Next-Generation Sequencing Reveals Increased Anti-oxidant Response and Ecdysone Signaling in STAT Supercompetitors in Drosophila

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ABSTRACT Cell competition is the elimination of one viable population of cells (the losers) by a neighboring fitter population (the winners) and was discovered by studies in the Drosophila melanogaster wing imaginal disc. Supercompetition is a process in which cells with elevated JAK/STAT signaling or increased Myc become winners and outcompete wild-type neighbors. To identify the genes that are differentially regulated in STAT supercompetitors, we purified these cells from Drosophila wing imaginal discs and performed next-generation sequencing. Their transcriptome was compared to those of control wing disc cells and Myc supercompetitors. Bioinformatics revealed that STAT and Myc supercompetitors have distinct transcriptomes with only 41 common differentially regulated genes. Furthermore, STAT supercompetitors have elevated reactive oxygen species, an anti-oxidant response and increased ecdysone signaling. Using a combination of methods, we validated 13 differentially expressed genes. These data sets will be useful resources to the community.

Competitive interactions between cells are ubiquitous, and the resolution of such interactions regulates a broad range of biological processes (Amoyel and Bach 2014; Johnston 2014; Claveria and Torres 2016; Baker 2017; Nagata and Igaki 2018). Cell competition was discovered by studies in developing epithelia of Drosophila (Morata and Ripoll 1975; Simpson 1979; Simpson and Morata 1981). Animals harboring mutations in ribosomal genes were viable in a homotypic environment but were eliminated when grown in a heterotypic environment with more robust wild-type cells. The eliminated cells were referred to as ‘losers’, and the cells that outcompete them were termed ‘winners’. This context-dependent elimination of a viable cell population was termed ‘cell competition’. Since these pioneering studies, additional types of competitive interactions have been reported. These include the context-dependent elimination of viable cells with decreased metabolism or signal transduction, or with aberrant polarity (Moreno et al. 2002; Brunmy and Richardson 2003; Pagliarini and Xu 2003; Del Cova et al. 2004; Moreno and Basler 2004; Igaki et al. 2006; Tyler et al. 2007; Neto-Silva et al. 2010; Tamori et al. 2010; Ziosi et al. 2010; Ohsawa et al. 2011; Vincent et al. 2011; Rodrigues et al. 2012; Schroeder et al. 2013). Importantly, cell competition is conserved in mammals and is triggered by differences in common factors like Myc. In mammals, competitive interactions between cells occur during embryogenesis and in adulthood and are important in both regenerative and homeostatic processes (Oliver et al. 2004; Claveria et al. 2013; Sancho et al. 2013; Martins et al. 2014; Villa Del Campo et al. 2014; Villa Del Campo et al. 2016; Diaz-Diaz et al. 2017; Liu et al. 2019).

Wild-type cells can become losers and be eliminated when confronted by cells with elevated activity or levels of certain proto-oncogenic pathways, including JAK/STAT, Myc, Wingless (Wg)/Wnt or Yorkie (Yki)/YAP (De La Cova et al. 2004; Moreno and Basler 2004; Neto-Silva et al. 2010; Ziosi et al. 2010; Vincent et al. 2011; Rodrigues et al. 2012). The elimination of wild-type cells by cells with higher levels of proto-oncogenic factors has been termed “supercompetition”. Winners eliminate less fit cells through direct contact and by the production of short-range soluble factors that kill losers at a distance (De La Cova et al. 2004; Li and Baker 2007; Martin et al. 2009; Ohsawa et al. 2011; Rodrigues et al. 2012; Ballesteros-Arias et al. 2014; Meyer et al. 2014; Kucinski et al. 2017; Yamamoto et al. 2017;

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Alpar et al. 2018). While the identities of these latter soluble factors are largely unknown, recent work from the Johnston lab has shown that Myc supercompetitors secrete serine proteases to create a local burst of active Spätzle (Spz), triggering Toll signaling and consequently apoptosis in less fit neighbors (Alpar et al. 2018). However, it is not clear whether other kinds of supercompetitors eliminate wild-type cells through a similar mechanism.

We previously reported that clones with higher levels of JAK/STAT signaling (termed STAT supercompetitors) eliminated neighboring wild-type cells by non-autonomously inducing hid-dependent apoptosis (Rodrigues et al. 2012). To gain insights into how STAT supercompetitors acquire their competitive advantages, we performed next generation sequencing on FACS-purified STAT supercompetitors and compared their transcriptome to that of FACS-purified Myc supercompetitors and FACS-purified control cells from wing imaginal discs. Analysis of these data sets reveal 1004 genes (P < 0.05) that were differentially regulated in STAT supercompetitors, including known JAK/STAT target genes Socs36E, chimino and domeless (dome) (Flaherty et al. 2009; Flaherty et al. 2010; Herrera and Bach 2019). Additionally, 328 genes (P < 0.05) were differentially regulated in Myc supercompetitors, including known Myc targets Nop60b, nop5 and Tif-1A (Grewal et al. 2005). There was limited overlap between these data sets with only 41 genes differentially regulated in both STAT and Myc supercompetitors, 24 upregulated in both conditions and 17 downregulated in both conditions. Of the differentially regulated genes in STAT supercompetitors, 210 had STAT binding sites in regulatory regions, suggesting that they could be directly regulated by JAK/STAT signaling. These include known JAK/STAT target genes, Socs36E, chimino and dome, as well as several in the edysone signaling pathway, including the Edysone receptor (EcR) and its targets Edysone-induced protein 75B (Eip75B), fitz-f1, and Edysone-inducible gene E1 (ImpE1). We validated 13 upregulated genes, 10 of which were increased only in STAT supercompetitors and 3 of which were upregulated in both STAT and Myc supercompetitors. Finally, we established a quantitative assay for supercompetition that can be used in future studies to test the functional significance of differentially regulated genes.

**MATERIALS AND METHODS**

**Fly Stocks**

We used dpp-gal4, UAS-gfp/ TM6B, Tb (a gift of Laura Johnston, Columbia University Medical Center, NY, USA), UAS-hop and UAS-Myc for FACS. We crossed y, w, act > y+w > gal4, UAS-gfp to y, w, hs-flp122, UAS-Dcr-2; +/+ to make GFP flip-out (GFP FO) clones or to y, w, hs-flp122; UAS-Dcr-2; UAS-hop/TM6B to make Hop flip-out (Hop FO) clones. We used PBac[cnc-EGFP.S]VK00037 (Bloomington Drosophila Stock Center (BDSC, BL-38631) to monitor endogenous expression of Nh2 (Drosophila Cap-n-collar (Cnc)). We used UAS-Stat92E_HM50035 RNAi (BDSC, BL-33637) (termed Stat-i) to deplete Stat92E from GFP FO or Hop FO clones in the cell competition assay (see below). We maintained crosses at 25° on standard food and in a 12-hour light/dark incubator.

**Time to pupariation**

To determine the time to pupariation, we used a protocol published by the Léopold lab (Colombani et al. 2015). We made 4-hour embryo collections from the cross dpp-gal4, UAS-gfp/ TM6B, Tb x Orc6 and the cross dpp-gal4, UAS-gfp/ TM6B, Tb x UAS-hop/ TM6B, Tb. We collected first instar larvae at 24 hr after egg deposition (AED) and reared 30 larvae per vial on standard food at 25°. At 90 hr AED, we monitored the time to pupariation every 6 hr. We calculated the average time to pupariation and the standard error of the mean for 30 dpp-gal4, UAS-gfp/+ and 30 dpp-gal4, UAS-gfp/UAS-hop larvae using Excel.

**Flow cytometry and RNA isolation**

We crossed dpp-gal4, UAS-gfp/TM6B, Tb to UAS-hop or to UAS-Myc. From non-Tb larvae, we dissect at least 60 third instar wing discs per genotype in triplicate at approximately 110-115 hr AED. The cells were dissociated and the GFP-positive cells were sorted by the Cytometry and Cell Sorting Core at NYU Langone Medical Center using a Sony SY3200 cell sorter per the protocol described in (De La Cruz and Edgar 2008). The sorted cells represent GFP-positive control cells from the dpp domain (referred to as “GFP” samples), GFP-positive cells from the dpp domain that had ectopic JAK/STAT signaling as a result of mis-expressing Hop (referred to as “Hop” samples) or GFP-positive cells from the dpp domain that had elevated Myc levels as a result of mis-expressing Myc (referred to as “Myc” samples). We isolated RNA from the sorted cells using TRIzol reagent (Ambion) and purified the RNA using RNeasy Mini Kit (Qiagen) per the manufacturer’s instructions.

**Quantitative PCR (qPCR)**

We performed qPCR using the SYBR Green PCR Mix (Applied Biosystems) protocol and a real-time PCR machine (ABI 7900HT) from Applied Biosystems. We isolated RNA as described above and synthesized cDNA using the SuperScript Reverse Transcriptase II kit (Invitrogen) per the manufacturer’s instructions. We measured the cDNA concentration using a Nanodrop ND-1000. We used 3 ng of cDNA per sample per reaction, 5 μM of each primer, and 1x SYBR. We performed the qPCR in triplicates per primer per sample. We normalized to tubulin (β-tub56d). The data were graphed using Excel, and statistical significance was determined using Student’s t-test in Excel. We used the following primers:

- Socs36F: GCTGCCGACGCAATATGTT and R: GACTGGCG-CAGCAAATGT
- dome F: CGGACCTTTCCGCTACTCCATC and R: GATCGATCATCGGAGATT
- Tif-1A F: GTAGCCGAGAACAGCAGAAGG and R: AATGCGAC-ATGATGGCTGTT
- β-tub56d: F: CTGAGTGCCTCGATGTTGCTCC and R: GCCAAGG-GAAGTGTTGAGATT

**RNA-seq**

The RNA sequencing was performed by the Genome Technology Center at the NYU Langone Medical Center. 10 ng of total RNA was used for library prep, and cDNA was amplified by using Nugen Ovation RNA-Seq System V2 kit (Part No. 7102-32), 100 ng of Covaris-fragmented cDNA were used as input to prepare the libraries, using the Ovation Ultralow Library system (Nugen, Part 0330-32), and amplified by 10 cycles of PCR. The samples were mixed into two pools and run in two 50-nucleotide paired-end read rapid run flow cell lanes on the Illumina HiSeq 2500 sequencer.

**Bioinformatics**

Bioinformatic analysis was performed by the Applied Bioinformatics Laboratories at NYU Langone Medical Center. Sequencing results were demultiplexed and converted to FASTQ format using Illumina Bcl2FastQ software. Reads were aligned to the dm6 release of the Drosophila melanogaster genome using the splice-aware STAR aligner. PCR duplicates were removed using the Picard toolkit (http://broadinstitute.github.io/picard). The HTSeq package (Love et al. 2014) was utilized to generate counts for each gene based on how many
aligned reads overlap its exons. These counts were then used to test for differential expression using negative binomial generalized linear models implemented by the DESeq2 R package (Anders et al. 2015). The adjusted p-value (padj) was generated by using the False Discovery Rate with the Benjamini-Hochberg method. Scatter (Volcano) plots were generated using Stata 15.1 (StataCorp LLC, College Station TX). For Table S1, we chose a cut-off at fold change $\geq 1.5$ (corresponding to log$_2$(fold change) 0.58) and an adjusted p-value $< 0.05$ because this data set would include known JAK/STAT target genes. For Table S2, we then decided to reciprocally apply this cut-off (fold change $\leq 0.667$ corresponding to log$_2$(fold change) -0.58) and an adjusted p-value $< 0.05$ to the downregulated genes. We then applied these cut-offs for differentially-regulated genes in the Myc supercompetitor RNA-seq in Table S2.

**Genome-wide analysis of Stat92E binding sites**

We obtained a positional weight matrix (PWM) for Stat92E from Jaspar (http://jaspar.genereg.net/matrix/MA0532.1) (Khan et al. 2018). We used a web-based program PWMScan (https://ccg.epfl.ch/pwmtools/pwmscan.php (Ambrosini et al. 2018)) to search the Drosophila genome for Stat92E binding sites that matched PWM with a stringent p value less than 1x10$^{-5}$ (recommended by developers of the PWMScan website (Ambrosini et al. 2018)). We then compared the list of locations of Stat92E binding sites with the list of genes and their locations (https://genome.ucsc.edu/cgi-bin/hgTables?command=start). We report in Tables S5 and S6 differentially regulated genes in STAT supercompetitors with at least one Stat92E binding site in non-coding regions, defined as 1,500 bps upstream of the transcription start site, introns, and 1,500 bps downstream of the termination sequence.

**Riboprobe synthesis**

We used these EST clones from the Drosophila Genomics Resource Center (DGRC) for riboprobe synthesis: hop (RH47993); Socs36E (SD04308); Ama (LD39923); dilp8 (IP06570); Mmp1 (RE62222); sas (LD44801); edl (LD15796); ftz-f1 (LD15303); ImpEl (IP15635); mld (SD03914); Mpcp2 (RE67391); mmd (LD25376). RNA probes were designed against the contiguous cDNA sequence of differentially expressed genes. The DGRC probes were synthesized using 1-5 µg of linearized plasmid in a 20 µl transcription reaction mix. We used a digoxigenin (DIG)-labeling kit (Roche) per the manufacturer’s instructions. The resulting labeled riboprobes were ethanol precipitated and resuspended in 100 µl of hybridization buffer (HB4) containing 50% formamide, 5x saline sodium citrate (SSC), 50 µg/ml heparin, 0.1% Tween-20 and 5 mg/ml of Tortula Yeast RNA extract.

**in situ hybridization**

Wandering, mid-third instar wing discs were dissected in cold 1x phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 20 min. They were subsequently washed three times in 1x PBS 0.1% Tween 20 (1xBST-T) for 10 min, rehydrated in decreasing concentrations of methanol and treated with 10 µg/ml proteinase K for 5 min. They were then fixed in 4% paraformaldehyde for 20 min, washed in 1xPBS-T, treated with acetylation solution (9.25 g triethanolamine HCL + 1.12 ml 10N NaOH in 500 ml H2O + 12.5 µl acetic anhydride) for 10 min and prehybridized for 1 hr at 65° in HB4. The discs were hybridized overnight in 100 µl of HB4 and 1 µl of the riboprobe that had already been denatured at 80° for 10 min in HB4 and then put on ice. After hybridization, the discs were washed two times for 25 min in a buffer containing 50% formamide, 50% 2x SSC with 0.1% Tween-20. They were rinsed in 1x PBS-T at room temperature three times for 10 min. Subsequently, they were incubated for 2 hr with anti-DIG (Roche; diluted 1:2000) and then washed three times for 10 min in 1x PBS-T. After this, they were rinsed once and washed for 5 min in alkaline phosphate buffer pH 9.5 containing 0.1 M NaCl, 0.05 M MgCl$_2$, 0.1 M Tris (pH 9.5) and 0.1% Tween-20. The reaction was developed by adding 40 µl of NBT/BCIP stock solution to 2 ml of 1x PBS.

**Antibody staining, ROS detection and TUNEL**

Immunofluorescence was performed as described in (Ekas et al. 2006). We used rabbit anti-Stat92E (1:500, (Flaherty et al. 2010)), rabbit anti-Dcp-1 (1:100) (Cell Signaling), rabbit anti-GFP (1:500) (Invitrogen), mouse anti- Patched Apal (1:10) (Development Studies Hybridoma Bank (DSHB)), mouse anti-Ptp10D 8B22F5 (1:5) (DSHB), Alexa647 Phalloidin (Invitrogen), fluorescent secondary antibodies at 1:250 (Jackson Laboratories), and Vectashield (Vector labs). We monitored ROS using CellROX Deep Red Reagent (Invitrogen) and followed the protocol in (Santabárbara-Ruiz et al. 2015). Briefly, we dissected third instar wing discs in Schneider’s medium containing 5 µM CellROX Deep Red Reagent and incubated the discs for 15 min, followed by three washes in Schneider’s medium. We then immediately analyzed the samples on a Zeiss LSM 510 confocal microscope at 25x. The samples were protected from light throughout the experiment. For terminal desynucleotidy transferase dUTP nick end labeling (TUNEL), we first stained with the pSTAT primary antibody and then with Cy5 Donkey anti-Rabbit secondary (1:250, Jackson Immunochemicals). After washing the fluorescent secondary antibody in 1x PBS, we prepared the TUNEL reaction (Roche # 12156792910) by adding the enzyme solution to label solution in a 1:10 dilution, with enough volume prepared to load 50 µl per sample. The solution was mixed well and kept on ice. We added 30 µl of label solution alone to the negative control. We added 50 µl of TUNEL reaction mixture to each sample tube. We incubated the negative control and the experimental samples at 37° for 1 hr. We then rinsed all samples twice with 1x PBS for 1 min. The samples were then mounted in Vectashield. We collected fluorescent images at 25x magnification using a Zeiss LSM 510 confocal microscope.

**Quantitative assay for supercompetition**

We made 4-hour timed embryo collections. Clones expressing GFP alone (labeled GFP flip-out (FO)) or GFP and Hop (labeled Hop FO) were randomly induced by hs-flp for 10 min at 48 hr AED. Wing imaginal discs were dissected at 72 hr after clone induction, and they were fixed, stained and imaged as described above. We used Image J to outline the flip-out clones and then to draw a second line at a distance of 10-cell diameters from the clone boundary using these values: 512 pixels = 509.12µm; 1 cell = 5µm; 1cell = 5pixels. We then counted the number of apoptotic (Dcp-1-positive) cells within the area delimited by the two lines. At least 15 clones per genotype were analyzed. The data were graphed using Excel, and statistical significance was determined using Student’s t-test in Excel.

**Data availability statement**

Strains and plasmids are available upon request. We obtained a PWM for Stat92E from Jaspar (http://jaspar.genereg.net/matrix/MA0532.1) (Khan et al. 2018). We used a web-based program PWMScan (https://ccg.epfl.ch/pwmtools/pwmscan.php (Ambrosini et al. 2018)) to search the Drosophila genome for Stat92E binding sites. Table S1 contains the list of genes that are differentially up- or downregulated (fold change 1.5 for upregulated genes, p-value < 0.05 and 0.667 for downregulated genes, p-value < 0.05) in STAT supercompetitors. Table S2 contains the list of genes that are differentially up- or downregulated
RESULTS

Ectopic activation of the JAK/STAT pathway induces cell competition

Cell competition is induced in the Drosophila wing disc when neighboring populations differ in levels of JAK/STAT activity (Rodrigues et al. 2012). To identify JAK/STAT pathway targets that may regulate cell competition, we carried out RNA-seq analysis of winners with elevated Stat92E activity, with elevated Myc, or GFP control cells (Figure 1A). We induced cell competition in the anterior compartment of the wing disc by mis-expressing the Drosophila Janus Kinase, Hopscotch (Hop), using UAS-hop and dpp-gal4, UAS-gfp transgenes in the anterior midline of the disc (Figure 1B,D). Ectopic mis-expression of Hop autonomously activates STAT, as evidenced by stabilized Stat92E (labeled “pSTAT”) in blue. Scale bar indicates 50 μM. (E) Quantitative PCR analysis of RNA isolated from FACS-purified, dpp-domain wing cells reveals that JAK/STAT targets dome (P < 0.05) and Socs36E (P < 0.05) are significantly increased in dpp > hop samples (purple) but not dpp > Myc samples (gray) compared to control dpp > gfp (blue) and that the Myc target Tif-1A (P < 0.1) is significantly increased in dpp > Myc samples (gray) but not in dpp > hop samples (purple) compared to controls (blue). The results were averages of 4 independent biological replicates. * P < 0.05; “ns” means not significant. (F) Principal component analysis for gfp (blue), hop (purple) and Myc (gray) triplicate samples. Genotypes (C) w/w, +/+; dpp-gal4, UAS-gfp/+ (D) w/w, +/+; dpp-gal4, UAS-gfp/UAS-hop (E,F) w/w, +/+; dpp-gal4, UAS-gfp/+ (gfp), w/w, +/+; dpp-gal4, UAS-gfp/UAS-Myc (Myc).

Figure 1 STAT supercompetitors outcompete wild-type neighbors. (A) Work-flow of the RNA-seq. Briefly, we purified GFP-positive cells from dpp > gfp (control), dpp > gfp+hop (STAT supercompetitors) or dpp > gfp+Myc (Myc supercompetitors) wing discs. We isolated total RNA from these cells and generated cDNA libraries, which were used for the RNA-seq. The reads were mapped to the Drosophila genome (dm6). 1004 genes were differentially expressed in dpp > gfp+hop cells compared to dpp > gfp cells, with 487 upregulated and 517 downregulated. (B) Cartoon of a third instar wing imaginal disc. The dpp expression domain (green stripe) resides within the anterior compartment (blue area). (C-D) In control dpp > gfp discs, few cells were undergoing programmed cell death (C, red cells) in either the anterior or posterior compartment, and Stat92E is not ectopically upregulated in the dpp domain (C’, white). By contrast, in dpp > gfp+hop discs, there were substantially more apoptotic cells in the anterior compartment (D, red cells), due to the competitive stress inflicted by STAT winners residing in the dpp stripe. The ectopic expression of Hop in the dpp domain ectopically activates Stat92E (D’, white). GFP is in green, TUNEL marking apoptotic cells is in red; activated Stat92E (labeled “pSTAT”) is in blue. Scale bar indicates 50 μM.
1.86 fold (calcium-activated potassium channel upregulated genes in STAT supercompetitors were CG30428 (Figure 2A and Table S1). We chose to impose the same fold change (1.5 fold, \( P < 0.0925 \)). Taken together, these results indicate that these transcriptome datasets accurately captures the expression profiles of JAK/STAT activation in STAT supercompetitors and of Myc mis-expression in Myc supercompetitors. There were only 41 genes differentially regulated in both types of supercompetitors, with 24 genes upregulated in both (Table S3), including Mpcp2, and 17 downregulated in both (Table S4).

We analyzed the differentially regulated genes in STAT supercompetitors for the presence of a Stat92E binding site in non-coding regions (see Materials and Methods). 133 significantly upregulated genes (fold change \( \geq 1.5 \) and \( P < 0.05 \)) in STAT supercompetitors had at least one Stat92E binding site (Table S5). These include established JAK/STAT target genes Sox36E with 8 binding sites and chinmo with 6 binding sites, as well as Jr38 with 2 sites. Jr38 has not been previously implicated as a possible JAK/STAT target. Of the differentially downregulated genes, 77 had at least one Stat92E binding site, including pnr (Table S6).

**STAT induces supercompetition by mechanisms distinct from other winners**

We surveyed the differentially regulated genes for factors known to regulate winner function in various types of cell competition. Wg supercompetitors secrete Notum, a conserved secreted feedback inhibitor of Wg signaling (Vincent et al. 2011). However, *notum* transcripts are not significantly altered in STAT winners (Table 2). Myc supercompetitors upregulate expression of *spz*, which encodes a Toll ligand, and *Spaetzle-Processing Enzyme (SPE)* and *modular serine protease* (*modSP*), which encode serine proteases that cleave Spz protein into an active form (Alpar et al. 2018). Cleaved Spz then triggers Toll signaling in losers, which activates NFKB proteins that induce apoptosis. STAT supercompetitors did not have an increase in *spz* genes (*spz*, *spz3*, *spz4*, *spz6*, SPE or *modSP* (Table 2). In polarity-deficient competition, the serine protease inhibitor Serpin 5 (Snp5) is required in wild-type winners to eliminate *scrib*-deficient cells (Katsukawa et al. 2018). Mechanistically, Snp5 prevents the cleavage of Spz into the active form. In the absence of Snp5 secreted from wild-type winners, active Spz is produced and it triggers the growth (not death) of *scrib*-mutant cells via Toll signaling. [Note that this is the opposite result from the role of Spz-Toll in Myc supercompetition. ] Snp5 is not upregulated in STAT winners (Table 2).

Wild-type winners also eliminate polarity-deficient neighbors by the Sas-Ptp10D system (Yamamoto et al. 2017) and Pvr-dependent engulfment (Ohawa et al. 2011). Stranded at second (Sas) is a transmembrane protein that acts as a ligand for the transmembrane phosphatase Ptp10D (Schonbaum et al. 1992; Lee et al. 2013). Wild-type winners require *sas* to eliminate polarity-deficient losers (Yamamoto et al. 2017). At the interface between wild-type winners and *scrib*-mutant cells, both Sas and Ptp10D relocalize from the apical domain to the lateral domain (Yamamoto et al. 2017). *sas* transcripts are significantly upregulated in STAT supercompetitors (1.5 fold, \( P < 0.00345 \)).
Table 1 Expression of differentially upregulated genes in STAT supercompetitors, in Myc supercompetitors, or in both STAT and Myc supercompetitors

| Gene   | FC (hop vs. gfp) | p-val value | FC (Myc vs. gfp) | p-val value |
|--------|-----------------|-------------|-----------------|-------------|
| hop    | 16.57 | 4.73 x 10^{-180} | 0.93 | 0.821 |
| Socs36E | 1.88 | 4.59 x 10^{-6} | 0.80 | 0.346 |
| chinmo | 4.74 | 5.44 x 10^{-23} | 1.48 | 0.0925 |
| dome   | 1.74 | 1.77 x 10^{-6} | 0.88 | 0.600 |
| zfh2   | 1.86 | 2.13 x 10^{-10} | 0.87 | 0.420 |
| Ama    | 2.31 | 4.11 x 10^{-6} | 0.74 | 0.283 |
| dipβ   | 3.91 | 8.01 x 10^{-13} | 1.50 | 0.182 |
| Mmp1   | 1.78 | 0.00254 | 0.86 | 0.704 |
| ftz-f1 | 1.59 | 0.00345 | 1.09 | 0.822 |
| edl    | 2.10 | 0.00210 | 1.29 | 0.556 |
| fti-1  | 1.82 | 0.00345 | 1.40 | 0.234 |
| ImpE1  | 1.65 | 0.0246 | 0.78 | 0.472 |
| Eip75B | 1.36 | 0.0130 | 0.82 | 0.217 |
| Hr38   | 2.42 | 2.89 x 10^{-9} | 1.14 | 0.737 |
| EcR    | 1.46 | 0.0417 | 1.06 | 0.906 |
| Mpcp2  | 1.50 | 0.00172 | 2.02 | 1.04 x 10^{-9} |
| mnd    | 1.59 | 0.0103 | 1.82 | 0.000432 |
| mid    | 1.62 | 4.10 x 10^{-4} | 1.52 | 0.00272 |
| Nop60B | 1.35 | 0.0306 | 1.86 | 1.11 x 10^{-7} |
| myc    | 1.32 | 0.161 | 2.46 | 2.79 x 10^{-9} |
| nop5   | 1.21 | 0.218 | 1.45 | 0.00448 |
| Tif-IA | 1.11 | 0.698 | 1.47 | 0.0210 |
| betaTub56D | 1.22 | 0.210 | 1.00 | 0.993 |

Legend: FC means “fold change”. p-val value is the adjusted p-value.

but not in Myc supercompetitors (Table 1), and the sas gene has one Stat92E binding site (Table S5). We used in situ hybridization to monitor sas transcripts in dpp > hop discs compared to dpp > gfp controls. sas mRNA is expressed at low levels in control wing discs, with the exception of a couple of patches at the notum-hinge interface (Figure 3A). sas mRNA is upregulated in STAT supercompetitors residing in the hinge and pouch (Figure 3B, arrow). In both control and dpp > hop discs, Ptp10D protein is localized to the apical domain as expected (Figure 3C,D). Importantly, in dpp > hop discs Ptp10D is not localized to the lateral interface between STAT winners and wild-type losers (Figure 3E), suggesting that the Sas-Ptp10D system does not function in JAK/STAT-dependent cell competition. In polarity-deficient competition, wild-type winners also upregulate Pvr, the Drosophila PDGF/VEGF receptor. However, Pvr is not changed in STAT winners compared to controls (Table 2). Taken together, these observations suggest that STAT supercompetitors are distinct from other kinds of winners.

STAT supercompetitors upregulate Nrf2

Our results indicate that STAT winners reside in an oxidizing environment caused by ROS production. We hypothesize that to protect themselves from this environment, STAT winners must upregulate a mild anti-oxidant response. Nrf2 (called Cap-n-collar (Cnc) in Drosophila) is a transcription factor that regulates numerous genes controlling oxidative homeostasis (Ma 2013). Under basal conditions, Nrf2 is sequestered in the cytoplasm through physical interactions with Keap1, which promotes Nrf2’s proteasomal degradation. Oxidants activate Nrf2 by modifying critical cysteine thiols on Keap1. This liberates Nrf2 to translocate to the nucleus, bind to anti-oxidant response elements and induce target gene expression, including Glutathione S-transferases (GSTs), the main cytosolic reducing agents (Taguchi et al. 2011; Sies et al. 2017). STAT winners have a transcriptional signature of an anti-oxidant response with a moderate but significant increase in cnc (1.30 fold, P < 0.0618) and a moderate but significant decrease in Keap1 (0.678 fold and P < 0.00726) (Table 3). These transcriptional changes should increase Nrf2 protein and decrease its inhibitor, resulting in a protective anti-oxidant response. Additionally, numerous Nrf2 target genes are significantly increased in STAT winners compared to controls (Table 3), include those encoding six cytosolic GSTs, two UDP-glucosyltransferases (Ugt), which reduce hydrophilic molecules, and three cytochrome P450s (Cyp), which reduce a large variety of substrates (Bock 2003; Coon 2005). To validate the increased cnc expression in STAT winners, we mis-expressed UAS-hop in the ptc domain in a genetic background that carried a bacterial artificial chromosome containing cnc under the control of endogenous regulatory elements C-terminally tagged with gfp. We find that Cnc-GFP is upregulated in STAT winners (Figure 4D, brackets).
most strongly in the dorsal and ventral hinge, which are the sites of highest endogenous JAK/STAT signaling in third instar wing discs (Bach et al. 2007; Ayala-Camargo et al. 2013). By contrast, Cnc-GFP is not observed in control ptc> discs (Figure 4C).

Ecdysone signaling is upregulated in STAT supercompetitors

Of the genes in Flybase that have been reported to part of the ecdysone pathway (Ihry and Bashirullah 2014; Jiang et al. 2018), 14 are differentially regulated in STAT supercompetitors. This group includes these upregulated genes, Eip75B, ftz-f1, EcR, ImpE1, Cyp18a1, Ecdysone Importer (EcI) (Flybase: Oatp74D), Iswi, swi2, E(bx), hid and rpr, and these downregulated genes, Blimp-1, let-7-C and DopEcR (Tables 4 and S1). Interestingly, several of these genes have STAT binding sites including ImpE1, ftz-f1 and Blimp-1 with 1 site each (Tables S5 and S6). Additionally, Eip75B has 4 Stat92E binding sites and EcR has 1, but neither was included in Table S5 because the fold change was below the 1.5 fold cut-off for upregulated genes. STAT winners could have increased ecdysone signaling compared to control dpp > gfp cells because the gene encoding the transporter required for ecdysone uptake EcI is differentially upregulated 1.68 fold (P < 7.1 x 10^-5) in STAT supercompetitors (Tables 4 and S1 and (Okamoto et al. 2018)). Since the EcI locus does not contain STAT binding sites, it remains unclear how EcI is upregulated in STAT winners, but it may be an indirect target.

We used in situ hybridization to validate some ecdysone pathway genes upregulated in STAT supercompetitors. As a proof of principle, we first assessed the expression pattern of hop (the mis-expressed gene in the STAT RNA-seq) and Socs36E, the best characterized JAK/STAT target gene (Bach et al. 2007). hop mRNA was expressed at low levels in control wing discs (Figure 5A) and, as predicted, was upregulated along the entire dpp domain in dpp > hop discs (Figure 5B, arrow). Socs36E mRNA is restricted to the presumptive hinge domain in control discs (Figure 5C). In dpp > hop discs, Socs36E mRNA is ectopically induced along the dpp stripe (Figure 5D, arrow).

Having proven the efficacy of in situ for validating differentially expressed genes in the RNA-seq, we next turned our attention to some ecdysone pathway genes. Ftz-f1 is a nuclear hormone receptor expressed normally at high levels in mid-prepupal stages, when it acts as a critical competence factor for the response to the late pupal ecdysone pulse (Woodard et al. 1994; Broadus et al. 1999). ftz-f1 is significantly upregulated in STAT winners (1.83 fold, P < 0.00345) but not in Myc winners (Table 1), and, as noted above, ftz-f1 has 1 Stat92E binding site (Table S5). Consistent with its induction in mid-pupariation, ftz-f1 mRNA is expressed at low levels in control third instar wing discs (Figure 5E). It is upregulated in STAT supercompetitors along most of the dpp stripe (Figure 5F, arrow). An early ecdysone response gene ImpE1 encodes a protein similar to a low-density lipoprotein receptor (Natzle et al. 1988; Natzle 1993). ImpE1 is upregulated 1.6 fold (P < 0.0246) in STAT supercompetitors but not in Myc supercompetitors (Table 1), and the gene has 1 Stat92E binding site (Table S5). ImpE1 mRNA is expressed in several distinct patches in a control third instar wing disc (Figure 5G), and it is upregulated in STAT supercompetitors located in the dorsal hinge in dpp > hop discs (Figure 5H, arrow).

### Table 2 Genes upregulated in other winners are not differentially expressed in STAT supercompetitors

| Gene     | Fold Change | Adjusted p-value |
|----------|-------------|------------------|
| notum    | 1.31        | 0.244            |
| spz      | 0.79        | 0.504            |
| spz3     | 1.04        | 0.884            |
| spz4     | 0.90        | 0.829            |
| SPE      | 1.03        | 0.952            |
| modSP    | 1.08        | 0.813            |
| Spn5     | 1.23        | 0.185            |
| Pvr      | 0.96        | 0.855            |

**Figure 2** Volcano plots of gene expression in STAT and Myc supercompetitors. (A,B) Scatter (Volcano) plot for genes in STAT supercompetitors (dpp > hop) compared to controls (dpp > gfp) in A and for genes in Myc supercompetitors (dpp > Myc) compared to controls (dpp > gfp) in B. The x-axis is the log_{2} of the fold change and the y-axis is the negative log_{10} of the adjusted p-value. Gray circles indicate genes with log_{2}(fold change) between -0.58 and 0.58 (corresponding to fold change between 0.667 and 1.5). Blue circles indicate genes with log_{2}(fold change) < -0.58 or > 0.58 (corresponding to fold change < 0.667 or > 1.5). The larger blue circles indicate the misexpressed genes (hop in A and Myc in B), known target genes (chinmo, zfh2, Socs36E, pnr in A and Nop60B in B) or highly differentially-regulated genes in the data sets (SK, CR46123, mth8, and CG30428 for A and Pdp1, ND-13A, Mpcps2 and Ec3 in B). Genotypes (A) w/w, +/++; dpp-gal4, UAS-gfp/UAS-hop (B) w/w, +/++; dpp-gal4, UAS-gfp/UAS-Myc.
cell-cell-adhesion (Seeger et al. 1988; Fremion et al. 2000; Zeev-Ben-Mordehai et al. 2009; Özkan et al. 2013). It is significantly upregulated (2.32 fold, \(4.11 \times 10^{-11}\)) in STAT supercompetitors but not in Myc supercompetitors (Table 1). Ama mRNA is observed in numerous discrete domains in the presumptive hinge and notum in control discs (Figure 5I). It is induced in the dpp domain in dpp > hop discs (Figure 5J, arrows). Drosophila insulin-like peptide 8 (Dilp8, FlybaseIlp8) is a relaxin-like protein that controls developmental timing by regulating the release of ecdysone by neuroendocrine cells in the brain (Colombani et al. 2012; Garelli et al. 2015; Garelli et al. 2015; Vallejo et al. 2015). Dilp8 transcripts are significantly upregulated (3.91 fold, \(P < 8.01 \times 10^{-11}\)) in STAT supercompetitors but not in Myc supercompetitors (Table 1). However, despite the upregulation of dilp8 transcripts in STAT winners, dpp > hop animals are not developmentally delayed (see above), possibly because the amount of ectopic dilp8 in dpp > hop discs is insufficient to activate Lgr3-expressing neurons in the brain (Colombani et al. 2015; Garelli et al. 2015; Vallejo et al. 2015). Dilp8 is present at low levels in control wing discs (Figure 5K) and is upregulated in several discrete areas along the dpp stripe in dpp > hop discs (Figure 5L, arrows). Matrix metalloproteinase 1 (Mmp1) is a secreted protease that cleaves substrates in the extracellular matrix and regulates tissue remodeling and wound healing (Page-McCaw et al. 2003). Mmp1 transcripts are augmented 1.78 fold (\(P < 0.00254\)) in STAT winners but not in Myc winners (Table 1). Mmp1 mRNA is expressed at low levels in a control third instar wing disc (Figure 5M), consistent with a prior report (Page-McCaw et al. 2003), and is increased in STAT supercompetitors located in the dorsal hinge (Figure 5N, arrow), the hinge being the site of highest endogenous activity of the JAK/STAT pathway.

We also validated upregulated genes in STAT supercompetitors that encode in intracellular proteins. ETS-domain lacking (Edl) acts downstream of MAPK to promote Epidermal growth factor receptor signaling (Baker et al. 2001; Tootle et al. 2003). edl transcripts are significantly upregulated in STAT supercompetitors (2.10 fold, \(P < 0.00209\)) but not in Myc supercompetitors (Table 1). edl mRNA is observed at moderate levels throughout the wing (Figure 5O) and is upregulated in STAT supercompetitors along the dpp domain (Figure 5P, arrow).

Finally, we validated genes differentially upregulated in both STAT and Myc supercompetitors. molting defective (mld) encodes a nuclear, zinc-finger domain protein required for ecdysone biosynthesis (Neubueser et al. 2005). mld is significantly increased in both STAT and Myc supercompetitors compared to controls (1.61 fold, \(P < 0.0004\) for STAT and 1.61 fold, \(P < 0.0004\) for Myc, Table 1). mld mRNA is expressed at low levels in a control third instar wing disc, higher in the anterior domain than the posterior (Figure 5Q). mld is increased in STAT supercompetitors located in the dorsal and ventral hinge (Figure 5R, arrows) and in Myc supercompetitors located in the dpp domain in the pouch (Figure 5S, arrow). As noted above, Mpcp2 is significantly upregulated in both STAT and Myc supercompetitors (1.50 fold, \(P < 0.00172\) for Hop; 2.02 fold, \(P < 1.036 \times 10^{-10}\) for Myc, and Table 1). Mpcp2 is a nuclear-encoded, mitochondrial inner membrane transporter that facilitates the movement of metabolites, nucleotides and cofactors across this mitochondrial membrane (Palmieri 2013). Mpcp2 is expressed at moderate and fairly uniform levels in a control third instar wing disc (Figure 5T) and is upregulated in dpp > hop and dpp > Myc discs in the dpp domain of the pouch and hinge (Figure 5U and V, arrows). minidiscs (mdn) encodes a leucine amino acid transporter (Martin et al. 2000; Reynolds et al. 2009). mdn is significantly upregulated in both STAT and Myc supercompetitors (1.59 fold, \(P < 0.0103\) for Hop; 1.82 fold, \(P < 0.000432\) for Myc and Table 1). mdn is expressed at a low level in a control disc (Figure 5W) but not in Myc supercompetitors (Table 1).

| Gene       | Fold Change (hop vs. gfp) | Adjusted p-value (hop vs. gfp) |
|------------|---------------------------|-------------------------------|
| cnc        | 1.30                      | 0.0618                        |
| Nox        | 0.684                     | 0.0957                        |
| Keap1      | 0.68                      | 0.00726                       |
| GstD5      | 2.45                      | 0.000933                      |
| GstD6      | 2.07                      | 0.0186                        |
| GstD3      | 2.05                      | 0.000392                      |
| GstD4      | 2.04                      | 0.0167                        |
| GstD10     | 1.70                      | 0.0138                        |
| GstD1      | 1.56                      | 0.000658                      |
| Ugt86Di    | 2.31                      | 1.23 \times 10^{-6}          |
| Ugt86Da    | 1.71                      | 0.00602                       |
| Cyp18a1    | 3.21                      | 4.26 \times 10^{-5}          |
| Cyp4aa1    | 1.98                      | 0.0222                        |
| Cyp9h1     | 1.88                      | 0.0669                        |
is upregulated along the dpp stripe in dpp > hop and dpp > Myc discs (Figure 5X and 5Y, arrows).

Establishing a quantitative assay for supercompetition

We developed an assay to quantify supercompetitor-induced apoptosis of wild-type neighbors (see Materials and Methods). We generated random clones mis-expressing GFP alone (i.e., control clones) or GFP plus Hop (i.e., STAT supercompetitor clones) precisely at 48 hr AED. We dissected wing discs 72 hr later (at 120 hr AED). After scanning the samples on a confocal microscope, we used Image J to outline the clone (Figure 6A-A’) and then to draw another line representing 10 cell-diameters from the clone boundary (Figure 6A”-A”). We then counted the number of apoptotic wild-type cells within the area between the two lines. There were significantly more dead wild-type cells neighboring STAT supercompetitors than those neighboring control clones (Figure 6F, compare purple to blue bar, P < 0.001). Clonal mis-expression of Hop induces STAT activation in a cell-autonomous manner (Figure 6C), whereas clonal mis-expression of GFP does not (Figure 6B). To prove that the competitive stress inflicted by supercompetitors was due to increased JAK/STAT pathway activity, we depleted Stat92E from both types of clones. This resulted in a robust autonomous decrease in STAT antibody reactivity in both types of clones (Figure 6D,E). Depletion of Stat92E from STAT supercompetitors substantially reduced their competitive properties, as assessed by significantly fewer apoptotic wild-type neighbors (Figure 6F, compare purple bar to red bar, P < 0.001). In fact, Hop clones depleted for Stat92E were now indistinguishable from control clones with respect to neighbor death (Figure 6F, no significant difference between the red and blue bars). As expected, depletion of Stat92E from control clones did not affect their wild-type neighbors (Figure 6F, no significant difference between yellow and blue bars).

**DISCUSSION**

Here we report the transcriptional profiling of highly purified STAT or Myc supercompetitors from wing imaginal discs. We demonstrate that the transcriptional profiles of these two type of competitors are largely distinct, with only 41 genes that are differentially regulated in both data sets. Our interest lies in identifying JAK/STAT target genes that regulate the competitive abilities of STAT supercompetitors. Using a combination of protein traps, immunofluorescence and in situ hybridization, we validated numerous upregulated genes in STAT supercompetitors, including several genes encoding secreted or transmembrane proteins. Our characterization of differentially regulated genes in STAT winners demonstrates that they have increased ROS generation, and, presumably as a result of this, an anti-oxidant response. Recent work has proposed that an anti-oxidant response is a hallmark of cells heterozygous for a ribosomal gene that can be outcompeted when confronted by wild-type cells (Kucinski et al. 2017). Our work demonstrates that STAT supercompetitors have a similar signature, suggesting that the anti-oxidant response is not a universal marker of less fit cells. In the future, we will need to determine whether Duox upregulation causes the anti-oxidant response and whether Duox and cnc are required for the competitive properties of STAT winners.

| Table 4 Expression of genes in the ecdysone pathway or ecdysone responsive genes in STAT supercompetitors |
|---|---|---|
| Gene | Fold Change (hop vs. gfp) | Adjusted p-value (hop vs. gfp) |
| Eip75B | 1.36 | 0.0131 |
| ftz-f1 | 1.82 | 0.00345 |
| EcR | 1.45 | 0.0417 |
| ImpE1 | 1.66 | 0.0246 |
| Cyp18a1 | 3.21 | 4.262 x 10^{-5} |
| Oatp74D | 1.69 | 7.191 x 10^{-5} |
| Iswi | 1.34 | 0.0106 |
| swi2 | 1.73 | 0.0478 |
| E(bx) | 1.33 | 0.0180 |
| hid | 1.50 | 0.0164 |
| rpr | 2.05 | 0.00657 |
| Blimp-1 | 0.53 | 0.00532 |
| let-7-C | 0.50 | 0.0249 |
| DopEcR | 0.61 | 0.0722 |

**Figure 4** ROS and Nrf2 are upregulated in STAT winners. (A-B) In a control ptc > gfp disc (A), cells in the ptc domain (labeled by GFP, green) have low levels of ROS as assessed by CellROX Deep Red Reagent (red) (A). In a ptc > hop disc (B), STAT winners (labeled by GFP, green) generated in the ptc domain have elevated levels of ROS (red) along the entire ptc domain (B). (C-D) Cnc-GFP, encoded by a bacterial artificial chromosome under endogenous regulatory sequences, is expressed at low levels in a control ptc-gal4 disc (C). Cnc-GFP is upregulated in STAT winners, particularly in the dorsal and ventral hinge (D, brackets). Ptc is red. Phalloidin, which marks F-actin, is in blue. Scale bar indicates 50 µM. Genotypes (A) w/w; ptc-gal4, UAS-gfp/+; +/- (B) w/w; ptc-gal4, UAS-gfp/+; UAS-hop/+ (C) w/w; ptc-gal4/ PBac(cnc-EGFP.S)VK00037; +/- (D) w/w; ptc-gal4/+; PBac(cnc-EGFP.S)VK00037; UAS-hop/+.
We report here that the established Jun N-terminal kinase (JNK) target Mmp1 is significantly upregulated in STAT winners, and this result suggests that JNK signaling in increased in STAT winners. Prior work has shown that ftz-f1 can be upregulated by JNK in imaginal discs (Külshammer et al. 2015). Consistent with increased JNK signaling in STAT winners, ftz-f1 is increased in STAT winners compared to control cells. JNK is required in wild-type winners to eliminate polarity-deficient losers (Ohsawa et al. 2011), and in the future, we will need to determine if JNK signaling facilitates the competitive properties of STAT winners. We will also need to address how JNK signaling is activated in STAT winners, particularly whether JNK is activated downstream of Duox or ROS generation in these cells.

STAT supercompetitors have increased edcsyne signaling and this is not shared with Myc supercompetitors. We suggest that the significantly increased expression of Ecl, the transporter required for edcsyne movement into cells, may underlie the heightened edcsyne responses in STAT supercompetitors, but future work will be needed to test this model. Recent work has shown that edcsyne signaling promotes growth of imaginal discs (Herboso et al. 2015; Neto et al. 2017). Work from the Casares and Aerts labs has shown that mis-expressing transcription factors Homothorax (Hth) and Teashirt (Tsh) in the early eye disc causes a significant increase in ftz-f1 and a significant decrease in Hormone receptor 3 (Hr3, also called Dhr3 or Hr46) and in Blimp-1. They reported that changes in these genes promote proliferation of undifferentiated eye disc progenitors (Neto et al. 2017). STAT winners share some elements of this Hth+Tsh proliferation and/or growth of STAT supercompetitors (Q-Y). At least 10 discs of each genotype were analyzed for expression pattern of each RNA probe, and the representative image of the expression pattern is shown. (A-B) hop is expressed at low levels in control (dpp > gfp) discs (A) but is upregulated along the dpp domain in dpp > hop discs (B, arrow). (C-D) Socs36E is expressed in several distinct patches in the dorsal, lateral and ventral hinge in a control disc (C) but is increased along the dpp domain in a dpp > hop disc (D, arrow). (E-F) ftz-f1 is expressed at low levels in a control disc (E) but is induced in STAT supercompetitors located in the dpp domain (F, arrow). (G-H) ImpE1 is expressed in numerous discrete patches in a control (dpp > gfp) disc (G) and is upregulated in the dorsal hinge in a dpp > hop disc (H, arrow). (I-J) Ama is expressed in many patches of cells in the hinge and notum in a control disc (I) but is upregulated along the dpp domain in a dpp > hop disc (J, arrow). (K-L) clip8 is expressed at low levels in a control (dpp > gfp) disc (K) but is induced in several distinct regions in the notum: hinge and pouch in a dpp > hop disc (L, arrow). (M-N) Mmp1 is expressed at low levels in a control (dpp > gfp) disc (M) but is upregulated within the dorsal hinge in a dpp > hop disc (N, arrow). (O-P) edl is expressed robustly and ubiquitously in a dpp > gfp disc (K) but is upregulated along the dpp domain in a dpp > hop disc (P, arrow) and along the entire dpp domain in a dpp > Myc disc (S, arrow). (T-V) Mpcp2 is expressed robustly in a control wing disc (T). Mpcp2 is upregulated in the dpp domain in a dpp > hop (U, arrow) and a dpp > Myc disc (V, arrow). (W-Y) mnd is expressed at low levels in a control wing disc (W). mnd is upregulated in the dpp domain in a dpp > hop (X, arrow) and a dpp > Myc disc (Y, arrow). Genotypes: (A, C, E, G, I, K, M, O, Q, T, W) w/w, +/-; dpp-gal4, UAS-gfp/+ (B, D, F, H, J, L, N, P, R, U, X) w/w, +/-; dpp-gal4, UAS-gfp/+.
should be valuable resources for others in the universal one, with different triggers and effectors. These transcriptomes concept that there are multiple types of cell competition, as opposed to a bar) does not alter the viability of wild-type neighbors. This in turn supports the Eip75B heme center. determine whether the anti-oxidant response in STAT winners impacts the Eip75B heme center.

**Figure 6** Quantitative assay for super-competition. (A) A representative wing disc with Hop flip-out clones (green) labeled with Dcp-1 (red) to mark apoptotic cells. We used Image J to outline the Hop flip-out clones (A') and then drew a second line 10-cell diameters away from the clone boundary (A‘‘). We counted the number of dying cells within the shaded area (A‘‘). (B) Control GFP flip-out (GFP FO) clones (green) do not have activated STAT (red, labeled pSTAT). (C) Hop flip-out (Hop FO) clones (green) ectopically activate STAT (C‘, arrows). (D) Control GFP flip-out clones expressing a UAS-Stat92E RNAi construct (green, labeled GFP FO + STAT-i) display autonomic loss of STAT (D‘, arrows). (E) Hop flip-out clones expressing a UAS-Stat92E RNAi construct (green, labeled Hop FO + STAT-i) display autonomic loss of STAT (E‘, arrows). Note: in B–E, the clones were not induced in timed embryo collections and hence they are of varying sizes. Scale bar indicates 50 μM. (F) Graph displays the number of apoptotic (Dcp-1-positive) wild-type neighbors per unit area (10^4 μM^2). There are significantly more apoptotic neighbors of STAT supercompetitors (Hop FO, purple bar) than control clones (GFP FO, blue bar). When Stat92E is depleted from STAT supercompetitors (Hop FO + STAT-i, red bar), there is a significant reduction in the number of apoptotic wild-type neighbors. By contrast, depletion of Stat92E from control clones (GFP FO + STAT-i, yellow bar) does not alter the viability of wild-type neighbors. ‘‘P < 0.001; “ns” is not significant. Genotypes (A, C) y, w, hs-flp^122/+; act > y>gal4, UAS-gfp/UAS-Dcr-2; UAS-hop/+ (B) y, w, hs-flp^122/+; act > y> gal4, UAS-gfp/UAS-Dcr-2; +/+ (D) y, w, hs-flp^122/+; act > y> gal4, UAS-gfp/UAS-Dcr-2; UAS-Stat92E^HMS00035/+ (E) y, w, hs-flp^122/+; act > y> gal4, UAS-gfp/UAS-Dcr-2; +/+ (F) y, w, hs-flp^122/+; act > y> gal4, UAS-gfp/UAS-Dcr-2; UAS-hop/UAS-Stat92E^HMS00035 y, w, hs-flp^122/+; act > y> gal4, UAS-gfp/UAS-Dcr-2; UAS-Stat92E^HMS00035/+ y, w, hs-flp^122/+; act > y> gal4, UAS-gfp/UAS-Dcr-2; UAS-hop/UAS-Stat92E^HMS00035.

**Figure 6** Quantitative assay for super-competition. (A) A representative wing disc with Hop flip-out clones (green) labeled with Dcp-1 (red) to mark apoptotic cells. We used Image J to outline the Hop flip-out clones (A') and then drew a second line 10-cell diameters away from the clone boundary (A‘‘). We counted the number of dying cells within the shaded area (A‘‘). (B) Control GFP flip-out (GFP FO) clones (green) do not have activated STAT (red, labeled pSTAT). (C) Hop flip-out (Hop FO) clones (green) ectopically activate STAT (C‘, arrows). (D) Control GFP flip-out clones expressing a UAS-Stat92E RNAi construct (green, labeled GFP FO + STAT-i) display autonomic loss of STAT (D‘, arrows). (E) Hop flip-out clones expressing a UAS-Stat92E RNAi construct (green, labeled Hop FO + STAT-i) display autonomic loss of STAT (E‘, arrows). Note: in B–E, the clones were not induced in timed embryo collections and hence they are of varying sizes. Scale bar indicates 50 μM. (F) Graph displays the number of apoptotic (Dcp-1-positive) wild-type neighbors per unit area (10^4 μM^2). There are significantly more apoptotic neighbors of STAT supercompetitors (Hop FO, purple bar) than control clones (GFP FO, blue bar). When Stat92E is depleted from STAT supercompetitors (Hop FO + STAT-i, red bar), there is a significant reduction in the number of apoptotic wild-type neighbors. By contrast, depletion of Stat92E from control clones (GFP FO + STAT-i, yellow bar) does not alter the viability of wild-type neighbors. ‘‘P < 0.001; “ns” is not significant. Genotypes (A, C) y, w, hs-flp^122/+; act > y>gal4, UAS-gfp/UAS-Dcr-2; UAS-hop/+ (B) y, w, hs-flp^122/+; act > y> gal4, UAS-gfp/UAS-Dcr-2; +/+ (D) y, w, hs-flp^122/+; act > y> gal4, UAS-gfp/UAS-Dcr-2; UAS-Stat92E^HMS00035/+ (E) y, w, hs-flp^122/+; act > y> gal4, UAS-gfp/UAS-Dcr-2; +/+ (F) y, w, hs-flp^122/+; act > y> gal4, UAS-gfp/UAS-Dcr-2; UAS-hop/UAS-Stat92E^HMS00035 y, w, hs-flp^122/+; act > y> gal4, UAS-gfp/UAS-Dcr-2; UAS-Stat92E^HMS00035/+ y, w, hs-flp^122/+; act > y> gal4, UAS-gfp/UAS-Dcr-2; UAS-hop/UAS-Stat92E^HMS00035.

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