A Roadmap to Understanding Toll Pathway Changes: An Educational Primer for Use with "Regulation of Toll Signaling and Inflammation by β-Arrestin and the SUMO Protease Ulp1"

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SUMMARY An article by Anjum *et al.* in the December 2013 issue of *GENETICS* can be used to illustrate reverse genetic manipulation in a model organism, targeted RNA interference, synergistic gene interaction, and biochemical regulation of gene expression using post-translational modification. This Primer provides background information, technical explanations of methods and genetic approaches from the study, an example approach for classroom use, and discussion questions to promote understanding of the research article.

Related article in *GENETICS*: Anjum, S. G., W. Xu, N. I. Nikkolgh, S. Basu, Y. Nie, *et al.*, 2013 Regulation of Toll signaling and inflammation by β-arrestin and the SUMO protease Ulp1. Genetics **195:** 1307–1317.

ANJUM and colleagues (Anjum *et al.* 2013) use *Drosophila melanogaster* to study activation of the innate immune system, the first line of defense against pathogens in living organisms and the primary means of defense in fungi, plants, and invertebrates. Using a variety of reverse genetic techniques and protein analysis, Anjum *et al.* (2013) show a previously unknown genetic and biochemical interaction between two genes and their protein products: the β-arrestin encoded by *kurtz (krz)* and a SUMO protease encoded by *Ulp1*. The authors demonstrate that the products of these two genes work synergistically to regulate the Toll immune pathway in *Drosophila*.

Drosophila as a Model System

The fruit fly *D. melanogaster* (*Drosophila*, or "the fly") has remained a popular genetic model organism since the 1930s (Rubin and Lewis 2000). Fruit flies are small and inexpensive to maintain on food mixtures that mimic rotten fruit. *Drosophila* has a life cycle consisting of embryo, larval, pupal, and adult stages that takes roughly 10–12 days to complete. The larvae hatch and go through three size stages (instars) before they pupate and finally metamorphose into the adult fly. Images of *Drosophila* larvae can be seen in Anjum *et al.* (2013, figure 1, A–C).

Drosophila has several advantages as a genetic model organism. First, *Drosophila* has only four chromosomes making it relatively easy to perform genetic screens and to map gene locations. Second, despite significant physiological differences, *Drosophila* and humans share many important gene families and cell signaling pathways. Thus, researchers can study certain essential gene functions in this simpler system. Third, *Drosophila* can be easily manipulated using both forward and reverse genetic approaches. Historic forward genetic mutant screens helped to define members of many central cell signaling pathways, including Ras, Hedgehog, and Toll, the pathway of interest in this article (St Johnston 2002). Anjum and colleagues use reverse genetic tools such as gene knockouts and RNAi knockdown to study gene loss-of-function and determine how two very different genes are used to control innate immunity.

The fly has a robust immune system with important similarities to the human innate immune response (reviewed in Hoffmann 2003; Lemaitre and Hoffmann 2007; Charroux and Royet 2010; Kounatidis and Ligoxygakis 2012). Like humans, flies have both humoral and cellular immunity. The humoral response involves antimicrobial peptide factors secreted into the fly's "blood" (called hemolymph) to circulate and defend against pathogens, while the cellular response involves phagocytic cells that consume

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or neutralize invaders, including bacteria, fungi, and parasites. Both the humoral and cellular responses are controlled by the highly conserved Toll/NF-κB signaling pathways and they reveal interesting comparisons between human and fly immunity.

Immune Signaling: The Toll and IMD Pathways

In both flies and mammals, activation of the transcription factor NF-κB is a central event in immune signaling. The Toll pathway, named for the cell surface receptor Toll, is one of two primary immune signaling pathways in *Drosophila* that work through NF-κB activation (Lemaitre *et al.* 1996). In mammals, the homologous Toll-like receptors (TLRs) directly detect microbial molecules that would be found during an infection. In contrast, the *Drosophila* Toll receptor is activated after other proteins detect a microbial infection; thus *Drosophila* Toll acts downstream of these proteins. Once activated, the Toll signaling cascade activates the fly homologs of NF-κB including Dorsal (Dl) and Dif. Normally, Dl and dorsal-related immunity factor (Dif) are located in the cytosol and are kept there by association with the IκB protein Cactus. When Toll or other immune signaling is active, Cactus is degraded, which allows Dl and Dif to move from the cytosol into the nucleus, where these transcription factors will bind to specific gene promoters and activate downstream immune responses. Downstream gene targets of Dl and Dif include antimicrobial peptides, described below. In this article, Anjum *et al.* (2013, figures 2 and 3, G and H) monitor the localization of Dif and Dl to determine whether NF-κB activation has taken place.

In addition to Toll signaling, flies have a second pathway that controls immune activation, called the immune-deficiency (IMD) pathway. This pathway is homologous to the mammalian tumor-necrosis factor alpha (TNFα) pathway, which contributes to the immune response and programmed cell death. IMD signaling results in activation of a third fly NF-κB homolog, Relish (Rel), that activates downstream effectors distinct from Dorsal and Dif. These different downstream effectors can be used to determine which immune pathway has been activated in a system. In figure 1G, Anjum *et al.* (2013) examine *Drosomycin* antimicrobial peptide (AMP) expression, controlled by Toll signaling, compared to *Diptericin* AMP expression mediated by IMD signaling. This comparison allows the authors to assess Toll-specific activation rather than general immune activation (See Figure 1.)

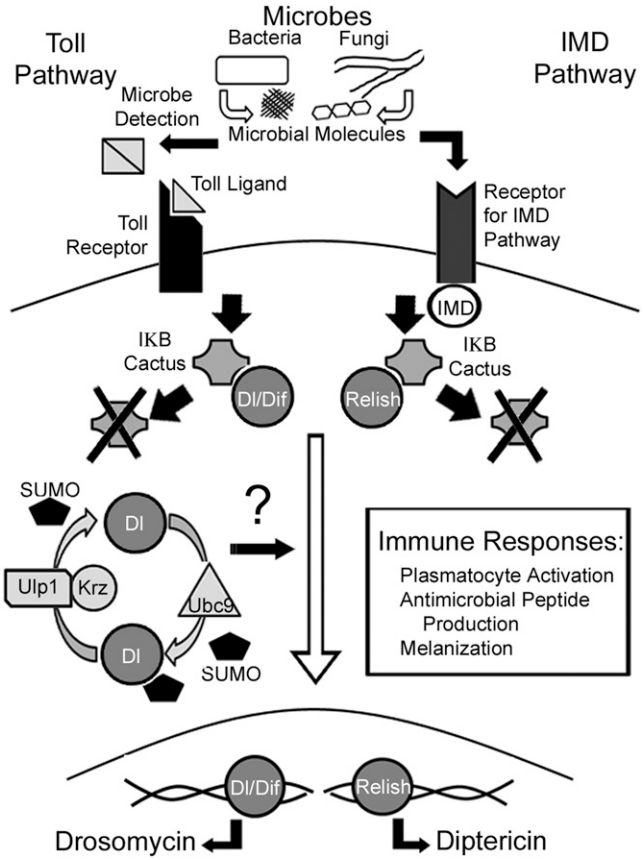


Figure 1 Toll and IMD signaling in *Drosophila* immune activation. The *Drosophila* immune response begins with detection of microbial molecules, proceeds through NF-κB signaling via two pathways, Toll and IMD, and results in defensive responses including plasmatocyte activation, antimicrobial peptide production, and melanization. This schematic highlights the players relevant to Anjum *et al.* (2013) but omits several intermediate steps and protein names for simplicity. Dorsal (Dl), Dif, and Relish are three fly homologs of NF-κB. Dl and Dif are activated by the Toll pathway, while Relish is activated by the IMD pathway. Each NF-κB is normally kept inactive by the IκB Cactus protein in the cytosol. Upon detection of microbial molecules, the Toll ligand is cleaved from its pro-form and binds to the Toll receptor. Toll receptor activation leads to degradation of Cactus and allows Dl/Dif to move into the nucleus to act as a transcription factor for the *Drosomycin* antimicrobial peptide. Alternatively, the receptor for the IMD pathway can detect microbial molecules and initiate signaling through the IMD adaptor protein. IMD signaling also results in Cactus degradation and permits Relish-mediated *Diptericin* antimicrobial peptide production. Dl activation can be modified by SUMOylation. The Ubc9 conjugation enzyme attaches SUMO to Dl, while Anjum *et al.* (2013) show that SUMO is removed from Dl by the cooperation of the Ulp1 SUMO protease and the β-arrestin Krz. The mechanism through which the Dl SUMOylation cycle affects immune response is currently under investigation.

The Humoral Response: Antimicrobial Peptides and Melanization

The *Drosophila* fat body is required for the humoral (noncellular) immune response. The fat body consists of two parallel structures running along the anterior–posterior body axis of a larval fly. GFP located in the larval fat body can be seen in Anjum *et al.* (2013, figure 1F). This organ is involved in energy storage and nutrient processing, much like the mammalian liver. It is also considered an immune organ because it is mainly responsible for secreting antimicrobial peptides into the hemolymph for host defense (Hoffmann 2003). Like blood, hemolymph transports nutrients, signals, and hemocytes (equivalent to white blood cells) around the body.

When Toll immune signaling is active in the fat body, Dl and Dif control production and secretion of powerful defensive AMPs (Lemaitre and Hoffmann 2007; Charroux and Royet 2010). AMPs are positively charged peptides that are attracted

to the generally negatively charged cell walls of bacteria and fungi. Some AMPs are thought to interfere with pathogen membranes, but the precise mechanism by which these antimicrobial peptides inactivate pathogens is still poorly understood. In this article, Anjum and colleagues focus on *Drosomycin (Drs)*, an AMP specifically controlled by Toll signaling. *Drosomycin* production at both the RNA and protein level is used to measure Toll pathway activation in this study (as in Anjum *et al.* 2013, figure 1). *Diptericin* (*Dpt)* is a second AMP that is specifically produced by IMD signaling, the non-Toll pathway mentioned above. Anjum *et al.* (2013) use *Dpt* levels as a control to ensure that they are observing Toll-pathway-specific effects of *krz* and *Ulp1* rather than the IMD pathway.

Melanization is an insect immune defense phenomenon that "walls off" damage and also produces toxic molecules that can act against invading microbes (Lemaitre and Hoffmann 2007; Charroux and Royet 2010; Kounatidis and Ligoxygakis 2012). Melanization can thus be compared to blood clotting and the complement cascade of the mammalian innate immune system. In the fly, melanization can be visible in the larvae as small black spots where melanin (a black pigment) has been deposited (Minakhina and Steward 2006). While melanization can be activated by both Toll and IMD signaling, in this article melanization is used to show general immune and Toll pathway activation.

The Cellular Immune Response

The cellular immune response in flies is controlled by hemocytes, the equivalent of vertebrate white blood cells. In larvae, hemocyte progenitor cells differentiate into three classes of hemocytes with specific roles in the fly immune response: plasmatocytes, crystal cells, and lamellocytes (Hoffmann 2003; Lemaitre and Hoffmann 2007). Plasmatocytes are phagocytic "clean-up" cells, while crystal cells are involved in melanization. Lamellocytes are large flat cells that encapsulate, or surround, damage or invaders (such as parasitic wasp eggs) that are too big to be phagocytosed by plasmatocytes (Hoffmann 2003). Lamellocytes also work with crystal cells in melanization, and sites of encapsulation can be seen as black spots due to accompanying melanization. Lamellocytes are rare and should be found only in larvae when they develop in the presence of a pathogen. Hence, Anjum *et al.* (2013) measure numbers of lamellocytes as an indication of immune activation in the mutant larvae. High levels of lamellocytes in the absence of an infection can result in "melanotic pseudotumors," also called "melanotic masses" (Minakhina and Steward 2006). Like an autoimmune response, these black spots are seen only when there are enough melanized lamellocytes in the larva to indicate increased immune activation, as seen in Anjum *et al.* (2013, figures 1, A–C, 3D, and 5C).

β-Arrestins: Signal Adaptor Proteins

β-Arrestins are cell signal-regulating proteins. They were first described as proteins that dampened the response of G-protein-coupled receptors. By binding to these receptors, β-arrestins can prevent further signaling from the receptor complex, thereby reducing the cellular response to an external signal (Luttrell and Lefkowitz 2002). Later studies showed that β-arrestins are also involved in "tuning" the responses of other important cell signaling pathways. Humans have two β-arrestin homologs, β-arrestin 1 and β-arrestin 2, while flies have a single homolog, *Kurtz* (*krz*) (Roman *et al.* 2000). Previous work from the Veraksa lab showed that the *Drosophila* β-arrestin *krz* is involved in regulating signals from both mitogen-activated protein kinase (MAPK) and NF-κB downstream of the Toll pathway in the *Drosophila* embryo (Tipping *et al.* 2010). It was previously unknown how *krz* might affect Toll signaling in *Drosophila*. Anjum *et al.* (2013) connect *krz* activity to SUMOylation, a method of post-translational protein modification.

SUMOylation

SUMO (small ubiquitin-like modifier) is a small protein that can be covalently linked to other proteins to regulate their activity (reviewed in Flotho and Melchior 2013). SUMOylation of a protein can result in changes to the protein's location and trafficking in the cell, increase or decrease a protein's activity, or affect the protein's ability to bind with other signaling partner proteins. SUMOylation has been shown to be involved in multiple cellular processes including transcription. In *Drosophila*, the enzyme Ubc9/Lwr is known to SUMOylate the transcription factor Dorsal (see above). SUMOylation is a reversible process, and SUMO modifications on proteins can be removed through the activity of SUMO proteases such as *Ulp1*, studied in this article.

The Technical Breakdown

Indications of active Toll signaling

Anjum *et al.* (2013) observe multiple phenotypes to directly and indirectly measure Toll pathway activation in *krz* and *Ulp1* mutants. The first phenotype examined is formation of melanotic masses (Anjum *et al.* 2013, figures 1 and 3). Since melanization usually indicates an infection or wound, the mutant larvae with visible black spots indicate hyperactive immune signaling. Antimicrobial peptide production is also considered in Figure 1. The authors use Drosomycin production to indicate Toll-specific signaling and Diptericin to act as a control for immune-deficiency-specific signaling. AMP production is estimated using flies expressing transgenic reporter constructs. In these constructs, AMP promoter elements are used to direct the expression of green fluorescent protein (GFP) or LacZ galactosidase, an enzyme that can produce a colored signal. Therefore, during immune signaling the cells expressing antimicrobial peptides also produce green fluorescent protein or LacZ, making them visible to the researchers and "reporting" gene activation. The authors use *Drosomycin*–GFP and *Diptericin*–LacZ transgenic flies to indirectly monitor

production of *Drosomycin* and *Diptericin*, as seen in Anjum *et al.* (2013, figure 1F). Antimicrobial peptide production is also measured directly through RT–PCR and Western blot to determine AMP expression at the mRNA and protein levels. Translocation of Dl and Dif into the nucleus during immune activation is detected using immunofluorescence in Anjum *et al.* (2013, figures 2 and 3). In this technique, fluorescent tags are attached to antibodies that bind specifically to Dl and Dif. The antibodies find their targets in the prepared samples, and the fluorescence allows the researchers to visualize the cellular location of the target proteins—in this case, nuclear *vs.* cytoplasmic. In Anjum *et al.* (2013, figures 4 and 5), the authors examine SUMOylation of the transcription factor Dl using Western blot and co-immunoprecipitation (Co-IP), described below. While the effect of SUMOylation of Dl is not yet completely decoded, the authors infer that these SUMOylation changes indicate a connection between SUMO signals and the Toll pathway.

Gene loss-of-function and genetic rescue

Anjum *et al.* (2013) focus on loss-of-function studies to examine the roles of *krz* and *Ulp1*. Two loss-of-function approaches are used in this article: gene knockout, which results in the absence of functional gene product, and gene knockdown through RNA inhibition (RNAi).

P-element gene knockout

Geneticists use gene knockout to help determine the function of a gene in a living organism. The authors examine *krz* knockout using the krz^{c01503} mutant flies in which the *krz* gene is disrupted by insertion of a transposon. Transposons are mobile genomic elements, and if a transposon inserts into the coding sequence of another gene, the disrupted gene is usually no longer functional. The *P* element is a natural *Drosophila* transposon (reviewed in Castro and Carareto 2004) that has been modified and used extensively in flies to create random mutations in the genome for forward genetic screens. krz^{c01503} was made during one such screen (Thibault *et al.* 2004). The authors compare their results using krz^{c01503} to phenotypes seen using a second independent *P*-element *krz* knockout mutant, krz^{1}, which results in the same reported effects (Tipping *et al.* 2010). Anjum and colleagues then crossed the knockout flies to *krz5.7* flies that are transgenic for a region of the fly genome that includes a working copy of *krz*. The *krz5.7* mutation was used to rescue the phenotypes that occurred in the *krz* knockouts.

GAL4–UAS-mediated RNAi

In RNAi knockdown, specific messenger RNA molecules are targeted for degradation by expression of complementary RNA so that even though the corresponding gene is transcribed into mRNA, the mRNA is not translated and therefore not expressed at the protein level. Anjum and colleagues used RNAi paired with the elegant GAL4–UAS system to knockdown genes in a particular time and place in the fly embryo (as reviewed in Duffy 2002). GAL4 is a yeast transcription factor that binds to sites in the upstream activating sequence (UAS) gene promoter. Specific promoters attached to the coding region of the GAL4 gene can be used to direct GAL4 transgene expression in particular tissues. In this article, the GAL4 transcription factor was expressed in the whole fly (*da–GAL4)* or in limited tissues (*Cg–GAL4* in the fat body and lymph gland, and *ppl–GAL4* in the fat body only). The GAL4 transgenic flies were then crossed to flies that were transgenic for a target gene downstream of the UAS promoter. This allows the UAS-driven gene to be expressed only when/where GAL4 is active. In this study, the UAS promoter was paired with sequences that when expressed form small hairpin RNA constructs specific for a gene of interest (UAS–*krz*, UAS–*Ulp1)*. When the hairpin RNA is processed by Dicer and RISC proteins, the processed sequence is used to "seek and destroy" the RNA message for the target gene, resulting in "knocked down" expression. The article (Anjum *et al.* 2013) uses UAS–*krz* and UAS–*Ulp1* paired with *da–GAL4* to knock down these genes in the whole larva, or with *Cg–GAL4* or *ppl–GAL4* to limit gene knockdown to the fat body and lymph node immune organs.

RNAi rarely results in an absolute inhibition of a gene, but the varying amount of inhibition can be used to examine dose-dependent gene effects. Anjum *et al.* (2013) increased the efficiency of RNA knockdown by coexpressing extra *Dicer-2*. By having more than enough Dicer available, the authors ensured that a higher level of RNA knockdown would be achieved for each target. This is an important experimental element as the level of knockdown was later "dialed back" by removing the extra availability of Dicer. Under these conditions, the partial knockdown allowed the authors to see more subtle interactions between *krz* and *Ulp1*. The reduced knockdown of both genes together had a similar effect as the greater knockdown of either gene on its own, revealing their synergistic effect on the Toll pathway.

Biochemical studies

In this article, Anjum *et al.* (2013) relate the synergistic effects of *krz* and *Ulp1* to biochemical interactions between the Krz and *Ulp1* proteins. This interaction was first identified in a proteomic screen but was confirmed here using Co-IP. In Co-IP, an antibody against an epitope found on one protein of interest is used to "grab" the protein out of a cell lysate solution. If that protein of interest is also part of a larger complex, then the associated proteins will also be grabbed during this interaction and can be detected by Western blot. Interactions between the SUMO protease *Ulp1* and Krz/β-arrestin homologs are examined in Anjum *et al.* (2013, figure 3).

Since Toll activity was previously shown to be modified through SUMOylation of Dorsal (Bhaskar *et al.* 2000), the authors of Anjum *et al.* (2013) examined how SUMOlyation patterns were affected by *Ulp1* and Krz working together, shown in Anjum *et al.* (2013, figures 4 and 5F). Anjum *et al.* (2013, figure 4B) examine how SUMOylation of a Dl can be altered in the presence of different doses of Krz and Ulp1.

The labels at the top right of the figure show what conditions or variables were included (+) or omitted (−) from each experimental sample. The labels to the right of each gel image list the antibody used to detect the protein in the corresponding Western blot. Thus, "IB: SUMO" reveals levels of SUMO protein, while "IB: arrestin" shows detection of krz, the fly arrestin homolog. Other proteins are detected through epitope tags. In this experiment, "IB: V5" shows levels of Dorsal tagged with the V5 epitope and "IB: SBP" shows levels of SBP-tagged Ulp1. HSP70 is a protein-folding chaperone expressed at similar levels among the experimental samples (see question 8 below). When a protein is SUMOylated, the SUMO is attached through a covalent link to the original protein. This increases the total molecular size of the SUMO-protein complex, and therefore the SUMOylated-Dorsal can be seen in Anjum *et al.* (2013, figure 4B) in the top row (IB: V5) as a faint band a little higher than the main Dorsal band. (See discussion questions 8 and 9, below.)

Protein SUMOylation can be effective even when very low levels of the target protein are modified. Thus, the authors were not able to detect changes in the Dorsal SUMOylation patterns using fly larvae. However, by using *Drosophila* cells lines that were also expressing an abundance of SUMO (Smt3) and the Ubc9/Lwr attachment enzyme, they were able to amplify the SUMO signal and detect changes in the Dorsal SUMOlyation in the presence or knockdown of Ulp1, as seen in Anjum *et al.* (2013, figure 4B). Students accustomed to looking at protein Western blots may note that the authors show an unusually large portion of the gel for the SUMO immunoblots. Since SUMO is covalently linked to its targets, SUMO banding appears as a "ladder" in Western blots of lysates due to the many different sized proteins that may be SUMOylated at any given time.

Connections to Genetic Concepts

This article by Anjum and colleagues highlights the use of *D. melanogaster* as a genetically tractable model organism. Previous forward genetic screens (Thibault *et al.* 2004) have led to the reverse genetic investigations of the β-arrestin homolog, *krz*, in Anjum *et al.* (2013) and earlier articles by this group (Mukherjee *et al.* 2005; Tipping *et al.* 2010). The link to humans and potentially to disease applications can be seen in Anjum *et al.* (2013, figure 3), where the interaction in the fly is confirmed using the human homologs. This example and the conserved homology of the NF-κB pathway can be used to illustrate evolutionary relationships.

Anjum *et al.* (2013) provide an opportunity to examine a signaling pathway using multiple observations. The combination of striking visual changes in melanotic spots seen in larvae and immunofluorescence imaging of transcription factor localization pair well with graphical measures of Toll activation by lamellocyte counts and quantification of gene expression levels. This variety of figures should give students multiple opportunities to grasp the effects of *krz* and *Ulp1* gene knockdown on the Toll pathway. The experiments are also well controlled (see the comparison of Toll-dependent *Drosomycin* expression to IMD-dependent *Diptericin* expression and use of β-lactamase RNAi in Anjum *et al.* 2013, figure 4B) and use statistical *t*-tests and *P*-values to support conclusions, providing a good model for student scientists.

The phenotype amplification techniques in Anjum *et al* (2013) (co-expression of *Dicer-2* in RNAi, co-expression of SUMO and its conjugating enzyme in biochemical studies) are a good entry into discussions of experimental limitations as well as gene dosage effects. Without the *Dicer-2* amplification of the RNAi phenotype for single *krz* and *Ulp1* knockdowns, the authors may not have identified the individual phenotypes of these genes. Moreover, this article shows a direct link between genetic synergy and biochemical interaction of the protein products. The immunofluorescent images of Dorsal and Dif translocation into the nucleus in Anjum *et al.* (2013, figure 2) can be used to demonstrate the physical movement of proteins that may be needed to affect changes in gene expression.

Both RNAi and SUMOylation are topics that can be used to address changes to gene expression in the absence of DNA changes. RNAi is an example of post-transcriptional modification of gene expression that has been coopted as a powerful research tool. While the effects of SUMOylation are less well characterized than the related ubiquitination, these post-translational protein modifications affect gene expression and other cell processes.

Approach to Classroom Use

It is suggested that this primer article be provided to students concurrently with the Anjum *et al* (2013) article. Students would prepare for discussion of the article by reading the introduction, results, and at least the first two paragraphs of the discussion. To enhance the usefulness of this article as a teaching aid, instructors may apply the C.R.E.A.T.E. approach of Hoskins *et al.* (2007) in which students consider, read, elucidate hypotheses, analyze and interpret the data, and think of the next experiment for this article in isolation. Alternatively, instructors may wish to use the sequential approach of Hoskins *et al.* (2007) in which multiple articles from the same research area are considered. While the following suggestions are not all from the same lab, a potential sequence could be Thibault *et al.* (2004) in which the piggyback *P*-element forward genetic screen that generated the krz^{c01503} mutant was made, followed by Tipping *et al.* (2010) in which the link between β-arrestin *kurtz* and Toll signaling was first described by the same group as in Anjum *et al.* (2013). To compare the role of *krz* in an another developmentally important signaling cascade, Notch, instructors may provide Mukherjee *et al.* (2005).

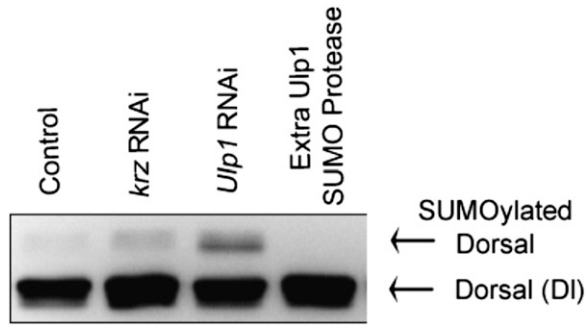
A discussion of the GAL4–UAS RNAi system for targeted gene knockdown in specific tissues, reviewed in Duffy (2002) would be a useful opening in examining this article. Once students are able to explain how tissue-specific GAL4

leads to UAS-driven RNAi knockdown in specific areas of the fly larvae, class discussion can focus on analyzing the figures in Anjum *et al* (2013). Anjum *et al* (2013, figures 1–3 and 5) should be approachable to students. Anjum *et al* (2013, figure 4) may be added with further discussions of the SUMOylation process, or instructors may wish to have students focus on the modified figures presented in the discussion questions below. Instructors may also wish to divide the class into small groups to consider individual figures in more detail or be assigned additional specific thought questions below. The main findings supporting the conclusion of a synergistic interaction between *kurtz* and *Ulp1* may be reviewed as a class.

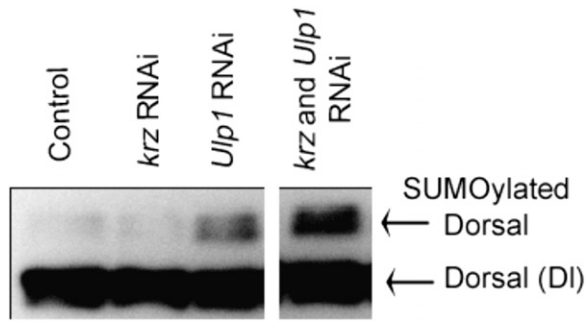
Further Thought Questions

These questions can be used to stimulate class or small group discussion as well as individual deep thinking about this research.

1. The authors use several outcomes to assess Toll pathway activation: Melanotic masses, GFP reporter of antimicrobial peptide expression (*Drosomycin)*, protein Western blot, lamellocyte numbers, and RT–PCR. Rank these assays from the least to most direct measurement of Toll-specific activation.
2. Compare the krz^{c01503} knockout *vs.* the RNAi knockdown in Anjum *et al.* (2013, figure 1B). Which had the greater effect on lamellocyte numbers? Why do you think this may be? What does it tell you about knockdown efficiency, even with extra Dicer-2? What happened to the lamellocyte number phenotype when the knockout was combined with *krz5.7* rescue?
3. What does the GFP in Anjum *et al.* (2013, figure 1F) indicate? What does this tell you about AMP production in the "wild-type" DD1 larvae compared to the DD1–krz^{c01503} knockout? Compare this to the Western blot data in Anjum *et al.* (2013, figure 1G). Why do you think the authors are showing results of both experiments? What might be some pros and cons associated with each type of measurement?
4. Why is Diptericin (*Dpt*) used as a control in Anjum *et al.* (2013, figure 1G)? What does figure 1, E and H, tell you about the role of *Dif* in Toll activation with *krz*?
5. Examine Anjum *et al.* (2013, figure 2). Why are Dorsal and Dif localized to the nucleus in Figure 2, B and D, but not in figure 2, A and C? What might they be doing there, and why does that make sense after Toll activation? In Anjum *et al.* (2013), compare figure 2 to figures 3, G and H, and the results of Figure 5—What might the Figure 2, B and D, series look like if the Dicer-2 was not also added?
6. In Anjum *et al.* (2013), Figure 3, A and B, illustrates biochemical interactions with HA epitope-tagged *krz* and V5-epitope-tagged *Ulp1*. Draw a diagram illustrating the co-immunoprecipiations in Figure 3, A and B, showing how the two experiments differ from each other.
7. In Anjum *et al.* (2013), figure 3C uses the human homologs for *krz*, ARRB-1 and ARRB-2 (β-arrestin 1 and β-arrestin 2). What does this add to the article? These homologs of *krz* were tested for biochemical interaction with the human SUMO protease SENP. Did both β-arrestin homologs interact with the SUMO protease equally? What does this illustrate about specialization after gene duplication among homologs?
8. Examine the figure taken from Anjum *et al.* (2013, figure 4B). The bands on the left indicate the "normal" level of Dorsal SUMOylation in a *Drosophila* cell line. How do *krz* RNAi, *Ulp1* RNAi, and co-expression of additional *Ulp1* SUMO protease affect Dorsal SUMOylation? HSP70 is a protein folding chaperone, a "cell-housekeeping" protein that is not expected to vary in response to changes in Toll signaling. Why, then, is the IB: HSP70 immunoblot shown in this figure?



9. Examine the figure taken from Anjum *et al.* (2013, figure 5F). What does the level of Dl SUMOylation tell you about the genetic activities of *krz* and *Ulp1* individually and working together?



10. The authors report that *krz* and *Ulp1* act synergistically on the Toll pathway. What does this mean? Do you think the authors would have observed a synergistic effect when they combined *krz* and *ulp1* knockdown with the *Dicer-2* expression booster? Why or why not?
11. Examine the figure comparing Toll and IMD pathway activation in this primer. Where does the RNAi against *krz* and *Ulp1* fit in? Diagram the effect of *Ulp1* knockdown on the Toll pathway activation by Dl SUMOylation. *Ulp1* is a SUMO protease that removes SUMO from proteins. Do the results of this study indicate that Dl SUMOylation generally increases or decreases Toll pathway activation?

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