The RED domain of Paired is specifically required for *Drosophila* accessory gland maturation

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1. Summary

The evolutionarily conserved paired domain consists of the N-terminal PAI and the C-terminal RED domains, each containing a helix–turn–helix motif capable of binding DNA. Despite its conserved sequence, the physiological functions of the RED domain remain elusive. Here, we constructed a *prd* transgene expressing a truncated Paired (Prd) protein without the RED domain, and examined its rescue ability in *prd* mutants. We found that the RED domain is specifically required for the expression of Acp26Aa and sex peptide in male accessory glands, and the induction of female post-mating response. Our data thus identified an important physiological function for the evolutionarily conserved RED domain.

2. Introduction

The *Drosophila* gene paired (*prd*) belongs to the pair-rule gene family that exhibits a pair-rule expression pattern and participates in the determination of anterior–posterior axis during early embryogenesis [1–4]. Together with other pair-rule genes, *prd* regulates the expression of segment-polarity genes, including *wingless* (*wg*), *gooseberry* (*gsb*) and *engrailed* (*en*) [3,5–7]. In addition to the embryonic functions, *prd* is necessary for postembryonic viability, and male fertility through the regulation of accessory gland (AG) development [8–12]. The *Drosophila* male AG is a pair of dead-end tubes composed of a single-cell layer of two kinds of secretory cells: the ‘main cells’ spreading all over the lumen of the glands, and the ‘secondary cells’ restricted only to the distal region of each tube [13]. The AG is a secretory organ that is functionally analogous to the human prostate and seminal vesicle whose secretions (seminal fluid), together with sperm from the testes, play important roles in regulating female post-mating response (PMR) [11,13–17].

*prd* is the founding member of the Pax genes, which encode an evolutionarily conserved family of transcription factors with multiple DNA binding motifs and play key roles in animal development [18–21]. All members of the Pax family are defined by the presence of a highly conserved 128-amino-acid paired domain (PD) [22], which was first identified in Paired (Prd) [23]. The crystal structure demonstrates that PD is a bipartite module, which is further divided into the N-terminal PAI and the C-terminal RED subdomains (PAI + RED = PD) [24]. Each subdomain contains a helix–turn–helix (HTH) motif and has the ability to bind to DNA independently [24,25]. In addition to PD, some Pax proteins also contain a paired-type homeodomain (HD). According to the different combinations of these domains, the Pax proteins are classified into five subgroups [26]. Prd is homologous to the mammalian Pax3/Pax7 subgroup containing both a complete PD and an intact HD. It has been reported that both HD and PAI subdomain are simultaneously required within the same molecule to execute the early embryonic pair-rule function of Prd [8], whereas the RED subdomain appears dispensable for these functions either in vitro [27] or in vivo [8].
To investigate whether the RED subdomain is important for other Prd functions, we introduced a transgene, **prd-PrdΔPBC**, which encodes a truncated Prd protein with a deletion of the RED domain under the control of the complete cis-regulatory region, into a **prd** null mutant background. We found that the RED domain is dispensable for most Prd functions, including embryonic segmentation, postembryonic viability, AG development and male fertility, but is specifically required for the expression of Acp26Aa (also called Ovulin) and sex peptide (SP), two seminal fluid components essential for the induction of female PMR. Consequently, **prd** mutant males rescued by **prd-PrdΔPBC** failed to induce increased egg laying and decreased receptivity in wild-type females. Thus, we have characterized a specific function for the RED domain of Prd protein, which has shed light on the evolution of Pax genes.

### 3. Results and discussion

#### 3.1. The RED domain is dispensable for the embryonic functions of Prd

**prd**, initially identified as a pair-rule gene involved in embryonic segmentation, has been shown to perform multiple functions in development, including: (i) activation of segment-polarity genes and proper segmentation of the larval cuticle [5–7]; (ii) postembryonic viability [10]; (iii) AG development [10]; and (iv) male fertility [10,12]. A **prd** transgene consisting of the entire **prd** coding region as well as the full cis-regulatory elements, **prd-Prd**, was able to rescue all **prd** functions in **prd** mutants (figure 1a) [28]. To examine the specific contribution of RED domain to Prd functions in vitro, we introduced another transgene, **prd-PrdΔPBC**, into **prd** mutants and examined its ability to rescue the mutant phenotypes (figure 1a). **prd-PrdΔPBC** replaces the Prd coding region in **prd-Prd** by a truncated version with a deletion of the RED domain (amino acids 75–125, figure 1a). According to the crystal structure [24] and the in vitro experiments [27], this deletion of the RED domain should not affect the DNA-binding property of the PAI domain [24].

The **prd** mutants used in this study are heterozygous for the deficiency **Df(2L)Prl** and the **prd** allele, which has a 1.1 kb insertion after amino acid 45 of the **Prd** and acts as a null allele [29]. Compared with the heterozygous control (figure 1b), **prd** mutants lose half of the segmental equivalents and exhibit the classical pair-rule phenotype in larval cuticle (figure 1c) [1]. We found that **prd-PrdΔPBC** could fully rescue the cuticle phenotype of **prd** mutants (figure 1d), indicating that the RED domain is not essential for the cuticle function of Prd.

Prd is required for the activation of segment polarity genes in every other parasegment in early embryos. In **prd** mutant embryos, the expression patterns of Gsb, Wg and En are abolished with a double-segment periodicity (figure 1f,j,l) when compared with the control (figure 1e,l,k). Consistent with its ability to rescue the **prd** mutant cuticle phenotype, **prd-PrdΔPBC** is able to fully rescue the expression patterns of Gsb, Wg and En (figure 1g,i,m), demonstrating that the RED domain is dispensable for Prd to activate the transcription of segmental polarity genes in embryonic development.

We have shown in previous work that an evolutionary allele of **prd**, **prd-Pax3**, in which the Prd mouse homologue Pax3 is placed under the control of the entire **prd** cis-regulatory region, is able to rescue larval cuticles and target segment-polarity genes expression, but not the embryonic lethality, in **prd** embryos [10]. Therefore, **prd** has a viability function independent of its cuticle functions to ensure the survival of embryos to adults [9,10]. Furthermore, **prd** embryos rescued by two copies of **prd-Gsb**, another evolutionary allele of **prd**, are able to develop into adulthood, yet some of the adult flies display a distorted segment phenotype in the abdominal cuticle [10], suggesting a role of Prd in regulating adult segmentation. We found that **prd-PrdΔPBC** is able to rescue the lethality in **prd** mutant embryos to a similar extent as that of **prd-Prd**, and there is no significant difference when compared with that of heterozygous controls (figure 2a), demonstrating that the RED domain is unnecessary for the viability function of Prd. However, **prd** mutant flies rescued by **prd-PrdΔPBC** produce a distorted segmentation phenotype in adult cuticles (electronic supplementary material, figure S1b,e), which phenocopies that of **prd** mutants rescued by **prd-Gsb** [10], suggesting that the RED domain contributes to the adult cuticle function of Prd.

#### 3.2. The RED domain is dispensable for the male fertility function of Prd

Previous work reported that another transgene, **prd-Res**, in which the downstream sequence of **prd-Prd** is deleted, is sufficient to rescue **prd** mutants to adulthood, yet all males are sterile [8]. Additional studies confirmed that **prd** is required for the development of AGs, which secrete seminal fluid necessary for *Drosophila* male fertility [8,9,11,12]. The male fertility function of **prd** depends on a 0.8 kb **Prd** male fertility enhancer (PMFE) located in the **prd** downstream region [12], as **prd** mutant males rescued by **prd-Res**, which does not include PMFE, produces a loss of AG phenotype (figure 2f) [9,12]. We then examined the rescue ability of **prd-PrdΔPBC** in **AG** development and male fertility in **prd** mutants. We found that **prd-PrdΔPBC** could rescue AG development to a similar extent as that of **prd-Prd** (figure 2g,h). Furthermore, about 70% of **prd** mutant males rescued by **prd-PrdΔPBC** are fertile, which is not statistically different from the case with **prd-Prd** (figure 2h). Hence, we conclude that the RED domain is dispensable for the male fertility function of Prd.

However, **prd** males rescued by **prd-PrdΔPBC** display a significantly reduced fecundity as they produce far less progeny than the heterozygous controls or **prd** males rescued by **prd-Prd** (figure 2c,d), suggesting the RED domain, though not necessary, still contributes to the male fertility. Prd is known to play a later function in AG maturation by regulating the expression of a variety of AG products, including SP and Acp26Aa, which stimulate female egg laying [12]. As shown below, these factors are significantly reduced in the AGs of **prd** mutant males rescued by **prd-PrdΔPBC**, which might account for the reduced number of eggs/progeny produced by their mates.

In addition, we also noted that **prd** males rescued by **prd-PrdΔPBC** exhibited a lower mating success rate compared with those rescued by **prd-Prd** (electronic supplementary material, figure S2a), yet there is no significant difference in the climbing ability between these two groups of flies (electronic supplementary material, figure S2b), suggesting factors other than the locomotion skill are responsible for the different copulation outcome.
Figure 1. The RED domain is dispensable for the embryonic functions of Prd. (a) Schematic of the coding structure of prd-Prd and prd-PrdΔPBC, and their ability to rescue Prd functions in Drosophila development. The rescue ability is scored by + if the transgene is sufficient for rescue or by − if no rescue is obtained. (b–d) The cuticle of a prd+/− (b), or a prd−/− (c) or a prd−/−; prd-PrdΔPBC/+ (d) embryo is shown under dark-field illumination with anterior up. prd-PrdΔPBC is able to rescue the cuticle phenotype in prd− (d). Expression patterns of Gsb (e–g), Wg (h–j) and En (k–m) in prd+/− (e,h,k), prd−/− embryo carrying no (f,i,l) or one copy of prd-PrdΔPBC (g,j,m) are shown during the extended germ band stage of embryogenesis. Embryos are oriented with their anterior to the left and dorsal side up. Expression of Gsb, Wg and En in prd mutants (f,i,l) is fully rescued by prd-PrdΔPBC (g,j,m).
Reproduction is one of the fundamental missions of life. Hence, many species have evolved an intricate variety of mechanisms to guarantee the success of procreation. In most insects, including Drosophila melanogaster, male-derived substances transferred during mating induce significant behavioural changes, so-called PMR, leading to an increased egg laying and decreased sexual receptivity in their mated partners [15,30–32].

Virgin females exhibit a high receptivity (figure 3a) and low rejection (figure 3b) towards males, and lay less than 10 eggs per day (figure 3c). The females show dramatically increased oviposition (up to about 50 eggs within 24 h) after mating to control males, and maintain this rate in the next few days with a gradual reduction (figure 3c). Meanwhile, mated females display a low receptivity (figure 3a) and high rejection (figure 3b) towards males after first copulation. However, females mated to prd mutant males rescued by prd-PrdΔPBC behave like virgins with low egg-laying (figure 3c), high receptivity (figure 3a) and low rejection (figure 3b). In contrast, prd mutant males rescued by prd-Prd are able to elicit PMR in their mates to a similar extent as control males (figure 3a–c). To rule out the possibility that the rescue failure of prd-PrdΔPBC is due to its lower expression level, we checked the transgene expression in male AGs by qRT-PCR, and found that prd-PrdΔPBC was expressed at a level comparable with that of prd-Prd or endogenous prd (figure 4g). Furthermore, adding another copy of prd-PrdΔPBC to prd mutant males does not improve PMR in their mates (electronic supplementary material, figure S3a–c).
Figure 3. The RED domain is imperative for female post-mating response (PMR). (a,b) Females mated with prd−/−; prd-PrdΔPBC/+ males exhibit a virgin-like behaviour with a high receptivity (a) and low rejection (b) to second mating, whereas those that copulated with the heterozygous controls or prd+/−; prd-PrdΔ/+ males exhibit an opposite behaviour with a low receptivity (a) and high rejection (b). (a) The percentage of virgin females (17/20) mated to naïve w1118 males within 1 h or the percentage of non-virgin females previously mated to prd+/− (2/20), prd−/−; prd-PrdΔPBC/+ (19/20) or prd+/−; prd-PrdΔ/+ (3/20) males successfully re-mating within 1 h. (b) The percentage of virgin females (2/20) not mated within 2 h or the percentage of non-virgin females previously mated to prd+/− (16/20), prd−/−; prd-PrdΔPBC/+ (1/20) or prd+/−; prd-PrdΔ/+ (14/20) males not re-mated within 2 h. For statistical analysis: *p < 0.05; **p < 0.01; n.s., not significant. (c) Egg-laying of virgin females or females mated to (i) prd−/−; (ii) prd−/−; prd-PrdΔPBC/+ or (iii) prd+/−; prd-PrdΔPBC/+ males (n = 20, respectively). Females mated with heterozygous controls or prd+/−; prd-PrdΔ/+ show dramatic increase in egg-laying that lasts for a few days with a gradual reduction. However, females mated with prd−/−; prd-PrdΔPBC/+ males fail to trigger the increased oviposition and lay few eggs per day, as do virgin females. The statistical analysis of egg laying using one-way ANOVA followed by Bonferroni’s multiple comparison test is shown on the right. Significant differences are indicated as *p < 0.05; **p < 0.01; ***p < 0.001.

3.4. The RED domain is necessary for Prd to activate the expression of Acp26Aa and sex peptide in accessory glands

Female PMRs have been correlated with substances derived from the male AG [32,33], and among them Acp26Aa and SP have been extensively studied. Meanwhile, a dual role of prd in male AG development has been characterized [12]. Prd is required at an early developmental stage to promote cell proliferation and later for the expression of secretions, including Acp26Aa and SP, to ensure the maturation of the AG [12]. Acp26Aa stimulates the release of oocytes from the ovary [16], which is the first step of the egg-laying process essential for the initial increase of oviposition in females after mating [34]. Recent research indicated that Acp26Aa increases ovulation and egg laying through the OA neuronal signalling [35]. SP, a key component of AG secretions that is transferred into females with sperm, downregulates the excitability of SP sensory neurons (SPSNs) in the female reproductive tract and their input onto SAG neurons of the abdominal ganglion, which results in an increased oviposition and reduced sexual receptivity [36–38]. SP also binds to the sperm and induces a prolonged PMR in females by a gradual release from the sperm stored in mated females [39].

Owing to the virgin-like behaviour of females copulated to prd mutant males rescued by prd-PrdΔPBC (figure 3a–c and electronic supplementary material, figure S3), we examined the expression of Acp26Aa and SP in male AGs. In control AGs, Acp26Aa is expressed in the whole organ (figure 4a), whereas SP is detected only in the main cells by using a sp-LacZ reporter transgene (figure 4d) [12]. Although prd-PrdΔPBC is able to rescue AG development and male fertility in prd mutant males, it fails to rescue the expression pattern of Acp26Aa (figure 4b) and SP (figure 4e), when compared with prd-Prd (figure 4c,f). These results were confirmed by qRT-PCR assay (figure 4g). Thus, we conclude that the RED domain is indispensable for Prd to activate the expression of AG secretions, which are essential for PMR but not for male fertility.

Gsb, first identified as a member of segment-polarity genes [1] and activated by Prd during embryogenesis [3,40], is required for the establishment of positional information along the anterior–posterior axis in the epidermis. In addition, Gsb also plays an important role in the Drosophila central nervous system (CNS), including the specification of embryonic neuronal cell fate [41–45] and the maintenance of postembryonic synaptic homeostasis [46]. We previously found that Gsb is specifically expressed in the secondary cells located in the distal region of each AG tube (electronic supplementary material, figure S4a) [12], though the function of Gsb in the AG has remained unknown. We found that Gsb expression was rescued in prd− males by prd-Prd, but not prd-PrdΔPB (electronic supplementary material, figure S4b,c), which is
confirmed by qRT-PCR assay (electronic supplementary material, figure S4d). Thus, the RED domain is also required for Prd to activate gsb expression in the AG.

It was proposed that the RED domain within the C-terminal of PD, though containing a functional DNA-binding motif, is dispensable for the physiological functions of Prd. In this study, we demonstrate that the RED domain is dispensable for most functions of Prd in development, yet it is specifically required for Prd to activate the expression of AG products Acp26Aa and SP, and thus to trigger PMR in mated females. However, the underlying mechanism by which the RED domain modulates Acp26Aa and SP expression, either through a direct transcriptional activation by binding to the cis-regulatory region or indirectly, requires further investigation.

4. Material and methods

4.1. Fly strains

All flies were raised on standard Drosophila media and maintained at 25°C. prd^{2.45}, Df(2L)Prl, prd-Prd [9] and prd-Res [12] were described previously. Prd-PrdΔPBC was produced by inserting the coding sequence from hs-PrdΔPBC [47] into prd-0 [10]. The plasmid was injected with Δ2–3 helper plasmid into ry^{506} embryos and ry^{+} transformants were selected. All the strains are in the same ry^{506} background.

4.2. Immunostaining of embryos and cuticle preparation

Embryo collection, fixation and immunostaining were carried out as described [48]. Double-labelling of embryos for β-galactosidase and Gsb, Wg or En protein was performed as described [49]. Cuticles were prepared essentially as described [8].

4.3. Dissection, immunostaining and X-Gal staining of male accessory glands

AGs were dissected [11] and stained with anti-Acp26Aa [50] and anti-Gsb [45] as described. X-Gal staining was performed according to Bertram et al. [13].

4.4. Male fertility, fecundity, egg laying and receptivity assay

All flies were aged for 3 days after eclosion and then analysed. For male fertility assay, w^{1118} females, which were successfully mated by (i) prd^{2.45}/+, (ii) Df(2L)Prl/prd^{2.45}; prd-PrdΔPBC/+...
or (iii) Df(2L)PrP/prd²⁴⁵; prd-Prd/+ males, were maintained separately on standard medium. The male was scored as fertile or sterile depending on the presence or absence of offspring, respectively. The data were calculated and presented as the ratio of fertile males. For male fecundity assay, each cross was of two virgin w¹¹¹⁸ females placed by one (i) prd²⁴⁵/+; (ii) Df(2L)PrP/prd²⁴⁵; prd-PrdΔPBC/+ or (iii) Df(2L)PrP/prd²⁴⁵; prd-Prd/+ male and transferred into fresh vials with standard media every 24 h for 10 days. The number of progenies produced per vial was scored (figure 2c), and also the total number in 10 days was calculated (figure 2d). For egg laying, virgin or none virgin (successfully mated by the indicated males) w¹¹¹⁸ females were transferred into fresh vials with standard media every 24 h and allowed to lay eggs for 5 days. The number eggs laid by each individual female was scored every day. For receptivity assay, virgin or none virgin (successfully mated by the test males) w¹¹¹⁸ females were housed individually for 24 h after first mating and then examined in a receptivity assay with naive w¹¹¹⁸ males. The receptivity was classified as remating within 1 h, and rejection was categorized as no remating within 2 h, respectively. Final data were calculated as the ratio of each classification.

4.5. Rapid iterative negative geotaxis (climbing) assay

A modified version of Nichols’s climbing assay was used [51] (electronic supplementary material, figure 2b). Briefly, 2-day-old flies were collected separately by gender within 24 h after eclosion. Ten to 15 flies per genotype were placed in a vertical vial (20 cm height, 2.5 cm diameter), and the vials were tapped at the bottom regularly. A picture was taken 5 s after each tapping and the average heights of flies climbing were calculated. Each analysis was repeated five times with 60 s resting intervals. The number of flies tested per genotype was n = 30 for females or n = 15 for males.

4.6. Statistical analysis

Statistical analysis of viability, male fecundity and egg laying assays was performed using one-way ANOVA followed by Bonferroni’s multiple comparison test. Statistical analysis of male fertility, receptivity and rejection assays was performed using Fisher’s exact test.

4.7. qRT-PCR

About 10 AGs were collected from 1-day-old males of indicated genotypes, and RT-PCR was performed as previously described [52]. Total RNA was extracted using the Ambion PureLink RNA mini kit according to the manufacturer’s instructions. Primers used for qRT-PCR are as follows:

| Primer Name | Forward Sequence | Reverse Sequence |
|-------------|------------------|------------------|
| actin88F    | sense 5'-ATCGAGCACGGGATCATAC-3' | antisense 5'-CAGCGGCGACTTGTTGTAA-3' |
| paired      | sense 5'-CAGTACGACCAATTCC-3' | antisense 5'-ACCCGGCAATTAGTACTGCG-3' |
| Acp26Aa     | sense 5'-TCAGATCTACAAAGAGCTG-3' | antisense 5'-ACGTGCTGCTCTGAAACTG-3' |
| SP          | sense 5'-GAATGGCCTGGAATAGGAA-3' | antisense 5'-GGCAACACTTACACGGGATT-3' |
| gbb         | sense 5'-ATGACAATCTTCTGGCGG-3' | antisense 5'-TGCTGCCATTCCTCACAGATT-3' |

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material.

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Conflict of interest. The authors declare no conflict of interest.

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