A Novel Ecdysone Receptor Mediates Steroid-Regulated Developmental Events during the Mid-Third Instar of Drosophila

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Abstract

The larval salivary gland of Drosophila melanogaster synthesizes and secretes glue glycoproteins that cement developing animals to a solid surface during metamorphosis. The steroid hormone 20-hydroxyecdysone (20E) is an essential signaling molecule that modulates most of the physiological functions of the larval gland. At the end of larval development, it is known that 20E—signaling through a nuclear receptor heterodimer consisting of EcR and USP—induces the early and late puffing cascade of the polytene chromosomes and causes the exocytosis of stored glue granules into the lumen of the gland. It has also been reported that an earlier pulse of hormone induces the temporally and spatially specific transcriptional activation of the glue genes; however, the receptor responsible for triggering this response has not been characterized. Here we show that the coordinated expression of the glue genes midway through the third instar is mediated by 20E acting to induce genes of the Broad Complex (BRC) through a receptor that is not an EcR/USP heterodimer. This result is novel because it demonstrates for the first time that at least some 20E-mediated, mid-larval, developmental responses are controlled by an uncharacterized receptor that does not contain an RXR-like component.

Introduction

During metamorphosis in Drosophila melanogaster, pulses of the 20E steroid hormone, stimulate diverse tissue-specific responses such as the histolysis of many larval tissues and the simultaneous differentiation of adult structures from imaginal discs [reviewed in 1]. In addition, multiple pulses of 20E, that occur during the last larval instar (L3) trigger different responses within the same target tissue, raising the interesting question of how a generalized developmental signal is manifested into distinct physiological responses that are separated by time. The larval/prepupal salivary gland is an ideal assay system in which to investigate the molecular mechanisms responsible for such temporally specific developmental specifications. In a 36-hour period, the gland responds to three distinct pulses of 20E in three fundamentally different ways.

During most of larval life, the salivary gland is engaged in the synthesis of non-digestive enzymes that most likely aid in the lubrication of the food through the gut [2–4]. However, about midway through the L3 stage, the pattern of gene expression is altered dramatically by the synchronous activation of a small number of genes (~8) that are abundantly expressed in the salivary gland [5]. These are known to encode components of the glue mix that cements animals to a solid surface during metamorphosis, and they were first identified because their induction is responsible for the “intermolt” puffs formed on the giant polytene chromosomes of the gland [6,7].

Approximately 18 hours later, in response to the pulse of 20E that occurs at the end of L3, glue synthesis abruptly ceases [5,8] because the hormone represses transcription from these genes [9,10]. At this time, the salivary gland begins to express another set of genes, many of which were originally described because they formed “early” and “late” puffs on the polytene chromosomes [reviewed in 11]. The end result of this 20E-mediated response is that glue granules are secreted into the lumen of the gland [12,13].

Finally at the end of prepupal development 10–12 hours later, the salivary gland responds to yet another pulse of 20E to initiate the programmed cell death of the tissue via a pathway that involves components of both autophagy and caspase activation [14,15].

The details of how 20E initiates glue secretion and gland histolysis are well understood. The hormone is known to bind to a receptor consisting of a heterodimer of EcR (FBgn0000546) and USP (FBgn0003964) proteins [16–18]. Both receptor components
Author Summary

During animal development the physiological response of individual tissues is often "reprogrammed" in response to signaling molecules. One important example is the activity of nuclear-hormone receptors that are controlled by small lipid compounds such as steroids and retinoids. Thus, understanding how tissue-specific developmental and physiological responses are regulated by these systemic ligands is a fundamental question of cell biology. *Drosophila* is an important model system in which to investigate this question because of its 100-year history of analyzing mutants that affect complex biological processes, and because researchers possess a powerful "toolkit" that allows for precise tissue- and temporal-specific expression and silencing of almost any gene in the genome. Furthermore, during the metamorphosis of *Drosophila*, the body plan is completely reorganized from that of a larva (specialized for growth and feeding) to that of an imago (specialized for reproduction and dispersal) by a single steroid hormone. Here we examine the molecular events that control different physiological responses within a single target tissue to different pulses of 20E. We show for the first time that these temporally specific events within the same tissue are controlled in part by different 20E receptors.

Here we examine the requirements of EcR and USP for the induction of the glue genes at mid L3. By employing the GAL4/UAS binary expression system [20] with transgenic inducible dominant-negative and RNAi constructs, we are able to limit perturbations of 20E signaling specifically to the salivary gland at defined developmental stages. We show that 20E is responsible for inducing a tagged glue transgene as a secondary response to the hormone, and that the 20E-inducible primary-response genes of the Broad Complex (BRC) (FBgn0000210) are sufficient to initiate transcriptional activation of the glue genes [29]. However, we clearly demonstrate that the mid-instar hormone response requires a receptor that has not yet been characterized. The receptor consists of EcR but not USP. These results challenge the traditional model that most developmental events triggered by 20E must signal through a heterodimer of EcR and USP, and they support an alternative explanation in which either EcR homodimers or other members of the nuclear-hormone receptor superfamily play an active role in the diversity of responses to 20E during *Drosophila* development.

**Results**

*An Sgs3 Transgene Is Induced by 20E*

It is generally assumed that the glue genes [Sgs1 (FBgn0003372), Sgs3 (FBgn0003373), Sgs4 (FBgn0003374), Sgs5 (FBgn0003375), Sgs6 (FBgn0003376), Sgs7 (FBgn0003377), Sgs8 (FBgn0003378), and T71-7 (FBgn0004592)] are induced by a pulse of 20E that occurs midway through the third instar. This inference is based on the dramatically coordinated developmental induction at mid L3 of most of these genes [5], and on studies in which Sgs expression is examined in backgrounds mutant for genes thought to be involved in 20E production or transport [21,22]. The model further proposes that induction of the glue genes occurs as a secondary response to 20E because Sgs expression is significantly perturbed in mutants defective for BRC and E74 (FBup0000567), which are known to be direct targets for the hormone/receptor complex [23–25]. However, an Sgs3-derived reporter transgene is induced when temperature-sensitive ecdf [26] mutants—known to produce low circulating levels of 20E [26]—are shifted to the non-permissive temperature before the L3 stage, and the same GFP reporter is also induced in animals that are mutant for USP, EcR-B1, and EcR-B2 receptor components [13].

Thus, the literature contains contradictory reports concerning the role of 20E in inducing the glue genes. Therefore, we began this analysis with an *in vitro* culture of salivary glands dissected from mid L3 because we are not aware of any published reports that directly test if glue-gene transcription can be induced by 20E in glands cultured from wildtype animals. To simplify the analysis, we dissected salivary glands from a line of flies in which the coding information for Sgs3 had been tagged with GFP (glueGRN) (FBst0005884). This stock (previously called SgsGFP) contains adequate regulatory information for the proper temporal, spatial, and high-level expression of the Sgs3 gene. It has also been extensively characterized and shown to be an accurate reporter for the secretion and expectation of endogenous SGS3 glue protein [13].

Larvae were synchronized at hatching and raised to the early-L3 stage approximately 4–5 hours prior to the normal transcriptional induction of the glue genes. Salivary glands were then dissected and exposed to media containing different concentrations of 20E (ranging 10^{-10} to 10^{-8} M) or in medium without hormone. Under these circumstances glueGRN accumulation was detected 4–6 hours later in cultures incubated with low 20E concentrations (Figure 1), but not in untreated cultures or those incubated with higher concentrations of the hormone. It should be noted that this response is not robust because only ~30% of the dissected glands produce glueGRN when treated. Presumably the results are variable because the culture conditions have not been optimized and/or the animals are not staged in a precise enough manner. However, it is significant to note that the only cultures in which glueGRN was detected were those incubated in either 10^{-10} M (8 out of 20) or 10^{-9} M (4 out of 20). Thus, the concentration needed for production of glueGRN is 2–3 orders of magnitude lower than the titer (~10^{−6} M) reported to trigger "early" polytene puff formation, imaginal disc eversion, and glue secretion [13,27,28]—all developmental events that occur near puparium formation in response to a much better characterized pulse of 20E. The result is consistent with the concentration of a small pulse of hormone that has been reported to occur in the hemolymph of developing larvae a few hours prior to the transcriptional activation of the glue genes [29].

**Sgs3-Derived Transgenes Require Functional EcR for Expression**

The *EcR* gene encodes three different protein isoforms, *EcR*-A, *EcR*-B1, and *EcR*-B2. All three contain the same DNA- and ligand-binding domains, but they contain different amino terminal A/B sequences due to the use of alternative promoters and differential splicing [16]. Null mutations for *EcR* die early in development and cannot be assayed for glue synthesis [30]. However, mutations that remove *EcR*-B1 and *EcR*-B2 [31] do produce glue [13]. These observations raise the possibility that either EcR is not required for the induction of Sgs3, or that any
EcR isoform is sufficient for the process. To distinguish between these possibilities and to take advantage of more powerful genetic tools that allow for tissue-specific manipulations of gene products, we utilized the GAL4/UAS binary expression system [20] to analyze glue-gene induction in developing salivary glands.

To perform this analysis in the most precise way, it was first necessary to identify a temporally and spatially restricted driver—a transgenic stock of flies in which the Gal4 transcription factor is under the control of specific Drosophila enhancers that limit its expression to larval salivary glands at least 10 hours preceding the normal induction of Sgs3. In our search for the best reagent, we noticed that a number of the drivers classified as salivary-gland specific were produced from the P{GawB} enhancer-trap element (FBtp0000352). Our lab and others have observed that GawB-derived elements display constitutive expression of GAL4 in larval salivary glands [32], perhaps because part of the GawB vector contains a cryptic larval salivary-gland-specific enhancer element. To test this hypothesis we used a hs-Gal4 stock that contains the Hsp70Bb (FBgn0013278) controlling elements driving Gal4 in a GawB vector. In the absence of heat stress these animals produced GAL4 in the L1 (first instar), L2 (second instar), and L3 salivary glands as indicated when they were crossed to the GFP.nls responder. In this stock GFP is expressed under GAL4 control (it contains UAS elements that are binding sites for the GAL4 transcription factor) and it is targeted to nuclei (Figure 2). Thus, in subsequent experiments we used hsGal4 (now referred to as sgGal4) to drive spatially restricted expression of UAS-transgenes only in larval salivary glands.

With a spatially restricted driver on hand, we now crossed sgGal4 to both UAS-dominant-negative (EcR-DN) and UAS-RNA-interference (EcRi) constructs of EcR. The EcR-DN protein is defective in ligand-activated transactivation so that it competes with endogenous EcR isoforms to block normal hormone responses [33]. The EcRi construct contains an inverted repeat of a DNA region common to EcR-A, B1, and B2 so that its expression silences all isoforms [34]. When either was crossed to a tester stock containing sgGal4 and glueGRN, no green fluorescence was detected in L3 larval glands. These results are consistent with a requirement that at least one EcR isoform must be present in the salivary gland for glueGRN synthesis (data not shown).

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Figure 1. Glue Genes are Induced by 20E in Cultured Glands. Mid-L3 animals were torn in half and incubated with ethanol as a control (A), or with 20E at a final concentration of 10⁻⁶ M (B). The induction of glue proteins in the salivary glands was detected by the expression of a GFP-tagged Sgs3 gene (glueGRN). Note that the positions of the salivary glands in (A) are outlined with dashed lines. The fluorescence detected in the pharynx (arrowheads) is non-specific and was used to standardize photographic exposures. Both photographs were taken at the same magnification indicated by the bar in A.

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Figure 2. sgGal4, a GawB-Derived Driver, Has an Expression Pattern Restricted to the Larval Salivary Glands. Flies containing sgGal4 were crossed to a GFP responder (UAS-GFP.nls) and all developmental stages were examined for the localization of GFP in the nuclei of live animals. The only tissues that contained green protein were the salivary glands. Depicted are a first-instar larva (A), a second-instar larva (B), an early-third-instar larva (C), and the anterior half of a late-third instar larva (D). Anterior is to the left in all photographs, and all were taken at the same magnification indicated by the bar in (D).

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One potential caveat with the above experiments is that by perturbing EcR in the salivary gland, we were killing it or causing it to develop too slowly to induce the Sgs3 transgene. To address this possibility, we utilized another tester stock containing three transgenic elements: glueRED; GFP.nls; sgGal4. The glueRED element is an endogenously tagged Sgs3 gene (under its own promoter/enhancer elements). It contains the same DNA sequence as glueGRN except the coding information for GFP is replaced with that of DsRED [35]. As with glueGRN, the glueRED element produces a protein that is synthesized (Figure 3A), secreted (Figure 3B), and expectorated in exactly the same manner as endogenous SGS3. Thus, when this tester stock was crossed to EcR-DN (Figure 3C) or EcRi (Figure 3D) no glueRED was produced, but GFP is still localized to nuclei that are similar in size to those of the control glands producing glueRED (Figure 3A,B). These results indicate that neither EcR-DN nor EcRi expression is killing the cells or preventing their normal nuclear polytenization. Thus, EcR function in the salivary gland is required for glueGRN and glueRED production.

Sgs3 Transgenes Can Be Induced by Any Isoform of EcR

To test the hypothesis that any isoform of EcR can be used to induce glue synthesis, we crossed each UAS-EcR isoform-specific transgene into a background in which EcR-DN was expressed in the salivary gland (under sgGal4 control) using the glueRED and GFP.nls transgenes to assay gland physiology. To confirm that extra copies of UAS-transgenes were not diluting the effects of EcR-DN in a non-specific manner, we included a UAS-control construct that contains a cassette of UAS/GAL4 binding sites. By itself, the expression of the UAS-control does not lead to a block in glueRED synthesis when driven by sgGal4 (data not shown). Furthermore when crossed into an animal producing EcR-DN and GFP.nls, it does not overcome the block in glueRED synthesis (Figure 4). This control eliminates the concern that the expression of EcR-DN may be reduced by the introduction of an additional transgene containing UAS elements.

In contrast to the UAS-control, introducing each of the known EcR-specific isoforms into the same genetic background completely rescues the block in the production of glueRED caused by EcR-DN. The rescue is fully penetrant and normal in the semi-quantitative scoring scheme that is presented in Figure 4. It is even more interesting because an artificially constructed EcR isoform—EcR-C, which contains only the common-regions of EcR because it is missing the isoform-specific A/B domain—also rescues the block in glueRED synthesis in approximately 90% of the animals examined. These results confirm an earlier conclusion that any isoform of EcR expressed in the salivary gland is capable of transmitting the 20E signal to induce the transcription of Sgs3-derived genes.

Proteins Encoded by the Broad Complex (BRC) Control the Expression of Sgs3 Transgenes

The BRC is a large transcription unit that produces several different isoforms of a transcription factor containing C4H2 zinc-fingers. Although multiple transcripts are derived from the locus [36], only four general types of proteins are produced. Each isoform contains an identical NH2 terminus, but it has a different combination of DNA binding domains [37]. The four proteins, referred to as BRC-Z1, BRC-Z2, BRC-Z3, and BRC-Z4, have been shown to play an important role in the production of SGS3 and other glue proteins. This conclusion is based on the phenotypic analyses of null- or isoform-specific hypomorphic mutants that either do not produce SGS3 or display a prolonged developmental delay in the accumulation of transcripts from the locus [13,23,24,38]. Because it has been shown that BRC is regulated as a primary response to 20E (the hormone/receptor complex directly binds to DNA elements within the gene and induction does not require de novo protein synthesis) [39], the above effects on Sgs3 activation have led to a model in which glue production occurs as a secondary response to the hormone. Thus, the BRC zinc-finger transcription factors are probably responsible for activating promoter/enhancer elements within the glue genes as suggested by DNA binding studies on Sgs3 [40].

To test this hypothesis in more detail, we utilized transgenic stocks in which each BRC isoform was expressed under UAS control in larvae also containing glueRED, sgGal4, GFP.nls; and EcR-DN. As indicated in Figure 4, each BRC-Z isoform is capable of partially rescuing the block in glue synthesis imposed by the production of EcR-DN. Rescue was scored using five categories that indicated the approximate percentage of cells within a gland that produced glueRED (none, few, ~25%, ~50%, 100%). However, not all BRC isoforms are equal in their ability to suppress the synthesis defect imposed by EcR-DN. BRC-Z2 (no glands were observed that were completely empty of glue, and 58% had full wildtype levels) and BRC-Z4 rescue the best; whereas, BRC-Z1 and BRC-Z3 (60% of the animals have glands with no glueRED) rescue poorly. The variability in rescuing the synthesis-blocked phenotype may reflect the partially redundant activities or regulatory dependencies that have been reported among the four types of BRC isoforms [37], or it may reflect the differences in expression levels among the different transgenes.

Figure 3. 20E-Mediated glueRED Induction Requires Functional EcR. Confocal images of salivary glands from stocks containing sgGal4; GFP.nls; glueRED transgenes are presented. Normally glueRED is synthesized during the mid-L3 stage and loaded into large granules that remain within the cell until 6–8 hours prior to pupariation (A). At the end of the instar (in response to another pulse of 20E), glue becomes secreted into the lumen (B). No glueRED is produced in animals expressing the EcR-DN (C) or EcRi (D) transgenes. Note that GFP is still expressed in both types of EcR-compromised glands. It marks nuclei that are able to survive and polytenize to the same degree in all cells presented. All photos were taken at the same magnification indicated by the bar in (C). Arrowheads and “L” mark the position of the lumen into which glue should be secreted.

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| Genotype (Transgene Addback) | A        | B        | C        | D        | E        | Number Assayed | Photographic Example of Selected Cells |
|------------------------------|---------|---------|---------|---------|---------|---------------|---------------------------------------|
| SgGal4                      |         |         |         |         |         |               |                                       |
| UAS-GFP.nls                 |         |         |         |         |         |               |                                       |
| UAS-EcR-DN glueRED          |         |         |         |         |         |               |                                       |
| UAS-Control                 |         |         |         |         |         |               |                                       |
| 100%                         | 0%      | 0%      | 0%      | 0%      | 0%      | (20)          |                                       |
| SgGal4                      |         |         |         |         |         |               |                                       |
| UAS-GFP.nls                 |         |         |         |         |         |               |                                       |
| UAS-EcR-DN glueRED          |         |         |         |         |         |               |                                       |
| UAS-EcR-A                   |         |         |         |         |         | (49)          |                                       |
| 0%                           | 0%      | 0%      | 0%      | 0%      | 100%     |               |                                       |
| SgGal4                      |         |         |         |         |         |               |                                       |
| UAS-GFP.nls                 |         |         |         |         |         |               |                                       |
| UAS-EcR-DN glueRED          |         |         |         |         |         |               |                                       |
| UAS-EcR-B1                  |         |         |         |         |         | (48)          |                                       |
| 0%                           | 0%      | 0%      | 0%      | 0%      | 100%     |               |                                       |
| SgGal4                      |         |         |         |         |         |               |                                       |
| UAS-GFP.nls                 |         |         |         |         |         |               |                                       |
| UAS-EcR-DN glueRED          |         |         |         |         |         |               |                                       |
| UAS-EcR-B2                  |         |         |         |         |         | (51)          |                                       |
| 10%                          | 0%      | 0%      | 0%      | 0%      | 90%      |               |                                       |
| SgGal4                      |         |         |         |         |         |               |                                       |
| UAS-GFP.nls                 |         |         |         |         |         |               |                                       |
| UAS-EcR-DN glueRED          |         |         |         |         |         |               |                                       |
| UAS-EcR-C                   |         |         |         |         |         | (49)          |                                       |
| 12%                          | 81%     | 5%      | 2%      | 0%      |         |               |                                       |
| SgGal4                      |         |         |         |         |         |               |                                       |
| UAS-GFP.nls                 |         |         |         |         |         |               |                                       |
| UAS-EcR-DN glueRED          |         |         |         |         |         |               |                                       |
| UAS-BRC-Z1                  |         |         |         |         |         | (43)          |                                       |
| 0%                           | 7%      | 7%      | 27%     | 58%     |         |               |                                       |
| SgGal4                      |         |         |         |         |         |               |                                       |
| UAS-GFP.nls                 |         |         |         |         |         |               |                                       |
| UAS-EcR-DN glueRED          |         |         |         |         |         |               |                                       |
| UAS-BRC-Z2                  |         |         |         |         |         | (55)          |                                       |
| 60%                          | 38%     | 2%      | 0%      | 0%      |         |               |                                       |
| SgGal4                      |         |         |         |         |         |               |                                       |
| UAS-GFP.nls                 |         |         |         |         |         |               |                                       |
| UAS-EcR-DN glueRED          |         |         |         |         |         |               |                                       |
| UAS-BRC-Z3                  |         |         |         |         |         | (42)          |                                       |
| 12%                          | 31%     | 19%     | 21%     | 19%     |         |               |                                       |
| SgGal4                      |         |         |         |         |         |               |                                       |
| UAS-GFP.nls                 |         |         |         |         |         |               |                                       |
| UAS-EcR-DN glueRED          |         |         |         |         |         |               |                                       |
| UAS-BRC-Z4                  |         |         |         |         |         | (48)          |                                       |
| 50 μm                      |         |         |         |         |         |               |                                       |
Figure 4. Any Isoform of EcR or BRC is Sufficient to Induce glueRED Synthesis. The percentage of glands displaying each synthesis phenotype is indicated below a low-resolution representative (all photographed at the same magnification as indicated in E). Categories include glands that are not producing any glueRED (A), glands in which only a few cells produce glueRED (B), glands in which approximately 25% of the cells are producing glueRED (C), glands in which approximately half the cells are producing glueRED (D), and glands in which all the cells are producing glueRED (E). Higher resolution images of cells representing each genotype (transgene addbacks) were all taken at the same magnification (indicated bottom right).

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Two additional points are worth noting. First, expression of all forms of UAS-BRC altered the expression/localization of GFP.nls in some cells, but this failure to localize GFP did not correlate with a defect in glueRED production. In all cases, a few cells producing glueRED were observed with large prominent nuclei that did not contain GFP. Because we never observe this effect in the experiments performed with EcR isoforms or the UAS-control, it is unlikely that extra transgenes containing UAS elements are titrating a limiting amount of GAL4 transcription factor.

Second, we sometimes observe the appearance of glueRED in L1 and L2 animals when BRC isoforms are ectopically expressed (data not shown). This early expression of glueRED or glueGRN is never observed in control animals or in crosses where EcR-specific isoforms are ectopically expressed. This result may indicate that BRC proteins are sufficient for SGS3 production at any stage of larval salivary gland development, but that critical levels of BRC isoforms are normally restricted to mid-to-late L3 stages in wildtype animals [5].

EcR Is Required for the Induction of Other Glue Genes

Glue is a mixture of at least eight different glycoproteins [41,42], which are coordinately induced midway through the third instar in a tissue-restricted fashion. To test whether perturbing EcR signaling disrupts the synthesis of most glue proteins, we assayed glue production in EcR-compromised glands in two different ways. First, we examined the glands directly. The cytoplasm of EcR-compromised cells is very small with no detectable secretory granules (Figure 3C,D). If other abundant non-tagged glue proteins were being loaded into granules, this result would not be expected. Second, when we examined the expression pattern of Sgs3, Sgs4, Sgs5, Sgs7, and Sgs8 transcripts by Northern analysis, we found very little signal for any of the five glue genes tested in animals in which EcR was compromised in the salivary gland (Figure 5). Note the normal developmental expression pattern in the control lanes (C-1; C-2). Transcript levels for all glue genes should be high in wandering larvae (L), and they should be low or undetectable at the time of pupariation formation (W).

Induction of the Glue Genes Does Not Require USP

Because all known 20E signaling pathways that control in vivo developmental events are thought to be mediated through an ecdysone receptor consisting of EcR and USP, we wanted to test the requirement for USP in the synthesis of glue. Thus, we utilized a transgenic RNAi construct that contains an inverted repeat of USP under UAS control (USPi). We expressed this construct using the sgGal4 driver and the reporter genes (glueRED; GFP.nls) described above in order to selectively silence USP in larval salivary glands. Under these circumstances glands were indistinguishable from parental stocks (compare Figure 3A with Figure 6A), and 100% of the glands produced wildtype levels of glueRED (Table 1). This result suggests that USP is not part of the receptor needed for glueRED expression. An alternative explanation is that the USP construct is not effectively knocking down USP levels in the salivary gland, but three lines of evidence make this possibility very unlikely.

First, we examined wildtype- and USP-i-compromised salivary glands for USP protein using a well-characterized USP antibody. As shown in Figure 6, no USP protein can be detected in the nuclei of salivary glands in which USP is expressed. This is in contrast to the wildtype glands of similar L3 stages (compare the tissues marked as SG in C-E with those outlined by a dashed line in C’-E’), and in contrast to USP-i animals where the fat body (FB), central nervous system (CNS), imaginal discs (ID) and midgut (MG) clearly display the expected nuclear staining. This result is consistent with sgGal4 driving USPi only in salivary glands and not in other tissues. In addition, no USP protein is detected in salivary-gland extracts when a Western-blot analysis is performed on glands expressing the USPi construct (Figure 7).

Second, because glue secretion (dumping of granules into the lumen of the gland) at the end of L3 has been shown to be 20E dependent [12] and to require functional EcR and USP [13], we expected that USP-i glands would not be able to secrete the glueRED that was produced at an earlier stage. This prediction is always supported by data. Note that the photograph of the gland in Figure 6A was taken at the time of pupariation formation and that no glueRED can be detected in the lumen (L) of the tissue. In wildtype parental glands, secretion of the tagged glue into the lumen (Figure 3B) always occurs by the white prepupal stage.

Third, because it has been reported that USP is necessary to repress the glue genes at the time of pupariation formation, we expect that transcript accumulation for each Sgs gene should decrease at the white prepupal stage. The data presented in Figure 5 (compare L with W in the USP-i lanes) support this hypothesis.

Another possible caveat for the observation that RNAi against USP does not prevent glueRED expression is that a small amount of USP protein may be very stable in the salivary gland and thus not subject to efficient silencing by the RNAi mechanism. Following this logic, the protein turn over might take 4 days to reach a critical threshold level. Thus, there would be enough USP protein for glueRED synthesis in 3-day old larvae (the age when glue genes are induced by 20E), but not enough in 4-day old larvae (the age when 20E causes glue secretion). To test the ability of the USP-i construct to silence USP effectively in a short time frame, we used the glueGal4 driver (FBst0006870) to express transgenes in the salivary gland from mid-L3 until pupariation formation. Under these circumstances glue secretion was blocked even though the USP-i responder was only being expressed for 24 hours prior to the assay (data not shown).

Because it is known that USP can heterodimerize with EcR at the end of the larval period, we predicted that an overproduction of USP at mid-L3 might prevent a critical amount of EcR from forming the functional receptor needed for glue-gene induction. However, if even a small amount of a receptor consisting of EcR and USP is required to induce the glue genes, overproducing the USP component at an earlier time should not affect the response. Thus, we generated transgenic flies in which the coding information for wildtype USP was placed under UAS controlling elements. When this transgene (USPs) was driven by sgGal4, a large amount of USP protein was detected on Western blots of salivary glands (Figure 7), and the production of glueRED was reduced (Figure 6B; Table 1). We verified that this construct produces functional protein by crossing it to flies carrying both the...
glueGal4 driver and USPi responder. Under these conditions the USP+ construct was able to rescue the block in glue secretion caused by USPi.

Although the overproduction of USP in the salivary gland perturbs glueRED expression (34% of the glands produce no product), the block was not complete because animals were able to express varying levels of glueRED in some salivary-gland cells (Table 1). To more precisely quantify the amount of glueRED produced under these conditions, we performed the Western blot presented in Figure 8. As expected, no DsRED-tagged protein can be detected in the lanes in which EcR-DN or EcRi are expressed in the salivary glands (Figure 8B). In addition, the levels of glueRED are not reduced when USPi is expressed in the salivary glands because both control lanes (w1118 x sgGal4: glueRED) and experimental lanes (USPi x sgGal4: glueRED) contain the same band intensities when quantified and adjusted for protein loading using α-Tubulin (Figure 8A,B). However, the levels of glueRED are reduced 3 fold when USP is overexpressed (USP+ x sgGal4: glueRED) in the salivary gland compared to the control and USPi lanes (Figure 8B).

One explanation for the reduction, but not elimination of glueRED, is that the amount of USP produced under these conditions is at a threshold level needed to antagonize the 20E-signaling pathway mediated by EcR. To test this hypothesis, we

![Figure 5. EcR, but Not USP, is Needed for Glue Synthesis.](https://www.plosgenetics.org/doi/figure-pdf/10.1371/journal.pgen.1000102.g005)
crossed the USP line to sgGal4; GFP.nls; glueRED and raised the larvae derived from the cross at two different temperatures (25°C and 29°C). Because temperatures closer to 30°C are reported to produce higher GAL4 activities [43] (probably because GAL4 is a yeast transcription factor), we predicted that larvae raised at 29°C would produce less glueRED (due to the overproduction of more USP that should antagonize 20E receptor formation). As indicated in Table 1, these differences were observed when animals were raised at the two different temperatures (53% of the glands failed to produce any glue when raised at 29°C compared to 34% that failed to produce any glue when raised at 25°C). In addition, we confirmed that raising control animals at 29°C did not perturb glueRED production, and raising experimental animals at the elevated temperature did not cause a non-specific induction of the heat shock promoter in other tissues because GFP.nls was only detected in the nuclei of salivary glands (data not shown).

Finally, to ascertain the role of USP in the production of other glue proteins, we compared the overall pattern of protein synthesis using Coomassie staining of SDS-PAGE. As shown in Figure 8C, the appearance of most of the glue proteins can be identified when whole salivary-gland-protein extracts are stained because the Sgs genes are abundantly expressed in this tissue. We were able to confirm the presence of the major glue bands by comparing extracts of secreted glue plugs [6] that were prepared as ethanol precipitates from the lumens of white prepupae (data not shown). As expected, the accumulation of most glue proteins is reduced drastically in glands in which EcRi and EcR-DN are expressed. Also as expected, they are not reduced when USPi is expressed, but they are affected when USP is overproduced.

Taken together these results are very compelling, and they indicate that USPi is very efficient at gene silencing in the salivary gland when driven by sgGal4. Therefore, USP is not needed for the 20E-mediated induction of the glue genes through the BRC.

**Discussion**

**The Glue Genes Are Induced by 20E**

Previous reports using mutants that are defective in 20E production or signaling yielded contradictory results concerning the role of 20E in the induction of the glue genes in the salivary gland. Here we demonstrate that a glue-gene reporter derived from the Sgs3 gene can be induced by 20E in cultured glands

| Genotype (Transgene Addback) | Empty | Few | Quarter | Half | Full | Number Assayed |
|------------------------------|-------|-----|---------|------|------|----------------|
| SgGal4 UAS-GFP.nls glueRED UAS-USPi | 0%    | 0%  | 0%      | 0%   | 100% | 50             |
| SgGal4 UAS-GFP.nls glueRED UAS-USPi(+) (at 25°C) | 34%   | 2%  | 17%     | 25%  | 22%  | 56             |
| SgGal4 UAS-GFP.nls glueRED UAS-USPi(+) (at 29°C) | 53%   | 11% | 16%     | 13%  | 7%   | 61             |

Glue synthesis was assayed as described in Figure 4.

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Figure 6. Silencing USP in the Salivary Gland Does Not Block glueRED Synthesis. Confocal images of salivary glands in which RNAi against USP is triggered demonstrate that glueRED synthesis is normal but not secreted at the time of puparium formation (A). Overexpressing a wildtype USP transgene (USPi) produces a synthesis-defective phenotype (B) that is similar to that observed when EcR is compromised. Both images were taken at the same magnification, which is identical to that presented in Figure 3. Confocal images are presented of L3 tissues from wildtype animals (C-E) and larvae in which RNAi was induced against USP in the salivary glands (C’-E’). Tissues are stained with a USP antibody and visualized with a FITC-conjugated secondary. Fluorescence is detected in the nuclei of early-third (C, C’), mid-third (D, D’), and late-third (E, E’) instars. The positions of the salivary glands (SG and dashed outlines), central nervous system (CNS), imaginal discs (ID), fat body (FB), and midgut (MG) are marked for comparison to indicate that USP silencing is restricted to the salivary gland as expected with a tissue-specific driver. Note that the gain in E’ is increased to emphasize the lack of USP staining in the nuclei of the salivary glands. Photos C-E’ were taken at the same magnification indicated by the bar in E.

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Table 1. Overexpressing USP blocks the synthesis of glueRED.
The Induction of the Glue Genes Requires a Different 20E Receptor

The dogma for the action of 20E during Drosophila development is that EcR and USP are associated as a heterodimer and often bound to the EcREs of target genes. When not bound by ligand, the heterodimer associates with a repressor complex to prevent transcription from those genes. Hormone binding (to the ligand-binding domain of EcR) leads to a conformational change in the complex, the dissociation of the repressor complex, and the recruitment of co-activators for high-level transcriptional activation [reviewed in 19]. Although this model is well supported by evidence that both EcR and USP are required for the induction of the 20E-mediated events that occur at the end of the larval period, our study presents compelling evidence for the existence of another bona fide receptor for 20E that consists of EcR but does not use USP as its heterodimeric partner.

We have provided evidence that SGS3 production (and probably glue synthesis in general) is a 20E-mediated event. We have also demonstrated that EcR is required for the induction of the glue genes, and that any isoform of EcR can be involved in the activation of Sgs3. This result is interesting because EcR-B1 is reported to be the predominant form that is normally expressed in the larval salivary gland [45]. Also, because expression of BRC is necessary and sufficient for the induction of Sgs3, these experiments suggest that the A/B domain of EcR does not participate in the expression of BRC by the smaller pulse of 20E that occurs midway through the L3 stage.

In contrast to the results for EcR, we have provided convincing evidence that USP is not the other half of the heterodimer needed for the 20E-mediated initiation of glue synthesis. In a previous report [13] we confirmed that USP mutants can be rescued from embryonic lethality by providing exogenous USP from a heat-
shock driven transgene [46]. Furthermore, if these animals are not provided with a source of USP during the L2 and L3 stages (by being deprived of subsequent heat pulses that would induce the transgenic cDNA), they will not pupariate, but they will grow, molt, and express an Sgs3 derived reporter [13].

In the current study we have used strong tissue-specific drivers that are exclusively expressed in the salivary gland at two different time points. We have demonstrated that the USPs stock is an effective reagent for silencing endogenous USP in the salivary gland (Figures 6; 7), even if it is only produced for 24 hours before the assay (i.e. inducing it with glueGal4 blocks glue secretion). Thus, when it is driven during all larval stages (3 days before glue synthesis) no USP protein can be detected by immunostaining, and this absence of USP protein has no effect on the production of glue. To further confirm that USP is not needed for glue synthesis, we demonstrated that when wildtype USP is overexpressed in the salivary gland during the larval stages, glue protein production is drastically reduced. Because USP is known to heterodimerize with EcR at a later developmental stage, the simplest explanation for this observation is that extra USP protein is preventing EcR from forming the functional 20E receptor needed for glue synthesis in mid L3. Such a result is not expected if only a small amount of functional EcR/USP is needed to induce the glue genes.

Interestingly, other researchers have observed similar effects. One report generated clones of usp-/usp- mutant tissue in the salivary gland, and although they do not discuss the effects of glue production in mutant tissue, the presence of glue granules is apparent in the clones from late-L3 glands [47]. This and other studies also describe the developmental differences of clones of usp- tissue in imaginal discs.

For example, movement of the morphogenetic furrow—a 20E mediated event responsible for eye development [48]—is actually accelerated across a usp- patch of tissue [49,50]. In addition, others have noted that the 20E dependent differentiation of chemosensory neurons in the wing margin occurs precociously in the absence of USP function [51]. Furthermore, when target-gene expression is examined, transcripts from the BRC (BRC-Z1) accumulate earlier in development in mutant clones within the eye and wing discs [47,51]. These observations led to the hypothesis that in the absence of ligand, the EcR/USP heterodimer can act as a repressor in some tissues by binding to the response elements of a select group of target genes. The function of the hormone is to de-repress the target genes by removing the EcR/USP complex from the promoter region allowing other bound transcription factors to activate transcription [34]. Thus in a usp- clone, genes controlled by this mechanism should be precociously activated. We do not think that the induction of the glue genes is controlled by a de-repression of BRC through EcR/USP for two reasons. First, the glue genes are not induced (de-repressed) if EcR is silenced with an EcRi construct. Second, we do not see precocious activation of glue genes when a USPi construct is expressed.

Our model proposes that USP is acting as a repressor by heterodimerizing with EcR to prevent the association of EcR with another nuclear-hormone receptor (NR-X). Our hypothesis may also explain some of the data generated with the use of usp- clones in imaginal discs. For example, if we assume that movement of the morphogenetic furrow is induced by an earlier and lower pulse of 20E (as has been reported for Manduca) [52], we would speculate that furrow movement is controlled by EcR/NR-X regulating downstream genes including BRC-Z1. The normal presence of USP in this tissue at that time might serve to control the amount of functional EcR/NR-X available for high-affinity hormone binding. Thus in a usp- clone, we would expect the morphogenetic furrow to move faster over the patch and the induction of BRC-Z1 to be premature. Such observations were reported [47,49,50].

The normal expression of USP in the salivary gland at mid L3 (Figures 6; 7) may also be needed to ensure that the response of glue-gene induction is precisely regulated. In any case, the induction of a 20E regulated pathway that does not require USP as part of the receptor has no precedence in the Drosophila literature. Thus, a better characterization of this response at the molecular level is critical for our understanding of normal insect development.

Transcriptional Regulation of the Glue Genes

In this report we demonstrate that EcR is necessary for the expression of most of the glue genes at mid L3, and that USP is not needed for this expression. In addition, we show that any isoform of BRC can be sufficient for Sgs3 transgene expression even if the EcR component of the receptor is compromised with EcR-DN, and that overexpression of some BRC isoforms in first- and second-instar larvae is enough to induce expression of the Sgs3 transgenes days before they would normally be transcriptionally active.

However, it is interesting to note that although Sgs3 and Sgs4 appear to be coordinately expressed in mid-L3 salivary glands, different binding sites for regulatory proteins have been identified in their promoter/enhancer regions. These include response elements for EcR/USP, and binding sites for BRC [40], GEBF-1 (FBgn0013970) [53], Forkhead (FBgn000659) [54–56], and SEBP5 (FBgn0015295) [57]. The binding of different transcription factors to these sites may modulate the levels of expression of the two genes or they may contribute to their restricted expression patterns in the salivary gland or other tissues. For example, although we have shown that Sgs3 derived transgenes are exclusively restricted to the salivary glands of third-instar larvae, others have reported the expression of different glue genes in tissues outside this cell type. These include Sgs4 expression in the proventriculus [58] and I11-7 expression in the midgut and hemocytes [59]. Such expression patterns raise the interesting possibility that these highly glycosylated mucin secretions may perform other functions stemming from their propensity to form a sticky substance in aqueous solution. These functions could include the formation of the peritropic membrane around the food or the formation of extracellular aggregates that might be involved in antimicrobial responses [59].

What Is the Composition of the 20E Receptor Responsible for Inducing the Glue Genes?

If we assume that members of the nuclear-hormone receptor superfAMILY form dimers to produce the active receptor needed for glue-gene expression, we can formulate two hypotheses concerning the composition of that functional receptor. First, the active receptor may be a homodimer of EcR proteins. Homodimers are known to function as receptors for steroid hormones in vertebrates using a different mechanism of ligand activation than that observed with RXR heterodimeric receptors (USP is the insect homolog of RXR), but to our knowledge no biological activity has been ascribed to EcR homodimers during Drosophila development. Our analysis does not rule out the possibility that EcR homodimers are responsible for the induction of the glue genes. The second possibility is that another member of the superfAMILY may be able to complex with EcR to transmit the hormone signal. Many of these receptors have pre-existing mutations and many more have UAS-RXhl lines that are now available from the RNAi Stock Centers in Vienna (http://www.vdrc.at) and Japan (http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp). At this point we have assayed production of glueRED in...
mutants or RNAi lines that knock down DHR38 (FBgn0014859) and DHR76 (FBgn0013239), but no effects on glueRED synthesis were observed [A. Andres, unpublished observations]. However, the existence of transgenic RNAi lines should simplify the analysis because it is expected that when a specific nuclear receptor is silenced in the salivary gland, it should display a phenotype that is defective in glue synthesis. It would then be very interesting to test if this type of receptor could control other developmental events (perhaps molting of the instars or some aspect of early imaginal disc development) that are regulated by 20E during earlier larval stages.

Materials and Methods

Drosophila Stocks and Culture

All flies were raised on standard cornmeal-molasses medium supplemented with live baker’s yeast as recommended by the Bloomington Stock Center (Bloomington, Indiana, United States) [http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm]. w + [FBst0307124], GFP.nls [[UAS-GFP.nls]14 (FBst0004775), E2R [[UAS-E2R-RNAi]104 (FBst0009327)], E2R-DN [[UAS-E2R-B1-ΔC555.F645A]TP1 (FBst0006869)], and the E2R-isofrom stocks [E2R-A [UAS-E2R-A]3a (FBst0006470), E2R-B1 [UAS-E2R-B1]3B (FBst0006469), E2R-B2 [UAS-E2R-B2]3a (FBst0006468), and E2R-C [UAS-E2R-C]1p-1-4 (FBst0006688)] were obtained from the Bloomington Stock Center.

The following stocks were provided as generous gifts: UAS-hid [60] from Eric Baehrecke, the hsGal4 driver on the third chromosome [20] from Robert Holmgren, and the stocks containing specific isolomers of the BRC (UAS-BRC-Z1, UAS-BRC-Z2, UAS-BRC-Z3, and UAS-BRC-Z6) [61] from Xiaofeng Zhou.

Generation of Transgenic Flies

Transgenic flies containing glueRED were prepared by digesting pD:Red2-CI (Clonetech, Palo Alto, California, United States) with AgeI and KpnI restriction enzymes to isolate a DNA fragment containing the open reading frame for Dredd. This fragment was cloned into pBS-SgaΔGFP [13] that was digested with the same enzymes to remove the ΔGFP tag and generate a vector with compatible ends. The resulting intermediate construct was digested with AgeI and the 3′ recessed ends were filled in and religated to restore the open reading frame between Sg3 and Dredd. The Sg3-Dredd sequence was removed from the Bluescript vector (Stratagene, La Jolla, California, United States) as a NotI/KpnI fragment and inserted into the NotI and KpnI sites of the pCaSpeR-4 fly transformation vector (FBmc000178). DNA was sent to the vonKalm laboratory at the University of Central Florida for the generation of transgenic flies using standard techniques [62].

To produce the UAS-USP stock, a PCR fragment was amplified from a USP cDNA plasmid [63] using the primers AA-GAATTCGTTACAGTATCCGCCTAAACCTTG and TTA-GATCTCGTCCTACATTTTACCTGG. The resulting amplification product (corresponding to a 924 bp fragment between positions 467 and 1390 relative to the USP mRNA sequence) was cloned in the pUAST vector (FBm000083) using two steps. First, a reverse fragment was placed between the vector BglII and KpnI sites. A second forward-orientated fragment was cloned between EcoRI and BglII sites. Recombinant UAS-USP constructs were transformed at 30°C in Sure-competent bacteria (Stratagene) to minimize DNA recombination and screened using appropriate restriction enzyme digestions. Transgenic lines were generated as previously described using a w + strain as a recipient stock.

UAS-USP+ stocks were prepared as follows: The vector pUAST-USP+ was constructed by PCR amplification of the USP open reading frame with the forward primer TTGTTGCGGGCCGCCACCGTGACACGGTCGGCCAG and the reverse primer TTTTTCTTAGACTCACTCCCAGTTCAATCACGCAG using p2Z-1 cDNA as a template [63]. The NotI and XbaI restriction sites flanking the PCR product were used for subsequent ligation into the corresponding sites in the pUAST vector. The pUAST-USP+ vector was transformed into flies at the Duke University Medical Center.

The UAS-Control line (LA1216) contains an insert of the construct pMAR-UAS-6.11 (FBst0001327). This vector was designed for gene-mis-expression screens because it contains a copy of the UAS/GAL4 binding sequences oriented to express flanking genes when inserted into the genome [64].

Selecting a Salivary-Gland Specific Driver

We tested four Gal4 drivers obtained from the Bloomington Stock Center [AB1-Gal4 (FBst001824), G147-Gal4 (FBst0024396), T155-Gal4 (FBt0002598), and 34B-Gal4 (FBst0001967)] with expression patterns reported to be restricted to the larval salivary gland. To ascertain which of these was best for tissue-specific expression studies, we crossed them to a stock in which the hid/Wrinkled cell-death gene (FBst0003997) was expressed under UAS control. Because the major function of the salivary gland in the larval stages is the reported synthesis of mucin-like proteins that help lubricate the food as it moves through the gut [2,3], we reasoned that animals could survive without a salivary gland only if they were provided a diet of freshly produced moist yeast paste. Thus, by using UAS-hid we could ablate the salivary gland and test if such animals were viable when raised on soft food.

The initial analysis using the above listed drivers indicated that no larvae were able to survive, probably due to expression of the hid gene in other vital tissues. But because these are derived from the GauB vector, we used a heat shock 70-Gal4 driver that is also GauB derived. These animals were able to survive to puparium formation when crossed to UAS-hid and raised on a diet of freshly prepared yeast paste. Crossing hGal4 to a stock containing both UAS-hid and UAS-GFP.nls confirmed that larval salivary glands could not be detected and were ablated. Great care was exercised to raise the animals crossed to gsfGal4 at temperatures below 30°C to prevent exposing them to a stress that might induce Gal4 in all cells. Because the GFP.nls transgene was used in most of the experiments, a non-specific response could easily be detected by the presence of green nuclei in other tissues.

Salivary Gland Organ Culture

20E (Sigma, St. Louis, Missouri, United States) was prepared as a stock solution of 10−2 M in 100% ethanol and stored at −20°C. The stock solution was diluted to the proper working concentration in Schneider’s medium (Sigma).

Flies of the appropriate genotype were crossed and reared in a small population cage containing approximately 500 females and 500 males. The cage was presented with hard-agar plates (10% molasses, 3.5% agar) containing a dab of fresh yeast paste (prepared as a 1:1 mixture of dry baker’s yeast with water) 2–3 times per day to collect fertilized eggs. Collection plates were aged at 25°C and first-instar larvae were collected in 1-hour intervals as they hatched. The first-instar larvae were added in groups of 100 to vials containing standard cornmeal-molasses-yeast medium and aged at 25°C for approximately 68 hours (a developmental stage that precedes glue induction by approximately 4 hours) before being washed from the food with Schneider’s medium. Animals were then in half lengthwise using small dissecting forceps (Fine
Larvae prepared in this manner were transferred to clean microscope slides containing 25 μl of Schneider’s medium with or without 20E. A range of 20E dilutions (10⁻⁶ - 10⁻⁵, 10⁻⁴, 10⁻³ M) was prepared for each experiment. Small strips of Number 1 Whatman filter paper (Millipore, Billerica, Massachusetts, United States) were placed around the culture as spacers before adding a 22 mm² coverslip. The culture was placed on a platform shaker in a box into which O₂ was continuously infused during the culture period. Cultures were incubated at 25°C for 4-6 hours before being assayed for glue production as detected by the presence of green fluorescent protein from the glurGRN transgene.

**Microscopy and Imaging**

Whole larvae were selected from the food, washed 3× in water, blotted on filter paper, placed in a depression slide, and killed with a few drops of ether. After the ether evaporated, animals were mounted in glycerol between two slides using glass coverslips as spacers. Larvae were photographed within 30 minutes of preparation. For isolated tissues, animals were dissected in PBS (DPBS) or Schneider’s medium. Low-resolution images of whole animals or dissected tissues were obtained on a Leica fluorescent stereo microscope containing filter cubes for GFP and/or Ds-RED. Images were captured with the Spot Insight QE Model #4.2 digital camera (McBain Instruments, Chatsworth, California, United States) and prepared with Canvas (ACD Systems, Miami, Florida, United States) graphics software.

High-resolution images of dissected salivary glands were imaged on a LSM 510 Axioplan confocal microscope (Carl Zeiss SMT, Peabody, Massachusetts, United States) equipped with LSM 510 image-analysis software.

**Northern Blots**

Northern blots were prepared as previously described [5]. Briefly, RNA was isolated from larvae by grinding animals in SDS lysis buffer, digesting the homogenate with 250 μM Proteinase K (NEB, Ipswich, Massachusetts, United States), extracting the sample with phenol/chloroform, and precipitating the aqueous phase with ethanol. Ten micrograms of total RNA were fractionated on 1% formaldehyde/MOPS/agarose gels and blotted onto Duralon-UV membranes (Stratagene). Probes for each glue gene and the rp49 control were prepared as gel-isolated fragments from digested clones and hybridized with labeled random oligonucleotides using a Prime-it kit (Strategene) and 32P dCTP (GE Healthcare) and a Typhoon 8600 Variable Mode Phosphorimager (GE Healthcare).

**Protein Detection**

Dissected tissues were prepared for antibody staining as previously described [66]. Tissues were stained using the AB11 USP mouse monoclonal antibody [67] (gift from Carl Thummel) at a dilution of 1:30. Protein levels were visualized using a goat-anti-mouse secondary antibody conjugated to FITC (Jackson Immuno Research, West Grove, Pennsylvania, United States). To prepare protein extracts for Coomassie staining or Western-blot analysis, salivary glands were dissected in DPBS as described above. Typically 10–20 pairs of glands were collected in DPBS, pelleted in a microfuge, and resuspended in lysis buffer containing a cocktail of protease inhibitors [68]. Glands were homogenized and boiled for 5 minutes before being stored at −20°C for less than one week. Samples were divided in two and resolved on separate 12% SDS polyacrylamide gels that were run in the same electrophoresis rig. One was stained with Coomassie brilliant blue (J. T. Baker, Phillipsburg, New Jersey, United States) and the other was transferred to Immobilon P membranes (Millipore) as previously described [66]. Blots were incubated with the following antibodies: mouse anti-α-Tubulin primary (Sigma) diluted 1:15,000; rabbit anti-DsRED primary (Clontech) diluted 1:15,000; mouse anti-USP primary diluted 1:100; goat anti-mouse-HRP secondary (Jackson Immuno Research) diluted 1:40,000; and goat anti-rabbit-HRP secondary (Jackson Immuno Research) diluted 1:25,000. Protein levels were visualized and quantified using Chemiluminescence ECL+ Western-blotting detection system (GE Healthcare) and a Typhoon 8600 Variable Mode Phosphorimager (GE Healthcare).

**Accession Numbers**

The FlyBase (http://flybase.bio.indiana.edu/search/) identification numbers are used in this work to describe genes, gene products, vectors, and *Drosophila* stocks.

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**Author Contributions**

Conceived and designed the experiments: JM AA. Performed the experiments: BC DB KA KS AP AA. Analyzed the data: BC DB AA. Contributed reagents/materials/analysis tools: JM CA JC VH. Wrote the paper: BC AA.

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