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Cloning of the neurodegeneration gene *drop-dead* and characterization of additional phenotypes of its mutation

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Abstract

Mutations in the *Drosophila* gene *drop-dead* (*drd*) result in early adult lethality and neurodegeneration, but the molecular identity of the *drd* gene and its mechanism of action are not known. This paper describes the characterization of a new X-linked recessive adult-lethal mutation, originally called *lot's wife* (*lwf*^{1}) but subsequently identified as an allele of *drd* (*drd*^{lwf}); *drd*^{lwf} mutants die within two weeks of eclosion. Through mapping and complementation, the *drd* gene has been identified as *CG33968*, which encodes a putative integral membrane protein of unknown function. The *drd*^{lwf} allele is associated with a nonsense mutation that eliminates nearly 80% of the CG33968 gene product; mutations in the same gene were also found in two previously described *drd* alleles. Characterization of *drd*^{lwf} flies revealed additional phenotypes of *drd*, most notably, defects in food processing by the digestive system and in oogenesis. Mutant flies store significantly more food in their crops and defecate less than wild-type flies, suggesting that normal transfer of ingested food from the crop into the midgut is dependent upon the DRD gene product. The defect in oogenesis results in the sterility of homozygous mutant females and is associated with a reduction in the number of vitellogenic egg chambers. The disruption in vitellogenesis is far more severe than that seen in starved flies and so is unlikely to be a secondary consequence of the digestive phenotype. This study demonstrates that mutation of the *drd* gene *CG33968* results in a complex phenotype affecting multiple physiological systems within the fly.

Keywords

Drosophila; drop-dead; oogenesis; vitellogenesis; crop; defecation; digestion; adult-lethal; NRF; neurodegeneration

Introduction

Over the last four decades, the use of *Drosophila* as a model organism has expanded dramatically to include many aspects of behavior and physiology previously thought inaccessible to genetic analysis. The pioneering work in this area by Benzer and colleagues included the isolation of neurodegeneration mutants, beginning with *drd*.[1] Mutations in this gene cause an adult lethal phenotype, with flies dying within the first two weeks after eclosion, and death is accompanied by gross degeneration in the brain. Since this first report, a number of *Drosophila* neurodegeneration mutants have been characterized, including several with phenotypes similar to human diseases.[2] While the original neurodegeneration mutant *drd* was

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subsequently reported to affect glial morphology[3], its molecular identity and mechanism of action remain unclear.

In the current work, several additional phenotypes of *drd* are described, including defects in oogenesis and, most uniquely, in the transit of food from the crop into the midgut. The crop is a blind-ended muscular food storage organ that connects to the esophagus via the crop duct[4]; contractions of the crop musculature force the stored food up the crop duct and through the stomodaeal valve into the midgut, where digestion and nutrient absorption occur.[4,5] The frequency of crop contractions can be modulated by several different neuropeptides[6–10]; however, the control of digestive physiology in the intact adult fly is largely uncharacterized, and, excluding the current work, no mutants have been reported that disrupt food transit through the gut.

The cloning of the *drd* gene is also reported here. The *drd* gene is a member of a large (17 genes in *Drosophila*) gene family that, though it had been previously identified on the basis of common protein domains, has been almost completely uncharacterized. Interestingly, related genes in *C. elegans* have been identified as being important for certain actions of the antidepressant drug fluoxetine. The linkage of novel neurodegenerative and digestive phenotypes to a member of this gene family will aid in the ultimate determination of these proteins' biological functions, both in *Drosophila* and other species.

Results

The mutant line described in this work was originally isolated in a screen for X-chromosome mutations that cause sensitivity of adult *Drosophila* to dietary salt, although, as will be described, the line is not salt sensitive. Canton S males were treated with EMS and mated with attached-X females. A total of 399 F1 lines were screened for survival on food prepared with 0.4M NaCl, which is tolerated by wild-type flies.[11] For any line displaying significant lethality after seven days on the food, sub-lines were established from individual F2 males to remove potential mosaicism and the sub-lines were re-screened. From this screen, a single line was identified which showed an extremely short adult lifespan (median survival 4 days post-eclosion); this lethality was not dependent upon the presence of salt in the food as it was also observed on cornmeal-molasses-yeast food and instant fly food prepared with water (data not shown). The mutation in this line is X-linked and recessive, causing the rapid death of both hemizygous males and homozygous females (fig. 1), while heterozygous females are fully viable (data not shown). Because of the original purpose of the screen, the mutated gene was named *lot's wife* (*lwf*) and the allele was named lwf^1; these names were used in a preliminary report[12] and incorporated into FlyBase.[13] However, as detailed below, lwf^1 was subsequently found to be allelic to the previously described mutant *drd* and will therefore be termed drd^{lwf}.

In addition to being extremely short-lived, drd^{lwf} flies display abnormal digestive function. Mutant flies frequently have a "bloated" appearance; this is due to the accumulation of large amounts of liquid in the crop. Measuring the volume of the crop contents showed a significant effect of the mutation, with drd^{lwf} males having more than a 4-fold increase in crop volume compared with wild-type males ($p=0.01$, unpaired t-test) and homozygous females having a nearly three-fold increase compared with heterozygotes ($p=0.04$) (fig. 2A, groups 8 and 2, respectively). These differences were significant despite an extremely high degree of variability in the crop volumes of the mutant flies, with 4/20 males and 5/21 homozygous females having crop volumes below 5 nL (see below). This digestive phenotype is not restricted to the crop; mutant flies also show a significantly reduced rate of defecation. Figure 2B shows the number of fecal spots produced by pairs of wild-type and drd^{lwf} males during the first and second days post-eclosion. As is clear from the figure, defecation rate in the mutants is

profoundly lower on both days (p=0.007, day 1, and p=0.0001, day 2, unpaired t-test). No accumulation of food is observed in any portions of the gut other than the crop (data not shown). Thus the increased storage of food in the crops of mutant flies occurs at the expense of the movement of food through the remainder of the gut.

The lethality resulting from *drd*^{lwf} appears to be confined to adult flies. No gross abnormalities were observed in either the survival of mutants through pre-adult development or the timing of such development (data not shown). It was observed, however, that mutant adults are smaller than wild-type flies. As shown in figure 3 (groups 8 and 2), the mean mass on the day of eclosion of *drd*^{lwf} males and homozygous females was 25–30% smaller than wild-type males and heterozygous females, respectively (p=0.0004 for males and 0.001 for females, unpaired t-test).

A final phenotype of the *drd*^{lwf} mutation is female sterility. While mutant males are fertile, I observed very few eggs and no larvae in crosses with homozygous females. To determine whether this phenotype is a result of some defect in oogenesis, ovaries of *drd*^{lwf} homozygotes and heterozygotes were dissected and examined. Dissection of the ovaries revealed that homozygotes contained significantly fewer egg chambers that had undergone vitellogenesis compared with heterozygotes (p<0.0001, unpaired t-test); indeed, mutant flies, on average, had less than one yolk-containing egg chamber in each ovary (fig. 4, group 2). It has previously been shown that subjecting female *Drosophila* to physiological stressors, including starvation, inhibits oogenesis by promoting apoptosis of the oocyte at stage 8, which corresponds to the beginning of vitellogenesis.[14–16] I hypothesized that if the mutant flies were starving due to the food-processing phenotype described above, the female sterility of *drd*^{lwf} mutants could be a secondary consequence of that starvation. To test this possibility, *drd*^{lwf} heterozygotes were starved by moving pupae to vials containing only 1% agarose, as a water source. The day after eclosion, flies were dissected and the number of yolk-containing egg chambers counted. As shown in figure 4 (group 2), starvation did result in a significant disruption of oogenesis (unpaired t-test, p=0.002); however, this disruption was not nearly as severe as that observed in the *drd*^{lwf} homozygotes (p<0.0001).

Cloning of drd^{lwf}

In order to determine the location of *drd*^{lwf} on the X chromosome, meiotic recombination mapping was performed with increasingly closely-spaced markers, resulting in a determination that the mutation lay roughly midway between the genes *garnet* (*g*, map position 1–44.4, cytologic band 12B4) and *scalloped* (*sd*, 1–51.5, 13F) at a map position of 1–46.5 to 1–47.0. Ten different chromosomal deficiencies from this region (fig. 5A) were tested for complementation with *drd*^{lwf}, but none of them uncovered the lethal phenotype in trans-heterozygous females (data not shown). Based on the breakpoints of these deficiencies, not all of which have been precisely defined[13], I identified three potential gaps in the coverage (fig. 5A). The most distal of these gaps, between *Df(1)ED7294* and *Df(1)RK4*, seemed the most likely location for *drd*^{lwf} based on the recombination map position. The proximal edge of this gap was defined precisely by the distal breakpoint of *Df(1)ED7294*, while the distal extent of the gap was unknown due to uncertainty about the proximal breakpoint of *Df(1)RK4*; therefore, I focused initially on the most proximal genes in the gap. The first of these is the ribosomal gene *RpL37a*. Mutation of ribosomal genes results in a dominant *Minute* phenotype characterized by poor overall viability and short, thin bristles[17]; because the recessive *drd*^{lwf} phenotype does not appear to be a *Minute* mutation, *RpL37a* was not examined further. The next gene, *CG33968*, consists of 9 exons and encodes an 827-amino acid protein with multiple predicted transmembrane domains (fig. 5B, C). Sequencing of the exons of this gene revealed an A to T transversion in exon 3 which converts the codon for lysine-181 (AAA) to a stop codon (TAA) (fig. 5C). Additional sequencing showed the presence of this mutation in the

genomes of all five *drd*^{lwf} males tested but none of three Canton S males. Quantitative real-time RT-PCR showed a significant decrease in the level of *CG33968* mRNA in both *drd*^{lwf} males and homozygous females (figure 6), consistent with the nonsense-mediated transcript decay that would be predicted to result from the mutation.[18]

To support more fully the identification of *CG33968* as the location of the *drd*^{lwf} mutation, flies carrying *CG33968*$^{CB6275\text{-}3}$, in which a P-element transposon is inserted five bases upstream of the predicted transcriptional start site[13], were examined. Neither hemizygous males nor homozygous females carrying the insertion showed any decreased viability during the first week of adult life (data not shown); furthermore, homozygous females were fertile and did not differ significantly from wild-type in crop volume (fig. 2A, groups 1 and 3)) or body mass (fig. 3, groups 1 and 3) and had slightly more vitellogenic egg chambers (fig. 4, groups 1 and 3). However, trans-heterozygous *CG33968*$^{CB6275\text{-}3}$/*drd*^{lwf} females did display an adult-lethal phenotype, although it was not as severe as that of *drd*^{lwf} homozygotes (fig. 1, Mantel-Haenszel test, p=0.0002), and had significantly higher crop volumes then their *CG33968*$^{CB6275\text{-}3}$/*FM7a* siblings (fig. 2A group 4, p=0.003). In contrast to *drd*^{lwf} homozygotes, however, *CG33968*$^{CB6275\text{-}3}$/*drd*^{lwf} females did not have a lower body mass then their siblings (fig. 3 group 4) and were fertile, although they did show a small reduction in the number of vitellogenic egg chambers (fig. 4 group 4, p=0.03). The failure of *CG33968*$^{CB6275\text{-}3}$ to complement *drd*^{lwf} in both adult viability and crop volume provides strong evidence that the two mutations lie in the same gene. The milder phenotype of the transheterozygotes suggests that the insertion mutant is a hypomorph, although interestingly, measurement of *CG33968* mRNA levels by real-time RT-PCR shows levels of transcript in *CG33968*$^{CB6275\text{-}3}$ males and females approximately double that of Canton S (fig. 6). Expression of *CG33968* in *CG33968*$^{CB6275\text{-}3}$/*drd*^{lwf} females was not different from that observed in Canton S (fig. 6).

If the adult lethal phenotype of *drd*^{lwf} is due to the mutation of *CG33968*, then one would predict that disruption of *CG33968* by imprecise excision of the *CG33968*$^{CB6275\text{-}3}$ transposon would also result in an adult lethal phenotype. To test this, I mobilized the P-element in this line and screened the resulting w^- progeny for adult lethality. 33% (27/81) of the w^- progeny died within 7 days of eclosion. The survival curves of lines from two of these flies are shown in figure 1. Both lines are adult lethal, but neither is as severe as *drd*^{lwf} (Mantel-Haenszel test, *drd*^{lwf} vs. *CG33968*^{G3}, p<0.0001; *drd*^{lwf} vs. *CG33968*^{W3}, p=0.002). Molecular analysis revealed that the milder *CG33968*^{G3} allele was associated with a 40 bp insertion at the *CG33968*$^{CB6275\text{-}3}$ site just upstream of the transcriptional start, while *CG33968*^{W3} contains a 1774 bp deletion to the right of the original insertion site that removes the entire first exon and part of the first intron (fig. 5C).

The phenotypes and map position of the *drd*^{lwf} allele and molecular characteristics of the associated *CG33968* gene are strongly reminiscent of the previously identified neurodegeneration-linked gene *drd*. Mutants in this gene were isolated by Benzer and co-workers as adult-onset lethals; *drd* mutants die within two weeks of eclosion and exhibit defects in glial morphology, profound brain degeneration, female sterility, and premature expression of aging markers.[1,3,19] According to preliminary reports, *drd* maps to cytologic position 13A/B and was identified as a gene that encoded a protein of 825 amino acids with multiple predicted transmembrane domains.[20] To determine whether the mutant line that I isolated was an allele of *drd*, I obtained three previously described alleles: *drd*^{1}, *In(1)drd*^{x1}, and *drd*^{x4}. As shown in figure 1, all three alleles caused a severe adult-lethal phenotype in both males and homozygous females. The *drd*^{1} allele was chosen for complementation studies as the survival of females homozygous for this allele was significantly shorter than for *In(1)drd*^{x1} (Mantel-Haenszel test, p<0.0001) and *drd*^{x4} (p=0.001). Compared with their heterozygous siblings, *drd*^{1} homozygotes also had lower body mass (fig. 3 group 5, p<0.0001), were sterile, and contained fewer

vitellogenic egg chambers (fig. 4 group 5, p<0.0001), although the latter phenotype was not as severe as in *drd*^{lwf} homozygotes. Mean crop volume did not differ significantly between homozygotes and heterozygotes (fig. 2A group 5, p=0.14); however, the crop volumes from homozygotes did show a significantly higher variance (F-test, p<0.0001). Just as with the *drd*^{lwf} homozygotes, many *drd*^{1} homozygotes had empty crops and appeared to be extremely unhealthy, even at just two days post-eclosion.

Crossing of *drd*^{lwf} with *drd*^{1} revealed a failure of the latter allele to complement any of the phenotypes of the former. Transheterozygous *drd*^{lwf}/*drd*^{1} females were short-lived (fig. 1) and sterile and had significantly higher crop volumes (fig. 2A group 6, p=0.0009), smaller body mass (fig. 3 group 6, p=0.0009) and fewer vitellogenic egg chambers (fig. 4 group 6, p=0.0003) than their *drd*^{1}/*FM7a* siblings. Furthermore, *drd*^{1} showed essentially the same pattern of complementation with *CG33968*$^{CB6275\text{-}3}$ as did *drd*^{lwf}; compared to their siblings, *CG33968*$^{CB6275\text{-}3}$/*drd*^{1} females had normal body mass (fig. 3 group 7, p=0.87) and numbers of vitellogenic egg chambers (fig. 4 group 7, p=0.10) and were fertile but were also short-lived (fig. 1) and had elevated crop volumes (fig. 2A group 7, p=0.0001). It is notable that the crop volumes of the three transheterozygous genotypes--*drd*^{lwf}/*drd*^{1}, *CG33968*$^{CB6275\text{-}3}$/*drd*^{lwf}, and *CG33968*$^{CB6275\text{-}3}$/*drd*^{1}--were more consistently elevated than those of the parental lines, which might result from the outcrossed flies being healthier during the first few days of adult life.

Finally, to determine whether the three previously characterized *drd* alleles were associated with molecular lesions in the *CG33968* gene, I examined the gene by PCR and sequencing from genomic DNA prepared from males carrying the three *drd* alleles-- *drd*^{1}, *In(1)drd*^{x1}, and *drd*^{x4}. A large inversion was found in *In(1)drd*^{x1} with a proximal breakpoint in exon 1 of *CG33968*, between codons 73 and 74 (fig. 5C), and a distal breakpoint 6.4 Mb away in the second intron of the gene *CG11284*. The *CG33968* gene in *drd*^{x4} contains a 453 bp deletion which eliminates parts of exons 5 and 6 and all of intron 5 and is predicted to result in the production of a severely truncated protein (533 amino acids, diverging from the wild-type sequence after position 445). The mutation in the EMS allele *drd*^{1} was not discovered. Sequencing of the entire coding sequence revealed only one polymorphism that would cause a change in the protein sequence from that published in FlyBase: a C to G transversion that would change Ser-61 (AGC) to Arg (AGG). However, this same polymorphism was also found in *In(1)drd*^{x1} and in a wild-type line from the Benzer lab and so cannot be the *drd*^{1} mutation.

Discussion

In this work I report the identification of *CG33968* as the *drd* gene. Benzer and colleagues originally isolated several *drd* alleles based on their adult-lethal phenotype; adult mutant flies appear normal at first, but at some point during the first two weeks post-eclosion each fly begins to exhibit uncoordinated behavior and dies soon afterwards.[1,3,21] Subsequent work highlighted the importance of the brain in this lethality. At the time that the behavioral abnormalities are observed, the brains of mutant flies show significant signs of neurodegeneration.[1] Glia in *drd* mutant brains also appear to be abnormal—specifically, the glia appear morphologically immature—and this glial phenotype can be seen well before any behavioral changes or neurodegeneration manifest themselves.[3] Using mosaic flies, Hotta and Benzer identified the head as the locus for the death and neurodegeneration phenotypes.[21] Furthermore, they found that flies in which only one side of the head was mutant were phenotypically normal; this observation of non-cell autonomous behavior led to the suggestion that *drd* is necessary for the production or secretion of a factor that is normally required for neuronal survival. Finally, it has been observed that the expression of several molecular markers of aging is accelerated in *drd* mutants in proportion to their decreased lifespan.[19]

The conclusion that *drd*^{lwf} and the "classical" allele *drd*^{1} are allelic and are mutations in the *CG33968* gene is based on the complementation and molecular data presented above. Transheterozygous *drd*^{lwf}/*drd*^{1} females show the full array of phenotypes observed in each parental line, including adult lethality, bloated crop, small body mass, sterility, and reduced oogenesis, demonstrating that the two mutations are alleles of the same gene. Further evidence of this allelism is that both show that same pattern of complementation when crossed with *CG33968*$^{CB6275\text{-}3}$: adult lethality and bloated crops but fertility and normal body mass.

Do the aforementioned complementation data also prove that *CG33968* is the *drd* gene? Taken alone, they are not definitive, as it might be possible that a "second hit" on the *CG33968*$^{CB6275\text{-}3}$ chromosome could cause lethality and bloated crops when over a *drd* allele. However, I have also shown that excision of the P-element from this line generates adult-lethal flies at high frequency, and two such lines with very different molecular lesions also have very different survival curves. This result is not easily explained by a second hit on the chromosome. Furthermore, while the mutation in *drd*^{1} has not yet been identified, I have found mutations in *CG33968* not only in *drd*^{lwf} but also in *In(1)drd*^{x1} and *drd*^{x4}. Taken as a whole, my data support the conclusion that *CG33968* is *drd*.

The phenotypes associated with the *CG33968*$^{CB6275\text{-}3}$ insertion merit some additional comments. By itself, this insertion appears to have no phenotype compared with Canton S, although direct comparison of these two stocks is complicated by their having different genetic backgrounds, but the insertion does cause some phenotypes when placed over a more severe *drd* allele. These observations would be consistent with the insertion being a hypomorph. Surprisingly, however, quantitative RT-PCR showed a two-fold *increase* in *CG33968* expression associated with the insertion. The simplest interpretation of this result is that the *CG33968*$^{CB6275\text{-}3}$ insertion alters the expression pattern of *CG33968*, reducing expression in tissues where the gene is required for viability and digestive function and increasing expression elsewhere. Consistent with this, figure 6 shows that *CG33968*$^{CB6275\text{-}3}$/*drd*^{lwf} females have normal overall levels of *CG33968* mRNA while still showing some *drd* phenotypes.

As shown in figure 5B, the *drd* gene *CG33968* encodes a protein with multiple hydrophobic stretches that is likely to be an integral membrane protein. Database searches indicate that this gene belongs to a family, known as the NRF family (see following paragraph for the origin of this name), which contains 17 members in *Drosophila* and 19 members in *C. elegans* but only a single homolog in each vertebrate species examined, including humans. Figure 7 shows a phylogenetic tree displaying the relationships among the 17 *Drosophila* genes, two of the *C. elegans* genes described below, and some of the vertebrate genes. Proteins in this family all contain two recognizable domains: in the N-terminal half of the protein there appears a NRF repeat, a cysteine-rich protein domain of unknown function[22], and the C-terminal half of the protein contains a domain that is related to bacterial integral membrane acyl transferases.[22, 23] The possibility that eukaryotic NRF-domain proteins function as acyl transferases has not been tested experimentally. In additional to the genes shown in figure 7, the *Drosophila* genome contains two related genes, *CG4576* and *CG9447*, that include the acyl transferase region but lack the NRF domain.

The eukaryotic NRF-domain gene family was first identified in *C. elegans* in a genetic screen for mutations that cause resistance to the antidepressant serotonin reuptake inhibitor fluoxetine.[24] Treatment of *C. elegans* with fluoxetine induces a contraction of the nose muscles that is not dependent upon the presence of serotonin. Seven nrf (nose resistant to fluoxetine) genes were found in the screen. Two of these genes, *nrf-6* and *ndg-4*, are members of the NRF-domain family (figure 7), and their expression in the intestine is required for a normal response to fluoxetine.[25] Interestingly, mutation of either *nrf-6* or *ndg-4* also disrupts the accumulation of yolk into the eggs.[25] Because yolk is synthesized in the intestine in this species and then

transported into the eggs, it has been speculated that the NRF proteins might act as transporters, but there is not yet any direct experimental evidence to support this hypothesis.

Apart from *drd*, no mutant phenotypes of any of the 17 *Drosophila* NRF genes have been reported. The only characterization of genes in this family comes from a study of changes in larval gene expression following starvation or feeding on a sugar-only diet.[26] The expression of 8 of 14 NRF genes represented on the microarray showed significant dependence upon the nutritional state of the larvae, although the precise dependence was not consistent among the 8 genes. The *drd* gene was not represented on the microarray. One NRF-domain gene, *CG14205*, was the most highly regulated of all genes that responded to sugar but not to starvation; a four hour treatment of larvae with 20% sucrose caused a 20-fold increase in *CG14205* expression. *In situ* hybridization showed expression of the gene in several parts of the larval gut following sugar feeding.

I have described a new allele of *drd*, drd^{lwf}, which is likely to be a null allele as the predicted protein is severely truncated by a nonsense mutation. The new allele has been used to characterize several additional *drd* phenotypes, including small body size and defects in digestion and oogenesis. Some of these phenotypes—small body size and female sterility—have been briefly mentioned in previous work but not described in detail.[3] The digestive defect is the most intriguing of these phenotypes because no other *Drosophila* mutations have been reported to have similar effects. In drd^{lwf} flies, there is a striking increase in the amount of food stored in the crop, a decrease in the amount of food that passes through and is excreted from the remainder of the gut, and no buildup of material in the midgut or hindgut. Because the crop is a storage organ and not a site of digestion and nutrient absorption[5,27], it seems likely that the mutant flies are not digesting most of the food that they eat, and that the resulting malnutrition could be a contributing factor in their premature deaths. The storage of large amounts of material in the crop is rarely seen in wild-type, *ad lib* fed flies (figure 2A and ref. [28]). The only reported conditions under which wild-type flies will exhibit swollen crops is after they are starved for a significant period of time and then returned to food[28]; indeed, even under these conditions, ingested food is rapidly transferred to the midgut in wild-type flies (C. Peller and Blumenthal, unpublished results). Broadly speaking, the absence of a functional *drd* gene could disrupt the movement of food from the crop into the midgut either through a direct effect on the gut—for example by inhibiting the muscular contractions of the crop or the opening of the stomodaeal valve at the entrance to the midgut—or through an indirect effect on the gut—by disrupting a pathway that regulates gut motility in response to factors such as nutritional state or environmental conditions. Future experiments will be necessary to distinguish among these possibilities. It is important to note that the digestive phenotype reported here is observed during the first two days post-eclosion, well before the uncoordinated movements that immediately precede the deaths of *drd* mutants.

If disruption of *drd* causes a defect in digestion, it may not be limited to adult flies. The larval gut does not include a crop[29], so a specific defect in crop function would not be expected to affect larval physiology. However, newly eclosed *drd* mutants are significantly smaller than wild-type, despite there being no difference in the length of pre-adult development. This aspect of the *drd* phenotype is reminiscent of that seen following starvation or disruption of insulin signaling in older larvae, whereas starvation of young larvae results in a lengthening of time to metamorphosis.[30–32]

Female flies homozygous for drd^{lwf} and other *drd* alleles are sterile. In the present work I show that this sterility is associated with a reduction in the number of yolk-containing egg chambers. However, the absolute magnitude of this reduction varies widely among genotypes, from drd^{lwf} homozygotes, which produce almost no late-stage egg chambers, to drd^{lwf}/drd^{1} females, which, though reduced compared with their sibling controls, produce the same number of late-

stage egg chambers as Canton S. Indeed, drd^{lwf}/drd^{1} females do appear to lay appreciable numbers of eggs (data not shown) but are still sterile. It is possible, therefore, that mutation of *drd* might have multiple effects on female reproduction, causing both a variable disruption in oogenesis and an absolute sterility; future experiments will examine both of these phenotypes in more detail. The disruption in oogenesis could occur at the known developmental "check-point" at stage 8, which corresponds to the onset of vitellogenesis[15]; based on the data shown in figure 4, it appears that the large majority of oocytes in drd^{lwf} homozygotes fail to proceed through that check-point. Starved heterozygous females also showed a deficit in vitellogenic egg chambers, consistent with previous findings that poor nutrition induces apoptosis at stage 8.[14,16] However, the drd^{lwf} phenotype is far more severe than that of totally starved heterozygotes, suggesting that it is not simply a secondary consequence of any digestive defect. It is interesting that *nrf-6* and *ndg-4* mutants in *C. elegans* also fail to load yolk into their eggs[25], indicating a possible common function for this family of proteins in two distantly-related organisms.

A role for *CG33968* in oogenesis was also suggested from a study of the effects of injecting *Drosophila* embryos and adults with double-stranded RNA (dsRNA) directed against the gene[33] (note that at the time of this publication, *CG33968* was annotated as two separate genes, *CG5652* and *CG32589*, and the dsRNA was directed against *CG5652*). RNAi in adult females led to a reduction in ovarian size and in egg-laying, in accordance with my results. RNAi in embryos was found to be lethal, which is not consistent with my data. The authors found that embryonic injection of dsRNA caused a disruption in the formation of denticle belts and named the *CG5652* gene *beltless* (*blt*). As in the current study, however, the *drd* name should take precedence over any subsequent names.

The data presented here demonstrate the diverse phenotypes associated with the mutation of *drd* and suggest that *drd* should not be categorized only as a neurodegeneration gene. While the previously published mosaic analysis clearly demonstrated the central role of *drd* expression in the head in the adult lethal phenotype[21], it remains possible that the other phenotypes are independent of neurodegeneration. Focusing on the digestive phenotype, for example, the association, in both *Drosophila* and *C. elegans*, between NRF-domain proteins and the gut has already been mentioned.[25,26] Furthermore, microarray profiling of *CG33968* expression in the adult fly shows highest expression in the crop and hindgut and no enhanced expression in the brain.[34] With the cloning of the *drd* gene, it will now be possible to determine its expression pattern and to begin unraveling the causal relationships among the various phenotypes of the *drd* mutant flies.

Materials and Methods

Drosophila stocks and chemicals

Fly stocks were maintained on cornmeal-yeast-molasses food at 24°C on a 12h:12h light:dark cycle. For some experiments, flies were placed on Instant Fly Food (Carolina Biological, Burlington, NC) reconstituted with 2 mL of liquid and 0.6 g of food per vial. Stocks carrying alleles of *drd* (FBgn0000494) ($drd^{1}/FM7c$ (FBal0003113), $In(1)drd^{x1}/FM7c$ (FBab0025048), $drd^{x4}/FM7c$ (FBal0082994)), were kindly provided by Dr. Seymour Benzer. Other genes and alleles referenced in this study are *CG33968* (*lwf*): FBgn0086221, drd^{lwf} (lwf^{1}): FBal0193421, $CG33968^{CB6275\text{-}3}$ ($drd^{CB6275\text{-}3}$, $lwf^{CB6275\text{-}3}$): FBal0193420, *g*: FBgn0001087, *sd*: FBgn0003345, *RpL37a*: FBgn0030616, *CG10182*: FBgn0039091, *CG13325*: FBgn0033792, *CG33337*: FBgn0053337, *CG10183*: FBgn0039093, *CG16723*: FBgn0039092, *CG14204*: FBgn0031032, *CG14205*: FBgn0031034, *CG14219*: FBgn0031033, *CG5892*: FBgn0038873, *CG3106*: FBgn0030148, CG*5156*: FBgn0031326, *CG14343*: FBgn0031325, *CG11353*: FBgn0035557, *CG30471*: FBgn0050471, *CG12990*: FBgn0030859, *CG17707*:

FBgn0025835, *CG32645*: FBgn0052645, *CG4576*: FBgn0038366, *CG9447*: FBgn0033110, and *CG11284*: FBgn0030056.

Except where noted, all reagents were obtained from VWR (West Chester, PA).

Mutagenesis and mapping

Canton S males (3–4 days post-eclosion) were treated overnight with 5 mM ethane methylsulfonate (EMS) in 1% sucrose[35] and crossed with *C(1)DX, y^1 f^1* females (FBab0000080). F1 male progeny were then crossed individually with *C(1)DX, y^1 f^1* females; F2 males from these individual lines were screened for survival on instant food prepared with 0.4M NaCl. For any line showing >20% mortality after 7 days, sub-lines were established by mating 4 F2 males individually with *C(1)DX, y^1 f^1* females and the resulting F3 male progeny were re-screened. A total of 399 F1 lineswere screened, and F2 sub-lines from 74 of these were established and re-screened. Mapping of lwf was accomplished by meiotic recombination with *y^1 cv^1 v^1 f^1 car^1* (FBst0001515) and *t^1 v^1 m^{74f} wy^{74i} sd^1 os^s* (FBst0001294) (Bloomington *Drosophila* Stock Center) and *t^1 wy^2 g^2 sd^1 os^s*, which was created by recombination between the latter mapping stock and *y^2 wy^2 g^2* (FBst0000192) (Bloomington). The deficiencies and insertions tested for complementation were *Df(1)g* (FBab0000827), *Df(1)RK2* (FBab0000674), *Df(1)RK4* (FBab0000676), *$In(1)AC2^LAB^R$* (FBab0027023), *Df(1)KA9* (FBab0000486), *Df(1)Exel6248* (FBab0037814), *Df(1)Exel6251* (FBab0037817), and *Df(1) ED7294* (FBab0030905) (Bloomington), *$CG33968^{CB6275\text{-}3}$* (Szeged Drosophila Stock Center) and *Df(1)CO1* (FBab0027061) and *Df(1)CO2* (FBab0027062) (from Dr. Howard Nash).

Defecation assay

Male flies were collected on the day of eclosion and placed in pairs in vials containing instant food prepared with 0.5% Acid Blue 9. After 23 hours, the blue fecal spots on the side of vial were counted (day 1) and the flies were transferred to fresh vials of blue food. After another 23 hours, fecal spots were again counted (day 2).

Adult fly mass

Flies were collected on the day of eclosion, frozen on dry ice for three minutes, warmed to room temperature, and weighed in groups of 2–11. The average fly mass for each group of flies was then calculated by dividing the total mass by the number of flies. Large, newly eclosed flies were excluded. To reduce potential confounding effects of genetic background in comparing males, *drd^{lwf}* males for this experiment were progeny of Canton S ♂ × *drd^{lwf}/ FM7a* ♀.

Crop volume measurements

Flies collected on the day of eclosion were placed on instant food containing 0.5% acid blue 9 for 43–47 hours. Flies were dissected under mineral oil, the crop contents were pulled into a 0.25 uL capillary (Drummond Scientific, Broomall, PA), the length of the blue column was measured with an ocular micrometer, and the volume was calculated.

Ovarian morphology

Female flies were collected on the day of eclosion and placed in vials with Canton S males. The following day, flies were dissected under PBS and the numbers of egg chambers containing visible yolk deposition in each ovary were counted. To determine the effect of starvation on ovarian development, pupae were placed in vials containing 1% agar. Females were collected on the day of eclosion and paired with Canton S males in fresh 1% agar vials, and ovaries were dissected the following day as above.

Fertility testing

Females carrying various drd alleles were collected on the day of eclosion and crossed with Canton S males. Crosses were checked daily for 7 days for the presence of larvae. At least three crosses were studied before a genotype was determined to be sterile.

Generation of imprecise excision alleles of CG33968$^{CB6275-3}$

Homozygous *CG33968*$^{CB6275-3}$ females were mated with $y^1\ w^{1118}$; *PBac{*w^{+mC}*=Delta2–3.Exel} 106* males (FBst0008200), and male progeny were mated individually with *C(1)DX,* $y^1\ f^1$ females. Individual w^- male progeny from these crosses were mated with *C(1)DX,* $y^1\ f^1$ females; for any w^- male that subsequently died within 7 days of eclosion and one male that died later, its progeny were maintained as a potential deletion line. 28 lines representing at least 12 independent excision events were generated, two of which were characterized further as described in the results.

Molecular techniques

Drosophila genomic DNA was isolated as described from groups of flies[36] or individual flies[37] and amplified by PCR using standard techniques, primers purchased from Integrated DNA Technologies (Coralville, IA), and Platinum Taq polymerase (Invitrogen, Carlsbad, CA). PCR products were purified (Qiagen, Valencia, CA) and sequenced commercially (Agencourt Bioscience, Beverly, MA). Sequences were analyzed using the Vector NTI software suite (Invitrogen). For quantitative real-time RT-PCR, whole-fly RNA was isolated on the day after eclosion (Trizol Reagent, Invitrogen) and reverse transcribed (Superscript, Invitrogen or iScript, Bio-Rad, Hercules, CA). The resulting cDNA was amplified (iQ SYBR Green supermix and MyiQ thermocycler, BioRad) and the data were analyzed using MyiQ software with the Canton S levels defined as 1 (Bio-Rad) and normalized to the ribosomal gene *rp49* (FBgn0002626). To avoid potential contamination of the signal from genomic DNA, both primer sets for real-time RT-PCR spanned introns.

Identification of the In(1)drd^{x1}breakpoints

Initial PCR analysis of genomic DNA prepared from *In(1)drd*^{x1} males showed a failure of multiple primer sets to amplify across the second half of exon 1 of *CG33968*, suggesting that an inversion breakpoint lay in that region. To identify the breakpoint, inverse PCR was performed as described.[36] Genomic DNA was digested with the blunt cutters StuI and ScaI and circularized with T4 DNA ligase, and PCR was performed with primers designed to sites within the beginning of intron 1. A single product was obtained and sequenced to reveal sequences from both *CG33968* and *CG11284*, as described in the results. As a control, DNA from *drd*^{1} flies was subjected to the same procedure and gave only *CG33968* sequences. Based on the hybrid sequence from *In(1)drd*^{x1}, primers were designed that would amplify directly across the breakpoints. PCR was performed across both breakpoints of the inversion and gave the expected size products, and the products were sequenced to confirm the breakpoints.

Statistics and data analysis

Data were graphed and analyzed using either Origin 7.5 for Windows (OriginLab, Northampton, MA) or GraphPad Prism 4.03 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com) software. All statistical analyses and calculations of median survival were performed with GraphPad Prism 4.03 software. For the crop volume, body mass, and vitellogenesis data sets, Bartlett's test indicated unequal variances of the data from different genotypes ($p<0.0001$ for all three data sets), precluding the use of an ANOVA. Therefore pair-wise comparisons of genotypes were carried out using an unpaired t-test; when necessary, Welch's correction for unequal variances was utilized. Real-time PCR data were analyzed

using a 1-way ANOVA and Tukey's post-hoc test. Survival curves were compared in a pair-wise fashion using a Mantel-Haenszel logrank test.

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References

1. Benzer S. From the gene to behavior. JAMA 1971;218(7):1015–22. [PubMed: 4942064]
2. Driscoll M, Gerstbrein B. Dying for a cause: invertebrate genetics takes on human neurodegeneration. Nat Rev Genet 2003;4(3):181–94. [PubMed: 12610523]
3. Buchanan RL, Benzer S. Defective glia in the Drosophila brain degeneration mutant drop-dead. Neuron 1993;10(5):839–50. [PubMed: 8494644]
4. Miller, A. The internal anatomy and histology of the imago of Drosophila melanogaster. In: Demerec, M., editor. Biology of Drosophila. Plainview, NY: Cold Spring Harbor Laboratory Press; 1950. p. 420-534.1994 Facsimile edition of original 1950 edition
5. Nation, JL. Insect Physiology and Biochemistry. Boca Raton: CRC Press; 2002.
6. Duttlinger A, Berry K, Nichols R. The different effects of three Drosophila melanogaster dFMRFamide-containing peptides on crop contractions suggest these structurally related peptides do not play redundant functions in gut. Peptides 2002;23(11):1953–7. [PubMed: 12431733]
7. Price MD, Merte J, Nichols R, Koladich PM, Tobe SS, Bendena WG. Drosophila melanogaster flatline encodes a myotropin orthologue to Manduca sexta allatostatin. Peptides 2002;23(4):787–94. [PubMed: 11897399]
8. Richer S, Stoffolano JG Jr, Yin CM, Nichols R. Innervation of dromyosuppressin (DMS) immunoreactive processes and effect of DMS and benzethonium chloride on the Phormia regina (Meigen) crop. J Comp Neurol 2000;421(1):136–42. [PubMed: 10813776]
9. Duve H, Audsley N, Weaver RJ, Thorpe A. Triple co-localisation of two types of allatostatin and an allatotropin in the frontal ganglion of the lepidopteran Lacanobia oleracea (Noctuidae): innervation and action on the foregut. Cell Tissue Res 2000;300(1):153–63. [PubMed: 10805084]
10. Predel R, Rapus J, Eckert M. Myoinhibitory neuropeptides in the American cockroach. Peptides 2001;22(2):199–208. [PubMed: 11179813]
11. Huang X, Huang Y, Chinnappan R, Bocchini C, Gustin MC, Stern M. The Drosophila inebriated-encoded neurotransmitter/osmolyte transporter: dual roles in the control of neuronal excitability and the osmotic stress response. Genetics 2002;160(2):561–9. [PubMed: 11861562]
12. Blumenthal EM. Cloning and characterization of *lot's wife* (*lwf*), a mutation that disrupts food processing and digestion. Ann Dros Res Conf 2007;48:862A.
13. Wilson RJ, Goodman JL, Strelets VB. the FlyBase Consortium. FlyBase: integration and improvements to query tools. Nucleic Acids Res 2008;36:D588–D593. [PubMed: 18160408]
14. Drummond-Barbosa D, Spradling AC. Stem cells and their progeny respond to nutritional changes during Drosophila oogenesis. Dev Biol 2001;231(1):265–78. [PubMed: 11180967]
15. McCall K. Eggs over easy: cell death in the Drosophila ovary. Dev Biol 2004;274(1):3–14. [PubMed: 15355784]
16. Terashima J, Bownes M. Translating available food into the number of eggs laid by Drosophila melanogaster. Genetics 2004;167(4):1711–9. [PubMed: 15342510]
17. Marygold SJ, Roote J, Reuter G, Lambertsson A, Ashburner M, Millburn GH, Harrison PM, Yu Z, Kenmochi N, Kaufman TC, Leevers SJ, Cook KR. The ribosomal protein genes and Minute loci of Drosophila melanogaster. Genome Biol 2007;8(10):R216. [PubMed: 17927810]
18. Valencia-Sanchez MA, Maquat LE. An enemy within: fly reconnaissance deploys an endonuclease to destroy nonsense-containing mRNA. Trends Cell Biol 2004;14(11):594–7. [PubMed: 15519847]

19. Rogina B, Benzer S, Helfand SL. Drosophila drop-dead mutations accelerate the time course of age-related markers. Proc Natl Acad Sci U S A 1997;94(12):6303–6. [PubMed: 9177212]
20. Kretzschmar, D.; Buchanan, RL.; Benzer, S. Cloning of the *drop-dead* gene. Cold Spring Harbor Neurobiology of Drosophila Meeting; 1993. p. 201
21. Hotta Y, Benzer S. Mapping of behaviour in Drosophila mosaics. Nature 1972;240(5383):527–35. [PubMed: 4568399]
22. Ponting CP, Mott R, Bork P, Copley RR. Novel protein domains and repeats in Drosophila melanogaster: insights into structure, function, and evolution. Genome Res 2001;11(12):1996–2008. [PubMed: 11731489]
23. Slauch JM, Lee AA, Mahan MJ, Mekalanos JJ. Molecular characterization of the oafA locus responsible for acetylation of Salmonella typhimurium O-antigen: oafA is a member of a family of integral membrane trans-acylases. J Bacteriol 1996;178(20):5904–9. [PubMed: 8830685]
24. Choy RK, Thomas JH. Fluoxetine-resistant mutants in C. elegans define a novel family of transmembrane proteins. Mol Cell 1999;4(2):143–52. [PubMed: 10488330]
25. Choy RK, Kemner JM, Thomas JH. Fluoxetine-resistance genes in Caenorhabditis elegans function in the intestine and may act in drug transport. Genetics 2006;172(2):885–92. [PubMed: 16118202]
26. Zinke I, Schutz CS, Katzenberger JD, Bauer M, Pankratz MJ. Nutrient control of gene expression in Drosophila: microarray analysis of starvation and sugar-dependent response. EMBO J 2002;21(22): 6162–73. [PubMed: 12426388]
27. Dimitriadis VK, Papamanoli E. Functional morphology of the crop of Drosophila auraria. Cytobios 1992;69(278–279):143–52. [PubMed: 1505206]
28. Edgecomb RS, Harth CE, Schneiderman AM. Regulation of feeding behavior in adult Drosophila melanogaster varies with feeding regime and nutritional state. J Exp Biol 1994;197:215–35. [PubMed: 7852903]
29. Bodenstein, D. The postembryonic development of Drosophila. In: Demerec, M., editor. Biology of Drosophila. Plainview, NY: Cold Spring Harbor Laboratory Press; 1950. p. 275-367.1994 Facsimile edition of original 1950 edition
30. Kramer JM, Davidge JT, Lockyer JM, Staveley BE. Expression of Drosophila FOXO regulates growth and can phenocopy starvation. BMC Dev Biol 2003;3:5. [PubMed: 12844367]
31. Mirth CK, Riddiford LM. Size assessment and growth control: how adult size is determined in insects. Bioessays 2007;29(4):344–55. [PubMed: 17373657]
32. Beadle GW, Tatum EL, Clancy CW. Food level in relation to rate of development and eye pigmentation in *Drosophila melanogaster*. Biol Bull Wood's Hole 1938;75:447–62.
33. Dzitoyeva S, Dimitrijevic N, Manev H. Identification of a novel Drosophila gene, beltless, using injectable embryonic and adult RNA interference (RNAi). BMC Genomics 2003;4(1):33. [PubMed: 12914675]
34. Chintapalli VR, Wang J, Dow JA. Using FlyAtlas to identify better Drosophila melanogaster models of human disease. Nat Genet 2007;39(6):715–20. [PubMed: 17534367]
35. Grigliatti, TA. Mutagenesis. In: Roberts, DB., editor. Drosophila: A Practical Approach. Oxford: Oxford University Press; 1998. p. 55-83.
36. Huang, AM.; Rehm, EJ.; Rubin, GM. Recovery of DNA Sequences Flanking P-element Insertions: Inverse PCR and Plasmid Rescue. In: Sullivan, W.; Ashburner, M.; Hawley, RS., editors. *Drosophila* Protocols. Cold Spring Harbor: CSHL Press; 2000. p. 429-37.
37. Gloor GB, Preston CR, Johnson-Schlitz DM, Nassif NA, Phillis RW, Benz WK, Robertson HM, Engels WR. Type I repressors of P element mobility. Genetics 1993;135(1):81–95. [PubMed: 8224830]
38. Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. ExPASy: The proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res 2003;31(13):3784–8. [PubMed: 12824418]
39. Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein. J Mol Biol 1982;157(1):105–32. [PubMed: 7108955]

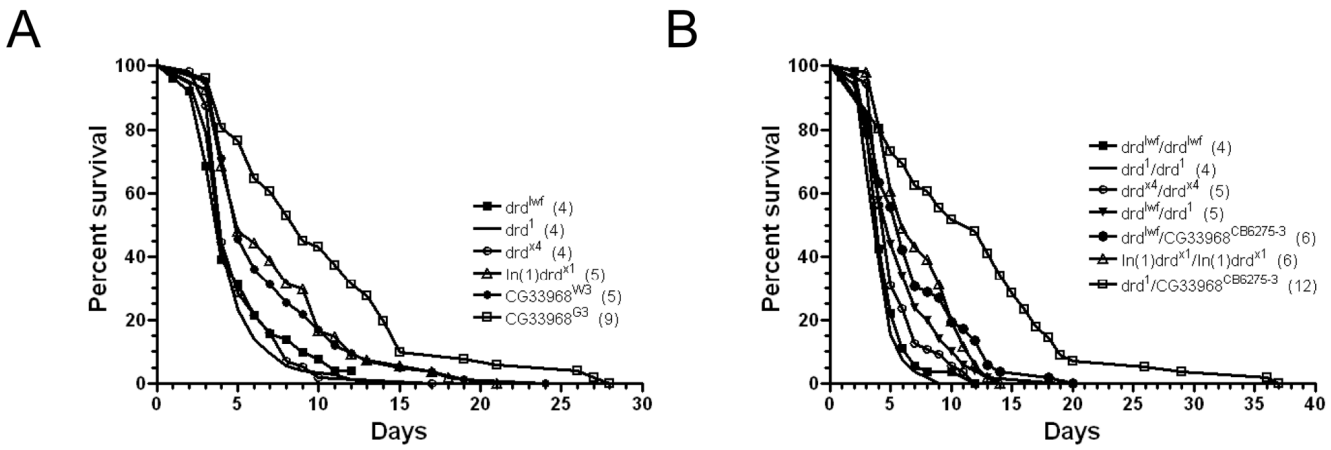

Figure 1.
Effect of *drd* alleles on male (A) and female (B) adult survival. P0–1 flies were placed in vials in groups of 1–28 flies and monitored daily. The median survival of each genotype is indicated in parentheses. n=50–83 flies/genotype

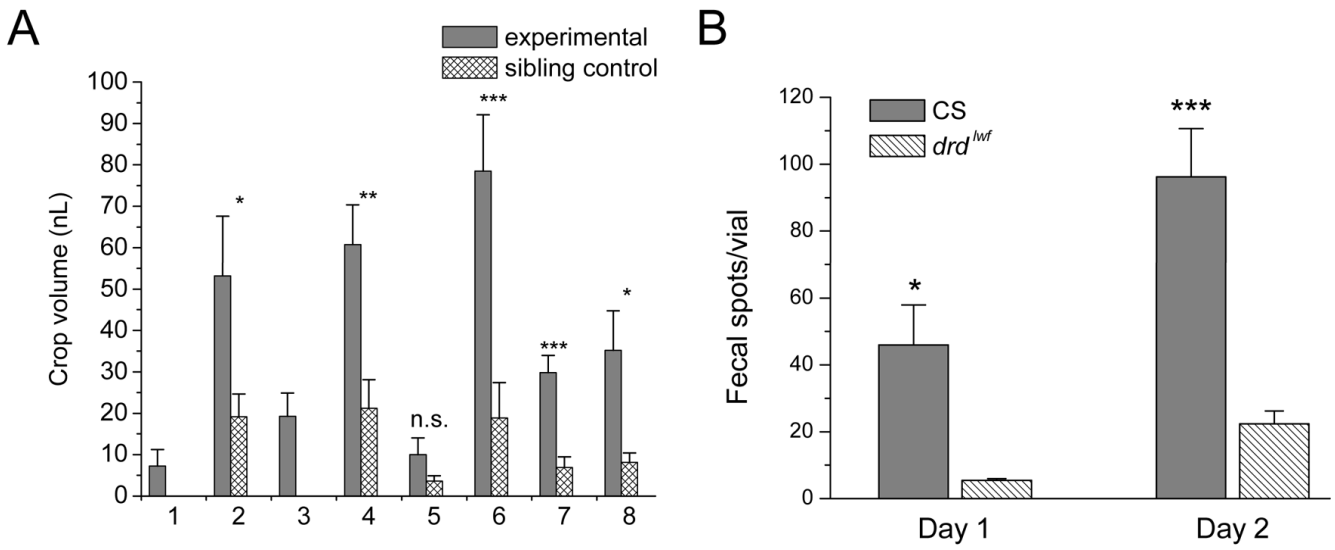

Figure 2.

Effect of *drd* alleles on gut function. A: Effect of *drd* alleles on crop volume. Groups of P0 flies were placed on instant food prepared with 0.5% acid blue 9 for 43–47 hours and their crop volumes measured. Genotypes: 1: Canton S females; 2: drd^{lwf}/drd^{lwf} experimental and $drd^{lwf}/FM7a$ control; 3: $CG33968^{CB6275\text{-}3}/\ CG33968^{CB6275\text{-}3}$; 4: $drd^{lwf}\ /\ CG33968^{CB6275\text{-}3}$ experimental and $CG33968^{CB6275\text{-}3}/FM7a$ control; 5: $drd^{1}/\ drd^{1}$ experimental and $drd^{1}/FM7c$ control; 6: $drd^{lwf}\ /\ drd^{1}$ experimental and $drd^{1}/FM7a$ control; 7: $drd^{1}/CG33968^{CB6275\text{-}3}$ experimental and $CG33968^{CB6275\text{-}3}/FM7c$ control; 8: drd^{lwf} males experimental and Canton S males control. Error bars represent SEM. n=9–21 flies/genotype. Sibling genotypes were compared by unpaired t-tests as described in the methods. *: $p<0.05$; **: $p<0.003$; ***: $p<0.001$; ****: $p<0.0001$. B: effect of drd^{lwf} on defecation rate. Pairs of flies of the same genotype were placed on blue food and the number of fecal spots produced during the first and second days post-eclosion were counted. p=0.007 (day 1) and 0.0001 (day 2) (Student's t-test), n=7–8 vials on day 1, 11–12 vials on day 2, error bars represent SEM.

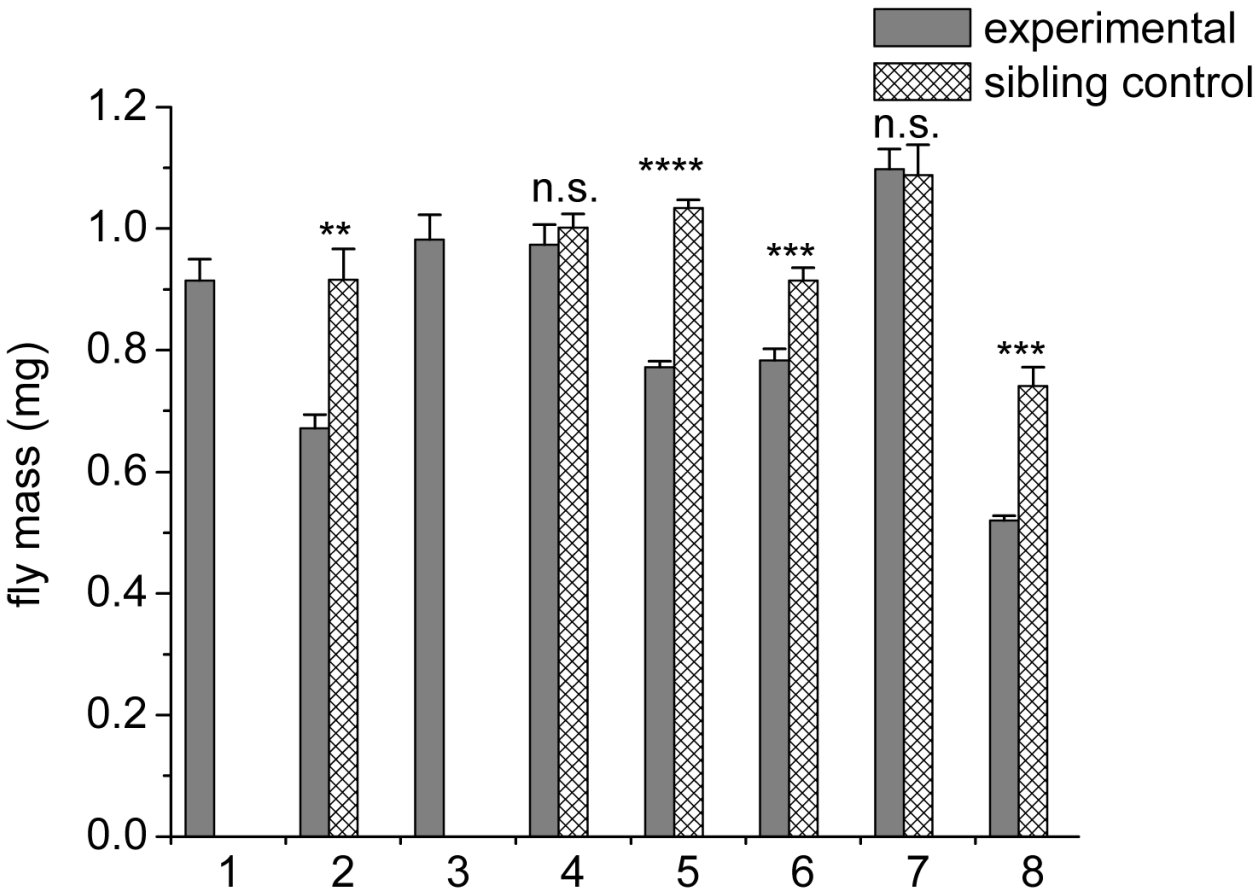

Figure 3.

Effect of *drd* alleles on body mass. Groups of P0 flies were weighed and the average mass per fly calculated. Genotypes: 1: Canton S females; 2: drd^{lwf}/drd^{lwf} experimental and $drd^{lwf}/FM7a$ control; 3: $CG33968^{CB6275\text{-}3}/CG33968^{CB6275\text{-}3}$; 4: $drd^{lwf}/CG33968^{CB6275\text{-}3}$ experimental and $CG33968^{CB6275\text{-}3}/FM7a$ control; 5: drd^{1}/drd^{1} experimental and $drd^{1}/FM7c$ control; 6: drd^{lwf}/drd^{1} experimental and $drd^{1}/FM7a$ control; 7: $drd^{1}/CG33968^{CB6275\text{-}3}$ experimental and $CG33968^{CB6275\text{-}3}/FM7c$ control; 8: drd^{lwf} males experimental and Canton S males control. Error bars represent SEM. n=5–7 measurements composed of 29–49 total flies/genotype. Sibling genotypes were compared by unpaired t-tests as described in the methods. *: $p<0.05$; **: $p<0.003$; ***: $p<0.001$; ****: $p<0.0001$

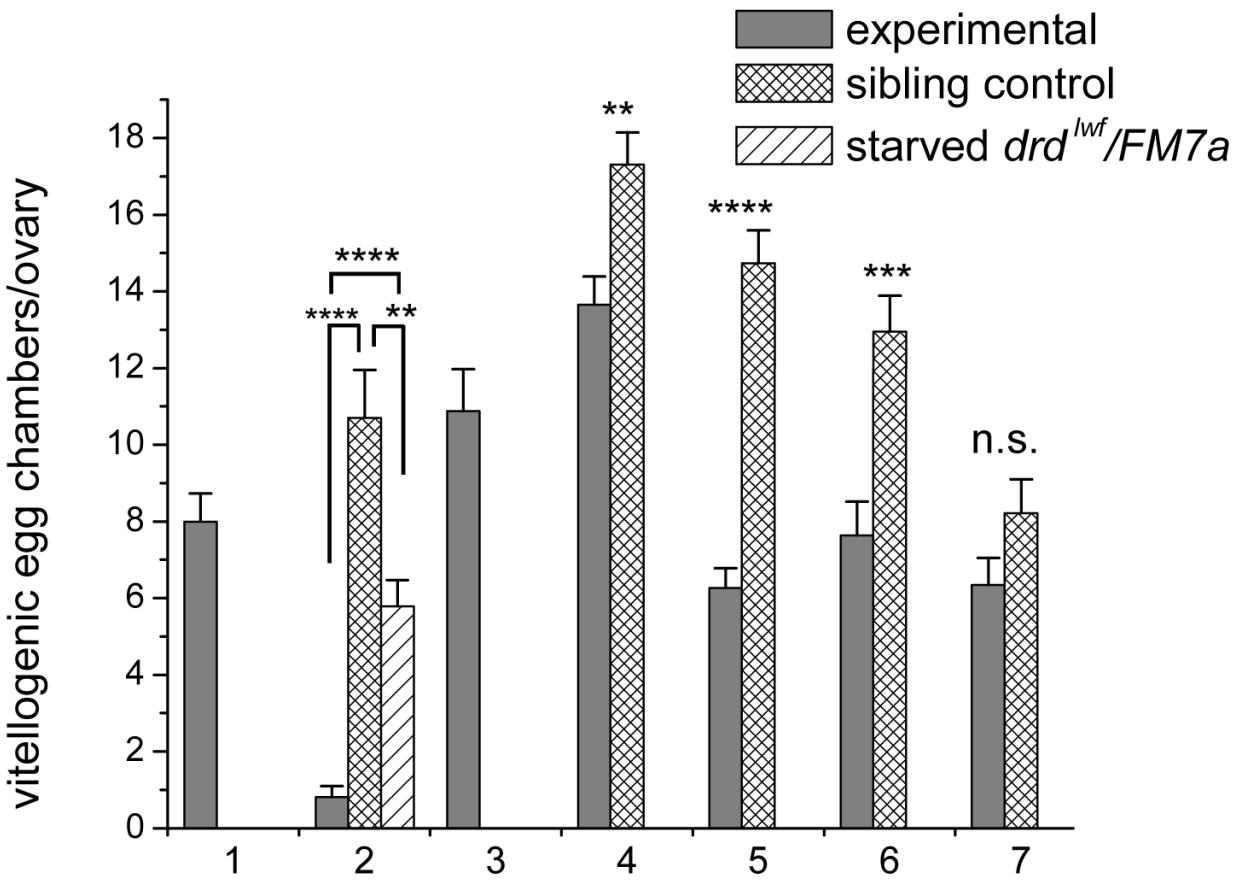

Figure 4.

Effect of *drd* alleles on oogenesis. Counts of vitellogenic egg chambers from P1 females of various genotypes are shown. Genotypes: 1: Canton S; 2: drd^{lwf}/drd^{lwf} experimental and $drd^{lwf}/FM7a$ control; 3: $CG33968^{CB6275\text{-}3}/\ CG33968^{CB6275\text{-}3}$; 4: $drd^{lwf}\ /\ CG33968^{CB6275\text{-}3}$ experimental and $CG33968^{CB6275\text{-}3}/FM7a$ control; 5: $drd^{1}/\ drd^{1}$ experimental and $drd^{1}/\ FM7c$ control; 6: $drd^{lwf}\ /\ drd^{1}$ experimental and $drd^{1}/FM7a$ control; 7: $drd^{1}/\ CG33968^{CB6275\text{-}3}$ experimental and $CG33968^{CB6275\text{-}3}/FM7c$ control. Error bars represent SEM. n=8–15 flies/genotype. Sibling genotypes were compared by unpaired t-tests as described in the methods. *: $p<0.05$; **: $p<0.003$; ***: $p<0.001$; ****: $p<0.0001$

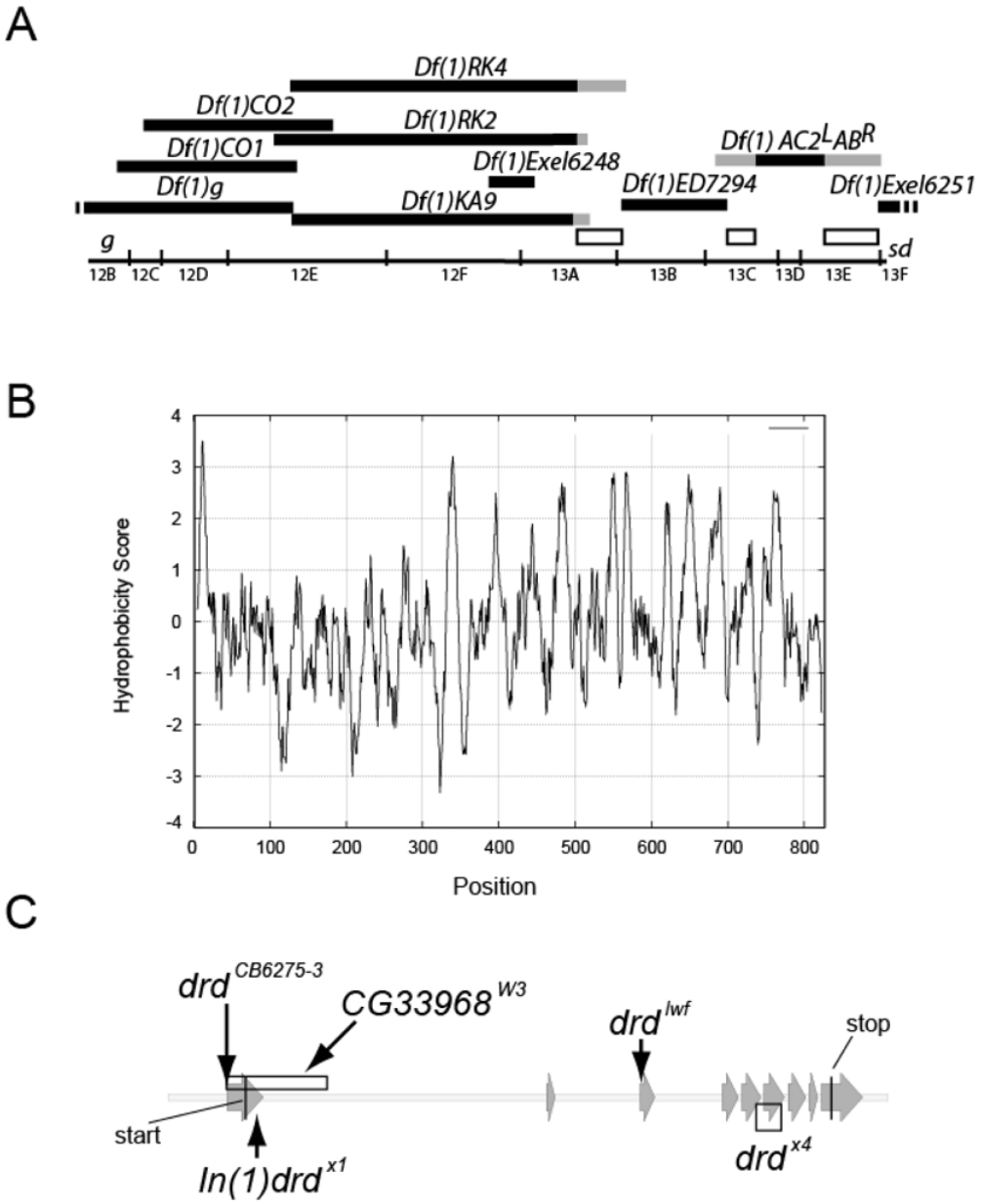

Figure 5.

A: mapping of *drd*^{lwf}. The region of the X chromosome between *g* and *sd* is shown, along with the known (black) and uncertain (gray) extent of the deficiencies that failed to uncover *drd*^{lwf}. Possible gaps in the deficiency coverage are shown as open boxes. B: hydrophobicity profile of the CG33968 gene product, showing multiple hydrophobic domains. Plot was generated on the ExPASy server[38] using the Kyte & Doolittle scale[39]. C: schematic of the *CG33968* gene indicating the nine exons, the translational start and termination sites (vertical lines), the position of the *drd*^{lwf} point mutation, *CG33968*$^{CB6275\text{-}3}$ insertion and proximal breakpoint of the *In(1)drd*^{x1} inversion (arrows), and the *drd*^{x4} and *CG33968*^{W3} deletions (open boxes). Not shown is the *CG33968*^{G3} mutation, which is an insertion of 40 bp of residual transposon sequence at the *CG33968*$^{CB6275\text{-}3}$ site. Approximately 12 kb of genomic DNA is shown.

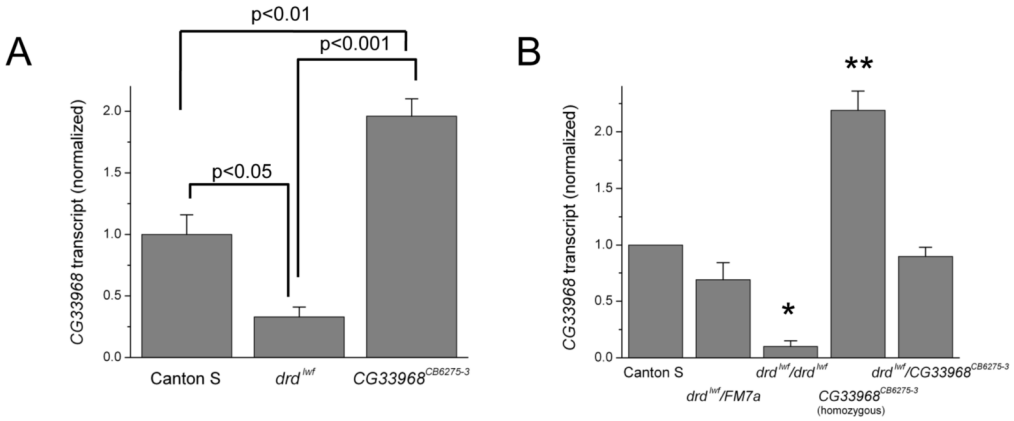

Figure 6.

Effects of *drd*^{lwf} and *CG33968*$^{CB6275\text{-}3}$ on *CG33968* transcript levels. Quantitative real-time RT-PCR was performed on whole-fly cDNA from males (A) and females (B) as described in the methods. The mean SEM from triplicate reactions is shown. Genotypes were compared using a 1-way ANOVA and Tukey's post-hoc test. *: significantly different from all other genotypes, p<0.01 vs. *drd*^{lwf}/*FM7a* and *drd*^{lwf}/*CG33968*$^{CB6275\text{-}3}$, p<0.001 vs. Canton S and *CG33968*$^{CB6275\text{-}3}$ homozygotes. **: significantly different from all other genotypes, p<0.001.

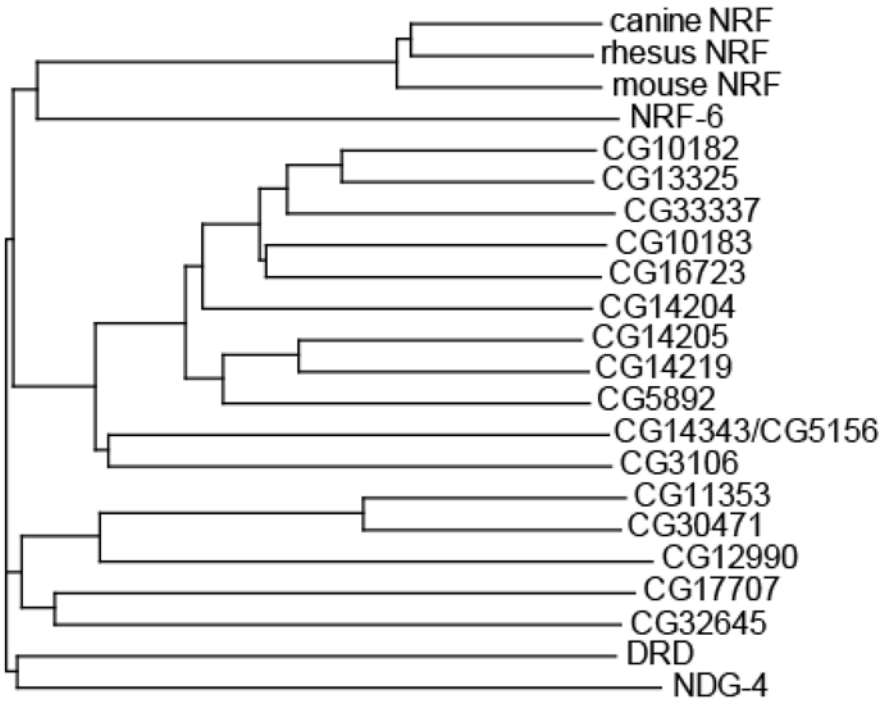

Figure 7.

The NRF-domain protein family. The tree was generated from a multiple alignment (Vector NTI) of DRD and the 16 other *Drosophila* NRF-domain proteins, the products of the *C. elegans* genes *nrf-6* and *ndg-4*, and the NRF domain proteins from Canis familiaris (XP_852671), Mus musculus (NP_796002) and Macaca mulatta (XP_001089574). For this analysis, the products of the adjacent genes *CG14343*, which encodes a short NRF-domain containing protein, and *CG5156*, which encodes an acyl transferase domain containing protein, were concatenated under the assumption that they are actually parts of the same gene. There are currently no EST or cDNA sequences listed in FlyBase that would either support or oppose merging the two genes.[13]