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Repressor activity of Headless/Tcf3 is essential for vertebrate head formation

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Abstract

The vertebrate organizer can induce a complete body axis when transplanted to the ventral side of a host embryo¹ by virtue of its distinct head and trunk inducing properties. Wingless/Wnt antagonists secreted by the organizer have been identified as head inducers^{2–4}. Their ectopic expression can promote head formation, whereas ectopic activation of Wnt signalling during early gastrulation blocks head formation^{5–7}. These observations suggest that the ability of head inducers to inhibit Wnt signalling during formation of anterior structures is what distinguishes them from trunk inducers that permit the operation of posteriorizing Wnt signals⁸. Here we describe the zebrafish *headless* (*hdl*) mutant and show that its severe head defects are due to a mutation in T-cell factor-3 (Tcf3), a member of the Tcf/Lef family^{9,10}. Loss of Tcf3 function in the *hdl* mutant reveals that *hdl* represses Wnt target genes. We provide genetic evidence that a component of the Wnt signalling pathway is essential in vertebrate head formation and patterning.

The *hdl* mutant was isolated as part of a screen for ethyl nitrosourea (ENU)-induced mutations that disrupt early neurogenesis in zebrafish¹¹. Mutant embryos obtained from *hdl* heterozygous parents, however, display a weak phenotype and are characterized by a slight reduction in eye size. Their weak phenotype allowed a subset of homozygous *hdl* fish to be grown to adulthood. Here, *hdl* mutants refers to maternally and zygotically homozygous mutant embryos. Our examination of *hdl* mutants with the early neuronal marker, *huC*¹², revealed an aberrant pattern of trigeminal neurons (Fig. 1a, b). The mutation, however, derives its name — *headless* — from the head defect in embryos that is characterized by complete loss of eyes, forebrain and part of the midbrain (Fig. 1c, d, e). Analysis of the head skeleton reveals cranial-specific defects (Fig. 1f, g); the pharyngeal arches appear relatively unaffected (Fig. 1h, i).

Changes in the expression of genes that are early markers for the forebrain and eye are consistent with morphological changes observed in *hdl* mutants. A homeobox gene *anf*/

Hesx1, normally expressed in the anterior neural plate (Fig. 2m), is almost absent in mutants (Fig. 2s). Mutational analysis has shown that *Hesx1* is required for normal forebrain development in mice and humans¹³. Transcripts of *six3*, another anterior brain marker¹⁴, are also reduced in the anterior neural plate, but its expression in the underlying prechordal plate is not changed (Fig. 2n, t). This suggests that the *hdl* phenotype is not related to loss of the underlying prechordal plate. Expression of *rx3*, a marker for the presumptive retina and ventral forebrain, is also strongly reduced in mutants (Fig. 2o, u). Mouse embryos carrying a null allele of the *Rx* gene have severe defects in eye and forebrain formation¹⁵. The reduction in expression of these anterior neural-specific genes is accompanied by a rostral expansion of midbrain-hindbrain boundary (MHB) genes such as *pax2* (Fig. 2p, v) and *engrailed2* (*eng2*) (Fig. 2q, w) but not *krox20* (*krx20*), expressed in rhombomeres 3 and 5 (Fig. 2w).

To investigate how early changes in the vertebrate organizer contribute to the genesis of the mutant phenotype, we examined a number of genes expressed in it during early gastrulation. There is little change in the expression of *gooseoid* (data not shown) and *chordin* (Fig. 2a, g) in *hdl* mutants. In contrast, expression of the Wnt antagonist, *dickkopf-1* (*dkk1*)^{3,16}, is increased in the organizer of mutants (Fig. 2b, h). These changes suggest that the *hdl* mutation leads to changes in head induction that start as early as the beginning of gastrulation.

Previous studies have shown that ectopic activation of Wnt signalling after the midblastula transition can mimic some aspects of the *hdl* phenotype⁵⁻⁷, so we examined whether there is ectopic expression of Wnts in the *hdl* mutants. During early gastrulation, *wnt8* is expressed in the ventrolateral margin and excluded from the dorsal margin⁷ (Fig. 2d, e). In *hdl* mutants, *wnt8* expression expands into the dorsal margin (Fig. 2j, k). In addition, rostral expansion of *wnt1* (Fig. 2f, l) and *wnt8b* (data not shown) expression is observed in late-gastrula-stage mutant embryos. These data suggest that the loss of anterior neural structures and the rostral expansion of MHB domain in *hdl* mutants may result from ectopic Wnt signalling. To test whether inhibition of Wnt signalling can reduce the *hdl* phenotype, we ectopically expressed the Wnt inhibitors, Cerberus⁴ and Dkk1 (ref. 16). Microinjection of their synthetic RNAs into *hdl* mutant embryos showed that neither of these Wnt inhibitors rescues the mutants (data not shown). This suggests that the normal function of *hdl* is necessary for effective function of these genes.

To clone the *hdl* mutation, we first established its position on the genetic map, using single-strand length polymorphism markers¹⁷. Bulk segregant analysis with centromeric markers localized *hdl* to linkage group 10 (Fig. 3a). Further genetic mapping placed the *hdl* locus between the SSLP markers z6183 and z9701. The proximity of *hdl* to z14193 revealed that the previously isolated *tcf3* gene, a Tcf/Lef family high mobility group (HMG)-box-containing transcription factor¹⁰, mapped close to the *hdl* locus. This gene has maternal expression and is involved in the Wnt pathway, so we investigated *tcf3* as a candidate for the *hdl* gene. We first compared the wild-type sequence of *tcf3* cDNA with that isolated from *hdl* mutants. The wild-type *tcf3* gene encodes a transcription factor with a β -catenin-binding domain, a single HMG DNA-binding domain and a nuclear localization signal (NLS) motif¹⁰. Sequence analysis revealed a seven-base-pair (bp) insertion in the *tcf3* cDNA from

hdl mutants that causes a frame-shift and is predicted to produce a truncated protein. The truncated Hdl protein retains its β -catenin-binding site but lacks the HMG box as well as the NLS motif (Fig. 3e). Myc-epitope tagged versions of the wild-type and mutant Hdl show that the truncated Hdl proteins are not specifically localized in the nuclei, but the wild-type Tcf3 protein is specifically transported into nuclei (Fig. 3f).

As ENU-based mutagenesis is not likely to cause a 7-bp insertion directly¹⁸, we predicted that the insertion in *tcf3* cDNA from *hdl* mutants was the result of a splicing defect caused by a point mutation. Thus, we isolated the *tcf3* gene from bacterial artificial chromosome (BAC) clones and analysed its genomic structure (Fig. 3b). Sequence analysis revealed the identical 7-bp sequence in the intron flanking exon V. In addition, we identified a T-to-A transversion in the *hdl* mutant genomic DNA. This point mutation is expected to cause altered splicing in the *hdl* transcripts (Fig. 3c). We used a polymorphism associated with the novel *MseI* site created by the point mutation for fine linkage analysis and showed that no recombinants were seen in 1,018 mutant embryos (Fig. 3d). Sequence analysis also revealed that the structure of the zebrafish *tcf3* gene (Fig. 3b) is similar to the human *Tcf1* gene¹⁹.

The *tcf3* gene is maternally expressed in *Xenopus*⁹ and zebrafish¹⁰, having very wide distribution during the blastula stage. However, despite the broad expression of *tcf3* in the early gastrula, its expression is excluded from the ventrolateral margin (Fig. 2c), where *wnt8* is expressed (Fig. 2d). Strong expression of the *tcf3* transcripts is detectable in the neurectoderm during gastrulation. By the tail bud stage, *tcf3* is expressed in a graded pattern in the anterior neurectoderm, with the highest levels at the anterior edge of the neural plate (Fig. 2r). Tcf3 is absent at the MHB where *wnt1* (Fig. 2f) and *wnt8b* (data not shown) are expressed. These expression patterns of *tcf3* are consistent with its potential early role in head induction and later in patterning the anterior neurectoderm. In *hdl* mutants, *tcf3* expression is strongly reduced both at early blastula and late gastrula stages (Fig. 2i, x).

To confirm that the *hdl* mutation in the *tcf3* gene is responsible for the mutant phenotype, we attempted to rescue the mutant embryos by expressing wild-type *tcf3* RNA. The injection of wild-type *tcf3* RNA (50 pg per embryo) rescued most of the mutants injected (90%; $n = 50$) (Fig. 4a). Ectopic expression of an equivalent amount of the mutant *hdl* RNA could not rescue the headless phenotype ($n = 50$). To test whether the truncated Hdl protein has dominant-negative effects¹⁰, we injected up to a tenfold higher concentration of *hdl* mutant RNA (500 pg per embryo) in both mutant and wild-type embryos. No significant effect was seen in either of these embryos, which suggests that *hdl* is functionally a null mutation.

It is known that Tcf can act as a repressor of Wnt target genes^{20,21} through its association with Groucho^{22,23} and/or CtBP²⁴, or as an activator through its association with β -catenin^{25,26}. Our functional analysis of the *hdl* mutants indicates, however, that the phenotype may be associated with the loss of a mechanism that is critical for repressing Wnt signalling, not facilitating it. To test this idea, we examined the effects of N-Tcf3, a deletion of Tcf3 that lacks β -catenin binding (Fig. 4f) but is known to retain its repressor activity⁹. Injection of RNA encoding N-Tcf3 (50 pg per embryo) rescued the *hdl* mutant phenotype (90%; $n = 50$). Embryos injected with plasmid DNA encoding N-Tcf3 also rescued mutants (30%; $n = 50$, Fig. 4b), indicating that zygotic expression of N-Tcf3 is

sufficient for rescue. Injection of 50 pg of N-Tcf3 RNA into one blastomere of two-cell-stage embryos suppressed expression of a known target of β -catenin/Tcf signalling²⁷, *eng2*, demonstrating that N-Tcf3 can repress Wnt target genes (Fig. 4c).

To provide further evidence that the activity of Tcf3 as a repressor is critical for head formation, the HMG box DNA-binding domain of Tcf3 was combined with the repressor domain of the Engrailed protein²⁸ (Fig. 4f). Injection of RNA (10 pg per embryo) encoding Eng-Tcf3 rescued the *hdl* mutants (80%; $n = 40$, Fig. 4f). Conversely, we investigated whether a chimaeric transcription activator²⁹ VP16-Tcf3 (Fig. 4f) could rescue *hdl* mutants. None of the mutants were rescued by injection (10 pg per embryo) of VP16-Tcf3 ($n = 50$), although higher doses (>50 pg per embryo) caused severe defects (data not shown). Most wild-type embryos injected with 10 pg of VP16-Tcf3 RNA display a headless phenotype (85%; $n = 19$, Fig. 4e), mimicking the effects of late Wnt activation^{5–7} and the *hdl* mutation.

These results indicate that function of Tcf3 as a repressor may be critical for head formation. Basal repression of Wnt targets by Tcf3 during early gastrulation seems to be essential for repressing the genes responsible for the formation of the MHB domain and for permitting the expression of genes responsible for formation of the forebrain, eyes and the midbrain. Repression of Wnt target genes in the prospective neurectoderm by Tcf3 defines a primary condition for function of head inducers. In the absence of this basal repression, secreted Wnt antagonists are ineffective, because they operate by preventing Wnt signals from de-repressing Wnt target genes in the anterior neurectoderm. The graded pattern of *tcf3* expression at the anterior edge of the neurectoderm and *wnt* genes at the MHB suggest a continuing role for Wnts and their antagonists in patterning the anterior neurectoderm during late gastrulation.

Methods

Genetic mapping

For mapping, an AB-background heterozygous *hdl* carrier was crossed to two polymorphic strains, WIK and HK. Linkage analysis was performed on embryos obtained by crossing an *hdl* AB homozygous female and an *hdl* AB/HK or *hdl* AB/WIK heterozygous male. Genomic DNA was used for genotyping embryos by polymerase chain reaction (PCR). The genetic distance between SSLP markers and the *hdl* locus was determined by assaying DNA polymorphisms in individual mutant embryos. Zebrafish BAC (192G1 and 103L24, Genome Systems, Inc.) and YAC (76G7, Research Genetics, Inc.) clones were screened by PCR as pools according to the manufacturer's instructions. Restriction enzyme-digested BAC DNA was subcloned into the pBluescript vector and sequenced by standard procedures. For fine linkage analysis, we amplified genomic DNA from individual mutant embryos with specific oligonucleotide primers (P2, 5'-TCAGTCATTCGCTATCACCTCTGTC-3' and P3, 5'-ACAATGGCGGGGTGAGGGATGC-3') and digested it with the restriction enzyme *MseI*.

Cloning of mutant *hdl* cDNA

RNA was extracted from wild-type embryos or maternally homozygous mutant embryos at the tail bud stage and PCR was carried out after reverse transcription of RNA. Multiple

clones were sequenced and compared with wild-type sequences to detect the mutation. To confirm the existence of a 7-bp insertion in the mutant *hdl* cDNA, we amplified a partial cDNA fragment expected to contain the mutated site using the primers P1 (5'-CCTCT TGGATGGTTGGTTCCTCAG-3') and P3. In high-percentage gel electrophoresis, a single predicted 177-bp band, slightly larger than the 170-bp wild-type band, was detected in *hdl* mutant embryos. There were no additional bands detectable in the gel, indicating that the acquired 3'-splice acceptor is preferred for RNA splicing in mutant embryos.

DNA constructs

To generate Myc-tagged Tcf3 (pCS3Tcf3), first the amino-terminal region, starting from the second amino-acid proline, was generated by PCR using primers flanked by *Bam*HI and *Eco*RI sequences. The PCR product was then subcloned into the *Bgl*II and *Eco*RI site of the pCS3 expression vector. Second, the *Bam*HI- and *Spe*I-digested fragment, containing the Myc epitope and the N-terminal β -catenin-binding domain, was subcloned into the CS2+Tcf3a plasmid (a gift from R. Dorsky). pCS3 N-Tcf3 was created by blunt-ligation of the *Nco*I- and *Spe*I-digested pCS3Tcf3, deleting the first 47 amino acids of β -catenin-binding domain. The pCS2Eng-Tcf3 construct was generated by fusing the Tcf3 DNA-binding domain (amino acids 297–446) to a fragment encoding amino acids 1–298 of the *Drosophila* Engrailed protein²⁸. pCS2VP16-Tcf3 was made by fusing the same Tcf3 DNA-binding domain to the activator domain (amino acids 410–490) of the VP16 from the *Herpes simplex* virus²⁹. RNA for injection was prepared from linearized plasmid DNAs using the SP6 mMessage Machine kit (Ambion).

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Figure 1.

Morphological analysis. **a, b**, *huC* expression at the neural plate stage shows abnormal rostral extension of trigeminal neurons (arrow). Dorsal-anterior view. **c, d**, 34-hour embryos show complete loss of eye, forebrain (fb) and part of the midbrain in *hdl*. mb, midbrain; hb, hindbrain; mhb, midbrain-hindbrain boundary; ot, otic vesicle. **e**, *hdl* larvae at 8 days show a severe head defect. **f-i**, Skeletal preparations of day-5 larvae stained with Alcian blue. In mutants, the ethmoid plate (ep) is reduced (asterisk), whereas ventral pharyngeal arches (p1–p7), including Meckel's cartilage (mc), are less affected. Fused pterygoid process of the palatoquadrate (pq) in *hdl* (arrowhead).

Figure 2.

Gene expression analyses. Animal pole is to the top for 60–80% epiboly embryos. Anterior is to the left for tail bud (TB) and 3-somite-stage embryos (3ss). **a, g**, *chd* is relatively unaffected in the organizer. **b, h**, increased *dkk1* expression in *hdl*. **c, i**, Widespread *tcf3* expression, excluded from the ventrolateral margin in the wild type and reduced in *hdl*. Arrowheads indicate the organizer region. **d, e** and **j, k**, *wnt8* is excluded from the dorsal margin (arrowheads) in the wild type but not in *hdl*. **f, l**, Anterior expansion of *wnt1* expression in *hdl*. **m–o** and **s–u**, Reduced expression of anterior neural markers in *hdl*. **p, q** and **v, w**, Anterior expansion of MHB markers. **q, w**, *krox20* is relatively unaffected in *hdl*. **r, x**, Graded anterior neural plate *tcf3* expression, reduced in *hdl*.

Figure 3.

Mapping of the *hdl/tcf3* gene. **a**, Genetic map of linkage group 10 (LG10) showing position of *hdl* in relation to previously mapped genes and SSLP markers. **b**, Structure of the *tcf3* gene. Arrowhead indicates the site of the *hdl* mutation. **c**, T-to-A transversion (green) produces an aberrant splice acceptor site (AG). The 7-bp insertion is indicated in red. **d**, *Mse* I digestion of the genomic PCR product (P2–P3), showing a polymorphism. **e**, Structure of Headless (Hdl). The truncated Hdl protein lacks the high mobility group (HMG) box DNA-binding motif as well as the nuclear localization signal (NLS). **f**, Subcellular localization of the wild-type Tcf3 and truncated Hdl proteins is changed from nuclear to cytoplasmic.

Figure 4.

Rescue by Tcf3 expression. **a**, Two-day-old *hdl* mutant injected with wild-type Tcf3 RNA (bottom). Top, uninjected control mutant. **b**, An *hdl* mutant injected with the Δ N-Tcf3 plasmid DNA which is expressed after the MBT. **c**, Δ N-Tcf3 suppresses expression of *eng2*. Blue X-gal staining shows the injected side of embryo. **d**, Rescue of *hdl* by Eng-Tcf3 RNA injection. Higher doses (>50 pg) cause trunk defects. **e**, Opposite effects of VP16-Tcf3 on head formation. A wild-type embryo injected with VP16-Tcf3 RNA, showing a headless phenotype. **f**, Structure of proteins used for injection experiments.