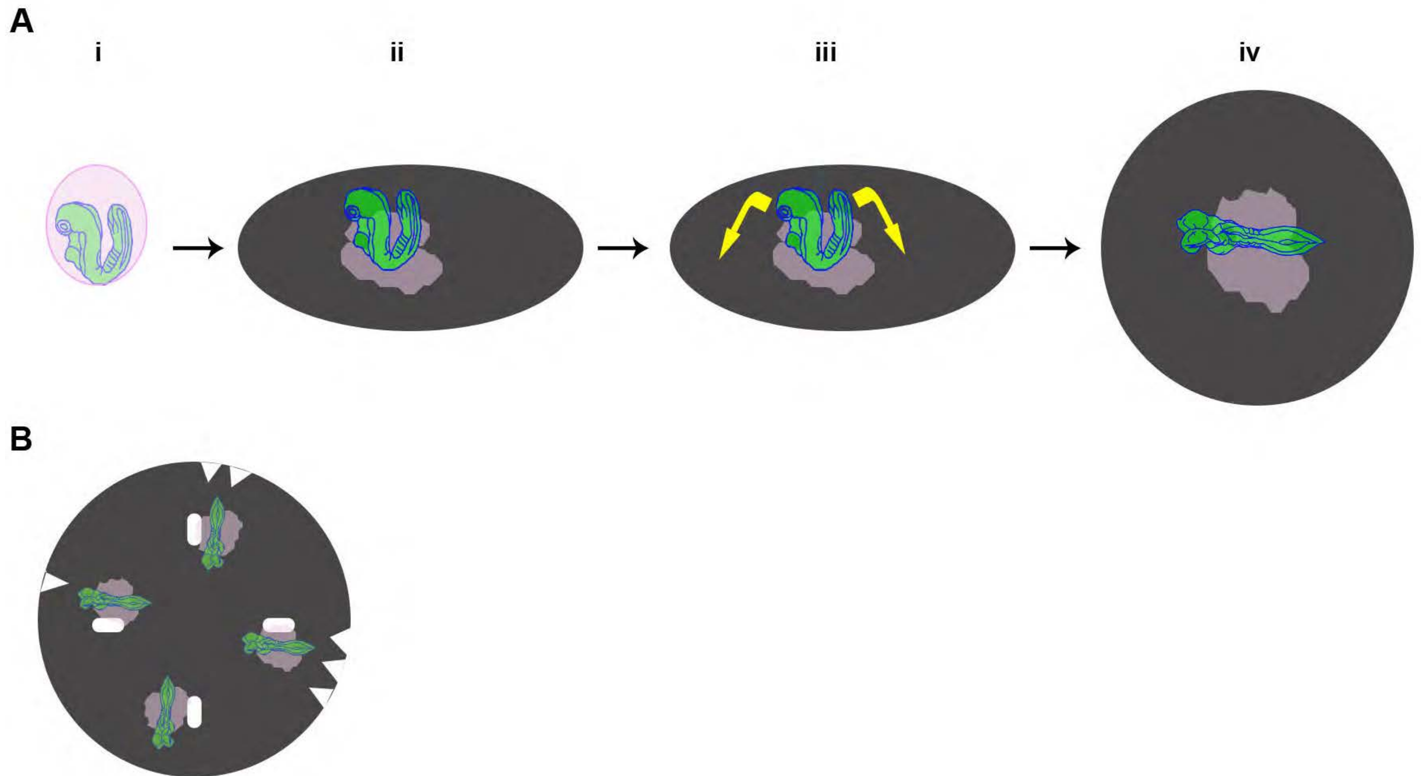
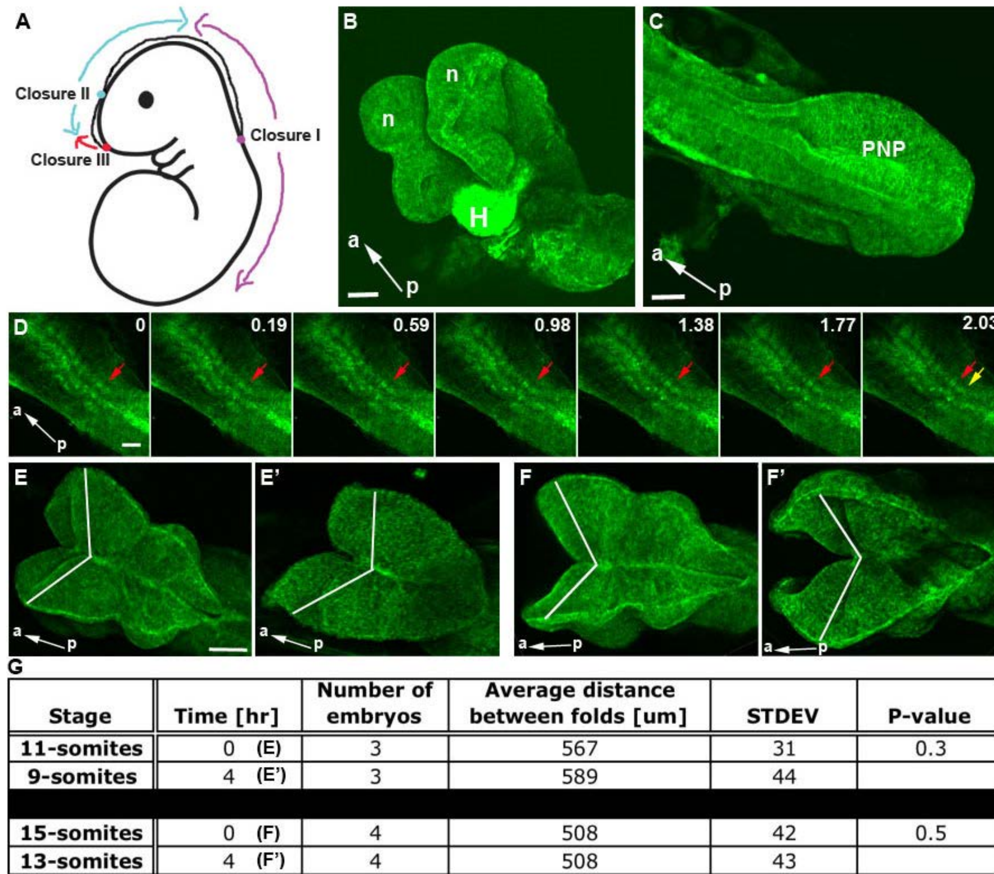


Fig. S1. A novel *in toto* imaging system for mouse embryogenesis. (**A**) The dissected embryo inside the yolk sac (i) is added to the drop of freshly prepared culture media on the surface of the filter paper (ii). The yolk sac is opened starting at the top away from the embryo and peeled down around the embryo, maintaining its connection with the ventral side of the embryo. The yolk sac is then adhered to the surface of the filter as shown in ii, to anchor the embryo to the filter. (iii) The embryo is extended by gently pushing both the head and the tail down onto the filter in the direction of the yellow arrows. The tissue will adhere to the surface of the filter allowing the neural tissue to be visualized. (iv) A view from the top with the embryo mounted on the filter paper for imaging. (**B**) Once the embryo is mounted as shown in A, cuts are made in the filter paper using a small blade (Moria, Fine Science Tools, item number 10316-14). The cuts beside the mounted embryos help to locate the embryos on the inverted microscope. The cuts at the edge of the filter near the embryo provide a method to mark and track the different embryos before, during and after live imaging and to correlate with the movies.



Stage	Time [hr]	Number of embryos	Average distance between folds [um]	STDEV	P-value
11-somites	0 (E)	3	567	31	0.3
9-somites	4 (E')	3	589	44	
15-somites	0 (F)	4	508	42	0.5
13-somites	4 (F')	4	508	43	

Fig. S2. Visualizing NTC in the living mouse embryo. (**A**) Schematic representation of an embryo at E9.5. Initial closure points are schematically shown as colored dots: closure points I, II and III as purple, turquoise and red, respectively. Closure of the neural folds proceeding from the different closure points is represented by the different colored arrows. (**B**) A transgenic Ven^{myr} embryo was dissected at the 8-somite stage and mounted for live imaging to observe closure point III. The image shows the embryo in supplementary material Movie 3 at time point zero. This ventral view of the head also allows visualization of the facial tissues. H, heart; n, neural folds. (**C**) A transgenic Ven^{myr} embryo at E10 was mounted for imaging of posterior neuropore (PNP) closure in the tail region. (**D**) A transgenic Ven^{myr} embryo at the 9-somite stage was mounted for imaging to visualize somite addition (supplementary material Movie 8). Red arrow shows the border of the most recently added somite at the beginning of imaging, and after 2 hours of imaging, the yellow arrow shows the border of the new pair of somites that were added. (**E**,**E′**) The distance between the neural folds, perpendicular to the midline, was measured in a transgenic Ven^{myr} embryo freshly dissected at the 11-somite stage (E) and in an embryo dissected at the 9-somite stage following 4 hours of imaging (E′). Results are summarized in G and indicate no significant difference in NTC. (**F**,**F′**) The distance between the neural folds, perpendicular to the midline, was measured in a transgenic Ven^{myr} embryos freshly dissected at the 15-somite stage (F) and in an 11-somite embryo following 4 hours of imaging (F′). Results are summarized in G and indicate no significant difference in NTC. (**G**) Table shows statistical analysis for the experiments described in E-F′. No significant difference was observed in the progression of neural tube closure between embryos that were imaged and embryos dissected at an equivalent developmental stage, indicating that the culture and imaging procedure did not affect neural tube closure progression. a, anterior; p, posterior. Scale bars: 100 μm.

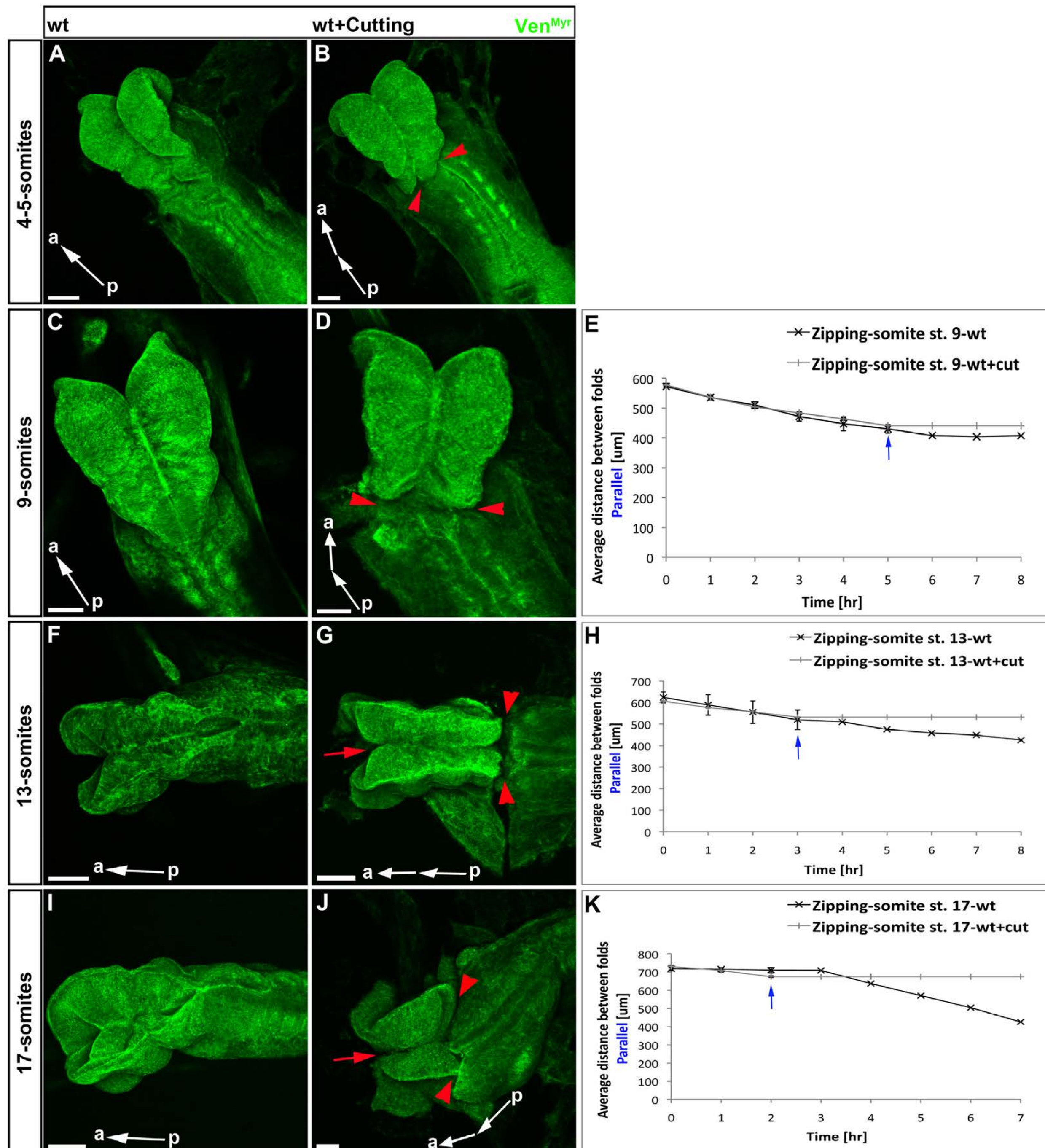


Fig. S3. Zipping proceeds normally up to the cut site in severed wild-type embryos. All embryos are transgenic for Ven^{Myr}. Red arrowheads show the cut site just rostral to the closing neural folds proceeding from closure point I. Red arrows show the vertical cut along the midline and separation of the neural folds to prevent closure from closure point II/ III. (**A**,**B**) Dorsal view of a wild-type uncut embryo (A) and a wild-type severed embryo (B) at 4- to 5-somites at the onset of the experiment and imaging. supplementary material Movie 9 shows that formation of closure point I is completed in cut embryos with similar dynamics to uncut embryos. (**C**,**D**) Dorsal view of a wild-type uncut embryo (C) and a wild-type severed embryo (D) at 9-somites at the onset of supplementary material Movie 10. (**E**) Zipping was measured and quantified in the severed embryos and compared with uncut embryos. No differences in zipping dynamics were observed until the closing neural folds reached the cut site. Zipping did not proceed rostral to the cut site (blue arrow). (**F**,**G**) Dorsal view of a wild-type uncut (F) and a severed (G) 13-somite embryo. (**H**) Zipping was measured and quantified in severed embryos (supplementary material Movie 11) and compared with uncut embryos. There were no differences in zipping dynamics until NTC reached the cut site wherein zipping did not proceed in cut embryos but did proceed in uncut embryos (blue arrow). (**I**,**J**) Dorsal view of a wild-type uncut (I) and a severed (J) 17-somite embryo imaged in supplementary material Movie 12. (**K**) Zipping was measured and quantified in severed embryos. There were no differences in zipping dynamics until NTC reached the cut site wherein zipping did not proceed in cut embryos but did proceed in uncut embryos (blue arrow). a, anterior; p, posterior. Error bars represent s.d. Scale bars: 100 μm.

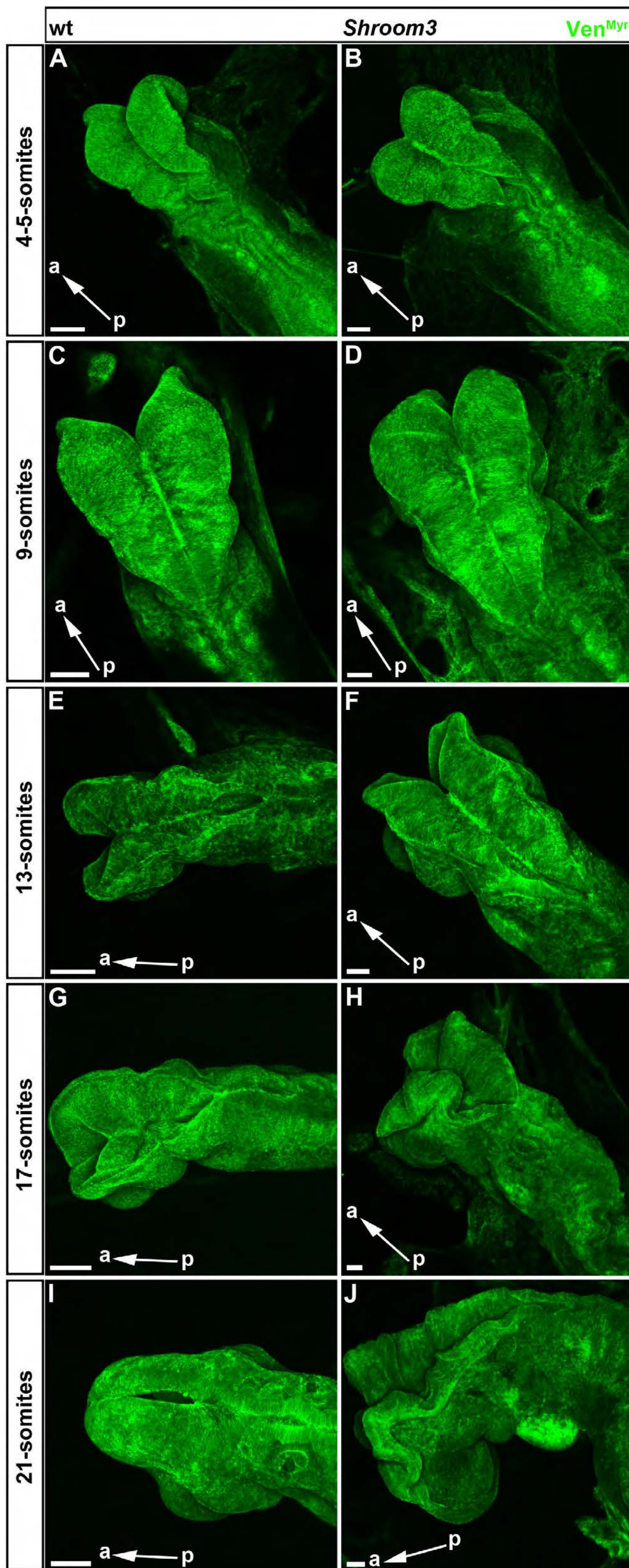


Fig. S4. *Shroom3* mutation disrupts the morphology of the neural folds during later stages of cranial NTC. All embryos are transgenic for Ven^{Myr} expressed constitutively in all embryonic tissues. (**A**,**B**) Dorsal view of a 4- to 5-somite wild-type embryo (A) and *Shroom3* mutant embryo (B) imaged to observe the formation of closure point I (supplementary material Movie 13). The morphology of the mutant embryos at this stage is normal and closure point I was formed similarly in mutant and wild type. (**C**,**D**) Dorsal view of a wild-type embryo (C) and a *Shroom3* mutant embryo (D) at 9-somites at the onset of supplementary material Movie 14. At this stage, *Shroom3* mutants show a slight change in morphology at the head region. (**E**,**F**) Dorsal view of a wild-type embryo (E) and a *Shroom3* mutant embryo (F) at 13-somites at the onset of supplementary material Movie 15. Morphologically, *Shroom3* mutants are different from wild-type embryos, in that the open part of the neural tube is wider and longer than in wild type. (**G**,**H**) Dorsal view of a wild-type embryo (G) and a *Shroom3* mutant embryo (H) at 17-somites at the onset of supplementary material Movie 16. Morphologically, *Shroom3* mutant embryos are severely abnormal. The neural folds in the mutant embryos were folded over themselves on each side of the brain region, and the open cranial neural folds was very irregular and misshapen in the mutant, whereas in the wild-type embryo the open neural folds were smooth and elliptically shaped. (**I**,**J**) Dorsal view of a wild-type embryo (I) and a *Shroom3* mutant embryo (J) at 21-somites at the onset of supplementary material Movie 17. Morphologically, *Shroom3* mutant embryos are severely abnormal compared with the wild-type embryos. The neural folds in the mutant embryos remain widely open in the brain region, and the gap that is still open in the head region is significantly wider and longer than the small opening in the wild type. a, anterior; p, posterior. Scale bars: 100 μm.