Supporting Information

Detailed Materials and Methods

Animal rearing and PRG-ortholog isolation

0-1 day AEL embryos (30 °C) were homogenized in TRIzol (Invitrogen) using a disposable RNase-free plastic tissue grinder (Fisher Scientific). Total RNA was extracted using a Qiagen RNeasy mini kit (Qiagen). After genomic DNA removal with RNase-free DNase (Qiagen), total RNA was purified using RNeasy spin columns. RNA quality and concentration were determined by spectrophotometry and gel electrophoresis. Embryonic cDNA was prepared using a QuantiTect Reverse Transcription kit (Qiagen). Primers were generate to amplify the following motifs: ***eve***, Homeodomain; ***odd***, Zn-finger double domain and RNA helicase (UPF2 interacting domain); ***run***, Runt domain; ***h***, basic helix-loop-helix; ***slp***, fork head domain; ***opa***, Zn-finger domain; ***ftz***, homeodomain (Ftz-specific N-terminal arm and conserved region QIKIWFQN); ***ftz-f1***, (conserved motifs in first Zn-finger, FF1 box and AF2 domain). After a gene-specific region had been isolated by degenerate PCR, 3' RACE was performed to isolate additional gene sequence using the FirstChoice RLM-RACE kit (Ambion). Generally, two rounds of PCR were performed to increase specificity. Additional amplification was performed if necessary to isolate sequence at least to the stop codon and usually including 3'UTR. Fig. S8 shows the schematic representation of the genes. Restriction enzyme cutting sites were also included at the 5' end of both primers for insertion into KS vectors. These KS-based vectors were used as templates for *in situ* probe and dsRNA synthesis.

RNAi

We have described detailed methods for RNAi in Dermestes previously (Xiang et al., 2016). Those methods were used for the current study. DNA templates for dsRNA synthesis were amplified from KS vectors described above,

with gene specific primers containing the T7 promoter sequence at both 5' ends. Complementary ssRNA was synthesized using MEGAscript T7 Transcription kit (Ambion) and then annealed to make dsRNA. After ethanol precipitation, dsRNA was dissolved in injection buffer (0.1 mM NaH_2PO_4, 5 mM KCl, pH 6.8). Data reported in Table S1 were compiled from embryos laid on the 3rd and the 4th day after dsRNA injection, as frequency of phenotypes decreased dramatically after the 5th day (see also (Xiang et al., 2015).

Gene expression in embryos and ovaries

Overnight embryo collections were used to perform *in situ* hybridization, using methods previously described (Xiang et al., 2015). Phenotypic analysis of embryos and larvae was carried out as described (Xiang et al., 2016). For antibody staining and SYTOX Green nuclear staining, after eggshell removal, fixed embryos were incubated in primary antibody at 4°C overnight using either mouse 4D9 anti-Engrailed 1:5, (Developmental Studies Hybridoma Bank), rabbit anti-cleaved *Drosophila* Dcp-1 1:100 (Cell signaling, #Asp216) or rabbit anti-phospho Histone H3 (PH3) 1:200 (EMD Millipore, #06-570) followed by the appropriate secondary antibody at room temperature for 2 hours (biotinylated anti-mouse antibody 1:500 (Vector Laboratories; #BA-9200); biotinylated goat anti-rabbit 1:200 (Vector Laboratories; #BA-1000); Alexa Fluor 488 goat anti-rabbit 1:200 (Thermo Fisher Scientific; #A11008). SYTOX Green staining was performed by 20 min room temperature incubation in SYTOX Green 1: 1000 (Thermo Fisher Scientific; #S7020). Blocking with 1% BSA and 1% NGS in PBS for one hour at room temperature was included for Dcp-1 and PH3 antibody staining. For analysis of ovarian morphology, ovaries were dissected from injected females and transferred to cold PBS immediately. After 20 min fixation in 4% PFA and several PBS/PBST washes, ovaries were incubated in Alexa Fluor 488 phalloidin 1:200 (Life technologies, #A12379) at 4 °C overnight. Ovaries were then placed on slides in 70% glycerol with DAPI and visualized with Leica 501007 microscopy.

qPCR methods

0-6 hour AEL embryos from 3' dsRNA-injected females were collected on the 3rd day after injection and aged for another 2 hours at 30 °C to reach blastoderm to germband stages (Xiang et al., 2015) RNA was extracted as described above. cDNA synthesis was performed using an iScript cDNA synthesis kit (BioRad) following manufacturer's instructions. 1 µg of total RNA was used for each reverse transcription reaction. To avoid amplifying dsRNA instead of endogenous mRNA transcripts, primers used for amplification did not completely overlap the RNAi target regions. All amplicons' sizes were below 200 bp. Two reference genes, COI and 16s rRNA, were included when performing qPCR reactions to normalize Ct values. A 1:10 dilution of cDNA generated from each 20 µl reverse transcription reaction was used as template for qPCR using a Roche LightCycler 480 with SYBR Green Master Mix with settings: 95 °C incubation, 10 min; 60 °C annealing temp. Abs Quant/Fit Points analysis was used to calculate Ct value. To calculate relative expression of the gene of interest in pRNAi offspring to *gfp* pRNAi offspring, we used the $2^{-\Delta\Delta Ct}$ method. Results are shown in Figure S4.

Table S1. Summary of egg yield, hatch count and penetrance after *Dermestes* PRG RNAi.

	Average egg yield per female	Hatch count*	Penetrance (hatched larvae with segmentation defects)
gfp dsRNA control 1st	24	83.7% (241/288)	0% (0/241)
gfp dsRNA control 2nd	33.7	46.6% (220/472)	0% (0/220)
eve 5' dsRNA	33.5	16.0% (96/602)	51.0% (49/96)
eve 3' dsRNA	44.2	24.0% (212/884)	12.7% (27/212)
odd 5' dsRNA	39.7	11.8% (84/714)	20.2% (17/84)
odd 3' dsRNA	40.2	13.7% (110/803)	23.6% (26/110)
run 5' dsRNA	32.0	10.2% (72/703)	48.6% (35/72)
run 3' dsRNA	44.3	23.7% (189/797)	29.6% (56/189)
h 5' dsRNA	26.2	44.5% (373/839)	0.8% (3/373)**
h 3' dsRNA	40.0	27.2% (239/879)	12.6% (30/239)
slp 5' dsRNA	35.1	14.6% (113/773)	44.2% (50/113)
slp 3' dsRNA	32.2	14.2% (55/386)	85.5% (47/55)
opa 5' dsRNA	30.0	86.5% (416/481)	0.7% (3/416)
opa 3' dsRNA	35.0	82.5% (520/630)	1.0% (5/520)
ftz 5' dsRNA	28.6	83.2% (476/572)	0.4% (2/476)
ftz 3' dsRNA	35.1	81.3% (570/701)	0.4% (2/570)
ftz-f1 5' dsRNA	4.9	57.7% (45/78)	0% (0/45)
ftz-f1 3' dsRNA	0.2	0% (0/2)	NA
gfp/*gfp* double	26.2	75.2% (394/524)	0.51% (2/394)
gfp/*slp* 3' double	34.25	13.7% (94/685)	30.85% (29/94)
prd 3'/*slp*3' double	30.35	8.7% (53/607)	43.4% (23/53)

**Dermestes* hatched larvae slaughter unhatched larvae. Hatch count above ~ 45% is considered as normal.

**Combined data from all four daily collections

Table S2. Summary of PRG expression, PRG mutant phenotypes or defects after RNAi knockdown in *Drosophila*, *Tribolium* and *Dermestes*.

		eve		***run***		***odd***		***h***		***prd***		***slp***		***ftz***		***ftz-f1***		***opa***	
		Expr.	**Func.**	**Expr.**	**Func.**	**Expr.**	**Func.**	**Expr.**	**Func.**	**Expr.**	**Func.**	**Expr.**	**Func.**	**Expr.**	**Func.**	**Expr.**	**Func.**	**Expr.**	**Func.**
Simultaneous segmentation	***Drosophila* (long-germ)**	PR-SS	PR	PR-SS	PR	PR-SS	PR	PR	PR	PR-SS	PR	PR-SS	PR	PR	PR	U	PR	U-SS	PR
Sequential segmentation	***Tribolium* (short-germ)**	P-PR-SS	PR?&T	P?-PR	PR?&T	P-PR-SS	PR?&T	PR-SS	NS	PR-SS	PR	PR-SS	PR	PR	NS	PR	PR	B-SS	NS
	***Dermestes* (intermediate-germ)**	P-PR-SS	PR&T	P-PR	PR&T	P-PR-SS	PR&T	PR-SS	D	PR-SS	PR	PR-SS	PR	PR	PR (m)	PR	NS	U-B-SS	NS

Abbreviations: **Expr.**, expression; **Func.**, function; **PR**, pair-rule pattern or defect; **SS**, segmental striped pattern; **P**, cap-like dynamic posterior expression; **U**, ubiquitous expression; **B**, broad posterior expression; **T**, truncated defect; **D**, disrupted segmentation; **NS**, no segmentation defects in trunk; (m), the defects are mild - indicates that an early expression pattern resolves into a later expression pattern. For example, P-PR-SS indicates that dynamic posterior expression resolves into PR striped expression. Later, secondary segmental stripes appear. & indicates that different defects were reported in one study.**?** indicates that the pattern was mentioned by personal communication (SJ Brown) but not reported in literature. Note that for expression pattern, only segmentation-related patterns are listed here. Many PRG orthologs have head or CNS expression, which are not included in this figure.

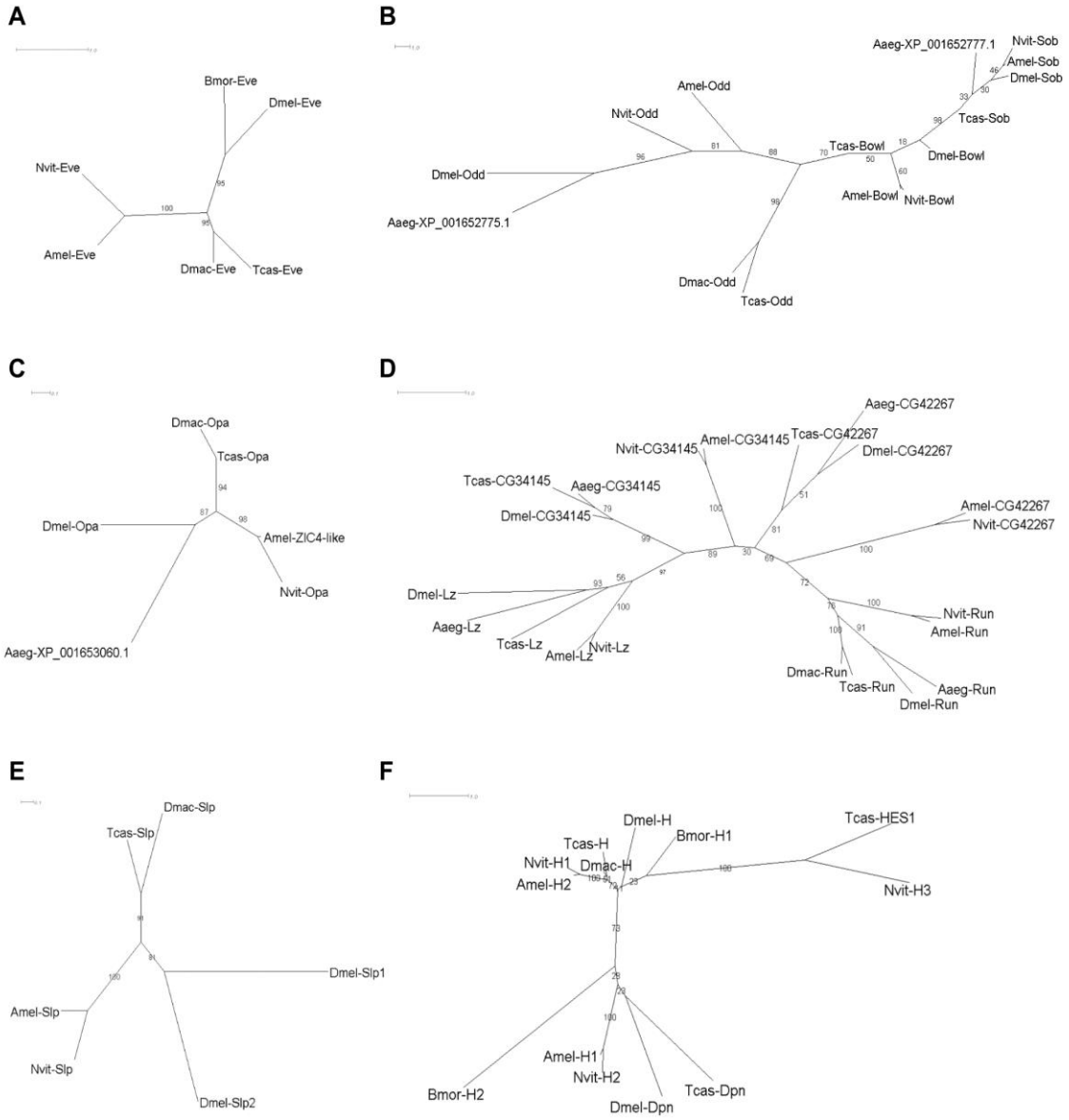

Figure S1. Phylogenetic analyses of isolated *Dermestes* PRG-orthologs. Phylogenetic analyses of *eve* (A), *odd* (B), *opa* (C), *run* (D), *slp* (E) and *h* (F) protein sequences. Isolated individual *Dermestes* PRG-orthologs closely grouped with counterparts in *Tribolium*. Bootstrap values (1,000 replicates) are shown adjacent to branches. Abbreviations: *Dmac*: *Dermestes maculatus*; *Dmel*:

Drosophila melanogaster; *Tcas*: *Tribolium castaneum*; *Nvit*: *Nasonia vitripennis*; *Amel*: *Apis mellifera*; *Bmor*: *Bombyx mori*; *Aaeg*: *Aedes aegypti*.

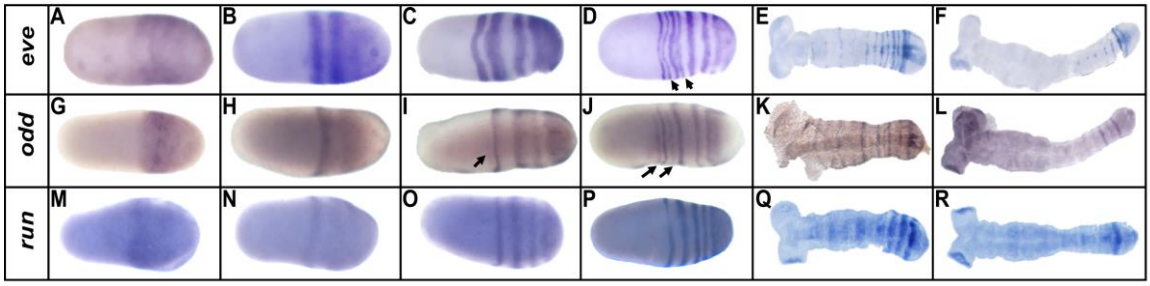

Figure S2. Expression of *Dmac-eve*, *-odd,* and *-run*. *In situ* hybridization was carried out for *Dmac-eve* (A-F), *Dmac-odd* (G-L) or *Dmac-run* (M-R). Embryos from early blastoderm to late germband elongation are shown; anterior, left. (A) Initial *Dmac-eve* expression in the posterior half of the embryo. (B) The first *Dmac-eve* stripe has settled in the center, and the second has resolved from the posterior cap-like expression. (C) 3 *Dmac-eve* primary stripes and posterior cap-like expression. (D) Embryo at late blastoderm stage with 4 *Dmac-eve* primary stripes and an emerging fifth stripe. Arrows indicate split secondary stripes. (E) Mid-germband stage embryo showing fading *Dmac-eve* stripes in the anterior. (F) Strong *Dmac-eve* in newly patterned posterior segments in late germband stage embryo. (G) *Dmac-odd* early expression in the posterior. (H) The first *Dmac-odd* primary stripe has appeared and weak posterior expression is visible. (I) Embryo with 3 primary *Dmac-odd* stripes and posterior cap-like expression. Arrow shows a weak secondary stripe anterior to the first primary stripe. (J) 2 weak *Dmac-odd* secondary stripes (arrows) have emerged while the fourth primary stripe is resolving from the posterior. (K) Growing germband with *Dmac-odd* primary and secondary stripes. Note that intensity alternates between primary and secondary stripes. (L) Extended germband with *Dmac-odd* stripes in posterior segments. (M) *Dmac-run* early broad expression. (N) Embryo showing the first stripe and weak posterior expression of *Dmac-run*. (O) 3 *Dmac-run* stripes and posterior cap-like expression. (P) 4 *Dmac-run* stripes and an emerging fifth stripe. (Q) Mid-germband stage embryo with faint anterior expression and 3 posterior stripes. (R) Late-germband stage embryo with one clear posterior *Dmac-run* stripe.

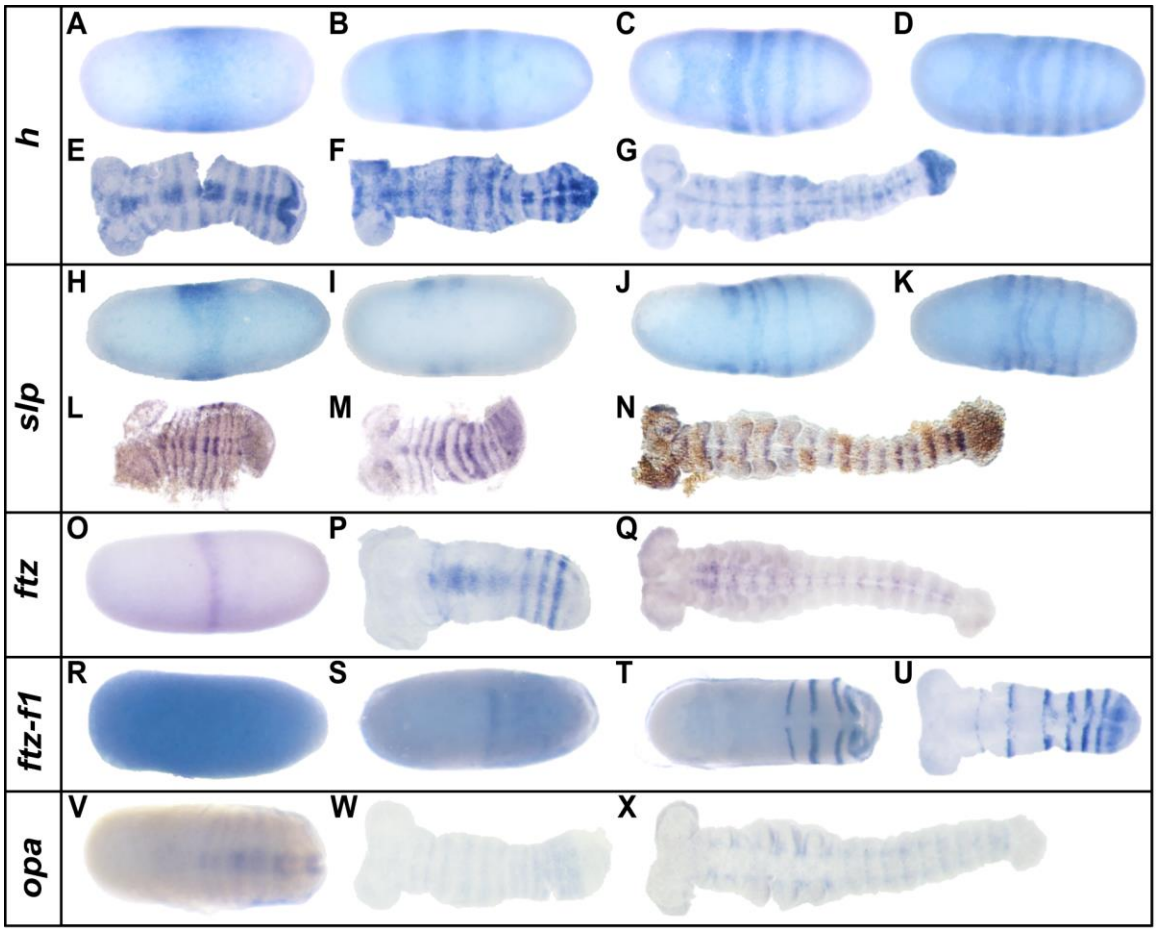

Figure S3. Expression of *Dmac-h*, *-slp*, *-ftz*, *-ftz-f1*, and *-opa*. *In situ* hybridization was carried out for *Dmac-h* (A-G), *Dmac-slp* (H-N), *Dmac-ftz* (O-Q) *Dmac-ftz-f1* (R-U), or *Dmac-opa* (V-X). Embryos from early blastoderm to late germband elongation are shown; anterior, left. (A) *Dmac-h* is initially expressed in a broad region in the center then (B) resolves into stripes. (C-F) Posterior stripes are added sequentially at both blastoderm and germband elongation stages. (G) *Dmac-h* striped expression fades during germband elongation and midline expression appears in late germband stages. (H) Lateral *Dmac-slp* striped expression in a broad central stripe. (I) Two distinct stripes emerge from this broad region. (J-M) Posterior *Dmac-slp* stripes arise from the posterior and secondary stripes appear in the anterior. (N) Striped expression of *Dmac-slp* persists until late germband stage. Note that the gold color in this embryo is background from the yolk. (O) *Dmac-ftz* is first detectable as a single, central stripe. (P) 3 *Dmac-ftz* stripes present in the posterior half in an embryo at mid

germband elongation. (Q) *Dmac-ftz* CNS expression in late germband stage embryo. (R) Uniform *Dmac-ftz-f1* expression in an early embryo. (S) One *Dmac-ftz-f1* stripe in the center. (T) *Dmac-ftz-f1* stripes during gastrulation. Note that expression was absent from the ventral furrow. (U) Embryo with 6 well-defined *Dmac-ftz-f1* stripes and a newly arising 7th stripe at the posterior. (V) Marginal striped *Dmac-opa* expression in embryo during gastrulation. (W) Weak striped *Dmac-opa* expression in anterior segments during germband elongation. There is a broad *Dmac-opa* expression domain in the posterior, anterior to the SAZ. (X) *Dmac-opa* is expressed segmentally in fully elongated germband stage embryo.

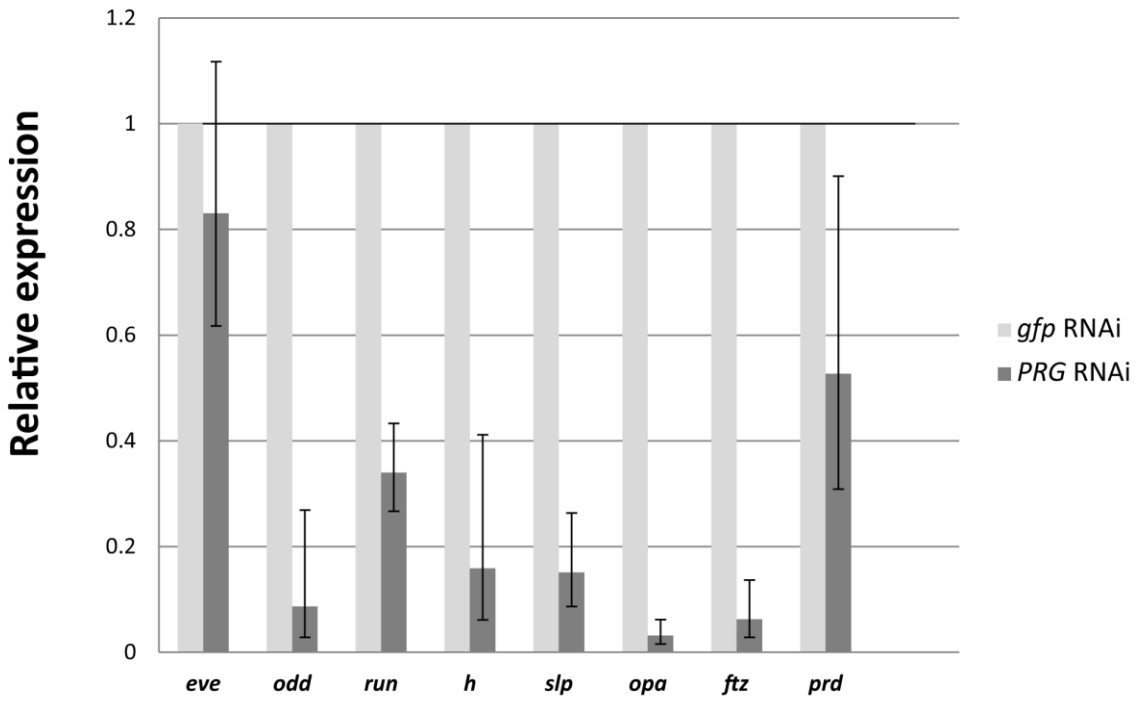

Figure S4. Validation of knockdown using qPCR. *Dermestes* embryos between blastoderm and late germband stages from RNAi treated females were collected for qPCR analysis. Expression levels of the target gene, as indicated, in the control group (*gfp* RNAi) was normalized to 1.0 (light gray bars). Relative expression levels of target genes in experimental groups (PRG-ortholog RNAi) was calculated using the $2^{-\Delta\Delta Ct}$ method (dark gray bars). Three biological replicates were carried out for each RNAi. Error bars indicate standard error. Note there was not a strict correlation between extent of knockdown and severity of defects observed.

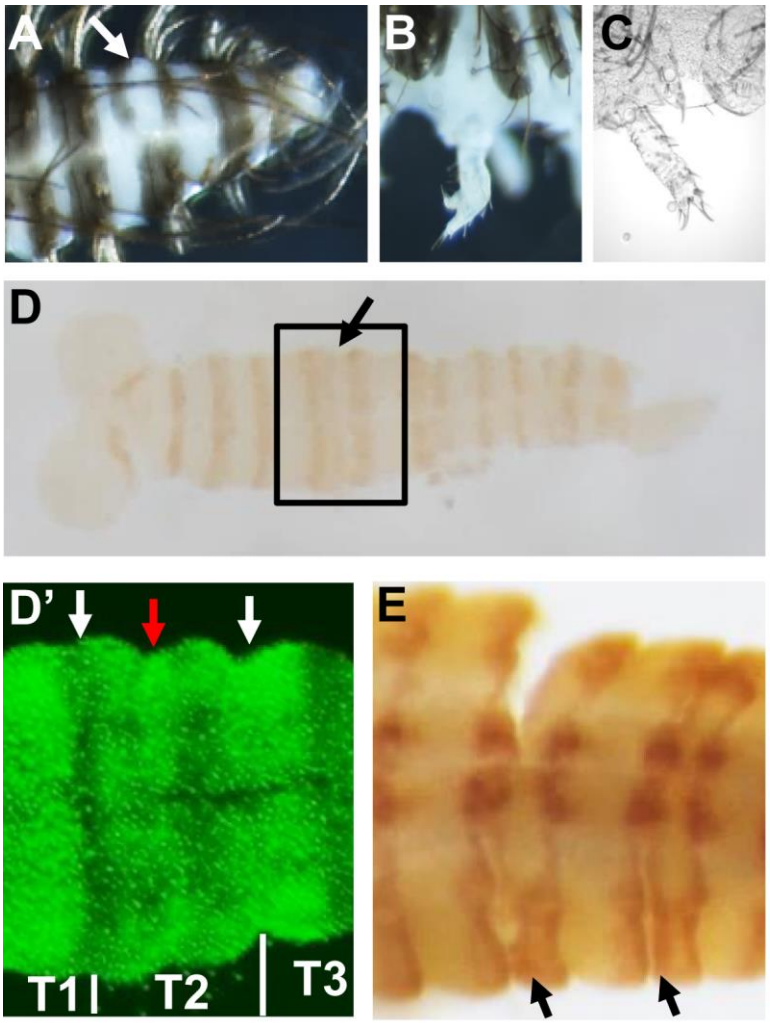

Figure S5. *Dmac-odd* RNAi results in deletions and duplications. Offspring of *Dmac-odd* dsRNA-injected females were analyzed. (A) Hatched larva with pigmented stripe disrupted by non-pigmented tissue (arrow). (B) Duplicated T2 leg claw in hatched larva. (C) Duplicated T2 leg claw in unhatched larva. (D) En expression in germband stage embryo. Arrow indicates an extra weak En stripe between T1 and T2 stripes. (D') SYTOX Green staining of the boxed region in (D). White arrows indicate segmental boundaries. Red arrow shows an extra segmental furrow. (E) Arrows show extra En stripes in embryo after germband retraction.

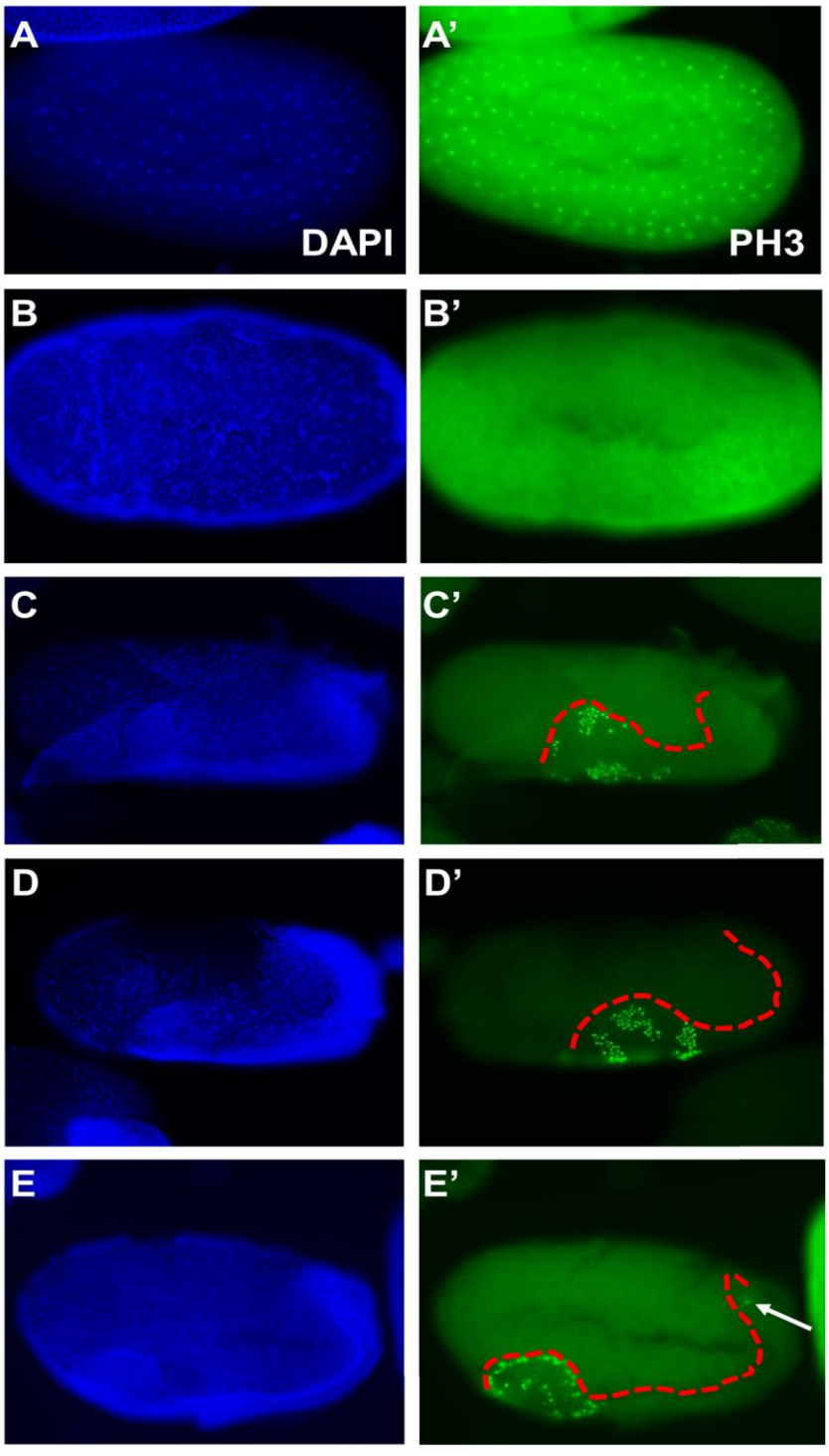

Figure S6. Mitotic activity in early *Dermestes* embryos. (A-E) DAPI staining was carried out for visualization of nuclei. (A'-E') Mitotic activity was examined using anti-PH3 antibody. (A, A') Dividing nuclei at syncytial blastoderm. (B, B') No detectable mitosis in embryo at late blastoderm. (C, C' and D, D') Embryos at early-germband stages show mitosis in the head region. (E, E') Mitotic cell detected in the posterior region at early- to mid-germband elongation (arrow). Dashed red line in C'-E' outlines the germband.

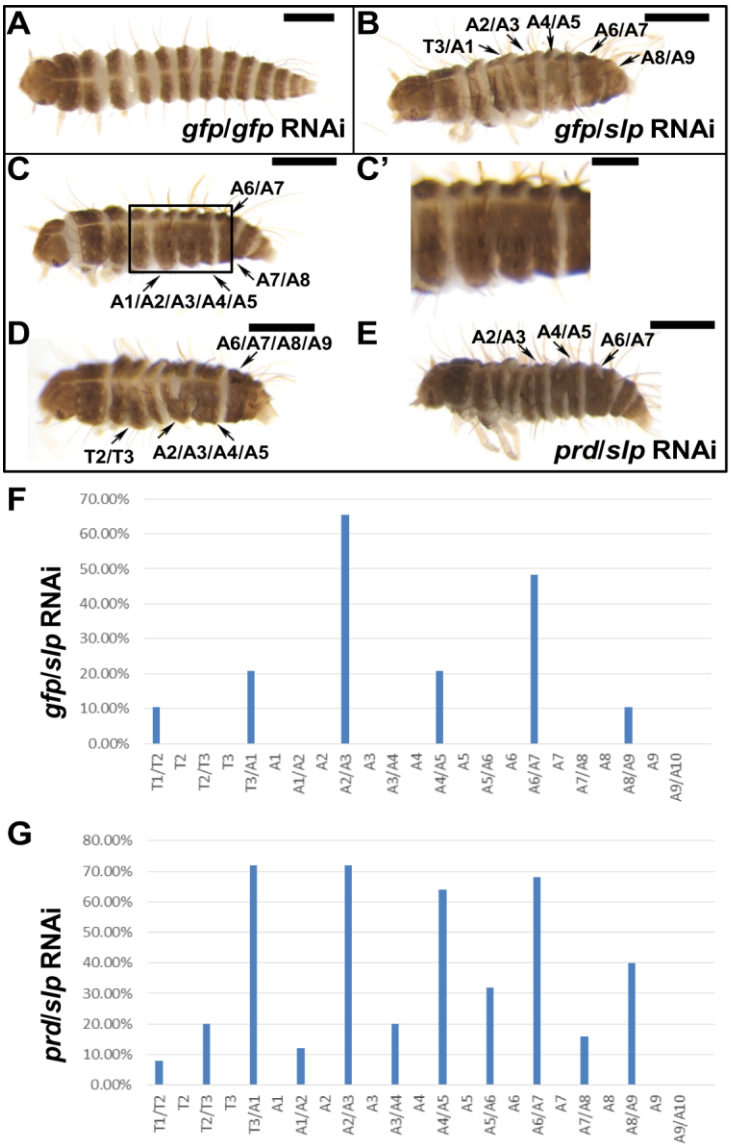

Figure S7. Defects in hatched offspring after *Dmac-prd* and -*slp* double knockdown. Females were injected with *Dmac-prd* and *Dmac-slp* dsRNA. Hatched offspring were screened for segmentation defects. Females injected twice with *gfp* dsRNA and females injected with *gfp* and *Dmac-slp* dsRNA were included as controls. (A) Hatched larva from *gfp*/*gfp* RNAi with wild type-like cuticle. (B) Hatched larva from *gfp*/*slp* RNAi with defects at the boundaries between T3/A1, A2/A3, A4/A5, A6/A7 and A8/A9, showing a typical defective

pattern after *Dmac-slp* RNAi. (C-E) Representative hatched larvae after *prd*/*slp* RNAi. (C, D) Hatched offspring with fused consecutive segments, combining defective patterns seen after *Dmac-prd* or *Dmac-slp* single knockdown. (C') Magnified view of the boxed region in (C). (E) Mildly affected hatched larva after double knockdown with typical *Dmac-slp* RNAi-like defects. (F, G) indicates (Y-axis) the frequency of defects seen in (X-axis) hatched affected larval segments, anterior to posterior, as indicated. (F) T1/T2, T3/A1, A2/A3, A4/A5, A6/A7 and A8/A9 most frequently affected. (G) Except for A9/A10, all segmental boundaries along trunk region affected. Scale bars represent 200 μm for C', or 500 μm for all other panels.

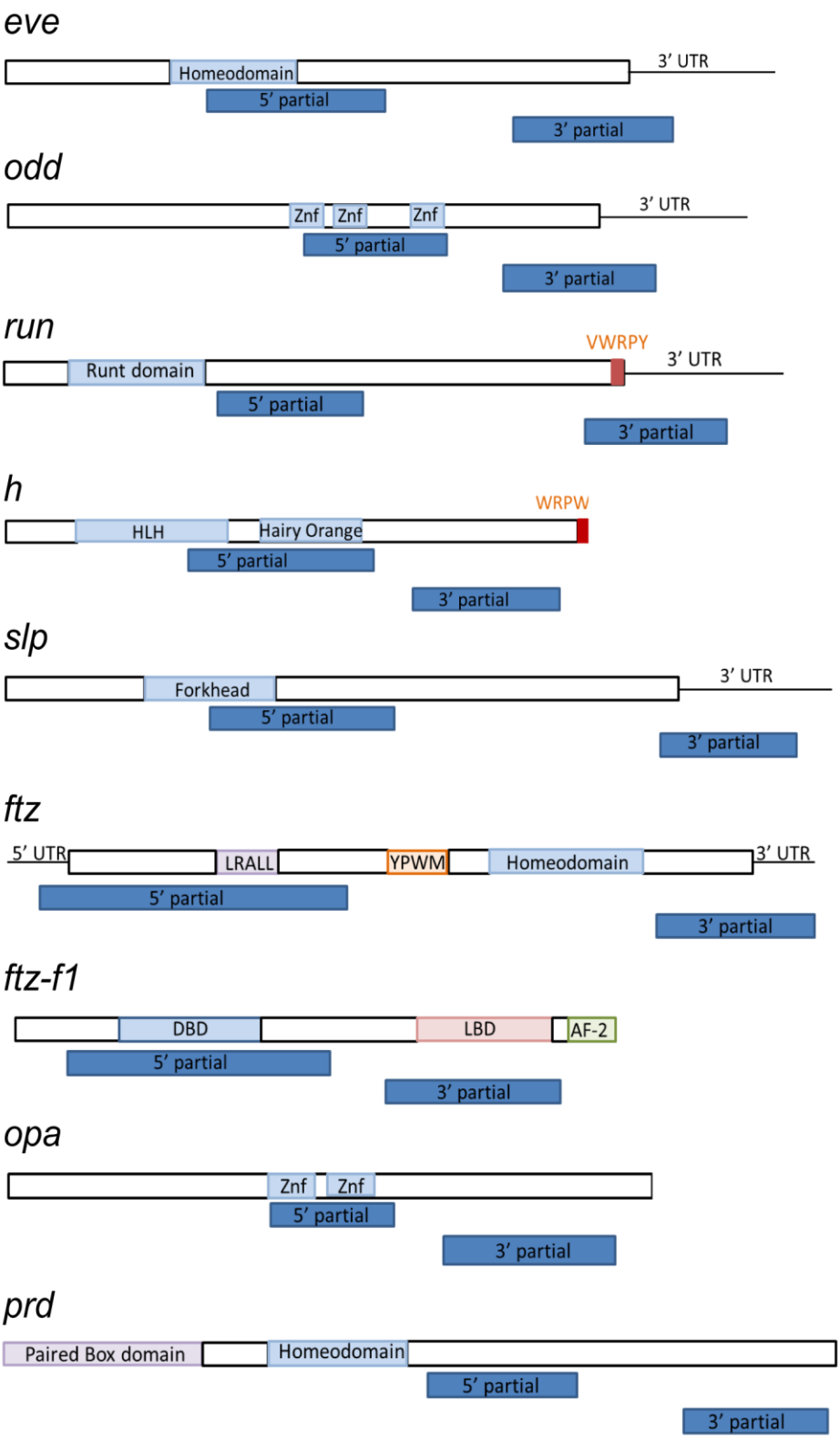

Figure S8. Gene regions targeted by *Dmac*-PRG-ortholog RNAi. Schematic drawings of genes examined in this study (not to scale). Conserved domains/motifs are shown for each *Dermestes* PRG-ortholog. Two dsRNAs targeting non-overlapping regions were used to ensure specificity (dark blue boxes). 5' partial dsRNAs targeted upstream coding regions, while 3' partial dsRNAs targeted downstream coding regions, sometimes including partial 3' UTR. For each gene knockdown, experiments using two dsRNAs gave very similar results, including defective patterns shown in hatched larvae, though the penetrance and severity varied.

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