Int. J. Dev. Biol. 61: 319-327 (2017)
doi: 10.1387/ijdb.160250ao

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

The SUMO-targeted ubiquitin ligase, Dgrn, is essential for *Drosophila* innate immunity

BELLA KOLTUN[1], ELIZA SHACKELFORD[1], FRANÇOIS BONNAY[2], NICOLAS MATT[2], JEAN MARC REICHHART[2] and AMIR ORIAN*[,1]

[1]Rappaport Research Institute and Rappaport Faculty of Medicine, Technion Integrated Cancer Center, Technion - Israel Institute of Technology, Haifa, Israel and [2]UPR9022 du CNRS, Institut de Biologie Moléculaire et Cellulaire Université de Strasbourg, France

ABSTRACT The ability of metazoans to combat pathogenic infection involves both systemic and local responses to the invading pathogens. Ubiquitin and SUMO pathways molecularly regulate the response to infection, immune signaling and gene expression. Here, we report that Degringolade (Dgrn, CG10981), a SUMO-targeted ubiquitin ligase connecting the two pathways, is essential for the innate immunity response in *Drosophila*. *dgrn*^{DK} null and heterozygous mutant adult flies are severely immune-compromised and succumb rapidly to both pathogenic bacteria and fungi infections. The sensitivity to infection stems from the inability to produce multiple anti-microbial peptides, and transcriptional analyses suggest that the overexpression of Dgrn enhances the transcriptional output of the NF-κB related Toll and immune deficiency (IMD)-pathways. Moreover, expression of Dgrn alleviated the inhibitory impact of the cytoplasmic NF-κB inhibitor Cactus and the nuclear co-repressor Groucho/TLE (Gro). Additionally, we found that Dgrn is required for the local regenerative response of the mid-gut following infection. Upon oral infection, *dgrn* mutant flies fail to activate the Delta-Notch pathway in stem cells and enteroblasts, and are unable to regenerate and replace the damaged and dying enterocytes. Interestingly, the ubiquitin-specific protease CG8334 (dUSP32/dUSP11) antagonizes Dgrn activity in the gut, and halving the dose of CG8334 restores Delta-Notch signaling and rescues the lethality observed in *dgrn* mutants. Collectively, our data suggest that Dgrn is essential for both systemic and local tissue response to infection.

KEY WORDS: *Drosophila, innate-immunity, STUbL, SUMO, ubiquitin*

Introduction

Post-transcriptional modifications (PTMs) by ubiquitin (Ub) and ubiquitin-like proteins (UbLs) regulate both innate and adaptive immune responses (van der Veen *et al.,* 2012; Everret *et al.,* 2013; Popovich *et al.,* 2014; Heaton *et al.,* 2016). In mammals and *Drosophila*, NF-κB pathway and REL-transcription-factors (TFs) form the core network that regulates the host response against infection. REL TFs are essential for development and their deregulation contributes to a wide variety of human diseases (Ghosh *et al.,* 2012). The *Drosophila* NF-κB protein family is comprised of three REL TFs: Dorsal, Dif and Relish. Specifically, in adult flies, activation of Toll pathway genes is mediated by TF Dif, in response to Gram-positive bacteria and fungal infections. On the other hand, the immune deficiency (IMD) pathway and TF Relish are required to cope with Gram-negative bacteria (Buchon *et al.,* 2014). In un-stimulated cells, REL TFs are sequestered in the cytoplasm by IκB/Cactus inhibitory proteins. Upon a variety of signals (e.g., infection, cytokines, etc.), receptor-mediated activation initiates a signaling cascade involving multiple events of ubiquitylation, leading to the degradation of the inhibitory proteins (Kanarek *et al.,* 2012). Subsequently, REL proteins translocate to the nucleus where they regulate the expression of target genes (Wan *et al.,* 2010). In *Drosophila* exposure to microbial threats at the level of epithelia drives a potent local immune response based on anti-microbial peptides

Abbreviations used in this paper: AMP, anti-microbial peptide; Dgrn, degringolade; DSS, dextran sulfate sodium; Gro, groucho; IMD, immune deficiency; PTM, post-transcriptional modification; SUMO, small ubiquitin-like modifier; UbL, ubiquitin-like protein.

***Address correspondence to:** Amir Orian. 11th floor, Rappaport Faculty of Medicine, Technion – Israel Institute of Technology, 1 Efron St., Bat-Galim, POB 9649, Haifa 31096, Israel. e-mail: mdoryan@techunix.technion.ac.il -Tel.: 972-4-829-5448/9. Fax: 972-4-852-394. http://orcid.org/0000-0002-8521-1661

Supplementary Material (one figure) for this paper is available at: http://dx.doi.org/10.1387/ijdb.160250ao

Accepted: 10 August, 2016.

ISSN: Online 1696-3547, Print 0214-6282

(AMPs) synthesis and activation of resilience mechanisms such as enterocyte-renewal in the gut epithelium (Ferrnadon D, 2013). In addition, invasion of the body-cavity by microbes activates the systemic innate immune response, which consist in the massive release of AMPs by the fat-body (the equivalent of the mammalian liver) (Ferrandon *et al*., 2007).

Similar to ubiquitylation, UbL pathways regulate innate response, and one such UbL pathway is SUMOylation. SUMOylation is initiated by the activation of SUMO, a ubiquitin-like molecule, by the SUMO-specific activating enzyme (E1). It also requires the activity of the SUMO conjugating E2 (Ubc9) and E3-SUMO ligases. SUMOylation is reversible by the activity of SUMO-specific peptidases SENPs (Flotho *et al.,* 2013). SUMOylation regulates NF-κB signaling and NF-κB-dependent transcription in the nucleus. It also regulates the activity of other immune pathways involved in the response to infection (Liu 1998; Lee *et al.,* 2011; Lee 2009; Decque *et al.,* 2016). Indeed, mutants deficient for the SUMO pathway exhibit an abnormal immune response, and targeted inactivation of the SUMO-conjugating E2, Ubc9, was developed by *Listeria* to evade recognition by the immune system and promote efficient infection (Ribet *et al.,* 2010; Paddibhatla *et al.,* 2010).

Ubiquitylation and SUMOylation are connected molecularly in part by SUMO-targeted ubiquitin ligases (STUbL). Conserved from flies to humans, STUbLs are a unique group of RING proteins: they bind non-covalently to the SUMO moiety of SUMOylated proteins via their N-terminal SUMO Interacting Motifs (SIMs) domains, and subsequently target the SUMOylated protein for ubiquitylation via their C-terminal RING domain. Therefore, they are able to "sense" SUMOylated proteins and modify them by ubiquitylation. STUbL-mediated ubiquitylation in many cases leads to proteasomal degradation, but also affects protein-protein interactions, protein localization and activity (Sriramachandran and Dohmen. 2014).

In the fly, a single STUbL exists termed Degringolade (*dgrn*, CG10981), which is essential for embryonic development (Abed *et al.,* 2011; Berry *et al.,* 2011). Dgrn is required for proper response to DNA damage/replication stress, and the regulation of gene expression during segmentation and sex determination (Ryu *et al.,* 2015; Abed *et al.,* 2011; Barry *et al.,* 2011). We previously found that *dgrn*^{DK} null adult males are fertile, yet *dgrn*^{DK} *null* females are viable however sterile, laying embryos that do not hatch (Barry *et al.,* 2011). Termed the maternal effect female sterile, this phenotype is also observed in mutants of the Toll pathway that are characterized by a defective immune system (Anderson *et al.,* 1985).

Here, we report that Dgrn is a new key ubiquitin ligase required for both systemic and local immunity in *Drosophila*. First, *dgrn* adult flies are immune-compromised and sensitive to multiple pathogenic threats. We found that Dgrn is essential during systemic immune response to infection essential for the transcription of Toll- and IMD-dependent gene expression. Second, Dgrn was required for a Notch-dependent, local stem cells regeneration upon gut infection. We also identified *CG8334*, an ubiquitin hydrolase, as a gene that antagonizes *dgrn* function in the gut.

Results

dgrn^{DK} *null adults are immuno-compromised and hyper-sensitive to infection*

In the adult fly, the Toll pathway is required for coping with Gram-positive bacteria and fungal infections (Buchon *et al.,* 2014). Therefore, we first tested the ability of *dgrn*^{DK} *null mutants* to resist

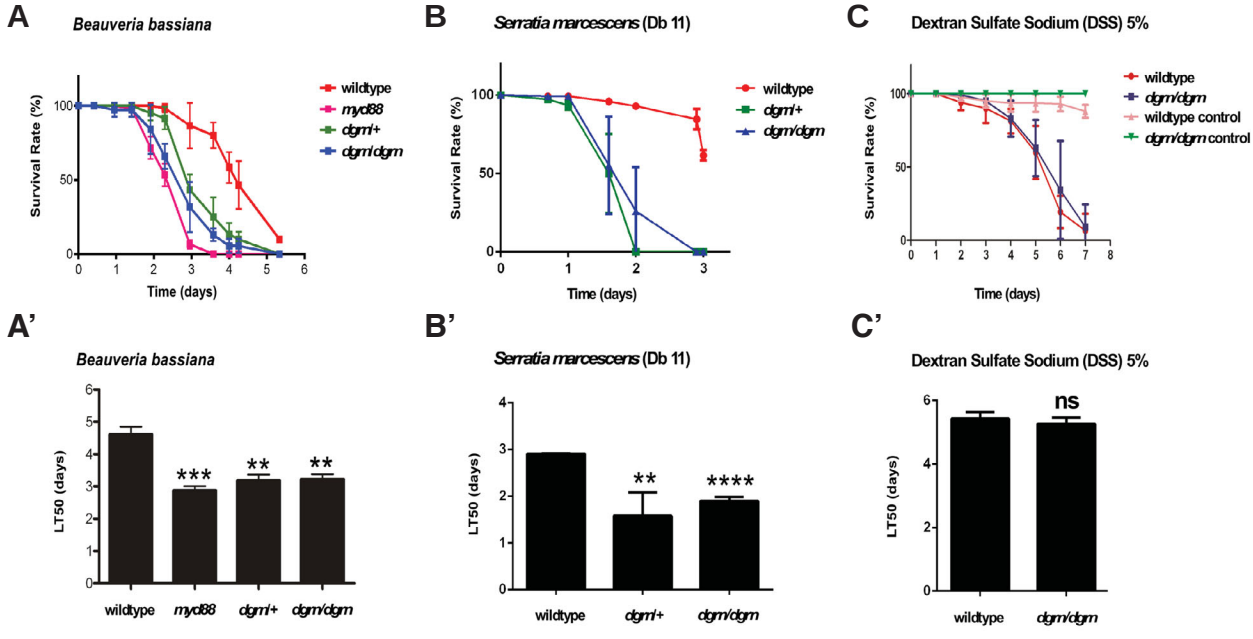


Fig. 1. *dgrn*^{DK} mutant flies are immune-compromised and sensitive to infection by diverse pathogenic microorganisms. (A-C) *Survival analysis of adult flies upon infection with the indicated fungus, bacteria or challenges by Dextran Sulfate Sodium (DSS) was performed as described under Materials and Methods.* **(A, A')**. *Infection with* Beauveria bassiana *fungal spores,* myd88c^{03881} *serves as a positive reference.* **(B, B')** *Oral infection with the Gram(-) bacteria* Serratia maracescans *(*Sm, *Db11).* **(C, C')** *Exposure to 5% DSS in A'-C' LT50 is determined as the time in days when 50% of the infected flies succumbed to infection. For pathogenic challenges n=20 for each genotype, and n=50 in DSS exposure in each individual experiment. Three independent biological repeats were performed in each set of experiments.*

fungal infection by performing natural *Beauveria bassiana* infection, a classical experiment to assess the Toll-pathway (Lemaitre *et al.*, 1997). We observed that under non-challenged conditions, the survival of adult *dgrn* mutants was identical to that of wild-type flies. However, exposure to *Beauveria bassiana* spores resulted in earlier lethality of $dgrn^{DK}$ heterozygous and homozygous adults to the fungus compared to wild-type flies. This sensitivity was similar to that observed with mutants for Myd88, an essential Toll pathway scaffold protein (Fig. 1A,A', data not shown).

The enhanced susceptibility of $dgrn^{DK}$ mutants to infection was also observed upon exposure to Gram-negative bacteria, known agonists of the IMD pathway. $dgrn^{DK}$ mutant flies exhibit enhanced sensitivity to intestinal infection with the Gram-negative bacteria *Serratia marcescens* (*Sm* DB11) and *Pseudomonas aeruginosa* (*PA*14; Fig. 1 B,B',S1A). This sensitivity was similar to that observed in $kenny^{1}$ mutants, deficient for a central kinase within the IMD pathway (not shown). Nonetheless, the survival of $dgrn^{DK}$ null mutants that were exposed to a chemical intestinal stressor (5% Dextran Sulfate Sodium, DSS) was identical to that of wild-type flies (Fig. 1 C,C'), ruling out the possibility that the observed *dgrn* susceptibility to pathogenic infections originated from an epithelial weakness.

dgrn^{DK} *null adults fail to express AMP genes upon infection*

A hallmark of the systemic immune response is the production of anti-microbial peptides (AMPs, Ferrandon *et al.*, 2007). We hypothesized that the increased sensitivity to infection observed in $dgrn^{DK}$ mutants stems from the inability to express AMP genes. Therefore, we monitored the expression of endogenous mRNA of AMP genes upon various pathogenic challenges. In contrast to wild-type flies, $dgrn^{DK}$ mutants failed to express Toll-related AMPs (*drosomycin, IM-1, metchnikowin* and *defensin*) in response to infections with the Gram-positive bacteria *M. luteus* or the fungus *Beauveria Bassiana* (Figs. 2 A-F). These decreased AMPs mRNA levels were comparable to those of *myd88* mutant flies. Likewise, we observed that Dgrn was essential for the expression of the IMD-dependent AMP genes *attacin-A, -B, -C, -D*, *diptericin* and *drosocin* upon infection with the Gram-negative bacteria *E. coli*. Similar decreased AMPs levels were observed in IMD-deficient *kenny* mutant flies (Fig. 2 G-L). Altogether, these results indicate that *dgrn* is required for *Drosophila* innate immune response. *dgrn* mutants are immune-compromised and fail to express AMP genes in response to infection with pathogens that activate either the Toll or IMD signaling pathways.

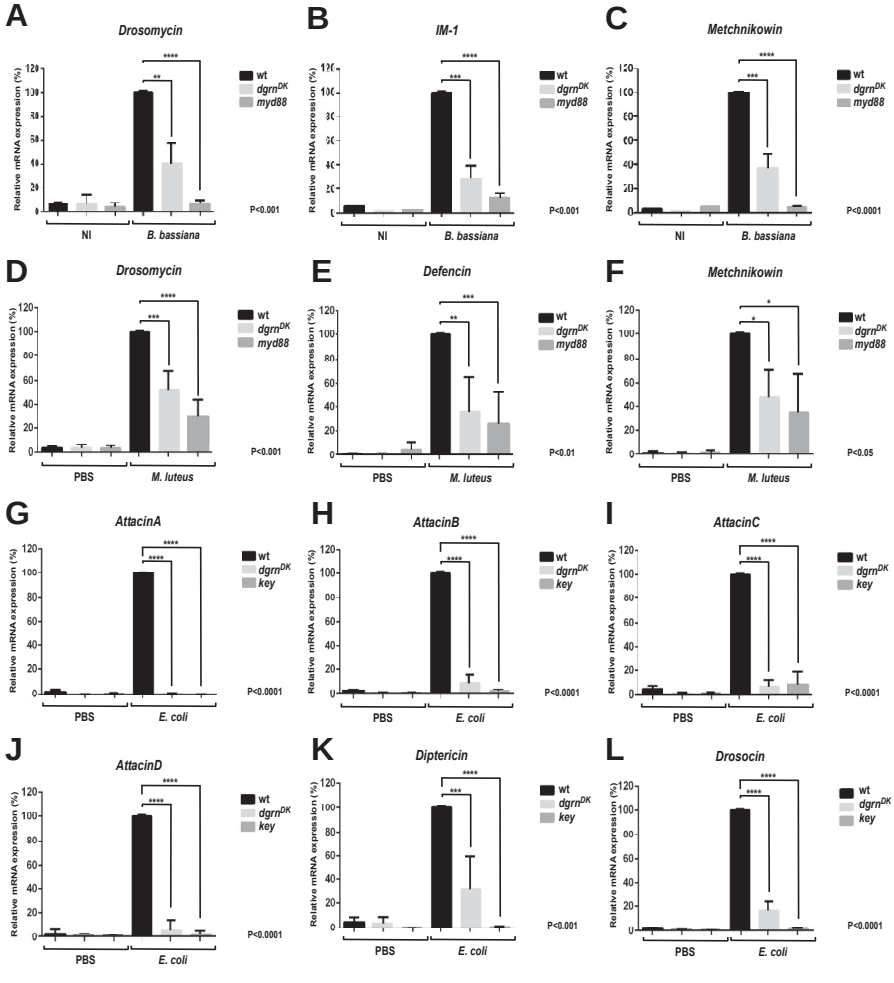


Fig. 2. *dgrn* is required for the expression of Toll- and immune deficiency (IMD)-dependent anti-microbial peptides (AMPs) upon infection. *Real-time qPCR analyses of mRNA levels of the indicated AMPs. Adult flies with the indicated genotypes were infected with* B. bassiana *spores* **(A-C)**, *pricked with PBS (control), or either with Gram(+) bacteria* M. luteus, **(D-F)**, *or the Gram(-) bacteria* E. coli. **(G-L)** *AMP tested:* drosomycin *(A, D),* IM-1 *(B),* metchnikowin *(C, F),* defencin *(E),* attacinA *(G),* attacinB *(H),* attacinC *(I),* attacinD *(J),* diptericin *(K),* drosocin *(L).* myd88 $c038^{81}$ *is a mutant within the Toll pathway (A-F).* keyc0283^{1} *is a mutant of the obligatory kinase* kenny *within the IMD pathway (G-L). n=10 for each genotype, and three biological repeats for each set of experiments were performed.*

Dgrn enhances NFκB-dependent gene expression in S2 Drosophila *cells*

To gain insight into the role of *dgrn* in the transcriptional activation of AMP genes, we used NF-κB luciferase reporter assays in S2 cells. We activated the IMD or Toll pathway by over-expressing the constitutively active IMD pathway PGRP-LCa receptor or the constitutively active truncated Toll receptor ($Toll^{\Delta LRR}$), respectively (Goto *et al.,* 2008) (Fig. 3A). Co-expression of Dgrn enhanced the transcriptional activation of the κB reporter by 2-3 fold compared to constitutively active receptors alone (Fig. 3A). Next, we studied the transcriptional effect(s) of Dgrn on the activation of the Toll pathway and its TF Dif. In unchallenged cells, Dif is sequestered predominantly in the cytoplasm by the IκBα-like inhibitor, Cactus. Toll-induced phosphorylation of Cactus results in the ubiquitylation and degradation of Cactus, enabling the nuclear translocation of Dif. In the nucleus, Dif induces the transcription of AMP genes such as drosomycin (Meng *et al.,* 1999). As expected, ectopic expression of Cactus inhibited a Toll-mediated activation of

drosomycin-Luc (Drs-Luc; Fig. 3B). However, co-expressing Dgrn alleviated Cactus inhibition and fully restored luciferase expression. Importantly, this function of Dgrn required its ligase activity as a catalytically inactive mutant in the RING domain ($Dgrn^{HC/AA}$) was unable to alleviate Cactus-mediated repression (Fig. 3B). These results suggest that Dgrn functions in parallel or downstream of Cactus in the Toll pathway signaling cascade.

One potential downstream nuclear substrate of Dgrn in this process would be the co-repressor Groucho that, during development Gro binds to REL transcription factor Dorsal (Cinnamon *et al.,* 2008). Moreover, we previously described that Dgrn inactivates the SUMOylated co-repressor Gro during development (Abed *et al.,* 2011). Indeed, we observed that Gro expression inhibited the Toll-mediated activation of the Drs-Luc reporter (Fig. 3C). Remarkably, the co-expression of Dgrn, but not its catalytically inactive mutant, alleviated Gro-dependent repression (Fig. 3C).

Next, we tested whether Dgrn's potentiation of *Drs* transcriptional activity in this setting required the TF Dif. Suppression of Dif via RNAi, but not control GFPi, resulted in reduced Dif protein levels and a concomitant decline in $Toll^{\Delta LRR}$-dependent transcriptional activation (Figs. 3D, D'). Remarkably, over-expression of Dgrn in these conditions restored $Toll^{\Delta LRR}$-dependent activation (Figs. 3D). Furthermore, the RNAi-dependent reduction in Dif protein levels were restored by the expression of Dgrn (Fig. 3D'). Taken together these results suggest that the residual Dif protein observed in Dgrn expressing cells are more potent in activating transcription, or that Dgrn functions in a Dif-independent parallel pathway. Collectively, this set of gain of function experiments suggests that Dgrn potentiates Toll- and IMD-dependent transcriptional activation and likely antagonizes the co-repressor Gro at the level of Toll pathway target genes.

Dgrn is required for local regenerative response of the gut to infection

In addition to the systemic response to infection, the local tissue response to infection is central for combating pathogens invasion (Lemaitre *et al.,* 2007). In both vertebrates and flies, the gut is the habitat of commensal bacteria but also the entry gate for pathogenic micro-organisms. Upon infection, enterocytes are lost and the integrity of the epithelial tissue is hampered. Subsequently, a regenerative response is initiated by signals secreted by the dying enterocytes, and results in hyper-proliferation of intestinal stem cells (ISCs) that are required to replace the damaged/dying enterocytes (ECs). One signaling pathway that is involved in regeneration in response to infection is Notch. In this case, infection of the gut epithelia results in enhanced expression of the Notch ligand Delta receptor on the surface of intestinal stem-cells (ISC) and the subsequent activation of Notch signaling and expression of Notch target genes in the adjacent cells, the enteroblasts (Jianian *et al.,* 2012).

Since $dgrn^{DK}$ mutants are sensitive to oral infection with *Sm Db11* (Fig. 1B), we monitored the local changes and activity of Notch signaling in the adult mid-gut in mock or *Sm* challenged flies. Upon infection, control guts showed an increase in the expression of the Notch ligand, Delta, on the surface of ISC and hyper-activation of the Notch-reporter transgene in enteroblasts (EBs) (Figs. 4 A-B'). These changes were also accompanied by an increase in mitosis (cells positive for the mitotic marker p-histone-3) (Figs. 4 A",B"). In sharp contrast, *dgrn* mutants failed to enhance delta expression on the surface of ISC and showed only minimal expression of the

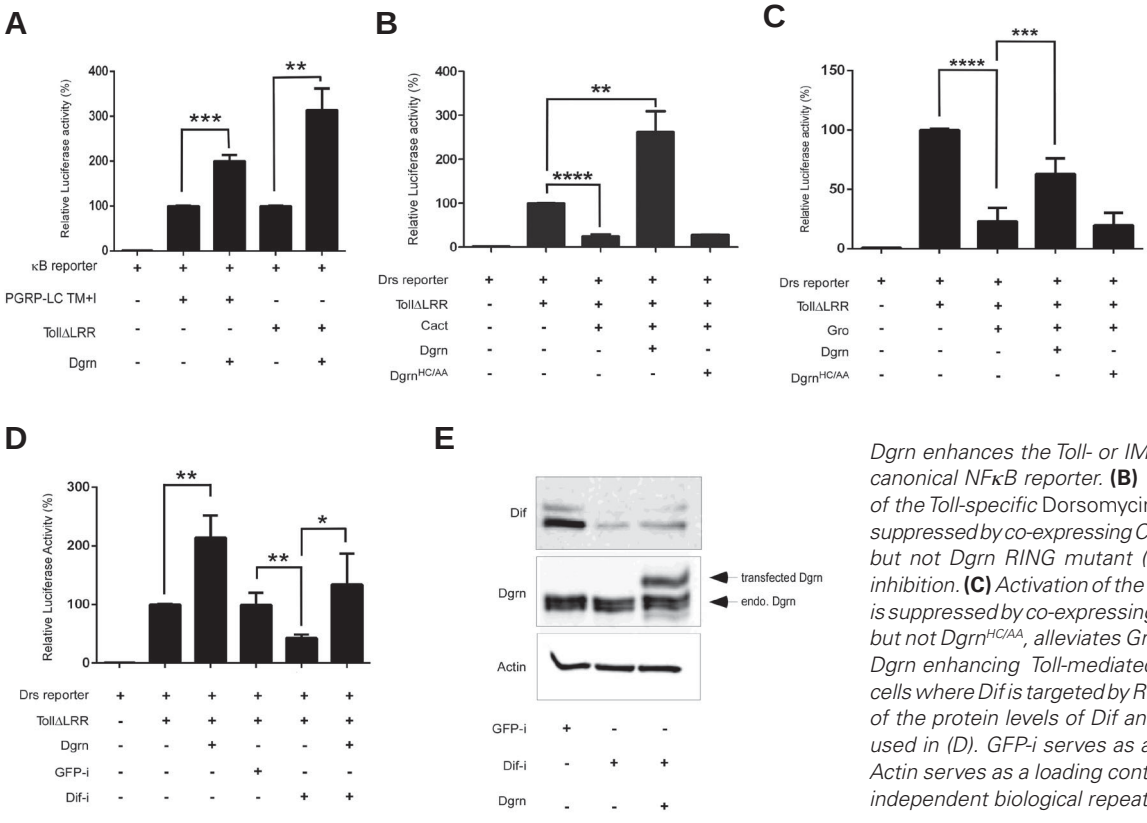


Fig. 3. Dgrn enhances Toll- and IMD-dependent transcriptional activation in S2 *Drosophila* cells. (A-D) *Relative NFκB-luciferase reporter activity in S2 cells transfected with the indicated plasmids and luciferase reporters as described under Materials and Methods.* **(A)** *Expression of Dgrn enhances the Toll- or IMD-dependent activation of a canonical NFκB reporter.* **(B)** *$Toll^{\Delta LRR}$-dependent activation of the Toll-specific* Dorsomycin *(Drs)-Luciferase reporter is suppressed by co-expressing Cactus. Co-expression of Dgrn, but not Dgrn RING mutant ($Dgrn^{HC/AA}$), alleviates Cactus inhibition.* **(C)** *Activation of the* Dorsomycin *(Drs)-Luciferase is suppressed by co-expressing Gro. Co-expression of Dgrn, but not $Dgrn^{HC/AA}$, alleviates Gro-dependent repression.* **(D)** *Dgrn enhancing Toll-mediated activation is reduced in S2 cells where Dif is targeted by RNAi.* **(E)** *Western blot analysis of the protein levels of Dif and Dgrn in RNAi treated cells used in (D). GFP-i serves as a non-specific RNA-i control. Actin serves as a loading control. In all experiments, three independent biological repeats were performed.*

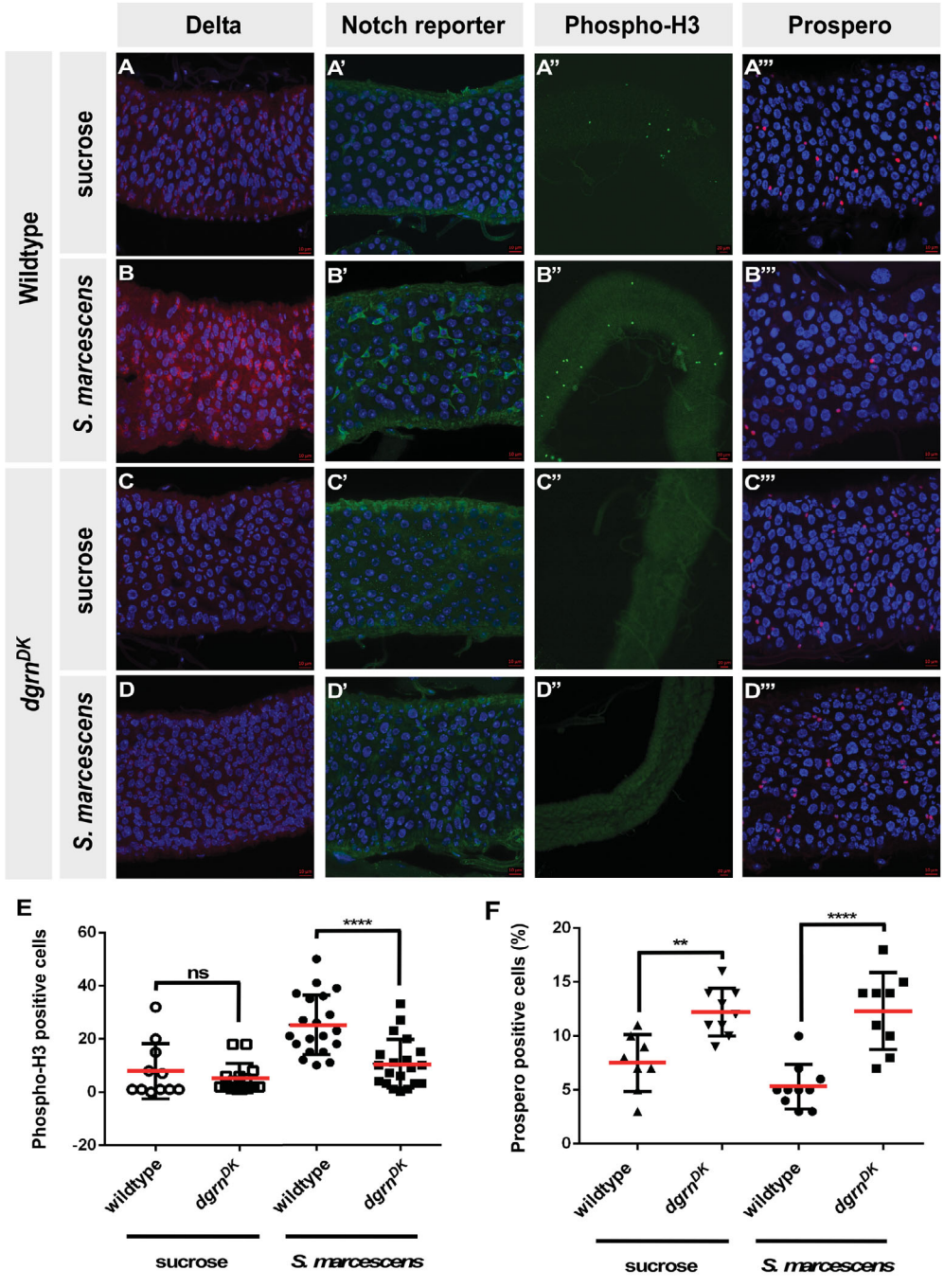


Fig. 4. Dgrn is required for local gut response to oral infection with *S. marcescens*. (A-D''') *Representative confocal images of* Drosophila *adult mid-gut of the indicated genotypes wild-type (A-B''') and* dgrnDK *(C-D'''). (A-A''', C-C''') are guts derived from sucrose-fed adult flies. (B-B''', D-D) are guts derived from flies orally infected with* Sm(Db11). *Forty-eight hours after infection, guts were analyzed for the expression of the Notch ligand and ISC marker, Delta (Red, A-D) and Notch reporter activity (Green, Gbe-Su(H)>lacZ; A'-D'). Mitosis was determined by phospho-Histone-3 (Green, Phsopho-H3; A''-D'') and α-Prospero antibody (Red) marks entero-endocrine cells (A'''-D'''). DAPI (Blue) marks DNA.* **(E)** *Quantification of mitotic cells (p-H3 positive) in wild-type and* dgrnDK *guts following oral infection with* Sm *or sucrose control. Each dot represents the number of phsopho-Histone-3 positive cells in whole gut. In all experiments at least 7 individual guts were examined for each genotype, and three biological independent repeats were performed.* **(F)** *Quantification of entero-endocrine cells (Prospero$^{(+)}$) in wild-type and* dgrnDK *guts following oral infection with* Sm(Db11) *or sucrose control. Individual guts are represented as a single dot on the graph. The number of positive cells per gut section was normalized to total number of cells observed in the same section.*

Notch-reporter in EBs (Figs. 4 C-D'). Likewise, *dgrn* mutants showed overall less mitosis in comparison to wild-type derived guts (compare Fig. 4A" and 4 B",C",D", and quantitated in 4E). In addition, we evaluated the number of entero-endocrine cells (EEs, Prospero positive cells) in control or *Sm*-infected guts. We found that the number of EEs was higher in *dgrn* mutants than in wild-type flies even without infection. However, we did not observe a dramatic change in the average number of EEs upon infection (Figs. 4 A''',B''',C''',D''',4F). We concluded that Dgrn is required for the Notch-dependent-regenerative response in the gut upon infection.

The iso-peptidase CG8334 *antagonizes Dgrn during the local gut response to infection*

Ubiquitylation is reversible by the activity of ubiquitin-specific proteases (Heride *et al.*, 2014). In this regard, of interest is *CG8334*, which is closely related to both dUSP11 and dUSP32. USP11 is a known component of the DNA damage response (Yu *et al.*, 2016) and was recently identified as an RNF4-associated protein capable of counteracting RNF4 function in human cancer cells (Hendriks *et al.*, 2015). Therefore, we hypothesized that the fly ortholog *CG8334* could potentially antagonize Dgrn function *in vivo*. Performing a genetic epistasis assay, we found that while *dgrn* heterozygous mutant flies (+;+;$dgrn^{DK}$/+) rapidly succumb to *Sm* infection, this lethality was greatly suppressed by halving the dose of *CG8334* (+ $dgrn^{DK}$/$CG8334^{p}$ +) (Figs. 5 A, A' and S1B). Moreover, halving the dose of *CG8334* (+ $dgrn^{DK}$/$CG8334^{p}$ +) restored the ability of ISCs to express Delta in the absence and upon *Sm* infection (Figs. 5 B-D''' and quantitated in 5E). These data fit well with the observation that in human glioma-derived cells USP11 expression is repressed by Notch (Wi *et al.*, 2014), implying that *CG8334* is a negative regulator of Notch signaling and Dgrn during the local response to infection and gut homeostasis. Yet, halving the dose of *CG8334* did not restore the inability of *dgrn* mutants to express the anti-microbial peptide *att* upon

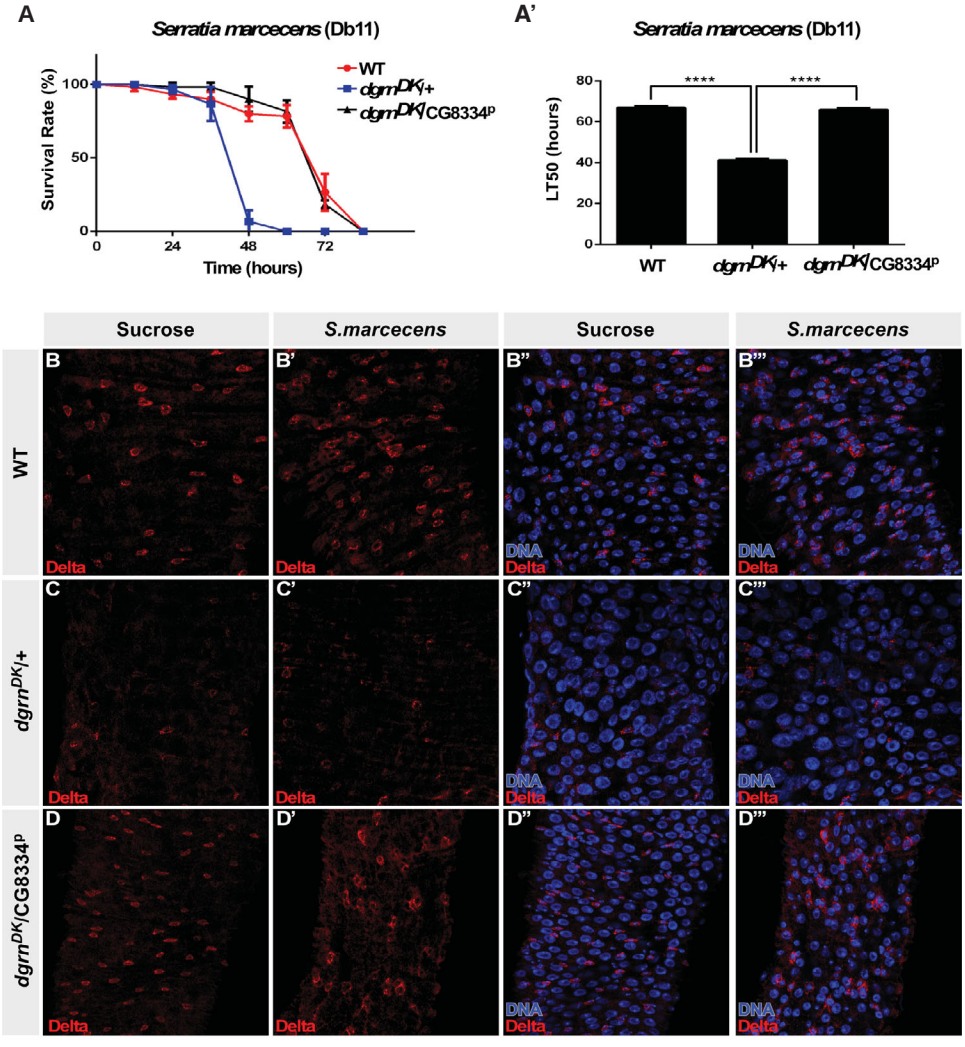
pricking with *E. coli* (not shown). Suggesting that the activity of *CG8334* is context specific, antagonizing Dgrn activity in the gut in the context of Notch signaling, but not in the context of the IMD pathway and the transcription of AMPs upon pathogenic challenges.

Discussion

The *Drosophila* SUMO-Targeted ubiquitin ligase Dgrn connects ubiquitylation and SUMOylation. Here, we report that Dgrn is required for both systemic and local responses to infection. *dgrn* heterozygous and null mutant flies are immuno-compromised and unable to express AMP genes upon infection, and rapidly succumbed to various pathogens. We found that Dgrn enhances the transcription of Toll and IMD target genes, and alleviated the repressive activity of the cytoplasmic inhibitor Cactus, as well as the nuclear co-repressor Gro. In addition, Dgrn is required within the adult mid-gut for the Notch-dependent stem cell regenerative response to infection, an activity that is counteracted by the ubiquitin-specific peptidases *CG8334*, which is highly similar to the mammalian USP11/USP32.

Dgrn transcriptional activity

We previously found that Dgrn functions as a "selector" that determines co-repressors recruitment during development (Abed *et al.*, 2011). Our current study reveals that Dgrn activity is not limited to transcriptional repression, but that Dgrn is required for pathogen- and signal-induced transcriptional activation of genes essential for innate immune response. However, while *dgrn* null mutants failed to express all endogenous AMPs tested, we found that *dgrn* null mutants express established IMD- and Toll-reporter transgenes indifferently from control flies (data not shown). This difference suggests that the signaling pathways and basal transcriptional machinery are intact and are not the molecular target of Dgrn activity. Moreover, the inability to express endogenous AMP genes is in agreement with our previous report and unpublished data that during embryogenesis, *dgrn* null embryos fail to express mRNA of zygotic genes like *twist* and *zen* but not maternal genes like nanos (Barry *et al*., Koltun, Orian and Paroush, personal communication). Taken together, we suggest that Dgrn activity may be related to a yet-to-be determined chromatin role of Dgrn, such as inhibiting the formation of local facultative heterochromatin, or the rapid opening of regulatory regions in



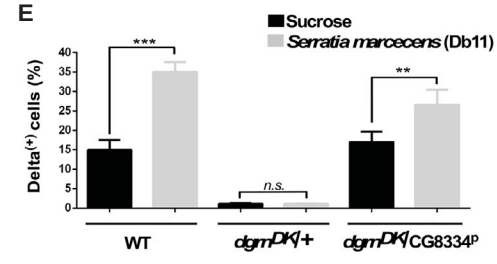


Fig. 5. The iso-peptidase *CG8334* antagonizes *dgrn*. (A,A') *Survival of adult flies with the indicated genotypes upon infection with* Sm (Db11). **(A)** *Halving the dose of* CG8334 (+ dgrnDK/CG8334^{P} +) *rescues the lethality observed in* DgrnDK/+ *heterozygous mutants.* **(A')** *LT50 is determined as the time in days when 50% of infected flies succumbed to infection. For pathogenic challenges n=20 for each genotype, and three independent biological repeats were performed in each set of experiments.* **(B-D'").** *Representative confocal images of* Drosophila *adult mid-gut of the indicated genotypes and α-Delta antibody (RED) and DAPI (Blue) to visualized nuclei. Wild-type (B-B'");* dgrnDK/+ *(C-C'"); and +* dgrn^{DK}/CG8334^{P} *+ (D-D'"). (B-D) Oral challenge with sucrose, (B'-D') Oral challenge with* Sm. *B"-D" and B'"-D'" are the merged images of (B-D) and (B'-D'), respectively, together with staining by DAPI (Blue) to visualized nuclei. In all experiments at least 7 individual guts were examined for each genotype, and three biological independent repeats were performed. (E) Quantification of % Delta positive cells 48 h following oral infection with* Sm(Db11) *(gray bar) or sucrose (control, black bar). n=250 P<0.01.*

a "pioneer-like" manner (Liang *et al.,* 2008; Harrison *et al.,* 2011). This is in agreement with growing evidence of a role for chromatin remodelers and modifiers in the dampening of the innate immune response in *Drosophila* and in mammals (Bonnay *et al*., 2014, EMBO.J; Tartey el al., 2014, EMBO.J).

The protein substrates of Dgrn

It was previously shown that like Cactus, UBC9 and SUMOylation counteract immune response preventing inadequate activation and are required for limiting and terminating the immune response (Chiu *et al.,* 2005; Paddibhatla *et al.,* 2010). While the REL protein Dorsal is SUMOylated and this SUMOylation site is conserved in Dif and Relish (Bhashkar *et al.,* 1997), our observations suggest Dif and Relish are not the targets for Dgrn activity. For example, the processing of Relish was intact in Dgrn-depleted S2 cells, and expression of Dgrn partially restored Toll-induced reporter activity in Dif-depleted cells. It is well established that in many cases, SUMOylation negatively regulates transcription, and many chromatin-related proteins are SUMOylated. Indeed, using *Drosophila* cells recent work identified proteins that undergo SUMOylation in response to LPS/Peptidoglycan challenge identifying nuclear proteins like HP1 that is involved in genes silencing (Handau *et al.,* 2016). However, the identity of the proteins targeted by Dgrn during immune response is yet unknown, and we are pursuing an effort to identify these substrates.

Dgrn and the local response to infection

The regenerative response of the adult gut that is induced by infection is mediated by several signaling pathways such as Notch, JAK-STAT and EGFR (Jiang *et al.,* 2012). We identified that *dgrn* is required for both basal and regenerative Notch signaling in the gut at the organismal level. However, we do not know the exact identity of the gut cell(s) where Dgrn is required, as expression of Dgrn in specific gut cells in the background of a *dgrn* null mutant was not sufficient for enabling the flies to survive infection or restore Delta expression (BK and AO, personal communication). Thus, Dgrn may be required in multiple cells in the gut or for gut regeneration in cells outside the gut epithelia that have a critical impact on ISC. Yet, reducing the level of *CG8334*, an iso-peptidase whose human orthologs of USP11 is associated with RNF4 and antagonized RNF4 function, suppressed *dgrn* associated lethality and Delta expression, suggesting that wherever Dgrn is required for gut regeneration, its activity is antagonized by *CG8334.* Moreover, Dgrn likely regulates the activity of other pathways involved in regeneration in addition to Notch. For example, the small SUMO ligase PIAS1 (Protein inhibitory of JAK-STAT) negatively regulates JAK-STAT-dependent gene expression (Liu *et al.,* 1998). Thus, targeting PIAS-SUMOylated substrates by Dgrn may relive the inhibitory activity of PIAS. Similarly, the transcription factors Yan and Pointed that mediate EGFR signaling were found to be SUMOylated (Handu *et al.,* 2016) and are therefore potential nuclear substrates for Dgrn. Thus, Dgrn-dependent ubiquitylation may serve as a molecular tool to relieve the inhibitory effect of SUMOylation on multiple substrates to enable gene activation.

To conclude, our study characterized the requirement for Dgrn in both systemic immune response and local regeneration upon infection in the gut. However, the exact mechanisms and specific substrates by which Dgrn acts during pathogen infection require further studies.

Materials and Methods

Fly strains

w^{1118}; +;+ were used as control, *w[1118]*; *Mi{ET1}Usp32[MB11462]* were from the Bloomington stock center. w^{1118};+; $dgrn^{DK}$ and UAS-Dgrn were as previously described (Berry *et al.,* 2011). $kenny^{c02831}$ and $myd88^{c03881}$ were described in (Tausszig-Delamasure *et al.,* 2002), *w; AttacinA::GFP;+, P[w+mC Diptericin::LacZ, Drosomycin::GFP]* were a kind gift of JL Imler (UPR9220, CNRS), the Notch reporter *w*;*3.37 Gbe-Su(H)::Lac z;TM2/TM6B* was a kind gift of S. Bray. Flies were raised on standard cornmeal-yeast-agar medium at 25°C unless otherwise indicated.

Plasmids

pPac Drs-Luc, pTal-LacZ, pPGRP-LCa, pPac $Toll^{\Delta LRR}$ were as previously described (GOTO *et al.,* 2008). pNFκB-Luc-reporter was from Promega. Cu^{++}-inducible pRMHA3 Dgrn, $Dgrn^{CH/AA}$, $Dgrn^{\Box SIM}$ and pMTVB Groucho were cloned Kpn/Xba from the corresponding UAS-Dgrn and UAS-Gro vectors (EMBO) (Abed *et al.,* 2011). pMTVB Cactus was cloned from the original *Cactus* cDNA bluescript vector, a kind gift of Ruth Steward. All plasmids were verified by sequencing.

Primers

Primers sets used for q-PCR (from applied biosystems):

Dm01822006_s1	*Drs*	*Dros*omycin
Dm01818074_s1	*Def*	*Defensin*
Dm01821460_s1	*Mtk*	*Metchnikowin*
Dm02362218_s1	*AttA*	*Attacin-A*
Dm02362226_s1	*AttB*	*Attacin-B*
Dm01821391_g1	*AttC*	*Attacin-C*
Dm02135981_g1	*AttD*	*Attacin-D*
Dm01821557_g1	*DptA*	*Diptericin A*
Dm01821449_s1	*Dro*	*Drosocin*

Antibodies

Mouse polyclonal α-Dgrn (1:500, [Abed *et al.,* 2011]); rabbit α-Dif (1:5000, a kind gift from Tony IP); mouse α-Actin (1:2000, MP Biomedicals 691001); Donkey α-mouse IgG-HRP (1:10000; 715-035-150) and Goat α-Rabbit HRP (1:10000, #715-035-152) were from Jackson Laboratory. Mouse α-Delta and α-Prospero (1:50 and 1:100, respectively) were from DSHB. α-Histon H3 (p-S10) 1:100 was from Abcam #ab5176); Rabbit α-βGal (1:500, MP Biomedicals #55976), Alexa Fluor® 568 goat α-mouse IgG1 (1:1000, Invitrogen) and Alexa Fluor® 488 goat α-rabbit were from Invitrogen (1:1000, #A21124 and #A11008, respectively).

Microbial strains, pathogenic challenge and exposure to Dextran Sulfate Sodium (DSS)

Escherichia coli (1106) and *Micrococcus luteus* (CIPA270) bacteria for septic injuries were as described in (Reichhart *et al.,* 2011, n=30). Natural *Beauveria bassiana* infections were performed as previously described (Lemaitre *et al.,* 1997). *Serratia marcescens* Db11 and *Pseudomonas aeruginosa* PA14 were used for oral infection (Bonnay *et al.,* 2012).

Oral exposure to Sm and DSS challenge

Twenty 3- to 5-day-old flies of each genotype were assayed each time. Flies were transferred into an empty vial containing three absorbent 3 mm filters soaked with sucrose alone (control) or with sucrose and *Sm* bacteria. For DSS challenge, 400 µl of 5% DSS in sucrose were used. Viability was counted daily, and surviving flies were transferred to new vials with fresh feeding media daily. For each pathogenic (pricking or oral challenges), or chemical challenge, at least three independent biological repeats were performed.

S2 cell culture, transient transfections and RNAi targeting

S2 were maintained in glutamine-enriched S2 Schneider media supplemented with 10% heat inactivated bovine calf serum. Transient transfections

were performed using FuGENE-HD® (Promega), and RNAi was performed using MEGAscript RNAi Kit (Ambion AM1626) as previously described (Abed *et al.,* 2011). The following specific primers were used:
GFP-i forward:
5'-gaattaatacgactcactatagggtgagcaagggcgaggagctg-3'
GFP-i reverse:
5'-gaattaatacgactcactatagggttgaagttcaccttgatgcc-3'
Dif-i forward:
5'- cggaattccgaattaatacgactcactatagggacaacaaactttgctaaatactagt-3'
Dif-i reverse:
5'- cggaaattccgaattaatacgactcactatagggaaattctaaagttatattttattta-3'

Luciferase reporter assay

Reporter assays in S2 *Drosophila* cells were performed similar to that described in (Abed et. al. 2011), with the following modifications: 3 x 10^6 cells were transfected where indicated with the following amounts of DNA: 100 ng pPac Drs-Lue, 100 ng pTAL NFkB-Lue, 100 ng pelican LacZ, 150 ng pPac PGRP-LCa, 50 ng pPac TollΔLRR (on Drs reporter) or 1000 ng (on kB reporter), 300 ng pRMHA3 vectors (Dgrn and its derived mutants), pMT/V5 Cactus, 150 ng pMT/V5 Groucho, and pCDNA3 was used in order to make an equal amount of DNA. Eight hours post-transfection, 0.8mM Cu^{+2} was added, and 48 hours later, Luciferase activity was measured and normalized to β-galactosidase activity (n=4).

Western blot analysis

Cell extracts were prepared using RIPA buffer, and 200 μg protein extract were resolved over SDS-PAGE and subjected to western blot analysis using the indicated antibodies similar to that described in (Abed *et al.,* 2011).

Determination of mRNA level of endogenous AMPs

RNA extraction was performed using TRI reagent (T9424 Sigma). Total RNA was converted to cDNA using qScript cDNA Synthesis kit (95047-100, Quanta Bioscience) according to manufacturer's instructions. Expression levels of each Amp gene were detected using TaqMan® with the indicated primers. qPCR was performed according to manufacturer's instructions. All samples were analyzed in triplicate, and the levels of detected mRNA were normalized to control Rp49 mRNA values. Normalized data were used to quantify the relative levels of a given mRNA according to cycling threshold analysis. Target gene expression in the uninfected flies was set arbitrarily as 1, and results were presented as relative expression levels.

Dissection of Drosophila gut and indirect immunofluorescence

Gut dissection and immunofluorescence detection: gut fixation and staining was carried out similar to that previously described (SHAW et. al. 2010). Guts were then incubated overnight at 4°C with 100μl first antibody diluted in blocking buffer (for immunostaining) with gentle rocking. The following day, guts were washed x4 in PTX (15 min per wash with gentle rocking) at room temperature and then incubated for 1 h with 100 μl fluorescent secondary antibody (1:1000) diluted in blocking buffer (for immunostaining). DAPI (Sigma D9542, 1:1000 of a 1 μg/μl stock solution) was added to the secondary antibody solution and covered with foil. Guts were washed with PTX-x2 (15'/wash), and an additional time with PBSx1 for 15 min. Subsequently, guts were mounted onto a microscope slide with two drops of mounting solution (Fluoromount, Southern Biotech, 0100-01) and analyzed using the Zeiss LSM 710 META laser scanning confocal microscope.

Statistical analysis

Reporter assays were analyzed statistically using SEM and t-test, and comparisons were performed using the GraphPad Prism 5.00 (GraphPad Software, San Diego, CA, USA). Significance is indicated by ***= P<0.001 and **= P<0.01. n=5.

LT50 (lethal time in days at which 50% of the flies died) analysis was calculated using the GraphPad Prism 5.00 (GraphPad Software, San Diego, CA, USA). Log-rank (Mantel-Cox) test was performed. Survival experiments shown are representative of at least three independent experiments where each 25 individuals were tested for each genotype.

Acknowledgments

We thank members of the Orian lab for their comments and assistance. We are grateful to Z. Paroush, D. Chamovich, B. Edgar, S. Bray and JL Imler for sharing their antibodies and fly lines. This research was published under the frame work of the LABEX:ANR-10-LABX-0036_NETRNA, and funding of the state managed by the French National Research Agency as part of the Investments for the future program to NM and JMR. The Orian lab was supported by ISF #739-2015, the Goldy Family Research Fund, and Rappaport Research Fund.

References

ANDERSON K V, JURGENS G, NUSSLEIN-VOLHARD C (1985). Establishment of dorsal-ventral polarity in the *Drosophila* embryo: genetic studies on the role of the Toll gene product. *Cell* 42: 779-789.

ABED M, BARRY K C, KENYAGIN D, KOLTUN B, PHIPPEN T M, DELROW J J, PARKHURST S M, ORIAN A (2011). Degringolade, a SUMO-targeted ubiquitin ligase, inhibits Hairy/Groucho-mediated repression. *EMBO J* 30: 1289-1301.

BARRY K C, ABED M, KENYAGIN D, WERWIE T R, BOICO O, ORIAN A, PARKHUTST S M (2011). The *Drosophila* STUbL protein Degringolade limits HES functions during embryogenesis. *Development* 138: 1759-1769.

BONNAY F, NGUYEN X H, COHEN-BERROS E, TROXLER L, BATSCHE E, CAMONIS J, TAKEUCHI O, REICHHART J M, MATT N. (2014) Akirin specifies NF-κB selectivity of *Drosophila* innate immune response via chromatin remodeling. *EMBO J.* 33: 2349-2362.

BUCHON N, SILVERMAN N, CHERRY S (2014). Immunity in *Drosophila melanogaster* – from microbial recognition to whole-organism physiology. *Nat Rev Immunol* 14: 796-810.

CINNAMON E, PAROUSH Z (2008). Context-dependent regulation of Groucho/TLE-mediated repression. *Curr Opin Genet Dev* 18: 435-440.

CHIU H, RING B C, SORRENTINO R P, KALAMATZ M, GARZA D, GOVIND S (2005). dUbc9 negatively regulates the Toll-NF-kappa B pathways in larval hematopoiesis and drosomycin activation in *Drosophila*. *Dev Biol* 288: 60-72.

DECQUE A, JOFFERE O, MAGALHAES J G, COSSEC J C, BLECHER-GONEN R, LAPAQUETTE P, SILVIN A, MANEL N, JOUUBERT P E, DEJEAN A. *et al.,* (2016). Sumoylation coordinates the repression of inflammatory and anti-viral gene-expression proGrams during innate sensing. *Nat Immunol* 17: 140-149.

EVERRET R D, BOUTELL C, HALE B G (2013). Interplay between viruses and host sumoylation pathways. *Nat Rev Microbiol* 11: 400-411.

FERRNADON D, IMLER J L, HETRU C, HOFFMANN J A. (2007). The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections. *Nat Rev Immunol.* 7: 862-874.

FERRANDON D. (2013) The complementary facets of epithelial host defenses in the genetic model organism *Drosophila melanogaster*: from resistance to resilience. *Curr Opin Immunol.* 1: 59-70.

FLTHO A, MELCHIOR F. (2013). Sumoylation: a regulatory protein modification in health and disease. *Annu Rev Biochem* 82: 357-385.

GHOSH S, HAYDEN M S (2012). Celebrating 25 years of NF-κB research. *Immunol Rev* 246: 5-13.

GOTO A, MATSUSHITA K, GESELLCHEN V, El CHAMY L, KUTTENKEULER D, TAKEUCHI O, HOFFMANN J A, AKITA S, BOUTROS M, REICHAART J M. (2008) Akirins are highly conserved nuclear proteins required for NF-kappaB-dependent gene expression in *Drosophila* and mice. *Nat Immunol* 9: 97-104.

HEATON S M, BORG N A, DIXIT V M (2016). Ubiquitin in the activation and attenuation of innate antiviral immunity. *J Exp Med* 213: 1-13.

HANDU M, KADUSKAR B, RAVINDRANATHAN R, SOORY A, GIRI R, ELANGO V B, GOWDA H, RATNAPARKHI G S (2015). SUMO-enriched proteome for *Drosophila* innate immune response. *G3* 5: 2137-2154.

HARRISON M M, LI X Y, KAPLAN T, BOTCHAN M R, EISEN M B (2011). Zelda binding in the early *Drosophila melanogaster* embryo marks regions subsequently activated at the maternal-to-zygotic transition. *PLoS Genet* 7:e1002266.

HENDRIKS I A, SCHIMMEL J, EIFLER K, OLSEN J V, VERTEGAAL A C (2015).

Ubiquitin-specific protease 11 (USP11) deubiquitinates hybrid small ubiquitin-like modifier (SUMO)-ubiquitin chains to counteract RING finger protein 4 (RNF4). J *Biol Chem* 290: 15526-15537.

HERIDE C, URBE S, CLAGUE M J (2014). Ubiquitin code assembly and disassembly. *Curr Biol* 24: R215-220.

JIANG H, EDGAR B A (2012). Intestinal stem cell function in *Drosophila* and mice. *Curr Opin Genet Dev*. 22: 354-360.

KANAREK N, BEN-NERIAH Y (2012). Regulation of NF-κB by ubiquitination and degradation of the IκBs. *Immunol Rev* 246: 77-94.

LEE J H, PARK S M, KIMOS, LEE C S, WOO J H, PARK S J, JOE E H, JOU I (2009). Differential SUMOylation of LXRalpha and LXRbeta mediates trans-repression of STAT1 inflammatory signaling in IFN-gamma-stimulated brain astrocytes. *Mol Cell* 35: 806-817.

LEE M H, MABB A M, GILL G B, YEH E T, MIYAMOTO S (2011). NFκB induction of the SUMO protease SENP2: a negative feedback loop to attenuate cell survival response to genotoxic stress. *Mol Cell* 43: 180-91.

LEMAITRE B, HOFFMANN J (2007). The host defense of *Drosophila melanogaster*. *Annu Rev Immunol* 2007 25: 697-743.

LIANG H L, NIEN C Y, LIU H Y, METZSTEIN M M, KIROV N, RUSHLOW C (2008). The zinc-finger protein Zelda is a key activator of the early zygotic genome in *Drosophila*. *Nature* 456: 400-403.

LIU B, LIAO J, RAO X, KUSHNER S A, CHUNG C D, CHANG D D, SHUAI K (1998). Inhibition of Stat1-mediated gene activation by PIAS1. *Proc Natl Acad Sci USA*. 95: 10626-10631.

MENG X, KHANUJA B S, IP Y T. (1999) Toll receptor-mediated *Drosophila* immune response requires Dif, an NF-kB factor. *Genes Dev* 13: 792-797.

PADDIBHATLA I, LEE M J, KALAMRTZ M E, FERRERESE R, GOVIND S (2010). Role for SUMOylation in systemic inflammation and immune homeostasis in *Drosophila* larvae. *PLoS Pathog*. 6:e1001234.

POPOVIC D, VUCIC D, DICKIC I (2014). Ubiquitination in disease pathogenesis and treatment. *Nat Med* 20: 1242-1253.

RIBET D, COSSART P (2010). *Listeria monocytogenes* impairs SUMOylation for efficient infection. *Nature* 464: 1192-1195.

RYU T, SPATOLA B, DELEBARE L, BOWLIN K, HOPP H, KUNITAKE R, KARPEN G H, CHIOLO I (2015). Heterochromatic breaks move to the nuclear periphery to continue recombinational repair. *Nat Cell Biol.* 17: 1401-1411.

SHAW R L, KOHLMAIER A, POLESELLO C, VEELKEN C, EDGAR B A, TAPON N (2010). The Hippo pathway regulates intestinal stem cell proliferation during *Drosophila* adult midgut regeneration. *Development* 137: 4147-4158.

SRIRAMACHANDRAN A M, DOHMEN R J (2014). SUMO-targeted ubiquitin ligases. *Biochim Biophys Acta* 21843: 75-85.

TARTEY S, MATSUSHITA K, VANDENBON A, ORI D, IMAMURA T, MINO T, STANDLEY D M, HOFFMANN J A, RERICHHHART J M, AKIRA S, TAKEUUCHI O.(2014) Akirin2 is critical for inducing inflammatory genes by bridging IκB-ζ and the SWI/SNF complex. *EMBO J.* 33: 2332-4238.

TAUSZIG-DELAMASURE S, BILAK H, CAPOVILLA M, HOFFMANN J A, IMLER J L. (2002) *Drosophila* MyD88 is required for the response to fungal and Gram-positive bacterial infections. *Nat Immunol* 2002 3: 91-97.

WAN F, LENARDO M J. (2010). The nuclear signaling of NF-κB: current knowledge, new insights, and future perspectives. *Cell Res* 20: 24-33.

WI H C, LIN Y C, LIU C H, CHUNG H C, WANG Y T, LIN Y W, MA H I, TU P H, LAWLER S E, CHEN R H (2014). USP11 regulates PML stability to control Notch-induced malignancy in brain tumours. *Nat Commun* 5: 3214.

YU M, LIU K, MAO Z, LUO J, GU W, ZHAO W (2016). USP11 is a Negative Regulator to γH2AX Ubiquitylation by RNF8/RNF168. *J Biol Chem* 291: 959-967.

van der VEEN A G, PLEOGH H L (2012). Ubiquitin-like proteins. *Annu Rev Biochem* 81: 323-357.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

Ontogeny of the *Drosophila* larval hematopoietic organ, hemocyte homeostasis and the dedicated cellular immune response to parasitism
Joanna Krzemien, Michele Crozatier and Alain Vincent
Int. J. Dev. Biol. (2010) 54: 1117-1125
http://dx.doi.org/10.1387/ijdb.093053jk

The deubiquitylating enzyme Cops6 regulates different developmental processes during early zebrafish embryogenesis
William K.F. Tse, May-Su You, Steven Hao-Kee Ho and Yun-Jin Jiang
Int. J. Dev. Biol. (2011) 55: 19-24
http://dx.doi.org/10.1387/ijdb.103089wt

Mammalian hibernation: differential gene expression and novel application of epigenetic controls
Pier Morin Jr. and Kenneth B. Storey
Int. J. Dev. Biol. (2009) 53: 433-442
http://dx.doi.org/10.1387/ijdb.082643pm

Role of polycomb proteins Ring1A and Ring1Bin the epigenetic regulation of gene expression
Miguel Vidal
Int. J. Dev. Biol. (2009) 53: 355-370
http://dx.doi.org/10.1387/ijdb.082690mv

Regulation of cell fate determination by Skp1-Cullin1-F-box (SCF) E3 ubiquitin ligases
Christopher J. Hindley, Gary S. McDowell, Helen Wise and Anna Philpott
Int. J. Dev. Biol. (2011) 55: 249-260
http://dx.doi.org/10.1387/ijdb.103171ch