The EGF Repeat-Specific O-GlcNAc-Transferase Eogt Interacts with Notch Signaling and Pyrimidine Metabolism Pathways in *Drosophila*

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**Abstract**

The O-GlcNAc transferase Eogt modifies EGF repeats in proteins that transit the secretory pathway, including Dumpy and Notch. In this paper, we show that the Notch ligands Delta and Serrate are also substrates of Eogt, that mutation of a putative UDP-GlcNAc binding DXD motif greatly reduces enzyme activity, and that Eogt and the cytoplasmic O-GlcNAc transferase Ogt have distinct substrates in *Drosophila* larvae. Loss of Eogt is larval lethal and disrupts Dumpy functions, but does not obviously perturb Notch signaling. To identify novel genetic interactions with eogt, we investigated dominant modification of wing blister formation caused by knock-down of eogt. Unexpectedly, heterozygosity for several members of the canonical Notch signaling pathway suppressed wing blister formation. And importantly, extensive genetic interactions with mutants in pyrimidine metabolism were identified. Removal of pyrimidine synthesis alleles suppressed wing blister formation, while removal of uracil catabolism alleles was synthetic lethal with eogt knock-down. Therefore, Eogt may regulate protein functions by O-GlcNAc modification of their EGF repeats, and cellular metabolism by affecting pyrimidine synthesis and catabolism. We propose that eogt knock-down in the wing leads to metabolic and signaling perturbations that increase cytosolic uracil levels, thereby causing wing blister formation.

**Introduction**

Glycosylation is the most common post-translational modification of proteins. Different classes of glycans or individual sugars within a glycan have been shown to regulate cell-cell recognition, cell migration, cell proliferation, the binding of pathogenic viruses and bacteria, growth factor and cytokine signaling, and Notch signaling [1]. Most sugars are transferred to proteins within the secretory pathway. For example, Fuc, Man, Glc, Xyl, GalNAc and GlcNAc may be transferred to Ser or Thr, and subsequently substituted with additional sugars to generate O-glycans. O-glycans, as well as single sugar residues, may confer a variety of functions on glycoproteins. For example, in *Drosophila*, Fringe adds a GlcNAc to O-Fuc on Notch (N) epidermal growth factor-like (EGF) repeats, thereby altering the binding of Notch ligands Delta (Dl) and Serrate (Ser), and up- or down-regulating Notch signaling, respectively [2,3]. This is critical for controlling Notch signaling during boundary formation in the wing imaginal disc, in leg development, and in the eye [4,5]. Another important protein modification is the addition of O-GlcNAc to cytoplasmic and nuclear proteins, the only glycosylation reaction known to occur outside the secretory pathway in vertebrates [6]. In *Drosophila*, cytosolic O-GlcNAc regulates the activities of cytoskeletal proteins, transcription factors and enzymes. It also links metabolism to epigenetics through histone modification [7], and is an efficient UDP-GlcNAc/nutrient sensor [8]. *Drosophila* Ogt is encoded by the *super sexcomb (sxc)* gene, and sxc null mutants are late pupal lethal [9].

The addition of O-GlcNAc to proteins sequenced within the secretory pathway was first reported as a modification of *Drosophila* Notch EGF repeat 20 (EGF20) [10]. The enzyme that catalyzes this transfer was subsequently identified as an EGF repeat-specific O-GlcNAc transferase and termed Eogt [11]. Eogt is resident in the endoplasmic reticulum (ER) [11] and generates β-linked GlcNAc on Ser or Thr of EGF repeats in the consensus sequence C_{2}XXGXT/SGXXG_{6} [12]. *Drosophila* Notch has 17 EGF repeats with this consensus site. Dumpy (Dp), a transmembrane protein largely in the extracellular matrix without a clear mammalian homologue [13], has 86 of 300 EGF repeats [14]. Dp mutants die at around larval stage 2, and phenocopy lethal dmp mutants [11]. Loss of function mutations of *dmp* or *eogt* in the adult wing result in blistering [11,14]. Several other loci are known to genetically interact with *dmp* [15]. For example, *dmp* cooperates with genes encoding the Zona

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Pellucida domain proteins papillote (pot) and pinio (pin) in maintaining the structural integrity of the aECM, by mediating its attachment to the epidermis in the pupal wing, and in anchoring the aECM to the transalar array, the cytoskeletal and junctional support important in integrin-mediated basal adhesion [16]. Furthermore, dp mutants genetically interact with mutants of pyrimidine metabolism [17–19].

To identify novel genes that interact with eogt, we performed dominant genetic interaction assays of an eogt RNAi-induced wing phenotype. Unexpectedly, even though eogt mutants do not show an X phenotype [11], reduction of X activity by removing one allele of various pathway members, including the transcription regulators Suppressor of Hairless (Su(H)) and mastermind (man), strongly suppressed the wing blister phenotype caused by loss of eogt. In addition, loss of one allele of genes encoding enzymes of the pyrimidine pathway that produce uridine nucleosides and UDP-GlcNAc, suppressed the eogt RNAi phenotype, while loss of alleles of uracil catabolic enzymes enhanced it. We propose that uracil toxicity, previously implicated in causing wing blisters in dp mutants [11], is a likely basis of the blistering phenotype observed in the absence of eogt.

Results

Human EOGT has a DXD Motif Important for Catalysis

We previously identified Drosophila CG9967 as a putative glycosyltransferase gene required for viability of Drosophila melanogaster [20]. This gene was recently shown to encode Eogt [11], and to be conserved in mouse [21]. We now show that one of the splice forms of the human gene C30sf64 cloned from HEK 293T cells (GenBank accession number KC347596.1), is an active human EOGT homologue. To establish that it has EOGT activity, human EOGT homologue. To establish that it has EOGT activity, to establish that it has EOGT activity, we transfected together with either a plasmid containing Notch (EGF repeats 3, 5, 9, 11–17, 19, 20, 22, 25–28). N(EGF1-20)-AP was transfected into S2 cells treated with eogt dsRNA. Compared to wild-type, mutant EOGT(AVA) was considerably less efficient at generating an O-GlcNAc signal on co-transfected N(EGF1-20)-AP, even though expression of the mutated protein was reproducibly much higher (Fig. 1C). The low residual activity of the mutant indicates that the DXD motif is not absolutely required for EOGT function, consistent with the observation that low levels of Drosophila Eogt activity were detected in vitro in the absence of divalent cations [11].

Several protein sequences in Drosophila other than Notch contain the EOGT recognition consensus sequence C-XXGTX/ SGXXC in EGF repeats [11,12]. For example, Notch ligands Dl and Serr contain five and seven perfect matches, respectively (Fig. 1D and 1E), and their ability to be modified with O-GlcNAc by Eogt in S2 cells was tested. Dl was readily modified with O-GlcNAc by endogenous Eogt in S2 cells, and the modification was increased by co-transfection with human EOGT. While O-GlcNAc was not detected on Ser exposed to endogenous Eogt in S2 cells, co-expression of human EOGT resulted in O-GlcNAcylation of Ser (Fig. 1F).

Drosophila Eogt has Predominantly High Molecular Weight Targets in Larvae

To assess substrates of Eogt in vivo, an eogt mutant was made using imprecise excision of a nearby P element and mapped by PCR and sequencing. Excision eogtex10 lacks the first 0.8 kb of exon 1, which includes the start codon (Fig. 2A). Using a GFP-marked second chromosome balancer, the eogtex10 allele was shown to be lethal at larval instar L2 (Fig. 2B), a stage at which the larvae can remain for up to 48 hours before dying, with a few escapers reaching L3. All surviving adults were CyO (n = 89), showing that no eogtex10 homoyzogotes survived. Homozygous eogtex10 mutants were rescued to adulthood upon ubiquitous expression of Drosophila UAS-eogt under the control of tubulin-Gal4 (ub-Gal4)>UAS-eogt, from here on termed tub>eogt, Fig. 2C). Rescue attempts with heat-shock-Gal4- or actin-Gal4-driven UAS-eogt were unsuccessful (not shown). Furthermore, the human ortholog EOGT driven by the tubulin promoter (ub-EOGT) also rescued Drosophila eogtex10 lethality (Fig. 2C and 2D). Rescued animals showed no visible phenotypes. In contrast, mouse Ago61 driven by the tubulin promoter (ub-Ago61) did not rescue eogtex10 (Fig. 2C), even though it was robustly expressed (Fig. 2D).

In order to analyze eogtex10 mutant phenotypes and to compare them with dp mutants [26], eogtex10 and a lethal allele of dp (dpb) were recombined onto Frt40A chromosomes, and the consequences of loss of eogt or dp in clones of homozygous mutant cells induced by Ubx-Flp [27] were analyzed. Mutant clones of eogtex10 or dpb in the thorax caused the formation of vortices in both cases, whereas none were observed in control clones (Fig. 3A–3C).

Consistently, knock-down of eogt using RNAi under control of the apterous promoter (ap-Gal4) led to disorganization of thoracic bristles and a vortex phenotype (not shown), as well as a comma phenotype in ap-Gal4>eogt(1)RNAi (Fig. 3E). Targeted knock-down of dp in the thorax by RNAi (ap-Gal4>dpb(1)RNAi) also caused vortices to develop (Fig. 3F). However, the penetrance of this dp vortex phenotype was weak at 31°C, even when a dicer2 transgene (UAS-
dcr2) was included to increase knock-down efficiency. Importantly, unmarked mutant eogt clones in the wing resulted in a severely deformed wing with blisters, similar to the phenotype observed in dplv clones (Fig. 3G–3I).

To assess Eogt modification of Dp, we used a dp-targeted RNAi construct that caused the expected dpshape (dp) phenotype when expressed at 18°C in the wing under en-Gal4 (not shown). RNAi knock-down of dp under the strong and ubiquitously expressed tub-Gal4 promoter at 31°C was late pupal lethal, precluding analysis at this stage. At 2nd instar, larval lysates showed no difference in O-GlcNAc signal between dp knock-down and control (not shown), presumably reflecting maternal contribution of dp. However, in early pupal control lysates (GFP-positive), a high molecular weight O-GlcNAc signal was observed that was absent from dp knock-down pupal lysates, suggesting that Dp is a major target of Eogt (Fig. 4A). An additional O-GlcNAc signal of ~75 kDa that was detected at stages beyond late L3, served as a loading control (Fig. 4A).

In order to identify general targets of Eogt in larval extracts, it was important to differentiate between targets of Ogt, which...
transfers O-GlcNAc to proteins of the cytoplasm and nucleus, and Eogt, which acts only on proteins that traverse the secretory pathway. We therefore recombined the Ogt mutant allele sxc\(^6\) [9] and the eog\(^{ex10}\) mutant allele to obtain double mutants, for comparisons with sxc\(^6\) or eog\(^{ex10}\) mutants and wild-type. Few O-GlcNAc-positive bands were detected in lysates of control or mutant L2 larvae under conditions optimized for detection of O-GlcNAc on EGF repeats (Fig. 4B). However, at molecular weights >250 kDa, control extracts gave a broad smear that aggregated at the interface of the stacking and running gels. There was a small reduction of O-GlcNAc signal in zygotic eog\(^{ex10}\) mutants, and a very strong reduction of the O-GlcNAc signal in zygotic eog\(^{ex10}\) double mutant lysates. The signal remaining in double mutant lysates is probably due to O-GlcNAc added by maternally provided Eogt or Ogt. Therefore, most O-GlcNAcylated proteins of >250 kDa, including dp, are substrates of Eogt.

**Eogt Knock-down is Sensitive to Dominant Modification**

To identify additional pathways affected by Eogt, we investigated RNAi-mediated eogt knock-down phenotypes for use in genetic interaction analyses. Ubiquitous expression of two overlapping RNAi lines VDRC/44572 and Shigen/R-3 (Fig. 2A) under the control of the tubulin promoter at 30°C, was late pupal lethal. When RNAi expression was driven by act-Gal4 at 30°C, 94% of the predicted VDRC/44572 animals hatched (n = 248), 57% of VDRC/44572 animals with a UAS-dcr2 transgene hatched (n = 136), and no Shigen/R-3 animals (n = 162) were found. Knock-down of eogt using en-Gal4 expressed in the posterior compartment, or ap-Gal4 expressed in the dorsal compartment, induced blistering of the wing.

To establish genetic interaction assays, we tested whether the wing blister phenotype of eogt provided a sensitive baseline to identify dominant modifiers. An en-Gal4-driven RNAi knock-down with Dicer (en-Gal4, UAS-VDRC44572, UAS-dcr2; from here on designated en\(\geq\)eogt\(^{B}\)), caused the formation of wing blisters specifically within the posterior compartment. No blisters were seen in the anterior compartment. The phenotype was temperature sensitive with virtually complete penetrance at 27.0°C, while hardly any blisters were found at 22.5°C (Fig. 5A and Table 1). Importantly, an eogt\(^{ex10}\) heterozygous background enhanced the frequency of flies with wing blisters up to 30% at 22.5°C (Fig. 5C and Table 1), while co-expression of human EOGT completely reverted the blister phenotype at 27°C, even in the absence of one gene dose of eogt (Fig. 5F and Table 1). Co-expression of Ago61 did not suppress blisters (Fig. 5D), consistent with biochemical data (Fig. 1).

Alleles of known dp interactors, as well as alleles encoding EGF repeat-containing proteins with blister phenotypes, were investigated for their ability to dominantly enhance or suppress the wing blister phenotype caused by en\(\geq\)eogt\(^{B}\). dp mutants are classified
according to three phenotypic classes: \( dp^{\text{null}} \) (\( dp^{\text{pl}} \)) mutants carry null alleles and are homozygous lethal, \( dp^{\text{oblique}} \) (\( dp^{\text{po}} \)) alleles show an oblique wing, and \( dp^{\text{vortex}} \) (\( dp^{\text{pv}} \)) alleles show vortices or commata of macrochaete on the thorax \[26,28\]. As expected \[11\], we observed an enhancement with lethal \( dp \) alleles (\( dp^{\text{vR}} \) and \( dp^{\text{l}} \)) (Fig. 5E and Table 1). However \( dp^{\text{v}} \), a non-lethal vortex class allele of \( dp \), in conjunction with the \( dp^{\text{v}} \) enhancer \( \zeta \), did not interact (Table 1).

Wing blisters may arise due to separation of dorsal and ventral wing surfaces, and we therefore tested \( \text{mysopheroid} \) (\( \text{mys} \)), a beta integrin linking the ventral and dorsal epithelia of the fly wing \[29\]. \( \text{mys}^{1} \) did not interact with \( \text{eogt} \) in the wing (Table 1), and no integrin-like phenotypes were observed during embryogenesis or larval development of \( \text{eogt}^{ex10} \) mutants. Nevertheless, removal of one copy of \( \text{wingblister} \) (\( \text{wbl} \)), that encodes the ECM component laminin \( \alpha \) chain \[30\], dominantly increased blister frequency (Table 1). Interestingly, laminin \( \alpha \) has one EGF O-GlcNAc consensus site at T1299 and also contains a putative lectin domain, similar to the GlcNAc binding domain of the mammalian intermediate filaments desmin and vimentin \[31\]. Therefore, loss of O-GlcNAc due to \( \text{eogt} \) knock-down might lead to altered adhesive properties of laminins, and thus promote blistering.

Surprisingly, \( \text{pot} \) and \( \text{pio} \), two well-documented \( dp \) genetic interactors implicated in the formation of the aECM \[15,16\], gave no discernible interaction with \( en>\text{eogt}^{IR} \) (Table 1). Neither misexpression of the EGF domains of the apical domain protein Crumbs (\( \text{Crb} \)) \[32\], which carries five O-GlcNAc consensus sites (EGF repeats 8, 10, 11, 13, and 26), nor absence of \( \text{crb} \) \[33\] are reported to result in a wing blister phenotype. Nevertheless, we detected weak but significant suppression of the \( en>\text{eogt}^{IR} \) blister phenotype by loss of a \( \text{crb} \) allele (Table 1; see Discussion).

### Reduced Notch Signaling Suppresses Wing Blisters Due to \( \text{eogt} \) Knock-down

EGF repeats in N, DI and Ser are substrates of EOGT (Figs. 1A, 1C and 1E). In addition, several independent \( dp \) alleles interact with the \( \gamma \)-secretase Presenilin (\( \text{psn} \)) \[34\], a crucial component of Notch pathway activation. However, embryos lacking both zygotic and maternal \( \text{eogt} \) do not show neurogenic phenotypes character-
istic of N mutants ([11]; and this work). In addition whereas overexpression of Ofut1, that transfers O-fucose to Notch EGF repeats, causes dramatic N phenotypes [35], tubulin-driven or restricted overexpression of eogt did not appear to affect Notch signaling in the wing or eye (not shown). However, effects of glycan removal on Notch signaling can be subtle, as observed in rumi mutants [36]. We therefore investigated interactions with Notch pathway mutants. Genetic interactions were detected with several mutant alleles of N including NotchSplit (NSpl-1) with a point mutation in EGF14 [37,38] that leads to an additional O-fucose site [39], NotchAbruptex alleles (NAx16, N AxE2, N Ax9B2) [40] which harbor mutations in EGF24 or EGF29, and the loss-of-function N55E11 allele. Each N mutant efficiently suppressed the blister phenotype (Table 2). The dominant L5 vein phenotype of NAXE2 was not modified by knock-down of eogt. In addition, removal of one copy of several other Notch pathway members, including the ligands Ser and Dl, the transcriptional repressor Suf(H), and the transcriptional co-activator nam, also suppressed the en>eogtIR-induced wing blister phenotype by about 30% to 50%, indicating that Notch signaling promotes blistering due to loss of eogt (Table 2).

Importantly, deficiencies uncovering these loci also suppressed wing blisters to corresponding degrees (Table 2).

To verify that the suppression caused by N55E11 was indeed due to a reduction of Notch activity, we attempted to revert suppression by adding back a dose of N from a genomic DNA construct integrated in the attP2 site [41]. This construct in the N55E11/+; en>eogtIR background significantly reverted suppression of the blister phenotype caused by removal of one N allele (Figs. 6 and 7B). The reversion was not complete, however, presumably because the attP2 control chromosome alone gave significant suppression (Fig. 6). Interestingly, an additional dose of N did not enhance the blister phenotype (Fig. 6), regardless of whether a Notch duplication Dp(1;2)51b or a Notch genomic transgene was used, but rather suppressed blister formation. This may be because flies with an additional N allele exhibit reduced Notch signaling in certain cell types (reviewed in [42]).

Mutations in Pyrimidine Synthesis and Uracil Catabolism Modulate eogtIR-Induced Wing Blisters

Previous studies have shown that mutant dp alleles and the pyrimidine biosynthetic pathway functionally interact. Pyrimidine
Eogt Interacts with Notch and Pyrimidine Pathways

Figure 5. Temperature-sensitive wing blister assay for eogt interactors. Wings of flies raised at 22.5°C (A, C, E) or 27°C (B, D, F). en>eogtIR wings were normal at 22.5°C (A) but blistered in the posterior compartment at 27°C (B). At the low temperature, blistering was induced when one gene dose of eogt (eogtx10/) or dp (dpolvR) was removed (C and E, respectively). While blistering at the higher temperature was not affected by co-expression of Ago61 (D), it was suppressed by co-expression of human EOGT (F), even when one gene dose of eogt had been removed.

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synthesis inhibitors fed to dp* mutant flies revert the oblique phenotype [43], and dp wing phenotypes are strongly enhanced in homozygous mutants in the pyrimidine catabolic enzyme suppressor of rudimentary (su[r]) [19]. In addition several pyrimidine biosynthetic activities are increased in dp mutant larvae [17,18]. We therefore tested mutations in enzymes of pyrimidine metabolism for modification of the en>eogtIR wing blister phenotype (Fig. 7 and Table 3). Pyrimidines are synthesized from glutamine (Gln) leading to the production of uridyl-derivatives including UTP, which is used in the synthesis of UDP-GlcNAc, the donor substrate of Eogt (Figs. 8A and S1). De novo biosynthesis is initiated by the multi-functional enzyme Rudimentary (R), followed by dihydroorotate dehydrogenase (Dhod), and a third enzyme Rudimentary-like (R-l), that encodes orotidine-5’-phosphate decarboxylase activity and generates UMP. Alleles of r, Dhod and r-l robustly suppressed the en>eogtIR-induced wing blister phenotype at 27°C (Fig. 7E for Dhod* and Table 3). When en>eogtIR flies were maintained at 31°C to further increase the expression of the RNAi transgene, r still suppressed the wing blister phenotype of en>eogtIR animals, albeit to a lesser extent, corroborating the dosage sensitivity of the interaction (Table 3). Importantly, we were able to partially revert the suppression of en>eogtIR by r in In(1)R106/+ animals (from 0% to 39% blisters) by rescuing r with a transgene encoding the UTP feedback-insensitive, thus hyperactive allele, Rsu(b) [44,45] (Table 3 and Fig. 7F). The wild-type R rescue construct was probably expressed at insufficient levels to revert the suppression [J. Rawls, personal communication]. In addition, enhancer of rudimentary (e(r)), originally identified as a mutation that enhances the wing phenotype of r mutants [46], robustly suppressed the en>eogtIR phenotype (Table 3). Therefore, mutations that cause a reduction in the synthesis of UMP suppressed the wing blister phenotype induced by knock-down of eogt.

Since a reduction of pyrimidine neo-synthesis suppressed the en>eogtIR wing phenotype, we hypothesized that a reduction of pyrimidine degradation should enhance it. Pyrimidines are converted to uracil that is further metabolized to β-alanine (Fig. S1). We therefore tested loss-of-function alleles corresponding to pyrimidine catabolic enzymes as interactors. Dihydropyrimidine dehydrogenase, which metabolizes uracil to dihydrouracil and is encoded by suppressor of rudimentary (su[r]), has been shown to
### Table 1. Specificity of $en>eogt^{IR}$ Interactions.

| Allele | % Flies with Blisters (22.5°C) | % Flies with Blisters (27°C) |
|--------|-------------------------------|-----------------------------|
| $w^{1118}$ | 0 (0/161) | 100 (212/212)  |
| $w^{1118}$ | 0 (0/125) | 95 (300/316)  |
| P-BG00673 | 0 (0/55) | 100 (39/39)   |
| $eogt^{IR}$ | 16 (23/144)* | 99 (70/71)    |
| $eogt^{IR}$ | 30 (32/108)* | 95 (174/183)  |
| $eogt^{IR}$; tub-EOGT | 0 (0/104) | 0 (0/70)*     |
| $eogt^{IR}$; tub-EOGT | 0 (0/164) | 0 (0/180)*    |
| $eogt^{IR}$; tub-Ago61 | 30 (11/118)* | 100 (82/82)  |
| $dpolvR$ | 58 (51/88)* | 100 (82/82)  |
| $dpolvR$ | 34 (38/113)* | 100 (157/157) |
| $dpolvR$ | 22 (35/160)* | 99 (134/135) |
| $pio2R-1$ | 0 (0/45) | 100 (49/49)   |
| $pioMB03570$ | 0 (0/31) | 100 (34/34)   |
| $pio2R-1$ | 0 (0/113) | 85 (134/135)  |
| $pio2R-1$; ed($pio2R-1$) | 12 (11/91)* | 100 (83/83)  |

$en>eogt^{IR}$ flies were crossed with indicated alleles. Three strains initially tested gave similar results so the data presented are from a single, triple recombinant $en>eogt^{IR}$ strain. The percentage of flies with wing blisters (n animals with blisters/total flies of appropriate genotype) is indicated. *Baseline of two independent experiments; data for each allele were compared to appropriate control. **$p<0.0001$ or $p<0.02$ by two-proportion Z-test in comparison to control.

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### Table 2. Notch Pathway Mutants Dominantly Suppress the Wing Blister Phenotype Induced by $en>eogt^{IR}$.

| Allele | % Flies with Blisters (22.5°C) | % Flies with Blisters (27°C) |
|--------|-------------------------------|-----------------------------|
| $NAx16$ | 0 (0/155) | 52 (57/110)*  |
| $NAxE2$ | 1 (1/131) | 13 (26/197)*  |
| $NAx9B2$ | 0 (0/132) | 35 (34/98)*  |
| $NSpl-1$ | 0 (0/88) | 40 (54/135)*  |
| $Df(1)N-264-105$ | 0 (0/78) | 41 (57/138)*  |
| $N55E11$ | 0 (0/93) | 37 (38/102)*  |
| $Df(3R)ED6237$ (Dl) | 0 (0/80) | 41 (24/59)*  |
| $Df(3R)ED6237$ (Dl) | 0 (0/44) | 100 (31/31)   |
| $Df(3R)ED6237$ (Dl) | 0 (0/71) | 48 (10/21)*  |
| $Df(3R)ED6237$ (Dl) | 0 (0/98) | 44 (45/103)*  |
| $Df(3R)ED6237$ (Dl) | 6 (7/124) | 70 (67/95)*  |
| $Df(3R)ED6237$ (Dl) | 0 (0/92) | 68 (92/135)*  |
| $Df(3R)ED6237$ (Dl) | 0 (0/120) | 76 (93/123)*  |
| $Df(3R)ED6237$ (Dl) | 0 (0/88) | 69 (81/118)*  |
| $Df(3R)ED6237$ (Dl) | 0 (0/73) | 54 (72/134)*  |
| $Pnh^{1}$ | 0 (0/165) | 40 (25/62)*  |
| $Pnh^{1}$ | 0 (0/105) | 52 (53/101)*  |
| $Df(2L)TES358C-4$ (SuH) | 2 (2/113) | 80 (96/120)*  |
| $SuH^{2}$ | 1 (1/148) | 73 (55/75)*  |
| $Df(2R)BC3583$ (man) | 1 (1/130) | 43 (21/49)*  |
| $man^{2}$ | 0 (0/137) | 45 (52/116)*  |

$en>eogt^{IR}$ flies were crossed with indicated alleles. The percentage of flies with wing blisters (n animals with blisters/total flies of appropriate genotype) is indicated. +Two independent experiments; data for each allele were compared to appropriate control. *$p<0.0001$ by two-proportion Z-test in comparison to control.

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aggravate uracil toxicity in wild-type larvae and is highly 5-fluorouridine (5-FU) sensitive [47,48]. Strikingly, and similar to dp; su(r) double mutants [19], removal of one gene dose of su(r) in the presence of en>eogtIR was lethal at 22.5°C, even though su(r) mutants are homozygous viable. Crosses of su(r) with control chromosomes lacking either the eogt dsRNA hairpin or the en-Gal4 driver hatched, indicating that the observed synthetic lethality of en>eogtIR with su(r) was dependent on the expression of the eogt RNAi construct. We also examined the subsequent step, the conversion of dihydrouracil to 3-ureidopropionate by dihydropyrimidinase encoded by Collapsin Response Mediator Protein (CRMP; Fig. S1), using the small deficiency Df(3R)noi-B, which deletes CRMP along with other genes [49,50]. Again, this deficiency was synthetic lethal with en>eogtIR. Further downstream in the pathway of uracil catabolism, removal of one allele of pyd3 led to a few flies with wing blisters at 22.5°C (Fig. 7G), as well as enhancement of the wing blister phenotype at 27°C (Table 3). Conversely, overexpression of wild-type pyd3 from a transgene suppressed the wing blister phenotype of en>eogtIR from 100% to 51% at 27°C, reflecting an increased metabolic flux towards β-alanine (Table 3). In Drosophila, β-alanine is also synthesized through decarboxylation of aspartate [51]. The black (b') mutation is a null allele for aspartate 1-decarboxylase (Fig. S1) [52], rendering pyrimidines the lone source of β-alanine in a b' background. We hypothesized that in the absence of b', more uracil would be metabolized to β-alanine, reducing uracil levels that might cause blisters. Consistent with this, the b' mutant suppressed the blister phenotype of en>eogtIR at 27°C (Table 3). The b' allele was also present in the su(r) and pyd3 mutant stocks available, but it did not prevent the synthetic lethality of su(r) with en>eogtIR, consistent with the fact that mutants in pyrimidine catabolism are epistatic to the suppression of b' [48].

Discussion

In this paper we identify a transcript of the human EGF-specific O-GlcNAc transferase EOGT that encodes O-GlcNAc transferase activity (Genbank KC347596.1). It is identical in sequence (527 aa) to the conceptual protein deduced from the proposed C3orf64 transcript b in AceView [53]. We show that transfection of this EOGT cDNA causes O-GlcNAcylation of Drosphila N, Dl and Ser EGF repeats; it also requires a conserved DXD motif for optimal activity, and it is primarily responsible for the transfer of O-GlcNAc to high molecular weight proteins, including Dp, in Drosophila larvae. Another cDNA in Genbank (NM_173654.1; [21]) lacks an internal segment that contains the DYD motif, and is therefore predicted to have low or no activity. In vivo, the human EOGT cDNA fully rescued homozygotes of a new P-element excision mutation of eogt (eogtex10). Both eogt RNAi knock-down and mutant clones exhibited blistered wing and vortex phenotypes, similar to the vortex class of dp mutations (dpv). The hallmark of the removal of eogt in the posterior wing in en>eogtIR flies was the temperature-dependent development of blisters, with essentially 100% frequency at 27°C (Table 1 and Fig. 5; [11]). Importantly, wing blister formation was enhanced at 22.5°C by the eogtIR mutation, and fully rescued at 27°C by the human EOGT transgene, but not a transgene encoding the related mouse gene Ago61 (Table 1).
To investigate the potential origin of \textit{en>\textit{eogt}}-induced wing blisters, we used a candidate genetic interaction strategy. The reduction in O-GlcNAc transfer caused by loss of \textit{eogt} physically affects EGF-repeat containing proteins of the secretory pathway. Thus, we examined genetic interactions with mutants of \textit{Notch}, \textit{crumbs}, \textit{dumpy} and \textit{wingblister} (Laminin \textit{\alpha}), which all contain EGF repeats with the consensus site for recognition by \textit{Eogt}. We also investigated integrins known to cause wing blisters. If a reduction in O-GlcNAc caused by \textit{en>\textit{eogt}} affects function due to loss of \textit{eogt} activity, stability, or altered localization of a membrane glycoprotein, the development of a wing blister may be a direct consequence. In that case, the additional loss of one dose of that glycoprotein should further reduce activity, and thus enhance blister formation due to \textit{en>\textit{eogt}} knockdown. If, on the other hand, integrins are involved, the additional loss of one dose of a membrane glycoprotein may not further reduce activity, and thus not enhance blister formation.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure7}
\caption{Wing blister phenotypes of \textit{en>\textit{eogt}} interactions with \textit{N} and pyrimidine metabolism mutant alleles.} \label{fig:7}
\end{figure}

(D) Example of a blistered wing of a fly overexpressing \textit{pyd3} in an \textit{en>\textit{eogt}}; \textit{pyd3}^{Lb10+/+} background.

\begin{flushright}
doi:10.1371/journal.pone.0062835.g007
\end{flushright}
Figure 8. Interactions between Eogt, pyrimidine metabolism and Notch signaling in the posterior wing. (A) The diagram shows the Eogt-catalyzed addition of O-GlcNAc from UDP-GlcNAc (UDP-blue square) to EGF-containing proteins Dp and N, and key steps of the pyrimidine synthesis and catabolism pathways in a wild-type wing cell. Repression of pyrimidine neo-synthesis by Dp was shown biochemically in dp mutant larvae [17,18]. Protein products of genes tested for interaction with en>eogtIR flies are indicated. (B) In en>eogtIR wings, reduced Eogt leads to loss of O-GlcNAc on Dp, N, Dl, Ser and other EGF-containing substrates. Genetic interactions with mutant alleles that resulted in suppression of wing blisters at 27°C are in green, while those that caused enhancement of wing blisters at 22.5°C are in magenta. Enhanced activity of initial enzymes in pyrimidine synthesis due to reduced Dp function is indicated by gray lines. The combined data suggest the unifying model that an increase in cytoplasmic uracil concentration is a likely cause of wing blisters when Eogt levels are reduced. The loss of O-GlcNAc from Dp and N may also contribute to the wing blister phenotype by reducing signals that influence pyrimidine biosynthesis.

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Table 3. Mutants in Pyrimidine Metabolism Interact with en>eogtIR.

| Mutant allele | % Flies with Blisters (22.5°C) | % Flies with Blisters (27°C) |
|---------------|---------------------------------|-------------------------------|
| eogtIR/gd266  | 0 (0/111)                       | 41 (31/76)*                   |
| eogtIR         | 0 (0/121)                       | 1 (1/114)*                    |
| eogtIR         | 54 (52/96)*                     |                               |
| hs[wg1]; P(1R) | 0 (0/87)                        | 1 (1/99)*                     |
| hs[wg1]; P(1G/ubd) | 0 (0/124) | 39 (41/104)*                 |
| DhoD3(0-775)  | 0 (0/70)                        | 0 (0/81)*                     |
| r-f2          | 73 (84/115)*                    |                               |
| su(l)/+; b1    | 2 (2/95)*                       |                               |
| Df(3R)ru0192 (CRMP) | synth. lethal | synth. lethal |
| b1; pyd3185    | 3.5 (3/85)                      | 100 (98/98)*                  |
| b1; pyd3185    | 0 (0/77)                        | 51 (67/132)*                  |
| b1            | 4 (3/85)*                       |                               |

en>eogtIR flies were crossed with indicated alleles. The percentage of flies with wing blisters (n animals with blisters/total flies of appropriate genotype) is indicated.

*p<0.0001 by two-proportion Z-test in comparison to control.

Cross was maintained at 31°C.

*100% represents a significant increase (p<0.02) from the appropriate experimental series that had 95% baseline wing blisters (Table 1).

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hand, activity is not altered by the loss of O-GlcNAc, the consequences of removal of one allele of a substrate would be independent of O-GlcNAc status. Alternatively, the lower amount of protein might not be limiting, and thus no dosage sensitivity would be observed. If protein function was enhanced by the loss of O-GlcNAc, leading to promotion of blister formation, removal of one allele should suppress blister formation.

In the case of Dumpy, a very large protein of the aECM [13], reduced O-GlcNAc on Dp EGF repeats might not be limiting, and thus no dosage sensitivity would be observed. If protein function was enhanced by the loss of O-GlcNAc, leading to promotion of blister formation, removal of one allele should suppress blister formation.

In the case of Notch, suppression of the en>eogtIR wing blister phenotype was observed in a Ns(3)ExE1/+, Ns(3)ExE2/+, or Ns(3)ExE3/+ background (Table 2). Importantly, the suppression observed in Ns(3)ExE1/+ heterozygotes was largely reversed by the addition of a genomic copy of Ns, the product of which would carry little if any O-GlcNAc in an en>eogtIR wing. Thus, Notch signaling may promote blister formation whether or not it carries O-GlcNAc, so that loss of Notch signaling would suppress blister formation. This conclusion is consistent with the observations that mutations in Su(H), Dl, and mam, as well as deficiencies of these alleles, suppressed en>eogtIR-induced blister formation (Table 2). Unfortunately, expression of N[+/CD] or N[neutral] in the en>eogtIR background were both lethal, and it was thus not possible to assess the effect of constitutively active N on en>eogtIR-induced blister formation. Similar results were obtained when blister formation obtained upon removal of one of N allele suppresses blisters of dp clones. Although only one mutant allele of Crb was investigated, the slight suppression in wing blister formation obtained upon removal of one of Crb allele may be due to relief of Crb inhibition of Presenilin-induced Notch activation [33], rather than to loss of Crb regulation of epithelial apical–basal polarity [54].

A potential unifying hypothesis that may tie defective Dp function and Notch signaling to opposite effects on the development of wing blisters in en>eogtIR flies, is that both pathways interact with the pyrimidine synthesis pathway. Biochemical data show that at 72 hr several dp mutants have increased aspartate transcarbamylase (ATC; one of the activities encoded by r), orotate phosphoribosyltransferase (OPRT) and orotidine-5'-phosphate decarboxylase (ODC) activities, both encoded by r-l [17,18] (Fig. S1). In addition, mutations in r that decrease ATC activity, suppress the development of dp mutant wing phenotypes, i.e. they normalize truncated oblique (dp) mutant wings [17]. Administration of the ATC inhibitors 6-azauracil and 6-azauridine to inhibit pyrimidine synthesis, causes phenotypes that mimic r mutation but normalize dp phenotypes [43,55]. This suggests that the loss of one allele of dp enhances en>eogtIR blisters, loss of one allele of r suppresses the formation of these blisters (Table 3; Fig. 7). In fact, complete and reversible suppression of the en>eogtIR wing blister phenotype was obtained with several mutants in pyrimidine neo-synthesis, including r, r-l and DhoD. The suppression of wing blister formation by reduced pyrimidine biosynthesis, along with the synthetic lethality (that we interpret as enhancement) observed when uracil is not removed by catabolism in en>eogtIR flies, suggest that increased levels of uracil promote or cause blister formation. Thus, the interaction of en>eogtIR with ada(l) recapitulates a strong
enhancement of dp wing phenotypes by su(r) that leads to blistered wings [19]. Also consistent is that the pyrimidine catabolic pathway activator black suppresses the en>egfR 

blister phenotype (Table 3; Fig. 8). On the other hand, flies homozygous for certain alleles of r can develop wing blisters as part of a vastly smaller wing, in spite of reduced pyrimidine synthesis [48]. The multiple mechanisms of wing blister formation are clearly varied and complex. In this context, it is worth mentioning that the reduction in wing size of dp and some r mutants have different origins, with the first being due mainly to changes in cell size [50], and the latter predominantly to a reduction in cell number [57].

Taken together, the facts that Dp is a substrate of Eogt, that dp and eogt mutants phenocopy each other, and that both exhibit genetic interactions with pyrimidine biosynthesis mutants, suggest a model in which Dp-O-GlcNAc slows the production of pyrimidines, while reduction of Dp or Dp that lacks O-GlcNAc enhances de novo pyrimidine synthesis. Consequently, overproduction of a toxic UMP metabolite such as uracil leads to the eogt RNAi blister phenotype (Fig. 8). This uracil toxicity model does not discount Dp-mediated, adhesion-dependent mechanisms of action during wing development. Due to chitin synthesis, UDP-sugar concentrations in the hemolymph of insect wing discs are enormous [58], and a corresponding amount of UDP liberated by chitin synthases in a short time might well upset baseline pyrimidine synthesis regulation, and require a temporally active, tissue specific regulatory mechanism.

The involvement of the EGF-O-GlcNAc modification in pyrimidine synthesis is consistent with published data on dp mutants. In contrast, the fact that decreased Notch signaling of a toxic UMP metabolite such as uracil leads to the eogt RNAi blister phenotype (Fig. 8). This uracil toxicity model does not discount Dp-mediated, adhesion-dependent mechanism of action during wing development. Due to chitin synthesis, UDP-sugar concentrations in the hemolymph of insect wing discs are enormous [58], and a corresponding amount of UDP liberated by chitin synthases in a short time might well upset baseline pyrimidine synthesis regulation, and require a temporally active, tissue specific regulatory mechanism.

Materials and Methods

Antibodies

Polyclonal mouse anti-human EOGT (AER61) was from ABCAM (ab69389). Mouse monoclonal anti-O-GlcNAc IgM (CD110.6; O7764), rabbit anti-mouse Ago61 (AV48972), and anti-α-tubulin (T5168) were from Sigma. Rabbit anti-GFP was from Invitrogen (A11122). Goat anti-human PLAP was from Santa Cruz (L-19, sc-15065) and mouse anti-His was from Roche (#1192416001). All antibodies were diluted in 3% bovine serum albumin (BSA) in Tris buffered saline pH 7.4 (TBS), and 0.1% Tween 20.

Plasmids

pMT-Notch/[EGF1-20-AP] , pMT-WB-Delta-AP-6His, pMT-WB-Serrate-AP-6His were a kind gift from Ken Irvine (HHMI and Waksman Institute, Piscataway, NJ). pCaspTubPA was a kind gift of Stephen Cohen (Institute of Molecular and Cell Biology, Singapore). pCMV-SPORT6 mouse Ago61 (MMM1013-7512204) was purchased from Open Biosystems (Thermo Scientific). Drosophila eogt cDNA (GH04522) in pOT2 was obtained from DGRG (Indiana University, IN). Human EOGT cDNA was cloned from HEK 293T cells that were extracted with Trizol (Invitrogen) to obtain total RNA from which polyA+ RNA was purified with the Geneutech mRNA Miniprep Kit (Sigma) according to the manufacturer’s recommendations. Reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen). EOGT was amplified using primers PS1427 and PS1428 (Table 4) and cloned into pCR2.1TOPO (Invitrogen). For this study, we used an isoform that was active in GlcNAc transfer and had an amino acid sequence identical to chimp Eogt (NP_001009171). The Genbank accession number is KC347596.1. Chinese hamster Eogt cDNA was amplified by RT-PCR of total RNA from Chinese hamster ovary cells (CHO; clone Pro 5 and two independent clones) using primers PS1271 and PS1166r (Table 4). The Genbank accession number is KC347595.1.

PCR products of full-length mouse Ago61 obtained with PS1444 and PS1448, human EOGT (PS1446 and PS1449) and Drosophila eogt (PS1450 and PS1452) coding sequences were cloned into pSC-A vectors (Agilent) introducing a 5’ NotI site and a ‘CCACC’ Kozak sequence [64] and a 3’ XhoI site (Table 4). They were further subcloned into pMT-V5/His-A (Invitrogen) and pCaspTubPA. pUASTeogt was made by inserting eogt as a BglII and XhoI fragment from pOT2 GH04522 into pUAST [63]. Genetic Services, Inc. (Cambridge, MA) injected DNA transgenes. To identify a DXD motif conserved across species, Eogt sequences were compared using CLUSTAL W [66]. Accession numbers for Eogt from the respective species were: Chinese hamster ovary cells (CHO Pro 5): KC347593.1; Trichoplax adhaerens: XP_002117650.1; Drosophila melanogaster: NP_608678.1; Ciona intestinalis: NP_001027841.1; Caenorhabditis elegans:
Table 4. Oligonucleotide Primers.

| PS1427 | hEOGT | GAGGTTCGAGCTTGTAGCTGAT | GAGGTTCGAGCTTGTAGCTGAT |
| PS1428 | hEOGT | GTCGTAGTGTGGAAGTGTTC | GTCGTAGTGTGGAAGTGTTC |
| PS1271 | CHO Eogt | ACTTARAGGGTCCTGCAGGGTCTGCT | ACTTARAGGGTCCTGCAGGGTCTGCT |
| PS1166r | CHO Eogt | TCTGACGCTGMAAGGACACAG | TCTGACGCTGMAAGGACACAG |
| PS1444 | mAgo61 | ATAAGAATGGCCGCGAGAAAATGACCTATGCGGTTACATC | ATAAGAATGGCCGCGAGAAAATGACCTATGCGGTTACATC |
| PS1448 | mAgo61 | CGCCTGAGTCATGCTGCGGTTACATCGGTTTGG | CGCCTGAGTCATGCTGCGGTTACATCGGTTTGG |
| PS1450 | CG9867 | ATAAGAATGGCCGCGAGAAAATGACCTATGCGGTTACATC | ATAAGAATGGCCGCGAGAAAATGACCTATGCGGTTACATC |
| PS1452 | CG9867 | CGCCTGAGTCATGCTGCGGTTACATCGGTTTGG | CGCCTGAGTCATGCTGCGGTTACATCGGTTTGG |
| PS1446 | hEOGT | ATAAGAATGGCCGCGAGAAAATGACCTATGCGGTTACATC | ATAAGAATGGCCGCGAGAAAATGACCTATGCGGTTACATC |
| PS1449 | hEOGT | CGCCTGAGTCATGCTGCGGTTACATCGGTTTGG | CGCCTGAGTCATGCTGCGGTTACATCGGTTTGG |
| PS1550 | AYA N-term | GAGGAATCTTGGAGTATGAAATGCGGCCGCTTAATGTTGTTTGTCTTTG | GAGGAATCTTGGAGTATGAAATGCGGCCGCTTAATGTTGTTTGTCTTTG |
| PS1454 | AYA N-term | CAAATGTATAAGAATGCGGCCGCAAAATGTTAATGTTGTTTGTCTTTG | CAAATGTATAAGAATGCGGCCGCAAAATGTTAATGTTGTTTGTCTTTG |
| PS1453 | AYA C-term | GCATTACTTTGATTGGTATATTTG | GCATTACTTTGATTGGTATATTTG |
| PS1551 | AYA C-term | GTATCTAGTATATATAGCTATGTGATTTCTTCTTAATG | GTATCTAGTATATATAGCTATGTGATTTCTTCTTAATG |
| PS7T1429 | eogt dsRNA | TAATACGACTCACTATAGGG | TAATACGACTCACTATAGGG |
| PS7T1430 | eogt dsRNA | TAATACGACTCACTATAGGG | TAATACGACTCACTATAGGG |
| PS1378 | eogt excision site | GCAGTAAATGCAGATACCCGCTGCAGTTAATGTTGTTTGTCTTTG | GCAGTAAATGCAGATACCCGCTGCAGTTAATGTTGTTTGTCTTTG |
| PS1380 | eogt excision site | CAAATGTATAAGAATGCGGCCGCAAAATGTTAATGTTGTTTGTCTTTG | CAAATGTATAAGAATGCGGCCGCAAAATGTTAATGTTGTTTGTCTTTG |
| 345for | sxc6 point mut. | GCTGATGACGACAGCAGAGCCACAGTGGGACGAC | GCTGATGACGACAGCAGAGCCACAGTGGGACGAC |
| 345rev | sxc6 point mut. | GAAGATGAAATGTCGAGTGCAGTGAAATGTTGTTTGTCTTTG | GAAGATGAAATGTCGAGTGCAGTGAAATGTTGTTTGTCTTTG |

Upper and lower sequences are forward and reverse primers, respectively. Italicized bases are T7 extensions.

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NP_506677.3: Family 61 protein from Arabidopsis thaliana: NP_565952.1; DUF563 protein from Cyanothece sp. NP_506677.3; Family 61 protein from Arabidopsis thaliana.

Site-directed mutagenesis of human EOGT to change DYD to EOGT was performed by overlap extension PCR [67] using primers PS1550 and PS1454, for the N-terminus and primers PS1453 and PS1551 for the C-terminus (Table 4). Wild-type EOGT was amplified using PS1550 and PS1551. PCR products were digested with BglII and XbaI and cloned into the BglII/XbaI sites of pMT-Bip-V5/HisA (Invitrogen) in frame with the Bip signal sequence. Mutations were confirmed by DNA sequencing.

Cell Culture

S2 cells were cultured in Schneider’s Drosophila Medium (Invitrogen, CA) supplemented with 10% heat inactivated fetal calf serum (Gemini Bio Products, CA) at 25°C. Transient transfection with plasmid DNA per 35 mm dish. Where appropriate, protein expression was induced with 1 mM CuSO4 and cells and cell-free supernatant was harvested after 24 h or 48 h at 25°C.

To generate RNAi constructs that targeted eogt, DNA template corresponding to VDRC/44572 (Fig. 2A) was generated from w1118 genomic DNA by PCR with primers PST71429 and PST71430 (Table 4), containing a T7 promoter at each end. The PCR products were cloned into pSG-A (Agilent) to create pSG-A-T7-44572-T7. A gel-purified T7 fragment from the plasmid pSG-T7-44572-T7 was used as template for PCR with the same primers, products were gel-purified using the QIAquick Gel extraction kit (Qiagen), and used as templates for in vitro transcription reactions using a Megascript cRNA kit (Ambion) following the manufacturer’s instructions. The dsRNA was purified using Qiagen’s RNeasy kit following the manufacturer’s protocol except that β-mercaptoethanol was omitted. S2 cells seeded onto a 10 cm dish in S2 medium. After cells adhered, the medium was replaced with serum-free medium, 200 μg of dsRNA or water was added, and the dish shaken every 20 min. After 1 h, heat-inactivated serum was added to 10% final concentration. On day 5 after treatment, the cells were split 1:4 into new 10 cm plates, and the dsRNA treatment was repeated. On day 9 of dsRNA treatment, the cells were transfected with 10 μg appropriate plasmid DNAs using CaPO4. Post nuclear supernatant was obtained after lysis in TBS, 1% TX-100 and 50% of the immunoprecipitated sample was loaded onto the SDS-PAGE.

Protein Extraction and Gel Electrophoresis

For Western analysis, sxc6 and eogt60 mutants were balanced over CyO-sectorGal4>UAS-GFP [68]. Embryos were collected for 2 hours on apple plates. At 60–62 h AEL, GFP-negative larvae were selected, flash frozen and stored at −80°C. Larvae (100 per genotype) were homogenized for 50 sec using a tissue homogenizer (Kontes) in PBS lacking Ca2+ and Mg2+ and containing 100 μM of the Ogt inhibitor PUGNaC (Sigma) and 1× Complete Proteinase Inhibitor (Roche). Samples were centrifuged twice at 3000 g for 5 min and the supernatant transferred to a new tube. The supernatant was cross-linked to homoygous UAS-Gal4>TM3,Sb,act-5C-GFP flies and the embryos were aged on apple plates
at 31°C to ensure homogeneous knock-down. After 5 days, 7 GFP-positive (controls) and 7 GFP-negative (dp knock-down) pupae were lysed and processed as above, except that lysates were centrifuged at 1000 g for 20 min at 4°C. Pupae extract (~100 μg protein) was separated on a 7.5% SDS-PAGE with a 3.5% stacking gel and processed for Western analysis.

Fly Stocks and Transgenic Lines

Drosophila RNAi lines UAS-eogP{DRC44572} and UAS-dpV{DRC44029} were obtained from the VDRC (Vienna, Austria) and UAS-eogP1563T (9067R-3) was obtained from the National Institute of Genetics (Mishima, Japan). Other lines were obtained from the Bloomington Stock Center. en-Gal4, heat-shock-Gal4, eog-Gal4, act-Gal4, and tub-Gal4 are described in Flybase (http://flybase.org/). eogt mutants were generated by imprecise P-element excision of Df(2L)Exel7011 presence of the alleles by backcrossing to standard procedures. Recombinants were screened for the dplv1224 aa, including the start codon; Fig. 2A).

The IUBMB names of pathway enzymes in pyrimidine biosynthesis and catabolism are shown with the product generated by Figure S1 Pyrimidine anabolic and catabolic pathways.

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Supporting Information

Figure S1 Pyrimidine anabolic and catabolic pathways. The IUBMB names of pathway enzymes in pyrimidine biosynthesis and catabolism are shown with the product generated by each reaction. Green bars signify steps for which reduced enzyme activity caused suppression of wing blisters in eogR wings; magenta bars signify steps for which reduced enzyme activity caused enhancement of wing blisters in eogR wings. (TIF)

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Author Contributions

Conceived and designed the experiments: RM AJ PS. Performed the experiments: RM AJ PS. Analyzed the data: RM AJ PS. Contributed reagents/materials/analysis tools: RM AJ PS. Wrote the paper: RM AJ PS.
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