing Myc levels through RNAi. Despite the RNAi expression being transient in our system, and only occurring in cells that survive ablation, RNAi-mediated persistent knockdown has worked for multiple genes, likely due to the shadow RNAi effect (Bosch et al., 2016). Two RNAi lines could significantly reduce Myc levels during normal development when expressed during early third instar (Figure S5E). However, when Myc RNAi was expressed during the 24-hour ablation period, Myc levels were not reduced at either R0 or R24, with one Myc RNAi line showing significantly higher levels of Myc compared to the control (Figure S5F). Thus, compensatory regulation of Myc

expression during regeneration prevented us from testing whether reduction in Myc could rescue the *brat*/+ phenotype.

Interestingly, animals that overexpressed Myc in the wing pouch during ablation did not undergo a regeneration-induced pupariation delay (Figure S5G), suggesting that Brat regulates the entry into metamorphosis independently of its regulation of Myc. Therefore, not all effects of loss of Brat are mediated through Myc.

Enhanced proliferation does not disrupt margin cell-fate specification during regeneration

Myc is an important driver of regenerative growth, and yet, we found that cell-fate specification during regeneration can be negatively affected if Myc levels are not constrained. To test whether the aberrant patterning was a specific result of high Myc levels or whether increases in growth and proliferation could, in general, cause margin defects, we also increased proliferation during regeneration by overexpressing both *cyclinE* (*cycE*) and *string* (*stg*).

Overexpressing the cell cycle genes in the wing imaginal disc during the 24-hour ablation period caused the resulting adult wings to be much larger than controls that had also undergone damage and regeneration (Figure 6A), though not larger than a normal wing. No significant regeneration-induced pupariation delay was seen, making

the enhanced regeneration even more remarkable (Figure 6B). Intriguingly, we did not observe many margin defects for wings that had experienced *cycE* and *stg* overexpression during regeneration (Figure 6C-E). Thus, pattern disruption during regeneration appears to be specifically associated with Myc overexpression and does not appear to be caused by increased proliferation alone.

Loss of cell-fate specification may be due to elevated expression of Chinmo

Given that driving growth by overexpressing Cyclin E and String does not cause loss of wing margin cell fates in regenerating tissue, this phenotype might be caused by misregulation of one or more targets of the Myc transcription factor. We have previously identified the gene *Chronologically inappropriate morphogenesis (chinmo)* as a novel regulator of regeneration (Khan et al., 2017). Chinmo is a transcription factor that regulates the balance between a proliferative self-renewal state and a differentiated state in stem cells (Dillard et al., 2017; Flaherty et al., 2010). Recent work has shown that *chinmo* also maintains wing epithelial cells in an unspecified state during development by inhibiting *ct* expression, and enhances regenerative potential (Narbonne-Reveau and Maurange, 2019). While *chinmo* mRNA can be a direct Brat target (Loedige et al., 2015), *chinmo* is regulated at the level of transcription in the wing imaginal disc (Narbonne-Reveau and Maurange, 2019). Therefore, we wondered whether *chinmo* could be misregulated downstream of Myc in the *brat*^{1/+} regenerating discs. Interestingly, the model organism Encyclopedia of Regulatory Networks (modERN) data show Myc binding near the *chinmo* promoter, supporting this

hypothesis (Kudron et al., 2017). Chinmo levels were not significantly different in undamaged control and *brat*^{1/+} discs (Figure 7A, 7B and 7E). However, Chinmo levels were significantly higher in *brat*^{1/+} regenerating discs compared to control regenerating discs at R24 (Figure 7C, 7D and 7F). Thus, the loss of *ct* expression and loss of margin cell fates in *brat*^{1/+} regenerating discs may be due, at least in part, to upregulation of *chinmo*.

To confirm regulation of *chinmo* downstream of Myc, we examined Chinmo levels in regenerating discs over-expressing Myc. Chinmo levels were elevated in Myc-overexpressing discs at R0, when Myc overexpression was the highest (Figure 5F and Figure 7G-I). However, Chinmo levels were restored to control levels by R24 in Myc-overexpressing discs, consistent with the return of Myc levels to normal at this time point (Figure 5F and Figure S6A-C). Interestingly, Myc and Chinmo expression almost perfectly co-localized, consistent with the hypothesis that Myc regulates Chinmo expression (Figure S6A-B'''). Additionally, we observed a high correlation between Myc and Chinmo expression levels in individual discs (Figure S6D and S6E). Undamaged discs overexpressing Myc did not show elevated Chinmo levels (Figure S6F-H), possibly explaining why Myc overexpression during normal development did not cause margin defects. Importantly, other unidentified Myc targets may also contribute to *ct* misregulation in regenerating discs with reduced Brat or overexpressed Myc.

Based on our findings, we propose a model in which pro-growth factors are important for coordinating regenerative growth, but can lead to deleterious side effects by perturbing cell-fate gene expression and patterning. Brat prevents a prolonged proliferative and unspecified state in regenerating wing discs by inhibiting Wg, Ilp8, Myc and Chinmo to enable cessation of growth, induction of cell-fate specification, and entry into metamorphosis (Figure 8).

Discussion

Here we have shown that Brat acts as a protective factor during regeneration by constraining levels of transcription factors such as Myc and Chinmo, which promote growth and proliferation but also inhibit cell-fate specification. If Brat is unable to perform its protective function during regeneration, Myc levels increase unchecked, resulting in misregulation of its targets, including Chinmo and subsequently Ct, causing loss of proper cell fates at the wing margin.

Myc is broadly used across organisms to promote proliferation and prevent differentiation (Amati and Land, 1994; Takahashi and Yamanaka, 2006), and Myc is strongly activated in the regenerating wing imaginal discs and is required for efficient regeneration. Importantly, increased Myc levels can enhance regeneration in both younger discs as well as mature discs that normally regenerate poorly (Harris et al., 2016; Smith-Bolton et al., 2009). Nevertheless, we have found that while these abnormally high Myc levels can enhance regenerative growth, they also perturb

differentiation by misregulating target genes such as Chinmo. Thus, enhanced regeneration happens at the expense of correct cell-fate specification.

Brat promotes differentiation in *Drosophila* larval neuroblasts and ovarian germline stem cells by asymmetrically segregating to one of the daughter cells where it post-transcriptionally inhibits Myc (Betschinger et al., 2006; Harris et al., 2011). This daughter cell is then able to differentiate while the other daughter cell remains a stem cell. In *brat* mutants, progeny of stem cells are unable to differentiate, resulting in an abnormal expansion of the stem-cell population, which can form tumors in the brain (Arama et al., 2000; Betschinger et al., 2006; Harris et al., 2011; Lee et al., 2006). Thus, Brat protects these tissues from overproliferation of stem cells. Importantly, wing imaginal disc regeneration is not stem-cell based, but in wing disc regeneration Brat also inhibits Myc to allow correct cell-fate specification. Based on these similarities in function, Brat likely acts as a protective factor across different biological contexts, including regeneration that does not employ stem cells.

We have previously shown that JNK signaling can induce posterior-to-anterior fate changes in regenerating wing discs, which can be prevented by the protective factor Taranis (Schuster and Smith-Bolton, 2015). We have now identified a second protective factor, Brat, which is needed specifically for correct patterning of the regenerating wing margin. Interestingly, while elevated JNK signaling causes anterior markers to appear in the posterior wing compartment, it does not cause margin loss, indicating that posterior

fate and margin fate are regulated in distinct ways (Schuster and Smith-Bolton, 2015). Protective factors such as Tara and Brat are important for maintaining the balance between fate specification and regenerative potential, but they do so by using very different mechanisms. While the molecular function of Tara is unknown, genetic interactions in *Drosophila* coupled with the demonstrated functions of its vertebrate homologs suggest it regulates gene expression at the level of transcription and chromatin (Calgaro et al., 2002; Hayashi et al., 2006; Hsu et al., 2001; Watanabe-Fukunaga et al., 2005). By contrast, Brat acts as a translational repressor, and suppresses its targets through mRNA degradation (Komori et al., 2018; Laver et al., 2015). Tara is required to prevent fate changes induced by JNK signaling, which is necessary for wound repair and regeneration but is not required for the normal development of the wing. By contrast, Myc is required for both development and regeneration of the wing disc, but is constrained by Brat only during regeneration.

An important open question in the field of regeneration is how patterning and cell-fate specification are regulated in regenerating tissue, and whether these mechanisms are different from the developmental program. Many studies have highlighted that regeneration must be distinct from development in some ways, because the damaged tissue already has complex patterning, and the wound-healing response causes strong activation of signaling pathways, some of which are not normally present in developing tissue (Bosch et al., 2010, 2005; Harris et al., 2016; Luttrell et al., 2016; McCusker and Gardiner, 2013; Myohara, 2004; Schuster and Smith-Bolton, 2015; Sun and Irvine, 2014; Vizcaya-Molina et al., 2018). We are just beginning to identify regulators like Brat

that are crucial for attenuating regenerative growth signaling and shielding the regenerating tissue from the harmful side effects of such signaling. Identification of these regulators highlights the fact that the regenerating tissue is distinct from normally developing tissue. Since regeneration signaling is complex and comprised of many signaling pathways, additional factors that play protective roles during regeneration likely exist. Identification of these factors will be important for the development of clinical therapies targeted at tissue repair, enabling these therapies to protect against the deleterious side effects of exogenous and unconstrained pro-growth signaling.

Materials and Methods

Ablation and Regeneration experiments

Ablation experiments were done as previously described (Schuster and Smith-Bolton, 2015). Briefly, cell death was induced by driving *UAS-reaper* under *rotund-GAL4*, with *GAL80^{ts}* for temporal control. Animals were raised at 18°C for 7 days after egg lay (AEL) (early third instar) before they were shifted to a 30°C circulating water bath for 24 hours. Animals were brought back to 18°C to allow regeneration. Wing discs were dissected at different time points after the end of ablation, or the animals were allowed to grow to adulthood to observe the adult wing phenotype. Undamaged control wing discs were the same genotype as the experimental animals but kept at 18°C and dissected on day 9 after egg lay, which is mid-late third instar. For undamaged adult wings, the animals were kept at 18°C until after eclosion. Any other undamaged conditions used are mentioned specifically in the figure legends.

510

511 Fly stocks

512 The following *Drosophila* stocks were used: *w¹¹¹⁸* (wild type)(Hazelrigg et al., 1984),
 513 *w¹¹¹⁸* ; *mGAL4*, *UAS-rpr*, *tubGAL80ts/TM6B*, *tubGAL80* (Smith-Bolton et al., 2009), *brat¹*
 514 (Wright et al., 1976)(FBst0003988), *brat¹⁹²* and *brat¹⁵⁰* (Luschnig et al., 2004)(a gift from
 515 Juergen Knoblich, Austrain Academy of Science), *brat¹¹* (Wright et al., 1981)(a gift from
 516 Chen-Yu Lee, University of Michigan), *Df(2L)Exel8040* (Parks et al.,
 517 2004)(FBst0007847), *Df(2L)TE37C-7* (Stathakis et al., 1995)(FBst0006089), *mGAL4*,
 518 *tubGAL80ts/TM6B* (Smith-Bolton et al., 2009), *P{Trip.HM05078}attP2* (called *bratRNAi*
 519 in the text)(FBst0028590), *P{CaryP}attP2* (called *attP2* control in the
 520 text)(FBst0036303), *{PZ}ap^{rK568}* (Cohen et al., 1992)(FBst0005374), *NRE-GFP* (Saj et
 521 al., 2010)(FBst0030727), *UAS-Nintra* (a gift from Gary Struhl, Columbia University),
 522 *aph-1^{D35}* (Littleton and Bellen, 1994)(FBst0063242), *UAS-Myc* (Johnston et al., 1999)
 523 (FBst0009674), *UAS-cycE*, *stg* (a gift from Laura Buttitta, University of Michigan), *dm⁴*
 524 (Pierce et al., 2004), *P{GD1419}v2947* (called *MycRNAi#1* in the text)(VDRC ID# 2947)
 525 and *P{GD1419}v2948* (called *MycRNAi#2* in the text)(VDRC ID# 2948),
 526 *P{GD6000}v15293* (called control in the text) (VDRC ID# 15293)(93). All fly stocks are
 527 available from the Bloomington Drosophila Stock Center unless stated otherwise.

528

529 Pupariation timing

530 Pupariation experiments were performed in a similar manner to the ablation
 531 experiments. Starting at day 9, newly formed pupal cases were counted in each vial.

Pupal cases were counted every 24 hours, up until day 15. Pupariation rates from three independent experiments were used to calculate the average plotted in the graphs.

Immunohistochemistry

Immunostaining was carried out as previously described (Smith-Bolton et al., 2009). Primary antibodies were rat anti-Brat (1:200) (Sonoda and Wharton, 2001) (a gift from Robin Wharton, Ohio State University), mouse anti-Nubbin (1:500) (Averof and Cohen, 1997) (a gift from Steve Cohen, University of Copenhagen), rabbit anti-Phospho-Histone H3 (1:500) (Millipore), mouse anti-Wingless (1:100) (The Developmental Studies Hybridoma Bank [DSHB]), rabbit anti-dMyc (1:500) (Santa Cruz Biotechnologies), mouse anti-βgal (1:100) (DSHB), mouse anti-Cut (1:10) (DSHB), mouse anti-Achaete (1:10)(DSHB), rat anti-Chinmo (1:500) (a gift from Nick Sokol, Indiana University). 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.

Secondary antibodies were AlexaFluor probes (1:1000) (Life Technologies). DNA was marked using TO-PRO3 (1:500) (Life Technologies) or DAPI (1:5000 of 0.5 mg/mL stock) (Sigma). Discs were mounted in Vectashield mounting medium (Vector Laboratories).

Discs were imaged on a Zeiss LSM 510 or a Zeiss LSM 700 confocal microscope. Parameters for imaging were identical for quantified images. Images were processed using ZEN lite (Zeiss), ImageJ (NIH) and Photoshop (Adobe). Maximum intensity projections were created for the confocal images. Fluorescence intensity was measured within the wing pouch as marked by anti-Nubbin or by using the morphology of the undamaged wing disc. Myc and Chinmo intensities were measure by outlining the region expressing elevated Myc or Chinmo levels. *NRE-GFP* intensity was measured by outlining the GFP-expressing region at the DV boundary.

Adult wing quantifications

Adult wings were mounted in Gary's Magic Mount (Canada balsam [Sigma] dissolved in methyl salicylate [Sigma]). Images were taken with an Olympus SZX10 microscope with an Olympus DP21 camera using the CellSens Dimension software (Olympus).

All adult wings that were 75% or 100% the size of a normal wing were used to quantify the loss of the wing margin. The wing margin was divided into five segments defined by where the wing veins intersect the margin. Each wing was scored for the number of segments with missing margin to assess the extent of the patterning defect.

Percentages from the three independent experiments were used to calculate averages plotted in the graphs. The area of undamaged and regenerated wings was measured using ImageJ (NIH). ImageJ was also used to measure the percentage of linear length of margin lost for the entire perimeter of the wing. Graphs were plotted using Excel and Graphpad Prism 7.

576

577 **qPCR**

578 For quantitative PCR (qPCR), 40-60 wing imaginal discs were collected in Schneider's
579 medium and stored at -80°C. RNA was extracted using the Qiagen RNeasy Mini Kit
580 (#74104), and cDNA synthesis was performed using the Superscript III First Strand
581 Synthesis kit (#11752-050). qPCR reactions using the Power SYBR Green MasterMix
582 (ABI) were run on the ABI Step One Plus Real Time PCR System. The experiment
583 consisted of 3 biological replicates. For each biological replicate there were three
584 technical replicates. Gene expression was analyzed by the $\Delta\Delta C_t$ method and
585 normalized to *Gapdh2* expression. The following primers were used: *Gapdh2* forward
586 primer (GTGAAGCTGATCTCTTGGTACGAC), *Gapdh2* reverse primer
587 (CCGCGCCCTAATCTTTAACTTTTAC) (Classen et al., 2009), *ilp8* primers used from
588 Qiagen (QT00510552), *dmyc* forward primer (AACGATATGGTGGACGATGG), and
589 *dmyc* reverse primer (CGGCAGATTGAAGTTATTGTAGC) (Mitchell et al., 2010). For
590 qPCR experiments undamaged controls were *rnGAL4*, *tubGAL80ts/TM6B* females
591 crossed to *w¹¹¹⁸* males and shifted to 30°C for 24 hours at 7 days AEL. Discs were
592 dissected either immediately or 24 hours after shifting the animals back to 18°C for R0
593 and R24 time points, respectively.

594

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Author Contribution Section

Experiments were designed by SNFA and RKSB, and conducted and analyzed by SNFA. The manuscript was written by SNFA and RKSB.

Competing interests

The authors declare no competing interests.

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Figure 1**A** **B** **C**

H

Frequency of margin defects after disc regeneration

Figure 1. Enhanced regenerative growth and wing margin cell-fate specification defects in *brat*^{1/+} during regeneration.

(A) The protocol used to study regeneration. Animals were raised at 18°C and shifted to 30°C for 24 hours during early third-instar larval development on day 7 after egg lay (AEL). Larvae were returned to 18°C and were dissected at the time points noted during recovery (R) or allowed to pupariate and eclose. Representative wings depicting the range of adult wing sizes observed after regeneration compared to the size of a normal wing are shown. (B) Adult wing sizes observed after disc regeneration for control (*w*¹¹¹⁸) (n = 317) and *brat*^{1/+} (n = 208) wings, from three independent experiments. (C) Adult wing area after disc regeneration, measured using ImageJ after mounting and imaging wings, for control (*w*¹¹¹⁸) (n = 309) and *brat*^{1/+} (n = 195) wings. p = 2.5158E-119. Wings in (C) are from the same experiments as (B). Note that number of wings in (C) is less for both control and *brat*^{1/+} due to some wings being damaged during the mounting process. (D) Undamaged control (*w*¹¹¹⁸) wing. (E) Undamaged *brat*^{1/+} wing. (F) Adult control (*w*¹¹¹⁸) wing after disc regeneration. (G) Adult *brat*^{1/+} wing after disc regeneration. (H) Frequency of margin defects seen in adult wings after disc regeneration for control (*w*¹¹¹⁸) (n = 93) and *brat*^{1/+} (n = 218) wings, from three independent experiments. The wing margin was divided into five segments based on where the veins intersect the margin as shown in the diagram. Each wing was scored for the number of segments that had some margin tissue missing, with wings with a perfectly intact margin scoring at zero. Wing shown in (G) had tissue missing in four segments. (I) Margin tissue lost as a percentage of total wing perimeter for control (*w*¹¹¹⁸) (n = 76) and *brat*^{1/+} (n = 221) wings. p = 9.947E-08. The margin perimeter and

the length of margin tissue lost were measured using ImageJ after mounting and imaging wings. Wings in (I) are from the same experiments as (H). Note that number of wings in the two quantifications is different because we did not quantify wings with length <1.1 mm for males and <1.7 mm for females, to ensure analysis was being carried out on nearly fully regenerated wings. (I). Percentage of wings with no defects fell from 79.0% to 77.6% for control and from 56.3% to 53.8% for *brat¹/+* wings due to the increased ability to detect lost margin tissue at the higher magnification and resolution achieved by imaging the wings. Wing shown in (G) had 33.9% of margin tissue missing. Error bars mark standard error of the mean (SEM). Student's T-test used for statistical analyses. Scale bars are 0.5 mm.

Figure 2

Figure 2. *brat*^{1/+} animals have a regenerative growth advantage.

(A) Pupariation rates after disc regeneration for control (*w*¹¹¹⁸) (n = 384) and *brat*^{1/+} (n = 107) animals, from three independent experiments. (B) Relative expression levels of *ilp8* for undamaged control, R24 control (*w*¹¹¹⁸) and R24 *brat*^{1/+} discs. (C) Anti-Nubbin immunostaining in an undamaged control disc. (D-E) Anti-Nubbin immunostaining in an R48 control (*w*¹¹¹⁸) disc (D), and an R48 *brat*^{1/+} disc (E). (F) Quantification of area of Nubbin-expressing cells for control (*w*¹¹¹⁸) and *brat*^{1/+} discs at R0 (n = 10 and 10), R24 (n = 12 and 12) and R48 (n = 10 and 10). * p < 0.03. (G-H) Anti-PH3 immunostaining in an R0 control (*w*¹¹¹⁸) disc (G), and an R0 *brat*^{1/+} disc (H). The yellow dashed lines outline the Nubbin-expressing wing pouch. (I) PH3-positive nuclei were counted within the regenerating tissue as marked by Anti-Nubbin co-immunostaining. Quantification of PH3-positive nuclei in Nubbin area for control (*w*¹¹¹⁸) and *brat*^{1/+} discs at R0 (n = 16 and 18) and R24 (n = 15 and 16). ** p < 0.002. Error bars represent SEM. Student's T-test used for statistical analyses. Scale bars are 100 μm.

Figure 3

Figure 3. *brat*^{1/+} animals experience elevated regeneration signaling.

(A) Anti-Wg immunostaining in an undamaged control (*w*¹¹¹⁸) disc. (B) Anti-Myc immunostaining in an undamaged control (*w*¹¹¹⁸) disc. (C-D) Anti-Wg immunostaining in an R0 control (*w*¹¹¹⁸) disc (C) and an R0 *brat*^{1/+} disc (D). (E) Quantification of Wg fluorescence intensity in R0 control (*w*¹¹¹⁸) (n = 13) and R0 *brat*^{1/+} (n = 17) discs. *** p < 0.0006. (F) Quantification of Wg fluorescence intensity in R24 control (*w*¹¹¹⁸) (n = 12) and R24 *brat*^{1/+} (n = 11) discs. Area for fluorescence intensity measurement was defined by the Wg expression domain in the wing pouch. (G-J) Anti-Myc immunostaining in an R0 control (*w*¹¹¹⁸) disc (G), an R24 control (*w*¹¹¹⁸) disc (H), an R0 *brat*^{1/+} disc (I) and an R24 *brat*^{1/+} disc (J). (K) Quantification of Myc fluorescence intensity in R0 control (*w*¹¹¹⁸) (n = 13), R0 *brat*^{1/+} (n = 12), R24 control (*w*¹¹¹⁸) (n = 13), and R24 *brat*^{1/+} (n = 12) discs. Area for fluorescence intensity measurement was defined by the elevated Myc expression domain in the wing pouch. R0 *** p < 0.0003, R24 *** p < 0.0001. Error bars represent SEM. Student's T-test used for statistical analyses. Scale bars are 100 μm.

Figure 4

Figure 4. *Brat* regulates margin cell-fate specification.

(A) Drawings of a wing imaginal disc and an adult wing. D = dorsal and V = ventral compartments of the wing disc, with the dorsoventral boundary marked in purple. Notch signaling and Cut expression are present at the dorsoventral boundary, which forms the adult wing margin, also marked in purple. Achaete-expressing cells, marked in green, give rise to the sensory bristles at the anterior half of the margin in the adult wing, also marked in green. (B) *ap-lacZ* expression in an undamaged control disc from a third-instar *ap-lacZ/CyO* animal. (C-D) *ap-lacZ* expression in an R24 control (*w¹¹¹⁸*) disc (C) and an R24 *brat^{1/+}* disc (D). (E-F) *NRE-GFP* expression in an undamaged control (*w¹¹¹⁸*) disc (E) and an undamaged *brat^{1/+}* disc (F). (G-J) *NRE-GFP* expression in an R0 control (*w¹¹¹⁸*) disc (G), an R24 control (*w¹¹¹⁸*) disc (H), an R0 *brat^{1/+}* disc (I) and an R24 *brat^{1/+}* disc (J). (K-L) Anti-Ct immunostaining in an undamaged control (*w¹¹¹⁸*) disc (K) and an undamaged *brat^{1/+}* disc (L). (M-O) Anti-Ct immunostaining in an R72 control (*w¹¹¹⁸*) disc (M) and an R72 *brat^{1/+}* discs (N-O). Arrowheads point to loss of Ct expression in (O). (P-Q) Anti-Ac immunostaining in an undamaged control (*w¹¹¹⁸*) disc (P) and an undamaged *brat^{1/+}* disc (Q). (R-S) Anti-Ac immunostaining in an R72 control (*w¹¹¹⁸*) disc (R) and an R72 *brat^{1/+}* disc (S). Scale bars are 100 μ m.

Figure 5

Figure 5. High Myc expression causes margin defects.

(A-A') Anti-Myc and Anti-Brat co-immunostaining in an undamaged control disc.

mGAL4, GAL80^{ts}/attP2 animals were shifted to 30°C on day 7 AEL and dissected 24 hours later. (B-C) Relative expression levels of *myc* for undamaged control, regenerating control (*w¹¹¹⁸*), and regenerating *brat^{1/+}* discs at R0 (B) and R24 (C). P values for comparison between regenerating control and *brat^{1/+}* discs: $p > 0.1$ at R0, and $p > 0.3$ at R24. (D-E) Anti-Myc immunostaining in an R0 control (*w¹¹¹⁸*) disc (D) and an R0 *UAS-Myc/+* disc (E). (F) Quantification of Myc fluorescence intensity in R0 control (*w¹¹¹⁸*) ($n = 13$), R0 *UAS-Myc/+* ($n = 12$), R24 control (*w¹¹¹⁸*) ($n = 13$), and R24 *UAS-Myc/+* ($n = 12$) discs. Area for fluorescence intensity measurement was defined by the elevated Myc expression domain in the wing pouch. *** $p = 1.2E-11$. (G-H) Anti-PH3 immunostaining in an R0 control (*w¹¹¹⁸*) disc (G), and an R0 *UAS-Myc/+* disc (H). The yellow dashed lines outline the Nubbin-expressing wing pouch. (I) PH3-positive nuclei were counted within the regenerating wing pouch as marked by Anti-Nubbin co-immunostaining. Quantification of PH3-positive nuclei in the Nubbin area for R0 control (*w¹¹¹⁸*) ($n = 15$) and *UAS-Myc/+* ($n = 15$) discs. *** $p < 0.00002$. (J) Adult control (*w¹¹¹⁸*) wing after disc regeneration. (K) Adult *UAS-Myc/+* wing after disc regeneration. (L) Frequency of margin defects, as quantified in Figure 1H, seen in adult wings after disc regeneration for control (*w¹¹¹⁸*) ($n = 134$), *brat^{1/+}* ($n = 193$) and *UAS-Myc/+* ($n = 200$) wings, from three independent experiments. (M-N) *ap-lacZ* expression in an R24 control (*w¹¹¹⁸*) disc (M) and an R24 *UAS-Myc/+* disc (N). (O-P) Anti-Ct immunostaining in an R72 control (*w¹¹¹⁸*) disc (O) and an R72 *UAS-Myc/+* disc (P). (Q) Adult undamaged control (+; *mGAL4, GAL80^{ts}/+*) wing from animals shifted to 30°C on day 7 AEL and

maintained at 30°C until eclosion. (R) Adult wing from discs continuously overexpressing Myc from day 7 AEL onwards (*UAS-Myc/+; rnGAL4, GAL80ts/+*). (S) Quantification of Myc fluorescence intensity in undamaged discs dissected 31 hours after animals were shifted to 30°C on day 7 AEL. Control (+; *rnGAL4, GAL80ts/+*) (n = 14) and *UAS-Myc* (*UAS-Myc/+; rnGAL4, GAL80ts/+*) (n = 14). Area for fluorescence intensity measurement was defined by the Myc expression domain in the wing pouch. *** p = 1.2E-11. (T-U) Anti-Myc immunostaining in an undamaged control disc (T) and an undamaged *UAS-Myc/+* disc (U). (V-W) Anti-Ct immunostaining in an undamaged control disc (V) and an undamaged *UAS-Myc/+* disc (W). Error bars represent SEM. Student's T-test used for statistical analyses. Scale bars are 100 µm. Scale bars for adult wings are 0.5 mm.

Figure 6

Figure 6. Overexpression of cell cycle genes does not cause patterning defects.

(A) Adult wing sizes observed after disc regeneration for control (w^{1118}) (n = 280) and *UAS-cycE*, *UAS-stg/+* (n = 194) wings, from three independent experiments. (B) Pupariation rates after disc regeneration for control (w^{1118}) (n = 174) and *UAS-cycE*, *UAS-stg/+* (n = 115) wings, from three independent experiments. (C) Adult control (w^{1118}) wing after disc regeneration. (D) Adult *UAS-cycE*, *UAS-stg/+* wing after disc regeneration. (E) Frequency of margin defects seen in adult wings after disc regeneration for control (w^{1118}) (n = 118), *UAS-Myc/+* (n = 146), and *UAS-cycE*, *UAS-stg/+* (n = 188) wings, from three independent experiments. Error bars represent SEM. Scale bars for adult wings are 0.5 mm.

Figure 7

Figure 7. Chinmo levels are elevated in *brat*^{1/+} and Myc-overexpressing regenerating discs.

(A-B) Anti-Chinmo immunostaining in an undamaged control (*w*¹¹¹⁸) disc (A) and an undamaged *brat*^{1/+} disc (B). (C-D) Anti-Chinmo immunostaining in an R24 control (*w*¹¹¹⁸) disc (C) and an R24 *brat*^{1/+} disc (D). (E) Quantification of Chinmo fluorescence intensity in undamaged control (*w*¹¹¹⁸) (n = 11) and undamaged *brat*^{1/+} (n = 10) discs. (F) Quantification of Chinmo fluorescence intensity in R24 control (*w*¹¹¹⁸) (n = 13) and R24 *brat*^{1/+} (n = 14) discs. p < 0.006. Area for fluorescence intensity measurement was defined by the elevated Chinmo expression domain in the wing pouch. (G) Merge of anti-Nubbin, anti-Myc and anti-Chinmo immunostaining in an R0 control (*w*¹¹¹⁸) disc. (G'-G'') Same disc as (G) showing anti-Myc and anti-Chinmo immunostaining, respectively. (H) Merge of anti-Nubbin, anti-Myc and anti-Chinmo immunostaining in an R0 *brat*^{1/+} disc. (H'-H'') Same disc as (H) showing anti-Myc and anti-Chinmo immunostaining, respectively. (I) Quantification of Chinmo fluorescence intensity in R0 control (*w*¹¹¹⁸) (n = 10) and R0 *UAS-Myc*⁺ (n = 11) discs. p < 0.02. Area for fluorescence intensity measurement was defined by the elevated Myc expression domain in the wing pouch. Error bars represent SEM. Student's T-test used for statistical analyses. Scale bars are 100 μm.

Figure 8

Figure 8. Brat restricts pro-regeneration factors and ensures correct margin cell-fate specification.

Model describing the network of Brat targets in the regenerating wing imaginal disc. Importantly, Brat restricts Myc levels, limiting expression of Myc's targets, including Chinmo, to allow correct margin cell-fate specification.

Figure S1

Figure S1. Loss of *brat* does not cause enhanced growth or margin defects during normal development (Related to Figure 1).

(A) Adult wing area measured using ImageJ after mounting and imaging wings, for undamaged control (w^{1118}) (n = 63 female and 70 male) and $brat^1/+$ (n = 38 female and 48 male) wings. *rnGAL4*, *GAL80^{ts}/TM6B* females were crossed to w^{1118} or $brat^1/SM6-TM6B$ males and taken through the protocol shown in Figure 1A. (B) Adult wing sizes after disc regeneration for control (w^{1118}) (n = 599), $brat^1/+$ (n = 199), $brat^{192}/+$ (n = 237), $brat^{150}/+$ (n = 235) and $brat^{11}/+$ (n = 188) wings, from three independent experiments. (C) Margin defects detected in adult wings from undamaged control (w^{1118}) and $brat^1/+$ discs. *rnGAL4*, *GAL80^{ts}/TM6B* females were crossed to w^{1118} or $brat^1/SM6-TM6B$ males and taken through the protocol shown in Figure 1A. Margin defects detected in the undamaged wings were never as severe as the ones seen in $brat^1/+$ wings after disc regeneration. A representative wing with margin defects is shown. (D) Anti-Brat immunostaining in undamaged control (*attP2*) and *bratRNAi/+* discs. *rnGAL4*, *GAL80^{ts}/TM6B* females were crossed to *attP2* or *bratRNAi* males. Larvae were kept at 18°C and shifted to 30°C on day 7 AEL. Discs were dissected 24 hours after the shift to 30°C. Quantification of Brat fluorescence intensity in undamaged control (*attP2*) (n = 15) and *bratRNAi/+* (n = 15) discs. Area for fluorescence intensity measurement was defined by wing pouch morphology and Anti-Myc co-immunostaining. * p = 0.02. (E) Margin defects detected in adult wings from undamaged control (*attP2*) and *bratRNAi* discs. *rnGAL4*, *GAL80^{ts}/TM6B* females were crossed to *attP2* or *bratRNAi* males. Larvae were kept at 18°C and shifted to 30°C on day 7 AEL and kept there until eclosion. (F) Frequency of margin defects seen in adult wings after disc regeneration for control

(*w¹¹¹⁸*) (n = 240), *brat¹/+* (n = 191), *brat¹⁹²/+* (n = 196), *brat¹⁵⁰/+* (n = 213) and *brat¹¹/+* (n = 152) wings. Wings in (G) are from the same experiments as (B). Error bars represent SEM. Student's T-test used for statistical analyses. Scale bars are 100 μ m. Scale bars for adult wings are 0.5 mm.

Figure S2

Figure S2. Loss of *brat* delays pupariation in a regeneration-specific manner
(Related to Figure 2).

(A) Pupariation rates in undamaged control (w^{1118}) (n = 221) and *brat*^{1/+} (n = 110) animals, from three independent experiments. (B) Pupariation rates after disc regeneration for control (w^{1118}) (n = 384), *brat*^{1/+} (n = 107), *brat*^{192/+} (n = 131), *brat*^{150/+} (n = 114) and *brat*^{11/+} (n = 113) animals. Pupariation rates are from the same experiments as in Figure 2A. (C) PH3-positive nuclei were counted within the regenerating tissue as marked by Anti-Nubbin co-immunostaining. Total number of nuclei were also counted in the Nubbin expressing region. Ratio of PH3-positive nuclei and total Nubbin-positive nuclei for control (w^{1118}) and *brat*^{1/+} discs at R0 (n = 16 and 18). *** p < 0.0005. Error bars represent SEM. Student's T-test used for statistical analyses. Error bars represent SEM.

Figure S3

Figure S3. Effects of loss of *brat* on Wg and Myc expression are regeneration-specific (Related to Figure 3).

(A-B) Anti-Wg immunostaining in an undamaged control (w^{1118}) disc (A) and an undamaged *brat*^{1/+} disc (B). (C-D) Anti-Myc immunostaining in an undamaged control (w^{1118}) disc (C) and an undamaged *brat*^{1/+} disc (D). (E) Quantification of Myc fluorescence intensity in undamaged control (w^{1118}) (n = 10) and *brat*^{1/+} (n = 10) discs. Area for fluorescence intensity measurement was defined by wing pouch morphology and the elevated Myc expression domain in the wing pouch. Error bars represent SEM. Student's T-test used for statistical analyses. Scale bars are 100 μ m.

Figure S4

Figure S4. Elevated Notch signaling does not cause margin defects (Related to Figure 4).

(A-B) *ap-lacZ* expression in an R72 control (*w¹¹¹⁸*) disc (A) and an R72 *brat^{1/+}* disc (B). Dashed yellow lines are drawn next to the DV boundary to highlight it. (C-D) Anti-Wg immunostaining in an R48 control (*w¹¹¹⁸*) disc (C) and an R48 *brat^{1/+}* disc (D). (E-F) *NRE-GFP* expression in an R24 control (*w¹¹¹⁸*) disc (E) and an R24 *UAS-Nintra/+* disc (F). (G) Frequency of margin defects seen in adult wings after disc regeneration for control (*w¹¹¹⁸*) (n = 84) and *UAS-Nintra/+* (n = 357) wings, from five independent experiments. (H-I) *NRE-GFP* expression in an undamaged control (*w¹¹¹⁸*) disc (H) and an undamaged *aph-1^{D35/+}* disc (I). *NRE-GFP/+* and *NRE-GFP/aph-1^{D35}* animals were raised at room temperature and dissected during third instar. (J) Quantification of GFP intensity in undamaged control (*w¹¹¹⁸*) (n = 15) and *aph-1^{D35/+}* (n = 15) discs. *** p < 0.0006. (K-L) *NRE-GFP* expression in an R24 control (*w¹¹¹⁸*) disc (K) and an R24 *aph-1^{D35/+}* disc (L). (M) Quantification of GFP intensity in R24 control (*w¹¹¹⁸*) (n = 13) and R24 *aph-1^{D35/+}* (n = 11) discs. * p < 0.02. (N) Frequency of margin defects in adult wings after disc regeneration for control (*w¹¹¹⁸*) (n = 21), *brat^{1/+}* (n = 137), *aph-1^{D35/+}* (n = 38) and *brat¹/aph-1^{D35}* (n = 80) wings. Error bars represent SEM. Student's T-test used for statistical analyses. Scale bars are 100 μ m.

Figure S5

Figure S5. Compensatory regulation prevents reduction of Myc expression during regeneration (Related to Figure 5).

(A) Margin defects detected in adult wings from undamaged control (w^{1118}) and *UAS-Myc/+* discs. *rnGAL4*, *GAL80^{ts}/TM6B* females were crossed to w^{1118} or *UAS-Myc* males and taken through the protocol shown in Figure 1A. (B) Frequency of margin defects in adult wings after disc regeneration for control (w^{1118}) (n = 103), *brat¹/+* (n = 203), *dm⁴/+* (n = 94) and *dm⁴/+; brat¹/+* (n = 94) wings, from three independent experiments. (C) Quantification of Myc fluorescence intensity in undamaged control (w^{1118}) (n = 12) and *dm⁴/+* (n = 11) discs. w^{1118} females were crossed to w^{1118} or *dm⁴/FM7i*, *ActGFP* males and dissected when the animals were third instar. Area for fluorescence intensity measurement was defined by wing pouch morphology and the elevated Myc expression domain in the wing pouch. (D) Quantification of Myc fluorescence intensity in R0 control (w^{1118}) (n = 13), R0 *dm⁴/+* (n = 10), R24 control (w^{1118}) (n = 13), and R24 *dm⁴/+* (n = 10) discs. Area for fluorescence intensity measurement was defined by the elevated Myc expression domain in the wing pouch. (E) Quantification of Myc fluorescence intensity in undamaged control (VDRC genetic background line, called control) (n = 14), *MycRNAi#1/+* (n = 12), and *MycRNAi#2/+* (n = 13) discs. *rnGAL4*, *GAL80^{ts}/TM6B* females were crossed to the control, *MycRNAi#1*, or *MycRNAi#2* males. The animals were shifted to 30°C during early third instar and kept there for 28 hours then dissected. *MycRNAi#1/+* *** p < 0.000007, *MycRNAi#2/+* *** p < 0.00002. Area for fluorescence intensity measurement was defined by wing pouch morphology. (F) Quantification of Myc fluorescence intensity in R0 control (n = 13), R0 *MycRNAi#1/+* (n = 15), R0 *MycRNAi#2/+* (n = 13), R24 control (n = 13), R24 *MycRNAi#1/+* (n = 13), and R24

MycRNAi#2/+ (n = 13) discs. Fluorescence intensity was measured in the area marked by Anti-Nubbin immunostaining. *** p < 0.00007. (G) Pupariation rates after disc regeneration for control (*w¹¹¹⁸*) (n = 216), *brat¹/+* (n = 114) and *UAS-Myc/+* (n = 209) animals, from three independent experiments. Error bars represent SEM. Student's T-test used for statistical analyses.

Figure S6

Figure S6. Myc regulates Chinmo expression (Related to Figure 7).

(A) Merge of anti-Nubbin, anti-Myc and anti-Chinmo immunostaining in an R24 control (w^{1118}) disc. (A'-A'') Same disc as (A) showing anti-Myc and anti-Chinmo immunostaining, respectively. (A''') Same disc as (A) showing an enlarged merge of anti-Myc and anti-Chinmo immunostaining. (B) Merge of anti-Nubbin, anti-Myc and anti-Chinmo immunostaining in an R24 *UAS-Myc/+* disc. (B'-B'') Same disc as (B) showing anti-Myc and anti-Chinmo immunostaining, respectively. (B''') Same disc as (B) showing an enlarged merge of anti-Myc and anti-Chinmo immunostaining. (C) Quantification of Chinmo and Myc fluorescence intensity in R24 control (w^{1118}) ($n = 13$) and R24 *UAS-Myc/+* ($n = 14$) discs. Area for fluorescence intensity measurement was defined by the elevated Myc expression domain in the wing pouch. Note that Myc and Chinmo expression co-localize. (D-E) Scatter plot showing correlation between Myc and Chinmo expression levels at R0 (D) and R24 (E). Pearson correlation coefficient for R0 = 0.93 and R24 = 0.94. (F) Quantification of Chinmo fluorescence intensity in undamaged discs dissected 31 hours after animals were shifted to 30°C on day 7 AEL. Control (+;*rnGAL4, GAL80ts/+*) ($n = 14$) and *UAS-Myc (UAS-Myc/+; rnGAL4, GAL80ts/+)* ($n = 14$). Area for fluorescence intensity measurement was defined by the Myc expression domain in the wing pouch. (G-H) Anti-Chinmo immunostaining in an undamaged control disc (G) and an undamaged *UAS-Myc/+* disc (H). Error bars represent SEM. Student's T-test used for statistical analyses. Scale bars are 100 μm , unless stated otherwise.