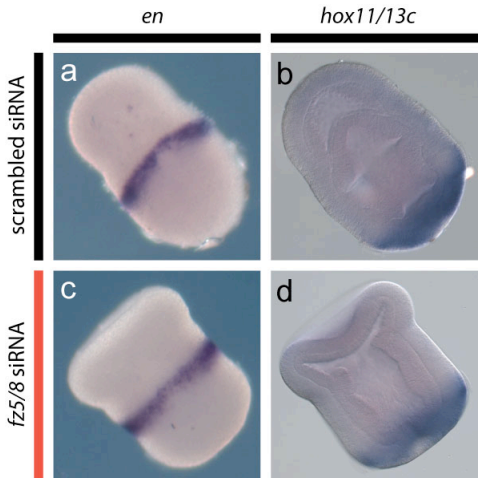
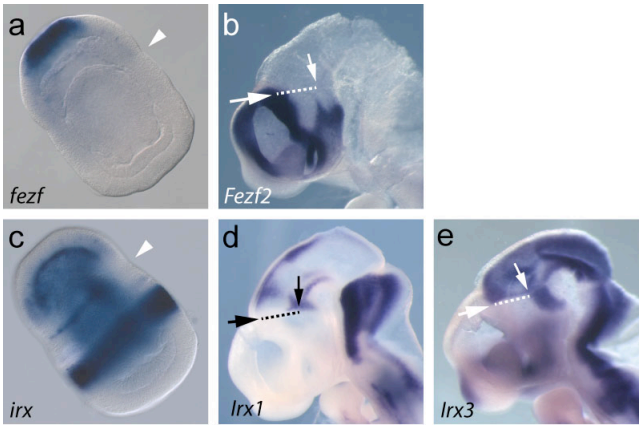


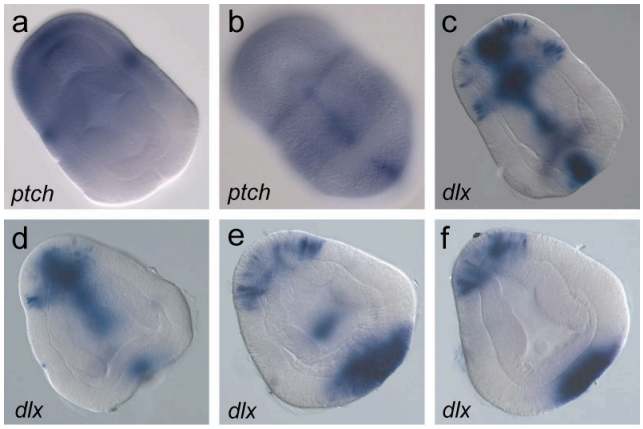
Supplementary figure 1. *Fz5/8* siRNA injection does not affect expression of collar and trunk markers.

a, b, expression of *en* (**a**) and *hox11/13c* (**b)** in embryos injected with a scrambled, control siRNA. **c, d**, expression patterns of *en* (**c**) and *hox11/13c* (**d**) are unaffected in embryos injected with *fz5/8* siRNA.



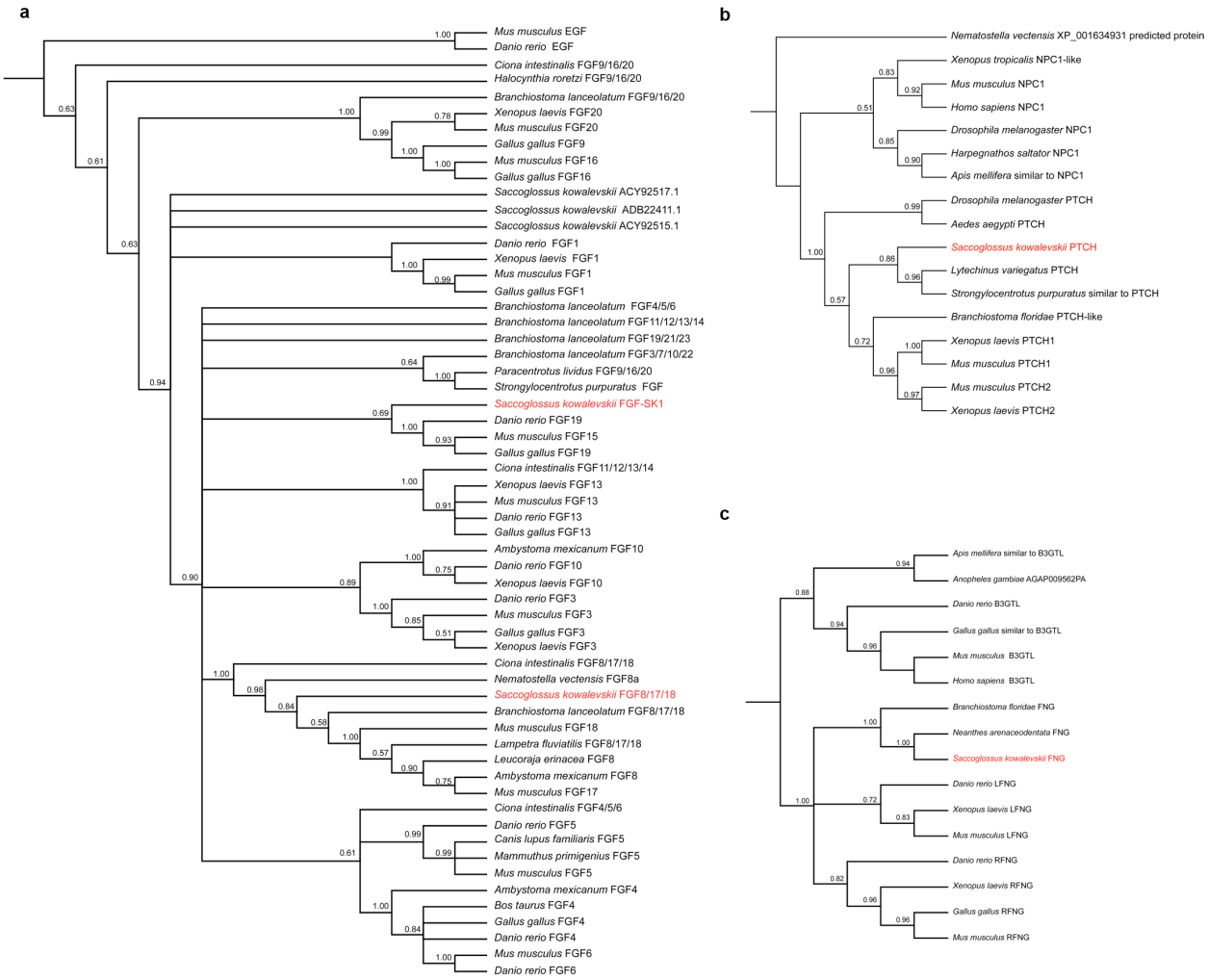
Supplementary figure 2. Expression patterns for *S. kowalevskii* and mouse homologues of additional genes involved in *Shh* regulation at the ZLI.

a, *fezf* is expressed in the *S. kowalevskii* anterior proboscis ectoderm. **b**, *Fezf2* expression in the mouse brain at E10.5. **c**, *irx* is expressed in anterior proboscis mesoderm, ventral proboscis-collar boundary ectoderm, collar-trunk boundary ectoderm, and collar endoderm in *S. kowalevskii*. **d, e**, mouse *Irx1* (**d**) and *Irx3* (**e**) are expressed in the diencephalon posterior to the ZLI with additional domains in the midbrain and hindbrain. Arrowheads mark the proboscis-collar boundary in hemichordates. In hemisected mouse heads, dashed lines indicate the ZLI with arrows additionally denoting its extent.



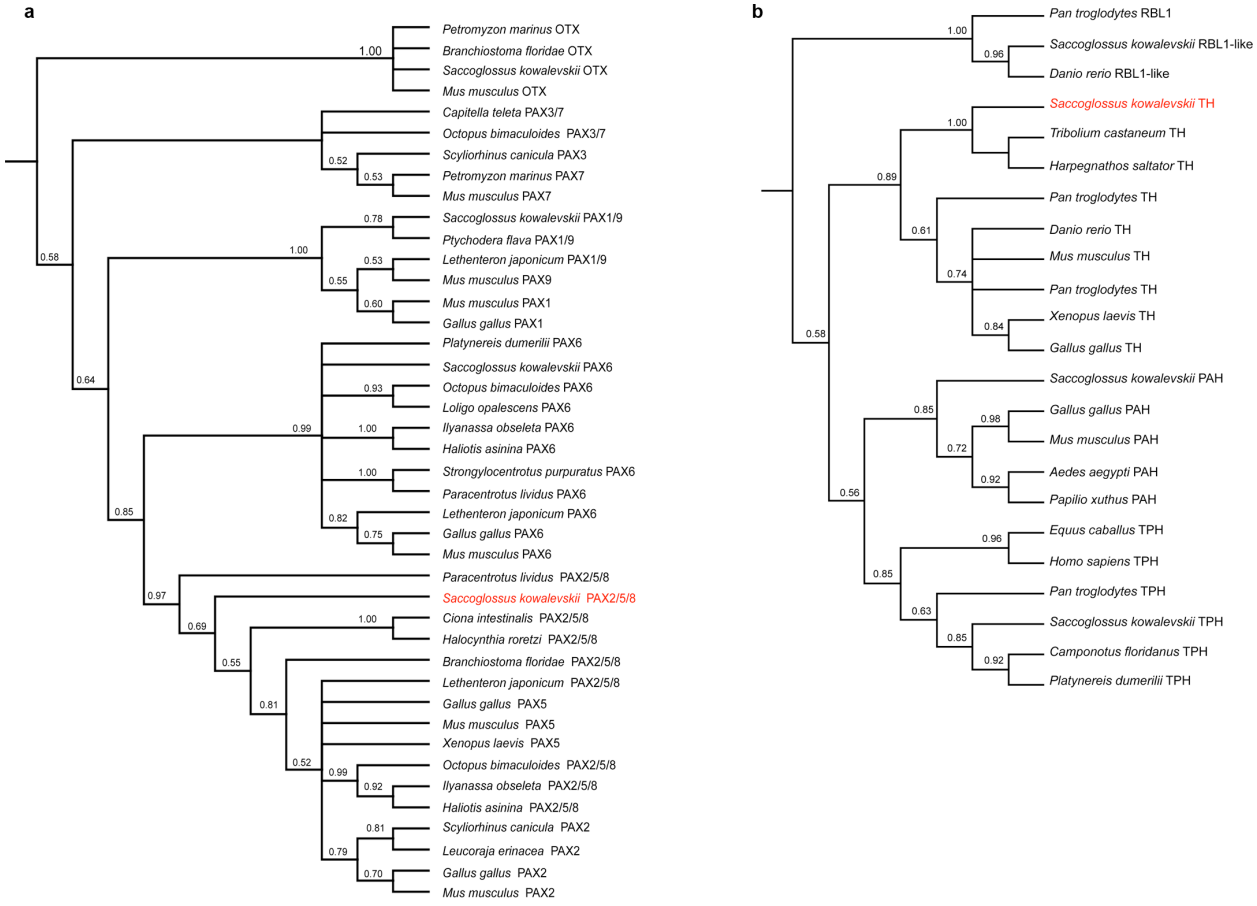
Supplementary figure 3. *Ptch* expression in wild-type embryos and spectrum of phenotypes caused by *hh* siRNA.

Images show optically cleared embryos oriented with anterior to top left. **a, b**, *ptch* expression with images focused through the embryo (**a**) or on the dorsal surface (**b**). Note expression in proboscis mesoderm and the ectodermal midline. **c**, *dlx* expression in an embryo injected with a scrambled, control siRNA. (**d-f**), *dlx* expression in embryos injected with *hh* siRNA. Milder phenotypes exhibit *dlx* downregulation in the apical ectoderm and proboscis base (**d**) while more severely affected embryos show progressive loss of the anterior proboscis and *dlx* dorsal midline expression (**e, f**).



Supplementary figure 4. Phylogenetic trees for *S. kowalevskii* FGFs, PTCH, and FNG.

a, phylogenetic tree for *S. kowalevskii* FGFs. *S. kowalevskii* FGF8/17/18 was placed within a robustly supported FGF8/17/18 clade, but the placement of FGF-SK1 was unstable, and it could not be confidently assigned to any FGF subfamily. Putative PTCH (**b**) and FNG (**c**) homologues were placed within well-supported clades. Posterior probabilities are shown above each node.



Supplementary figure 5. Phylogenetic trees for *S. kowalevskii* PAX2/5/8 and TYROSINE HYDROXYLASE (TH).

Putative *S. kowalevskii* PAX2/5/8 (**a**), and TH (**b**) homologues were placed within robustly supported clades that corroborated earlier predictions. Posterior probabilities are shown above each node.

Supplementary table 1. siRNA sequences.

Target gene	Sense	Antisense
β-catenin	ACAUGCUGUUGUAAAUCUUuu	AAGAUUUACAACAGCAUGUuu
fgf8/17/18	AAAGCGGUACAAUUUAUGAtt	UCAUAAAUUGUACCGCUUUtt
fgf8/17/18 scrambled	GAGAAUAACUGAACGUAUAtt	UAUACGUUCAGUUAUUCUCtt
fz5/8	GCUGAUACCUUCCGUGAAAtt	UUUCACGGAAGGUAUCAGCtt
fz5/8 scrambled	GCGUUCCAUCGAUCAAGUAtt	UACUUGAUCGAUGGAACGCtt
hh	GGAGCGGACCGATTAATGAtt	UCAUUAAUCGGUCCGCUCCtt
hh scrambled	ACAAGATGGTAGGCGTGCCtt	GGCACGCCUACCAUCUUGTtt
otx	CCGCCGAUAGCUGCUUAGAtt	UCUAAGCAGCUAUCGGCGGtt

Supplementary table 2. *S. kowalevskii* gene accession numbers.

Gene	NCBI accession number
β-catenin	ACH73219.1
dlx	AAP79300.1
en	AAP79298.1
fgf-Sk1	ACY92516.1
fgf8/17/18	ADB22412.1
fng	JN084014.1
foxa	ACG76356.1
foxg	AAP79301.1
gbx	AAP79285.1
hh	ABD97267.1
hox11/13c	AAP79288.1
otx	AAP79293.1
pax2/5/8	ADB22664.1
pax6	AAP79294.1
ptch	ADB22662.1
rx	AAP79282.1
sfrp1/5	ACY92646.1
six3	AAP79281.1
th	ADB22647.1
wnt1	ACH68427.1
wnt8	ACY92691.1