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Role of *caudal* in hindgut specification and gastrulation suggests homology between *Drosophila* amnioproctodeal invagination and vertebrate blastopore

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SUMMARY

During early embryogenesis in *Drosophila*, *caudal* mRNA is distributed as a gradient with its highest level at the posterior of the embryo. This suggests that the Caudal homeodomain transcription factor might play a role in establishing the posterior domains of the embryo that undergo gastrulation and give rise to the posterior gut. By generating embryos lacking both the maternal and zygotic mRNA contribution, we show that *caudal* is essential for invagination of the hindgut primordium and for further specification and development of the hindgut. These effects are achieved by the function of *caudal* in activating different target genes, namely *folded gastrulation*, which is required for invagination of the posterior gut primordium, and *fork head* and *wingless*, which are required to promote development of the internalized hindgut primordium. *caudal* is not sufficient for hindgut gastrulation and development, however, as it does not play a significant role in activating expression of the genes *tailless*, *huckebein*, *brachyenteron* and *bowel*. We argue that *caudal* and other genes expressed at the posterior of the *Drosophila* embryo (*fork head*, *brachyenteron* and *wingless*) constitute a conserved constellation of genes that plays a required role in gastrulation and gut development.

Key words: Cdx, *fork head*, HNF-3, *wingless*, Wnt, *brachyenteron*, Brachyury, *folded gastrulation*, *Drosophila*, *caudal*, Gastrulation, Hindgut

INTRODUCTION

Gastrulation begins at three hours of embryogenesis in *Drosophila*. During this process (described by Campos-Ortega and Hartenstein, 1997), cells of the ventral midline invaginate, forming the ventral furrow; the invaginated cells go on to form the mesodermal derivatives. At either end of the ventral furrow, cells invaginate to give rise to the anterior and posterior portions of the gut. At the posterior, a cap of approximately 450 cells begins moving dorsally, forming a plate and then a cup of cells referred to as the amnioproctodeal invagination. During invagination of the amnioproctodeum, the central domain of cells sinks in first, becoming the posterior midgut primordium. The ring of cells surrounding the posterior midgut primordium then comes together – forming the borders of a slit – and moves inside, completing gastrulation of the posterior gut primordia. The invaginating ring of approximately 300 cells is the hindgut primordium, which later gives rise to the hindgut proper and the Malpighian tubules.

Present understanding is that the patterning of the embryo posterior necessary for amnioproctodeal invagination and posterior gut development is initiated solely by activation of the maternal terminal system (reviewed by St Johnston and Nüsslein-Volhard, 1992; Perrimon et al., 1995). The critical component of the terminal system is the Torso receptor tyrosine kinase; activation of this receptor leads to transcription of only two known target genes, *tailless* (*tll*) and *huckebein* (*hkb*) (Weigel et al., 1990; Pignoni et al., 1992). Both of these genes encode transcription factors, and, acting together or separately, control the expression of additional transcription factor genes required for development of the posterior gut, namely *fork head* (*fkh*) (Weigel et al., 1989a,b), *brachyenteron* (*byn*, originally called *Trg*) (Kispert et al., 1994; Singer et al., 1996) and *bowel* (*bowl*) (Wang and Coulter, 1996).

tll and *hkb*, in addition to controlling genes required for specification and continued development of the posterior gut primordia, work together with *fkh* to activate *folded gastrulation* (*fog*) (Costa et al., 1994). *fog* encodes a secreted molecule that is required for gastrulation, specifically for coordinating the constriction of cells within the epithelial plate that gives rise to the posterior gut (Sweeton et al., 1991; Costa et al., 1994). Available data thus support the notion that the terminal system, acting solely through the genes *tll* and *hkb*, is responsible for initiating all of the changes in gene activity that are required to establish and maintain the posterior gut primordia, and to promote gastrulation.

A potential, but largely overlooked player in this network of gene activity controlling gastrulation and gut development is *caudal*. The *caudal* (*cad*) gene encodes a homeodomain transcription factor expressed, as a result of both maternal and zygotic transcription, at the posterior of the embryo (Mlodzik et al., 1985; Macdonald and Struhl, 1986). The domain of *cad* expression overlaps those of the above described genes, during the period when they are being transcriptionally activated.

While a requirement for *cad* in the development of epidermal, external structures that arise from the posterior of the embryo has been described (Macdonald and Struhl, 1986), the role of *cad* in gastrulation or in establishing posterior gut primordia has not been investigated.

To determine if *Drosophila cad* is required for gastrulation and/or posterior gut development, we generated embryos in which *cad* activity was removed maternally, both maternally and zygotically, or only zygotically. We find that embryos completely devoid of *cad* activity are unable to carry out gastrulation normally and, at older stages, lack the hindgut. By examining gene expression in these *cad* mutant embryos, we deduce that their gastrulation defect is due to a decrease in expression of *fog*, and their lack of hindgut due to loss (by apoptosis) of the misspecified hindgut primordium as a result of diminished expression of *fkh* and *wg*. These results establish *cad* as a key regulator in pathways required for gastrulation and gut development.

MATERIALS AND METHODS

Fly stocks

The strongest alleles available were used, namely: tll^{l49} (Pignoni et al., 1990), hkb^{A423R1} (Gaul and Weigel, 1990), fkh^{XT6} (Weigel et al., 1989a), wg^{7L74} (Van den Heuvel et al., 1993), byn^{5} (Singer et al., 1996), $bowl^{2}$ (Wang and Coulter, 1996), cad^{2} and cad^{3} (Macdonald and Struhl, 1986), fog^{114} (Schalet and Finnerty, 1968), and fog^{4a6} (Sweeton et al., 1991).

Generation of *cad* germline clones

Embryos were derived from mosaic females carrying germline clones (GLCs) homozygous for either cad^{2} or cad^{3}, obtained by using the FLP-DFS technique of Chou et al. (1993), as applied to *cad* by Rivera-Pomar et al. (1995). To generate GLCs, males carrying the FRT *ovoD* chromosome and an X-linked hs-FLP chromosome were crossed to females carrying an FRT *cad* chromosome. Progeny of this cross were heat-shocked at 37˚C for 2 hours on each of days 3, 4 and 5 after egg lay (AEL), and allowed to develop at 25˚C. These GLC females were mated to males heterozygous for *cad* to obtain progeny embryos, half of which lacked both maternal and zygotic *cad* activity (denoted cad^{m-z-} embryos, and also referred to as '*cad*-deficient embryos', or 'embryos lacking *cad* activity'), and half of which lacked maternal *cad* activity but carried one zygotically active wild-type *cad* gene (denoted cad^{m-z+} embryos). These two genetic classes of embryos were easily distinguished at later stages on the basis of their previously described phenotypes (Macdonald and Struhl, 1986): cad^{m-z-} embryos have severe segmentation defects particularly in the posterior, while cad^{m-z+} embryos look relatively normal but have smaller anal pads. Embryos lacking only zygotic *cad* were obtained by crosses between heterozygous parents, and are denoted cad^{m+z-}. Similar phenotypes were obtained with both *cad* alleles.

Antibody staining

Antibody staining was performed using standard techniques (Ashburner, 1989). Anti-Crumbs antibody Cq4 (which labels the apical surface of all ectodermally derived epithelia; Tepass et al., 1990) was used at a dilution of 1:100 to outline the lumina of the hindgut and Malpighian tubules. Streptavidin conjugated to horseradish peroxidase secondary antibody was used at 1:2000. Horseradish peroxidase activity was detected by adding $NiCl_2$ to 0.07% to the diaminobenzidine reaction, leading to a black precipitate. Embryos were dehydrated in ethanol and mounted whole in Epon/Araldite. Embryos were staged according to Campos-Ortega and Hartenstein (1997).

In situ hybridization

For in situ hybridization to whole embryos, embryos were collected, devitellinized, fixed and stored in 100% ethanol at −20˚C until needed. DNA probes were labeled by random priming with digoxigenin-dATP (Genius kit, Boehringer Mannheim). Hybridization and detection was carried out by the procedure of Tautz and Pfeifle (1989).

Histology

Embryos were fixed, embedded and sectioned at 2 μm with a LKB 2088 Ultratome V, stained with toluidine blue (Ashburner, 1989) and mounted in Epon under coverslips. Whole-mount embryos and sections of embryos were photographed on a Zeiss Axiophot microscope equipped with differential interference contrast optics.

RESULTS

cad expression pattern

Maternally produced *cad* mRNA is deposited in the oocyte during oogenesis; very early during embryogenesis this uniformly distributed maternal mRNA is differentially degraded under control of the maternal effect gene *bicoid* to produce a posterior-to-anterior gradient (reviewed by Rivera-Pomar et al., 1996; Fig. 1A). By the beginning of the cellular blastoderm stage (stage 5), maternal *cad* mRNA has been largely degraded. As a result of zygotic gene activity, a

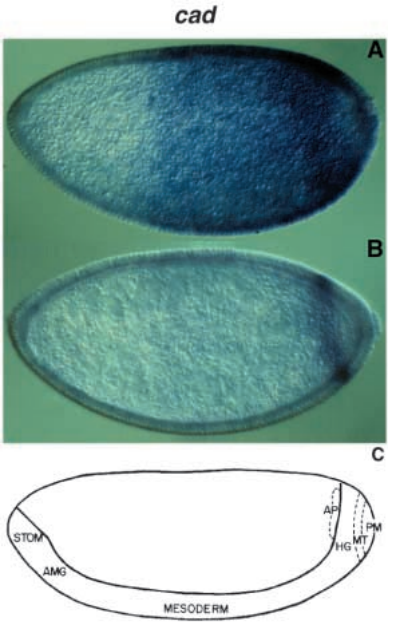

Fig. 1. Posterior-to-anterior differential in *cad* expression. (A) The maternally uniformly deposited component of *cad* expression is rendered, via translation repressing activity of Bicoid, into a posterior-to-anterior gradient that is evident in the stage 4 embryo. (B) The zygotic component of *cad* expression is seen in the posterior of the stage 5 embryo from approximately 10 to 55% EL (Rivera-Pomar et al., 1995; not shown); in lightly stained embryos it can be seen that the strongest part of this staining is in a stripe at 11-17% EL. (C) Fate map of blastoderm stage embryo; primordia shown are AP, anal pads; HG, hindgut; MT, Malpighian tubules; PM, posterior midgut; AMG, anterior midgut; STOM, stomodeum [based on data reviewed by Campos-Ortega and Hartenstein (1997) and redrawn from Pignoni et al. (1990)]. In this and all other figures, embryos are shown in lateral view.

posterior domain of *cad* mRNA now appears (Rivera-Pomar et al., 1995). Zygotic *cad* expression is highest at the posterior of this domain, in a stripe at approximately 11 to 17% egg length (EL, measured at 50% dorsal-ventral) (Fig. 1B), which corresponds with the position in the blastoderm fate map of the anlagen of hindgut and anal pads (Campos-Ortega and Hartenstein, 1997).

cad is required to establish the hindgut

To assess the requirement for *cad* in establishing the structures that arise from the posterior ~15% of the blastoderm embryo, namely the posterior midgut, Malpighian tubules and hindgut, we examined mature embryos lacking *cad* activity (maternal and/or zygotic contributions). Embryos lacking both maternal and zygotic *cad* activity (referred to as cad^{m-z-}, or *cad*-deficient), as well as embryos lacking maternal *cad* activity but carrying one zygotically active wild-type *cad* gene (cad^{m-z+}) and embryos lacking only zygotic *cad* (cad^{m+z-}) were generated as described in Materials and Methods.

Staining with anti-Crb antibody, which labels the apical surface of the hindgut epithelium (Fig. 2A), reveals that *cad* activity, provided either maternally or zygotically, is essential for formation of the hindgut. When *cad* is provided only maternally (cad^{m+z-}), the posterior gut appears essentially normal (not shown). When *cad* is provided only zygotically (cad^{m-z+}), a hindgut tube is formed; this can range from almost normal to somewhat reduced in length and twisted (Fig. 2B). When *cad* activity is completely lacking (cad^{m-z-}), the hindgut (as well as the anal pads and the eighth abdominal segment) is almost entirely absent (Fig. 2C). We conclude that *cad* activity is essential for normal hindgut formation, and that while some of this activity must be provided maternally, most can be provided either maternally or zygotically.

The Malpighian tubules and posterior midgut also arise from domains expressing *cad*, but form relatively normally in *cad*-deficient embryos. The posterior midgut arises from the most posterior region of the embryo, where the maternally derived *cad* mRNA level is highest (Fig. 1A); additionally, starting during stage 10 and continuing to the end of embryogenesis, *cad* is expressed in a posterior-to-anterior gradient in the posterior midgut (Fig. 2D). Despite this suggestive expression pattern, however, in *cad*-deficient embryos the posterior midgut primordium forms normally, expresses *cad* in the characteristic posterior-to-anterior gradient and appears normal in size and development (Fig. 2E). The Malpighian tubules and the hindgut both arise from the same blastoderm primordium (Diaz et al., 1996); also, similar to what is seen for the posterior midgut, *cad* expression is reinitiated in a proximal-to-distal gradient in the tubules during stage 13 (Liu and Jack, 1992). Nevertheless, as revealed by their expression of *fkh*, the tubule primordia form relatively normally in embryos lacking *cad* activity (although their elongation is incomplete, as noted by Liu and Jack, 1992) (Fig. 2G).

Thus, even though it is expressed maternally in all of the primordia giving rise to posterior gut, in terms of the generation of a normal-appearing structure in the embryo, *cad* is only required in the hindgut primordium.

fkh and *wg* mediate the *cad* hindgut phenotype

The absence of the hindgut primordium from *cad*-deficient embryos suggests that Caudal regulates genes required for establishing and/or maintaining the hindgut primordium. *tll*, *fkh*, *byn*, *bowl* and *wg* are likely targets for *cad* regulation, as all are required for some aspect of hindgut development: the hindgut is missing from both *tll* and *fkh* embryos, and severely reduced in *wg*, *byn* and *bowl* embryos (Fig. 3A-F; Pignoni et al., 1990; Skaer and Martinez Arias, 1992; Harbecke and Lengyel, 1995; Diaz et al., 1996; Singer et al., 1996; Wang and Coulter, 1996). Furthermore, since maternally provided Caudal, which persists only through the blastoderm stage (Macdonald and Struhl, 1986), is sufficient for essentially normal hindgut formation, the fact that all of these genes are expressed at the posterior of the embryo during the blastoderm stage (Fig. 4A-D,F; Baker, 1988; Pignoni et al., 1990; Weigel et al., 1990; Kispert et al., 1994; Wang and Coulter, 1996) means that they are potential targets for regulation by Caudal.

The effect of absence of maternal and/or zygotic *cad* activity on the expression of these genes was assessed by in situ hybridization with appropriate probes. For *tll*, *byn* and *bowl*, absence of *cad* activity did not result in a detectable effect on

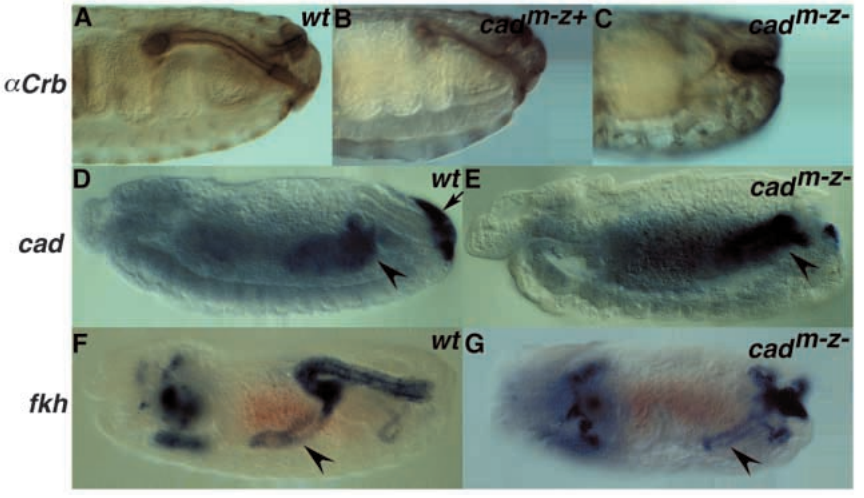

Fig. 2. *cad* is required for hindgut, but not posterior midgut and Malpighian tubule formation. (A) Hindgut of wild-type stage 16 embryo labeled with anti-Crb. In embryos lacking zygotic *cad* activity, the hindgut is indistinguishable from wild type (not shown). (B) Embryos lacking the maternal *cad* contribution (cad^{m-z+}) develop hindguts that are variably somewhat reduced in size and twisted. (C) The hindgut in embryos completely lacking *cad* activity (cad^{m-z-}) is severely reduced or undetectable. (D) In wild-type embryos at stage 13, *cad* expression (detected by in situ hybridization) labels the anal pads (arrow) and the posterior midgut in a posterior-to-anterior gradient (arrowhead). (E) In the cad^{m-z-} embryo, identified by lack of anal pads, expression of *cad* reveals the presence of a normal amount of posterior midgut tissue (arrowhead). (F) In the stage 15 embryo, the elongating Malpighian tubules are recognizable by their expression of *fkh* (arrowhead, detected by in situ hybridization). (G) In the cad^{m-z-} embryo, *fkh* staining reveals the presence of normal size (although incompletely elongated) tubules (arrowhead).

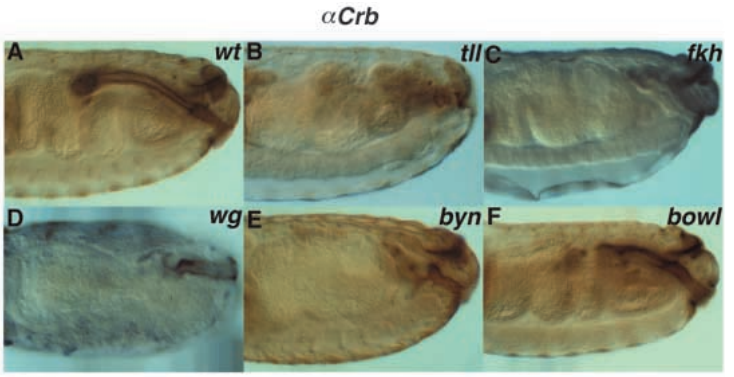

Fig. 3. Hindgut phenotype of *tll*, *fkh*, wg, *byn* and *bowl* mutant embryos. Hindgut of stage 16 embryos labeled with anti-Crb. (A) Wild-type embryo. (B) Hindgut is virtually absent from a *tll* embryo. (C) A very small remnant of hindgut is seen in *fkh* embryos; the hindgut is severely reduced in *wg* (D) and *byn* (E) embryos; hindgut is reduced to about one-half normal length in *bowl* (F) embryos.

expression (Fig. 4A-C; data not shown). As described below, however, we found that *cad* activity is essential for expression of *fkh* and *wg*.

Both maternal and zygotic *cad* contributions are necessary for posterior *wg* expression. During early stage 5, just prior to its expression in 14 stripes that are required to establish the segmental pattern, *wg* is expressed in two domains at the anterior, and in a broad posterior stripe (Baker, 1988; Fig. 4D). This terminal *wg* stripe is located at approximately 8-12% EL, overlapping with the posterior of the zygotic *cad* stripe and with the position of the hindgut and Malpighian tubule primordia in the blastoderm fate map (Campos-Ortega and Hartenstein, 1997). Expression of the *wg* terminal stripe has been shown to be independent of other segmentation genes, but has not been otherwise characterized (Ingham and Hidalgo, 1993). We find that all embryos from *cad* germline mothers (both cad^{m-z-} and cad^{m-z+}) fail to express the terminal stripe of *wg* (Fig. 4E). These results demonstrate that maternal *cad*

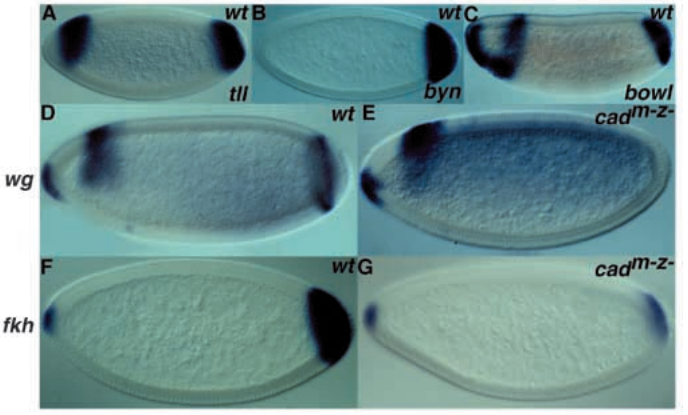

Fig. 4. *cad* is required for *fkh* and *wg* expression. Wild-type blastoderm stage expression is shown for *tll* (A), *byn* (B), *bowl* (C), *wg* (D) and *fkh* (F). Expression of *tll*, *byn* and *bowl* is not detectably altered in embryos lacking *cad* (not shown). (E) In embryos lacking maternal *cad* activity (both cad^{m-z-} and cad^{m-z+}) the *wg* terminal stripe is missing. Also, embryos lacking *cad* only zygotically (cad^{m+z-}) do not express the terminal *wg* stripe (not shown). (G) In embryos lacking maternal and zygotic *cad*, *fkh* expression is severely reduced. Note that the anterior expression domains of *fkh*, *wg* and *bowl* provide internal controls for normalization of the posterior expression pattern. The posterior *wg* stripe is also missing from embryos mutant for either *fkh* or *tll*, but is present in *byn* and *bowl* embryos (not shown). Embryos are shown at stage 5.

activity is essential for the transcription of *wg* in the terminal stripe. Among embryos from *wg* heterozygous parents, approximately one-quarter (presumably those lacking only the zygotic component of *cad* expression) lack the terminal *wg* stripe (not shown). Thus both maternal and zygotic *cad* activities are required for expression of the terminal *wg* stripe.

The expression of the early cap of *fkh* also requires *cad* activity; approximately half of the embryos from *cad* germline females mated to *cad* heterozygous males (i. e., cad^{m-z-} embryos) show a dramatic reduction in both the size and intensity of the posterior cap of *fkh* expression (Fig. 4G). If *cad* is supplied either maternally or zygotically, however, *fkh* expression is normal (cad^{m-z+}, cad^{m+z-}, not shown). Thus expression of the posterior cap of *fkh* requires *cad* activity, which can be provided either maternally or zygotically. Later, by stage 10 (Fig. 6D), *fkh* expression is expressed as strongly in *cad*-deficient as in wild-type embryos, indicating that this later expression is independent of *cad* activity. Since *tll* and *hkb* are required to activate early *fkh* expression (Weigel et al., 1990) but are not themselves regulated by *cad* (data not shown), *cad* must act combinatorially with these two genes to promote early *fkh* expression.

cad also regulates *wg* in combination with other genes. In addition to the demonstrated requirement for *cad*, expression of the posterior *wg* stripe requires positive input from *fkh* and *tll*, as the stripe is absent from the respective mutant embryos (data not shown). Since embryos lacking either maternal or zygotic *cad* fail to express the posterior *wg* stripe, but still express *fkh* and *tll*, *cad* must act combinatorially with *fkh* and *tll* to promote formation of the posterior *wg* stripe. Expression of the terminal *wg* stripe thus requires combinatorial action of *cad*, *tll* and *fkh*; the posterior limit of the stripe is defined by repression by *hkb* (Mohler, 1995).

Ectopic cell death in hindgut primordium of *cad*-deficient embryos

The fact that *tll*, *byn* and *bowl* expression at the blastoderm stage are apparently normal in *cad*-deficient embryos suggests that a hindgut primordium is established in the absence of *cad* activity. The lack of proper blastoderm stage expression of *fkh* and *wg*, however, indicates that this hindgut primordium is not properly specified. We showed previously in *byn* mutant embryos that one of the earliest phenotypic manifestations of an abnormally specified hindgut primordium is ectopic expression of the cell death gene *reaper* (*rpr*) (Singer et al., 1996).

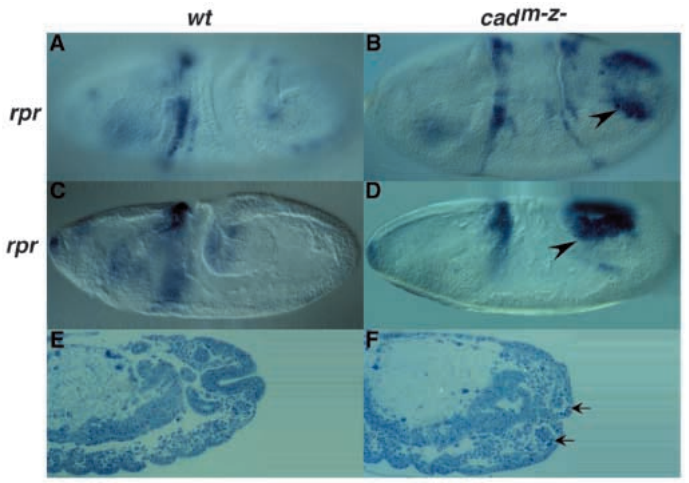

Fig. 5. Ectopic *rpr* expression and cell death in *cad*-deficient embryos. (A,B) *rpr* mRNA expression in wild-type (A) and cad^{m-z-} (B) embryos during early stage 7. Normal *rpr* expression in strong and weak stripes immediately posterior to the cephalic furrow (A) provides an internal control for comparison with the embryo lacking *cad* activity (B). (C,D) *rpr* mRNA in wild-type (C) and cad^{m-z-} (D) embryos at stage 8; the extreme anterior spot and the stripe just anterior to the cephalic furrow again provide internal controls for staging. Ectopic *rpr* in B and D is indicated by arrowheads. (E,F) Transverse sections of stage 13, wild-type (E) and cad^{m-z-} embryos (F) reveals ectopic apoptotic cells in *cad*-deficient embryos at the posterior of the embryo, in the region around the exit of the hindgut remnant (arrows).

To ask whether the extremely reduced hindgut in *cad*-deficient embryos might result from a similar course of programmed cell death, we examined expression of *rpr* in embryos lacking *cad*. We observed a striking pattern of ectopic *rpr* expression in cad^{m-z-} embryos, beginning during stage 7 and continuing into stage 8 (gastrulation), in a ring at the circumference of the amnioproctodeal plate (Fig. 5B,D). The actual loss of cells that is presumably initiated by this ectopic *rpr* expression does not begin until after early stage 10, however, as the hindgut primordium is present at this stage in *cad*-deficient embryos, indicated by its expression of *byn* and *fkh* (see below). By stage 13, the *cad*-deficient embryo has a very short hindgut and no detectable anal pads (Fig. 2C,E); in sections of stage 13 embryos there are numerous apoptotic cells in the region of the hindgut remnant (Fig. 5F).

Incomplete germband extension in *cad*-deficient embryos

The amnioproctodeal plate of cad^{m-z-} embryos, distinguished by the fact that it ectopically expresses *rpr*, becomes increasingly retarded in its anteriorward movements during stages 7 and 8 (Fig. 5B,D, arrowheads). By early stage 10 (~4.5 hours AEL), when the germ band in the wild-type embryo is almost fully extended, the germ band in the cad^{m-z-} embryo has extended only about 40% of the normal distance and the embryo appears twisted (Fig. 6B). This phenotype is particularly obvious when embryos are labeled for *fkh* expression (which is reestablished in *cad*-deficient embryos after the blastoderm stage): the hindgut primordium, which strongly expresses *fkh*, remains near the posterior of the embryo (Fig. 6D). Thus, in the absence of *cad* activity, there is a severe defect in germband extension.

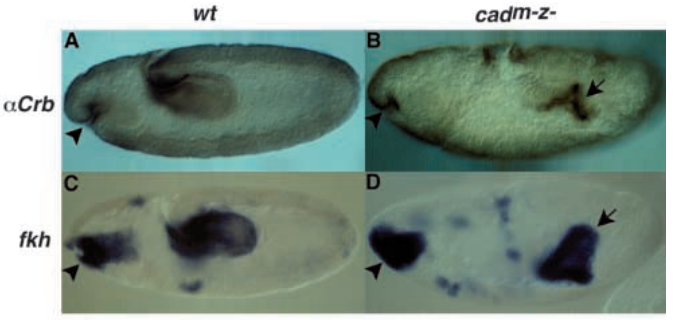

Fig. 6. Incomplete germband extension in embryos lacking *cad*. Anti Crb staining shows that, compared to wild type (A), the germband is incompletely extended in *cad*-deficient (cad^{m-z-}) embryos (arrow) (B); note that the extent of the stomodeal invagination (indicative of early stage 10) is equal in both embryos (arrowheads). Failure of germ band extension is also observed by comparing stage 10 wild-type (C) and *cad*-deficient (cad^{m-z-}) (D) embryos hybridized with *fkh* probe.

Hindgut does not invaginate (gastrulate) in *cad*-deficient embryos

Gastrulation can be followed by using expression of *byn* as a marker for the hindgut primordium. In the wild-type embryo, *byn* is expressed in a ring at the circumference of the amnioproctodeal plate. The edges of this ring fold together as the posterior midgut primordium invaginates during stages 6 and 7; the ring of hindgut primordium then sinks inward during stage 8 and is completely internalized by the end of stage 9 (Fig. 7A-C,F,G). The zygotically expressed *cad* stripe and the posterior *wg* stripe are also expressed in the bordering ring (i.e., the hindgut primordium) of the invaginating amnioproctodeal plate (Fig. 7D,E). Strikingly, in *cad*-deficient (cad^{m-z-}) embryos, the *byn*-expressing ring of hindgut primordium draws together, but fails to invaginate, remaining on the outside of the embryo (Fig. 7H). Thus, although internalization of the Malpighian tubule and posterior midgut primordia is normal in *cad*-deficient embryos (Fig. 2E,G), the gastrulation movements necessary for internalization of the hindgut primordium do not occur in embryos lacking *cad* activity.

fog mediates the *cad* gastrulation phenotype

The failure of the hindgut to become internalized in cad^{m-z-} embryos raises the question of whether *cad* might regulate a zygotically expressed gene required for the invagination of the amnioproctodeal plate. One gene known to be required for gastrulation is *fog*; *fog* mutant embryos lack not only the posterior midgut, but, as revealed by anti-Crb staining, the Malpighian tubules and hindgut as well (Fig. 8A). In the blastoderm stage embryo, *fog* expression is first activated in the region that will become the ventral furrow; shortly thereafter, expression is initiated in a posterior cap, in the region that will become the amnioproctodeal invagination (Costa et al., 1994; Fig. 8B). In cad^{m-z-} embryos, *fog* expression in the prospective ventral furrow is normal, but is significantly reduced in the posterior cap (Fig. 8C). Thus, *cad* is required for the normal level of expression of *fog* in the prospective amnioproctodeal plate; decreased *fog* expression in cad^{m-z-} embryos is likely responsible for the failure of the hindgut primordium to be internalized during gastrulation.

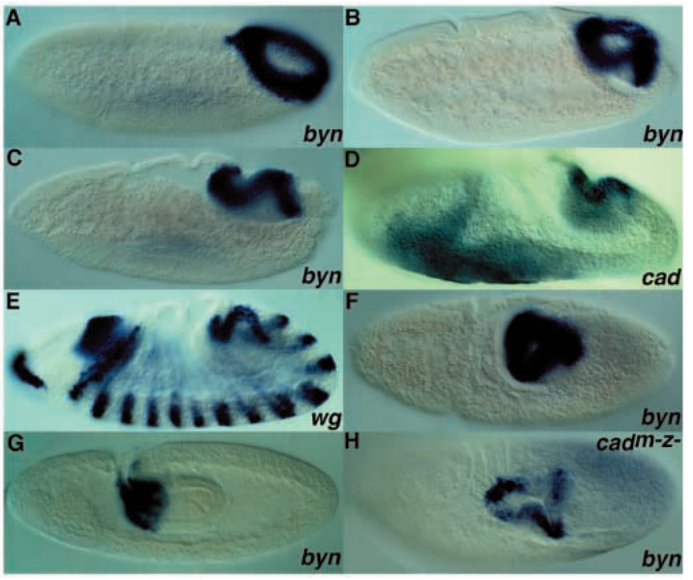

Fig. 7. Gastrulation defect in *cad*-deficient embryos. (A-C,F,G) Expression of *byn* in wild-type embryos is shown at stages 6 (A,B), 7 (C), 8 (F) and 9 (G). (D,E) Expression of *cad* (D) and *wg* (E) in the hindgut primordium at stage 7. (G) By end of stage 9 the hindgut is completely invaginated in wild-type embryos as is revealed by the internally localized expression of *byn*. (H) In cad^{m-z-} embryos of early stage 10, the hindgut primordium is not internalized, but remains as a *byn*-expressing ring around the exterior opening of the invaginated posterior midgut primordium.

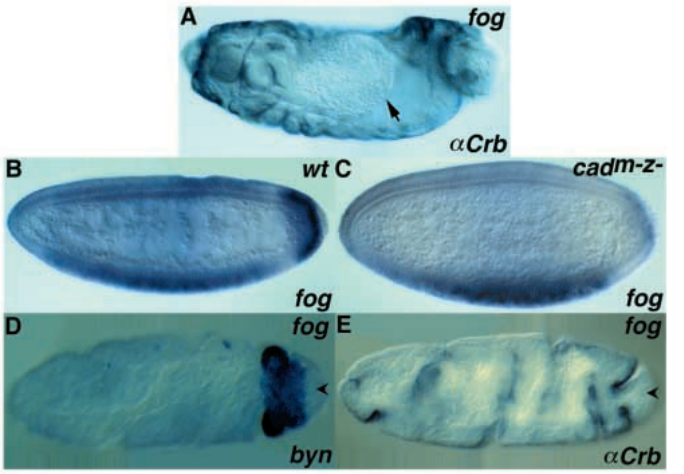

Fig. 8. *fog* is required for hindgut formation and is regulated by *cad*. (A) As revealed by αCrb staining, *fog* mutant embryos lack hindgut and Malpighian tubules; in addition, the posterior portion of the yolk is not surrounded by the posterior midgut epithelium (arrow). (B) At the beginning of gastrulation (stage 5 to 6 transition), *fog* is expressed in a strong posterior cap, in the region that will invaginate as the amnioproctodeal plate. (C) In cad^{m-z-} embryos, this posterior *fog* expression is significantly reduced (although not completely eliminated). (D) The hindgut primordium does not invaginate in *fog* mutant embryos, as can be seen by expression of *byn* in a ring of surface cells at the posterior; the labeled cells in two lobes anterior to this ring are presumably the anal pads, which also express *byn*. (E) A Crb-stained embryo of the same stage; note that in the stage 10 embryos shown in both D and E, the posterior midgut primordium remains uninvaginated (arrowheads).

As *fkh* or *wg* mutant embryos do not display detectable defects in gastrulation (Weigel et al., 1989b; data not shown), *fog* is the only gene presently known to mediate effects of *cad* on gastrulation. In *fog* mutant embryos, none of the posterior gut primordia invaginate (Fig. 8D,E), while in *cad*-deficient embryos the posterior midgut and Malpighian tubule primordium do invaginate; thus, consistent with the in situ hybridization results (Fig. 8C), a low level of *fog* activity is present at the posterior of embryos lacking *cad*.

DISCUSSION

Using germline clones to remove both the maternal and zygotic contribution of the homeodomain transcription factor Caudal, we have shown that *cad* activity is required to establish the hindgut. This is explained, at least in part, by the required role of *cad* in activating blastoderm stage expression of the genes *fkh* and *wg*, which are required for hindgut commitment and development. *cad* is also required for invagination of the hindgut primordium, a function that is largely explained by its role inducing posterior expression of *fog*, a secreted protein required for cellular constriction during gastrulation. Thus via regulation of *fkh*, *wg* and *fog*, and possibly other downstream genes, *cad* plays an essential role both in specifying the hindgut and in its invagination.

Combinatorial gene activity controlling hindgut patterning and gastrulation

The genetic data presented here demonstrate that *cad* acts together with a number of zygotically functioning genes to activate transcription at the posterior terminus. *fkh* expression requires input from *cad* (either maternal or zygotic) plus *tll* and *hkb*, while expression of the posterior *wg* stripe requires both maternally and zygotically provided *cad*, as well as *tll* and *fkh*. Previous work showed that transcription of *fog* requires *tll*, *hkb* and *fkh* (Costa et al., 1994); the significant decrease in *fog* expression in embryos lacking *cad* activity adds another required gene activity to this combination. Thus, as has been described for patterning of the central segmented trunk ectoderm (reviewed by Rivera-Pomar and Jäckle, 1996), different combinations of transcription factors – including Caudal – act combinatorially at the posterior of the embryo to pattern the hindgut and to control gastrulation.

The network of regulatory interactions controlling hindgut formation and gastrulation, deduced from previous work and results described here, is summarized in Fig. 9. The regulatory interactions involving Caudal, indicated by solid arrows, are most likely direct. First, all of the genes involved in these interactions encode transcription factors. Second, all of the interactions shown occur within a period of about 1 hour (stage 5, cellularization) allowing little time for the expression and action of unknown, potentially intervening transcription factor encoding genes.

Are the activities of *fkh*, *wg* and *fog* sufficient to mediate the function of *cad* in hindgut specification and gastrulation? Reduction in the domain of expression of *fog* has been reported to cause a corresponding reduction in the size of the posterior

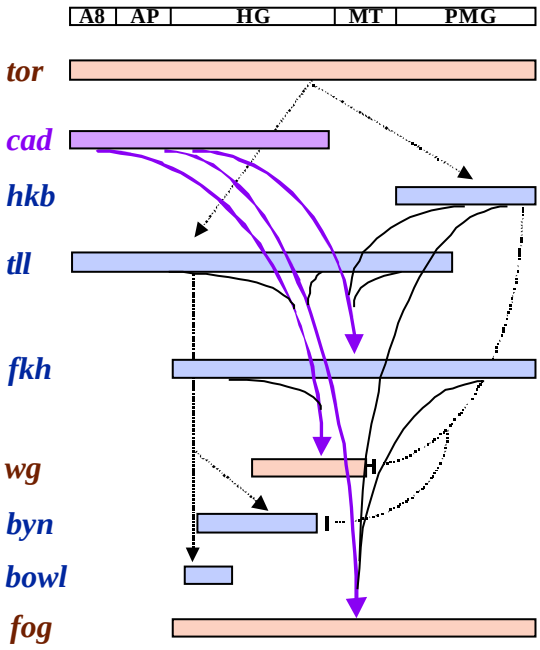

Fig. 9. *cad* regulatory interactions in gene network controlling hindgut development and gastrulation. Interactions are based on experiments and references described in the text. Activations by *cad* are shown with solid purple arrows, combinatorial interactions with *cad* with thinner solid black arrows. Interactions not involving *cad* are indicated with dotted lines. Activating interactions are indicated by lines ending in arrowheads, repression by lines ending in bars. The upper bar is a linear fate map indicating the structures formed from the posterior of the embryo (A8, eighth abdominal segment; AP, anal pads; HG, hindgut; MT, Malpighian tubules; PMG, posterior midgut). The colored bars below this map indicate domains where particular genes are required, deduced from the structures that are lacking when the gene is mutated. Domains for genes encoding transcription factors are blue/purple, those for genes encoding signaling molecules (*wg* and *fog*) or a receptor (*tor*) are brown.

Fig. 10. Conserved expression of *cad*, *fkh*, *wg* and *byn* in *Drosophila* and *Xenopus*. Outlines are shown of a dorsal view of the pre-gastrula *Drosophila* and the early gastrula (stage 10) *Xenopus*, with anterior and animal pole, respectively, to the left. Color code for expression of different genes is shown below. *Drosophila* expression patterns are from data described here; *Xenopus* expression patterns are from references in the text. Note that, while Wnt8 expression (shown) is largely excluded from the dorsal lip, expression of Wnt11 (not shown) is throughout the marginal zone, with strongest expression in the dorsal lip (Ku and Melton, 1993).

gut primordium that is invaginated (Costa et al., 1994); the observed reduction in the domain of *fog* expression (Fig. 8C) seems sufficient to explain the partial gastrulation defects in *cad*-deficient embryos. Thus *cad* appears to be required together with *tll*, *hkb* and *fkh* to promote *fog* expression in the hindgut primordium, but is not necessary for the *fog* expression required to internalize the primordia of the posterior midgut and Malpighian tubules. The effect of *cad* on germband extension may be a result of its effect on gastrulation, since non-gastrulating (e.g., *fog*, *tor* and *DRhoGEF2*) embryos fail to elongate the germband (Fig. 8E; Schüpbach and Wieschaus, 1986; Häcker and Perrimon, 1998).

Whether the effect of *cad* on hindgut specification is mediated entirely through *fkh* and *wg* is less easily determined, since, late in embryogenesis, *fkh* and *cad*-deficient embryos lack most, and *wg* embryos lack much, of the hindgut. The reduced hindgut is likely a result of cell death in the misspecified primordium. While ectopic *rpr* expression is seen in the hindgut primordium of *cad*-deficient embryos beginning at stage 7, we did not detect this ectopic expression in either *fkh* or *wg* embryos (although there is ectopic expression of *rpr* later in the hindgut of stage 12 *fkh* embryos) (not shown). Thus *cad* may regulate at least one gene in addition to *fkh* and *wg* that is required for proper hindgut specification (and hence prevention of ectopic apoptosis).

cad homologs: conserved expression and function

cad homologs are highly conserved across several phyla. There is one gene known in *C. elegans* and *Drosophila*, while there are three *cad* homologs in vertebrates, known as X-cad in frog and Cdx in mouse and chick (reviewed by Marom et al., 1997).

Early expression of *cad* homologs is at the posterior of the embryo. In the 4-cell *C. elegans* embryo, the *cad* homolog (pal-1) is required in the most posterior cell for its correct specification (Hunter and Kenyon, 1996). In a number of other embryos, at the beginning of gastrulation, *cad* homologs are expressed at the posterior, in a ring or line of cells about to invaginate (see Fig. 10 for *Drosophila* and *Xenopus*). This region, which can be considered the 'blastopore equivalent', consists of the hindgut/posterior midgut primordium in *Drosophila*, the germ ring in fish, the marginal zone/blastopore lip in frog, and the node/primitive streak in chick and mouse. In chick and mouse, Cdx genes are expressed in the primitive streak, where cells are about to ingress into the interior of the embryo (reviewed by Marom et al., 1997). In a number of cases (*Drosophila cad*, chick Cdx-B and Cdx-C), the earliest *cad* expression is as a posterior-to-anterior gradient (Macdonald and Struhl, 1986; Marom et al., 1997).

An additional common feature in the expression of many *cad* homologs is a later expression in the intestine (gut). *Drosophila cad* is expressed in the posterior midgut and the Malpighian tubules of the older embryo (Mlodzik et al., 1985; Macdonald and Struhl, 1986; Fig. 2D); chick and mouse Cdx genes are expressed in the posterior gut, which is the only tissue of expression in the adult (reviewed by Marom et al., 1997). Echoing the early expression pattern, this gut expression is also usually found as a posterior-to-anterior gradient; this is seen for *cad* in the *Drosophila* midgut (Fig. 2D), and has been described for all three mouse Cdx genes in the adult intestine (James and Kazenwadel, 1991; James et al., 1994; Gamer and Wright, 1994).

Conserved constellation of genes involved in gastrulation

In addition to *cad*, three other genes required at the posterior of the *Drosophila* embryo for formation of the hindgut, *fkh*, *byn* and *wg*, are related to genes found throughout the metazoa, known as HNF-3 (α, β, and γ), Brachyury (also known as T) and Wnt, respectively. In many cases, these homologs are expressed in portions of the 'blastopore equivalent' at the posterior of the embryo that overlap with domains of expression of *cad* (Cdx).

In *C. elegans*, a Wnt homolog is expressed, and required for proper posterior development, in the same posterior blastomere where the *cad* homolog pal-1 functions (reviewed by Han, 1997). In sea urchin, HNF-3 and Brachyury homologs are expressed in the vegetal plate just prior to gastrulation (Harada et al., 1996). In fish and frog, Caudal, Brachyury and Wnt (Wnt8 and Wnt11) are initially expressed around most or all of the blastopore lip while HNF-3 expression is dorsally localized (Joly et al., 1992; Kelly et al., 1995; Schulte-Merker et al., 1992; Strähle et al., 1993; Northrop and Kimelman, 1994; O'Reilly et al., 1995; Horb and Thomsen, 1997; Smith and Harland, 1991, Christian and Moon, 1993; Ku and Melton, 1993) (see Fig. 10 for expression in *Xenopus*). As gastrulation proceeds, the expression of these genes becomes more restricted and non-overlapping, with HNF-3 and Brachyury expression becoming localized to the notochord and Wnt8 expression retreating from the dorsal position and becoming exclusively ventral. Patterns of expression of HNF-3 and Brachyury consistent with this general description have been found in ascidians, amphioxus, chick and mouse (Olsen and Jeffery, 1997; Yasuo and Satoh, 1993; Corbo et al., 1997; Holland et al., 1995; Zhang et al., 1997; Shimauchi et al., 1997; Shimeld, 1997; Tam and Behringer, 1997; Ruiz i Altaba et al., 1993).

Required roles for some of these genes have been demonstrated by analysis of mutants: mouse HNF-3ß knockouts reveal requirements in formation of the node, notochord and head process (reviewed by McMahon, 1997); fish *no tail* and mouse *T* mutants reveal a requirement for Brachyury in migration of mesoderm through the primitive streak and in formation of the notochord (reviewed by Hermann and Kispert, 1994; Tam and Behringer, 1997).

There is thus a constellation of conserved genes – *cad* (Cdx), *fkh* (HNF-3), *wg* (Wnt8 and Wnt11) and *byn* (Brachyury) – whose overlapping expression patterns in the blastopore equivalent suggests function in a related process. The phenotypes of the available mutations in these genes suggest that the common function is to specify cell fate at the blastopore; in most cases, an essential part of this fate is internalization and forward migration during gastrulation.

Evolutionary implications

The striking conservation in expression, and likely in function, of *cad* suggests that the regulation of posterior terminal development in *Drosophila* by Caudal may represent a more ancient regulatory mechanism than the *tor* receptor and the two genes that it activates, *tll* and *hkb*. Of these three genes, a vertebrate homolog is known only for *tll*; the function of this vertebrate gene, Tlx, is related to that of *Drosophila tll* not in the posterior, but rather in the anterior, in the establishment of the brain (Pignoni et al., 1990; Monaghan et al., 1997). Thus the Torso receptor pathway and its activation of *tll* and *hkb* has probably been superimposed relatively recently (in evolutionary terms) upon a more ancient, Caudal-regulated network of gene activity controlling gastrulation and gut formation.

The fact that the same four genes are expressed at the blastopore equivalent of chordates and the amnioproctodeal invagination of *Drosophila* suggests that these two highly dynamic domains are homologous. Given the regulatory hierarchy that we have demonstrated in *Drosophila*, we propose that, in embryos of the proximate ancestor to arthropods and chordates (reviewed by DeRobertis and Sasai, 1996), the posterior was defined by a posterior-to-anterior gradient of Cad activity. Cad then activated expression of downstream network of genes that controlled invagination (gastrulation) and gut specification. Cad expression in the archenteron probably continued and played an essential role, as this structure differentiated into the gut. Going beyond the bilaterian ancestor to chordates and arthropods, it is worth considering that this nexus of gene expression may have evolved even more basally in the metazoa.

The foregoing, by homologizing the insect amnioproctodeal invagination with the echinoderm and vertebrate blastopore, does not fit with the classical definition of protostomes and deuterostomes. This view categorizes arthropods as protostomes, in which the mouth is derived from the primary invagination of gastrulation, and chordates as deuterostomes, where the mouth arises from a secondary invagination (reviewed by Willmer, 1990). More recently, comparisons of gastrulation patterns in many different species, as well as construction of molecularly based cladograms, has called into question the utility of these classically defined groups (Willmer, 1990; Kirschner and Gerhart, 1997). While there continues to be uncertainty in our understanding of 'protostome' and 'deuterostome' phyla, the significant conclusion of the data presented here is that there may be a homology between the blastopore of vertebrates and the amnioproctodeal (posterior) invagination of insects.

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