**extra macrochaetae**, encoding *Drosophila* Id, controls apical cell shape in the hindgut epithelium

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**Abstract**

Inhibitor of DNA-binding (Id) transcription factor regulates the balance of cell differentiation and proliferation and is involved in organ morphogenesis in various species. Previously, we revealed that *extra macrochaetae* (*emc*), encoding the only Id protein in *Drosophila*, controls chirality of cell shape in hindgut epithelium. Here, to further understand functions of *emc* in cell-shape regulations, we analyzed apical cell shape in the hindgut epithelium of *emc* mutant embryos. We found that *emc* mutants showed expansion of their apical surface, but no abnormalities in cell differentiation and proliferation. Therefore, our results demonstrate that Id can control cell morphology without affecting specification and propagation of cells.
Figure 1. The apical area of epithelial cells and the epithelial-tube diameter increased in the embryonic hindgut of an emc mutant.

(A) A diagram showing imaging of the Drosophila embryonic hindgut epithelium (orange) under a confocal microscope. The embryo (shown in gray) is viewed from the right side (A: anterior; P: posterior; D: dorsal; V: ventral). The hindgut (hg) and its epithelium are shown in white and orange, respectively. We imaged the dorsal side of the hindgut luminal (apical) surface (the lateral view is indicated by a green line) and determined apical-surface cell boundaries. (B-F) Representative images showing apical-surface cell boundaries at stage 12 (before hindgut rotation) in the following genotypes: (B) wild-type (WT), (C) an emc\textsuperscript{AP6} homozygote (emc\textsuperscript{AP6}), (D) an emc\textsuperscript{AP6} homozygote overexpressing UAS-emc::GFP (NP2432\textsuperscript{>emc::GFP} in emc\textsuperscript{AP6}), (E) a double homozygote of da\textsuperscript{10} and emc\textsuperscript{AP6} (da\textsuperscript{10}, emc\textsuperscript{AP6}), and (F) wild-type overexpressing UAS-da (NP2432\textsuperscript{>da}). Scale bars: 5 μm. (G) A bar graph showing the mean apical cell-surface area in the hindgut of stage 12 embryos with the indicated genotypes (described in B-F). The number of embryos (N) and the number of cell boundaries (n) analyzed is shown above each genotype. ****: p < 0.001; n.s: not significant; significance was determined by Tukey's multiple comparison test with a 5% significance level. (H) Typical images of the hindgut nuclei, stained with an anti-Lamin C antibody, in the WT embryo at stage 13 (after hindgut rotation) and (I) in the emc\textsuperscript{AP6} homozygous (emc\textsuperscript{AP6}) embryo at stage 13 (corresponding to the period when the WT hindgut is completing its rotation). The dotted orange lines indicate the outline of the hindgut). Scale bars: 25 μm. (J, K) Bar graphs showing (J) the number of cells (values show mean ± standard error) in the hindgut epithelium and (K) the mean luminal (white) and outer (grey) diameters of the hindgut in WT and emc\textsuperscript{AP6} embryos at stage 13. The number of embryos analyzed (N) is shown above each genotype. ****: p < 0.001; *: p < 0.05; n.s: not significant; significance was determined by likelihood-ratio test with a 5% significance level. Error bars indicate standard error.

Description

The basic helix-loop-helix (bHLH) transcription factor is a representative class of transcriptional regulators conserved in a wide range of eukaryotes (Gyoja, 2017). The bHLH proteins form homo- or heterodimers and bind DNA, thereby controlling cell proliferation and differentiation. The first reported bHLH proteins were found to bind E-box DNA sequences and were thus named E proteins (Murre et al. 1989). E proteins are essential for mesodermal and neural development in various species (Wang & Baker, 2015).

Another class of HLH proteins, known as antagonists of bHLH transcription factors, are called Inhibitor of DNA-binding (Id) proteins. Id proteins form a heterodimer with bHLH proteins and thereby inhibit their ability to bind DNA (Murre et al. 1989). Thus, the functions of the E and Id proteins are relatively easy to study in Drosophila compared with those in vertebrate model organisms, which have multiple E and Id genes (Gyoja, 2017). In Drosophila, Da forms a homodimer or a heterodimer with bHLH proteins and upregulates the expression of downstream genes, while Emc antagonizes Da by binding it in an inactive heterodimer (Garrell & Modolell, 1990). Thus, the functions of the E and Id proteins are relatively easy to study in Drosophila compared with those in vertebrate model organisms, which have multiple E and Id genes.

Here, we analyzed the morphology of hindgut epithelial cells in Drosophila embryos in which emc and da were genetically modulated (Fig. 1 A). The hindgut epithelial tube is formed with monolayer epithelium whose luminal side corresponds to the apical surface of each epithelial cell. We found that the area of the apical surface was significantly larger in emc\textsuperscript{AP6} homozygotes than in the wild-type embryos at stage 12, which corresponds to the period just before hindgut rotation begins in the wild-type embryo (Fig. 1 B, C, G; p < 0.001), and that the lumen of the hindgut was markedly larger in emc\textsuperscript{AP6} homozygotes than in wild-type embryos in all cases examined (N=7) (Fig. 1 C). The tissue-specific overexpression of UAS-emc::GFP in the hindgut epithelium, driven by NP2432, significantly rescued the apical-area expansion in emc\textsuperscript{AP6} homozygotes (NP2432\textsuperscript{>emc::GFP} in emc\textsuperscript{AP6}) at stage 12, demonstrating that the expansion of the apical area can be attributed to the absence of emc function (Fig. 1 D, G; p < 0.001), and also rescued luminal expansion in all cases examined (N=6) (Fig. 1 D). We previously revealed that the emc mutation did not affect the tissue-specification of the hindgut (Ishibashi et al. 2019). Thus, the expansion of the apical area in emc\textsuperscript{AP6} mutants may not be associated with cell-fate changes in the hindgut epithelial cells.
We previously showed that a proper Da–Emc balance is essential for the LR-asymmetric development of the embryonic hindgut (Ishibashi et al. 2019). The embryonic hindgut forms first as a bilaterally symmetric structure at early stage 12, and its anterior part curves toward the ventral side of the embryo (Hayashi & Murakami, 2001). At this stage, the hindgut epithelial cells are intrinsically chiral. The subsequent dissolution of cell chirality induces the 90° counterclockwise rotation of the hindgut, causing the anterior part of the hindgut to curve to the right at stage 13 in wild-type embryos (Fig. 1 H) (Taniguchi et al. 2011). Our previous study revealed that in emcAP6 homozygotes, hindgut LR symmetry becomes randomized when cell chirality disappears at stage 13 (Fig. 1 I) (Ishibashi et al. 2019; Ishibashi et al. 2020). This LR randomization and loss of cell chirality associated with homozygous emcAP6 was completely suppressed in combination with homozygous da10, because da hyperactivation causes LR randomization in emc mutants (Ishibashi et al. 2019; Ishibashi et al. 2020). In contrast, we here found that the expansion of the apical area associated with homozygous emcAP6 was not significantly suppressed in combination with homozygous da10 at stage 12 (da10; emcAP6 in Fig. 1 E, G; p > 0.05). Thus, the expansion of the apical area in the emcAP6 homozygote is independent of da. This is further supported by our finding that although tissue-specific da overexpression in the hindgut epithelium of otherwise wild-type embryos rescued the condition of the emc mutant hindgut, it did not increase the apical surface area of the hindgut epithelium, whereas our previous study revealed that such da overexpression induces random LR asymmetry of the hindgut and the loss of cell chirality (NP2432>da in Fig. 1 F, G) (Ishibashi et al. 2019; Ishibashi et al. 2020). These data indicate that emc is required for regulating apical area independently of da.

Emc/Id proteins control cell proliferation in various species (Wang & Baker, 2015). Therefore, the expansion of the apical area in the emc homozygote may be indirectly caused by changes in the number of hindgut epithelial cells. To test this possibility, we visualized the nuclei in the hindgut epithelium by immunostaining with an anti-Lamin C antibody, since Lamin C is expressed specifically in the epithelium of the embryonic hindgut at stage 13 (Fig. 1 H, I) (Riemer et al. 1995), and manually counted the nuclei in the hindgut epithelium. The number of cells in the hindgut epithelium stays the same during hindgut rotation (stage 12-13). We found that the number of nuclei in the hindgut epithelium was almost the same in wild-type and emcAP6 homozygote embryos (Fig. 1 J), suggesting that the expansion of the apical area in the emcAP6 homozygote is caused by morphological changes in the hindgut epithelial cells, not by a change in the number of cells. We then speculated that such structural abnormalities in individual cells might be coupled with alterations in the overall structure of the hindgut. To examine this possibility, we measured the luminal and outer diameters of the hindgut tube in wild-type embryos and emc homozygotes. We found that the mean luminal and outer diameters of the hindgut were significantly larger in the emcAP6 mutants (Fig. 1 K), demonstrating that the hindgut tube became thicker in the emcAP6 mutant compared with the wild-type embryo.

Taken together, we concluded that emc regulates the apical area of hindgut epithelial cells independently of da. Our results also show that emc controls the shape of hindgut epithelial cells, but not their proliferation, to produce proper hindgut architecture (such as luminal and outer diameters). However, the mechanisms by which emc affects the shape of hindgut epithelial cells and the global architecture of the hindgut remains unknown.

**Methods**

Fly stocks: we used Canton-S as a wild-type line. We used emcAP6 as an amorphic allele of emc (Bloomington #36544) and da10 as an amorphic allele of da (Bloomington #5531) (Ellis, 1994; Wülbeck et al. 1994). We used the following UAS lines: UAS-emc::GFP (Popova et al. 2011) and UAS-da (Bloomington #51669) (Giebel et al. 1997). As a hindgut epithelium-specific gal4 driver, we used NP2432 (Kyoto DGRC #104201) (Hayashi et al. 2002). Mutations on the second chromosome were balanced with CyO, P[en1]wg em111. Mutations on the third chromosome were balanced with TM3, P[ftz-lacZ.ry+]TM3, Sb1 ry+, or TM6B, P[iab-2(1.7)lacZ]6B, Tb1. All genetic crosses were carried out at 25 °C on a standard Drosophila culture medium.

Drosophila embryos were collected and dechorionated in 3% hypochlorous acid solution. The dechorionated embryos were fixed with 6% paraformaldehyde in PBS (130 mM NaCl, 7 mM Na2HPO4, and 3 mM NaH2PO4) for 30 minutes and the vitelline membrane was removed by washing in 100% methanol. The embryos were fixed in 100% methanol at −20 °C.

The fixed embryos were treated with 2% Block Ace (Yukijirushi) in PBT (130 mM NaCl, 7 mM Na2HPO4, 3 mM NaH2PO4, and 0.1 % (w/v) Triton-X-100). The embryos were then washed with PBT and incubated in the appropriate primary antibody solutions in Can Get Signal Solution B (TOYOBO) in 2 mL tubes (Eppendorf) at room temperature for 3 hr or at 4 °C overnight. The embryos were then washed with PBT and incubated in the appropriate secondary antibody solutions at room temperature for 3 hr or at 4 °C overnight. After washing, the embryos were dehydrated with ethanol and mounted with clearing reagent (methyl salicylate). The following primary antibodies were used at the dilutions indicated: mouse anti-
phosphotyrosine antibody (PY20) (1:1,000, BD Biosciences), chicken anti-β-galactosidase antibody (1:500, Abcam), and mouse anti-Lamin C antibody (1:1000, DSHB). We used the following secondary antibodies at the indicated dilutions: anti-chicken IgY-Alexa 488 (1:1,000, Jackson ImmunoResearch) and anti-mouse IgG-Cy3 (1:1000, Jackson ImmunoResearch). We processed images of the immunostained hindgut using Adobe Photoshop CS3 (Adobe Systems) or Adobe Illustrator CS3 (Adobe Systems).

To analyze apical cell size, we obtained images of the apical cell boundaries at stage 12. We manually defined the cell boundaries and measured the apical cell area with ImageJ Fiji (Schindelin et al. 2012).

To count nuclei in the hindgut, we stained stage 13 embryos with anti-Lamin C antibody. Images of the whole hindgut were captured with an LSM880 confocal microscope (Carl Zeiss) in wild-type and emcAP6 homozygous embryos. We manually selected the centroids of Lamin C-positive nuclei in three-dimensional space using ImageJ Fiji (Schindelin et al. 2012) and calculated the number of nuclear centroids.

To measure the luminal and outer perimeters of the hindgut, we visualized hindgut cell boundaries by immunostaining with anti-PY20 antibody. Images of the whole hindgut were captured with an LSM880 confocal microscope (Carl Zeiss) in WT and emcAP6 homozygous embryos at stage 13. We examined serial optical sections located halfway between the anterior and posterior ends of the hindgut, and selected a representative image showing the maximal diameter of the middle portion along the gut tube. We then measured the luminal and outer perimeters manually.

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