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An ancient defense system eliminates unfit cells from developing tissues during cell competition

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Abstract

Developing tissues that contain mutant or compromised cells present risks to animal health. Accordingly, the appearance of a population of suboptimal cells in a tissue elicits cellular interactions that prevent their contribution to the adult. Here we report that this quality control process, cell competition, uses specific components of the evolutionarily ancient and conserved innate immune system to eliminate *Drosophila* cells perceived as unfit. We find that Toll-related receptors (TRRs) and the cytokine Spätzle (Spz) lead to NF κ B-dependent apoptosis. Diverse “loser” cells require different TRRs and NF κ B factors and activate distinct pro-death genes, implying that the particular response is stipulated by the competitive context. Our findings demonstrate a functional repurposing of components of TRRs and NF κ B signaling modules in the surveillance of cell fitness during development.

Successful development of multicellular animals requires cooperative cell-cell interactions that ensure tissue integrity. Mechanisms exist to enforce this behavior (1–4). One such mechanism monitors genetic identity so that potentially noncooperating mutant cells are prevented from contributing to the tissue (5, 6). How genetic disparities are recognized is unknown, but evidence points to cell vigor or fitness as a critical component. For instance, mutation of genes encoding ribosomal proteins (Rp), known in *Drosophila* as *Minute* mutants, or of the Myc transcriptional regulator, which controls numerous genes involved in metabolism and growth, can occur without inherently compromising cell viability. However, when surrounded by wild-type (WT) cells, the mutant cells are recognized and actively eliminated (7–10). This cell selection process, known in *Drosophila* and in mammals as cell competition, promotes tissue fitness by recognizing and eliminating genetically different or suboptimal somatic cells. Supercompetition, a variation of cell competition, occurs when

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SUPPLEMENTARY MATERIALS

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Figs. S1 to S8

Tables S1 and S2

cells with activated oncogenes like Myc compete with neighboring WT cells (11–18). Short-range signaling between Myc supercompetitor cells and WT cells instructs the latter to die, whereas the supercompetitors “win” and colonize the tissue. Cell competition can therefore be homeostatic (WT versus unfit cells) or potentially pathologic (supercompetitors versus WT cells, which may promote cancer). Myc-regulated cell competition has been documented in *Drosophila* wing imaginal discs and among mouse epiblast cells, where fluctuations in Myc expression precede the transition of cells to a more limited developmental potential and correlate with survival (9, 11, 14, 19).

The Toll signaling pathway was first identified as a developmental regulatory module that patterns the embryonic dorsoventral axis in *Drosophila* but was subsequently recognized to also function in host defense against infection. Toll-like receptors and NF κ B transcription factors are key signaling and transcriptional mediators of the ancient and broadly conserved innate immune recognition system activated in response to non-self (e.g., microbial infection) or altered-self cells (e.g., viruses, cancer) (20–24). The early appearance of these factors in metazoan evolution and their conserved use in altered-self recognition in a process akin to cell competition led us to probe whether they mediate the response to mutant or otherwise compromised cells in developing tissues. We report here that components of the innate immune system function to eliminate cells recognized as unfit during cell competition.

Mutations in the Toll and IMD pathways prevent Myc-induced cell competition

To test for involvement of innate immune factors in cell competition, we used a simple genetic assay in wing discs for Myc-induced competition, wherein cells constitutively express a Myc transgenic FRT cassette ($>Myc<$), increasing the expression of Myc ~1.5-fold above the endogenous level (11, 13). Removal of this cassette via Flp/ FRT-mediated recombination generates clones of WT cells and concomitantly allows expression of the Gal4 transcriptional activator and the Gal4-regulated upstream activating sequence–green fluorescent protein (UAS-GFP) (Fig. 1A). Cells in these clones are WT for Myc expression but because they are surrounded by cells that retain the Myc cassette, they become “losers” and are competitively eliminated via apoptosis. Within 24 hours of clone induction, cell death increased within the loser clones (fig. S1, A and B). Over time, this led to a significant reduction in clone size relative to control clones generated in a noncompetitive environment (Fig. 1B, green versus gray bars, respectively) (11, 12, 25). Genetic studies have shown that in the absence of the proapoptotic factor, Hid, WT loser clones grow to the same size as controls, implicating Hid as a critical mediator of loser cell elimination (11, 12, 25).

In *Drosophila*, two signaling pathways and three NF κ B family members govern the innate immune response. Infection by Gram-negative bacteria induces the immune deficiency (IMD) pathway (26, 27), which activates the NF κ B homolog Relish (Rel) (28), whereas the Toll pathway is activated upon infection by Gram-positive bacteria and fungi and leads to activation of the other NF κ B homologs Dorsal (dl) and Dorsal-related immunity factor (Dif) (Fig. 1D) (29, 30). We induced loser clones in genetic backgrounds mutant for genes in the

IMD or Toll pathways and measured their size after a defined period of time. Loss of the receptor PGRP-LC, the adaptor BG4/FADD, Dredd, the apical caspase implicated in Rel cleavage and activation, and Rel itself suppressed the loss of loser cells and increased the size of the clones (Fig. 1B), indicating that their competitive elimination was blocked (11, 12, 25). However, mutations in *key* (*kenny*, encoding IKK- γ), *ird5* (*immune response deficient-5*, encoding IKK- β), and *dTak1*, all necessary for infection-induced Rel activation, did not prevent elimination of loser cells (Fig. 1B). Because dTak1 is a homolog of transforming growth factor- β activated kinase and is an activator of Jun N-terminal kinase (JNK) signaling (31), this result is consistent with the previous demonstration that, although activated, JNK is not essential for Myc-induced competition (11). Loss of *imd*, encoding a protein that links FADD and Dredd to PGRP-LC, also did not prevent loser cell elimination (Fig. 1B). From the Toll pathway, Myc-induced competition was not suppressed in genetic backgrounds that lacked the NF κ B factors Dorsal or Dif, the kinase Pelle, or the Toll/interleukin-1 receptor (TIR)-domain containing adaptor MyD88 (Fig. 1B). By contrast, mutation of *Ect4*, which encodes the TIR-domain protein dSarm, completely suppressed loser cell elimination without affecting the growth of control clones (Fig. 1C). *tube* encodes an IRAK-4 homolog in the Toll pathway (32), and heterozygotes displayed dominant genetic interactions with *Rel* that were specific to the competitive context (table S1), suggesting that Tube also functions during cell competition.

Activation of innate immune pathways in cell competition involves noncanonical receptors and the ligand Spz

Loser cells were also not eliminated in Spätzle (*spz*) mutant backgrounds (Fig. 1B and table S1). Spz is a secreted cytokine and a ligand for the Toll receptor in innate immunity and in embryonic dorsoventral patterning (30, 33–35). However, RNA interference (RNAi)-mediated knockdown of *Toll* itself (*Toll-1*) had little effect on competition (fig. S2C). The *Drosophila* genome encodes nine Toll-related receptors (TRRs), but most are poorly characterized. Notably, loss of *Toll-2* (*18wheeler*), *Toll-3* (*Mst-Prox*), *Toll-8* (*tollo*), and *Toll-9* blocked elimination of the loser cells and significantly increased clone size (Fig. 1, E and F, and fig. S2A) but did not affect growth of control clones (Fig. 1E and fig. S2A). RNAi-mediated knockdown specifically in the loser population indicated that the receptors were required within the loser cells (fig. S2A). By contrast, RNAi directed against Toll-4, -5, and -7 had little effect on competition (fig. S2C).

These observations suggest that a cohort of components of the IMD pathway—PGRP-LC, Fadd, Dredd, and Rel—could be united in a functional circuit with four noncanonical TRRs, the Toll ligand Spz, the Tube adaptor, and the TIR domain protein dSarm to eliminate WT loser cells in response to Myc-induced competition. *Toll-8* (*tollo*) mutants were especially efficient at suppressing the loser fate (Figs. 1, E and F, and 2, A and B). Consistent with this, Toll-8 LRR, an activating truncation of Toll fused to Toll-8 trans-membrane and intracellular domains (36), enhanced competition when expressed in loser cells, without altering control clones (fig. S2B). This outcome suggests that Toll-8 activity is specific to the competitive context and implies that factors not present under noncompetitive conditions are also required. It is possible that if, as our data suggest, a Toll-8-dependent signaling

pathway leads to death of loser cells, it requires heterotypic interactions with Toll-2, Toll-3, or Toll-9. Notably, expression of Toll-2, Toll-8, and Toll-9 mRNA was down-regulated in Myc-expressing S2 cells (fig. S2D), which could bias signaling to the loser cells.

Transcriptional up-regulation of *hid* via the nuclear mediator Rel

Based on the TRR signaling components required for the elimination of WT loser cells, we postulated that Rel could be the nuclear mediator of their death. Consistent with this idea, loser clones generated in a *Rel* mutant background contained many fewer dying cells than WT loser clones (Fig. 2, A and B). To determine whether Rel functioned within the loser cells, we expressed *Rel*-RNAi (or *Rel*-RNAi and Dicer-2 to increase RNAi efficiency) specifically in that population. Indeed, *Rel*-RNAi suppressed the loss of loser cells, consistent with a role as a regulator of their fate (Fig. 2C). Moreover, Rel's effect on the cells was specific to the competitive condition, because neither a *Rel* mutant nor *Rel*-RNAi altered growth of control clones (Fig. 2C and fig. S3A). Rel undergoes proteolytic cleavage by the caspase Dredd after pathogenic infection, which releases an active, N-terminal Rel68 fragment and allows its nuclear translocation (37). Null alleles of *Dredd* suppressed the elimination of loser cells (Fig. 2D), as did overexpression of the Dredd inhibitor Caspar (Casp) (38) in loser cells, whereas mutants of *casp* enhanced their loss (fig. S3, B and C). Again, these treatments did not affect cells in a noncompetitive environment and thus were specific to the competitive context. In addition, dominant genetic interactions between *Dredd* and *Rel* compound heterozygotes blocked elimination of loser cells as effectively as complete loss of *Dredd* (Fig. 2D). Thus, loser cells have heightened Dredd activity, which could lead to Rel activation during cell competition. *Dredd* overexpression in *Drosophila* S2 cells leads to cleavage of Rel (39), and expression of UAS-*Dredd* in cell clones in WT wing discs induced cell death and reduced clone size (Fig. 2E and fig. S3, D to F), phenocopying cell competition. Dredd's effect on clone size required Rel, as it was prevented by concomitant expression of *Rel*-RNAi (Fig. 2E). These results suggest that TRR activation increases Dredd activity and leads to activation of Rel, which results in the elimination of loser cells.

To determine whether cell competition led to nuclear translocation of Rel and activation of antimicrobial target genes, as occurs during innate immunity, we used a cell-based competition assay (12). Conditioned medium (CM) from cocultures of competing WT and Myc-expressing *Drosophila* S2 cells contains diffusible factors that confer the outcomes of the competitive interactions, including Hid-dependent cell death, on naïve S2 cells (12, 25). Consistent with our genetic results in vivo, double-stranded RNA (dsRNA)-mediated knockdown of *Rel* prevented the death of naïve S2 loser cells (Fig. 3A). To examine the subcellular localization of Rel, we transfected S2 cells with GFP-tagged Relish (GFP::Rel). In control experiments, treatment of the transfected cells with bacterial peptidoglycan (PGN) increased the fraction of cells with nuclear GFP::Rel (Fig. 3B) and also induced expression of several AMP genes (fig. S4A). Treatment of the transfected S2 cells with competitive CM (cCM) also increased nuclear translocation of GFP::Rel, but cCM was unable to activate expression of AMP genes, in contrast to PGN challenge (fig. S4A). Likewise, AMP gene expression was not activated in wing disc cells or in any larval tissue in animals in which cell competition was induced in vivo (fig. S4F). Disc cells may lack factors necessary to

activate an immune response, because although infection of larvae with *Erwinia carotovora* induced expression of several AMP genes (fig. S4, B and C) and reporter transgenes in larval immune tissues (fig. S4, D and E), they were not expressed in wing disc cells. IKK β phosphorylation of Rel is required for induction of AMP gene expression during the immune response (39); thus, the lack of AMP induction may reflect Rel-dependent transcriptional programs that are accessed by diverse signaling pathways: IKK-dependent for immune response activation of AMPs and IKK-independent for cell competition. The absence of requirements for *ird5*/IKK- β and *key*/IKK- γ in cell competition (Fig. 1B) is consistent with this notion.

In considering relevant transcriptional targets of Rel during cell competition, we examined *hid*, given its role in elimination of WT loser cells (11, 12, 25). Bioinformatic analysis identified consensus NF κ B binding sites in the *Wrinkled* (*W*) locus, which encodes Hid (fig. S5). Expression of *Rel68* (the active form of Rel) in wing disc cells activated *hid*^{P-lacZ}, a transcriptional reporter inserted in the *W* locus (Fig. 3, C and D). *Rel68*-expressing cell clones were small and underrepresented in the disc, as expected because Hid expression leads to cell death (Fig. 3, E and F). Notably, the presence of the *hid*^{P-lacZ} insertion, not only a transcriptional reporter but also a mutant allele, allowed clones to grow as well as GFP-expressing control clones (Fig. 3, E to G). Expression of *Rel68* in wing discs up-regulated *hid* mRNA approximately threefold above the WT level (Fig. 3H) but did not induce expression of *reaper* (*rpr*), a proapoptotic gene in the same chromosomal region, indicating that Rel induces death of wing disc cells by the selective activation of Hid. Both Rel and Hid are required in the loser cells for their elimination; thus, we infer that Rel activation during competition triggers loser cell death via its induction of Hid expression (11, 12, 25).

Elimination of *Minute* loser cells requires a partially overlapping signaling module

A defining feature of cell competition is its context dependence, so that cells perceive themselves as more or less fit as a direct result of interactions with their neighbors (5, 6). To determine whether the TRR-NF κ B signaling module that we identified was specific to competition induced by Myc or was a more general mechanism, we examined the *Minute* competition paradigm. We used larvae in which all cells carried one mutant copy of the *RpL14* gene (*M^{RpL14}/+* cells) but were rescued to WT by expression of a genomic *RpL14* trans-gene (*gRpL14*) (Fig. 4A). In this background, we generated GFP-marked wing disc clones of *M^{RpL14}/+* cells through Flp-FRT-mediated removal of the *gRpL14* transgene. These clones were competitively eliminated at high frequency, as scored by counting how many GFP-positive *M^{RpL14}/+* clones persisted in larvae (Fig. 4, A and B). Using this assay, we targeted individual genes in the Toll and IMD pathways with RNAi to determine if their loss prevented the competitive elimination of the GFP-*M^{RpL14}/+* loser cells (Table 1 and fig. S6A). We identified a group of innate immune genes that prevented loss of *M^{RpL14}/+* loser cells that were also required for Myc-induced competition [including *spz*, *Toll-3*, *Toll-9*, and *Rel* (Table 1)], indicating that this signaling mechanism was not restricted to Myc-induced competition or due to oncogenic effects of Myc supercompetitor cells. However, the assay also revealed an interesting difference between the two competitive

contexts: RNAi targeted against *dl* or *Dif* or expression of their inhibitor, *cact*, strongly suppressed *MRpL14^{-/+}* clone loss. Likewise, compound heterozygotes carrying the *dl^l* allele and a deficiency that removed both *dl* and *dif* suppressed their elimination, whereas RNAi against *cact* enhanced it (Table 1 and Fig. 4B). Thus, although in each competitive context the outcome for the loser population was elimination, the signaling module required for this to occur was only partially overlapping.

Competitive context determines selection of NFκB factor and apoptotic inducer

Because the loser cells were WT in one of our competitive paradigms and in the other they were *MRpL14^{-/+}*, we considered the possibility that the specific signaling module activated within the loser cells was influenced by the identity of the competing populations, similar to the response of innate immune signaling to diverse pathogens. In particular, the NFκB factor required for the elimination of the loser population in the two contexts appeared to be different. To investigate this further we measured clone size and cell number in GFP-*MRpL14^{-/+}* clones in wing discs and found that *Rel*/RNAi only mildly suppressed competition against *MRpL14^{-/+}* cells. Loss of both *Dif* and *Dorsal* activity (achieved by *cact* overexpression) strongly blocked elimination of *MRpL14^{-/+}* losers, and coexpression of *Rel* RNAi with *cactus* enhanced this effect (fig. S7, A and B). *Dif* and *Dorsal* therefore appear to be the predominant NFκB mediators of *MRpL14^{-/+}* loser elimination, in contrast to the exclusive requirement for *Rel* in triggering death of WT loser cells (Figs. 1B and 2, A to C). Elimination of the *MRpL14^{-/+}* cells depended upon apoptosis and was suppressed by expression of *cact* (Fig. 4, C and D), as well as a chromosomal deficiency called *H99* that removes *hid*, *rpr*, and other pro-death genes or expression of the caspase inhibitor p35 (Fig. 4E). However, whereas expression of RNAi targeted specifically against *rpr* prevented loss of *MRpL14^{-/+}* loser cells, RNAi against *hid* did not (Fig. 4E). This suggested that distinct apoptotic regulators were induced by the selective activation of a NFκB factor within each loser population. Like *Rel*, *dl* expression reduced clone size in wing discs (Fig. 4F) and induced pro-death gene expression, except that *dl* (and *Dif*) led to preferential expression of *rpr* rather than *hid* (Fig. 4G). In contrast, *Hid* is required for *Rel68*-mediated elimination of WT cells (Fig. 3, G and H) and WT loser cells in *Myc*-induced competition (11, 12, 25). Thus, in two genetically distinct competitive contexts, partially overlapping signaling components activate different NFκB factors and prompt expression of distinct proapoptotic target genes yet trigger the same outcome: the death and elimination of the loser cells.

Conclusions

Altogether, our results demonstrate that the conceptual resemblance between cell competition and innate immunity is matched with genetic and mechanistic similarities. Thus, cells within developing tissues that are recognized as mutant or compromised are competitively eliminated via a TRR- and NFκB-dependent signaling mechanism. Although similar core signaling components are activated in both processes, cell competition culminates in local expression of proapoptotic genes rather than systemic induction of antimicrobial genes. Because cell competition is initiated by the emergence of cells of

different fitness than their neighbors in a tissue, we surmise that the initiating signal is common to many competitive contexts. Our genetic data lead us to propose a model for how this signal is detected and transduced. Our results point to a role for Spz in signal detection, as it is a secreted protein that is required for the killing activity of cCM (fig. S8A), is a known ligand for the Toll receptor, and is produced by several tissues in the larva (fig. S8B). Thus, we speculate that Spz functions as a ligand for one or more TRR in cell competition. Because Spz must be activated through a series of proteolytic steps, the relevant proteases may respond directly to the initiating signal in cell competition. We propose that the genetic identity or context of the competing populations influences activation of different TRR signaling modules and that the precise configuration of TRRs on loser cells dictates which of the three *Drosophila* NF κ B proteins is activated (Fig. 5). How signaling to the NF κ Bs is restricted to the loser cells is not known, but higher expression of *Toll-2*, *Toll-8*, and *Toll-9* in loser cells (fig. S2D) could bias signal transduction. PGRP-LC, a receptor known to bind only bacterial products, also plays a role in Myc-induced competition. As commensal gut microflora is known to influence larval growth (40–42), this raises the possibility that it also contributes to the competitive phenotype.

Throughout evolution, signaling modules have adapted to fulfill different functions even within the same species. Here we have provided evidence for adaptation of TRR-NF κ B signaling modules in an organismal surveillance system that measures internal tissue fitness rather than external stimuli. It is noteworthy that the killing of WT cells by supercompetitor cells is a potentially pathological form of cell competition that could propel expansion of premalignant tumor cells. If so, activated TRR-NF κ B signaling modules in nonimmune tissues could be diagnostic markers, and their competitive functions could serve as therapeutic targets for cancer prevention.

Materials and methods

Bacterial infection

To elicit an immune response as a control for AMP gene induction, we orally infected larvae with *E. carotovora carotovora15* as in (43). 200 μ l of bacterial pellet was added to 400 μ l of crushed banana into a 2-ml microfuge tube. Thirty third instar larvae were placed inside the tube and fed the banana-bacteria mixture for 30 min at room temperature. The larvae and bacteria-banana were then transferred to standard molasses food and allowed to feed overnight at 29°C. At 24 hours after infection, larval tissues were examined for AMP gene expression by quantitative real-time polymerase chain reaction (qRT-PCR) or reporter activity. Similar results were obtained with septic infections of larvae with *Escherichia coli*.

Bioinformatics

Rel binding sites were identified in the *hid/W* locus using the Web-based program Genome Surveyor (44).

Cell culture and generation of conditioned medium

Drosophila S2 cells were maintained at 25°C in Schneider's *Drosophila* Medium supplemented with 10% fetal bovine serum, 0.5% yeast extract, and penicillin and

streptomycin (50 IU/ml each). cCM and noncompetitive cCM (ncCM) were generated as described in (12). Briefly, direct cocultures of either WT S2 cells with pMT-HA-Myc S2 cells or WT S2 cells with WT-S2 cells were plated at a density of 4×10^5 per ml per cell type for a total density of 8×10^5 cells per ml, as described (12). Conditioned medium was collected after 24 hours $\pm$ 125 μ M CuSO₄ treatment and centrifuged at 1200 revolutions per minute for 3 min. 125 μ M CuSO₄ was added to the conditioned medium without CuSO₄ to control for inducer effects (12). The CM was then used for treatments in experiments. Activity of the CM was verified by C3 activity measurements as described (12). For Rel-GFP assays, S2 cells were transfected with a pMT-Rel-GFP transgene (gift from E. Foley) as follows: WT S2 cells were seeded in a 12-well plate with one coverslip per well at 8×10^5 cells per ml and cultured at 25°C overnight. Transfection was done using Cellfectin II Reagent as directed (Invitrogen). Subcellular location of pMT-Rel-GFP in WT-S2 cells or Myc-S2 cells was scored after treatment $\pm$ 50 μ g/ml LPS as a source of PGN (Sigma) + 125 μ M CuSO₄, cCM, or ncCM. At least 200 cells per condition were scored per experiment. The ratio of cells positive for nuclear localization of GFP-Relish to total GFP-positive cells was calculated.

Drosophila stocks and care

The following mutant strains were used in this study: *Rel^{E20}* (gift from D. Hultmark); *imd^{shadok}* and *imd^l* (gifts from D. Ferrandon); *key^l*, *tak1^l*, *tak1²*, and *Dif^l* (gifts from B. Lemaitre); *FADD^{f06954}* (*BG4*), *Dredd^{B118}*, *ird5^{KG08072}*, *pl²*, *pl⁷*, *tube²*, *spz²*, *d^l*, *d¹*, *Df(2L) TW119*, *Df(2L)J4*, *cact¹*, *cact⁴*, *hid^{W05014}* (Plac-Z), *Ect4⁴²⁷³*, and *Rel^{E38}* (from Bloomington Drosophila Stock Center, <http://flystocks.bio.indiana.edu>); *casp^{P1}* (gift from J. Chung); *Tollo¹⁴⁵* (gift of J. Kim); *Tollo^{R23B}* and *Tollo^{R5A}* (gifts from P. Simpson); *PGRP-LC^{Totem 5}* and *PGRP-LC^{E12}* (gifts from B. Matthey-Prevot); and *spz^{tm7}* (gift from C. Hashimoto). For Myc competition, control or loser clones were generated in wild type, mutants, or with UAS-transgenes using these strains: *yw hsf1p;;act>y>Gal4*, UAS-*GFP* (controls), *ywhsf1p;;tub>Myc>Gal4*, UAS-*GFP*, *ywhsf1p;UAS-GFP tub>Myc>Gal4* (11), and *ywhsf1p;UAS-Myc* (7). *ywhsf1p;;FRT82B ubi-GFP*, *ywhsf1p;;FRT82B Rel^{E20}/TM6B* was used for fig. S3. *FRT82B Rel^{E20}* was generated by recombination and screened for presence of the mutation by PCR using primers from (45). For *M^{RpL14}/+* competition, UAS-transgenes were driven in a *ywhsf1p UAS-GFP::CD8;;M^{RpL14}(w+)-/+salE>gRpl14(w+)>Gal4/TM6B* strain. UAS-*RNAi* lines were from the Vienna Drosophila Resource Center (<http://stockcenter.vdrc.at>) or the Transgenic RNAi Project (TRiP) collection (BDSC) and are listed in table S3. Other strains include UAS-*Toll-8 LRR* (gift from J.-L. Imler), UAS-*dicer2* (BDSC), *yw;;UAS-cactus-HA/TM3* and *yw;;UAS-cactus, yw UAS-GFP hsf1p; tub-Gal80 FRT40, Ubi-GFP FRT40; tub-Gal4*, UAS-*dorsal, w P(w⁺, Dros-LacZ)*, *yw P(w⁺, Dipt-LacZ)*, and UAS-*Dredd* (gifts from B. Lemaitre), *yw;;CecA-lacZ* (gift from S. Stöven), UAS-*Rel68* (gift from N. Silverman), and UAS-*caspar* (gift from J. Chung). Null mutants were used when possible. A complete list of mutant strains is included in table S1. Flies were raised at 25°C on cornmeal-molasses food supplemented with penicillin and streptomycin, as indicated below.

dsRNA treatment of S2 cells

The sequences for dsRNA against Relish and Spz were obtained from the Drosophila RNAi Screening Center (DRSC 37194 for Relish and DRSC 17065 for Spz). The dsRNA was synthesized in vitro with T7 RNA polymerase as directed (Roche). Single cultures of WT or pMT-HA-Myc S2 cells were seeded at 8×10^5 cell per ml in 12-well plates. Sixteen hours later dsRNA was transfected using Cellfectin II Reagent (Invitrogen), as described (12). Three days after the dsRNA transfection, WT S2 cells with pMT-HA-Myc S2 cells (cCM) or WT S2 cells with WT-S2 (ncCM) were seeded at a density $(4 + 4) \times 10^5$ cell per ml, and CM was obtained as described above. Naïve WT S2 cells were also transfected with dsRNA 72 hours before the treatment with CM. Efficiency of the dsRNA treatment was assayed by RT-PCR.

Heat shock induction of loser clones

In the Myc competition assays, eggs from appropriate crosses were collected on yeasted grape-agar plates for 2 to 4 hours. After hatching, larvae were transferred to standard molasses food vials (50 per vial) supplemented with Pen and Strep and fresh yeast and raised at 25°C. The *tub>myc>-Gal4* transgene was used to generate random UAS-*GFP*-marked *tub>Gal4* clones via FLPase-mediated recombination, as described (11). *tub>-Gal4* clones were induced by heat shock (HS) of larvae at 37°C for 10 to 15 min at 48 hours after egg-laying (AEL), and progeny were allowed to grow at 25°C for either 48 or 96 hours. The *act>y>Gal4* transgene was used to generate random UAS-*GFP*- or UAS-*Rel68*-expressing *act>Gal4* clones in WT wing discs. *act>Gal4* clones were induced by larval HS at 37°C for 6 min at 48 hours AEL and larvae were treated as above. These heat shock times were optimized to generate only a few clones per disc, to avoid merged clones. Clone area (in square pixels) was scored for clones in the central area of the wing disc (wing pouch and proximal hinge). For some genotypes, cell number per clone was also counted. The MARCM (mosaic analysis with a repressible cell marker) technique (46) was used to generate mitotic clones that expressed UAS-*GFP* as a lineage marker $\pm$ UAS-*dorsal*. Expression of *hs-CD2* was used to mark the sibling clones (11). Nonparametric Mann-Whitney tests were performed for statistical significance.

In the *M^{RpL14}/+* competition assays, the driver strain carried a *Minute* mutation in the gene *RpL14* (*M^{RpL14}/+*), the *salE>gRpL14(w+)>Gal4* flip-out transgene (*salE* is a wing pouch-specific enhancer), and *UAS-GFP::CD8* (CD8 targets GFP for membrane localization). In *M^{RpL14}/+* clones, Gal4 is expressed instead of the RpL14 genomic rescue construct, and GFP expression is activated. The driver strain was crossed to RNAi or overexpression transgene strains, and flies were allowed to lay eggs for 12 to 16 hours. Parental flies were removed, and larval progeny were heat-shocked 24 hours later for 15 min at 37°C and analyzed using a fluorescence binocular or confocal microscope 76 to 80 hours later (for larval clone assays) or 48 to 52 hours later (for clone measurements and cell counting assays). The UAS-*GFP*-marked *M^{RpL14}/+* clones are generally eliminated from larvae within 80 hours, resulting in the absence of GFP in late third instar wing discs. In cases where the induced transgene rescues the clones, GFP is readily visible through the larval cuticle. Each transgene was tested in biological triplicate, and the average number of larvae with surviving clones was compared to the WT control. *yw, yw;;UAS-RpL14(w+)/TM6B*,

and *yw;Sp/CyO;UAS-P35 (w+)* strains were used as controls. Nonparametric *t* tests were used to test for statistical significance.

Imaging, image analysis, and quantifications

Cell clones were imaged in larvae with a Zeiss MZFLIII and in fixed wing discs with a Zeiss Axiophot, Leica SP5, or Leica LSM710 confocal microscope. Clone area was measured (in square pixels) using ImageJ or Photoshop software. Cells per clone were counted in the GFP and/or DNA channels. Only clones in the wing pouch and proximal hinge area were measured; in the *M^{RpL14}/+* assays, this corresponded to the *salE* expression domain. Significance was determined using Student's *t* and Mann-Whitney tests.

Immunohistochemistry

Wing disc cells were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 20 min at room temperature and washed with PBS 0.01% Tween-20. Hoechst 33258 or 4',6-diamidino-2-phenylindole was used to stain DNA. The primary antibodies used include mouse anti-Relish (DHSB), rabbit anti- β -galactosidase (Cappel), and rabbit anticlaved Caspase-3 (Cell Signaling Technology). Secondary antibodies were Alexa-conjugated anti-mouse and anti-rat and Cy3-conjugated anti-rabbit (Molecular Probes). For cell culture, cells seeded on glass coverslips were fixed in 4% paraformaldehyde/PBS for 30 min. Coverslips were washed with PBS once, permeabilized in 0.5% TX-100/PBS for 5 min, then blocked for 30 min in 3% BSA, 0.1% Tween-20/PBS. After blocking, each coverslip was flipped onto 50 μ l of primary antibody in blocking buffer for 1 hour at room temperature. Coverslips were washed 3 $\times$ with PBS, and primary antibodies were incubated in AlexaFluor 546 goat anti-rabbit or goat anti-rat immunoglobulin G (Molecular Probes) for 45 min at room temperature. Coverslips were washed 3 $\times$ for 5 min each with PBS, counterstained for DNA with Hoechst 33258 for 4 min, washed 3 $\times$ for 5 min each, then mounted on slides. TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling) assays were carried out on fixed discs using either TUNEL TMR (Roche Diagnostics) or ApopTag Red (Chemicon).

RNA isolation and qRT-PCR

Total RNA was isolated from wing disc using the Nucleo SpinII RNA isolation kit (Machery Nagel) or Trizol (Invitrogen). RNA quantity was measured using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). For qRT-PCR of Rel-expressing cells, *yw;; tub>CD2>Gal4* flies were crossed either with *yw;; UAS-GFP* or *yw;;UAS-Rel68* and heat-shocked for 30 min at 37°C. At 24 hours after clone induction, the wing discs were dissected, and total RNA was isolated. qRT-PCR was performed using Applied Biosystems 7900HT Real-Time PCR Systems (Applied Biosystems, Foster City, CA). Primer sets were obtained from either Microsynth (Microsynth AG, Balgach, Switzerland) or MWG (Huntsville, AL). For qRT-PCR, total RNA was treated with RNase-free DNaseI, and cDNA was synthesized by using oligo-dT primers and Superscript RT-III (Invitrogen). Samples were then used for qRT-PCR with the ABI SYBR green system. Measurements of transcript level were normalized to *actin5c* and/or *tubulina1*. The following primers were used for qRT-PCR: *hid*, F: TCTACGAGTGGGTCAGGAT-GT, R: GCGGATACTGGAAGATTTGC; *reaper*, F: GAGCAGAAGGAGCAGCAGAT, R:

GGACTTTCT-TCCGGTCTTCG; *dipteracin*, F: GTTCACCATTGCC-GTCGCCTTAC, R: CCCAAGTGCTGTCCATATCCTCC; *drosomycin*, F: TTGTTCGCCCTCTTCGCTGTCTT, R: GCATCCTTCGCACCAGCACTTCA; *attacin*, F: GTG-GTGGGTCAGGTTTTTCGC, R: TGTCCGTTGATGTG-GGAGTA; *Toll*, F: GGTCTTTTGGCCGGTTTCAC, R: CTCCACATCTCCGATGTCCG; *Toll-2*, F: GAGGCTA-TAGGCTGCCCTTG, R: ATGTTGCGGCACACAACTC; *Toll-3*, F: CCTTCCAGCGAACTGGACT, R: ACT-TATGAGGACAGGGGGCT; *Toll-8*, F: CTCCCATGC-TGGAAATGGGT, R: GCGAAGATCGGGATCGAGTT; *Toll-9*, F: CTTTTGCCCATCTGGGGGAT, R: AAACAT-GGCGGGAGTGAGAG; *act5c*, F: TGTGACGAAGAA-GTTGCTGCT, R: AGGTCTCGAACATGATCTGG; *tuba1*, F: GCCAGATGCCGTCTGACAA, R: AGTC-TCGCTGAAGAAGGTGTTGA.

The following primers were used for dsRNA knockdown in S2 cells: *Relish*, F: GCATGGAA-CACATGGATCGC, R: CTGATGGGAATGTGGG-CTGT; *Spätzle*, F: CTCTCGCTGTCGTGTGTTCT, R: TTCCTTTGCACGTTTGCGAG.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Select components of the IMD and Toll pathways are required to eliminate WT loser cells in Myc-induced cell competition

(A) Schematic of the assay for Myc-induced cell competition. See the materials and methods section for details. (B) Mean clone size in wing discs of the indicated genotype. In all figures, gray bars show the mean size of control clones (generated in the absence of cell competition), green bars show the mean size of loser clones in WT wing discs, and white bars show the mean size of loser clones in wing discs of the indicated genotype. The number of clones scored for each genotype is indicated in each graph. Mutations in *PGRP-LC*, *BG4/FADD*, *Dredd*, and *Rel* of the IMD pathway and mutations in the Toll pathway gene *spz* suppress elimination of the loser cells, resulting in a significant increase in clone growth. Mutations in most genes in the Toll pathway (including *dl*, *Dif*, and *MyD88*) encoding a TIR-domain protein do not alter the outcome of WT loser cells. (C) The TIR-domain protein Ect4/dSarm is required to eliminate WT loser cells in wing discs. The *Ect4⁴²⁷³* mutant does not affect control wing disc clones but completely suppresses elimination of loser cells. (D) Simplified schematic of the *Drosophila* innate immune pathways. In the IMD pathway, signaling from the transmembrane protein PGRP-LC, IMD/RIP, FADD, and DREDD/Caspase-8 causes endo-proteolytic cleavage of Relish (Rel), removing an autoinhibitory domain and allowing the Rel-homology domain (RHD/Rel-68) to translocate to the nucleus and activate AMP genes. In the Toll pathway, binding of Spz to the Toll receptor recruits a complex consisting of Myd88, Tube/IRAK-4, and Pelle/IRAK-1, which phosphorylates Cactus/IκB and targets it for degradation. This releases Dorsal or Dif for activation of

distinct AMP genes in the nucleus. Components outlined in red denote those also required for Myc-induced cell competition. P, phosphorylation. (**E** and **F**). Null mutations in *Toll-3*, *Toll-8*, or *Toll-9* suppress elimination of loser cells, increasing loser clone size (**E**) and cell number per clone (**F**), but do not alter control clones. Error bars in this figure are SEM. All *P* values are relative to WT loser clones except in (**E**) at left—they are relative to WT control clones. ****P* < 0.001. ns, not significant (Mann-Whitney test).

Fig. 2. Dredd-mediated Rel activation eliminates loser cells

(A) Rel and Tollo are required for death of loser cells. TUNEL assays showing cell death in control clones, WT loser clones, and loser clones in *Rel^{E20}* and *tollo^{R23B}* mutant backgrounds 24 hours after clone induction. Many WT loser cells are TUNEL-positive, but their death is suppressed in the mutant backgrounds. (B) Quantification of data from (A). (C) Expression of *Rel*-RNAi (or *Rel*-RNAi and Dicer to enhance RNAi efficiency) within loser cells suppresses their loss but does not affect control clones. (D) Dredd and Rel are critical for elimination of WT loser cells. Loss of *Dredd* suppresses the loss of loser clones but does not affect the growth of control clones. Whereas *Dredd^{B118/+}* has little effect on loser death, in combination with *Rel^{E20/+}* it strongly suppresses death. (E) Expression of *Dredd* in the absence of competition reduces clone size, but this is blocked by coexpression of *Rel*-RNAi. The number of clones scored for each genotype is indicated. All *P* values are relative to WT loser clone size except in (C): ****P* < 0.001. ns, not significant (Mann-Whitney test).

Fig. 3. Rel triggers death of WT loser cells by inducing the proapoptotic factor Hid

(A) *Rel* is required to kill naïve S2 cells. cCM from control co-cultures or those treated with dsRNA against GFP increased death of naïve cells, as measured by activated caspase-3 staining. Cell death was prevented by dsRNA against *Rel*. Both cocultures and naïve cells in these assays were treated with the indicated dsRNA. Error bars denote SD. (B) Cell competition increases nuclear translocation of Rel. Nuclear localization of Rel-GFP in transfected WT-S2 cells treated $\pm$ PGN, noncompetitive (nc) CM, or competitive (c) CM for 4 hours is shown. Error bars indicate SD. (C and D) Wing disc with control, GFP-expressing cell clones (C) and cell clones expressing UAS-*Rel68* (activated Rel) and UAS-GFP (D) from animals carrying a *lacZ* reporter insertion in the *hid* locus (*hid-lacZ*, red). DNA is stained with Hoechst (blue). *hid-lacZ* is not induced in control clones (C and C'). *Rel68*-expressing clones activate expression of *hid-lacZ* (D and D'), grow poorly, and are small compared with controls (E and G) but are rescued by the mutation caused by the *hid^{PlacZ}* insertion (F and G). Clones in (D) are shown at higher magnification to facilitate resolution of *hid-lacZ* expression. (G) Average clone size of control clones (GFP), *Rel68*-expressing clones in a +/+ background, and *Rel68*-expressing clones in the *hid^{PlacZ}/+* mutant background. Error bars denote SEM. (H) Expression of UAS-*Rel68* in WT wing discs induces *hid* mRNA but not *rpr* mRNA in quantitative RT-PCR experiments on RNA isolated from wing discs. Error bars indicate SD. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. ns, not significant (Mann-Whitney test).

Fig. 4. Competitive context influences selection of the TRR-NF κ B signaling module and proapoptotic inducer. (A)

Scheme for larval screening of innate immune pathway components in competition between WT and $M^{RpL14/+}$ cells. GFP-positive wing disc clones ($M^{RpL14/+}$ loser cells) are generated in a background of essentially WT cells ($M^{RpL14/+}$ rescued with $gRpL14$); see materials and methods for details. The GFP- $M^{RpL14/+}$ loser clones are competitively eliminated and thus not present in larvae. If competition is suppressed by overexpression or RNAi, the GFP-positive wing disc clones persist in the larvae. **(B)** Competitive elimination of $M^{RpL14/+}$ loser cells is predominantly triggered by Dorsal and Dif. Overexpression of Cactus, RNAi against dl or Dif , or $dl^1/Df(2L)TW119$, a deficiency that removes both dl and Dif , each suppress loss of $M^{RpL14/+}$ loser cells. Over-expression of UAS- $RpL14$ was used as a control for clone survival. See fig. S6 for the full data set. **(C and D)** Cell death mediates $M^{RpL14/+}$ cell competition. **(C)** Apoptosis, marked by TUNEL staining, is high in $M^{RpL14/+}$ loser cells but is reduced when Dorsal and Dif activity are blocked by UAS-*cactus* expression. **(D)** TUNEL staining of wing discs with UAS-*GFP*-expressing clones, quantified in **(C)**. Left to right: control noncompetitive (nc) $M^{RpL14/+}$ wing disc rescued by $gRpL14$ expression; control nc *act>Gal4*, UAS-*GFP* clones generated in unrescued $M^{RpL14/+}$ wing disc; $M^{RpL14/+}$ loser clone generated in $M^{RpL14/+}$ *salE>gRpL14* disc; and $M^{RpL14/+}$ loser clone expressing UAS-*cactus* generated in $M^{RpL14/+}$ *salE>gRpL14* disc. **(E)** Competitive elimination of $M^{RpL14/+}$ loser cells requires cell death induced by Reaper (*Rpr*). Elimination of $M^{RpL14/+}$ loser clones is prevented by expression of the apoptosis inhibitor *p35*; by one copy of the *H99* deficiency that removes *hid*, *rpr*, and other proapoptotic genes; and by RNAi against *rpr*, but not RNAi against *hid*. Number of larvae scored per genotype is indicated. **(F)** *dorsal*-expressing clones are smaller than WT sibling

clones. Mitotic recombination generated GFP-positive clones that express UAS-*dorsal* and WTGFP-negative sibling clones. The graph shows the mean clone size of the indicated number of clones. (G) Expression of UAS-*dorsal* and UAS-*Dif* induces expression of *rpr*, but not *hid*, in quantitative RT-PCR experiments on RNA isolated from wing discs. Error bars in (B) and (E) to (G) denote SD; error bars in (C) indicate SEM. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ (Student's *t* test).

Fig. 5. A model of the role of TRR-NFκB modules in cell competition

A model for TRR-NFκB function in cell competition, incorporating data from both competitive contexts. See text for full description. We propose that in both *Minute*- and Myc-induced competitive contexts, loser cells are eliminated through a signaling pathway mediated by Spz, TRRs, and NFκB function. Context-and/or genetic-dependent interactions that cause Rel activation trigger expression of *Hid*, whereas those activating *Dif* and *Dorsal* trigger expression of *Rpr*. Apoptosis is the ultimate fate of both loser populations. Red denotes genes required for Myc-induced competition, blue indicates those required for competition in *M/+* mosaics, and orange denotes shared requirements.

Table 1
Suppression of $M^{RpL14-/+}$ loser clone elimination by Toll and IMD pathway genes

P values are compared with that of $M^{RpL14-/+}$ (*t* test).

Genotype	% larva with GFP ⁺ clones	SD	<i>P</i> value
$M^{RpL14-/+}$	15.5	3.7	
<i>M^{RpL14-/+} + overexpression</i>			
UAS- <i>RpL14</i>	80.5	2.5	4.30×10^{-5}
UAS- <i>p35</i>	43.8	2.3	4.40×10^{-7}
UAS- <i>cact</i>	49.2	1.4	0.03
<i>M^{RpL14-/+} + RNAi</i>			
UAS- <i>cact</i>	6.6	4.7	0.03
UAS- <i>dl</i>	41.8	7.1	0.00
UAS- <i>Dif</i>	37.0	11.9	0.03
UAS- <i>pelle</i>	24.3	1.4	0.01
UAS- <i>Toll-3</i>	39.3	13.3	0.03
UAS- <i>Toll-9</i>	30.1	8.0	0.03
UAS- <i>spz</i>	28.8	8.2	0.04
UAS- <i>imd</i>	27.0	4.2	0.01
UAS- <i>Rel</i>	29.6	6.8	0.02