The *IMD* innate immunity pathway of Drosophila influences somatic sex determination via regulation of the *Doa* locus

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**Abstract**

The *IMD* pathway induces the innate immune response to infection by gram-negative bacteria. We demonstrate strong female-to-male sex transformations in double mutants of the *IMD* pathway in combination with *Doa* alleles. *Doa* encodes a protein kinase playing a central role in somatic sex determination through its regulation of alternative splicing of *dsx* transcripts. Transcripts encoding two specific *Doa* isoforms are reduced in *Rel* null mutant females, supporting our genetic observations. A role for the *IMD* pathway in somatic sex determination is further supported by the induction of female-to-male sex transformations by *Dredd* mutations in sensitized genetic backgrounds. In contrast, mutations in either *dorsal* or *Dif*, the two other NF-κB paralogues of Drosophila, display no effects on sex determination, demonstrating the specificity of *IMD* signaling. Our results reveal a novel role for the innate immune *IMD* signaling pathway in the regulation of somatic sex determination in addition to its role in response to microbial infection, demonstrating its effects on alternative splicing through induction of a crucial protein kinase.

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1. Introduction

The innate immune response to infection in *Drosophila* depends upon the activation of the *Toll* or the *IMD* (Immune Deficiency) pathways, which can be simplistically summarized as responding to fungal or gram-positive (*Toll*) or gram-negative (*IMD*) bacterial infections (De Gregorio et al., 2002), despite some overlaps and complexities linking the pathways (Hedengren-Olcott et al., 2004). The transcriptional effects of the *Toll* and *IMD* pathways are mediated by three NF-κB transcription-factor paralogues: DORSAL (DL), and Dorsal-related immunity factor (DIF) in the *Toll* pathway, and Relish (REL) in the *IMD* pathway. DL, DIF and REL proteins transcriptionally activate loci encoding anti-microbial peptides (AMP) by directly binding the promoters of their target loci as homo- or hetero-dimers (Han and Ip, 1999; Petersen et al., 1995; Senger et al., 2004; Tanji et al., 2010).

The eleven loci of the *IMD* pathway were identified and defined by genetic and RNAi screens (see Myllymaki et al., 2014 for review). Among these are loci encoding a peptidoglycan receptor known as PGRP-LC, whose product is responsible for bacterial recognition; an internal adapter molecule, *IMD*; the *Drosophila* orthologue of mammalian caspase 8 known as *Dredd*; mitogen activated kinase TAK1; *Ird5* and *Key* which are members of an IKK complex; and finally the NF-κB family member, *Rel*.

REL is activated via at least two independent steps, phosphorylation on Ser528 and 529 by the IKK complex (Erturk-Hasdemir et al., 2009), and also by proteolytic cleavage by *Dredd* (Stoven et al., 2003). REL migrates to the nucleus following phosphorylation and cleavage, where it activates transcription from its target promoters.

In addition to the activation of innate immune responses, DL, DIF and REL play additional roles in *Drosophila* development and physiology. All three have been implicated in the regulation of the Achaete–Scute complex (Ayyar et al., 2007), which encodes a set of pro-neural transcription factors. The *Toll* pathway and its effector DL were first described as regulators of dorsal–ventral patterning in the early *Drosophila* embryo (Hong et al., 2008; Nusslein-Volhard et al., 1980; Perry et al., 2009). REL, on the other hand seems to have more subtle functions aside from its function in immune signaling. In contrast to *dl*, a *Rel* null allele is viable and fertile (Hedengren et al., 1999). However, *Rel* mutants display reduced sleep, and expression of *IMD* pathway mRNAs is induced by sleep deprivation (Williams et al., 2007). Consistent with these observations, sleep-deprived flies were more resistant to gram-negative bacterial infection, presumably due to induced expression of *IMD* signaling. Flies increase their sleep in response to a bacterial challenge, and *Rel* mutants fail to do so (Kuo et al., 2010).
DREDD has also been implicated in sperm individualization (Huh et al., 2004). However, specific IMD pathway targets in sleep or spermatogenesis remain to be identified. IMD signaling is also activated during larval molting by the hormone bursicon, apparently providing a type of prophylactic immunity (An et al., 2012).

LAMMER (or CLK) protein kinases are among the 347 eukaryotic “signature” proteins (Hartman and Federov, 2002), and are thus represented in the genomes of all eukaryotic species. These kinases phosphorylate and regulate the activity of SR and SR-like proteins, among other substrates (Rabinow, 2012). SR proteins integrate multiple steps of mRNA maturation, playing diverse roles from transcription initiation and elongation to RNA localization, translation and half-life regulation in the cytoplasm (Long and Caceres, 2009; Shepard and Hertel, 2009). The unique LAMMER protein kinase of Drosophila is encoded at the Darkener of apricot (Doa) locus, which produces a minimum of six different proteins whose mRNAs originate through the use of alternative promoters rather than from alternative splicing (Kpebe and Rabinow, 2008a). These isoforms possess at least three differentiable functions, as revealed by isoform-specific alleles, over-expression of wild-type cDNAs, and interfering RNAi constructs (Kpebe and Rabinow, 2008b; Serpinskaya et al., 2014). Moreover, different DOA isoforms populate either the cytoplasm or the nucleus (Yun et al., 2000), further implicating the kinase in diverse processes.

Doa is required for development of the embryonic nervous system, segmentation, photoreceptor maintenance (Yun et al., 1994) and somatic sexual differentiation, the last through phosphorylation of the SR and SR-like proteins RBP1, TRA, TRA2 (Du et al., 1998) and SRm160 (Fan et al., 2014).

We describe here regulation by the IMD pathway of female somatic sex determination in Drosophila. IMD signaling induces transcripts encoding two specific Doa isoforms required for the regulation of alternative splicing. Thus IMD signaling, like Toll, is essential for the regulation of a central developmental process in

![Fig. 1. Dredd alleles strongly enhance Doa-dependent somatic sex transformations.](image-url)
addition to its role in innate immunity. Transcriptional regulation of Doa additionally implicates IMD signaling as a possible determinant of alternative splicing and other post-transcriptional regulatory processes.

2. Materials and methods

2.1. Drosophila genetics

Drosophila were maintained on corn-meal agar medium. Crosses were performed at 25 °C. Doa alleles were described (Kpebe and Rabinow, 2008b; Rabinow et al., 1993). Standard segregation and recombination generated the allelic combinations described. Mutations are described in Flybase (http://flybase.org/). Df(2L)4/CyO removes the dl and Dif loci (Meng et al., 1999). It and additional mutants were obtained through the generosity of Tony Ip and Marc Dionne. Dan Hultmark kindly provided strains overexpressing REL68. Cell clones were generated by mating male hs-Flp122; UAS-FlpD1/CyO, Act-GFPMR11; ActSC > CD2 > Gal44, UAS-mCD8GFP15/15/TM6b with UAS-Rel68 to obtain UAS-Rel68/ hs-Flp122; UAS-FlpD1/+; ActSC > CD2 > Gal44, UAS-mCD8GFP15/15/+ females. A 20 min heat shock during the first larval instar induced salivary gland clones.

2.2. Immunohistochemistry

Salivary glands dissected from female larvae in PBS were fixed in 2% paraformaldehyde for 1 h at room temperature. Immunostaining was carried out as previously described (Viktorin et al., 2013) using rabbit anti-Doa (Yun et al., 2000) 1:2000, mouse anti-Dig 1:50 (4F3, DSHB), Alexa-conjugated goat anti-mouse 6471:300, and DyLight goat anti-rabbit 549 1:300 (LI-COR). Secondary antibodies were incubated for 1 h at room temperature.

2.3. Bacterial infections

C, RelE20 and Doa/DoaDEM flies were pricked under the wing with a needle previously dipped into a culture (OD 200) of the gram-negative bacterium Erwinia carotovora.

2.4. Scanning electron microscopy

Flies were dehydrated through 30%, 50% and 70% ethanol, frozen at −18 °C under partial vacuum (90 Pa) on a Peltier stage and observed under ESED mode on a Hitachi S3000N scanning electron microscope (acceleration voltage 10–12 kV).

2.5. Molecular biology

Total RNA from 0–24 h old adults was prepared with Trizol. qPCR used a StepOne instrument with Fast SYBR Green Master Mix according to the manufacturer’s protocol (Applied Biosystems). Primers are listed (Supplemental Table 1). rp49 was the control. Each reaction (95 °C for 20 s; 40 cycles at 95 °C, 3 s; 60 °C, 30 s) was performed twice using a 10 μl reaction mixture.

3. Results

3.1. Enhancement of Doa-induced sex transformation phenotypes by alleles of Dredd and Rel

Flies heteroallelic for two hypomorphic Doa alleles, Doa/DoaDEM or DoaHD/DoaDEM barely show any female-to-male somatic sex-transformation phenotypes (Fig. 1C), compared with wild-type (Fig. 1A), although previous molecular analysis demonstrated that female-specific alternative splicing of dsx transcripts is partially impaired in this genotype (Du et al., 1998). Females homozygous for three Dredd alleles (L23; EY08404; EY05906) also show completely normal cuticular morphology (Fig. 1D–F). In particular, Dredd(23) is a null (Leulier et al., 2000). However, approximately 20% of Dredd/+; Doa/DoaDEM females showed enhancement of Doa-induced female-to-male sex transformations (not shown). This phenotype was substantially enhanced in Dredd/Dredd; Doa/DoaDEM females of all three Dredd alleles tested (Fig. 1G–I, Table 1), producing a phenocopy of doublesex mutations. Two well defined claspers are present below the female vaginal plates in animals of these genotypes (arrowheads).

Dredd encodes an apical caspase related to vertebrate caspase 8. Its only known substrate is the NF-κB orthologue RELISH (Er-turk-Hasdemir et al., 2009; Stoven et al., 2000, 2003), the transcription factor at the terminus of the IMD innate-immunity pathway. We therefore tested whether RelE20, a null allele (Hedengren et al., 1999) would also affect Doa-dependent somatic sex transformations. Although female RelE20 homozygotes show no discernable sex-transformations (arrows, Fig. 2C, compare with wild-type, Fig. 2A), crosses generating RelE20/DoaHD/+ DoaDEM yielded ~20% of F1 females showing substantially enhanced female-to-male sex transformations (arrowheads, Fig. 2D). Crosses generating RelE20/Doa/DoaDEM homozygotes also produced ~20% of females with sex-transformations of the same magnitude, that were not enhanced relative to Rel/+ heterozygotes. We further tested RelE23, a revertant carrying a precise excision of the P-element whose excision generated the RelE20 deletion. Double-mutant RelE23/Doa animals showed no enhancement of the Doa/Doa sex transformation phenotype, confirming that the Rel genotype was responsible for the observed effects.

3.2. Somatic sex transformations induced by mutations in additional components of the IMD pathway

We investigated whether other members of the IMD pathway might also induce sex transformations in Doa mutant backgrounds by testing alleles of PGRP-LC, Imd, TAK1 (Fig. 2E) and ird5 (Fig. 2F). Crosses with alleles of each locus were performed to generate Dredd/DoaDEM double-mutants heterozygous and or homozygous for each of these IMD pathway loci as summarized in Table 1. In each case between 10–20% of double Doa and IMD-pathway mutant females revealed sex-transformations. These were generally less extreme than in the cases with Dredd and Rel alleles, with appearance of single male “clasper”-like appendages, even in the case of homozygotes. The somatic sex transformations observed in Dredd; Doa and Rel Doa double mutants are thus induced generally by reductions in IMD pathway signaling in Doa backgrounds.

3.3. Induction of female-to-male sex transformations by Dredd alleles

To further examine the role of Dredd in somatic sex determination and to determine whether its effects were invariably dependent upon reduction in function of Doa, we crossed alleles of Dredd with double-mutant tra29/CyO; tra/TM3, creating Dredd/ Dredd; tra29/+; tra/+ females. These flies possess backgrounds sensitized to subtle shifts in the efficacy of the somatic sex determination machinery. Under our conditions (genetic background, media, temperature), tra29/+; tra/+ XX females showed no evidence of somatic sex transformations (Fig. 3A). However, when also homozygous for any of three different Dredd alleles, tra29/+; tra/+ double heterozygous females reveal strong transformations towards dsx-like phenotypes (Fig. 3B–D and D’).

Moreover, homozygotes of two of the same Dredd alleles in
combination with the heteroallelic combination of \( \text{dsx}^1/\text{dsx}^{19} \) further enhanced the \( \text{dsx} \) sex-transformation phenotype (Fig. 3F, G, compare with \( \text{dsx}^1/\text{dsx}^{19} \), Fig. 3E), causing the appearance of additional male-like structures on XX; \( \text{Dredd}/\text{Dredd} \) animals. These results suggest a general role for \( \text{Dredd} \) and \( \text{IMD} \) signaling in establishing the robustness of the \( \text{Drosophila} \) somatic sex determination pathway.

### 3.4. Mutations in dorsal and Dif, encoding the two additional NF-\( \kappa \)B paralogues in \( \text{Drosophila} \), do not induce somatic sex transformations

\( \text{DORSAL} \) (DL) and Dorsal Immunity Factor (DIF) proteins bind core promoter elements similar in sequence to those recognized by REL, but they generally activate different genes, despite some overlap in the specificity and heterodimerization among these NF-\( \kappa \)B-like proteins (Han and Ip, 1999; Tanji et al., 2010). To test whether the \( \text{Toll} \) pathway acts similarly to the \( \text{IMD} \) pathway in the regulation of somatic sex determination, we generated \( \text{DoaHD}/\text{DoaDEM} \) females in backgrounds heterozygous, homozygous, trans-heterozygous and deficient for \( \text{dl} \) and \( \text{Dif} \) (Table 2). In no case were somatic sex transformations observed, even when \( \text{Doa} \) heteroallelic flies carried a \( \text{dl} \) or \( \text{Dif} \) allele in opposition to a deficiency of the region encoding both \( \text{dl} \) and \( \text{Dif} \) (e.g. \( \text{Df}(2L)\text{Exel 7068}/\text{dl}^1 \) or \( \text{Df}(2L)\text{J4}/\text{Dif} \)) (Table 2). We conclude that the \( \text{IMD} \) but not the \( \text{Toll} \) pathway specifically regulates somatic sex determination.

### 3.5. Effects of IMD signaling on sex determination are mediated through transcriptional regulation of specific Doa isoforms

Double-mutant \( \text{Dredd} \); \( \text{RelE20} \) homozygotes displayed no evident sex transformations (not shown), nor did homozygosity for mutations at either locus induce detectable changes in the

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### Table 1

Summary of interactions between alleles encoding \( \text{IMD} \) and \( \text{Toll} \) pathway members and \( \text{Doa} \).

<table>
<thead>
<tr>
<th>IMD pathway/genotype tested</th>
<th>Observation/figure</th>
<th>( \text{tra}^2+/+; \text{tra}^-/+ )</th>
<th>( \text{dsx}^1/\text{dsx}^{19} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Dredd}^{23}/\text{Dredd}^{23} )</td>
<td>Enhanced female-to-male sex transformation (Fig. 1G)</td>
<td>Enhanced female-to-male sex transformation (Fig. 3B)</td>
<td>Enhanced (Fig. 3F)</td>
</tr>
<tr>
<td>( \text{Dredd}^{23}/+ ); ( \text{Dredd}^{23}/ )</td>
<td>Enhanced female-to-male sex transformation (Fig. 1H)</td>
<td>Enhanced female-to-male sex transformation (Fig. 3C)</td>
<td>ND</td>
</tr>
<tr>
<td>( \text{Dredd}^{23}/\text{Dredd}^{108444}/ )</td>
<td>Enhanced female-to-male sex transformation (Fig. 1I)</td>
<td>Enhanced female-to-male sex transformation (Fig. 3D, E)</td>
<td>Enhanced (Fig. 3G)</td>
</tr>
<tr>
<td>( \text{Dredd}^{23}/\text{Dredd}^{108444} )</td>
<td>Enhanced female-to-male sex transformation (Fig. 2D)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>( \text{RelE20}^+/+ ); ( \text{RelE20}^+/ )</td>
<td>Enhanced female-to-male sex transformation (not shown)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>( \text{IMD}^{10191}/\text{IMD}^{10191} )</td>
<td>Enhanced female-to-male sex transformation (not shown)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>( \text{Tak1}^+/+ ); ( \text{Tak1}^+/ )</td>
<td>Enhanced female-to-male sex transformation (Fig. 2E)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>( \text{irds}^5+/+ )</td>
<td>Enhanced female-to-male sex transformation (Fig. 2F)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>( \text{PGRP-LC}/\text{PGRP-LC} )</td>
<td>Enhanced female-to-male sex transformation (not shown)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

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Fig. 2. \( \text{Relish} \) and other alleles of the \( \text{IMD} \) pathway enhance \( \text{DoaHD}/\text{DoaDEM} \) somatic sex transformations. Rows of female vaginal “teeth” are indicated with arrows in each panel. Male clasper-like appendages in the double-mutants are indicated with arrowheads. (A) \( \text{C}^+ \) female; (B) \( \text{DoaHD}/\text{DoaDEM} \) female; (C) \( \text{Relish}^2 \) female homozygote; (D) \( \text{RelE20}/+ \); \( \text{RelE20}/ \) female; \( \text{RelE20}/\text{DoaDEM}/\text{RelE20}/ \) females gave the same result (not shown); (E) \( \text{TK}^+/+ \); \( \text{DoaDEM}/\text{DoaDEM} \) female. \( \text{TK}^+ \) mutants show less severe enhancement of the \( \text{DoaHD}/\text{DoaDEM} \) phenotype than either \( \text{Dredd} \) or \( \text{Rel} \) alleles. The expressivity of this phenotype was not enhanced in \( \text{TK}^+ \) homozygotes, although the penetrance was increased (Table 1). (F) \( \text{irds}^5 \text{DoaHD}/\text{DoaDEM} \) female. \( \text{irds}^5 \) alleles encode the \( \text{Drosophila} \) orthologue of \( \text{IkB} \) kinase and also enhance \( \text{Doa} \)-induced sex transformations, even in heterozygotes.
Fig. 3. Dredd alleles enhance sex transformation in tra2 B: tra / + double heterozygotes and in ddx heteroallelic XX animals. (A) y w; tra2B+/ +; tra/ + XX females show normal female genital morphology (arrows); (B) Dredd275/Dredd275; tra2B+/ +; tra/ + XX animals demonstrate a dux phenotype, with both female (arrows) and male (arrowheads) structures. Dredd275 is a null allele (Leslie et al., 2006). (C) Dredd275/Dredd275, tra2B+/ +; tra/ + XX females also show strong sex transformations, with both female (arrows) and male genitalia (arrowheads) visible. (D) Dredd275/Dredd275, tra2B+/ +; tra/ + XX females show what appears to be partial female-to-male transformation and lack of extrusion of their external genitalia (arrows). (D’) is a high magnification view of (D). (E) +/+; ddx1/ddx19 (XX) show a typical dux phenotype, with female genitalia (arrows) in addition to well-developed male clasps (arrowheads). (F) Dredd275/Dredd275, ddx1/ddx19 (XX) double-mutants show even stronger transformation of female structures (arrows) towards male (arrowheads), with the appearance of an extra appendage (double-arrowhead) compared to the simple ddx mutant in (E). (G) dredd275/dredd275, ddx1/ddx19 (XX) double-mutants also reveal an enhanced dux phenotype (double-arrowhead), similar to the Dredd275/Dredd275, ddx1/ddx19 animals in (F).

Table 2
Toll pathway NF-κB mutation, allelic combination or deficiency.

<table>
<thead>
<tr>
<th>Allele Combination</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>df1/df1</td>
<td>No enhancement of sex transformation</td>
</tr>
<tr>
<td>df1/df3</td>
<td>No enhancement of sex transformation</td>
</tr>
<tr>
<td>df1/Df</td>
<td>No enhancement of sex transformation</td>
</tr>
<tr>
<td>df1/Df</td>
<td>No enhancement of sex transformation</td>
</tr>
<tr>
<td>Df(2L)Exel 7068/Df</td>
<td>No enhancement of sex transformation</td>
</tr>
<tr>
<td>Df(2L)Exel 7068/df1</td>
<td>No enhancement of sex transformation</td>
</tr>
<tr>
<td>Df(2L)j4/Df</td>
<td>No enhancement of sex transformation</td>
</tr>
</tbody>
</table>

All effects are described in F1 homozygous females of the listed innate-immune pathway mutations, unless otherwise indicated. Df(2L)Exel 7068 (36C7–36C10) completely removes both df1 and Df as well as several adjoining loci on each side. Df (2L)j4 removes only Df and Df. ND: Not Done.

alternative splicing of ddx transcripts (not shown). Thus, IMD pathway alleles visibly affect somatic sex determination only when central participants in the sex-determination cascade are already partially reduced in function. Given the relationships among these loci and their functions, we reasoned that REL might already partially reduced in function. Given the relationships when central participants in the sex-determination cascade are pathway alleles visibly affect somatic sex determination only.

We next asked whether binding sites for NF-κB were found to be enriched in males. The other four Doa isoforms fail to show this difference, and indeed, possess slightly elevated transcript levels in REL females and males compared with wild-type. A single exception is the 227 kD isoform in REL males, which is reduced in comparison with C5 males, similar to the situation in females for the 55 and 69 kD isoforms. Our observations thus suggest that REL is a transcriptional activator specifically of the 55 and 69 kD-encoding Doa isoforms in females and potentially of the 227 kD isoform in males.

We also examined whether REL expression increased Doa protein accumulation in third-instar larval salivary glands, by generating cell clones over-expressing the N-terminal REL transcriptional activation domain, REL68 (Wiklund et al., 2009). Fig. 4B reveals greatly increased DOA protein accumulation in a two-cell clone (GFP +, green), in which UAS-Rel68 expression is induced by FLP-out. This observation confirms the qPCR results, confirming that DOA protein expression is induced by REL. However, it does not identify the specific protein kinase(s) affected, because the available antibody detects the catalytic domain of the kinase (Yun et al., 2000), which is common to all isoforms (Kpebe and Rabinow, 2008a).

We further asked whether the effects of REL on Doa expression were mediated through promoter sequences. Flies carrying a UAS-Doa 69 kD construct under control of the retina-specific GMR-GAL4 element possess rough, shiny eyes, with variable expressivity depending upon their growth conditions (Kpebe and Rabinow, 2008a). We crossed these flies with REL 20 null mutants, anticipating that reduction in Doa function would suppress the kinase over-expression phenotype. However, no alterations of the kinase-induced phenotype were observed in REL 20 homozygotes (genotype: GMR > Doa69/Cyo; REL 20/REL 20; not shown), suggesting that UAS-driven Doa expression was immune from the effects of REL alleles. We conclude that the effects of REL signaling on Doa transcript levels are mediated through the gene’s promoter region.

We next asked whether binding sites for NF-κB-like factors had been repertoried by genomic analyses in or near the Doa locus, since these sites are related to each other, albeit not identical (Senger et al., 2004; Tanji et al., 2010). Interestingly, several groups examining early embryos (2–3 h after egg-laying (AEL), corresponding to embryonic stages 4 and 5, mapped binding sites for DL protein to the presumptive promoter regions of the 55 and 69 kD-coding transcripts (http://flybase.org/reports/FBsf000425392.
3.6. No evident functional role for Doa in the IMD immune response

Because Rel and IMD signaling induce innate immunity to gram negative bacteria, we tested whether reduction in Doa function would expose flies to higher susceptibility of infection. We tested wild-type (C), RelE20 and Dod80/Dod83 flies for resistance to infection against the gram-negative plant pathogen E. caratova (gift of F. Leulier) at both 25 °C and 29 °C. To briefly summarize our results at both temperatures, Dod80/Dod83 flies of both sexes die more quickly than either wild-type (C) or RelE20 animals in uninfected conditions (non-infected or "mock"-infected, i.e. pricked with a clean needle; see Supplemental Fig. 1). When infected, although there is a decrease in longevity of Doa flies of both sexes, they remain more resistant than RelE20 animals, although not as
resistant as C. We attribute the shortened longevity of uninfected/mock-infected Doa/Doa animals compared with C to interference with normal physiological processes in the mutants, while their relatively unchanged longevity following infection compared with RelE20 suggests that their resistance is not altered by lack of the kinase.

4. Discussion

Our data demonstrate that IMD signaling directs somatic sex-determination in Drosophila through regulating the abundance of transcripts encoding the 55 and 69 kD DOA kinase isoforms. Given the documented function of REL as a transcriptional activator, we speculate that these transcripts are induced through REL's direct binding and activation of their regulatory region(s). The 55 kD and 69 kD DOA kinases are those likely to be responsible for the effects of kinase mutations on sex determination, in part because of the sex transformation effects of alleles specifically affecting them (Kpebe and Rabinow, 2008b). Moreover, the transcripts encoding these isoforms, and the 55 kD protein itself are reduced in Doa/Doa mutants (Yun et al., 1994, 2000). The 55 kD protein is also nuclearily localized, consistent with a role in the regulation of splicing. Additionally, as revealed here, the 55 and 69 kD isoforms are more highly expressed in females. In contrast, alleles and RNAi constructs affecting expression of the 105 and 138 kD isoforms had no discernable effects on sex determination (Kpebe and Rabinow, 2008b). Tellingly, these transcripts are not affected by Rel. Our observations are also consistent with a report demonstrating significant up-regulation of Doa transcripts in S2 cells within 1–4 h following treatment with lipopolysaccharide (Kim et al., 2005). The basis for the female-specific effects of RelE20 on Doa transcript accumulation remains unaccounted for, but we note with interest that females are more susceptible than males to infection by the gram-negative bacteria Escherichia coli (Taylor and Kimbrell, 2007), and it is the IMD pathway which is primarily responsible for this response.

Strong enhancement of Doa-induced sex transformations observed in mutants of downstream IMD pathway components (Dredd, Rel), in contrast to weaker enhancement by mutations in the more upstream components PGRP-LC, IMD, ird5 and Tak1 when combined with Doa alleles, is presumably due to buffering by fully active proteins further downstream in the pathway. The function of IMD signaling in sex determination is evident only under conditions where sex-specific alternative splicing of dsx transcripts is partially compromised, such as in tra2+/+: tra+/+ double heterozygotes or Doa heteroallelic flies.

In contrast to Rel, mutations in either of the two NF-kB paralogues regulating the Toll branch of Drosophila innate immunity, Dif and dl, had no effects on somatic sex determination. This result is in apparent contradiction to the observation that DL binding was found for several fragments defining potential promoter regions of Doa, specifically including those in positions appropriate to regulate transcription of the 55 and 69 kD isoforms (Hoskins et al., 2011; MacArthur et al., 2009; Roy et al., 2010). However, REL and DL possess similar, albeit not identical binding sites (Senger et al., 2004; Tanji et al., 2010), and the DL binding studies were performed in embryos, whereas our observations reveal phenotypes determined only later in development. It is thus conceivable that DL binding to Doa sequences is replaced later in development by REL, although we did not test this possibility. Although Doa/Doa embryos display numerous defects, no dorsal–ventral abnormalites have been described.

Interestingly, ecysdnone signaling was recently described as inducing accumulation of transcripts encoding the IMD pathway receptor, PGRP-LC, thus integrating hormonal and immune signaling (Rus et al., 2013). We previously confirmed ecysdnone-dependent induction of transcripts encoding the 55 kD and 105 kD DOA proteins (Kpebe and Rabinow, 2008a), based upon the report of Gorski et al. (2003). They and others (Lee et al., 2003) also found that Doa transcripts are among those most highly induced during pupariation and salivary gland autophagy. Among the other most highly induced transcripts were those encoding Cecropin A1 and Cecropin B, which are also REL targets (De Gregorio et al., 2002; Senger et al., 2004). These results suggest that the integration of immune and ecdysosterone signaling may extend beyond that recently documented.

Finally, our work illustrates a connection between the innate-immune response and the regulation of alternative splicing of pre-mRNAs. DOA kinase influences sex-determination via phosphorylation of RBP1, TRA and TRA2 (Du et al., 1998), which are SR and SR-like proteins influencing the alternative splicing of dsx transcripts. Thus the induction of Doa by IMD innate-immune signaling and infection influences post-transcriptional regulation of transcripts targeted by these SR and SR-like proteins, as well as others, in addition to the direct transcriptional induction of loci encoding anti-microbial peptides. Global analysis of alternative splicing in Doa and IMD pathway mutants could thus reveal important observations concerning the immune response.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.09.013.

References


