Published in final edited form as:
Nature. 2005 September 29; 437(7059): 746–749.

WntD is a feedback inhibitor of Dorsal/NF-kB in *Drosophila* development and immunity

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Abstract

Regulating the Nuclear factor-κB (NF-κB) family of transcription factors is of critical importance to animals, with consequences of misregulation that include cancer, chronic inflammatory diseases, and developmental defects[1]. Studies in *Drosophila melanogaster* have proved fruitful in determining the signals used to control NF-κB proteins, beginning with the discovery that the Toll-NF-κB pathway, in addition to patterning the dorsal-ventral (D/V) axis of the fly embryo, defines a major component of the innate immune response in both *Drosophila* and mammals[2,3]. Here, we characterize the *Drosophila wntD* (Wnt inhibitor of Dorsal) gene. We show that WntD acts as a feedback inhibitor of the NF-κB homolog Dorsal during both embryonic patterning and the innate immune response to infection. *wntD* expression is under the control of Toll-Dorsal signaling, and increased levels of WntD block Dorsal nuclear accumulation, even in the absence of the IκB homolog Cactus. The WntD signal is independent of the common Wnt signaling component Armadillo (β-catenin). By engineering a gene knockout, we show that *wntD* loss-of-function mutants have immune defects and exhibit increased levels of Toll-Dorsal signaling. Furthermore, the *wntD* mutant phenotype is suppressed by loss of zygotic *dorsal*. These results describe the first secreted feedback antagonist of Toll signaling, and demonstrate a novel Wnt activity in the fly.

The D/V axis of the Drosophila embryo is initially patterned by a ventral-to-dorsal nuclear gradient of Dorsal protein activity under the control of Spatzle-Toll signaling[4,5]. Toll activates Dorsal primarily through the degradation of Cactus, thereby freeing Dorsal to enter the nucleus and activate or repress target genes[6]. The transcriptional profile that is regulated by Dorsal defines the spatial organization of tissues in the embryo, with ventral-most cells becoming mesoderm, flanked by the mesectoderm and neuroectoderm in more lateral regions, and gut primordia at the poles[7].

The gene *wntD* was identified as a member of the *Drosophila* Wnt family based on a genomic search for Wnt-related genes (synonym CG8458) [8]. Examination of *wntD* RNA *in situ* revealed that the first detectable expression is seen at the ventral poles of the blastoderm embryo, followed by sequential ventral-to-dorsal expression in the presumptive mesoderm, mesectoderm, and neuroectoderm (fig. 1). Embryos derived from mothers carrying a dominant activated allele of Toll express *wntD* RNA more broadly and at higher levels than wild type (fig. 1c,d). This demonstrates that *wntD* expression is induced by Toll signaling. Examination of WntD protein distribution shows that WntD is secreted and travels multiple cell diameters away from producing cells, suggesting that WntD is capable of signaling at a distance (fig. 1f,g).

Supplementary Information accompanies the paper on www.nature.com/nature.

To uncover the signaling activity of WntD in the embryo, we expressed WntD ectopically in the female germline, producing blastoderm stage embryos that contain high levels of WntD protein (data not shown). These embryos lacked detectable nuclear Dorsal (fig. 2b), although total cellular levels of Dorsal protein remained unchanged (fig. 2 inset b). Consequently, the mesodermal Dorsal target gene Twist was not expressed (fig. 2d), and the embryos produced only dorsal cuticle (fig. 2f). Furthermore, the observed defects were specific to dorsal-ventral patterning, as the anterior-posterior patterning gene *hunchback* was unaffected by WntD (data not shown). Following submission of this manuscript, similar results for the over-expression of WntD were reported by Ganguly *et al*.[9]

In order to determine the point of intersection between WntD activity and the Toll-Dorsal pathway, we constructed flies that over-express WntD and carry strong hypomorphic alleles of *cactus*. While maternal *cactus* mutants exhibited a ventralized phenotype (fig. 2g), those also over-expressing WntD were dorsalized, and indistinguishable from embryos over-expressing WntD alone (fig. 2h). These data demonstrate that WntD, a secreted growth factor, is capable of producing a signal that blocks Dorsal nuclear translocation downstream of, or in parallel to, Cactus. It has been shown previously that Dorsal undergoes Toll-dependent and –independent phosphorylation[10], and that Dorsal nuclear localization can be regulated independently of Cactus[11].

That WntD is a member of the Wnt family of growth factors raises the question of whether it signals through the well-characterized Frizzled-Armadillo/β-catenin pathway[12]. We suggest that it does not, based on two lines of evidence: First, germline clones of *axin*, a negative regulator of Armadillo, do not produce dorsalized embryos[13]; and second, over-expression of WntD in tissues sensitive to Armadillo signaling does not have any detectable effect (data not shown). These observations however, do not rule out the possibility that WntD signals through a Frizzled receptor in an Armadillo-independent manner.

In order to investigate the role of endogenous WntD, we constructed a loss-of-function mutation using “ends-out” gene targeting (fig 3a). The modified *wntD* locus produced no detectable protein, as assayed by western blot (fig. 3c). Analysis of flies homozygous for either of two $wntD^{KO}$ alleles revealed that *wntD* is not essential for viability or fertility.

Despite their viability, *wntD* mutant embryos show an expansion of nuclear Dorsal into the pole regions where endogenous WntD is first detected (fig. 3e). This indicates that the earliest role of WntD in the embryo is to restrict the field of Dorsal activation, thereby ensuring the establishment of the proper boundary between the developing ventral and terminal domains; Dorsal, along with A/P positional information, induces transcription of *wntD* at the ventral poles of the embryo, and WntD in turn feeds back to repress Dorsal nuclear translocation, and prevent improper spread of the ventral domain. This mechanism stands in contrast to another characterized mode of Dorsal pathway repression at the embryonic termini- that of signaling from the Torso (Tor) receptor tyrosine kinase[14]. In the case of Torso, signaling at the poles of the embryo selectively interferes with the ability of Dorsal to repress the expression of specific target genes, while exerting only a minor effect on those genes activated by Dorsal[14]. These data suggest that Torso signaling affects the activity of nuclear Dorsal, whereas WntD signaling affects Dorsal’s nuclear translocation.

In addition to its role in D/V patterning, it has been well-established that Toll-NF-κB signaling has a more evolutionarily conserved role in regulating the innate immune system[3,15]. During the immune response, Toll induces the nuclear translocation of two NF-κB family members: Dorsal and Dorsal-related immunity factor (Dif). Genetic analysis has suggested that Dif, while dispensable for development, is the major transcription factor involved in the Toll-mediated immune response[16]. In addition to Dorsal and Dif, the fly immune response also uses a third

NF-κB related protein, Relish, which is activated upon signaling by PGRP-LC and Imd[17, 18]. Together, these pathways regulate the expression of hundreds of genes following microbial infection[19].

In light of the interaction between WntD and Dorsal in the embryo, we asked if WntD could be playing a role later in the fly's life as a repressor of Toll/Dorsal-mediated immunity. RT-PCR was used to confirm expression of endogenous *wntD* RNA in adults (data not shown). *wntD* mutant adults appear normal, with the exception that at low frequency (1-2%), we have observed sites of ectopic melanization, most notably on the wing hinge (fig. 3g). This is consistent with a role for WntD in maintaining low basal levels of Toll-Dorsal signaling, as other mutations that hyper-activate Toll show increased levels of phenoloxidase-driven melanization[20,21]. Furthermore, Dorsal has been shown to be an essential component of the melanization response in larvae[22].

To investigate the role of WntD following septic injury, *wntD* and control flies were injected with a dilute culture of the gram-positive bacterium *Micrococcus luteus*, and the induction of antimicrobial peptide (AMP) transcripts were monitored over time using quantitative RT-PCR (fig 4b,c). We observed that some, but not all, AMPs showed aberrant expression in *wntD* mutants. *diptericin* was most severely affected, with *wntD* flies displaying dramatically elevated basal levels of expression (approximately 15-fold), and significantly higher mRNA levels following infection (fig 4b). In contrast, *drosomycin* mRNA levels were not significantly different from controls in either uninfected or infected *wntD* mutants. A third AMP, *defensin*, showed an intermediate pattern of expression, with elevated mRNA levels in *wntD* mutants at some time points (data not shown).

These results pose an apparent paradox, as previous experiments have characterized *diptericin* as a target of IMD-Relish, and *drosomycin* as a target of Toll signaling[15,23]. *Drosomycin* expression is reported to be primarily regulated by Dif in adult flies, and appears to be unaffected by increased Dorsal activity[16]. Thus, our results for *Drosomycin* are consistent with past work. The *diptericin* result initially appears puzzling, but existing data demonstrate that the signal transduction pathways regulating immunity are not as specific as initially described. For example, Relish is required for *diptericin* induction in response to infections *in vivo*, but constitutive activation of Toll signaling results in elevated levels of *diptericin* in adult flies[19]. Furthermore, Dorsal is sufficient to activate the *diptericin* promoter *in vitro*[24]. The simplest explanation for these observations is that *diptericin* transcription can be induced by Toll-Dorsal signaling. Taken together, these data support a model in which WntD signaling specifically represses Toll-Dorsal, and not –Dif signaling.

Given a role for WntD in the regulation of antimicrobial gene transcription, we sought to determine whether *wntD* mutants are immunocompromised. To test this, *wntD* and control adults were infected with the gram positive and lethal pathogen *Listeria monocytogenes*[25]. In response to infection, *wntD* mutants exhibited significantly higher levels of mortality when compared to parental lines (fig 4a). Importantly, this phenotype was suppressed by the introduction of *dorsal* mutations (fig 4a), with close to full suppression in the absence of both copies of dorsal and partial suppression in flies heterozygous for a dorsal mutation. These genetic interactions are consistent with our assertion that WntD specifically regulates Dorsal, and not other mediators of immunity. Recent reports have demonstrated that a fly's response to bacterial challenge includes factors that are damaging to the host[26], and that increased Toll signaling can render flies more susceptible to viral infection[27]. We therefore propose that it is the deleterious hyper-activation of specific Dorsal target genes that is responsible for the increased mortality seen in *wntD* mutants. Furthermore, the susceptibility of *wntD* mutants to a lethal infection suggests a reason for the positive selection of *wntD* during evolution; immune responses have a cost, and their appropriate downregulation would be expected to provide flies

with a selective advantage. While *wntD* flies appear healthy in a lab environment, it is easy to imagine that under the more stressful, and septic, conditions in the wild, flies lacking *wntD* would suffer the perils of a hyperactive immune system.

We have presented evidence that WntD, a Wnt family member, produces a signal that blocks the nuclear translocation of Dorsal. Furthermore, WntD is a target of Toll-Dorsal signaling, and creates a negative feedback loop to repress Dorsal activation. We have shown that *wntD* is not required for viability under lab conditions, but that *wntD* mutants show defects in embryonic Dorsal regulation, and the adult innate immune system. As the WntD signal in the embryo is not mediated by Armadillo, we suppose that the immune function of WntD is also Armadillo-independent, although Zettervall et al. have observed immune defects in flies expressing a dominant-negative form of the Aramdillo partner DTCF[28]. Further characterization of signaling events bridging WntD and Dorsal could yield valuable insight into the regulation of the therapeutically important NF-κB family of proteins.

Methods

Drosophila stocks.

Flies carrying a *P[UASp-wntD]* were generated using standard techniques. Other transgenic flies used were: *P[nos-Gal4:VP16]*[29].

Immunostaining and *in situ* hybridization.

All *in situ* hybridizations were performed using standard procedures, with the exception that Proteinase K treatment was omitted. All immunostainings and cuticle preparations were performed using standard procedures. Primary antibodies were used at the following dilutions: mouse anti-Dorsal (Developmental studies hybridoma bank; 1:10), rabbit anti-Twist (a gift from Siegfried Roth; 1:5000), rabbit anti-WntD (1:500). Photos were taken using a Zeiss Axiocam camera, images were processed with Adobe Photoshop, and figures were prepared using Adobe Illustrator.

Western and Southern blots.

Western blot for Dorsal protein was performed as previously described[10]. Lysates were prepared in TNT buffer (150mM NaCl, 50mM Tris-Hcl, 1% Triton X-100, pH 7.5). In order to detect WntD in embryonic lysates, a collection of 0-3 hour embryos was lysed in TNT buffer, and incubated overnight with 20mL Blue Sepharose beads (Amersham Biosciences). The beads were washed, and exposed to sample buffer (15mM Tris-HCl, 2.5% Glycerol, 5% SDS, 1% 2-β-mercaptoethanol, 0.006% bromophenol blue) prior to gel loading. Western Blot was performed using standard techniques with rabbit anti-WntD antibody (1:1000). Southern Blots were performed using standard techniques. *wntD* radio-labeled probe was generated to full length *wntD* cDNA using Rediprime II kit (Amersham).

Generation of anti-WntD antibodies.

See supplementary information.

Generation of *wntD* knockout.

See supplementary information.

Bacterial injections and Quantitative RT-PCR.

All injections were done using male flies aged one week post eclosion. A culture of *Listeria monocytogenes* was diluted to an OD(600) of 0.1, and a 25nL volume was injected abdominally using a pulled glass needle as previously described[30]. Groups of 20 flies of each genotype

were injected in an alternating manner to control for variability over time. Flies were maintained on nonyeasted, standard dextrose medium at 25°C, 65% relative humidity, and survival was monitored daily. *Micrococcus luteus* was injected as described for *L. monocytogenes*. RNA extraction and quantitative RT-PCR was performed as described[30],with the exception that 6 flies were used per sample.

Supplementary Methods.

Generation of *wntD* knockout.

The "ends-out" targeting scheme was a modified version of that described previously[1]. The donor vector was constructed using pP[EndsOut] (a gift from Jeff Sekelsky) in three steps: (1) A 3kb genomic fragment including the 5' portion of *wntD* was amplified from genomic DNA by PCR using the oligos: 5'- CCGCTCGAGGGGTGCCTCTAAGAGTTTGG-3' and 5'-ACATGCATGCAGATCACTGGAACAGGAATGC-3'. The product was digested with Xho-I and Sph-I, and cloned into the Xho-I, Sph-I sites of pP[EndsOut]. (2) pBS-70W (Jeff Sekelsky) was digested with Sph-I and Kpn-I to yield an *hsp70-white* fragment which was cloned into the Sph-I, Kpn-I sites of the plasmid made in step 1. (3) A 3kb genomic fragment including the 3' portion of *wntD* was amplified using the oligos: 5'-CGGGGTACCCGTCGATTGTGACCGATG-3' and 5'-CGGGGTACCTTTTGCAAACGTGACCTCCT-3'. The product was digested with Kpn-I, and cloned into the Kpn-I site of the vector made in step 2. Seven fly lines carrying independent insertions of the donor construct were generated using standard procedures. Virgin females heterozygous for each donor insertion were crossed to *yw; p[ry, 70FLP]11 p[70I-SceI, v$^+$]2B Sco/S2 Cyo* males[2], and the resulting larvae were heatshocked for 1 hr at 37C 0-3 days AEL. The resulting unbalanced females were mated in groups of 4 to *yw; p[ry, 70FLP]11 p[70I-SceI, v$^+$]2B Sco/S2 Cyo* males. The progeny from this cross were heat-shocked at 0-3 days AEL, and 700 vials were screened for red eyes upon eclosion. Non-mosaic, unbalanced flies were saved as FLP-insensitive integrations, and non-mosaic *Cyo* flies were subjected to a second cross to *yw; p[ry, 70FLP]11 p[70I-SceI, v$^+$]2B Sco/S2 Cyo*. Lines producing non-mosaic progeny were also saved as FLP-insensitive integrations. In total, 13 FLP-insensitive integrations were found that mapped to the 3rd chromosome. Southern Blot analysis revealed that 2 were homologous targeting events, each derived from a different donor line. *wntD^{KO1}* and *wntD^{KO2}* were backcrossed to the *yw* parental line for 5 generations through the female germline to allow for meiotic recombination with parental genome.

Generation of anti-wntD antibodies.

wntD protein was purified as described previously[3]. Highly concentrated wntD included wntD precipitate, which was collected by centrifugation and resuspended in 4.5M Urea to a concentration of 1.4 mg/mL. Concentrated wntD was injected into a Rabbit using standard procedures (Josman Labs).

Acknowledgements

We thank Jeff Brown for preparing WntD protein for antibody production; Chi-Hwa Wu and Kathy Xu for preliminary experiments with *wntD*; Matt Fish for p-element transformations; Jeff Sekelsky and Kent Golic for fly strains and vectors used in gene targeting; Siegfried Roth, Gregor Zimmermann, and the Developmental Studies Hybridoma Bank for antibodies; Alana O'Reilly and the Bloomington Stock Center for fly stocks; and Catriona Logan and Amanda Mikels for critical reading of the manuscript. MDG was supported by a Howard Hughes Medical Institute predoctoral fellowship and a Stanford Graduate Fellowship. This work was supported by a grant from the NIH and by the Howard Hughes Medical Institute. Correspondence to: Roel Nusse. Correspondence and requests for materials should be addressed to R.N. (rnusse@stanford.edu)

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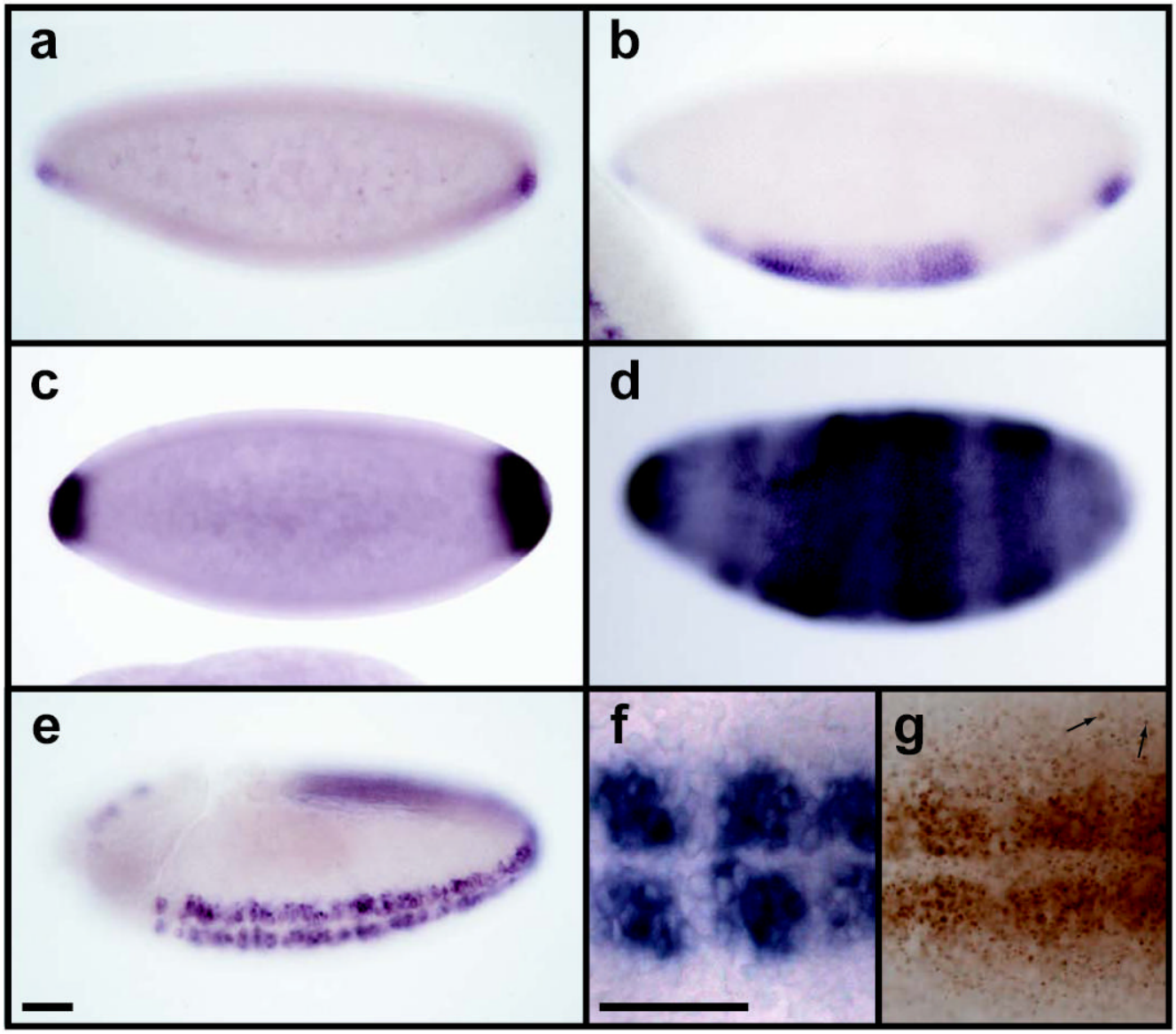

Figure 1.

wntD is expressed with D/V polarity, and is under the control of Toll signaling. **a-e**, *In situ* hybridization to *wntD* mRNA in wild type embryos **(a,b,e)** and those derived from *Toll*^{10b} mothers **(c,d)**. Wild type expression is seen in the ventral poles at stage 5 **(a)**, presumptive mesoderm at stage 6 **(b)**, and neurogenic ectoderm at stage 9 **(e)**. Stronger, expanded *wntD* expression is seen in *Toll*^{10b}-derived embryos at stages 5 and 6 **(c,d). f,g** Close-up ventral views (anterior left) of stage 9 wild type embryos stained for *wntD* mRNA (**f**) or with anti-WntD antibody (**g**). Arrows indicate examples of WntD antigen detected multiple cell diameters away from *wntD*-expressing cells. Scale bars indicate 50 μm. All embryos here and henceforth oriented anterior left, ventral down, unless otherwise indicated

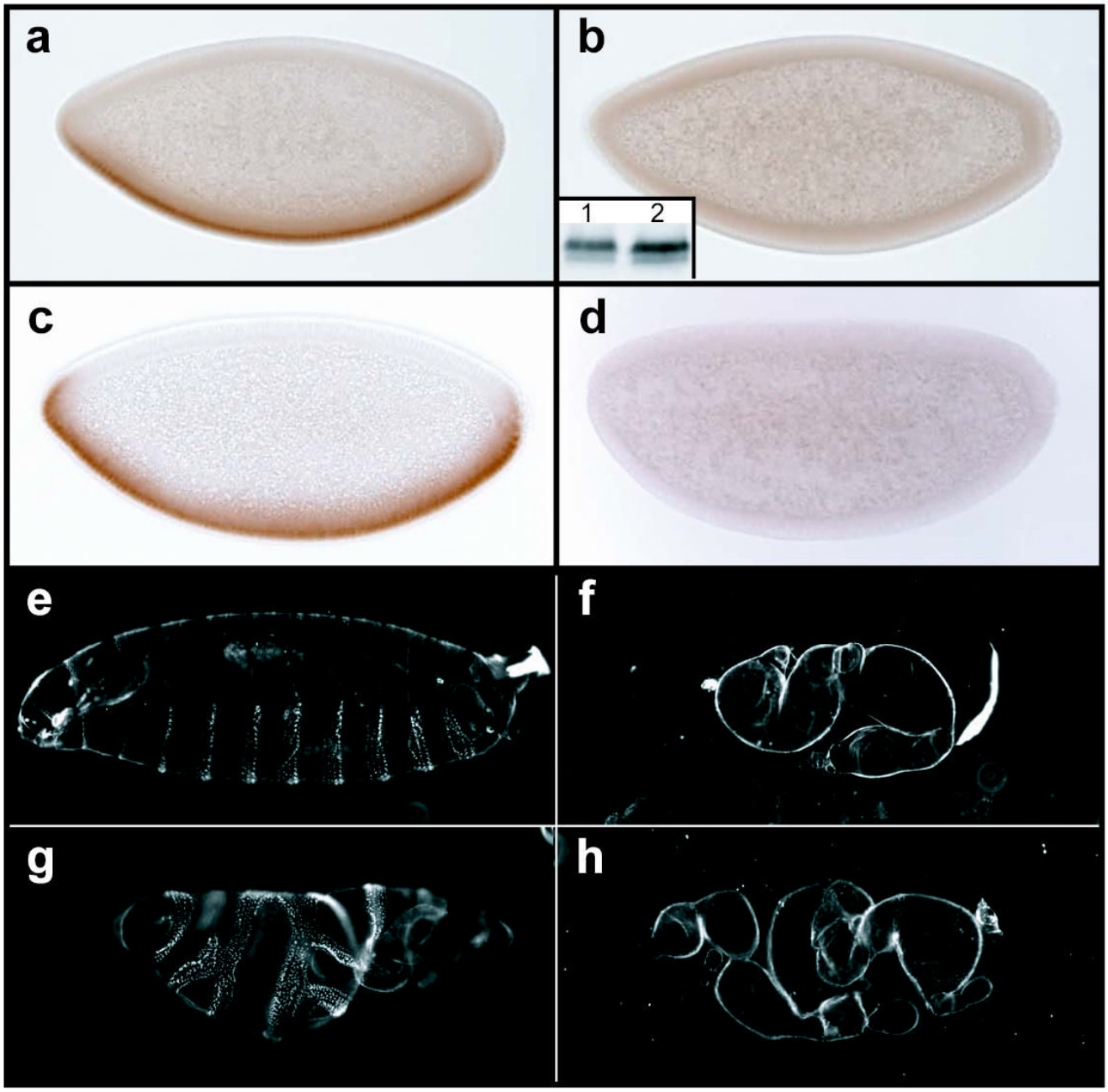

Figure 2.

Over-expression of WntD blocks Dorsal protein activation independently of Cactus. **a,c**, Wild type embryos stained with antibodies to Dorsal **(a)**, or Twist **(c). b,d**, Embryos from females carrying *P[nos-Gal4:VP16]* and *P[UASp-wntD]* transgenes, stained with antibodies against Dorsal **(b)**, or Twist **(d). inset b**, Total Dorsal protein levels (assayed by western blot) are equivalent in wild type embryos (lane 1) and those from *P[nos- Gal4:VP16]/P[UASp-wntD]* females (lane 2). **e-h** Cuticles of embryos with the maternal genotypes: wild type **(e)**; *P[nos-Gal4:VP16]/P[UASp-wntD]* **(f)**; $cact^{1}/cact^{4}$ **(g)**; and $cact^{1}/cact^{4}$; *P[nos-Gal4:VP16]/P[UASp-wntD]* **(h)**.

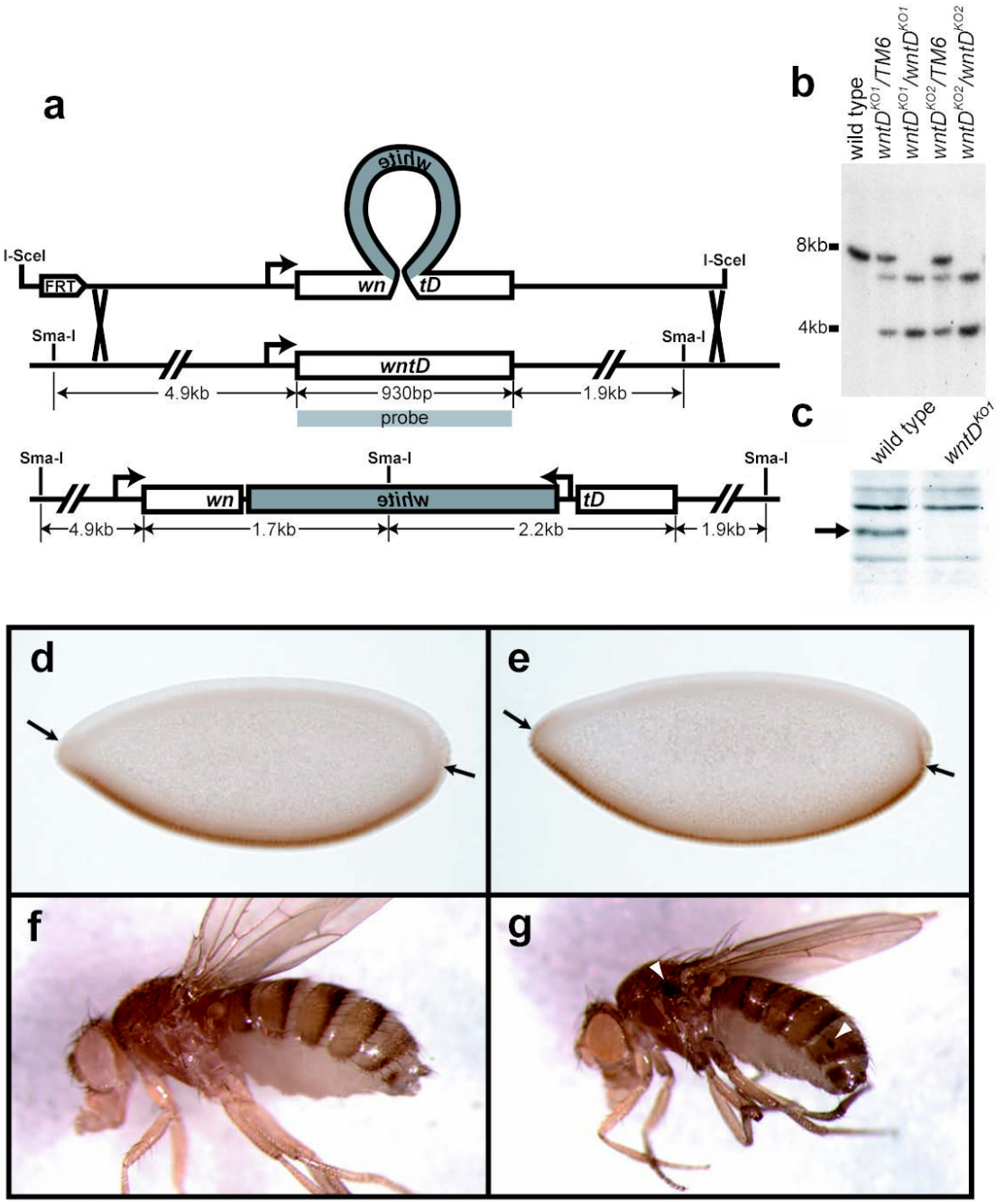

Figure 3.

wntD knockout flies exhibit ectopic Dorsal activation. **a**, "Ends-out" knockout targeting scheme, illustrating how a *white* mini-gene was used to interrupt the *wntD* open reading frame. **b**, Southern blot of Sma-I digested genomic DNA, confirming proper integration of targeting construct. **c**, Anti-WntD Western blot of lysate from wild type and $wntD^{KO1}$ embryos (arrow indicates size of WntD protein). **d,e**, *yw* **(d)** and *yw;* $wntD^{KO1}$ **(e)** embryos stained with antibodies against Dorsal. Arrows show point of ventral-most nuclear Dorsal seen in control embryos. **f,g** adult female *yw* **(f)** and *yw;* $wntD^{KO1}$ **(g)** flies. Arrowheads mark sites of ectopic melanization.

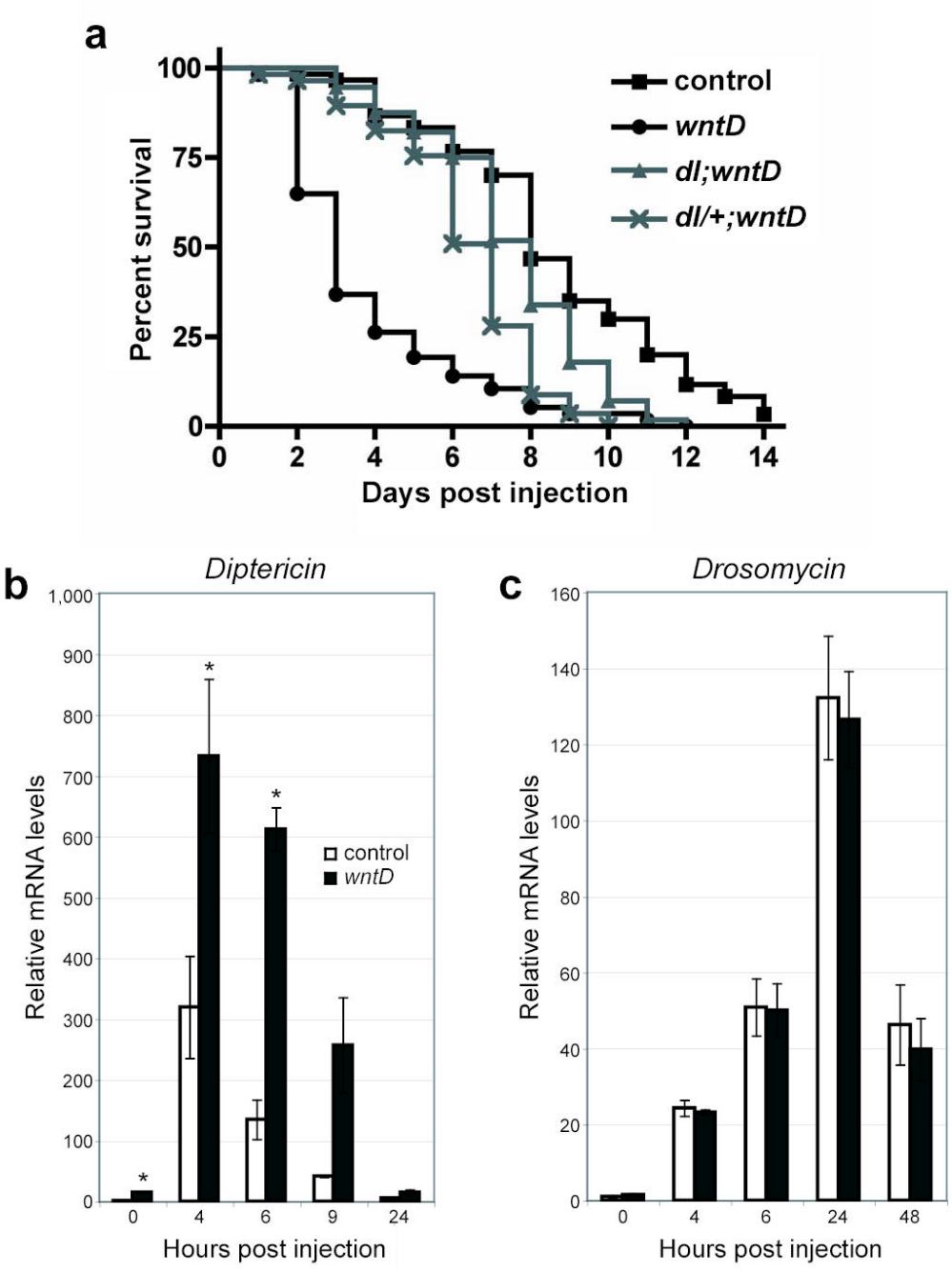

Figure 4.

wntD mutants show an aberrant response to microbial infection. **a**, One week old adult *yw* **(squares, n=60)**, *yw; wntD*^{KO1} **(circles, n=57)**, *yw; dl*^{1}*/dl*^{4}*; wntD*^{KO1} **(gray triangles, n=56), and** *yw; dl*^{4}*/+; wntD*^{KO1} **(gray crosses, n=57)** were injected with a dilute culture of *Listeria monocytogenes*, and survival was monitored. Log rank tests indicate that *wntD* mutant curve is significantly different from the other three, with p<0.0001. **b,c,** Real-time PCR was used to monitor *diptericin* **(b)** and *drosomycin* **(c)** mRNA levels in *yw* **(white bars)** and *yw; wntD*^{KO1} **(gray bars)** adults following injection with *Micrococcus luteus*. Results are means and s.e.m. Asterisks (*) denote significance level of p<0.05.