

Expression and regulation of *caudal* in the lower cyclorrhaphan fly *Megaselia*

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Abstract The homeobox gene *caudal* (*cad*) regulates posterior development in *Drosophila*. In early embryos, the *cad* protein (CAD) is expressed in a posterior-to-anterior concentration gradient, which contributes polarity to the developing embryo. The CAD gradient is complementary to and dependent on the anterior pattern organizer Bicoid (BCD), which represses the translation of ubiquitous maternal *cad* transcripts in the anterior embryo through a direct interaction with the *cad* 3' untranslated region (UTR). Here, we show that early embryos of the lower cyclorrhaphan fly *Megaselia* express the putative *cad* orthologue *Mab-cad* throughout the posterior three quarters of the blastoderm but lack maternal transcripts. In transgenic blastoderm embryos of *Drosophila*, *Mab-cad* cis-regulatory DNA drives the expression of a reporter gene in a similar pattern, while *Mab-cad* 3' UTR fails to mediate translational repression of a ubiquitously transcribed reporter. For another lower cyclorrhaphan fly (*Lonchoptera*) and two related outgroup taxa of Cyclorrhapha

(*Empis*, *Haematopota*), we report maternal *cad* expression in ovarian follicles. Together, our results suggest that BCD is not required for the translational repression of *Mab-cad*, and that maternal *cad* expression was lost in the *Megaselia* lineage.

Keywords Gene expression · Enhancer · Translational repression · Evolution · Brachycera

Introduction

The protein products of the homeobox gene *cad* (CAD) accumulate in a concentration gradient across early insect embryos and are an important factor of posterior patterning. *cad*-deficient *Drosophila* embryos exhibit severe defects in the abdomen and thorax (Macdonald and Struhl 1986; Wu and Lengyel 1998), and *cad* RNA interference (RNAi) in short-germ insects (*Tribolium*, *Gryllus*) yielded embryos that consist of the prognathal head only (Copf et al. 2004; Shinmyo et al. 2005). In *Drosophila*, the full complement of embryonic *cad* activity requires maternal and zygotic transcripts (Macdonald and Struhl 1986). Homozygous *cad* embryos from heterozygous mothers only lack terminal structures including anal tufts, parts of the anal pads, and posterior sensory organs. In these embryos, maternal *cad* expression is sufficient for the proper formation of segments. Conversely, ectopic *cad* expression in the anterior embryo causes head defects (Mlodzik et al. 1990; Niessing et al. 1999; Niessing et al. 2000; Cho et al. 2005).

The CAD gradient of *Drosophila* forms by translational repression of ubiquitous early *cad* transcripts in the anterior embryo, and is mediated by the protein products of *bicoid* (BCD) and *d4EHP* (Dubnau and Struhl 1996; Rivera-Pomar et al. 1996; Cho et al. 2005). The current model of

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this mechanism proposes that ubiquitous d4EHP binds to the 5' cap structure of *cad* mRNA and to BCD, which also directly binds to a BCD binding region (BBR) in the 3' UTR of *cad* mRNA, thereby circularizing the transcript and preventing translation initiation (Niessing et al. 2002; Cho et al. 2005). In other insects, a mechanism of CAD gradient formation has not been elucidated. In the parasitic wasp *Nasonia*, *cad* transcripts are localized at the posterior pole and a CAD protein gradient may be formed by diffusion (Olesnický et al. 2006). In the silk moth, *Bombyx*, and in the red flour beetle, *Tribolium*, translational repression of ubiquitous maternal *cad* transcripts occurs in the anterior embryo but these insects, like *Nasonia*, lack a BCD orthologue (Xu et al. 1994; Schulz et al. 1998).

Here, we report data on the expression and regulation of a *cad* homologue (*Mab-cad*) from the lower cyclorrhaphan fly *Megaselia abdita* (Phoridae). We show that *Mab-cad* expression in the blastoderm is similar to the expression of *cad* in the higher cyclorrhaphan fly *Drosophila*. Unlike *cad*, however, *Mab-cad* is not expressed in ovarian follicles, and early embryos therefore lack maternal transcripts. A *lacZ* reporter construct containing ca. 8 kb of *Mab-cad* cis-regulatory DNA upstream of the first *Mab-cad* exon essentially recapitulates the endogenous early *Mab-cad*

pattern in transgenic *Drosophila*, while the *Mab-cad* 3' UTR fails to suppress the anterior translation of a ubiquitously transcribed reporter gene. Finally, we report the expression of *cad* homologues in the ovarian follicles of three related dipterans (*Lonchoptera*, *Empis*, and *Haematopota*). Our results suggest the loss of maternal *cad* expression in the *Megaselia* lineage and that BCD is not required for the translational repression of *Mab-cad*.

Materials and methods

Sample collection

M. abdita Schmitz (Phoridae) was obtained from our laboratory culture. Adults of *Empis livida* L. (Empididae), *Haematopota pluvialis* L. (Tabanidae), and *Lonchoptera lutea* Panzer (Lonchopteridae) were caught in the surroundings of Göttingen (Germany).

Cloning procedures

Partial homeobox sequences of *cad* homologues were isolated by polymerase chain reaction (PCR) using the

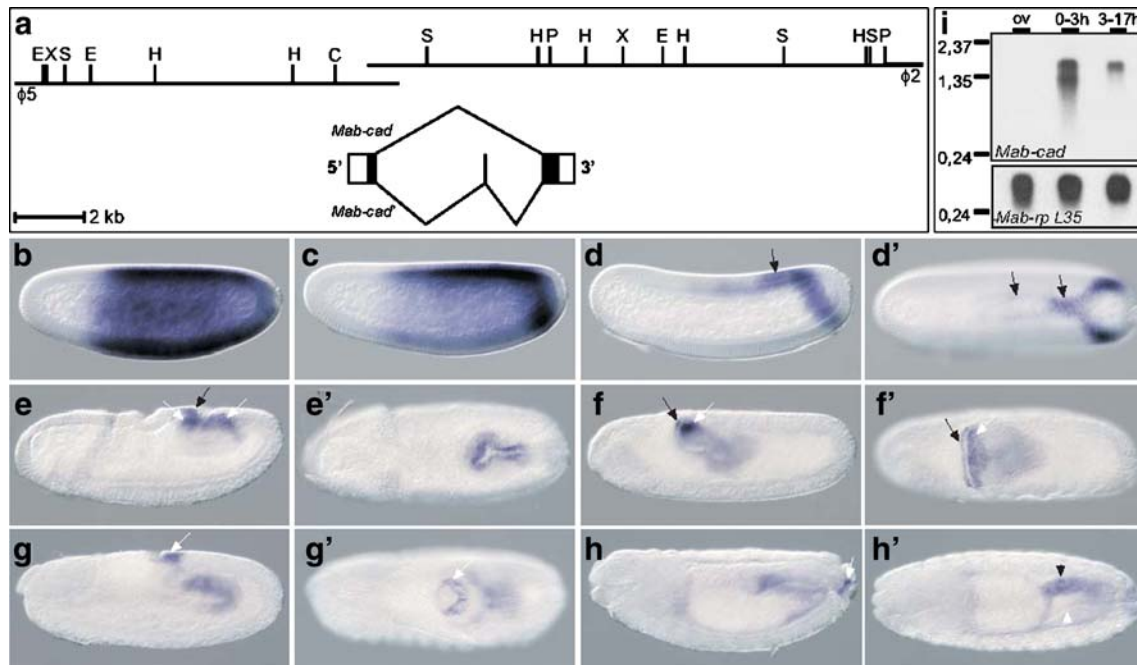


Fig. 1 Characterization of *Mab-cad*. **a** Genomic organization of the *Mab-cad* locus. Restriction sites of *Cla*I (C), *Eco*RI (E), *Hind*III (H), *Pst*I (P), *Spe*I (S), and *Xba*I (X) on two phage clones (Φ 5, Φ 2) are shown above alternative splice variants of *Mab-cad*. ORF sequence is shown as filled boxes. *Mab-cad* expression is shown at syncytial blastoderm (**b**), blastoderm cellularization (**c**), cellular blastoderm (**d**, **d'**), germband extension (**e**–**f'**), germband retraction (**g**, **g'**) stage, and shortly before dorsal closure (**h**, **h'**). All embryos are shown in lateral view with anterior to the left. Embryos at later stages are also shown in

dorsal view (**d'**, **e'**, **f'**, **g'**, **h'**). Amniotic expression (black arrows), posterior embryonic expression (white arrows), and posterior midgut expression (arrowhead) relative to the position of the unstained hindgut (white arrowheads) are indicated. **i** Northern Blot with poly (A) RNA from ovaries (ov), early (0–3 h) and late (3–17 h) embryos hybridized with *Mab-cad* probe (top) and subsequently with a *Mab-rpL35* probe (bottom). RNA standard sizes in kb are shown at the left margin

degenerate primer pair 5'AAR ACS CGB ACS AAR GAY AA / 5'VCG VCG RTT YTG RAA CCA RAT on genomic DNA. 5' and 3' rapid amplification of cDNA ends (RACE) reactions were performed using MARATHON and SMART cDNA Amplification Kits (Clontech) starting with ovarial (*Empis*, *Haematopota*), embryonic (*Megaselia*), and adult (*Lonchoptera*, *Megaselia*) poly(A⁺) RNA. A *Megaselia* riboprotein L35 gene fragment was accidentally recovered in a PCR with degenerate primers and used as a positive control in the Northern Blot analysis. All sequences have been deposited under EU295452–EU295458 in the GenBank database.

Two genomic phages, which include the *Mab-cad* locus, were obtained from a Lambda-Fix II (Stratagene) phage library of adult flies (unpublished). Genomic DNA for the reporter construct was PCR amplified using TaKaRa LA Taq polymerase (TaKaRa) on Lambda-Fix II phage clone Φ 5 using 5' TTT CGA TTG CAC TAA ATA A and M13 (–20). The product was cloned into pGEM-T Easy (Promega), excised with *NotI* and cloned into pCaSpeR-hs43-*lacZ* (Thummel and Pirrotta 1992). A clone with the insert in normal orientation relative to the transcription start was used for transgenic experiments.

To assay translational control in the *Drosophila* blastoderm, we generated a construct containing the *Mab-cad* open reading frame (ORF; 981 bp including start and stop codon) with an N-terminal hemagglutinin (HA)-tag (ATG TAC CCA TAC GAT GTG CCA GAT TAC GCC) as reporter gene. At the 5' end, the reporter gene was flanked by the maternal *hunchback* promoter (nucleotides 1 to 1512 of GenBank accession Y00274; Tautz et al. 1987). At the 3' end, *AscI*, *MluI* and *FseI* cloning sites and the SV40 small t antigen 3' UTR (853 bp) were added. The cassette was cloned into the vector pCaSpeR4 (Thummel and Pirrotta 1992) using *BamHI*. *Drosophila cad* 3' UTR (856 bp), truncated *cad* 3' UTR downstream of the BBR (343 bp) and *Mab-cad* 3' UTR (559 bp) were PCR amplified with the following primer pairs, respectively: 5'-GGC GCG CCA CGA CCA TTC CTG TTA TGC / 5'-GGC CGG CCG AGT TGC TTT ATC TAT GGT G, 5'-GGC GCG CCG TTA AAC ATA ATT TTA GTA CAA GC / 5'-GGC CGG CCG AGT TGC TTT ATC TAT GGT G, and 5'-GGC GCG CCA TAT TGG GAG AAC CAC GTC / 5'-GGC CGG CCG AAT GAT TAA AAA TTT TAT TTT TCA TCG. These primers contain *AscI* (upper primers) or *FseI* (lower primers) endonuclease restriction sites that were used for directional cloning of the 3' UTRs into the reporter cassette.

In situ and Northern hybridization and antibody staining

In situ and Northern hybridizations were done as described (Stauber et al. 2002; Rafiqi et al. 2008). Digoxigenin-labeled RNA probes were synthesized from 5' (*Empis*, *Lonchoptera*), or 3' *cad* RACE products (*Megaselia*,

Haematopota) and from the complete ORF (*lacZ*). Transcripts were detected using anti-digoxigenin-AP antibody (Roche) and NBT/BCIP (Roche). HA was detected using anti-HA rat monoclonal antibody (Roche) and standard *Drosophila* protocols.

Results and discussion

Genomic organization and expression of a *cad* homologue from *Megaselia*

A homeobox fragment of *Megaselia caudal* (*Mab-cad*) was obtained by degenerate PCR. This sequence was extended by 5' and 3' RACE experiments on cDNA templates from both embryos and adult flies. We obtained two transcripts, which differed in the presence of a small (54 bp) exon in

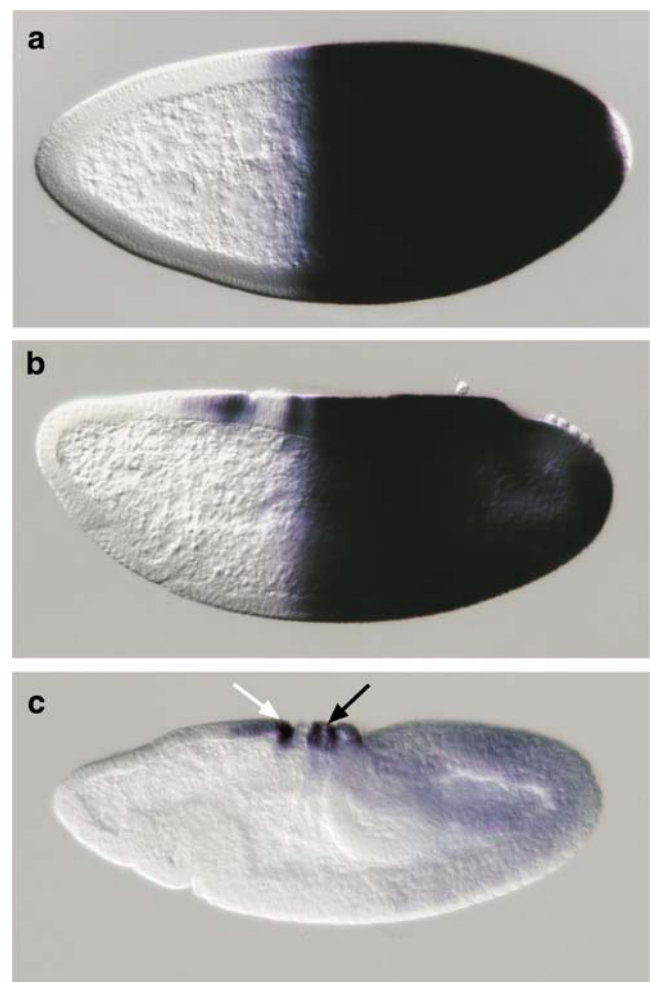


Fig. 2 Expression of *lacZ* transcripts under the control of *Mab-cad* cis-regulatory DNA in transgenic *Drosophila*. Embryos are shown at **a** blastoderm, **b** early gastrulation, and **c** germband extension. Anterior is left and dorsal up. The white arrow designates expression in the dorsal portion of the cephalic furrow and the black arrow expression in the posterior amnioserosa

the open reading frame of the embryonic transcript. Both *Megaselia* transcripts were mapped onto phage clones from a representative genomic library of *Megaselia*, revealing two alternative splice variants of the same locus (Fig. 1a; we refer to the sequence without the 54 bp exon as *Mab-cad* and to the sequence with this exon as *Mab-cad'*). The position of the miniexon is identical to the position of a miniexon that has been reported as an alternative splice variant of the *Tribolium cad* transcripts *Tc-cadA* and *Tc-cadB* (Schulz et al. 1998). In *Drosophila*, this splice variant may have been lost. The predicted full-length proteins of *Mab-cad* and *Mab-cad'* share 38% and 34% sequence similarity, respectively, with the predicted CAD protein of *Drosophila*. The homeodomains of *Mab-CAD* and CAD are 92% identical. These data suggest that the *Mab-cad* locus is orthologous to the *cad* locus of *Drosophila*.

Embryonic whole mount in situ hybridization experiments with *Mab-cad* probes against the 5' exon (not shown) and the 3' exon (Fig. 1b–h') gave identical patterns. Zygotic expression starts one nuclear cycle prior to the onset of cellularization and occurs throughout the posterior 75% of the blastoderm. During blastoderm cellularization, *Mab-cad* expression changes rapidly and becomes restricted to the amnion anlage (black arrows in Fig. 1; Rafiqi et al. 2008)

and a narrow posterior stripe (Fig. 1c–d'). During germband extension, the posterior expression marks the end of the germband, and the amniotic expression becomes restricted to a narrow stripe that invaginates with the proctodeum (Fig. 1e–g'). In addition, a new expression domain appears in the posterior midgut. This expression is strongest in the narrow posterior portion of the midgut, which bends dorsoanterior and connects to the hindgut, which does not express *Mab-cad*. In later embryos, we detected *Mab-cad* expression in this domain and in a ring around the posterior hindgut (Fig. 1h,h'). Unlike *cad* in *Drosophila* (Macdonald and Struhl 1986), *Mab-cad* is not expressed in the pole cells, malpighian tubules, and bilateral pairs of neurons. Also, we could not detect *Mab-cad* transcripts in ovaries and very early embryos, indicating that embryos lack maternal transcripts. To confirm this result, we examined the expression of *Mab-cad* in a developmental Northern blot. This experiment revealed two transcripts in 0–3 h old embryos (Fig. 1i). The longer transcript was also detected at later stages and matches the predicted length of *Mab-cad* (with and without the mini exon). In ovaries, the detection of residual amounts of the longer *Mab-cad* transcript required a 16 times longer exposure time than the detection of much stronger bands in embryos (not shown), confirming the

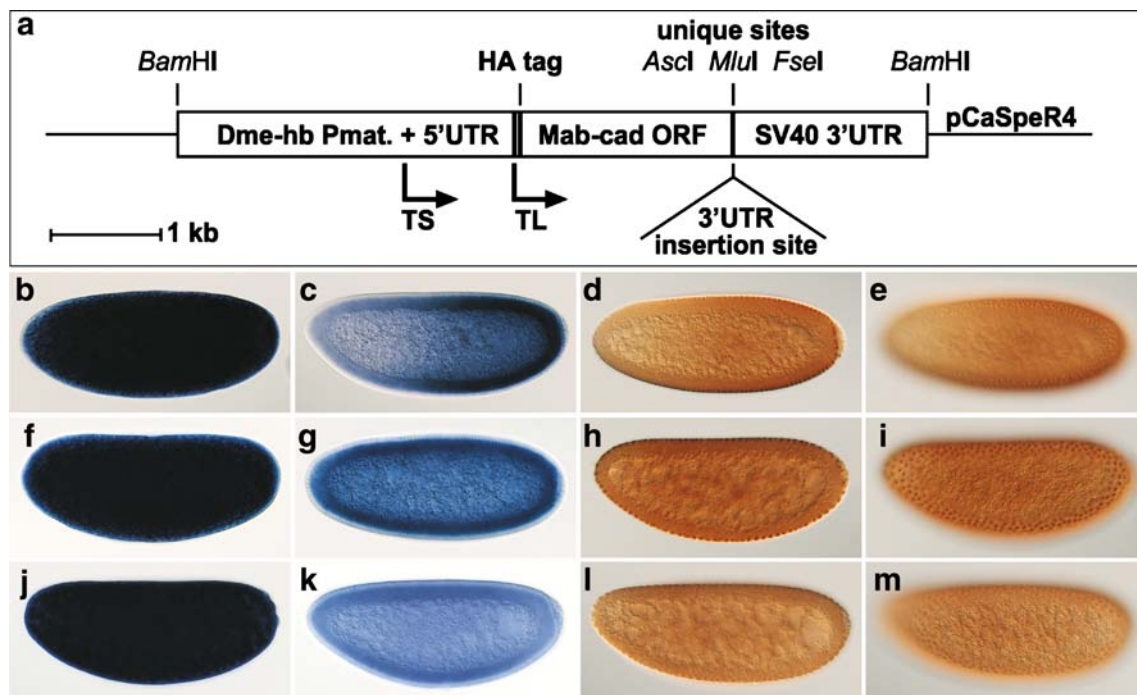


Fig. 3 Translational repression assay. **a** Schematic overview of the translation assay cassette. *TS*, transcription start, *TL* translation start. Transgenic *Drosophila* embryos containing the translation assay cassette with the complete *Drosophila cad* 3' UTR (**b–e**), a truncated *Drosophila cad* 3' UTR lacking the BBR (**f–i**), or the *Mab-cad* 3' UTR (**j–m**) are shown. *Mab-cad* in situ hybridizations of pre-blastoderm (**b, f, j**) and blastoderm embryos (**c, g, k**) as well as two focal planes of early blastoderm embryos stained with a hemagglutinin (*HA*) antibody (**d, e, h, i, l, m**) are shown for each of the three transgenic lines. Note that in

blastoderm embryos with the complete *Drosophila cad* 3' UTR *Mab-cad* transcripts disappear from the anterior pole (**c**) coincident with translational repression of the reporter (**d, e**). The distribution of *Mab-cad* transcripts and protein remains uniform in embryos carrying the cassette with the truncated *Drosophila cad* 3' UTR (**g–i**), or the *Mab-cad* 3' UTR (**k–m**). In situ hybridizations with an *SV40* anti-sense probe were negative, indicating that the polyadenylation signals (AAUAAA) of the added *cad* and *Mab-cad* 3' UTR sequences are functional. All embryos are shown with anterior to the *left* and dorsal *up*

absence of significant maternal *Mab-cad* transcription in nurse cells and oocytes.

Regulation and evolution of *Mab-cad* expression

The lack of maternal *Mab-cad* expression suggests that in *Megaselia*, transcriptional regulation might be sufficient for the anterior repression of *Mab-cad* in the early blastoderm. To test this prediction, we cloned 8 kb immediately upstream of the identified *Mab-cad* transcript sequence into a *lacZ* reporter vector and examined the expression of *lacZ* under the control of *Mab-cad* cis-regulatory DNA in transgenic blastoderm embryos of *Drosophila*. Two independent transgenic lines expressed *lacZ* in patterns reminiscent of endogenous *Mab-cad* expression (Fig. 2a; a third transgenic line showed no expression). Unlike early zygotic transcripts of endogenous *cad*, which accumulate in two broad stripes (Schulz and Tautz 1995), the transcripts of the transgene accumulated evenly throughout the posterior blastoderm like *Mab-cad* transcripts in *Megaselia*. Compared to *Mab-cad* expression in *Megaselia*, the anterior expression boundary of the transgene was shifted by about 10% egg length towards posterior. In addition, the transgene was expressed in the dorsal portion of the cephalic furrow and in the posterior portion of the amnioserosa (Fig. 2b,c). These observations are consistent with the hypothesis that the anterior repression of *Mab-cad* activity

in early *Megaselia* embryos is regulated at the transcriptional level, without BCD-dependent posttranscriptional regulatory input.

Surprisingly, BCD represses the translation of a ubiquitously expressed *cad* transgene from *Tribolium* (Wolff et al. 1998). The signal provided by the transcript of this *cad* homologue that mediates translational repression in transgenic *Drosophila* has not been identified, but the observation raises the question whether BCD can repress the translation of a broad range of *cad* homologues. To test the effect of BCD on the translation of maternally expressed *Mab-cad*, we generated a reporter cassette to test 3' UTRs for their ability to mediate translational repression in early *Drosophila* embryos. The cassette contains the maternal promoter and 5' UTR of the segmentation gene *hunchback*, the hemagglutinin (HA)-tagged *Mab-cad* open reading frame, and a cloning site for 3' UTRs (Fig. 3a). To ensure the stability of reporter transcripts, SV40 3' UTR was included downstream of the multiple cloning site. Using this cassette, we generated constructs containing the *cad* 3' UTR from *Drosophila*, either with or without the Bicoid Binding Region (BBR; truncation 5' of BBR) or the *cad* 3' UTR from *Megaselia*, and tested their expression, both at the transcript and at the protein level in transgenic blastoderm embryos of *Drosophila*. In embryos containing the reporter construct with the complete 3' UTR of *Drosophila cad*, early reporter transcripts were evenly distributed. However, the translation of

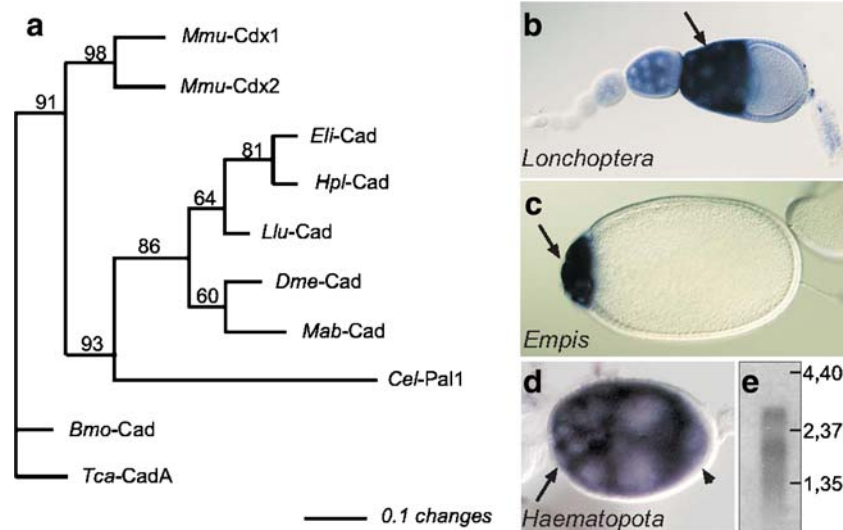


Fig. 4 Homeodomain tree of CAD homologues and expression in ovarian follicles. **(a)** Quartet Puzzling analysis (Strimmer and von Haessler 1996) with sequences from the dipterans *E. livida* (*Eli*), *H. pluvialis* (*Hpl*), *L. lutea* (*Llu*), *M. abdita* (*Mab*) and *Drosophila melanogaster* (*Dme*; accession no. NP_476954), the silk moth *Bombyx mori* (*Bmo*; NP_001037514), *Tribolium castaneum* (*Tca*; NP_001034498), the nematode *Caenorhabditis elegans* (*Cel*; NP_001021210) and the mouse (*Mmu*; Cdx-1: AAA37412; Cdx-2: AAA19645). The tree has been constructed with the homeodomain sequences using the default settings of software on <http://bioweb.pasteur.fr/>. Support for internal branches is shown in percent. Branch

lengths indicate the average number of amino acid changes per position (see scale). In situ hybridizations of ovarian follicles from *L. lutea* (**b**), *Empis livida* (**c**) and *H. pluvialis* (**d**), with anti-sense probes against the respective *cad* homologues. Expression is detected in the nurse cells (arrows) of *Lonchoptera* and *Empis* follicles, and in the nurse cells and the oocyte (arrowhead) of the *Haematopota* follicle. Note that the chorion may have prevented *cad* transcript detection in the oocytes of *Lonchoptera* and *Empis*. **e** Northern blot of poly(A) RNA from ovarian follicles of *Haematopota* hybridized with a radio-labeled *Haematopota cad* probe. RNA standard sizes are given in kb

the reporter was repressed in the anterior quarter of the blastoderm, and in late blastoderm stages, the reporter transcript had disappeared from the anterior pole (4 independent lines; Fig. 3b–e). These observations match with the sequence of events in *Drosophila*, in which translational repression of ubiquitous *cad* transcripts at the anterior pole precedes the disappearance of anterior transcripts (own observations; cf. Fig. 5e–h, and 7a, c, e in Mlodzik and Gehring 1987), and suggests that translational repression mediated by the *cad* 3' UTR causes transcript instability. The reporter construct with the truncated 3' UTR of *Drosophila cad* expressed the reporter transcript and protein throughout the blastoderm (three independent lines; Fig. 3f–i). Likewise, the reporter construct with the 3' UTR of *Mab-cad* expressed the reporter transcript and protein throughout the blastoderm, albeit at a lower level (three independent lines; Fig. 3j–m). These results suggest that, unlike the 3' UTR of *cad*, the 3' UTR of *Mab-cad* is insufficient to mediate BCD-dependent translational repression in *Drosophila*.

Finally, we tested whether the absence of maternal *cad* expression is the ancestral condition for cyclorrhaphan flies, i.e. a plesiomorphic trait of Cyclorrhapha. We cloned *cad* homologues from a second lower cyclorrhaphan fly (*L. lutea*, Lonchopterae), and two paraphyletic lower brachyceran taxa (*H. phivialis*, Tabanidae; *E. livida*, Empididae) (Fig. 4a), and tested ovaries for maternal expression by in situ hybridization (Fig. 4b–d). In all three species, the nurse cells of ovarian follicles expressed the respective *cad* homologue. In *Haematopota*, we detected *cad* transcripts also in the oocyte (Fig. 4d). Failure to detect *cad* transcripts in the oocytes of *Lonchoptera* and *Empis* follicles is most likely a staining artefact due to the developing eggshell. For *Haematopota*, we verified maternal expression in a Northern blot and detected two *cad* transcripts (Fig. 4e), like in *Megaselia* (Fig. 1i) and *Drosophila* (Mlodzik and Gehring 1987), suggesting that the occurrence of two *cad* transcripts is conserved in evolution. However, the developmental time course of their appearance is not. In *Drosophila*, alternative *cad* promoters express the shorter transcript (2.4 kb) during oogenesis and the longer transcript (2.6 kb) during blastoderm cellularization (Mlodzik and Gehring 1987), while in *Megaselia*, the two *cad* mRNAs are transcribed during early embryogenesis. We cannot exclude that a paralogue of *Mab-cad* provides maternal expression, but a very recent duplication of *Mab-cad* is unlikely because stringent hybridization of endonuclease digested genomic DNA with a *Mab-cad* probe in Southern blots is consistent with a single copy gene (not shown). Taken together, our results suggest that maternal *cad* expression was lost in the *Megaselia* lineage and that BCD is not required for the translational repression of *Mab-cad*.

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