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Regulation of Hemocytes in *Drosophila* Requires *dappled* Cytochrome b5

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Abstract A major category of mutant hematopoietic phenotypes in *Drosophila* is melanotic tumors or nodules, which consist of abnormal and overproliferated blood cells, similar to granulomas. Our analyses of the melanotic mutant *dappled* have revealed a novel type of gene involved in blood cell regulation. The *dappled* gene is an essential gene that encodes cytochrome b5, a conserved hemoprotein that participates in electron transfer in multiple biochemical reactions and pathways. Viable mutations of *dappled* cause melanotic nodules and hemocyte misregulation during both hematopoietic waves of development. The sexes are similarly affected, but hemocyte number is different in females and males of both mutants and wild type. Additionally, initial tests show that curcumin enhances the *dappled* melanotic phenotype and establish screening of endogenous and xenobiotic compounds as a route for analysis of cytochrome b5 function. Overall, *dappled* provides a tractable genetic model for cytochrome b5, which has been difficult to study in higher organisms.

Keywords Hemocytes · *dappled* · Cytochrome b5 · Melanotic tumors · Granulomas

GenBank Accession Number HQ864711 dappled.

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Introduction

Drosophila is well studied as a model of innate immunity in humans and has been instrumental in establishing principles of defense and gene signaling pathways that involve both immune induction and hematopoiesis (Lemaitre and Hoffmann 2007). The *Drosophila* Toll pathway mediates the response to microbial infections, and humans were subsequently found to have a family of Toll receptors that also respond to infections. Other shared immune signaling pathways are the Imd (immune deficiency)/TNF1 (tumor necrosis factor 1), JNK (Jun kinase), and JAK-STAT (Janus kinase-STAT transcription factor) pathways. Activation of NFkB transcription factors, which regulate target genes such as cytokines and antimicrobial proteins, is a key shared feature (Wang and Ligoxygakis 2006). Like the signaling pathways, the cell and tissue components of the *Drosophila* and human immune systems are analogous (Kimbrell and Beutler 2001). In *Drosophila*, the hemocytes and the fat body mediate, respectively, the major cellular and humoral arms of the response. Hemocytes participate in immune signaling to the fat body, phagocytosis, damaged tissue surveillance, encapsulation of foreign intruders, and remodeling of cells during development and also respond to aberrant self-cells to form melanotic tumors/pseudotumors (Babcock et al. 2008; Brennan et al. 2007; Gateff 1994; Rizki and Rizki 1983; Tepass et al. 1994; Vass and Nappi 2000). The fat body, like the mammalian liver, produces abundant humoral response molecules, including antimicrobial peptides released into the hemolymph (Aggrawal and Silverman 2007).

Drosophila produces three types of hemocytes: plasmatocytes, lamellocytes, and crystal cells (Crozatier and Meister 2007). Plasmatocytes account for $\sim$95% of total blood cells and most functions. In particular, plasmatocytes are responsible for the phagocytosis of microorganisms during an infection and apoptotic cells during development. Lamellocytes are produced only in response to parasites or by mutations that produce melanotic tumors. Crystal cells constitute $\sim$5% of total blood cells and are involved in melanization. As in vertebrates, *Drosophila* blood cells also have two developmental origins: one early in the embryonic head mesoderm and another during larval development in the lymph gland (Evans et al. 2003). Molecular cascades and factors regulating hematopoiesis are also shared with humans and involve many pathways, for example, Toll, JAK/STAT, Hedgehog, Notch, GATA, and Runx (Evans and Banerjee 2003; Mandal et al. 2007).

A major category of mutant hematopoietic phenotypes in *Drosophila* is that of melanotic tumors or nodules. Melanotic nodules may be in the hemocoel or associated with the lymph glands and consist of abnormal and overproliferated blood cells that are covered by lamellocytes and melanized, similar to the immune response to a parasitic wasp egg (Luo et al. 1995; Meister and Lagueux 2003; Minakhina and Steward 2006b; Stofanko et al. 2008). Mutations in a variety of genes and genetic pathways have been found to produce this phenotype, such as JAK–STAT, which is also associated with blood cell overproliferation in humans, the Toll pathway, and Ras/MAPK (Asha et al. 2003; Li 2008; Minakhina and Steward 2006a; Zettervall et al. 2004). Some other genes with this mutant phenotype are *air*^{8}, encoding ribosomal protein s6 (Watson et al. 1992); *black pearl*,

encoding a DnaJ homolog (Becker et al. 2001); *l(3)malignant blood* neoplasm, encoding a protein with human cytokeratin homology (Gateff 1994); *yantar*, encoding a conserved arginine-rich protein (Sinenko et al. 2004); and the Polycomb group gene *multi sex combs* (Remillieux-Leschelle et al. 2002). The melanotic tumor genes are an important category to identify and define in order to understand blood cell function and regulation.

In a P-element enhancer detector screen concentrating on immune-related tissues and phenotypes, we identified the melanotic tumor gene *dappled* (*dpld*) (Rodriguez et al. 1996). Enhancer detector expression of *dappled* was high in the fat body, oenocytes, ring gland, and gut. Lethal and viable mutations of *dappled* produced melanotic tumors, and lethal mutation was also associated with aberrant tissue morphology, for example, of the fat body and gut (Rodriguez et al. 1996). A distinguishing feature of *dappled* mutants is consistent melanotic tumor formation, in contrast to other melanotic tumor mutants, which typically have low and variable rates. We now report identification of *dappled* as a member of the cytochrome b5 family. Cytochrome b5 is a conserved, ubiquitous, small hemoprotein that participates in electron transfer in multiple biochemical reactions and pathways, such as fatty acid desaturation, cytochrome P450-catalyzed reactions, sterol metabolism, and conversion of methemoglobin (Schenkman and Jansson 2003). Cytochrome b5 is thus involved in processes such as metabolism of endogenous and xenobiotic compounds, maintenance of oxygen levels, and production of steroid hormones (Locuson et al. 2007; Miller 2005; Yamazaki and Shimada 2006). Cytochrome b5 genetics in higher organisms has not been very amenable to study, and *dappled* provides a new avenue of approach for study of cytochrome b5 functions. In this report, we characterize the *dappled* mutant hemocyte phenotype and present the potential to study cytochrome b5 function by screening for compounds that affect the *dappled* hemocyte phenotype.

Materials and Methods

Drosophila Stocks and Rearing

Oregon R flies were used as the standard wild-type strain. The *dappled* mutants are the $dpld^{MLB}$ (97/16) and $dpld^{EJL}$ (142/2) strains of Rodriguez et al. (1996). Experiments were performed using $dpld^{MLB}/dpld^{EJL}$ transheterozygotes or *dpld* mutants hemizygous with *Df(2R)ST1* (Bloomington Stock Center). The *hemese-Gal4* strain is that of Zettervall et al. (2004). The *UAS-dappled* transgenic strain was made using the cDNA clone RE66521 from the Bloomington Drosophila Genome Project, which matched our splice product for *dappled* mRNA (see below). The insert of plasmid RE66521 was isolated using the *Eco*RI and *Bam*HI restriction enzyme sites, ligated into the *Eco*RI and *Xba*I sites of the pUAST vector using *Bam*HI/*Xba*I linkers, and transgenic strains made by Genetic Services, Inc. Insertion strains of *UAS-dappled* into chromosomes X, 2, and 3 were recovered. All stocks were raised on the Bloomington Stock Center food recipe in a 25°C incubator. For the curcumin-raised flies, the food, before cooling, was supplemented with

Fig. 1 The *dappled* locus. **a** Four P-element insertions in *dappled*/CG2140 produce mutations with a melanotic tumor phenotype (k09716, k00107, k14202, and 01857). These four *dappled* insertions and the 03427 and 05643 insertions are included within the k08815 deletion. Insertions 03610 and 08492 are outside the *dappled* and k08815 regions. One *dappled* mRNA transcript has been detected, and it has one intron spanning from +120 to +1712 (*dotted line*). The numbers 55, 56, and 57 indicate the positions of PCR primers used to test for alternative mRNA splicing products; only the 55–57 pair produced a PCR product. *Black bars* indicate the *dappled* protein coding region. **b** Sequence of the *dappled*/CG2140 gene. The sequence of the PCR fragment obtained from primers 55 and 57 to total adult RNA was combined with the sequence data for CG2140 at Flybase (Tweedie et al. 2009). Transcribed sequence with 5′ UTR and 3′ UTR is shown in *capital letters*. The protein encoding sequence is *underlined*, with the ATG start site *shaded gray*, and the translated amino acid sequence is below the *underline*. *Bold* and *italicized* sequences indicate the primers 55, 56, and 57 ▶

20 mg/ml curcumin (Sigma), and parents were placed onto the food for mating and egg laying. Compare with Gupta et al. (2009), in which doses for mice were 5, 30, and 60 mg/kg orally, daily for 19 days, and note that at 100 mg/ml, flies had high lethality in addition to developmental delay.

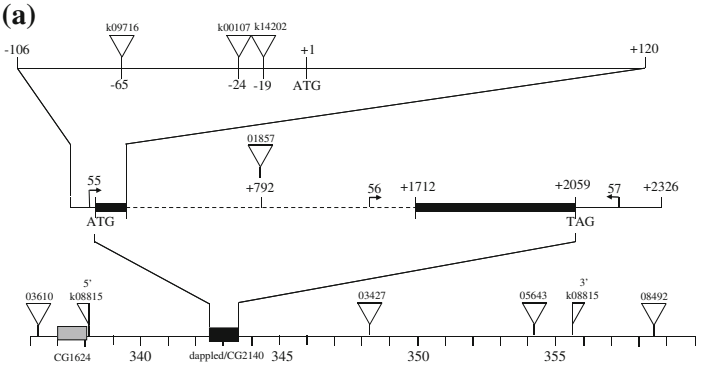
Molecular Genetic Analysis

Genomic DNA fragments flanking the *dappled* 97/16 (k09716) and 142/2 (k14202) P-element insertion sites were recovered by the plasmid rescue technique (Wilson et al. 1989), sequenced (Davis Sequencing), and aligned to the genome (Blast). The PCR primers used to compare the 88/15 (k08815) strain P-element insertion sequences AQ034113 and AQ025871 (Tweedie et al. 2009) to wild-type sequences were TATCGTCGCTGTGCTTACAGAT (60, forward) and CTAAACCTCCAA TTCGGCTATG (61, reverse) at the AQ034113 end and CTCCACGCGTAATA TACCCATT (58, forward) and AGTCATGGATGCGTAGTCACAC (59, reverse) at the AQ025871 end. In order to confirm the 88/15 insertion site, PCR fragments that resulted from the P-element end primer *Rsy*II (Tweedie et al. 2009) in combination with primers 59 and 60 were sequenced. The PCR primers used to test for possible alternative splicing of CG2140/*dappled* were sequences 55, 56, and 57 (Fig. 1). The sequence of the single transcript detected was translated to an amino acid sequence and placed into the cytochrome b5 family by Blast comparison. Clustal W was used to align the *dappled* cytochrome b5 sequence to selected other cytochrome b5 proteins.

Northern Blot Analysis

Total RNA was isolated using the Totally RNA kit (Ambion), and poly-A RNA was isolated using the Oligotex Direct mRNA Purification kit (Qiagen). The RNA was run on formaldehyde gels and transferred overnight using Ambion Northern Max reagents. The *dappled* probe was 642 bp defined by the primers 55 and 57 (Fig. 1). This 642 bp PCR fragment was subcloned using the TA cloning kit (Invitrogen), following the manufacturer's protocol. After blue/white screening to select the colony, PCR and sequencing were used to confirm the insert and determine orientation. Transcription used T7 polymerase and ^{32}P-labeled UTP and the Ambion Strip EZ kit following the manufacturer's protocol. The rp49 probe was made using

(a)



(b)

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- 140 agccatcttgctgcgcgccagtgtgaccgccggccaccAGTTCTGTGAGACCGTGCGCCC
-  80 TGGAGGAAGAGTGCGAGGCTGGACAAAAGTTTTTCCGTGACACAAATCAAACGCGCAGTA
-  20 GAAATC***AACAAGTGATCACCATGTCGAG***CGAGGAAACAAAGACTTTCACGCGGGCCGAGG
                                  M  S  S  E  E  T  K  T  F  T  R  A  E  V
+  40 TCGCCAAGCACAACACGAACAAGGACACCTGGCTGCTCATACACAACAACATCTACGACG
      A  K  H  N  T  N  K  D  T  W  L  L  I  H  N  N  I  Y  D  V
+ 100 TCACCGCCTTCCTCAACGAGGTGAGAATCGCACGGAACACGTCGCAGCTGAGTGCCACCG
      T  A  F  L  N  E
+ 160 CCCCGCACCCGCTCCCACCACTAAATGGGGGTCATCGTTTGCTATTGGGCCTTATCTGGG
+ 220 TTTCACTTGTCCCGATGAGCTAATGGGCCCAGCCCCAGCACAAGCCCAAGCCCACGTAGC
+ 280 CACATGTAGCGACTCTAACTTGCATCGCGGGGCAATTAGGTCTGTACTTTGACCCCCGGA
+ 340 CGGTAGTTTGACCTGCGTCCGGATTTCGCAGATTTGCTGCGACGTCGAAACTTCCAGCAG
+ 400 CCACTTTCGCCCAGCTGTCGAAAGAGCTCCACATGGCGAAACTATCCATTTGCATACATG
+ 460 TATATATGTGTGTGATGGGCGACGAGGGTCAAGTGTTAAGCGAAATCCGCGTCTGCATTT
+ 520 GCATCTCATTTGATTAACGGACTCCGATTTGAATTCGGATTCTTATGCAAGTGCACCACC
+ 580 GACAAACCGACTGCCGCCGGCTCCGAAAAGTGTATCACCCTGACGCAGCAGTAGGGTCAT
+ 640 CACCAAATCTATACATATAGACTACACAAGCATTCCCGCTATCTGCGGGATCTAAGATAA
+ 700 GCTCTGTGAAAATTCCGATCTAACGATGACGTTGCACTTTAGGTACACGCTGAAAATAAC
+ 760 AATAGTGAGAGTGACCAAGCATCTAATTTATAATTCCATTCCAAAGTTTCAGTCCGCCTA
+ 820 TCGACCGCAGCCATCCCCAGTCCATGTTTAAAACATGCCCCTAATCTGATCCGGCTTGGTC
+ 880 CTCTTCCATCTTAAGGAATAACATTAGCTACTGTTACCTTGCTATCTGCGTAACAGCATC
+ 940 ACCAGACAAATCGCAAATTAGACTTCCAGATTTTAGAGCGTTTGCTGGATCTAAAGATTA
+1000 TTTTTACGTGTTTATTCGTTATTTATTCAACATGATCAGTTTATTTTGACGAATCTTAT
+1060 TCAATGTGTTTCCTGCATATGTATGTTCCGATTTGTCATATATAAACCAACACAGTAATT
+1120 ACAATATAATCATCGAAATTAGATAAGGCTAAGTTGGACTATGTAAAAGTTTAAAGAATT
+1180 GTGAAAATAATTATGAGATACGCTTATCATCGATTCAATTGCACTCGGCCACCTTGAAGA
+1240 GTTTGGCTAAAATTACTTTAGCTTATCACTCAGCACAATTGAGGCGAAACAACTTGTTTG
+1300 CTTTTAATAGTAATTAATAACAATAATTACATCATACTTGCTCCGCGGCTTTTTCAGCAT
+1360 GACTCTAAGCTATAGTACAAAGTTTCAACATTATTATAATGGTACAATCAAAACGTAACT
+1420 TCGCTCGAGTTCTTAGGATCAAAGTTTCTGTAACTTTAT***TGGCTTATAAGTGCCAAATTA***
+1480 ***AA***TCTGTAACTGAAATTAGTTGTGATATTTTTACTTTCAACGCAACGGAAGTATAAAATA
+1540 TTACCATAAAGCTTCCGCATAAACGAATGTTTCGAAGCATTCCCGACTTTATACCTTGAT
+1600 ACCATACCCTCTGCTGTCTGGTCCATGAAATTCAGGGTCAGACGTCCATTAGCATTTATC
+1660 CAAGATCAGCCAACAGTTATTTATGCGAATATATTAATCTGTCTCTTTTCCTCATCCCGG
                                                              H  P  G
+1720 TGGCGAAGAGGTGCTCATCGAGCAGGCCGGCAAAGATGCCACGGAGAACTTTGAGGACGT
      G  E  E  V  L  I  E  Q  A  G  K  D  A  T  E  N  F  E  D  V
+1780 TGGCCACAGCAACGATGCCCGCGACATGATGAAGAAGTACAAAATCGGTGAGCTGGTGGA
      G  H  S  N  D  A  R  D  M  M  K  K  Y  K  I  G  E  L  V  E
+1840 GAGCGAAAGGACAAGCGTGGCCCAGAAGTCGGAGCCGACCTGGAGCACGGAACAGCAAAC
      S  E  R  T  S  V  A  Q  K  S  E  P  T  W  S  T  E  Q  Q  T
+1900 CGAGGAGAGTTCTGTGAAGTCGTGGCTGGTGCCCCTCGTCCTGTGCTTGGTGGCCACGCT
      E  E  S  S  V  K  S  W  L  V  P  L  V  L  C  L  V  A  T  L
+1960 CTTCTACAAGTTCTTCTTTGGCGGTGCCAAGCAGGAGCCACAATGCCCAGCAGAGACTTT
      F  Y  K  F  F  F  G  G  A  K  Q
+2020 CCAGCCCGCTCCGGAGTCGCTTAGTTAATCAACAAACGGAGAGCCCGAGAGTCACATGAG
+2080 GAGCGGGACAGTGGAGCGGGAAAGCTTTTTATAGTAAATTTGTGCTAAGTTTT***AACGATA***
+2140 ***ATTTTTGGCATTTGG***CGTTTTTGTACAACCCTCTACACGTGTAATTCCTTCAGACGGCGG
+2200 TTCTAGACTCTAAAAGTAATTTATCCCATTGATATTCCAACATTTGAGGCACGGAACGAA
+2260 AACGATAAGTTTTTATTCGCATGTATGTACATTAAACATATTTCCAAAAGCaagcaaaca
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the primers TCCTTCCAGCTTCAAGATGACC (forward) and AGAAGTTCCTG GTGCACAACGT (reverse). Hybridization was overnight, and a Fuji phosphoimager and ImageJ were used to analyze the hybridization signal.

Blood Cell Counts

Third-instar larvae were developmentally staged as early versus late wandering phase based on retention in the gut of food dye (Zettervall et al. 2004). The Bright-Line Counting Chamber (Fisher) hemocytometer was used to count blood cells stained with monochlorobimane dye (Tirouvanziam et al. 2004) or GFP-expressing blood cells as in Zettervall et al. (2004), except that instead of red food color we used blue (Deep Blue Shade, Esco Foods, Inc.). Siblings that were heterozygous for *dappled* were distinguished based on either a CyO balancer with the marker y+ (i.e., *y*; +/*dpld* vs. *y*; +/*CyO, y*+) or B-galactosidase staining to indicate presence or absence of a *dpld* enhancer trap (Rodriguez et al. 1996). Separate counts of blood cells in *y* versus *y*+ larvae established that *y* did not affect the blood cell counts. Determination of sex was by the size of the gonads or by marking the sexes with *y* versus *y*+. Hemocyte counts from different samples were compared using the *t* test, and *P* values were calculated using VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html).

Results

Cytochrome b5, the Encoded Product of *dappled*

The original *dappled* alleles, $dpld^{EJL}$ and $dpld^{MLB}$, result from the P-element insertions (P-lacW) in strains 142/2 (k14202) and 97/16 (k09716), respectively (Rodriguez et al. 1996). The $dpld^{EJL}$ allele is homozygous and hemizygous lethal, and $dpld^{MLB}$ is homozygous and hemizygous viable. Heteroallelic $dpld^{EJL}/dpld^{MLB}$ is also viable and, like $dpld^{EJL}$ *and* $dpld^{MLB}$ homozygotes and hemizygotes, has melanotic tumors (Rodriguez et al. 1996). In order to identify the gene associated with the melanotic tumor phenotype, we molecularly cloned and sequenced the *dappled* gene. We used the technique of plasmid rescue to isolate the genomic DNA flanking the k14202 and k09716 insertions. Sequencing of the flanking DNA and alignment to the genome placed both insertions into the 5′ UTR of CG2140 (Tweedie et al. 2009), with k09716 46 bp farther upstream than k14202 (Fig. 1).

In contrast to our results, genome annotation assigned *dappled* as CG1624 based on the position of the lethal P-element insertion 88/15 (k08815), which did not complement the 142/2 insertion (Spradling et al. 1999). We therefore investigated this discrepancy in the identification of *dappled.* The 5′ and 3′ flanking sequences for the k08815 insertion are 3′-AQ034113 and 5′-AQ025871. Alignment of these flanking sequences on the latest *Drosophila* genome sequence, however, places them 176,059 bp apart. There are several potential causes for this gap in the sequence: multiple insertions, an error in data reporting, or a large deletion. To test for the accuracy of the k08815 flanking sequence, primers were designed to (1) flank

the 3′ and 5′ insertion sites of the k08815 transposon and (2) extend from a P-element end primer (p*Rsy*II) into a flanking sequence. The resultant PCR fragments supported the published flanking sequences, as wild-type products were not observed in the mutant strain and both insertion sites were verified with the P-element end primer (Fig. 1). Finally, these PCR products were sequenced to provide confirmation of the flanking sequences. These results indicate that k08815 is not a simple insertion but is associated with additional disruption at its genomic position. We set out to differentiate between either multiple insertions or a large deletion by performing complementation tests with other lethal insertion strains in the region. Complementation tests confirmed that a large deletion accompanies the k08815 insertion (Fig. 1). Complementation and mapping also placed two more insertions, k00107 and 01857, into the 5′ UTR and intron of CG2140/*dappled*, respectively (Fig. 1).

We tested these various *dappled* mutants for alterations in transcription, further confirming that the *dappled* phenotype results from an effect on CG2140, by performing Northern blot analysis using RNA from *dappled* mutants. This analysis showed a decrease in CG2140 transcript levels in the *dappled* mutants compared with the Oregon R controls (Fig. 2). The *dappled* strains are hypomorphic, with the lethal alleles stronger. The lethal allele $dpld^{EJL}$ expresses approximately 10% of wild-type embryonic RNA levels, whereas the viable allele $dpld^{MLB}$ has ∼80% of wild-type adult RNA levels. Analysis of the transcripts by RT-PCR and sequencing showed one splice variant, with a single intron (Fig. 1).

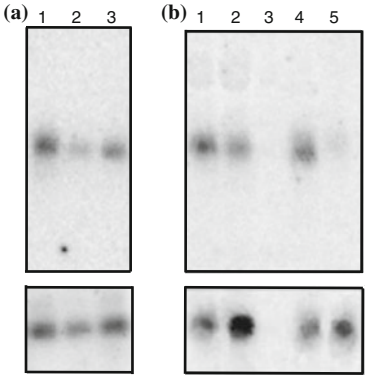

Fig. 2 The *dappled* transcript levels are reduced in *dappled* mutants. Northern blot analysis of *dappled* transcripts shows reduced levels in mutants compared with wild type. *Upper panels*: Northern blots after probing with labeled *dappled*. *Lower panels*: control for loading of RNA in each lane by reprobing the blots with labeled *rp49*. **a** Adult total RNA from Oregon R (*lane 1*), $dpld^{MLB}/dpld^{EJL}$ transheterozygotes (*lane 2*), and $dpld^{MLB}$ homozygotes (*lane 3*). **b** Embryonic total RNA from Oregon R (*lane 1*) and $dpld^{01857}$ homozygotes (*lane 2*); embryonic poly-A RNA from Oregon R (*lane 4*), and $dpld^{EJL}$ homozygotes (*lane 5*). *Lane 3* contains no RNA. Embryos from the homozygous lethal strains $dpld^{EJL}$ and $dpld^{01857}$ were sorted via a Balancer chromosome expressing GFP (*CyO, Kr-GFP*), in order to select for the homozygous mutant embryos. Quantitation after correcting for loading using *rp49* shows reduced levels of *dappled* RNA in all mutants. Compared with wild type, approximate levels are (**a**) 65% in *lane 2* and 80% in *lane 3*; (**b**) 40% in *lane 2* and 10% in *lane 5*

Based on sequence homology from our alignments and the Flybase Genome Annotation, *Drosophila* gene CG2140/*dappled* encodes a microsomal cytochrome b5 (Fig. 1; http://flybase.org/reports/FBgn0033189.html). Genome annotation has identified CG2140 as the only cytochrome b5 in *Drosophila*. One related *Drosophila* sequence has been identified, cytochrome b5-related (Cyt-b5-r; http://flybase.org/reports/FBgn0000406.html). Cyt-b5-r has 436 residues (there are only 134 for Dappled and 135 for human cytochrome b5), and only 30 of the Cyt-b5-r residues are identical to the Dappled sequence. In a comparison of the *dappled* encoded amino acid sequence with a selection of other cytochrome b5 proteins from mammals and insects, Dappled has 45% identity with human cytochrome b5 and 76% identity with housefly cytochrome b5 (Fig. 3). Overall, the selected mammals and insects have 32% identity. Cytochrome b5 is a highly conserved, ubiquitous family of small hemoproteins that participate in electron transfer in multiple biochemical reactions. These reactions are involved in many biological processes, such

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                          10         20         30         40         50         60
                           |          |          |          |          |          |
Rattus_norvegicus         MAEQSDKDVKYYTLEEIQKHKDSKSTWVILHHKVYDLTKFLEEHPGGEEVLREQAGGDAT
Mus_musculus              MAGQSDKDVKYYTLEEIQKHKDSKSTWVILHHKVYDLTKFLEEHPGGEEVLREQAGGDAT
Sus_scrofa                MAEQSDKAVKYYTLEEIQKHNNSKSTWLILHHKVYDLTKFLEEHPGGEEVLREQAGGDAT
Homo_sapiens              MAEQSDEAVKYYTLEEIQKHNHSKSTWLILHHKVYDLTKFLEEHPGGEEVLREQAGGDAT
Drosophila_dappled        ---MSSEETKTFTRAEVAKHNTNKDTWLLIHNNIYDVTAFLNEHPGGEEVLIEQAGKDAT
Musca_domestica           ---MSSEDVKYFTRAEVAKNNTKDKNWFIIHNNVYDVTAFLNEHPGGEEVLIEQAGKDAT
Anopheles_gambiae         ---MS--EVKTYSLADVKSHNTNKSTWIVIHNDIYDVTEFLNEHPGGEEVLLEQAGREAT
                             *   .* ::  :: .:: ....*.::*:.:**:* **:********* **** :**
Prim.cons.                MAEQSD2DVKYYTLEEIQKHNTSKSTWLILHHKVYDLTKFLEEHPGGEEVLREQAGGDAT

                          70         80         90         100        110        120
                           |          |          |          |          |          |
Rattus_norvegicus         ENFEDVGHSTDARELSKTYIIGELHPDDRSKIAKPSETLITTVES-NSSWWTNWVIPAIS
Mus_musculus              ENFEDVGHSTDARELSKTYIIGELHPDDRSKIAKPSDTLITTVES-NSSWWTNWVIPAIS
Sus_scrofa                ENFEDVGHSTDARELSKTFIIGELHPDDRSKIAKPSETLITTVES-NSSWWTNWVIPAIS
Homo_sapiens              ENFEDVGHSTDAREMSKTFIIGELHPDDRPKLNKPPETLITTIDS-SSSWWTNWVIPAIS
Drosophila_dappled        ENFEDVGHSNDARDMMKKYKIGELVESERTSVAQKSEPTWSTEQQTEESSVKSWLVPLVL
Musca_domestica           EHFEDVGHSSDAREMMKQYKVGELVAEERSNVPEKSEPTWNTEQKTEESSMKSWLMPFVL
Anopheles_gambiae         EAFEDVGHSSDAREMMKKFKVGELIEAERKQIPVKKEPDWKMDQQ-DDNQLKQWIVPLIL
                          * *******.***:: * : :***   :* .:    :.  .  :. ...  ..*::* :
Prim.cons.                ENFEDVGHSTDAREMSKTYIIGELHPDDRSKIAKPSETLITTV2STNSSWWTNWVIPAIS

                          130
                           |
Rattus_norvegicus         ALVVALMYRLYMAED--
Mus_musculus              ALAVALMYRLYMAED--
Sus_scrofa                ALVVSLMYHFYTSEN--
Homo_sapiens              AVAVALMYRLYMAED--
Drosophila_dappled        CLVATLFYKFFFGGAKQ
Musca_domestica           GLVATLIYKFFFGTKSQ
Anopheles_gambiae         GLLATILYRFYFTQ---
                           : .:::*:::
Prim.cons.                ALVV2LMYRFY2AED2Q
```

Fig. 3 Cytochrome b5 sequences. The sequences of Dappled and selected other cytochrome b5 proteins from insects and mammals are compared using Clustal W for alignment and consensus generation (Prim. Cons.). See Wang et al. (2007) for an X-ray crystal structure comparison of the heme binding domain, residues 6–95. Note the high sequence identity within this region, which is 82% for residues 40–73. Sequence accession numbers are *Rattus norvegicus* GenBank AAB67610.1; *Mus musculus* Swiss-Prot P56395.2; *Sus scrofa* Swiss-Prot P00172.3, *Homo sapiens* GenBank AAA35729.1; *Drosophila* dappled GenBank HQ864711; *Musca domestica* GenBank AAA56985.1; *Anopheles gambiae* GenBank AAO24766.1

as conversion of methemoglobin, sterol metabolism, fatty acid desaturation, and cytochrome P450-catalyzed reactions (Schenkman and Jansson 2003).

Hemocyte Misregulation and Melanotic Nodule Formation in *dappled* Mutants

The $dpld^{MLB}$ insertion has made analysis possible, as in addition to producing consistent melanotic tumor formation, it is the only viable allele. Maintenance of $dpld^{MLB}$ homozygotes as a stock, however, was not feasible because of effects on fertility. Homozygous $dpld^{MLB}$ or transheterozygous $dpld^{MLB}/dpld^{EJL}$ females and males produced few progeny, even when mated with wild-type flies (<5 progeny/*dappled* parent), and mutant females laid few eggs ($\sim$50/female). It is unlikely that this fertility problem is due simply to physical obstruction in the abdomen, because flies with less obtrusive tumors (i.e., small tumors near the thorax) also show low fertility. To avoid the effects of accumulation of second site mutations by maintaining the $dpld^{MLB}$ chromosome over a balancer, all experiments were done on progeny from crosses that produced transheterozygotes or hemizygotes.

The development of the Gal4–UAS system (Brand and Perrimon 1993) to include drivers for the hematopoietic system makes it more feasible to be able to investigate the blood cell phenotypes of mutant genes, for example, Kimbrell et al. (2002) and Zettervall et al. (2004). The *hemese-Gal4* construct drives expression in blood cells of the plasmatocyte and lamellocyte classes (Zettervall et al. 2004). Strains with *hemese-Gal4 UAS-GFP* thus have the blood cells marked with GFP, enabling hemocyte counting and phenotype analysis in various genetic strains (Zettervall et al. 2004). We used *hemese-Gal4 UAS-GFP* to investigate the blood cells of *dappled* melanotic tumor mutants. In melanotic tumors of $dpld^{EJL}/dpld^{MLB}$; *hemese-Gal4 UAS-GFP* larvae, all of the cells in the masses were found to fluoresce strongly for GFP (Fig. 4). Cell counts of circulating plasmatocytes and lamellocytes showed large numbers of circulating lamellocytes (Fig. 4). Lamellocytes are not found in normal, healthy larvae, whereas in melanotic nodule mutants lamellocytes are abundant (Crozatier and Meister 2007). The *dappled* melanotic tumors are thus specifically designated as melanotic nodules, which consist of hemocytes, as opposed to melanizations, which are non-blood cell tissues that have melanized (Minakhina and Steward 2006b).

Mutant *dappled* larvae were categorized based on the relative size of the melanotic nodules. Larvae with small versus large melanotic nodules showed different numbers of circulating plasmatocytes and lamellocytes (Fig. 4). Notably, lamellocytes are more prevalent in small tumors, whereas plasmatocytes are more abundant in large nodules. One possible interpretation of the higher number of lamellocytes in smaller nodules is that these nodules are still actively forming, and lamellocytes are still being recruited to the nodules. During normal larval development, the number of plasmatocytes typically increases two- to threefold from the early wandering (early) to the late wandering (late) stage of the third instar (Crozatier and Meister 2007; Zettervall et al. 2004). Plasmatocyte representation from early to late stage wandering was different for small and large tumors. Larvae with large tumors showed an increase from early to late stage, whereas larvae with

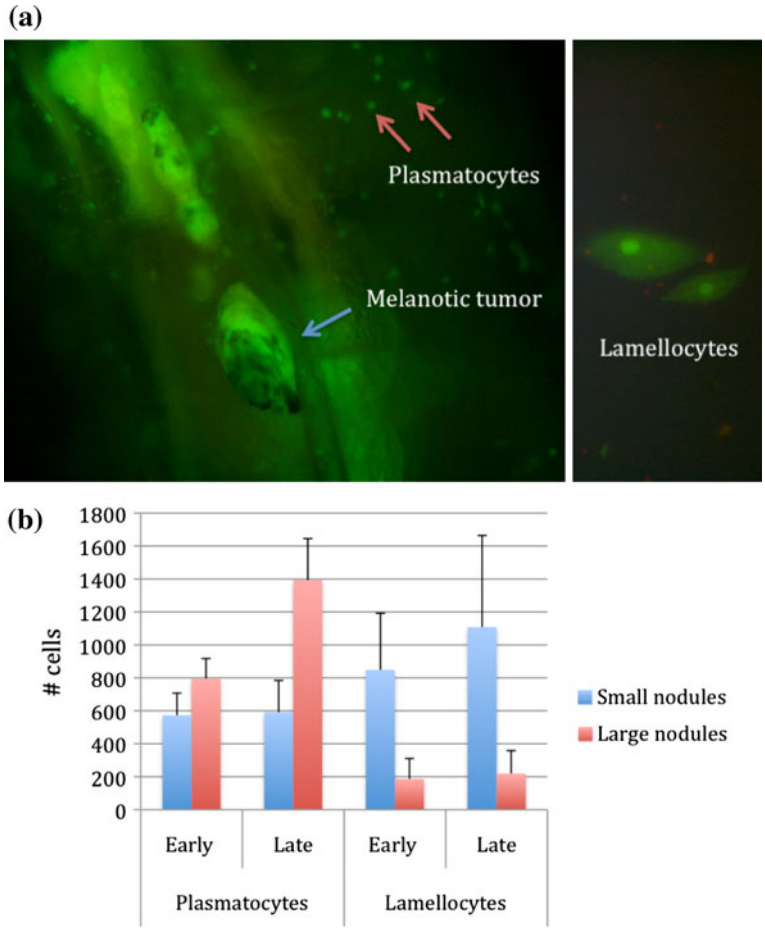

Fig. 4 Hemocytes of *dappled* viable mutants. **a** Hemocytes visualized by GFP expression show (*left*) melanotic tumors/nodules in a third-instar larva as clumps of hemocytes that are partially melanized, along with circulating plasmatocytes, and (*right*), at higher magnification, lamellocytes. Lamellocytes are approximately four times larger than plasmatocytes. GFP expression is driven by the *hemese-Gal4* driver. **b** Cell counts of circulating hemocytes in early and late wandering third-instar larvae show high levels of lamellocytes and characterize the dynamic process of melanotic tumor formation by comparing mutants with small (*left bars*) versus large (*right bars*) melanotic nodules. Error bars show 0.95 confidence limits. $N \geq 15$ for each category. (Color figure online)

small tumors did not (Fig. 4), and this may also reflect the dynamic nature of *dappled* melanotic nodule formation.

Heterozygosity of *dappled* does not produce tumors, but it does have a dominant effect on blood cell number. The *dappled* heterozygotes (Bal/*dpld*) have a significantly higher number of plasmatocytes than wild-type larvae (+/+). This difference was confirmed by counting hemocytes of *dappled* heterozygotes (*dpld*/+) and their control siblings (Bal/+) (Fig. 5). Further, counts of siblings showed that the increase in plasmatocytes is present both early and late in the third instar. The

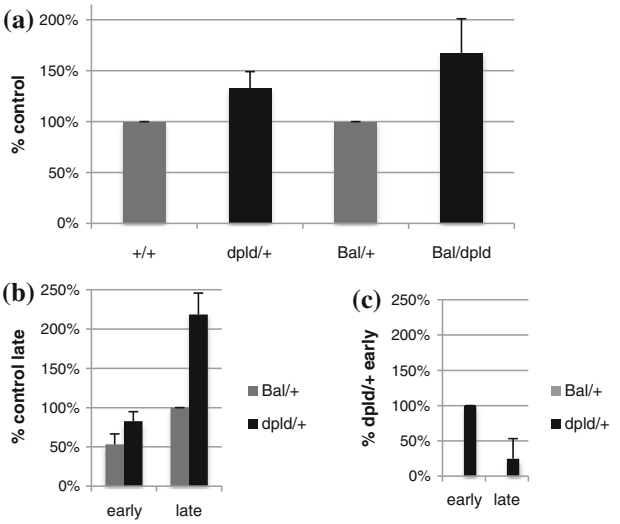

Fig. 5 Plasmatocytes and lamellocytes are increased in *dappled* heterozygotes. **a** Cell counts of plasmatocytes during late third instar of *dappled* heterozygotes, over a Balancer (Bal) or wild-type (+) chromosome, show significantly more plasmatocytes than wild-type (+/+) and Balancer (Bal/+) controls. Counts were normalized across three experiments to the percentage of controls. The average for control +/+ was 5,697 cells/larva and Bal/+ was 4,935 cells/larva. $P < 0.01$ for dpld/+ versus Bal/+, and $P < 0.05$ for Bal/dpld versus +/+. **b** Cell counts of plasmatocytes of *dappled* heterozygotes and controls that are siblings show that the increase in plasmatocytes has already occurred by early wandering third instar. Additionally, *dappled* heterozygotes show a normal increase, which is approximately two- to threefold, in plasmatocyte number from early to late stage. Counts were normalized across three experiments to the percentage of control Bal/+ late, which averaged 4,125 cells/larva. $P < 0.01$ for both early and late of dpld/+ versus Bal/+. **c** Cell counts of the larvae (**b**) show that lamellocytes are also increased. Control Bal/+ larvae had no lamellocytes in any sample. $P < 0.05$ for both early and late dpld/+ versus Bal/+. The average number of cells in early *dappled* heterozygotes was 5, with high variability and no significant difference from late *dappled* heterozygotes ($P > 0.05$). $N \geq 15$ per genotype for each experiment

relative numbers of lamellocytes in *dappled* heterozygotes is less clear, as the average was very low, many larvae had no lamellocytes, and the number did not increase from early to late stage (Fig. 5). Thus, although the lamellocyte data are statistically significant ($P < 0.05$), *dappled* heterozygosity at most yields a very weak induction of lamellocyte differentiation.

Rescue of Hemocyte Phenotype but Not Lethality by Wild-Type *dappled* in Blood Cells

We next asked if the *dappled* phenotype could be rescued by expressing wild-type *dappled* only in blood cells. We constructed a plasmid containing *UAS-dpld*, which was used to make transgenic strains with *UAS-dpld* in different chromosomal locations. The *hemese-Gal4* was then used to drive *UAS-dpld,* and the rates of melanotic nodule formation were compared in *dappled* mutants and controls. In addition to hemocytes, *hemese-Gal4* drives strong expression in the salivary glands,

Table 1 Melanotic tumor phenotype is rescued by *dappled* expression in hemocytes

Cell type	*Gal4; UAS*	Percentage (*n*) of adults[a] with melanotic nodules			Total *N*
		Negative control[b]	Positive control[c]	Rescue test[d]	
Blood cells	*hemese-Gal4; UAS-dpld* line 1	0% (352)	87.5% (32)	5% (121)	505
	hemese-Gal4; UAS-dpld line 3a	0% (526)	94.1% (43)	7.5% (105)	674
	hemese-Gal4; UAS-dpld line 9b	0% (353)	93% (17)	5.7% (106)	476
Control	*sgs3-Gal4; UAS-dpld*[e]	0% (514)	89.3% (75)	81.6% (103)	692

[a] Adults are progeny from crosses of *w; CyO/dpldMLB; Gal4* females with *w/Y; CyO/dpldEJL; TM3,Sb* or *UAS-dpld/UAS-dpld* males

[b] All genotypes with *CyO/dpldMLB* or *EJL*. Two thirds of the progeny are expected to be in this category

[c] *dpld* transheterozygotes with *Gal4* or *UAS*

[d] *dpld* transheterozygotes with both *Gal4* and *UAS*

[e] Results with all three *UAS-dpld* lines

so a control salivary gland driver, *sgs3-Gal4*, was also used to drive *UAS-dpld*. To test for rescue of lethality by ubiquitous expression, the *da-Gal4* driver (Pili-Floury et al. 2004) was used.

The *dappled* mutants with *hemese-Gal4*, but not *sgs3-Gal4*, driving *UAS-dpld* are rescued from the melanotic phenotype. Three different *UAS-dpld* insertions were tested with *hemese-Gal4*, and all showed similar rates of rescue (Table 1). Dissections were performed to determine if blood cell nodules might be forming but not melanizing. The dissections confirmed that blood cell nodule formation without accompanying melanization did not occur.

The dominant effect of heterozygous *dappled* on plasmatocyte number is also rescued by adding *dappled* expression to blood cells. Hemocyte counts of *dappled* heterozygotes with *hemese-Gal4* driving *UAS-dpld* had, on average, reduced numbers of hemocytes, compared with control *dappled* heterozygotes (Fig. 6). The number of lamellocytes in heterozygotes was also reduced on average, but with the small number and variability in lamellocytes, was not significantly different (Fig. 6). These results do not necessarily rule out other tissues as important for the *dappled* mutant blood cell phenotypes, but rather emphasize that the level of *dappled* expression in the blood cells regulates number and differentiation.

Expression of wild-type *dappled* in blood cells did not rescue the mutant lethality. In contrast, ubiquitous expression of wild-type *dappled* completely rescued the mutant lethality, producing normal adults with no tumors (Table 2). These results are consistent with the aberrant tissue morphology of lethal *dappled* (Rodriguez et al. 1996) and establish the importance of *dappled* transcript levels for normal function in other tissues.

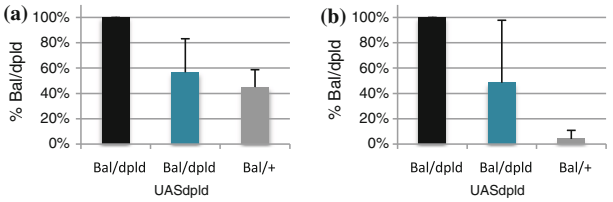

Fig. 6 Rescue of *dappled* heterozygote phenotype by expression of wild-type *dappled* in hemocytes. The *dappled* heterozygotes with *UAS-dpld* driven by *hemese-Gal4* (Bal/dpld UAS-dpld) were compared with controls without UAS and Gal4 (Bal/dpld and Bal/+). Counts were normalized across three experiments to the percentage of Bal/dpld. Plasmatocyte counts (**a**) are significantly reduced in rescued larvae compared with Bal/dpld ($P < 0.05$), but not compared with Bal/+ ($P > 0.05$). Lamellocyte counts (**b**) are reduced on average in rescued larvae, but not to a significant extent ($P > 0.05$, compared with both Bal/dpld and Bal/+). $N \geq 15$ per genotype for each experiment

Table 2 Hemocyte expression of *dappled* is insufficient for rescue of lethality

Expression type	*Gal4; UAS*	Percentage (*n*) survival of adults[a]		Total *N*
		Control[b]	Rescue test[c]	
Ubiquitous	*daughterless-Gal4; UAS-dpld*	84.6% (1503)	15.4% (273)	1776
Blood cells	*hemese-Gal4; UAS-dpld*	100% (1086)	0% (0)	1086

[a] Adults are progeny from crosses of *w; CyO/dpldEJL;TM3,Sb* or *UAS-dpld/UAS-dpld* females with *w/Y; CyO/Df1888;TM3,Sb/Gal4* males

[b] *CyO/dpldEJL* or *CyO/Df(2R)ST1* with *Gal4* or *UAS*. 85% of the total progeny are expected to be in this category

[c] *dpldEJL/Df(2R)ST1* with both *Gal4* and *UAS*. 15% of the total progeny are expected to be in this category

Effect of *dappled* on Hematopoietic Waves During Development

The *dappled* nodules form early in larval development, which is more consistent with an increase of plasmatocytes from the first hematopoietic wave (embryonic origin) rather than the second hematopoietic wave (lymph gland origin at the end of the third instar). As noted above, plasmatocytes in *dappled* tumors express the *hemese-Gal4* driver, which does not express in the lymph glands, further indicating an early developmental origin for these cells. Additionally, the lymph glands appear normal, at least at a gross level, and only one *dappled* enhancer trap expresses in the lymph glands (Rodriguez et al. 1996). We analyzed 1 kb of the *dappled* upstream region for potential binding by blood cell, lymph gland, and other transcription factors using the Transfac database (Wingender 2008) provided by Biobase Biological Databases (http://www.biobase-international.com/index.php?id=home). Among the transcription factor binding sites are many that are predicted to be T cell factor/pan binding sites (Brunner et al. 1997), consistent with blood cell expression, and tinman/Nkx2.5 binding sites (Kimbrell et al. 2002), suggesting lymph gland expression (Table 3).

We tested for *dappled* effects in both hematopoietic waves by counting plasmatocytes at a time in development when cells of both hematopoietic waves are present. Cells of the two waves were differentiated by (1) using *hemese-Gal4* to drive *UAS-GFP* in cells from the first wave of development, so that first-wave cells fluoresced green, and (2) staining all hemocytes with monochlorobimane dye, so that the second-wave hemocytes fluoresced blue but not green. The developmental stage selected to have both sets of hemocytes was white prepupae, age 0–3 h postformation as white prepupae. These results show that heterozygosity of *dappled* increases the number of plasmatocytes from both hematopoietic waves (Table 4).

Mutation of *dappled* affects both sexes similarly, and our results show both sexes together (Figs. 4, 5, 6). Similar numbers of the sexes are needed per sample,

Table 3 Predicted T cell factor/pangolin and Nkx 2.5/tinman sites

T cell factor/pangolin	
−730	(+) 1.000 0.994 gcgtaTCAAAagcaca TCF
−687	(−) 0.964 0.946 gcttagTTTGTtgtta TCF
−611	(−) 0.928 0.919 gactgtTTTTAttgaa TCF
−440	(−) 0.928 0.929 atacatTTTTAttaat TCF
−430	(+) 0.928 0.922 attaaTGAAAtggtct TCF
−408	(−) 0.957 0.938 aataaaTTTGCttacg TCF
−395	(−) 0.964 0.948 acgaatTTTGTttgta TCF
−383	(−) 0.928 0.922 tgtacaTTTCAtttga TCF
−170	(+) 0.964 0.944 tgtaaACAAAttataa TCF
Nkx 2.5/tinman	
−939	(−) 0.801 0.786 tgattgAAGTTggcaa NKX2.5
−935	(+) 0.869 0.828 tgAAGTT NKX2.5
−923	(−) 0.869 0.870 AACTTaa NKX2.5
−896	(+) 1.000 0.859 cccgtCACTTgaatta NKX2.5
−894	(+) 1.000 0.971 cgtCACTTga NKX2.5
−891	(−) 1.000 1.000 CACTTga NKX2.5
−795	(+) 0.844 0.805 taggcCACTCcaggat NKX2.5
−780	(+) 0.800 0.819 tgcCAATTgc NKX2.5
−761	(−) 0.869 0.823 AACTTac NKX2.5
−711	(+) 1.000 0.741 gccgtCACTTatcaat NKX2.5
−709	(+) 1.000 0.906 cgtCACTTat NKX2.5
−706	(−) 1.000 0.939 CACTTat NKX2.5
−677	(+) 0.801 0.732 ttgttAACTTgaattg NKX2.5
−672	(−) 0.869 0.884 AACTTga NKX2.5
−551	(+) 0.869 0.828 tgAAGTC NKX2.5
−429	(−) 0.788 0.751 ttaatgAAATGgtctg NKX2.5
−377	(+) 0.800 0.815 tttCATTTga NKX2.5
−374	(−) 0.783 0.808 CATTTga NKX2.5
−235	(+) 0.833 0.818 tgatcCACTAtacaat NKX2.5
−145	(+) 0.869 0.823 atAAGTT NKX2.5

Predicted by Transfac Professional Match 2009.3, provided by Biobase Biological Databases

Table 4 Hemocyte number differs by sex during development in wild type and *dappled*

Genotype	Sex	Number of hemocytes (±95% confidence interval)		
		Average for embryonic origin	Average for lymph gland origin	Total cells
Wild type	Male	3380 (±753)	4610 (±942)	7990 (±1237)
	Female	5413 (±1492)	6956 (±1505)	12369 (±2109)
	P, male vs. female	<0.01	<0.001	<0.01
dpld[a]	Male	6455 (±1128)	6675 (±1268)	13130 (±1716)
	Female	8628 (±1879)	8987 (±1374)	17615 (±3013)
	P, male vs. female	<0.05	<0.01	<0.01
Wild type vs. *dpld*[a]	*P*, male vs. male	<0.001	<0.01	<0.001
	P, female vs. female	<0.01	<0.05	<0.01

[a] *dpld* heterozygotes, *dpldEJL/+*

however, as the sexes differ in the number of hemocytes. For the experiment depicted in Table 4, we separated the sexes in order to demonstrate this finding and test for a sex difference in the second hematopoietic wave as well. The results are that in both wild type and *dappled* there is a significant difference between males and females in the number of hemocytes for both hematopoietic waves.

Curcumin Enhancement of the *dappled* Melanotic Phenotype

Cytochrome b5 is widely studied for its effects on drug metabolism, and conversely, various drugs are investigated for alteration of cytochrome b5 levels (El-Lakkany et al. 2004; Juvonen et al. 1992; Portoles et al. 1989; Schenkman and Jansson 1999; Sheweita 2004; Yamaori et al. 2003; Yamazaki et al. 2002). We hypothesized that modulation of the *dappled* phenotype could be used as an in vivo assay for cytochrome b5 related effects of endogenous and xenobiotic compounds. We are therefore screening compounds with a known connection to cytochrome P450s and/or anti-inflammatory and anticarcinogenic properties for enhancement or suppression of the *dappled* melanotic phenotype. Initial studies with aspirin and the corticosteroid dexamethasone are negative (data not shown), whereas curcumin, the active ingredient in the spice turmeric, has consistently shown an enhancement of the melanotic phenotype (Table 5; Fig. 7). Adult *dappled* mutants that have been raised (egg to adult) on curcumin-supplemented food have an enhanced melanotic phenotype, showing more and/or larger melanotic tumors (Table 5). The enhanced phenotype of the *dappled* mutants is already evident in the larval stages (Fig. 7). Adult *dappled* heterozygotes, however, do not develop a melanotic phenotype when raised on curcumin (Table 5). Since both the heterozygotes and heteroallelic mutants have some reduction in *dappled* transcript levels, the effects of curcumin may depend on the exact amount of cytochrome b5 protein that is produced.

Table 5 Curcumin enhances the melanotic tumor phenotype of *dappled*

Diet	Percentage of adults[a] with melanotic tumors							
	Genotype[b] (*N*)		Melanotic nodule severity (%)[c]					
	Balancer	*dpld*	1	2	3	4	5	6
Normal	0% (373)	90.3% (154)	0.6	3.9	59.1	25.3	1.3	9.7
Curcumin supplement	0% (762)	87.1% (310)	1.6	26.8	56.1	1.3	1.3	12.9

[a] Adults are progeny from crosses between *w; CyO/dpldMLB* and *w; CyO/dpldEJL*

[b] "Balancer" indicates heterozygous *CyO/dpldMLB* or *EJL*. "*dpld*" indicates transheterozygous *dpldMLB/dpldEJL*

[c] Severity decreases left to right. Category 1 is defined as multiple small nodules; 2, single large or >1 typical nodule; 3, typical nodule; 4, small nodule; 5, very small nodule; 6, no nodule

Discussion

We have identified *dappled* as a member of the conserved, ubiquitous cytochrome b5 family. The complexities of cytochrome b5 function, through its participation in electron transfer in multiple biochemical reactions, are reflected in the difficulties and limited study of its genetics in higher organisms. Characterizations in humans have shown that cytochrome b5 mutation causes methemoglobinemia, which interferes with the balance between hemoglobin and methemoglobin, thus affecting oxygenation and inflammation (Hegesh et al. 1986; Umbreit 2007). Mutation of cytochrome b5 reductase also causes methemoglobinemia and is more common (Davis et al. 2004). In addition, one case of cytochrome b5 methemoglobinemia may also have been associated with hormonal defects (Giordano et al. 1994). With the exception of a porcine mutation associated with an altered androstenone level (Lin et al. 2005), cytochrome b5 mutations in other organisms have not been identified. In mice, cytochrome b5 deletion is also lethal, but experiments with a tissue-specific knockout have allowed liver function to be addressed (Finn et al. 2008). With our studies of *dappled*, *Drosophila* now provides a tractable genetic model for the study of cytochrome b5 in blood cells and other tissues.

Hemocytes are particularly sensitive to mutation of *dappled* and show that cytochrome b5 functions to produce normal differentiation and numbers of hemocytes. Mutation of one copy of *dappled* results in a significant increase in the number of circulating hemocytes, and mutation of both copies results in the formation of melanotic nodules. The formation of *dappled* melanotic nodules is a Class 2 reaction in the classification of Watson et al. (1991), in which Class 2 represents alteration of immune cells and Class 1 represents normal immune cells responding to other cell types that are aberrant. The dominant effect on blood cell number emphasizes that wild-type levels of cytochrome b5 are needed for full normal functions. In humans, levels of cytochrome b5 have been implicated as important with respect to xenobiotic metabolism and health. Kurian et al. (2007) hypothesized that functionally significant genetic variability in cytochrome b5 occurs in individuals without clinical symptoms. They investigated processing of hydroxylamines, which are oxidative products of arylamine drugs and

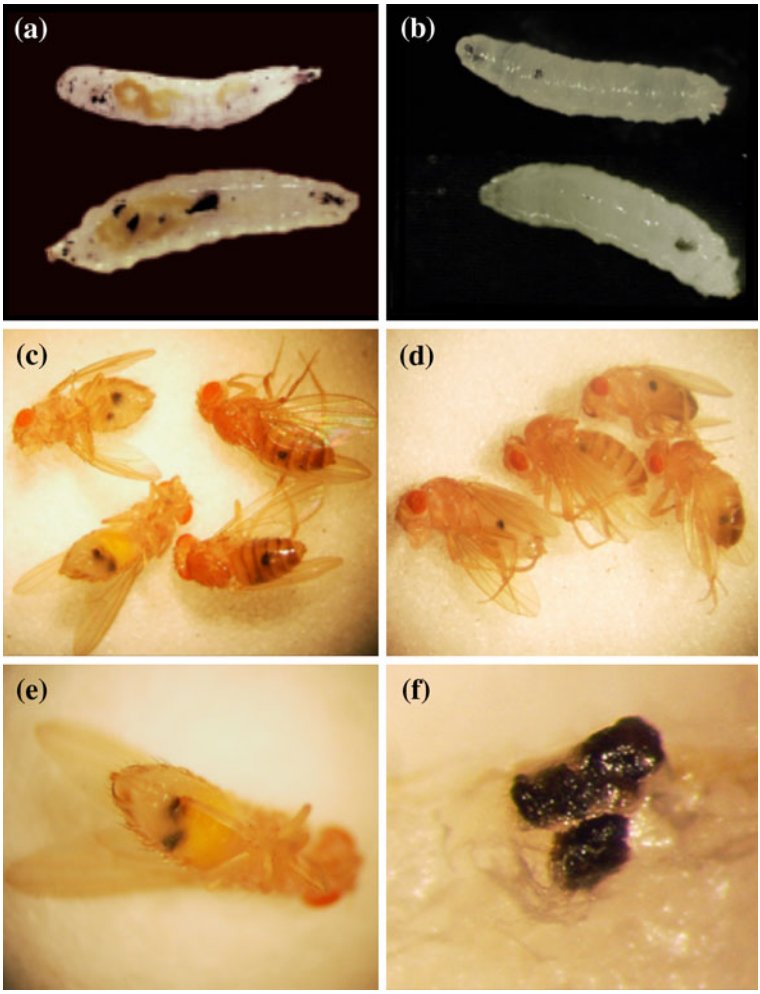

Fig. 7 Curcumin enhances the *dappled* melanotic phenotype. The *dappled* mutants on the *left* (**a**, **c**, and **e**) were raised on curcumin-supplemented food. Those on the *right* (**b** and **d**) were raised on normal food. **f** Free-floating melanotic tumors from a curcumin-fed adult. (Color figure online)

environmental carcinogens, by the mutant cytochrome b5 of one heterozygous individual and found, in vitro, altered affinity for hydroxylamine substrates and dramatically reduced cytochrome b5 expression (Kurian et al. 2007).

Expression of wild-type *dappled* in hemocytes rescued both the increase in blood cell number and the formation of melanotic nodules. The driver used was *hemese-Gal4*, which is active in circulating plasmatocytes and sessile hemocytes, but not the lymph glands (Markus et al. 2009; Zettervall et al. 2004). Interestingly, the sessile hemocytes are a newly discovered hematopoietic site and appear to be the main developmental origin of lamellocytes (Markus et al. 2009). This site of expression may be the key factor in rescuing the *dappled* melanotic nodule phenotype. Rescue

of the plasmatocyte increase in *dappled* heterozygotes is expected to result from the *hemese-Gal4* expression in circulating blood cells of the first hematopoietic wave, both because of the age of the larvae (prerelease of lymph gland cells) and because *hemese-Gal4* does not express in lymph glands. However, *dappled* also increased the number of hemocytes from the second hematopoietic wave. Hemocytes from both developmental origins, embryonic and lymph gland, require *dappled* in order to be produced in normal amounts. It is an interesting question as to whether the plasmatocyte increase in *dappled* mutants can be independently controlled in the first and second hematopoietic waves.

Does the increase in plasmatocytes indicate overproliferation or less apoptosis? Since *dappled* encodes a cytochrome b5, the obvious choice would be apoptosis rather than overproliferation. There are two problems with this choice: it is mitochondrial cytochrome b5 that is established in the canonical cytochrome C signaling apoptotic pathway, and the use of that pathway by *Drosophila* is controversial (Kornbluth and White 2005; Means and Hays 2007; Oberst et al. 2008; Ow et al. 2008). Microsomal cytochrome b5, however, has also been suggested to be involved in cytochrome C mediated apoptosis (Davydov 2001), and *Drosophila* may have its own version of apoptotic signaling that uses the *dappled* cytochrome b5. Further, blood cells are well described for their role in response to apoptotic cells, but the role of their own apoptosis in development and infection is not clear (Bruckner et al. 2004; Crozatier and Meister 2007; Lemaitre and Hoffmann 2007).

The sensitivity of hemocytes (compared with other cell types) to *dappled* mutation may result from blood cell mitotic cell division continuing during development (Crozatier and Meister 2007). There are many potential mechanisms for *dappled* to affect overproliferation, especially given the increasingly complex functions of plasmatocytes (Charroux and Royet 2009). One option consistent with cytochrome b5 function, and potentially the *dappled* effects on fertility, is that hormonal signaling of hematopoiesis is affected. In that case, however, rescue of the *dappled* blood cell phenotype would be predicted by adding back wild-type *dappled* expression in the steroid-producing ring gland (Gilbert 2004) rather than hemocytes. Alternatively, *dappled* expression could rescue a steroid-processing step in the hemocytes.

An intriguing option is that cytochrome b5 has a new, previously unidentified role in blood cell signaling pathways, for example, through JAK/Stat or JNK. More specifically, a candidate to mediate the effects of *dappled* is TNF-receptor associated factor 6 (Traf6), which is predicted from protein studies to interact with cytochrome b5 (Drosophila Interactions Database, http://www.droidb.org). Among its activities, Traf6 is an upstream activator of nuclear factor-kappaB, and mutants are immune response defective (Cha et al. 2003; Wu and Arron 2003). Cytochrome b5 involvement with Traf6 is a testable hypothesis through analysis of genetic interaction between *dappled* and *Traf6* mutants.

Our results with curcumin demonstrate that screening of endogenous and xenobiotic compounds for effects on the *dappled* phenotypes can be done as an in vivo approach for analysis of cytochrome b5. Curcumin is widely studied for its potential as a nutritional pharmaceutical (Duvoix et al. 2005; Jagetia and Aggarwal 2007). Among its many activities, which are mainly anti-inflammatory and

anticancer, curcumin inhibits cytochrome P450s, down-regulates NFkB, and affects apoptosis and the cell cycle (Alpers 2008; Appiah-Opong et al. 2007; Kwon et al. 2007; Sa and Das 2008). Curcumin enhanced the melanotic phenotype of *dappled* mutants. This effect of curcumin is consistent with NFkB involvement, but there are other alternatives, in particular, cytochrome P450 processing in the fat body.

Mutation of *dappled* showed similar effects in females and males, but an unexpected consequence of our studies is the finding that females and males differ significantly in the number of circulating blood cells. In humans, a difference between the sexes in the numbers of blood cell types is well established, but the effects are unclear (Blum and Pabst 2007; Huppert et al. 1998; Reichert et al. 1991). In both humans and *Drosophila*, there are differences in the immune responses between the sexes that affect health (Dale et al. 2006; Marriott and Huet-Hudson 2006; Shames 2002; Taylor and Kimbrell 2007; Ye et al. 2009; Zandman-Goddard et al. 2007), and the blood cell number differential may be part of mediating the immune response differences. Recently, *Drosophila* hemocytes have been shown to directly signal immune induction of the fat body, and a reduced number of hemocytes resulted in less immune induction (Shia et al. 2009). In *Drosophila*, the increase in hemocytes of *dappled* and females may result in higher immune induction, which may or may not provide a healthier condition. For example, human females typically have a more robust immune response but also a higher incidence of autoimmune disorders (Dale et al. 2006; Zandman-Goddard et al. 2007).

Cytochrome b5 expression is critical for survival, and its importance to hemocytes and additional tissues is emphasized by the finding that ubiquitous, but not hemocyte, expression of wild-type *dappled* rescued lethality. The functions of cytochrome b5 are open for testing in a wide range of tissue types using *dappled*. In all respects, genetic interaction and drug screens for suppressors and enhancers of *dappled* will be useful ways to discern the many roles of cytochrome b5 function.

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