Chato, a KRAB zinc-finger protein, regulates convergent extension in the mouse embryo

Maria J. García-García1,2,*, Maho Shibata1 and Kathryn V. Anderson2

In Xenopus and zebrafish embryos, elongation of the anterior-posterior axis from a spherical early embryo depends on the movement and intercalation of lateral cells towards the midline, a process called convergent extension (reviewed by Wallingford et al., 2002). Extensive studies on intact embryos and tissue explants using time-lapse imaging have confirmed that coordinated cell rearrangements mediate convergent extension in fish and frog embryos (Concha and Adams, 1998; Davidson and Keller, 1999; Elul and Keller, 2000; Jessen et al., 2002; Keller and Tibbetts, 1989; Tahinci and Symes, 2003; Wallingford et al., 2000; Wilson and Keller, 1991).

Non-canonical Wnt signaling is required for convergent extension in Xenopus and zebrafish (reviewed by Tada et al., 2002). Genetic and experimental disruptions of this signaling pathway, such as loss of function mutations in zebrafish van gogh-like 2 (vangl2; also known as trilobite) (Hammerschmidt et al., 1996; Jessen et al., 2002), or overexpression of mutated forms of Dishevelled in Xenopus (Goto and Keller, 2002; Moon et al., 1993; Tada and Smith, 2000; Wallingford et al., 2000), cause characteristic convergent extension defects, such as a short anterior-posterior axis, a wide notochord and a broad, open neural tube. Other genetic pathways are also important for convergent extension in zebrafish, including BMP gradients (von der Hardt et al., 2007), the zinc-finger protein Bloody fingers (Sumanas et al., 2005) and the ERRα orphan nuclear receptor (Bardet et al., 2005).

In the mouse, the morphogenetic events that create the elongated anterior-posterior body axis are not well understood. Elongation of the mouse embryo takes place during late gastrulation [embryonic day (E) 7.5-9.0], when extensive cell rearrangements/movements generate the germ layers and organ primordia (Kinder et al., 1999). As these cells reorganize and migrate, the embryo grows dramatically, from ~600 cells at pregastrula stages (E6.0) to nearly 14,000 at neurulation (E8.5) (Lawson, 1999). Recent time-lapse imaging studies showed that cell intercalation takes place in the axial midline of mouse embryos during the lengthening of the node along the anterior-posterior axis (Yamanaka et al., 2007). However, the importance of convergent extension movements to elongation of other embryonic tissues is not clear, in part owing to a lack of analysis of cell behavior during these stages.

Mouse mutants that lack components of the non-canonical Wnt signaling pathway show some of the features characteristic of Xenopus and zebrafish embryos with disrupted convergent extension, including a wide notochord and open neural tube (Greene et al., 1998; Kibar et al., 2001; Murdoch et al., 2001a). It has been proposed that defects in axial mesendoderm extension in mouse Vangl2 [also known as loop-tail (Lp)] mutant embryos are caused by defective midline cell intercalation in the node area (Ybot-Gonzalez et al., 2007). Although it is clear that non-canonical Wnt signaling contributes to the elongation of the mammalian embryo (Wallingford et al., 2002; Wang, J. et al., 2006), the phenotypes of mouse mutants that lack non-canonical Wnt signaling are not as severe as those of their zebrafish mutant counterparts. For example, elongation and convergence of non-axial mesoderm is not as severely affected in mouse Vangl2 embryos (Greene et al., 1998; Kibar et al., 2001; Murdoch et al., 2001a) as in zebrafish vangl2 mutants (Hammerschmidt et al., 1996; Jessen et al., 2002), even though the mutations disrupt orthologous genes. Mouse mutants that lack non-canonical Wnt signaling die at birth with severe neurological defects and disruption of planar cell polarity (PCP) in inner ear hair cells (Curtin et al., 2003; Montcouquiol et al., 2003; Wang, Y. et al., 2006), but their trunk length is similar to that of wild-type littersmates and the contribution of PCP defects to mouse axis elongation is not clear. To date, the results suggest that convergent extension mechanisms controlled by non-canonical Wnt signaling are important for elongation of some embryonic tissues such as the notochord (Ybot-Gonzalez et al., 2007), but the differences between mouse and zebrafish vangl2 mutant phenotypes argue that other pathways and/or mechanisms contribute to the elongation of non-axial tissues in the mouse embryo.
Here we report the identification and characterization of Chato, a novel KRAB zinc-finger protein required for mammalian convergent extension. Two independent recessive mutant alleles of chato cause morphogenetic defects similar to those of fish and frog embryos with defective convergent extension, including a shorter and wider body axis, open neural tube and mediolaterally expanded somites. To evaluate whether chato regulates convergent extension mechanisms similar to those seen in fish and frogs, we measured changes in the length and width of wild-type and mutant embryonic tissues during early development. Because of the relative simplicity of its morphogenetic movements, we focused our analysis on the definitive endoderm layer, the precursor of the gut. Morphometric analysis of wild-type embryos shows that the definitive endoderm narrows and elongates during embryogenesis and that convergent extension of this tissue is mediated by cell rearrangements. In chato mutants, the definitive endoderm is wider and cell rearrangements do not take place. Genetic experiments indicate that Chato regulates convergent extension events through a novel pathway independent of non-canonical Wnt signaling.

MATERIALS AND METHODS

Mouse (Mus musculus) strains

The chato mutation was generated by ENU-mutagenesis as described previously (Garcia-Garcia et al., 2005; Kasarskis et al., 1998). chato was analyzed in C3H/FeJ, CAST/Ei and 129Sv/ImJ genetic backgrounds. Nodal-lacZ and Lp mice were obtained from Dr Elizabeth J. Robertson (Collignon et al., 1996b) and from Jackson Labs (LPT/LeJ strain) respectively. Lp mice were outcrossed to C3H/FeJ and genotyped with D1Mit36 and D1Mit149 SSLP markers.

Physical mapping and sequencing of candidate genes

Genetic mapping of Zfp568chato was performed by linkage analysis of 981 opportunities for recombination with SSLP markers (www.informatics.jax.org and http://mouse.ski.mskcc.org). Physical map information was obtained from Ensembl (http://www.ensembl.org/Mus_musculus/index.html).

cDNAs of all candidate genes in the chato interval (Zfp27, Zfp74, Zfp568, Zip14, Zfp82 and Zfp260) were amplified by RT-PCR (Superscript One-Step RT-PCR, Invitrogen) using RNA from E8.5 chato and C57BL/6J (control) embryos. Amplification products were sequenced. A mutation, T to C, was identified at codon 64 of the chato (Superscript One-Step RT-PCR, Invitrogen) using RNA from E8.5 carrier animals. No mutations were found in any of the other genes in the polymorphism that was used to confirm linkage with ORF. This point mutation generated an et al., 1996b) and from Jackson Labs (LPT/LeJ strain) respectively. SSLP markers.

RESULTS

chato mutants fail to elongate the anterior-posterior axis

The chato mutation was isolated in a mutagenesis screen designed to identify recessive mutations that alter embryonic morphology at midgestation (Garcia-Garcia et al., 2005; see Materials and methods). chato mutant embryos arrested by E9.0 and remained unturned, with a short anterior-posterior body axis and an open gut tube (Fig. 1; see Fig. S1A,B in the supplementary material).

Analysis of mesodermal tissues in chato embryos showed that defects in axis elongation were accompanied by a failure of cells to properly localize with respect to the midline. Analysis of Twist1 expression, which marks somites and lateral plate mesoderm (Quertermous et al., 1994), showed that these mesodermal tissues were located further away from the midline of chato embryos than in wild-type littermates (Fig. 1A,B). Expression of a Nodal-lacZ reporter (Collignon et al., 1996b) also showed that the lateral plate mesoderm in chato mutants was shorter and wider than in wild-type embryos (Fig. 1C,D). Somitic mesoderm was specified in all chato mutants, but it showed defects in morphogenesis (Fig. 1A,B,E,F). Many chato embryos (n=61/184) showed condensed somites that were mediolaterally expanded and narrow in the anterior-posterior axis, as shown by expression of Meox1 (Candia et al., 1992) (Fig. 1E,F and Fig. 3C-E). Mesodermal precursors of the heart, which arise from lateral positions, failed to migrate and fuse at the midline of all chato mutants and remained in two separate domains at both sides of the embryo as shown by expression of the heart marker Nkx2.5 (Fig. 1G,H) (Lints et al., 1993); this cardiobifida phenotype is presumably responsible for the death of the embryos at E9.5-10. Altogether, these mesodermal defects are similar to those seen in zebrafish embryos in which convergent extension is disrupted (Matsui et al., 2005), but are different than those of mouse non-canonical Wnt pathway mutants.

Morphogenetic defects in the chato neural plate and notochord

Epithelial tissues in chato embryos also had morphogenetic defects. The chato headfolds failed to fuse to form a neural tube (Fig. 2A-G).

In the open neural plate, markers of specific cell-type populations, such as Kroxl20 (Egr2) (Wilkinson et al., 1989), were expressed in domains that were narrow along the anterior-posterior axis and laterally expanded when compared with wild-type littermates (Fig. 2A,B), a phenotype similar to zebrafish vangl2 mutants (Jessen et al., 2002). The neural tube also failed to close normally at more-posterior positions of the anterior-posterior axis. In some chato mutants, it completely failed to close (55%, Fig. 2E), whereas in
The basis of the defects in neural tube closure appeared, however, to be different between chato embryos and non-canonical Wnt pathway mutants. It is believed that the underlying cause of neurulation defects in Lp embryos is the abnormally wide floor plate, which might impair the formation of the medial hinge point and the apposition of the neural folds (Greene et al., 1998). The floor plate ventral hinge was morphologically normal in chato mutants (Fig. 2F,G). In addition, other markers of territories along the dorsal-ventral neural axis, including Shh (Echelard et al., 1993), Foxa2 (Ruiz i Altaba et al., 1993) and Olig2 (Zhou et al., 2001), were expressed in regions comparable to those of wild-type littermates (not shown).

chato embryos also showed other phenotypic differences from non-canonical Wnt signaling mutants. The notochord, a mesendoderm-derived tissue, is wider in fish, frog and mouse embryos in which the activity of this pathway is disrupted (Goto and Keller, 2002; Greene et al., 1998; Hammerschmidt et al., 1996). Analysis of brachyury (T) expression (Wilkinson et al., 1990) in whole-mount chato embryos at E8.5 revealed that the notochord was disrupted and was wider than in wild-type littermates in some regions, but narrower or absent in other positions (Fig. 2I,J). In transverse sections, analysis of T expression indicated that the characteristic notochord rod present in wild-type embryos at these stages had not been formed in chato mutants and, instead, the notochord was still part of the mesendoderm layer (Fig. 2F,G). Therefore, although the notochord irregularities of chato mutants indicate defects in the reorganization of this tissue, these defects are different than those of non-canonical Wnt signaling mutants (Fig. 2H).

**chato does not genetically interact with non-canonical Wnt signaling mutants**

To assess whether chato affected the activity of the non-canonical Wnt pathway, we tested for genetic interactions between chato and Lp. Mouse mutant embryos that lack Lp (Vangl2) display some of the hallmarks of convergent extension mutants, including a wider notochord and failure to close the neural tube (Greene et al., 1998; Murdoch et al., 2001a). Lp mutants show strong genetic interactions with other mutations that affect non-canonical Wnt signaling. For example, embryos that are doubly heterozygous for Lp and scribbled (Scrib; also known as circletail (Crc)) (Lp+/–; Crc+/+) (Murdoch et al., 2001b) or for Lp and Ptk7 (Lp+/–; Ptk7+/–) (Lu et al., 2004), as well as Lp+/–; Dvl1+/–; Dvl2+/– embryos (Wang, J. et al., 2006), all show the same neural tube closure defects seen in Lp homozygous embryos. By contrast, we found that Lp+/–; chato+/– double heterozygous animals were viable and fertile and had the curled tail typical of Lp heterozygotes (see Fig. S2 in the supplementary material). We also mated double heterozygous carriers to obtain more-severe mutant combinations and evaluated their phenotypes in mesoderm, neural tube and notochord. We did not observe any modification of the Lp mutant phenotype in embryos lacking one copy of chato (Lp+/–; chato+/–). Similarly, the chato mutant phenotype did not change in the absence of one copy of Lp (Lp–/–; chato+/–). Lp–/– chato double mutant embryos (Lp–/–; chato+/–) showed characteristics of both chato and Lp mutants, including elongated somites and an open neural tube (see Fig. S2 in the supplementary material). The lack of genetic interaction between the two mutants does not support a role of chato in non-canonical Wnt signaling.

To further test whether chato interferes with non-canonical Wnt signaling, we assayed expression of components of this pathway in chato mutants. We found that Vangl1, Vangl2, Celsr1, frizzled 3 (Fzd3), Dvl1, Dvl2 and Prickle1 were all expressed in chato mutants.

**Fig. 1. Mesoderm defects in chato embryos.** Wild-type (wt) (A,C,E,G) and chato mutant (B,D,F,H) mouse embryos were assayed by in situ hybridization with markers expressed in head mesenchyme/lateral plate mesoderm/somitic mesoderm (Twist1; A,B, dorsal and ventral views, respectively), somites (Meox1; E,F, ventrolateral views) and cardiac mesoderm (Nkx2.5; G,H, ventral views). Staining for β-galactosidase activity from a Nodal-lacZ reporter labeled lateral plate mesoderm and node of wild-type (C) and chato mutant (D) embryos (lateral views). Thirty-three percent of chato mutants (n=184) had condensed somites that appeared narrow and laterally extended (F). In 52% of chato embryos (n=184), somites were not clearly discernible morphologically, but somite markers Twist1 and Meox1 marked some imperfectly shaped somites. Only 15% of chato mutants showed normal somites. Arrowheads in A,B point to head mesenchyme. Brackets in C,D highlight the different width of the lateral plate mesoderm in wild-type and chato mutant embryos. Brackets in E,F highlight the different width of the somites. Arrowheads in H mark the cardiac mesoderm in chato mutants. LPM, lateral plate mesoderm; som, somites.

others it remained open only at some locations (45%, Fig. 2D), as visualized by expression of the pan-neural marker Sox2 (Collignon et al., 1996a). Failure to close the neural tube is a characteristic phenotype of zebrafish and Xenopus convergent extension conditions (Darken et al., 2002; Goto and Keller, 2002; Wallingford and Harland, 2002), as well as of mouse mutants in components of non-canonical Wnt signaling (Lp; Fig. 2H) (reviewed by Copp et al., 2003).
tube a wavy appearance. Transverse sections in F–H were hybridized with probes for both posterior and ventral views, respectively. In some chato mutants, parts of the neuroepithelium remained open (arrowhead in D), giving the neural tube a wavy appearance. Transverse sections in F–H were hybridized with probes for both T (arrowheads) and the somitic marker Meox1 (arrows). In chato mutants, the notochord was embedded in the mesendoderm layer (arrowhead in G) and never formed an individualized rod (arrowhead in F). The notochord of Lp mutants is wider than that of wild-type embryos (F–H, arrowheads) (Greene et al., 1998). Expression of T in chato mutants (I) shows areas where the notochord was wider (w), thinner (t) or absent (a) as compared with wild-type embryos (I, arrowhead). NT, neural tube; not, notochord; r3, rhombomere 3; r5, rhombomere 5; som, somitic mesoderm.

The chato mutation disrupts Zfp568, a novel KRAB zinc-finger protein

Meiotic recombination mapping localized the chato mutation to an interval of 209 kb on the proximal region of chromosome 7 (Materials and methods). Sequence analysis of all six genes in this interval revealed a single change: a missense mutation in Zfp568 (Fig. 3A). Both Zfp568chato/Zfp568RRU161 and Zfp568RRU161 homozygous embryos recapitulated the chato phenotype (Fig. 3C–F). Thus, the complementation test indicated that the ENU-induced chato mutation is a null allele of Zfp568.

Zfp568 (chato) showed a broad expression pattern during embryogenesis (Fig. 3G–L). At E7.5, chato was expressed in all cell types as assessed by in situ hybridization in whole-mount embryos and in sections (Fig. 3G, H). At later stages, expression was also ubiquitous in extraembryonic and embryonic tissues (Fig. 3J, L). Expression was highest in the extraembryonic ectoderm (Fig. 3G, J, arrowheads).

chato mutants fail to undergo convergent extension of definitive endoderm

Characterization of the cellular basis of the chato axis elongation defects was complicated by the architecture of the E8.5 mouse embryo, which consists of several cellular layers, some of which (e.g. the neuroepithelium) are folded. Compared with other germ layers, we found that the simple epithelial structure of the definitive endoderm made it amenable to straightforward and reliable analysis during the stages of axis elongation. Definitive endoderm cells arise from the primitive streak during gastrulation and form an epithelial
monolayer that is continuous with the extraembryonic visceral endoderm (VE) on the exterior of the embryo after E8.0 (reviewed by Lewis and Tam, 2006).

We measured the overall dimensions of the definitive endoderm in wild-type and chato mutant embryos during the stages of anterior-posterior axis elongation. Definitive endoderm and VE cells can be discriminated using markers expressed exclusively in the VE, such as transthyretin (Ttr) (Cereghini et al., 1992). At E7.5, some VE cells were still present in the embryonic region (Fig. 4A,B, arrowhead). After E8.0 (0-somite stage), the definitive (Ttr-negative) endoderm covered the entire embryonic region (Fig. 4C-J). Posterior views of wild-type embryos marked with Ttr revealed that the definitive endoderm narrowed between E8.0 and E9.5 (Fig. 4). Measurements of definitive endoderm in wild-type embryos showed that the total length of the definitive endoderm increased 50% between 0-somite and 10-somite stage embryos (Fig. 4K, blue columns; Fig. 5H). At the same stages, definitive endoderm width, measured as the lateral distance across the center of the embryo (Fig. 4H, red line), decreased 2.7-fold (Fig. 4K, green columns; Fig. 5H). These measurements demonstrate that elongation of the definitive endoderm of the wild-type mouse embryo is accompanied by narrowing of the tissue, and thus definitive endoderm undergoes convergent extension.

At early E8.5 (2- to 4-somite stage), the length of the chato definitive endoderm was not significantly different from that of wild-type littermates (P=0.31), but its width was 1.23-fold that of the wild type (P=0.019, Fig. 5F,H). At the 5- to 7-somite stage, the definitive endoderm of chato mutants was 14% shorter (P=0.031) and twice as wide (P=0.0002) as that of wild-type embryos of the same stage (Fig. 5E,F,H). The length and width measurements indicated that the chato mutant endoderm grew in both dimensions (Fig. 5E-H). However, the length-to-width ratio (LWR) of chato embryonic endoderm did not significantly change between the 2- to 4-somite and the 5- to 7-somite stages (P=0.23, Fig. 5G, red columns), whereas the wild-type LWR more than doubled (P=0.0007, Fig. 5G, gray columns). By the 5- to 7-somite stage, the LWR of wild-type definitive endoderm was 2.6-fold greater than that of chato mutants (P=0.0022, Fig. 5G). Thus, convergent extension of the mouse definitive endoderm requires the activity of the Chato protein.
Elongation and narrowing of the wild-type definitive endoderm is coupled to cell rearrangements

Convergent extension in zebrafish and Xenopus embryos depends on cell rearrangements, including mediolateral cell intercalation and polarized cell migration, which contribute to a decrease in the number of cells across the width of the embryo and to an increase in the number of cells along the anterior-posterior axis (reviewed by Wallingford et al., 2002). We therefore evaluated variations in the number of cells across the width of the mouse definitive endoderm to assess the contribution of cell rearrangements to convergent extension of the mouse endoderm.

We quantified the number of cells across the width of the definitive endoderm at different developmental stages by counting the number of Fast Red-stained nuclei in the outermost layer of transverse sections from E8.0 (0 somites) to E9.0 (10 somites) wild-type embryos (Fig. 4b, d, f, h, j). In headfold stage embryos (E8.0; 0-4 somites), the definitive endoderm layer was 47±6 cells wide at intermediate positions of the anterior-posterior axis (length) and narrowed laterally (width). The white tissue covering the embryonic region corresponds to definitive endoderm (DE). Arrows point to white extraembryonic tissue (ext). All images are at the same magnification. At E7.5, some VE cells (arrowhead in A, B) still overlay the exterior of the embryonic region; the line in A, B delimits embryonic-extraembryonic parts. Gut closure prevented visualization of all the definitive endoderm in I and J. (b, d, f, h, j) Representative transverse sections of the embryos in the columns above, counterstained with Fast Red. Sections correspond to intermediate levels along the anterior-posterior axis. Only half of each section is shown (midline at the right edge of panel). (K) Plot of length (blue) and width (green) definitive endoderm measurements in wild-type embryos of different stages; data in μm. Dimensions of the endoderm were taken as exemplified by dashed lines in G, H. Note that measurements were taken in non-Ttr-stained embryos, in which transparency of the tissue allowed for accurate measurements of the whole definitive endoderm. Error bars indicate s.d. See Fig. 5H for primary data.

A decrease in the number of cells across the width of the definitive endoderm could be the result of mediolateral cell intercalation. However, this number could also be influenced by proliferation, apoptosis and delamination of cells from the primitive streak. To evaluate the contribution of cell proliferation, we assayed the frequency of mitotic cells in transverse sections of the definitive endoderm using phospho-histone H3 antibodies (Fig. 7A, C, green signal). Between E8.0 (0 somites) and E9.0 (10 somites), the definitive endoderm contained 0-3 proliferating cells per section at all levels along the anterior-posterior axis where the gut remained open (n=21 embryos/380 sections, Fig. 7A, C and data not shown). By contrast, other embryonic tissues, such as the mesoderm or neuroepithelia, showed a higher mitotic index (Fig. 7A, C). Our results confirm previous reports indicating that the definitive endoderm is a relatively quiescent tissue during early developmental stages (Tremblay and Zaret, 2005) and indicate that the rate of proliferation in the endoderm plays a minor role in the growth of the definitive endoderm during these stages. We did not observe any apoptotic cells in the definitive endoderm at any of the stages analyzed (data not shown). Delamination of cells from the primitive streak plays important roles in the growth of the definitive endoderm at gastrulation stages (Lewis and Tam, 2006). Therefore, during the stages of anterior-posterior axis elongation, the number of cells in the endoderm might increase owing to the continued delamination...
of cells from the primitive streak, with a minor contribution from cell proliferation. Because we did not observe apoptosis in the endoderm, we conclude that cellular rearrangements (mediolateral cell intercalation or polarized cell migration) must be responsible for the observed decrease in the number of cells across the width of the definitive endoderm and for the decrease in the width of the tissue.

**chato mutants fail to undergo the cell rearrangements required for definitive endoderm convergent extension**

We also analyzed rearrangements of cells in the definitive endoderm of chato mutants using the approaches described above. Headfold stage (0-4 somites) chato mutants had approximately the same cell number across the width of the definitive endoderm as wild-type littermates (Fig. 6A,B). At the 5- to 7-somite and 8- to 10-somite stages, chato embryos contained on average ~70% more cells across the width of the definitive endoderm than wild-type embryos (P<0.0001, Fig. 6A,B), paralleling the increased width of the definitive endoderm in chato mutants (Fig. 5B,D). The rate of cell proliferation (n=3 embryos/56 sections) in the definitive endoderm of chato mutants was similar to that of the wild type (Fig. 7B-D). Also, no apoptosis was observed in the chato mutant endoderm and we did not detect any abnormality in the delamination of definitive endoderm from the primitive streak or in the migration of definitive endoderm cells at gastrulation (see Fig. S1C-F in the supplementary material). Therefore, we conclude that the definitive endoderm is wide in chato mutants because normal function of the chato gene is required for the cells to rearrange into a longer, narrower structure.

**DISCUSSION**

**Convergent extension in the definitive endoderm of the mouse embryo depends on Chato**

Although the contribution of convergent extension mechanisms to the elongation of zebrafish and *Xenopus* embryos has been well studied, evidence for a role for convergent extension in mammalian embryogenesis has been limited to the notochord and neural tube (Wang, J. et al., 2006; Yamanaka et al., 2007; Ybot-Gonzalez et al., 2007). Based on embryo morphology and the pattern of expression of...
molecular markers, chato mutants appear to have global defects in elongation of the body axis, with abnormalities in the neural plate, paraxial mesoderm, lateral plate mesoderm and definitive endoderm.

To test definitively whether chato affects convergent extension, we examined the morphogenesis of the definitive endoderm, a single-layered cell sheet that can be analyzed reliably. Our morphometric analysis provides evidence that the wild-type mouse definitive endoderm undergoes convergent extension. The definitive endoderm begins to narrow and elongate in headfold stage embryos and continues to do so until ~14-somite stage embryos, when definitive endoderm closes to form the gut tube. From the 0- to 10-somite stages, the width of the wild-type definitive endoderm narrows 2.6-fold (from 820 to 310 mm); at the same time it elongates 2-fold. Although delamination of cells from the primitive streak probably contributes to the elongation of the definitive endoderm, the cell rearrangements that we observed are likely to account for the narrowing of the definitive endoderm and to contribute to the anterior-posterior elongation of this tissue. By contrast, the definitive endoderm does not narrow in chato embryos.

The most dramatic change in dimensions of the definitive endoderm of wild-type embryos occurs between the 2- to 4-somite and 5- to 7-somite stages, when the length-to-width ratio more than doubles; at the same stages, the length-to-width ratio of the chato definitive endoderm does not change significantly. In parallel with the abnormal dimensions of the tissue, the chato mutation disrupts cell rearrangements in the definitive endoderm. We therefore conclude that the cell rearrangements that depend on Chato are responsible for convergent extension of the definitive endoderm.

The mechanisms underlying the cell rearrangements of convergent extension have been studied in vertebrate and invertebrate embryos. Mediolateral cell intercalation mediates the elongation of Xenopus embryos and animal cap explants (Elul et al., 1997; Keller and Tibbetts, 1989; Wilson and Keller, 1991), polarized cell migration is also important for zebrafish convergent extension (Concha and Adams, 1998; Jessen et al., 2002; Warga and Kimmel, 1990), and germ band elongation of Drosophila embryos is propelled by the directional generation and resolution of multicellular rosettes (Bertet et al., 2004; Blankenship et al., 2006). One or more of these mechanisms may mediate convergent extension of the mouse definitive endoderm. Because mouse definitive endoderm has an epithelial organization, where cells are held together by adherent apical complexes (Fig. 7, E-cadherin in red), we favor the hypothesis that mediolateral cell intercalation and/or multicellular rosettes, rather than cell migration, mediate definitive endoderm convergent extension. The development of new methods that enable observation of live mouse embryos at cellular resolution will be required to elucidate the precise mechanisms involved.

Chato is likely to act in convergent extension of all germ layers

Although our studies of convergent extension in chato focused on the definitive endoderm, the chato phenotype suggests that it also acts in other tissues to regulate convergent extension. Both chato lateral plate and somitic mesoderm are shorter in the anterior-posterior axis and wider in the mediolateral dimension than in wild-type embryos, similar to zebrafish convergent extension mutants (Hammerschmidt et al., 1996; Jessen et al., 2002). The neural plate in chato fails to close, which could be due to defects in cell rearrangement in this tissue layer. Because chato is broadly expressed, it seems likely that it acts autonomously in these tissues to control cell rearrangements. It is, however, possible that convergent extension of the definitive endoderm is required for the migration and/or reorganization of epithelial and mesenchymal tissues.

Most chato mutants (n=156/184) also show extraembryonic defects, including a ruffled VE (see Fig. S1A,B in the supplementary material). It is therefore possible that these extraembryonic defects could influence the reorganization of definitive endoderm, epithelial and mesenchymal tissues in chato mutants. However, the defects in embryonic morphogenesis precede the appearance of extraembryonic phenotypes in chato mutants (see Fig. S1C-F in the supplementary material). In addition, 16% of E8.5 chato mutants do not show obvious extraembryonic abnormalities but have strong convergent extension phenotypes. Therefore, we favor the hypothesis that the embryonic and extraembryonic defects in chato embryos represent distinct, autonomous requirements for chato. Further experiments assessing the phenotype of chato chimeric embryos or using conditional alleles will define the tissue requirements of this novel KRAB zinc-finger protein.

The role of the Chato KRAB zinc-finger protein in morphogenesis

The chato mutation defines the role of a novel KRAB zinc-finger protein in mammalian convergent extension. Although KRAB domain zinc-finger proteins represent one of the largest gene families in mammals (Urrutia, 2003), only a few mutants have been described. These mutants affect diverse processes, including fertility, pigmentation and embryonic growth (Casademunt et al., 1999; Krebs et al., 2003), but Chato is the first member of this family shown to be required for embryonic morphogenesis. Although the high degree of sequence conservation among members of the family suggests that the genes might be functionally redundant, the severity and specificity of the chato phenotype indicates that some KRAB domain proteins have distinct functions.

The KRAB domain seems to be a relatively recent evolutionary feature, as it has only been found in the genomes of tetrapod vertebrates (Urrutia, 2003) (www.ensembl.org). Nevertheless, the C-terminal zinc-finger-containing region of chato shows homology to genes found in other animals. The closest homolog of chato in

Fig. 7. Proliferation in definitive endoderm. Cryosections of wild-type (A, C) and chato mutant (B, D) mouse embryos at different embryonic stages were labeled with anti-E-cadherin (red) and phospho-histone H3 (green) antibodies. Mitotic cells (green) in the definitive endoderm (highlighted in red by localization of E-cadherin) are indicated by arrowheads. E-cadherin is also present in E7.5 epithelia (ep), embryonic surface ectoderm (se) and extraembryonic visceral endoderm (VE, dashed line). Proliferation of mesoderm and epithelial tissues was not significantly different between wild-type (n=21 embryos/380 sections) and chato mutant (n=3 embryos/56 sections) embryos at these stages.
Drosophila is crooked legs (crol), with 39% identity and 53% similarity to the Chato zinc-finger domain. crol mutant pupae die with twisted legs that fail to elongate (D’Avino and Thummel, 1998). Although the zebrafish genome does not encode any KRAB domain proteins, morpholinos that disrupt the activity of the zinc-finger gene bloody fingers (bfl) display shortened and widen axial tissue due to defective convergent extension (Sumanas et al., 2005). Bfl and Chato share similar zinc-finger domains, but, based on synteny, it is unlikely that Bfl is the zebrafish ortholog of Chato. Therefore, it is possible that Chato, Crol and Bfl derived from a common ancestral zinc-finger protein that controlled tissue elongation during morphogenesis.

Our results suggest that the Chato KRAB zinc-finger protein acts through a molecular pathway that is independent of non-canonical Wnt signaling. Although mutations in both the mouse chato and non-canonical Wnt signaling genes affect convergent extension, their phenotypes are fundamentally different. The defects in axis elongation in the chato mesoderm are more profound than those reported in mouse non-canonical Wnt signaling mutants (Figs 1, 2) (Greene et al., 1998; Wang, J. et al., 2006). Most clearly, our analysis shows that chato mutants fail to close the gut endoderm and fail to undergo convergent extension in the gut, phenotypes that are not present in Lp mutants (data not shown). By contrast, Lp mutants have more-dramatic defects in neural tube closure and in convergent extension of the notochord than chato mutants (Greene et al., 1998; Wang, J. et al., 2006; Ybot-Gonzalez et al., 2007). A specific role for non-canonical Wnt signaling in morphogenesis of axial tissues is supported by the high level of expression of Vangl2 and Vangl1 in the mouse neural tube (Tobran et al., 2006; Tobran et al., 2008). Altogether, the observations suggest that Chato and non-canonical Wnt signaling act in different tissues and regulate convergent extension through different molecular mechanisms.

Because chato mutants are blocked in both definitive endoderm convergent extension and the accompanying cell rearrangements, we conclude that these cell rearrangements drive convergent extension of the mammalian endoderm. KRAB zinc-finger proteins are believed to act as transcriptional repressors (Bellefroid et al., 1991), so Chato might regulate the transcription of genes that regulate specific aspects of cytoskeleton dynamics, components of the extracellular matrix (ECM) or chemotactic clues. Because mutations in mouse genes that have global effects on cytoskeleton organization or the ECM (Garcia-Garcia and Anderson, 2003; George et al., 1993; Rakeman and Anderson, 2006) cause phenotypes dramatically different from those of chato mutants, we infer that chato controls cellular processes that are specific to convergent extension.

**Chato might act in a common molecular pathway with Hand1 and Yap65**

Although the molecular mechanisms that implement Chato function remain to be discovered, additional information might come from the analysis of two other mouse mutants with phenotypes similar to chato. Mutants that lack Hand1, which encodes a bHLH transcription factor, arrest development at the 9- to 14-somite stage, fail to close the gut endoderm, have a kinked neural plate and show extremelyyonic defects similar to those of chato embryos (Firulli et al., 1998). Loss of mouse Yap65 (Yap1), which encodes a protein with a proline-rich domain, WW domains, SH3 binding motifs, a coiled-coil and a PDZ binding motif, also causes phenotypes similar to chato (Morin-Kensicki et al., 2006). Studies similar to those described here could test whether these mutants have convergent extension defects in epithelia, mesenchyme and endoderm and whether cell rearrangements underlie the Hand1 and Yap1 abnormalities. Future experiments will be able to test whether chato, Hand1 and Yap1 act in a common biochemical process that regulates convergent extension in the mouse.

We thank Andrew Recknagel, Maegan Harden, Nina Lampen and Carole Daugherity for help and technical support; Elizabeth Robertson, Scott Weatherbee, Tristan Rodriguez, Philippe Gros, Andre Godineau, Torditora Tumbar and Tony Bretscher for providing mouse strains, reagents and/or use of lab equipment; and Holger Sondermann, Jeffrey Lee and Isabelle Migeotte for helpful discussions and comments on the manuscript. This work was supported by NIH grant HD003545 to K.V.A. and a Basel O’Connor March of Dimes award to M.J.G.G.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/18/3053/DC1

**References**


