Genetic regulation of patterned tubular branching in *Drosophila*

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A common theme in organogenesis is the branching of epithelial tubes, for example in the lung, liver, or kidney. The later morphogenesis of these branched epithelia dictates the final form and function of the mature tissue. Epithelial branching requires the specification of branch cells, the eversion process itself, and, frequently, patterned morphogenesis to produce branches of specific shape and orientation. Using the branching of renal tubule primordia from the hindgut in *Drosophila*, we show that these aspects are coordinately regulated. Cell specification depends on Wnt signaling along the tubular gut and results in the spatially restricted coexpression of two transcription factors, Krüppel and Cut, in the hindgut, whose activity drives cells toward renal tubule fate. Significantly, these transcription factors also confer the competence to respond to a second signal; TGF-β induces branching to form the four renal tubule buds. Differential activation of the TGF-β pathway also patterns the tubules, resulting in the asymmetry in size and positioning that is characteristic of the two tubule pairs. High levels of TGF-β promote the expression of *Dorsocross1–3* and anterior tubule growth, whereas low levels allow the expression of the transcriptional repressor, Brinker, and thus promote posterior tubule identity. We show that patterning of the tubule primordium into two distinct pairs is critical for the eversion of tubule branches, as well as for their asymmetric morphogenesis.

branching morphogenesis | patterning | renal tubule | TGF-β signaling | Wnt

The branching of epithelial tubes is an underlying theme in the morphogenesis of internal organs, as well as the vascular system, allowing a prodigious increase in surface area for the exchange of physiologically important molecules. Although the signals that promote the branching process itself have been studied extensively (1–5), comparatively little is known about the networks that specify branch cells and subsequently orchestrate the cytoskeletal modifications and changes in cell adhesion that direct their morphogenesis. In many tissues, the patterning of epithelial branching and tubule elongation ensures coherence of the mature structure; for example, the asymmetric architecture of the two lobes of the human lung or the differential growth of the cortical and medullary renal tubules of the kidney. We use the origin of epithelial branches in the development of a simple renal system in the fly as a model to address the regulation of branch specification, eversion, and patterning.

The fruit fly, *Drosophila melanogaster*, has four renal (or Malpighian) tubules, paired structures that empty into the hindgut at its junction with the posterior midgut (PMG) (6). Each pair is distinctive in terms of the site of eversion (Fig. 1 *A* and *B*), the subsequent pattern of cell division (7), their final positions in the body cavity (Figs. 1 *C* and *D*; ref. 8) and, as their cells differentiate, the subdivision into regions expressing specific physiological markers (9, 10). The tubules arise from the embryonic hindgut at its junction with the PMG, where clusters of primordial cells can be identified through the expression of two transcription factors, Krüppel (Kr) and Cut (Ct) (Fig. 1 *A*, *E*, and *F*; refs. 11–14). Once specified, these clusters evert to form four bud-like branches from the hindgut tube, growing out in a stereotypical fashion to take up characteristic positions, with the anterior pair projecting forwards and the posterior backwards from their junction with the hindgut (Fig. 1*D*). Thus, as in vertebrate systems, branching results from the segregation of cells to the tubule fate, the morphogenesis of these cells to form primordial buds and patterning of the primordia to specify specific types of branches. Little is known about the regulation of these events.

In this paper, we aim to elucidate the intercellular signals, and their targets that control and coordinate cell activities that

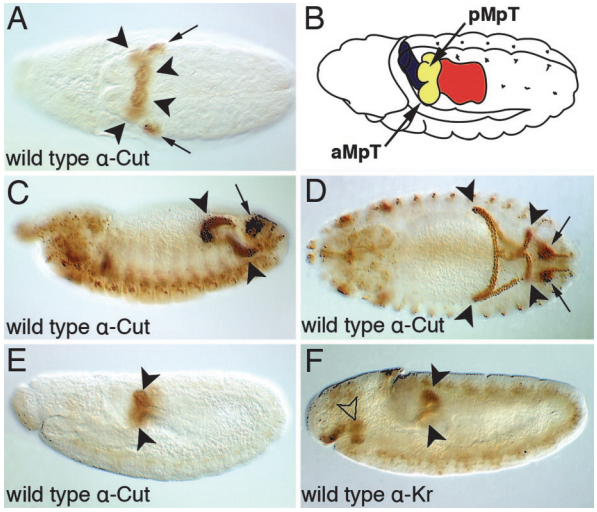

Fig. 1. Renal tubule development in wild type embryos. (*A*) The four tubule primordia (arrowheads) branch out from the hindgut at ≈5 h after egg laying (AEL). (*B*) The future anterior (aMpT) and posterior (pMpT) tubules branch from specific regions of the hindgut. (*C* and *D*) The tubules grow by cell division, projecting either backwards or forwards from the site of budding (*C*) and later elongate, coursing through the body cavity to take up stereotypic positions (*D*). (*E* and *F*) Both Ct (*E*) and Kr (*F*) are expressed in the tubule primordia (arrowheads). Note that, whereas Kr is expressed in the anterior gut (open arrowhead in *F*), Ct is not (*E*). *A* and *C–E*, immunostained for Ct; *F* for Kr; arrows indicate posterior spiracles.

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Abbreviations: PMG, posterior midgut; Kr, Krüppel; Ct, Cut.

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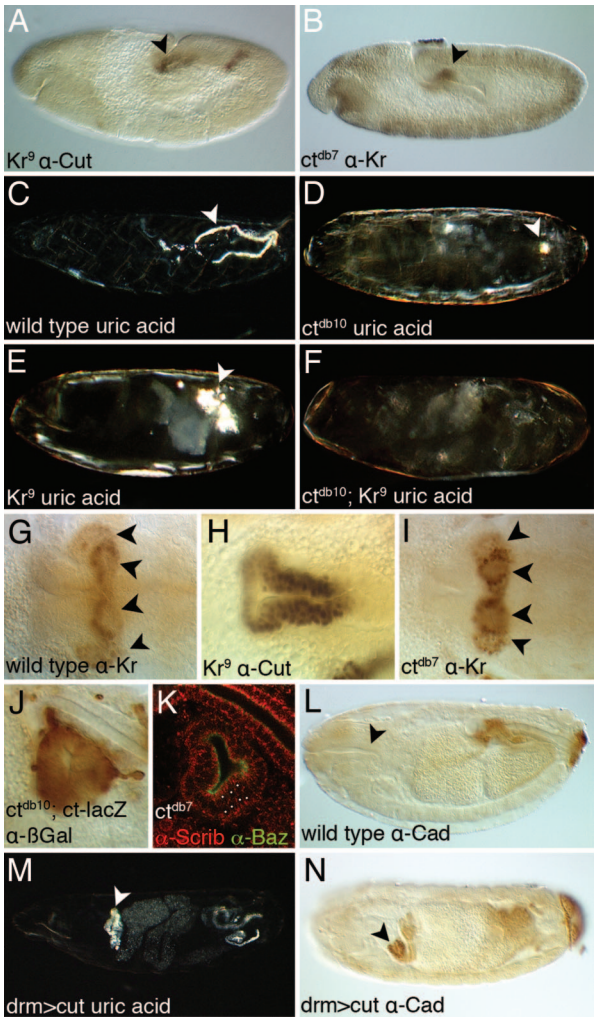

Fig. 2. Segregation of tubule cells. (*A* and *B*) *Kr* and *ct* are expressed independently of one another; Ct is expressed in the hindgut domain of a 5-h *Kr* null embryo (*A*, arrowhead). Similarly, *Kr* is expressed in the loss of Ct function (*B*). (*C–E*) Polarized light reveals precipitates of uric acid in the tubule lumen of wild-type (*C*) or in the deformed gut of *ct* (*D*) or *Kr* (*E*) mutant embryos. (*F*) Uric acid is not seen in the gut of double mutants. (*G–K*) Although hindgut branching fails in *Kr* mutants (compare *G* and *H*), normal buds form in *ct* mutants (*I*), but later tubule elongation fails and tubule cells form a multilayered blister (layers marked with white spots in *K*) adjacent to the hindgut (*J*; tubule cells revealed by β-Gal expression in the reporter line CtB2.1A ref. 62), *K*, kindly provided by S. Bunt (Department of Zoology, University of Cambridge) is labeled for apical Bazooka and basolateral Scribble, revealing that only cells of the inner layer are polarized). (*L–N*) Coexpression of Kr and Ct in the anterior gut induces the ectopic expression of *caudal* at the anterior midgut/foregut boundary (compare *L* and *N*, arrowheads). Later, uric acid precipitates in the lumen of the anterior gut (*M*, white arrowhead), as well as in the MpTs.

underlie patterned epithelial branching. We show that tubule morphogenesis depends on the combined activity of Wnt and differential TGF-β signaling. Wnt signaling is set up in the inner hindgut by interactions from the PMG and acts to specify cell fate through the expression of *Kr* and *ct* in the subset of hindgut cells that will branch out to form the Malpighian tubules. However, for these morphogenetic movements to take place, additional signaling through TGF-β is required. An "on-off" switch operates to define which pair of tubules will evert, and we show this results from the activation and repression of specific pathway targets.

Results

To understand the relationship between cell specification and bud eversion, we first analyzed the activity of Kr and Ct. Previous reports suggest that *ct* is a target of Kr (12–14). However, we find that each gene is expressed in the tubule primordia independently of the other (Fig. 2 *A* and *B*). The phenotype of *Kr* mutants has been described as the complete loss of renal tubule cells (15, 16) and of *ct* mutants as the failure of specified cells to branch out from the hindgut (12, 14, 17). Using the transport of urates as a diagnostic feature for differentiated tubule cell function, we show that it is only when the functions of both *Kr* and *ct* are removed that cell specification fails completely (Figs. 2 *C–F*). Kr and Ct therefore act in concert to specify tubule cell fate. Examination of early mutant embryos shows that the Ct-expressing tubule cells fail to evert in *Kr* mutants (see Fig. 2 *G* and *H*). However the Kr-expressing primordial cells branch out normally in *ct* mutants (Fig. 2*I*) but later fail to elongate, instead forming a multilayered structure adjacent to the hindgut and thus producing mature embryos apparently lacking tubules (Figs. 2 *J* and *K*).

To test whether Kr and Ct together are sufficient to specify tubule cells in the gut, we used the *drmGAL4* driver (18) to express Ct at the anterior midgut/foregut boundary, where Kr is normally expressed (Fig. 1*F*). We find that *caudal*, a later tubule cell marker (12), is ectopically expressed just in this *Kr*-expressing domain (see Fig. 2 *L* and *N*) and, later, uric acid precipitates in the anterior gut lumen (Fig. 2*M*). Even though *drmGAL4* drives expression more widely in the midgut and hindgut (18), these tubule cell characteristics are restricted to Kr-expressing domains in the gut (Fig. 1*F*). These results indicate that Kr and Ct together regulate tubule cell specification, leading to aspects of later tissue differentiation. Strikingly these respecified cells do not produce tubule branches in the foregut, indicating that Kr, although necessary for hindgut branching, is not sufficient.

We have shown that removal of two genes that are expressed in the midgut, *huckebein* (*hkb*) and *serpent* (*srp*), results in complete failure of tubule cell specification in the hindgut (19). This is unlikely to result from an autonomous requirement for these transcription factors in the inner regions of the embryonic hindgut, because *srp* is not expressed in this region (20, 21). Moreover, although *hkb* is transcribed in the hindgut anlage at stages 6–8 (22), it appears not to be translated [ref. 23; see also supporting information (SI) Fig. 7 *A* and *B*]. This suggests an alternative hypothesis in which *hkb* and *srp* together establish midgut cell fate and in which influences from the midgut induce Kr and Ct in the tubule primordia. Indeed the midgut is lost in *hkb*, *srp* double mutants (ref. 19; see also SI Fig. 7 *C–J*).

If the midgut signals to the neighboring hindgut to promote tubule cell fate, defects in the signaling pathway will reduce or remove the tubules. We have already shown that Ct expression in the tubule primordia is reduced when *wingless* signaling is lost (19). Here we extend this analysis and show that the number of primordial cells expressing both Kr and Ct is reduced to approximately half the wild-type number when Wg signaling is lost. In contrast, when the pathway is hyperactivated, the primordial cell number increases (Table 1; see also SI Fig. 8 *A–F*). The later tubule phenotype of embryos lacking *wg*, in which just two tubules bud out from the hindgut (7), confirms its role as an inducer for two of the four tubules (Fig. 3*D*). These observations suggest that Wg might act from the midgut to pattern the neighboring hindgut cells to form tubule primordia. However, *wg* is expressed not in the midgut when tubule cells are specified but in a band within the hindgut covering the future tubule primordium (24–26; Fig. 3*A*). This expression is reduced in *hkb* mutant embryos but lost in *hkb,srp* embryos (Fig. 3 *B* and *C*). These results suggest a cascade in which an as-yet-unidentified influence from the midgut sets up Wg signaling within the hindgut to

Table 1. Perturbing Wnt signaling alters the number of tubule cells allocated

Wild type	wg^{cx4}	arm^{XM19} glc	dsh^{V26} glc	sgg^{M11} glc	nkd^{7E}	hs-wg
77 ± 1 (n = 23)	42 ± 2 (n=28)	41 ± 3 (n=12)	47 ± 2 (n=28)	110 ± 4 (n=15)	89 ± 2 (n=7)	100 ± 4 (n=13)

Numbers of MpT cells at stage 10 (before mitosis 15) are given as means ± SEM. glc, germ-line clone.

prepattern tubule branch sites, through the expression of Kr and Ct. We tested this hypothesis by driving the expression of *Kr* in the hindgut primordium of *wg* mutant embryos and found rescue of the phenotype; four tubule buds evert (Fig. 3*E*). As in *wg* mutant embryos, the buds are unable to grow, because Wg signaling is required for division of tubule cells (7).

Significantly, although the coexpression of Kr and Ct in the foregut promotes the ectopic differentiation of cells with renal function in this tissue (Fig. 2*M*), branches do not arise in the anterior. Thus additional influences present in the hindgut must be required for the eversion of tubule buds. In embryos lacking TGF-β signaling, hindgut tube branching fails (27, 28); Kr and Ct are expressed in the hindgut, but these cells fail to evert from it (Fig. 4 *A* and *B*). To determine the role of TGF-β, we increased the activity of the pathway by overexpressing either the ligand Dpp or a constitutively active receptor, Thickveins (Tkv^{QD}) (29). We also analyzed embryos mutant for the pathway inhibitors, Short gastrulation (Sog) (30), or Brinker (Brk) (31). In each case, the phenotype is identical; a normal primordium of Ct-expressing MpT cells is established (Table 2), but just two hindgut branches form, later developing the characteristics of anterior tubules (≈144 cells in each tubule, which project forward through the body cavity; Table 2 and Fig. 4 *C–F*). The posterior tubule branches fail to develop, and Ct-positive cells remain in the hindgut (Fig. 4 *D* and *F*). We found a complementary phenotype in embryos overexpressing Brk in the hindgut; the MpT primordium is established (Table 2), and the posterior tubules develop normally (≈107 cells in each tubule, which project backward through the body cavity; Table 2 and Fig. 4*G*), but the anterior branches fail to form. These results are consistent with a role for the TGF-β pathway in patterning the tubule primordium, so that cells with high levels of activation evert and develop anterior characteristics, whereas those in which TGF-β signaling activity is low or absent evert and develop as posterior tubules.

This model for the patterning of the tubule primordium predicts that Brk is expressed in the posterior tubule buds. *In situ* hybridization and analysis of expression in the reporter line brk^{M12} reveals that Brk is indeed restricted to the posterior tubules (Figs. 4*I* and 6 *A–C*). Because *brk* is expressed in just two tubules, we asked whether its expression depends on the specification of tubule cells by Wg signaling. Staining for the brk-LacZ reporter in *wg* mutant embryos shows that *brk* is not expressed in the absence of Wg (Fig. 4*J*). Thus, it is the posterior MpTs that are lost in a *wg* mutant. The phenotype of *brk*;*wg* double mutants is consistent with this observation; two tubule buds develop, although they do not grow or manifest anterior morphogenesis, because of the absence of Wg (Fig. 4*H*).

We next addressed when patterning of the gut tube is required to differentiate between the anterior and posterior tubule pairs.

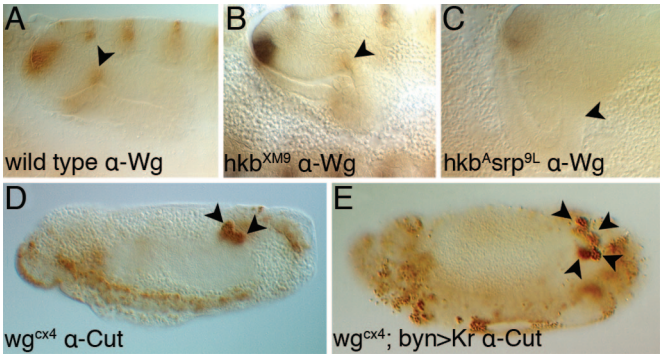

Fig. 3. The midgut induces *wg* expression in the hindgut. (*A*) At 5 h after egg laying (AEL), Wg is expressed in two rings of the hindgut, the inner covering the presumptive tubule primordia (arrowhead). (*B* and *C*) This inner ring is present, although weaker, in *hkb* mutant embryos (*B*, arrowhead) but is lost in *hkb, srp* double mutants (*C*, arrowhead). (*D*) In *wg* mutant embryos, only two tubule primordia develop (arrowheads, stage 14). (*E*) However, when Kr is driven in the hindgut of *wg* mutant embryos, eversion of the four primordia is rescued (arrowheads). Note that tubules in *wg* mutant embryos fail to grow because of a later requirement for Wg.

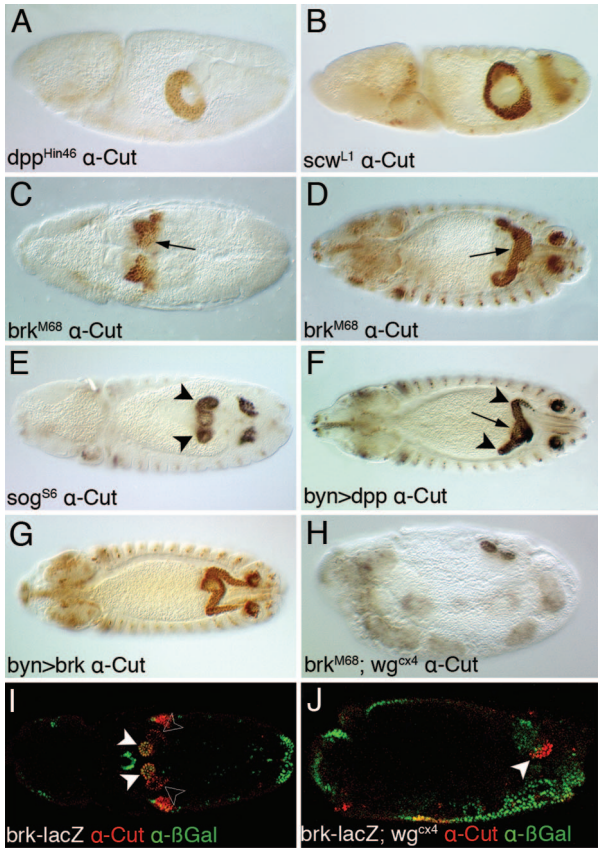

Fig. 4. Differential TGF-β signaling is required for hindgut branching to establish four tubule primordia. (*A* and *B*) In the absence of the ligands, Dpp or Scw Ct-expressing cells are found in the deformed hindgut, but tubule primordia do not evert. (*C–G*) If TGF-β signaling is deregulated, either by the ectopic expression of *dpp* (*F*) or through loss of the repressors Sog or Brk (*C–E*), normal numbers of Ct-expressing tubule cells are specified, but only two hindgut branches appear, which develop with characteristics of anterior tubules. In contrast, repression of TGF-β signaling by the ectopic expression of *brk* results in the eversion of just two primordia, which develop with characteristics of posterior tubules (*G*). (*H*) In *wg;brk* double mutants, only two tubules form, indicating that Wg and Brk affect the same tubule pair. (*I*) Brk (shown as the expression of a reporter line) is expressed only in the posterior tubules (white arrowheads, tubules marked by Ct; anterior tubules, open arrowheads). (*J*) In *wg* mutant embryos, Brk expression is lost from the tubules; the remaining, anterior pair express only Ct (arrowhead).

Table 2. Perturbing TGF-β signaling affects the number of tubule cells that evert from the hindgut

Cell line	Stage 10*	Stage 14†	Anterior cells/MpT	Posterior cells/MpT
Wild type	77 ± 1 (*n*=23)	484‡	144 ± 2 (*n*=21)	107 ± 2 (*n*=26)
brk^{M68}	76 ± 1 (*n*=13)	303 ± 9 (*n*=23)	++	–
byn-GAL4xUAS-dpp	73 ± 1 (*n*=20)	326 ± 9 (*n*=24)	++	–
bynGAL4xUAS-tkv^{QD}	74 ± 4 (*n*=19)	330 ± 5 (*n*=19)	++	–
byn-GAL4xUAS-brk	72 ± 4 (*n*=19)	221 ± 5 (*n*=22)	–	++
Df(3L)DocB	74 ± 1 (*n*=12)	220 ± 2 (*n*=12)	–	++

Numbers of MpT cells are given as means ± SEM.
*The anterior and posterior tubule primordia contain the same number of cells at this stage. Later differences result from specific patterns of cell division (7).
†Number of MpT cells in everted tubules (cells remaining in the HG not included).
‡Ref. 64; numbers given ± SD.

Dpp is expressed in the hindgut only from stage 11 (32), which is after the tubule buds have everted. We therefore analyzed the expression of Dpp and of the active phosphorylated form of the pathway transducer Mad (33) earlier in development. Dpp is expressed from blastoderm stages in a dorsal cap that extends posteriorly to cover the shared anlage of the hindgut and MpTs (34–36). The activation of the pathway, reflected by pMAD expression, correlates with this expression domain (Fig. 5 *A* and *B*). When the embryo gastrulates the expression of *dpp* and activation of the pathway in the amnioproctodeal invagination persists (Fig. 5 *C* and *D*) but then fades and is lost as hindgut branching is initiated to establish the tubule buds (Fig. 5 *E* and *F*).

These observations suggest that early events in dorsoventral patterning lead to the activation of anterior and posterior tubule-specific targets. Expression of *brk* in the blastoderm extends posteriorly to cover the ventrolateral regions of the embryonic hindgut (37, 38). After gastrulation, it is expressed strongly in the dorsal domain of the tubule primordium (Fig. 6*A*), so that, as they evert, its expression is limited to the posterior buds (Fig. 6 *B* and *C*). As in the trunk ectoderm, *brk* is repressed by Dpp, so that driving the expression of *dpp* in the shared HG/MpT primordium abolishes *brk* expression in all tubule cells (see Fig. 6 *D* and *E*; see also SI Fig. 9*D* and ref. 37). Together, these data suggest that ventrolateral cells of the shared HG/MpT anlage receive low levels of TGF-β signaling and therefore express *brk*, possibly in response to Dorsal (37). When the amnioproctodeal invagination sinks into the embryo during gastrulation, these cells come to lie dorsally in the hindgut tube (Fig. 5 *A*, *C*, and *E*), so that Brk expression is specific to the posterior tubule buds.

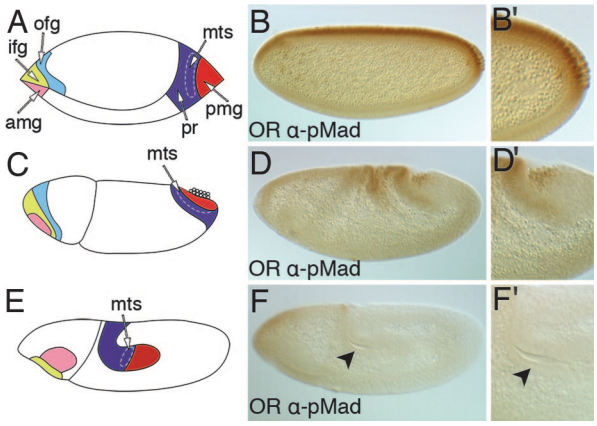

Fig. 5. Early dorsoventral TGF-β signaling patterns the hindgut. (*A*, *B*, and *B′*) At the blastoderm stage, the TGF-β pathway is activated, as shown by immunostaining for phosphorylated MAD, in a domain that includes the future amnioproctodeal invagination but not the whole anterior gut. (*C*, *D*, and *D′*) This distinction between pathway activation in the anterior and posterior gut primordia becomes more pronounced during gastrulation. (*E*, *F*, and *F′*) By the time the tubule cells are specified (*E*), pathway activation (*F* and *F′*) in the hindgut has disappeared. Fate maps (*A*, *C*, and *E*) are after ref. 63. Amg, anterior midgut; ifg, inner foregut; ofg, outer foregut; mts, Malpighian tubules; pmg, PMG; pr, proctodeum.

In a screen for genes that are differentially expressed in the anterior vs. posterior tubules (www.fruitfly.org/cgi-bin/ex/insitu.pl), we found that the Dorsocross family of transcription factors (Doc1–3) are expressed in the embryonic hindgut from stage 9 but are restricted to the anterior tubule buds, persisting as this tubule pair develops until midembryogenesis (Fig. 6 *F* and *G*). As in the dorsal ectoderm, the Doc genes are targets of high TGF-β signaling (SI Fig. 9 *A–C* and ref. 39). In *dpp* mutant embryos, or where *brk* is expressed throughout the tubule primordium, tubule cells fail to express Doc (Fig. 6 *H* and *I*).

To investigate the role of the Doc genes in anterior tubule development, we stained embryos carrying a deficiency that uncovers all three genes and found that, although the normal number of Ct-expressing primordial cells is specified, just two tubule buds evert, which later develop characteristics of posterior tubules (Table 2 and Fig. 6 *J* and *K*). Conversely, when Doc genes are ectopically expressed throughout the shared HG/MpT primordium, the posterior buds fail to evert, and just the anterior tubules develop (Table 2 and Fig. 6*L*). Thus, the Doc genes are both targets and effectors of information relayed through high levels of Dpp present in the dorsally derived HG/MpT primordium at the blastoderm stage. After gastrulation, this domain comes to lie ventrolaterally in the hindgut (Fig. 5 *A*, *C*, and *E*), where Doc activity is required for the branching events, which establish the anterior MpTs.

Discussion

Taken together, our results indicate that the morphogenesis of renal tubule branches from the hindgut depends on two patterning processes. First, cells are specified by signaling along the gut tube, in which the midgut promotes the expression of *wg* in neighboring hindgut cells. Wg signaling within this domain results in the segregation of a subgroup of cells in which the coexpression of Kr and Ct is established (Fig. 6*N*). These factors act together to specify MpT cell fate, regulating the differentiation of specific physiological capabilities such as the transport of urates. However the Wg-dependent subgroup accounts for only half the MpT primordium, indicating the existence of another inducing factor, also dependent on the presence of midgut cells and also required to establish the coexpression of Kr and Ct in a subpopulation of hindgut cells. We have not yet identified this pathway.

Although Kr is required for the morphogenetic movements, that result in the eversion of hindgut branches, it is not sufficient. Here the second patterning process, mediated by TGF-β signaling, acts

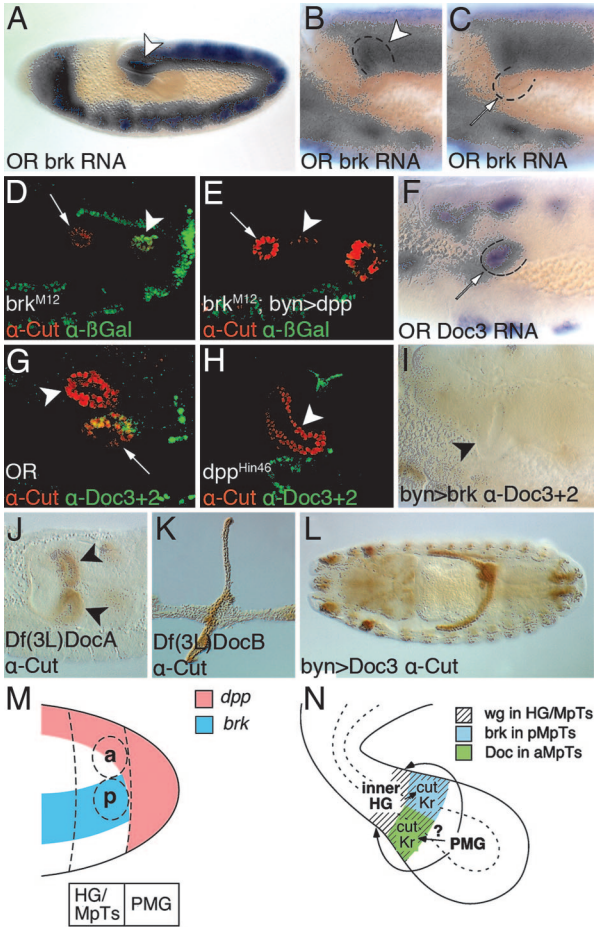

Fig. 6. Restricted expression of TGF-β targets promotes hindgut branching and determines tubule type. (*A–C*) At 4-h AEL, *brk* mRNA is expressed on the dorsal side of the embryonic hindgut (*A*) and later is confined to the everting posterior (*B*) tubule buds (compare anterior bud, *C*). (*D*) Accordingly, the expression of Brk in a reporter line is seen only in the posterior tubule pair (arrowhead, tubules stained for Ct, anterior tubule, arrow). (*E*) If *dpp* is ectopically expressed throughout the embryonic hindgut, Brk expression in the Ct-positive tubule cells remaining in the hindgut (arrowhead) is lost. (*F* and *G*) The Doc family of transcription factors is expressed only in the anterior tubule buds (arrows in *F* and *G*; arrowhead in *G*, posterior tubule). (*H* and *I*) The expression of the *Doc* genes depends on high levels of Dpp, so that expression in the Ct-positive cells in the embryonic hindgut is lost in *dpp* mutants (*H*) or if *brk* is ectopically expressed (*I*). (*J–L*) In embryos carrying a deficiency uncovering the *Doc* genes, only two primordia evert; the posterior pair (*J* and *K*), whereas if *Doc* is ectopically expressed, only the anterior tubules develop (*L*). (*M* and *N*) Summary diagrams to show the domains of high levels (pink, *dpp* expression) and the absence (blue, *brk* expression) of TGF-β signaling superimposed on the posterior blastoderm fate map (*M*; refs. 34 and 63). High levels of TGF-β signaling activate *Doc* (green) but repress *brk* (blue) (*N*). These genes distinguish between the prospective anterior and posterior tubule primordia. Influences from the PMG activate *wg* expression in the hindgut to promote Kr and Ct in the posterior tubule cells, whereas an unknown PMG-derived influence promotes the expression of these genes in the anterior tubule cells (*N*). Together, Kr with Doc (anterior) or Brk (posterior) acts to facilitate hindgut branching to establish the four tubule primordia.

with Kr to promote the eversion of four separate branches. Patterning around the hindgut derives from earlier establishment of the dorsoventral axis in the ectoderm (Fig. 6*M*), and we suggest that this patterning becomes manifest when cells receive inductive signals from the PMG. Only then do tubule buds branch out from the hindgut (Fig. 6*N*) and, significantly, buds form in the hindgut only where *Kr* and *ct* are coexpressed, indicating that these transcription factors confer the ability to respond to TGF-β signaling. In the foregut, even though Kr is expressed at its junction with the midgut, branches do not develop, and we suggest this is because the inner foregut anlage is more ventrally placed in the early embryo and therefore lies outside the domain of pMAD activation (compare Fig. 5 *A* and *C* with *B* and *D*).

Tubule eversion fails completely in mutants lacking TGF-β signaling. Posterior gut development is very abnormal in mutant embryos, making it impossible to determine whether defects in hindgut branching result directly from the loss of signaling. However, differential activation of the TGF-β pathway plays a direct role, by distinguishing the anterior from the posterior tubule pairs. Without this distinction, hindgut branching is perturbed. It is striking that uniform levels of pathway activation, whether high or low, do not transform buds between anterior and posterior fate; we do not find four anterior tubules when Dpp is deregulated. Instead one pair of tubule branches fails to form, revealing that the subdivision into anterior and posterior domains is a prerequisite for the activation of genes that drive the first steps of tubule morphogenesis. We have identified transcriptional regulators specific to and required for anterior (Doc) and posterior (Brk) tubule budding and development. It will now be important to characterize their targets.

Signaling of both Wnt and TGF-β has been implicated in branching morphogenesis during vertebrate organogenesis, for example in the kidney. Several Wnt genes are expressed during nephrogenesis, of which Wnt 4, 6, and 11 are required for normal branching of the ureteric buds (3, 40, 41). Wnt11 is expressed early in the ureteric buds as they grow out from the Wolffian duct (42) and then refines to the branching tips. This signal acts synergistically with glial-derived neurotrophic growth factor (GDNF) from the surrounding mesenchyme (43, 44). GDNF, originally described as a member of the TGF-β superfamily (45), is now known to signal through the receptor tyrosine kinase C-ret, which is expressed in the ureteric epithelium (46, 47). Although GDNF promotes branching, TGF-β ligands, BMP4, BMP7, and Activin A, repress branch formation (48–51), and the up-regulation of TGF-β1 has been reported in kidney disease (52, 53). As in the fly, the inhibitory effects of BMP4 are graded, strongly repressing ureteric branching in the posterior kidney but having little effect in anterior kidney (54, 55). The branch-promoting ligand, GDNF, activates the expression of Wnt 11 (43), but high concentrations of TGF-β repress its expression in the ureteric epithelium (51), suggesting that TGF-β signaling can dictate where the buds arise. In a similar way, we find that Wg combines with the absence of TGF-β signaling to promote posterior tubule buds in *Drosophila*, and that the levels of TGF-β signaling must be tightly regulated for the formation of the full complement of Malpighian tubule primordia.

We also demonstrate that TGF-β signaling acts to differentiate morphogenetic tubule subtypes. In the kidney, the medullary and cortical nephrons are of different lengths and take up distinctive positions in the final organ (56). How these differences are specified is not yet understood. Further analysis, at the molecular level, of the cellular activities that are regulated by the dose-dependent targets of TGF-β signaling, Doc and Brk, will increase our understanding of patterning in renal tubule development.

Materials and Methods

***Drosophila* Stocks.** The following lines were used: Oregon R (OR); brk^{M12} (*brk-LacZ*); brk^{M68}; cut^{db10}; cut^{db7}; *Df(3L)DocA*; *Df(3L)DocB*; dpp^{Hin46}; Kr^{9}; hkb^{XM9}; hkb^{2}, srp^{9L}; hkb^{A}, srp^{9L}; nkd^{7E}; scw^{1L}; sog^{S6}; srp^{6G}; srp^{9L};*hs-wg*; wg^{cx4}; *UAS-brk*; *byn-GAL4*; *drm-GAL4*; *UAS-cut*; *UAS-Doc1*; *UAS-Doc2*; *UAS-Doc3*; *UAS-dpp*; *UAS-Kr*; $UAS\text{-}tkv^{QD}$. *byn-GAL4* drives expression throughout the posterior gut from stage 8 (57) and *drm-GAL4* in proventriculus, anterior, and PMG tubules and small intestine from stage 9 (18).

Germ-line clones were generated according to ref. 58, by using dsh^{V26}, *FRT18A/FM7*; sgg^{M11}, *FRT101/FM7*; arm^{XM19}, *FRT101/*

FM7; and *ovo^{D}, FRT101; FLP38*. Females carrying clones were crossed to *FM7, ftz-lacZ/Y* males. Embryos were aged to 4–5 h before fixing.

Manipulating *wg* Expression. *hs-wg* embryos were collected for 1 h and aged for a further 2 h 45 min at 25°C before being subjected to a heat pulse at 37°C for 20 min. They were fixed after an additional 1 h (age equivalent to 4–5 h at 25°C).

Immunostaining and *in Situ* Hybridization. Standard protocols were used (59, 60). Primary antibodies: anti-Baz (rabbit, 1:1,000, A. Wodarz); anti-Ct (mouse, 1:200, K. Blochlinger); anti-Cad (rabbit, 1:500, P. MacDonald); anti-β-Gal (mouse, 1:1,000, Promega, Madison, WI); anti-β-Gal (rabbit, 1:10,000, Cappel, MP Biomedicals, Solon, OH); anti-Doc3 + 2 (guinea pig, 1:600, M. Frasch); anti-Peb [mouse, 1:20, Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Iowa City, IA]; anti-Hkb (mouse, 1:200, C. Doe); anti-Kr (guinea pig, 1:1,000, S. Small); anti-Scb (guinea pig, 1:500, D. Bilder, University of California, Berkeley, CA); anti-Srp (rabbit, 1:1,000, D. Hoshizaki); anti-Wg (mouse, 1:200, DSHB); secondary antibodies: biotinylated (1:200, Vector Laboratories, Burlingame, CA); FITC-and Cy3-conjugated (1:100, Jackson Immunoresearch, West Grove, PA) with Vectastain Elite ABC kit (Vector Laboratories) or dichlorotriazinylamine fluorescein-conjugated streptavidin (1:100, Jackson Immunoresearch) signal amplification.

Embryos were equilibrated in Araldite for capillary mounting (61) and viewed by using a Zeiss (Oberkochen, Germany) Axioplan with JCB KY-F55B camera or a Leica (Deerfield, IL) confocal microscope. Images were captured with Leica TCS software and processed by using Adobe Photoshop CS (Adobe Systems, San Jose, CA).

Assaying Tubule Function. Uric acid precipitation in the tubule lumen was assayed in 24-h embryos, dechorionated in bleach, mounted in 30% glycerol, and viewed under polarized light. For analysis of *ct^{db10};Kr^{9}* double mutants, unhatched (mutant) embryos were assayed firstly for cuticle pattern (to identify *Kr* mutant embryos, *ct* mutants have normal denticle bands), and then for uric acid. Twenty-five percent of embryos carrying a *Kr* mutation will also be mutant for *ct*. Accordingly, of 31 *Kr* mutant embryos, we found 7 (22.5%) lacked uric acid. Control embryos were positive for uric acid; *Kr^{9}* (97%), *ct^{db10}* (91%), and OR (100%).

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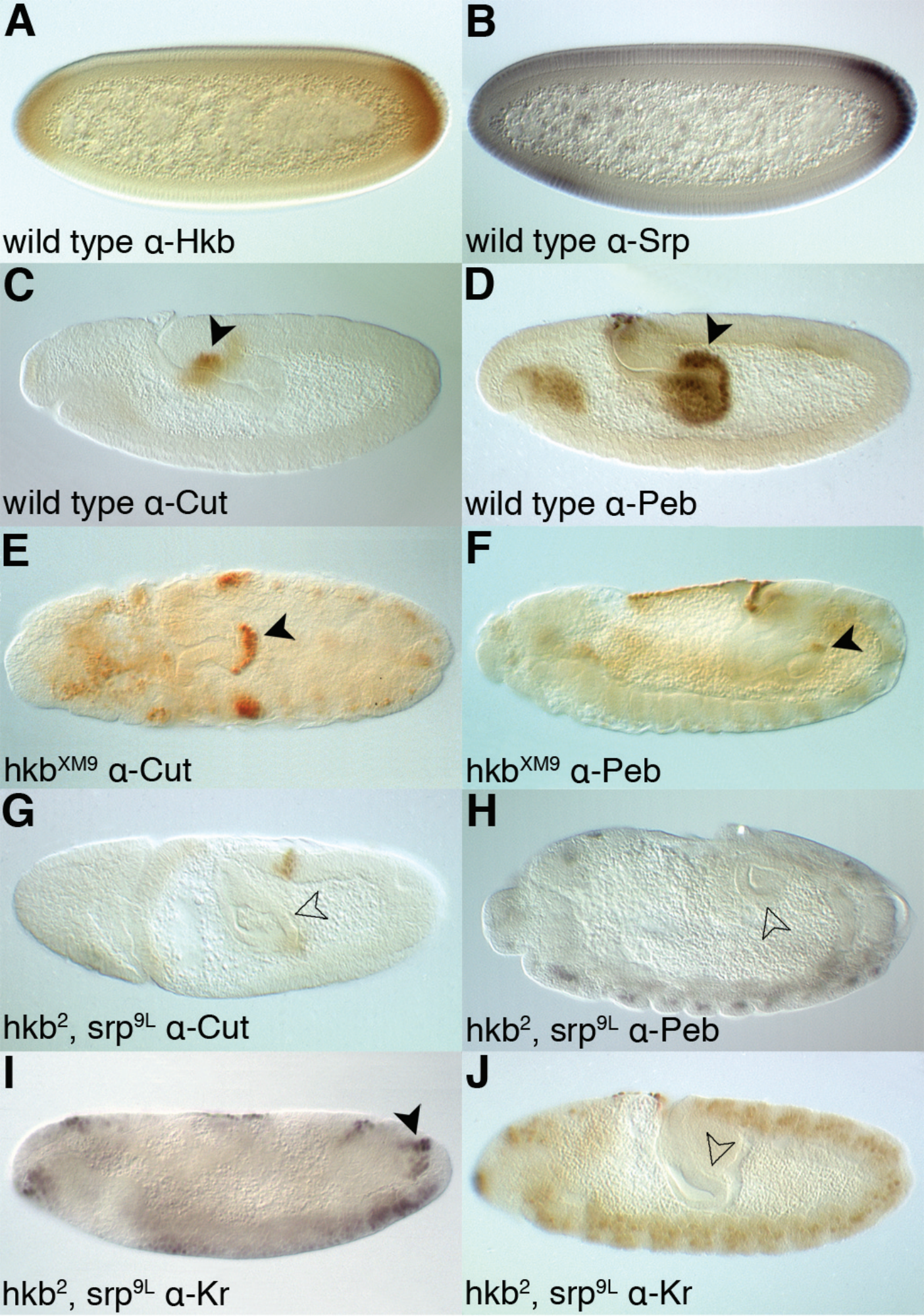
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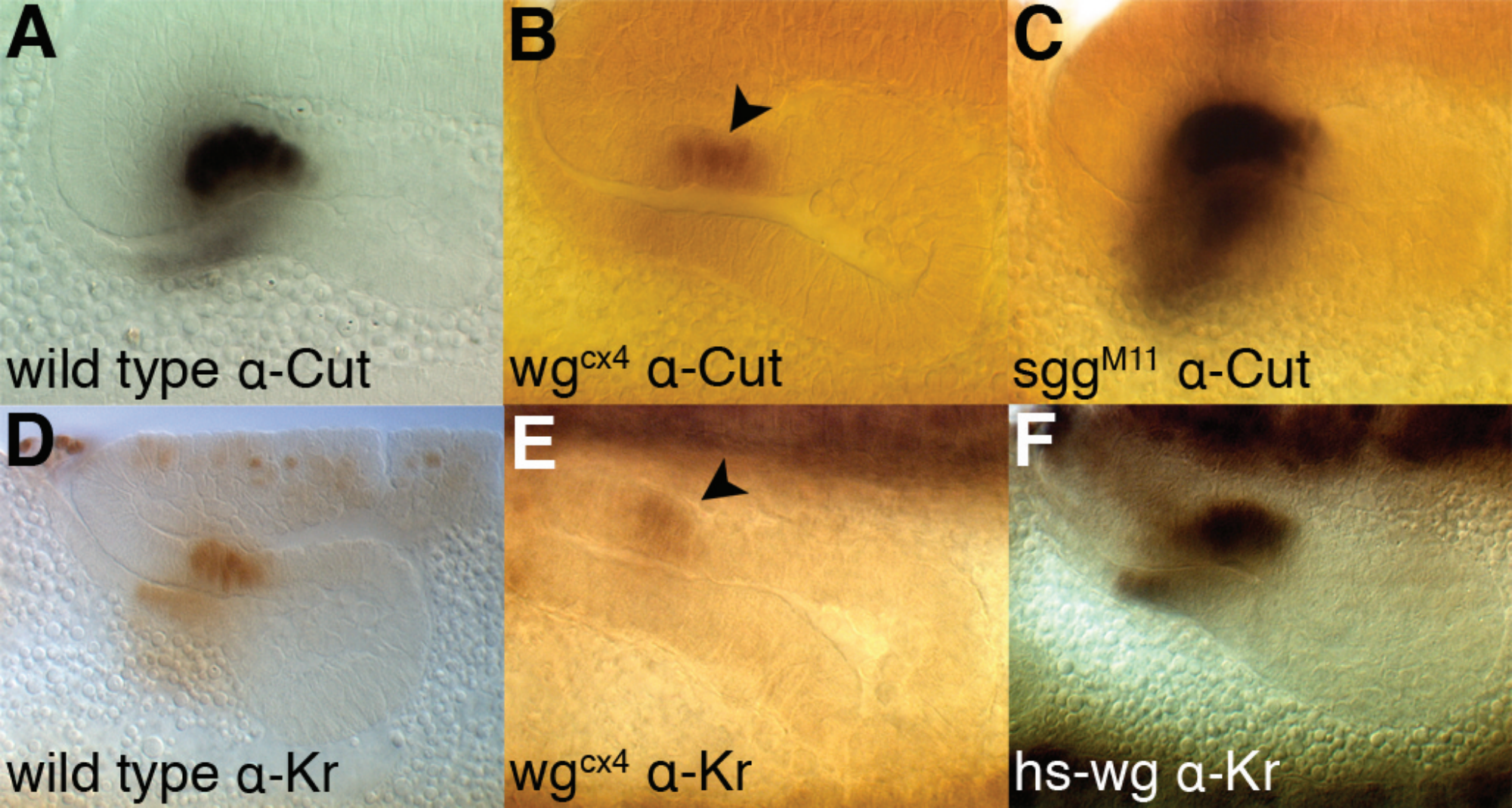
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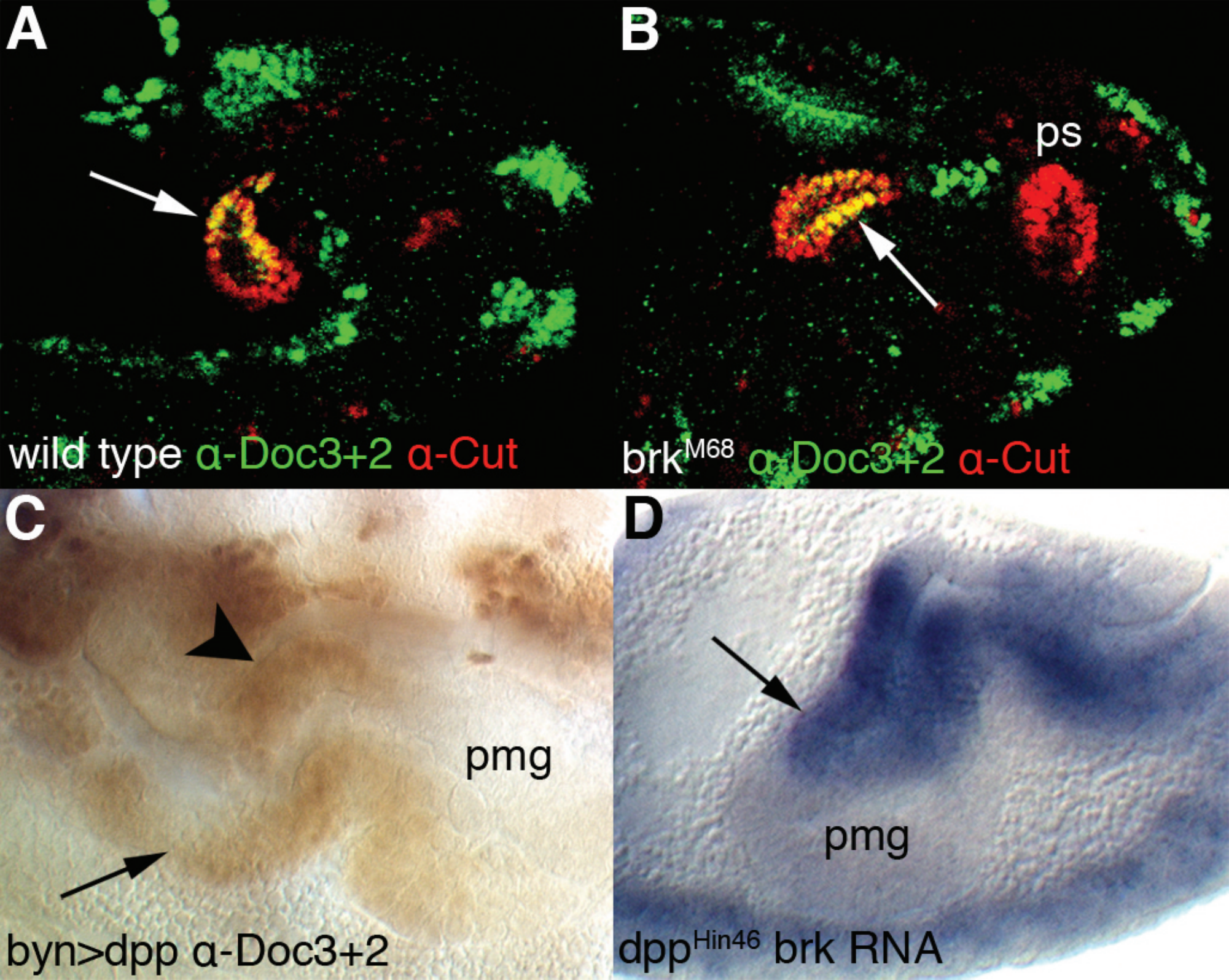
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