Functional Conservation of the *Drosophila gooseberry* Gene and Its Evolutionary Alleles

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**Abstract**

The *Drosophila* Pax gene *gooseberry* (*gsb*) is required for development of the larval cuticle and CNS, survival to adulthood, and male fertility. These functions can be rescued in *gsb* mutants by two *gsb* evolutionary alleles, *gsb-Prd* and *gsb-Pax3*, which express the *Drosophila* Paired and mouse Pax3 proteins under the control of the *gooseberry* cis-regulatory region. Therefore, both Paired and Pax3 proteins have conserved all the Gsb functions that are required for survival of embryos to fertile adults, despite the divergent primary sequences in their C-terminal halves. As *gsb-Prd* and *gsb-Pax3* uncover a *gsb* function involved in male fertility, construction of evolutionary alleles may provide a powerful strategy to dissect hitherto unknown gene functions. Our results provide further evidence for the essential role of cis-regulatory regions in the functional diversification of duplicated genes during evolution.

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**Introduction**

During early *Drosophila* embryogenesis, the antero-posterior axis is progressively defined by the activities of four classes of segmentation genes: maternal coordinate genes, zygotic gap genes, pair-rule genes, and segment-polarity genes [1–4]. In addition to their roles in patterning the embryonic epidermis, many segmentation genes participate in other developmental programs like neurogenesis [5], myogenesis [6], and development of segmentation genes: maternal coordinate genes, zygotic gap genes, pair-rule genes, and segment-polarity genes [1–4]. In addition to segment-polarity genes [1–4], their roles in patterning the embryonic epidermis, many segmentation genes participate in other developmental programs like neurogenesis [5], myogenesis [6], and development of segmentation genes [7].

The *Drosophila gooseberry* (*gsb*) gene, initially identified as a member of the segment-polarity gene class [1], is required after germ band extension to maintain the ventral epidermal expression of *wingless* (* wg*), which suppresses ubiquitous denticle formation, through a *wg-gsb* autoregulatory loop [8]. In the central nervous system (CNS), *gsb* is essential for the activation of *gooseberry neuro* (*gsbn*) in a segmentally repeated pattern [9], for the differentiation of certain neuroblasts, and for the formation of the posterior commissure in each segment [10–14]. Since all known *gsb* mutant alleles are embryonic lethal [11], possible postembryonic functions of *gsb* remain largely unknown. Recently, *gsb* has been found to sustain expression of synaptic homeostasis, indicating the existence of postembryonic functions [15].

*gsb* encodes a transcription factor including two DNA binding domains in its N-terminal moiety, a paired-domain and a *prd*-type homeodomain [16–18]. Both domains are highly conserved in the N-terminal halves of the *Drosophila* Paired (*Prd*) and mouse Pax3 proteins, whose C-terminal halves, however, seem unrelated in their primary sequences to the C-terminal portion of Gsb [17,19]. *prd* is a member of the pair-rule gene class, specifying position along the antero-posterior axis with a double-segment periodicity and regulating the expression of segment-polarity genes [20]. The Pax3 gene, a mutation in which is responsible for the *Splotch* phenotype in mice [21] and Waardenburg’s syndrome I in humans [22,23], plays a pivotal role in myogenesis [24].

Despite their divergent developmental functions, Gsb and Pax3 proteins are able to substitute for most functions of Prd when expressed under the control of the complete *prd* cis-regulatory region in *prd-Gsb* and *prd-Pax3* transgenes [25]. While *prd-Pax3* is able to rescue the cuticular phenotype of *prd* mutants, *prd-Gsb* can further rescue *prd* mutants to adulthood [25], though the rescued males show reduced accessory glands and are sterile [26]. Taken together, these results indicate that Gsb, and Pax3 proteins have retained most functions of Prd despite their highly diverged C-terminal halves and further point to the cis-regulatory region as an important determinant for the functional diversification of these three genes. However, these experiments left unanswered the question of whether Prd and Pax3 proteins could substitute for the normal functions of Gsb.

To address this question, we produced two “evolutionary alleles” [25] of *gsb*, namely *gsb-Prd* and *gsb-Pax3*, which express Prd or Pax3 proteins under the control of the complete *gsb* cis-regulatory region. We show that both transgenes are able to rescue *gsb* mutants to fertile adults, albeit at reduced efficiencies, which suggests that both Prd and Pax3 proteins have conserved all normal functions of Gsb. We conclude that the divergent functions of *gsb*, *prd*, and *Pax3* genes are predominantly determined by their different cis-regulatory regions and are further modified by their protein coding regions. These results provide additional evidence to our previous model that the acquisition of different *cis-
regulatory elements is the primary mechanism in the evolution of new functions [25]. Since some of the rescued males are sterile, gsb is important for male fertility. This discovery of a male fertility function of gsb suggests that the construction of “evolutionary alleles” may serve as a powerful tool to reveal the hitherto unknown functions of a gene.

Results
Characterization of two hypomorphic gsb alleles
The gsb gene was initially uncovered by two large deficiencies, Df(2R)IIX62 and Df(2R)KrSB1, obtained in a screen for embryonic segmentation mutants [1]. Transheterozygotes of the two deficiencies have lost at least two genes in addition to gsb (Figure 1A). Their cuticle shows a strong segment-polarity phenotype (Fig. 2C), which is indistinguishable from that of homozygous Df(2R)IIX62 embryos (Figure 2B) [1].

Two alleles affecting only the gsb gene were identified late, including a point mutation, gsb525, and a P-element insertion, gsbP1155 [11]. In gsb525, the codon of the first amino acid of the homeodomain is mutated to a TAA stop codon. In gsb525 embryos, the gsb mRNA level is much reduced by stage 11, presumably because gsb activity depends on the wg-gsb autoregulatory loop [8], and no Gsb protein is detected by immunostaining, while the protein product of the Gsb target, Gsbn, is barely detectable. The fact that gsb525/Df(2R)IIX62 embryos exhibit only a weak cuticular phenotype (Figure 2D), while that of gsb525 embryos (Figure 2E) is nearly wild-type (Figure 2A) implies that gsb525 is not a null allele [11]. Its hypomorphic nature might be explained in two not mutually exclusive ways: the cuticular function of gsb is provided either by a Gsb525 protein truncated before the homeodomain but including the entire paired domain, or by undetectable levels of wild-type Gsb protein generated by a low probability of read-through at the ochre nonsense mutation. To elucidate this question, we prepared two rescue constructs. gsb0-525 contains the same mutation as gsb525, whereas gsb0-DHC encodes only the truncated Gsb525 protein (Figure 1B). Both of these two constructs are under the control of the gsb upstream region including the gsb cuticle enhancers GEE and GLE [26]. Evidently, only gsb0-525 can rescue the cuticle phenotype (Figure 2G), whereas gsb0-DHC cannot (Figure 2H). This demonstrates that in gsb525 embryos an undetectable level of wild-type Gsb protein is produced that is nearly enough to rescue the cuticular function of gsb.

gsbP1155 is also an interesting allele. It is an insertion of a P element located only 54 bp upstream of the gsb transcription start site (Figure 1C). This P-element insertion leads to largely reduced gsb mRNA and protein levels in homozygous embryos. While these mutants show a wild-type cuticular phenotype (Figure 2F) and

Figure 1. Locus of the gsb gene. (A) gsb mutant alleles. The two deficiencies, Df(2R)IIX62 and Df(2R)SB1, as well as the two hypomorphic alleles, gsb525 and gsbP1155, are depicted. Neighboring genes uncovered by Df(2R)IIX62, zip, zip, CG3441, and gsbn upstream of gsb, gol and dTKR downstream of gsb, and their direction of transcription are indicated (the right telomere of the second chromosome is to the right). Exons are marked by black boxes in the enlarged portion of (A) and also in (B). (B) Map of gsb-525 and gsb-DHC transgenes. Both transgenes contain the upstream epidermis enhancers of gsb, GEE and GLE (Fig. 1A; Li et al., 1993), the gsb promoter, and the entire 3’ UTR of gsb. In gsb-DHC, 519 bp of coding region between the gsb525 mutation and a SacII site are deleted, resulting in a shift of the open reading frame after the gsb525 nonsense mutation. (C) Sequence surrounding the gsbP1155 insertion site. The negative numbers refer to nucleotides upstream of the transcription start site. The eight nucleotides, duplicated during insertion of the P-element, are underlined. doi:10.1371/journal.pone.0030980.g001
Conservation of Drosophila gooseberry Functions

Figure 2. Cuticular phenotypes of gsb mutants. (A) Df(2R)IIX62/CyO, (B) Df(2R)IIX62/CyO, (C) Df(2R)IIX62/Df(2R)KrSB1, (D) Df(2R)IIX62/gsb525, (E) gsb525, (F) gsbP1155, (G) Df(2R)IIX62; gsb525, (H) Df(2R)IIX62; gsb0-ΔHC. Note in strong gsb mutants (B, C), the ventral naked cuticle region of each segment is transformed into denticle belt, generating an overall denticle pattern, which is in contrast to wild-type (A). Scale bar: 50 um. doi:10.1371/journal.pone.0030980.g002

Generation of gsb-Prd and gsb-Pax3 transgenic flies

Previous work demonstrated that a gsb rescue construct, gsb-res, was able to perform all the known gsb functions and rescue gsb mutants to adulthood [9,11], which suggests that all essential gsb enhancer elements are included in this gsb transgene (Figure 3). To examine whether and to what extent the Prd and Pax3 proteins are able to substitute for the normal functions of Gsb, two rescue constructs, namely gsb-Prd and gsb-Pax3, were obtained by replacing the gsb coding region in gsb-res by that of prd and Pax3, respectively (Figure 3). Transgenic files were generated by P-element-mediated transformation in the Drosophila germlines [31]. Several independent lines were obtained for each construct. Only transgenic lines that were homozygous viable were selected for further investigation.

It has been previously shown that in wild-type embryos, Gsb protein is initially expressed during blastoderm at the end of cellularization in eight stripes in every other segment, which correspond to the odd-numbered Gsb stripes [9]. At gastrulation, the even-numbered Gsb stripes emerge between the odd-numbered stripes to generate a segmentally repeated expression pattern. Toward the end of germ band extension, Gsb protein reaches its highest levels in the ectoderm and becomes laterally restricted to the neuroectodermal region (Figure 4A). As expected, in gsb-Prd and gsb-Pax3 embryos, the Prd protein (Figure 4B) and Pax3 mRNA (Figure 4C) are expressed in patterns that are indistinguishable from that of endogenous Gsb protein (Figure 4A). At this time of development the endogenous Prd protein is barely detectable in the epidermis [27].

Rescue of gsb target gene expression by gsb-Prd and gsb-Pax3

Previous work has shown that Gsb is required to maintain late wg expression in the ventral epidermis through a wg-gsb autoregulatory loop [8]. In homozygous Df(2R)IIX62 embryos, Wg starts to decay in the ventral epidermis after 6 hours [8] and is no longer detectable at stage 13 (Figure 4D), while it remains expressed in wild-type embryos (Figure 5A). By introducing gsb-Prd or gsb-Pax3 transgenes into such gsb mutant embryos, the Wg expression pattern is fully rescued by one copy of either transgene (Figure 5C, D).

Beginning with stage 9, Gsb is expressed in delaminating neuroblasts, where it is required for the activation of gsbn [9]. This is apparent from a complete loss of Gsbn expression in Df(2R)IIX62/gsb525 embryos at the extended germ band stage (Figure 5F), while Gsbn expression is strongly expressed in the CNS of wild-type embryos at this stage (Figure 5E). Gsbn

Figure 3. Map of gsb-res, gsb-Prd and gsb-Pax3 transgenes. The gsb-res transgene corresponds to the enlarged 20-kb genomic fragment in Fig. 1A, which includes the gsb transcribed region as well as adjacent 14-kb upstream and 3-kb downstream sequences [9]. The upstream sequence also contains the 5’ portion of the gsbn up to part of the third exon. In gsb-Prd and gsb-Pax3 transgenes, the gsb coding region (except of a small region encoding the C-terminus) is replaced by prd and Pax3 DNAs, while upstream and downstream regions are retained. The gsb intron is also retained by inserting it between sequences of the gsb and prd or Pax3 leaders. Coding regions are indicated as black boxes except for the paired-domain (PD) and the prd-type homeodomain (HD) which are hatched. The gsb and gsbn introns are indicated as open boxes. The transcription start of gsb is marked by 0, and poly(A) addition signals AATAAA are indicated. doi:10.1371/journal.pone.0030980.g003
The left and dorsal side up. Embryos were collected from crosses between Df(2R)IIX62/CyO and were stained with β-galactosidase and anti-Wg monoclonal antibodies or rabbit anti-Gsbn antiserum. Embryos stained with β-galactosidase under control of the gsb cis-regulatory region. Expression of Gsb protein in wild-type embryos (yw65A), of Prd protein in transgenic gsb-Prd embryos (B), and of Pax3 mRNA in transgenic gsb-Pax3 embryos (C) at the extended germ band stage. Wild-type embryos were stained with anti-Gsb antiserum and transgenic embryos, collected from homozygous gsb-Prd or gsb-Pax3 stocks, were stained with anti-Prd antiserum or hybridized in situ with digoxigenin-labeled Pax3 cDNA. Unfolded embryos are shown and oriented with their anterior to the left. Scale bar: 100 um.

Figure 5. Rescue of Wg and Gsbn expression in gsb mutant embryos by gsb-Prd and gsb-Pax3 transgenes. Expression of Wg (A–D) and Gsbn (E–H) proteins in wild-type (A, E), homozygous Df(2R)IIX62 (B–D) or transheterozygous Df(2R)IIX62/gsb525 (F–H) gsb mutant embryos carrying no (B, F), one copy of the gsb-Prd (C, G) or gsb-Pax3 (D, H) transgene. Embryos at stage 13 (A–D) or stage 10 (E–H) are oriented with their anterior to the left and dorsal side up. Embryos were collected from crosses between Df(2R)IIX62/CyO, hb-LacZ; gsb-Prd+/+; or Df(2R)IIX62/CyO, hb-LacZ; gsb-Pax3+/ males and Df(2R)IIX62/CyO, hb-LacZ; gsb525/CyO, hb-LacZ; gsb-Pax3/+, and double stained for β-galactosidase and anti-Wg monoclonal antibodies or rabbit anti-Gsbn antiserum. Embryos stained with β-galactosidase have at least one copy of wild-type gsb allele and were used as control (A, E). One quarter of the embryos did not stain for β-galactosidase. Half of these embryos did not express Wg in the ventral epidermis and Gsbn in the CNS as expected for gsb mutants. The other half displayed rescued expression patterns, which suggested the presence of the transgenes. Scale bar: 100 um.

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eexpression in such mutants are rescued by gsb-Prd or gsb-Pax3 transgene, respectively (Figure 5G, H). Taken together, these results demonstrate that Prd and Pax3 proteins can substitute for Gsb function in the transcriptional activation of two essential target genes.

Rescue of gsb- cuticular phenotype by gsb-Prd and gsb-Pax3

One conspicuous feature of the Drosophila larva is the metameric ventral cuticular pattern, which crucially depends in each segment on the products of the segment-polarity genes. Embryos lacking gsb function exhibit a segment-polarity cuticle defect [1], which consists of mirror image duplications of denticle belts into the posterior portions of each segment where naked cuticle would develop in wild-type embryos (compare Figure 6A, B). This phenotype is caused by the loss of late Wg expression, which is required to repress the ubiquitous denticle formation in the ventral epidermis [6]. Consistent with the result that both gsb-Prd and gsb-Pax3 can rescue the late Wg expression in gsb mutants, both transgenes are able to fully rescue the cuticular phenotype of homozygous Df(2R)IIX62/gy65A embryos when present as a single copy (Figure 6C, D). It follows that Prd and Pax3 proteins are able to perform the cuticular function of Gsb.

Rescue of gsb- CNS phenotype by gsb-Prd and gsb-Pax3

In addition to its function in patterning the epidermis, gsb plays an important role in the development of the embryonic CNS [9–12]. Most prominently, posterior commissures (Figure 7A) are missing or reduced in each segment of Df(2R)IIX62/gy65A embryos (Figure 7B). This CNS phenotype can be fully rescued by one copy of the gsb-Prd (Figure 7C) or gsb-Pax3 transgene (Figure 7D), which indicates that Prd and Pax3 proteins are able to replace the Gsb function in the CNS.

Rescue of gsb mutants to adulthood by gsb-Prd and gsb-Pax3

To test if Prd and Pax3 proteins are able to substitute for all Gsb functions, we tested the ability of gsb-Prd and gsb-Pax3 transgenes to rescue gsb mutants to adulthood. For this purpose, a deficiency, Df(2R)IIX62, and two strong alleles of gsb, gsb677 and gsbP1155, were used. Homozygous or heterozygous combinations of these three alleles are lethal during embryogenesis, which shows that gsb is required for postembryonic viability. Although rescue efficiencies are less than half of that of gsb-res, one copy of gsb-Prd or gsb-Pax3 is able to rescue about a quarter of Df(2R)IIX62/gy65A embryos to adulthood (Table 1). For all three transgenes, two copies result in 50% higher rescue efficiencies than one copy (Table 1), which suggests that at least one gsb function required for the viability is dosage dependent. Consistent with this interpretation, one copy of the transgenes is able to rescue a much higher proportion of Df(2R)IIX62/gy65A or gsb677/gyP1155 embryos to adulthood (Table 1). Therefore, both Prd and Pax3 proteins are able to...
gsb is required for male fertility

Since all known gsb mutant alleles are lethal during embryogenesis [1,9,11], the adult functions of gsb remain unknown. Interestingly, most of the Df(2R)IIX62/gsb255 males rescued by one copy of gsb-Prd or gsb-Pax3 are sterile (Table 2), while females are fully fertile (data not shown). Therefore, gsb is endowed with a function that is essential for male fertility. Two copies of gsb-Prd or gsb-Pax3 result in significantly enhanced fertilities of Df(2R)IIX62/gsb255 males (Table 2), which suggests that this male fertility function is also dosage dependent. Consistent with this explanation, one copy of gsb-res rescues fertility in 39% of the Df(2R)IIX62/gsb255 males, while two copies rescue male fertility almost completely (Table 2). In addition, one copy of gsb-Prd or gsb-Pax3 is able to rescue fertility in about half of the Df(2R)IIX62/gsbP1155 males and in three quarters of the gsb255/gsbP1155 males (Table 2), whereas one copy of gsb-res suffices to fully rescue male fertility in these two mutant combinations (Table 2). We conclude that gsb is required for male fertility, a function for which both Prd and Pax3 proteins are able to substitute.

Discussion

Evolutionary alleles of gsb

The Drosophila gsb and prd and mouse Pax3 genes encode transcription factors that share in their N-terminal moieties two DNA binding domains, a paired-domain and a prd-type homeodomain [16–19]. The homology between the N-terminal parts of the three proteins suggests that they were derived from a common ancestor, and thus might have retained some same abilities, despite their divergent C-terminal sequences and apparently distinct developmental functions [32]. Indeed, gsb-Prd and gsb-Pax3, which express Prd or Pax3 protein under the control of the gsb cis-regulatory region, are able to execute all in vivo functions of gsb, though less efficiently. Hence, both Prd and Pax3 may be considered as leaky mutant proteins of Gsb, whereas gsb-Prd and gsb-Pax3 are hypomorphic or ‘evolutionary’ alleles of gsb, as the coding regions of the three genes have been derived from a common ancestral gene during the course of evolution. These two ‘evolutionary’ alleles are weaker than the weakest previously known gsb allele, gsbP1155, which generates a normal cuticular pattern but displays a weak CNS phenotype and is homozygous lethal during embryogenesis [11]. As these two new alleles have uncovered the previously unknown function of gsb required for male fertility, construction of evolutionary alleles may serve as an additional approach to discover unknown functions of a gene [25].

Although the N-terminal portions of the three proteins are rather conserved, their C-terminal parts have diverged to an extent that no obvious similarity in the primary sequences could be perceived [17,19]. Thus, it is particularly interesting that both Prd and Pax3 proteins have retained the potential to perform all the normal functions of Gsb, which suggests that all the important functional motives in the C-terminal part of Gsb have been conserved in the C-termini of Prd and Pax3, presumably in the 3D structures. It follows that the functional diversification of gsb, prd, and Pax3 reside in their cis-regulatory rather than their divergent C-terminal coding regions. Therefore, our results are consistent with, and add further weight to, the hypothesis that the acquisition of new enhancer elements by a gene plays a dominant role in evolution [25,26].

Evolutionary relationship between Gsb, Prd and Pax3 proteins

Our previous work has shown that Pax3 can perform only the cuticle function, but not the viability and male fertility functions of Prd [25,33]. Here we report that Pax3 is able to substitute for all Gsb functions in promoting embryonic CNS and cuticle development, postembryonic viability, and male fertility. Thus, in terms of functional conservation, Pax3 seems to be more closely related to Gsb than to Prd. It follows that Gsb and Pax3 are functionally also closer to the common ancestor than Prd. As an independent test of this conclusion, it would be interesting to see if Gsb is a better substitute for Pax3 functions than Prd.

In support of this hypothesis, Pax3 resembles Gsb better than Prd in primary sequences. For Gsb and Pax3, but not Prd, share an octapeptide that is located between the paired-domain and the prd-type homeodomain [16,19,32]. In addition, Prd possesses near...
survival to adulthood [25]. In addition, overexpression of Gsb displays strong dosage effects for all adult segmentation phenotype, and the cient [36], and overexpression of exhibit strong dosage effects, as most Pax genes encode transcription regulators characterized by the particular for the development of accessory glands [33,34]. Since (copy number of the transgenes or using weaker male fertility. This function appears dosage dependent, as better rescue efficiencies were scored in both cases by two copies of the transgene (Table 1, 2), which indicates a dosage dependence of gsb functions in promoting viability and male fertility. This interpretation was confirmed by the use of two different combinations of gsb mutants, and by two gsb evolutionary alleles, gsb-Prd and gsb-Pax3 (Table 1, 2). A dosage effect was also reported for gsb functions in embryonic cuticle and CNS development, as reflected by differences in penetrance of the cuticle and CNS phenotypes in various combinations of different gsb mutant alleles [11]. Since the hypomorphic gsb mutants, gsb125 and gsb1155, display a normal cuticle but defects in the CNS [11], and one copy of gsb-res is able to fully rescue the CNS phenotype but to rescue the viability and male fertility functions only partially (Table 1, 2) in Df(2R)IX62/gsb125 mutants, the cuticle function is least sensitive while the viability and male fertility functions are most sensitive to a decrease in the level of Gsb activity. The incomplete rescue of the viability and male fertility functions in Df(2R)IX62/gsb125 mutants by one copy of gsb-res may result from two effects. First, the deficiency Df(2R)IX62, which deletes, in addition to gsb, several other genes including gsbn [17,30], which is downstream of gsb, might affect the viability and male fertility. Second, gsb-res expresses Gsb protein at a subnormal level [9,11], which may result from a position effect of the P-element insertion or from the absence of additional gsb enhancer element(s) from the transgene.

The male fertility function of gsb
In addition to its embryonic functions, gsb is also required for the male fertility. This function appears dosage dependent, for better rescue efficiencies were achieved by either increasing the copy number of the transgenes or using weaker gsb mutant alleles (Table 2). gsb may get involved in male fertility via several means.

### Table 1. Rescue of gsb mutant embryos to viable adults by gsb-Prd and gsb-Pax3 transgenes.

| gsb-res (%) | gsb-Prd (%) | gsb-Pax3 (%) |
|-------------|-------------|-------------|
| 1 copy 2 copies | 1 copy 2 copies | 1 copy 2 copies |
| Df(2R)IX62/gsb125 | 62 (238/385) | 96 (194/203) | 21 (90/429) | 31 (104/339) | 27 (88/326) | 41 (96/234) |
| Df(2R)IX62/gsb1155 | 86 (607/707) | nd | 51 (144/284) | nd | 74 (192/250) | nd |
| gsb125/gsb1155 | 99 (344/346) | nd | 61 (230/376) | nd | 77 (226/293) | nd |

Percentage of rescued gsb- flies harboring one or two copies of gsb-res, gsb-Prd or gsb-Pax3 transgenes (actual numbers of rescued flies per total number of expected gsb mutants are given in parentheses). Df(2R)IX62/gsb125 flies carrying one or two copies of the transgenes were obtained as offspring of the crosses between Df(2R)IX62/SM1; P/P (P stands for the transgenes) males and gsb125/SM1 or gsb1155/SM1; P/P females. Df(2R)IX62/gsb1155 and gsb125/gsb1155 flies carrying one copy of the transgenes were obtained from the crosses between Df(2R)IX62/SM1; P/P or gsb125/SM1; P/P males and gsb1155/SM1 females. nd, not determined.

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### Table 2. Rescue of fertility of gsb mutant males by gsb-Prd and gsb-Pax3 transgenes.

| gsb-res (%) | gsb-Prd (%) | gsb-Pax3 (%) |
|-------------|-------------|-------------|
| 1 copy 2 copies | 1 copy 2 copies | 1 copy 2 copies |
| Df(2R)IX62/gsb125 | 39 (36/92) | 91 (20/22) | 9 (2/23) | 30 (6/20) | 15 (3/20) | 35 (6/17) |
| Df(2R)IX62/gsb1155 | 92 (90/98) | nd | 43 (16/37) | nd | 48 (16/33) | nd |
| gsb125/gsb1155 | 95 (74/78) | nd | 77 (23/30) | nd | 75 (21/28) | nd |

Percentage of fertile males among gsb mutant males that were rescued by one or two copies of gsb-res, gsb-Prd or gsb-Pax3 transgenes (actual numbers of fertile males per total number of rescued males are given in parentheses). Rescued males were obtained from crosses described in legend of table 1 and were placed individually with at least three wild-type virgin females in fresh vials to score fertility. nd, not determined.

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First, Gsb plays pivotal role in the development of ejaculatory duct that is required for the transfer of accessory gland secretions and sperm to females during copulation. Ejaculatory duct also secretes components of seminal fluid that might be essential for sperm fertility [39]. Second, Gsb is expressed in the secondary cells of adult accessory glands, suggesting a role of Gsb in the regulation of accessory gland secretions that are crucial for the male fertility [33]. Third, males heterozygous for Df(2R)IIX62, which deletes gsb and its downstream gene gsbn, behave less aggressive in copulation (data not shown). This phenotype can be rescued by adding one copy of gsb-res (data not shown), implying the impaired Gsh-Gsbn pathway is responsible for this behavioral defect. In support of this interpretation, both Gsb and Gsbn are expressed in the leg and antenna imaginal discs [W.L., L.X. and M.N., unpublished observation], suggesting a role of gsb and gsbn in the development of leg and antenna, both of which have been shown to be important for eliciting proper male sexual behavior [40].

Interestingly, prd is also required for male fertility, for prd mutant males rescued by two differently modified prd transgenes, prd-Gsb [25] and prdRes [41], are sterile, despite their capabilities to copulate and transfer sperm to females [33]. These males have severely reduced or no accessory glands [33,41], suggesting prd is essential for accessory gland development. Hence, prd and gsb, though both are required for male fertility, are involved in distinct developmental programs during metamorphosis. Since Gsb is able to substitute for all Prd functions that are required for survival to adulthood [25], but not its male fertility function [33], the male fertility function of Prd might have evolved after its separation from Gsb or have been subjected to strong selection during the course of evolution.

Materials and Methods

Plasmid constructions and generation of transgenic flies

Mutations were introduced into gsb-525 and gsb-ΔHIC by PCR mutagenesis. Taking pKSpl5-Gsb [27] as template, the following primers were used: gsb-8 (5′-GTC TAG AGT AAG CAC GAT CGC GGA CG 3′), gsb-11 (5′-GGG AAT AAA GGC GAT CGC GGA CG 3′), gsb-12 (5′-CTG CCG CGA TCG CCT TTA TTC CC 3′), T3 primer, and T7 primer. Fragments containing the mutations were cloned into gsb-0 [27], the gsb complete leader region and intron were also recovered.

The gsb-Prd and gsb-Pax3 constructs were derived from gsb-res [9] in three steps. First, the 1-kb gsb intron was obtained as a PCR product with the primer gmi1 (5′-GTC TAG AGT AAA GGC GAT CGC GGA CG 3′) and gmi2 (5′-GTC TAG ACT GGA AGT AGA GAA ACA 3′), digested with XbaI and inserted into the SpeI site of pKSpl5-Prd and pKSpl5-Pax3 [27] to generate pKSgint-Prd and pKSgint-Pax3, respectively. Subsequently, the 3.4-kb XbaI fragments from pKSgint-Prd and pKSgint-Pax3 were cloned into the AccII site of gsb-0 to produce gsb-int-Prd and gsb-int-Pax3. Finally, gsb-Prd and gsb-Pax3 were constructed by replacing the 3.6-kb NheI-Xhol fragment in gsb-res with the corresponding fragments from gsb-int-Prd and gsb-int-Pax3, respectively.

The gsb-Prd and gsb-Pax3 constructs were injected together with pUChspA2-3 helper plasmid into ry196 embryos and ry+ transformants were selected.

Immunostaining and in situ hybridization of embryos

Embryo collection, fixation, and immunostaining were carried out as described [28]. Polyclonal antibodies against Prd (1:500) [28] Gsb, and Gsbn (1:1000) [9], monoclonal antibody against Wg (1:100) [29], and monoclonal antibody BP102 (1:50), which reveals the patterns of the longitudinal and commissural axons in the CNS [11], have been described. Polyclonal anti-β-galactosidase antibody (1:1000) was obtained from Cappell.

In situ hybridization with digoxigenin-labeled Pax3 cDNA was performed essentially as described [25].

Cuticle preparation

Embryos were collected and allowed to develop for 24 h at 25°C before cuticles were prepared as described [1].

Fly strains and rescue experiments

Three gsb alleles were used in this work: Df(2R)IIX62, a gsb null allele that deletes gsb, gsbn, and five additional genes [17,30]; gsb525+, a strong hypomorphic allele in which the first amino acid of the homeodomain is converted to a stop codon [11]; and gsbP1153+, a hypomorphic allele with a P-element inserted into the gsb promoter region [11]. To rescue the cuticle, CNS, viability, and male fertility functions of gsb by the transgenes, we used the following fly stocks: (1) Df(2R)IIX62/SM1, (2) gsb525+/SM1, (3) gsbP1153+/SM1, (4) Df(2R)IIX62/SM1; gsb-res, (5) gsb525+/SM1; gsb-res, (6) Df(2R)IIX62/SM1; gsb-Prd, (7) gsb525+/SM1; gsb-Pax3, and (8) Df(2R)IIX62/SM1; gsb-Pax3, and (9) gsb525+/SM1; gsb-Pax3.

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Author Contributions

Conceived and designed the experiments: LX. Performed the experiments: WL LX. Analyzed the data: WL LX. Wrote the paper: WL LX.

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