

Eomesodermin is required for mouse trophoblast development and mesoderm formation

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The earliest cell fate decision in the mammalian embryo separates the extra-embryonic trophoblast lineage, which forms the fetal portion of the placenta, from the embryonic cell lineages. The body plan of the embryo proper is established only later at gastrulation, when the pluripotent epiblast gives rise to the germ layers ectoderm, mesoderm and endoderm. Here we show that the T-box gene *Eomesodermin*¹ performs essential functions in both trophoblast development and gastrulation. Mouse embryos lacking *Eomesodermin* arrest at the blastocyst stage. Mutant trophoblast does not differentiate into trophoblast, indicating that *Eomesodermin* may be required for the development of trophoblast stem cells². In the embryo proper, *Eomesodermin* is essential for mesoderm formation. Although the specification of the anterior–posterior axis and the initial response to mesoderm-inducing signals is intact in mutant epiblasts, the prospective mesodermal cells are not recruited into the primitive streak. Our results indicate that *Eomesodermin* defines a conserved molecular pathway controlling the morphogenetic movements of germ layer formation and has acquired a new function in mammals in the differentiation of trophoblast.

The T-box genes encode a family of transcription factors sharing an evolutionarily conserved DNA-binding domain first defined in the *Brachyury* (*T*) gene^{3–5}. The T-box gene *Eomesodermin* has been implicated as an important regulator of gastrulation in *Xenopus*¹. To investigate the role of *Eomesodermin* in mammalian development, we mutated the murine orthologue by homologous recombination.

Expression of mouse *Eomesodermin* (*Eomes*) is first detected in the trophoblast lineage, starting in the trophoblast of the blastocyst (Fig. 1a), and continuing in the extra-embryonic ectoderm of the early postimplantation embryo (Fig. 1b–h). In the embryo proper, expression of *Eomes* starts in the posterior part of the epiblast and then extends distally into the primitive streak and nascent mesoderm (Fig. 1c–h). *Eomes* is also expressed in the developing forebrain and the olfactory lobes (Fig. 1i, j)^{6–8}.

We disrupted *Eomes* by inserting a *LacZ* reporter gene into the locus (Fig. 2a–c). In heterozygous embryos, this allele (*Eo*^{LacZ}) shows the same expression pattern as seen by *in situ* hybridization,

albeit with a slight lag in developmental stage most probably reflecting differences between protein and messenger RNA half-lives (Fig. 1e–h). Although animals heterozygous for *Eo*^{LacZ} are healthy and fertile, no homozygous offspring were obtained, indicating embryonic lethality. Morphological analysis at 6.0 and 7.5 days post coitum (d.p.c.) revealed that mutant embryos arrest soon after implantation and fail to form organized embryonic or extra-embryonic structures (Fig. 3a–e). This impairment of peri-implantation development is probably due to a defect in the trophoblast lineage, as *Eomes* is only expressed in the trophoblast at this stage.

The differentiation of mutant trophoblast was further investigated by blastocyst culture *in vitro*. In control embryos (*n* = 99), trophoblast cells started to spread on the culture dish 15–24 h after hatching and usually formed extensive outgrowths by 48 h (Fig. 3f). In contrast, most homozygotes (30 out of 33) did not form outgrowths, although they hatched normally and maintained the typical blastocyst morphology for more than 7 days (Fig. 3g). Some mutants (3 out of 33) showed late (>96 h) spreading of small trophoblast-like cells, which then rapidly degenerated (not shown). Mutant blastocysts expressed the typical repertoire of integrins (Fig. 3h–k) and attached strongly to the substratum, indicating that the defect in trophoblast differentiation is not due to a lack of integrin-mediated adhesion^{9,10}.

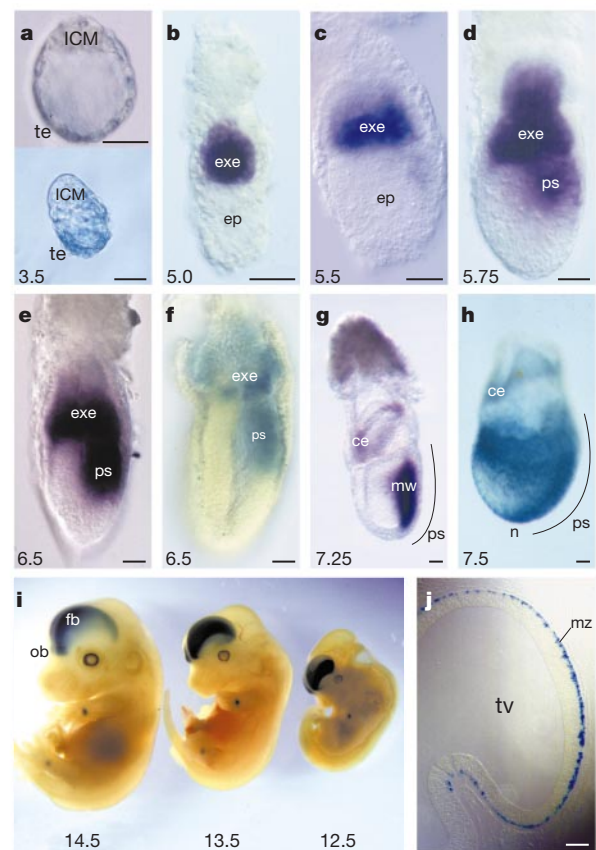


Figure 1 Expression of *Eomesodermin* from 3.5 to 14.5 d.p.c. as shown by β -galactosidase staining of *Eo*^{LacZ} heterozygotes (a, f, h–j) or whole-mount *in situ* hybridization (b–e, g), anterior aspects facing left. a–f, Expression in the trophoblast (a), extra-embryonic ectoderm and posterior epiblast (b–f). g, h, Expression in primitive streak, mesoderm wings and chorionic ectoderm. i, Expression in forebrain and limbs. j, Coronal section of forebrain at 12.5 d.p.c. te, trophoblast; ICM, inner cell mass; exe, extra-embryonic ectoderm; ep, epiblast; ps, primitive streak; mw, mesoderm wings; ce, chorionic ectoderm; n, node; fb, forebrain; ob, olfactory bulb; mz, mantle zone; tv, telencephalic vesicle. Scalebars, 50 μ m.

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A self-renewing population of diploid trophoblast cells can be derived *in vitro* from blastocyst outgrowths². These trophoblast stem (TS) cell lines strongly express *Eomes*, and their maintenance requires the presence of fibroblast growth factor 4 (FGF4). When blastocysts lacking *Eomes* were cultured in the presence of FGF4, the block in trophectoderm differentiation could not be overcome, indicating that *Eomes* may be required cell autonomously for the transition from trophectoderm to trophoblast.

We investigated the role of *Eomes* in gastrulation using chimaeras consisting of wild-type extra-embryonic and mutant embryonic tissues. In tetraploid host embryos, which can only generate extra-embryonic tissues, the phenotype of mutant epiblasts derived from homozygous embryonic stem (ES) cells or inner cell masses (ICMs) can be studied¹¹.

No gross abnormalities were observed in chimaeras at early gastrulation stages (6.5 d.p.c., Fig. 4a). At 7.5 d.p.c., the typical elongation of the primitive streak and a morphologically distinct node were absent (Fig. 4b). Histological sections show a posterior thickening of the epiblast with morphological signs of epithelial-to-mesenchymal transition, but no emergence of embryonic (Fig. 4e) or extra-embryonic mesoderm (Fig. 4f, g). At 8.5 d.p.c., the equivalent of early somite stages, mutant chimaeras still resembled enlarged egg cylinders lacking mesoderm (Fig. 4c, d).

This phenotype could be due to defects in axis specification and mesoderm induction, or due to an impairment in the formation or function of the primitive streak. To distinguish between these possibilities, we analysed the expression of marker genes. In mutant epiblasts, the targeted *Eo^{LacZ}* locus is initially expressed in its normal posterior domain (6.5 d.p.c., Fig. 4a), but subsequently shows broad ectopic activation in the lateral and anterior parts of

the embryonic ectoderm (Fig. 4b–d). The expression of *Brachyury/T* and *Fgf8*, two early markers of the primitive streak and prospective mesoderm, is similarly extended (compare Fig. 4b and 4h, i). This indicates that the anterior–posterior axis is specified and mesoderm inducing signals are present. The domain of ectopic marker expression is similar to the location of prospective mesoderm in the pregastrulation epiblast¹², suggesting that prospective mesodermal cells receive an inducing signal and activate early streak genes, but are unable to initiate the directed migration towards the streak. No expression of *Hnf-3β* is detected in mutants (data not shown), indicating that the differentiation of anterior streak and node is blocked.

Ectodermal patterning was visualized by *Otx2*, which is normally active in undifferentiated epiblast cells and neuroectoderm, and *Hesx1*, a marker of anterior visceral endoderm and neuroectoderm¹³. In mutant epiblasts, *Otx2* is expressed only in the anterior domain (Fig. 4j), indicating that its typical suppression by posterior signals is intact. No ectodermal expression of *Hesx1* was detected (Fig. 4l), suggesting that the mutant anterior ectoderm is undifferentiated, and that the specification of neuroectoderm is delayed or absent.

The anterior visceral endoderm (AVE) has been implicated as the main signalling centre for the anterior patterning of the mouse embryo¹⁴. Expression of *Hesx1* is reproducibly seen in the AVE, which is of wild-type origin in chimaeras (Fig. 4l), indicating that the specification and positioning of the AVE is not impaired in the presence of a mutant epiblast.

To test whether *Eomes* is required for mesoderm differentiation in general, or specifically for the recruitment of prospective mesoderm into the primitive streak, we examined the developmental potential

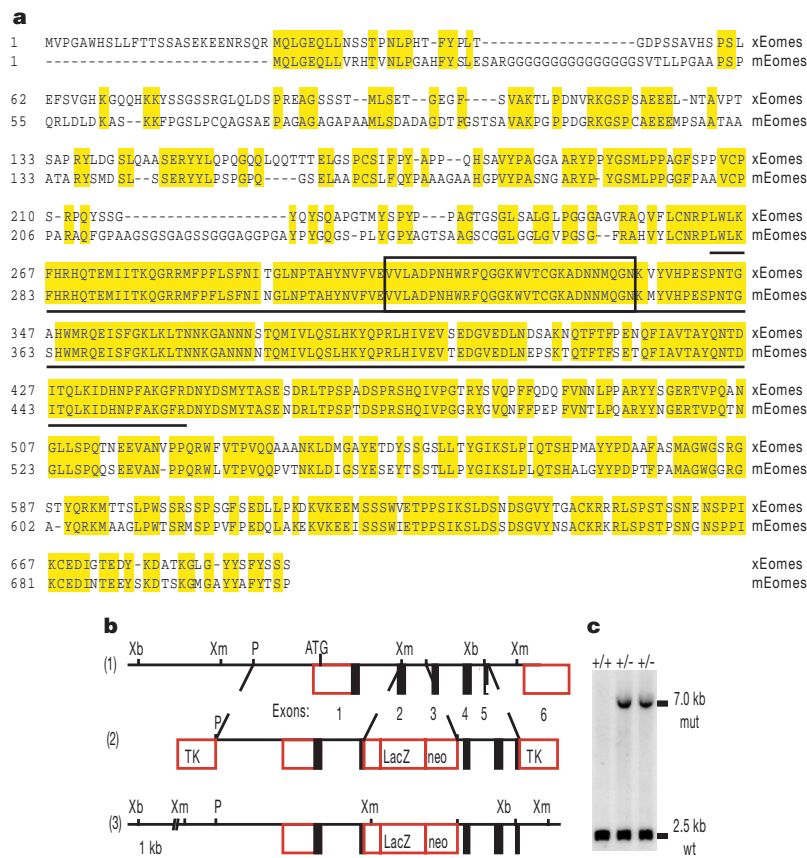


Figure 2 Primary structure and targeted disruption of mouse *Eomesodermin*. **a**, Alignment of the predicted amino-acid sequences of mouse (*mEomes*) and *Xenopus* (*xEomes*) proteins. Identical residues are shaded, the T domain is underlined. The region deleted in

the *Eo^{LacZ}* allele is boxed. **b**, Targeted disruption of the *Eomesodermin* locus: wild-type locus (1), targeting construct (2), mutant allele (3). The T-box coding region is shaded. Xb, *Xba*I; Xm, *Xmn*I, P, *Pst*I. **c**, Southern blot with 3' external probe, *Xmn*I digest.

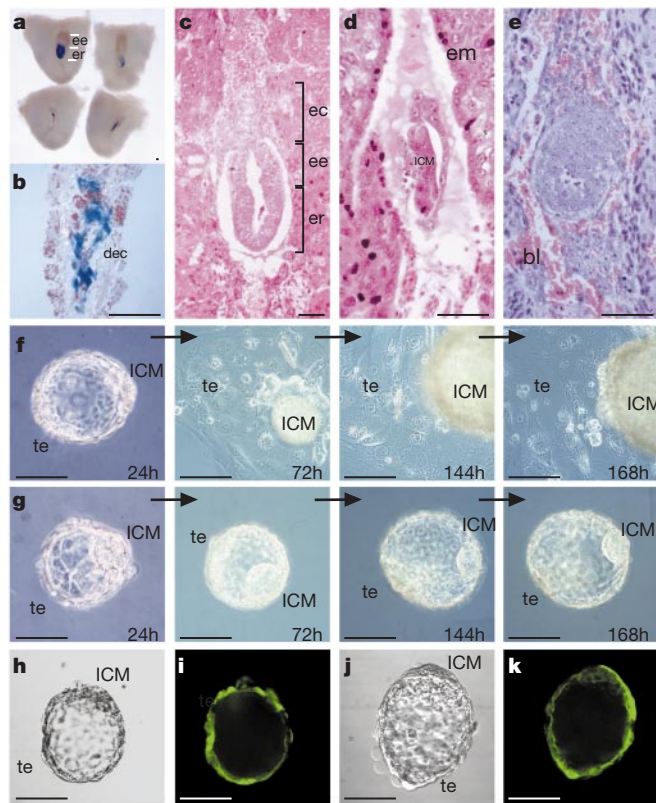


Figure 3 Morphology of mutant embryos. **a**, Heterozygous (top) and homozygous embryos (bottom) in decidua, *LacZ* stain. **b**, Longitudinal section through disorganized homozygous embryo in decidua, *LacZ* stain. **c–e**, Haematoxylin and eosin (HE)-stained longitudinal sections of embryos in decidua at 6.5 d.p.c. **c**, Wild-type littermate. **d**, Presumptive homozygous mutant arrested in a blastocyst-like stage. **e**, Disorganized embryo in a blood-filled implantation site (bl). **d, e** are depicted at higher magnification than **c, f, g**. **f, g**, Time Course of mutant blastocysts and control littermates cultured *in vitro*.

Phase contrast micrographs. **f**, Control embryo. Trophoctoderm-derived cells (te) are spreading over the dish, whereas ICM-derived cells form a central clump. **g**, Typical mutant blastocyst showing no sign of trophoblast outgrowth. **h–k**, Expression of $\alpha 5/\beta 1$ integrin in mutant blastocyst (**h, i**) and wild-type littermate (**j, k**) 36 h after hatching. Phase contrast (**h, j**); immunofluorescence (**i, k**). em, endometrium, er, embryonic region; ee, extra-embryonic ectoderm; dec, decidua; ec, ectoplacental cone; ICM, inner cell mass; te, trophoctoderm and derivatives. Scalebars, 50 μ m.

of homozygous mutant ES cells. Mesoderm formation is partially rescued in chimaeras with diploid host embryos, where mutant and wild-type epiblast cells are intermingling (Fig. 4m, n). Wild-type cells migrate efficiently through the primitive streak and become enriched in the mesoderm wings, whereas most mutant cells persist in the ectoderm (Fig. 4n). When injected into syngeneic hosts, mutant cells give rise to teratomas containing muscle, cartilage and haematopoietic tissue (Fig. 4o). This shows that *Eomes* is not absolutely required for the differentiation of mesodermal cell types, and indicates that the mutation may specifically affect the morphogenetic movement of cells from the epiblast into the primitive streak.

In *Xenopus*, the homeobox-containing genes *Bix1–4* have been identified as transcriptional targets for the T-box genes *Brachyury/Xbra* and *VegT* (refs 15, 16). A murine homologue of the *Bix/Mix* gene family is *Mml*, which is normally expressed in the visceral endoderm and the primitive streak¹⁷. To test whether *Mml* is a potential transcriptional target of T-box genes in the mouse, we analysed its expression in *Eomes*- and *Brachyury*-deficient (*T/T*) embryos. *Mml* expression is normal in *T* mutants (not shown). No transcription could be detected in epiblasts lacking *Eomes* (Fig. 4k), whereas the other early streak markers tested show enhanced expression (see above). This suggests that *Mml* is acting downstream of *Eomes*.

Our findings show that *Eomesodermin* performs independent essential functions in extra-embryonic and embryonic tissues. The expression of *Eomes* in trophoctoderm, diploid trophoblast and TS cell lines, and the phenotype of mutant embryos *in vivo* and *in vitro*, indicate that *Eomes* may be required for the differentiation of

trophoctoderm and the formation of trophoblast stem cells^{2,18}. Notably, although the differentiation of trophoctoderm into primary giant cells *in vitro* is dependent on *Eomes*, it does not require FGF4, as shown by mutations in the FGF pathway^{19,20}. This suggests that FGF4 is not absolutely required for the initial activation of *Eomes* in trophoctoderm, but that it might act to maintain *Eomes* expression and to expand the TS cell pool.

In the embryo proper, *Eomes* is required for mesoderm formation and the morphogenetic movements of gastrulation. In contrast to other mutations affecting gastrulation^{21–23}, lack of *Eomes* abrogates the formation of embryonic and extraembryonic mesoderm without apparent effects on the initial specification of the anterior–posterior axis, mesoderm induction, cell viability or proliferation. The spatiotemporal expression patterns of marker genes suggest that *Eomes* is specifically required for the directed movement of cells from the epiblast into the streak in response to mesoderm induction. The accompanying block in mesoderm differentiation is probably secondary to the defect in migration, as mutant cells can give rise to mesodermal tissues *in vitro* and in teratomas.

Previous work has implicated *Brachyury/T* (refs 24, 25) and FGF signalling^{26,27} in the control of cell migration through the primitive streak. Our data indicate that *Eomes* acts at an earlier stage than *T*, *Fgfr1* and *Fgf8*. A potential transcriptional target of *Eomes* is *Mml* (ref. 17), suggesting that, similar to *Xenopus*, a *Mix*-like homeobox protein might be acting downstream of a T-box gene^{15,16}.

It is intriguing that during evolution a conserved protein that controls mesoderm formation has acquired an additional function in the trophoblast—a ‘new’ tissue type, specific to placental mammals. The common denominator of *Eomes* function in

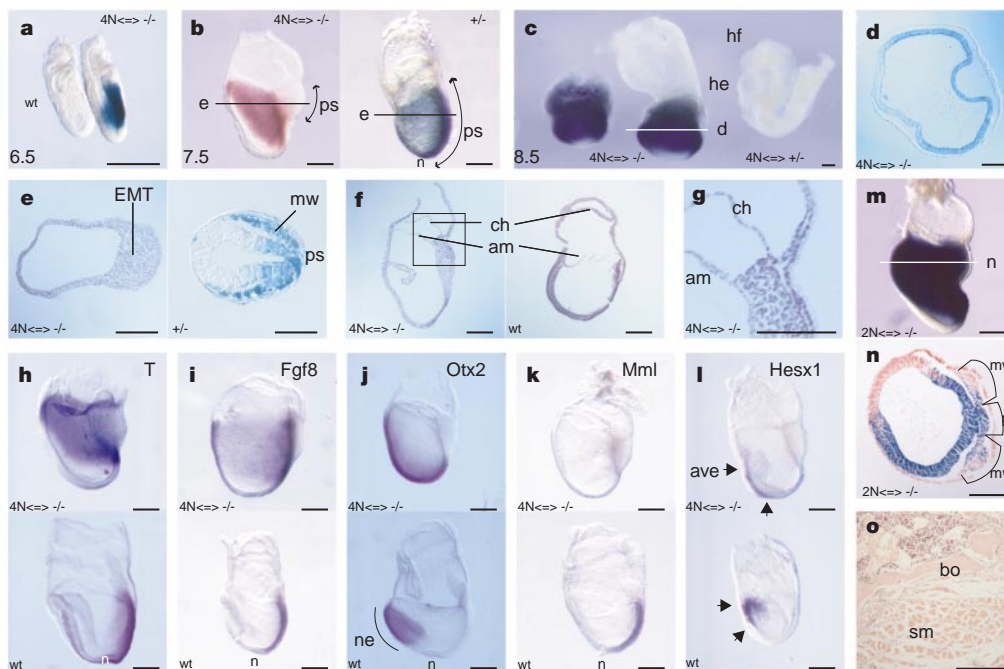


Figure 4 Analysis of chimaeric embryos. **a–l**, Chimaeras derived from homozygous mutant ES cells (**a, b, e–l**) or ICMs (**c, d**) in tetraploid host embryos. **a**, Chimaera (right) and control at 6.5 d.p.c., *LacZ* stain. **b**, Chimaera (left) and heterozygous control, 7.5 d.p.c. The mutant primitive streak is not elongated, *Eo^{LacZ}* is expressed in the posterior, lateral and anterior epiblast. **c**, Two chimaeras and control littermate (right) at 8.5 d.p.c., *LacZ* stain. hf, headfolds; he, heart. **d, e**, Transverse sections as indicated in (**c, b**). Chimaeras lack mesoderm and posterior, lateral and anterior epiblast express *Eo^{LacZ}* (**d**). The control (**e**, right) expresses *Eo^{LacZ}* in the primitive streak and mesoderm wings. **f**, Longitudinal sections, 7.5 d.p.c., HE stain. Posterior thickening of the epiblast and irregular anterior ectodermal fold in the chimaera (left). Amnion (am) and chorion (ch) consist only of ectodermal cell layers, extra-embryonic mesoderm is absent. **g**, Higher magnification of area boxed in **f**. **h–l**, Analysis of marker gene expression by *in situ* hybridization in chimaeras (top) and controls (bottom). **h, i**, *T* (*Brachyury*) (**h**) and *Fgf8* (**i**)

are expressed in the same extended posterior–lateral domain as *Eo^{LacZ}*. Controls show normal expression in the primitive streak. **j**, *Otx2* is repressed in the posterior–lateral epiblast. Expression is confined to neuroectoderm (ne) in control. **k**, *Mml* expression is not detected in the mutant epiblast. **l**, In the chimaera, *Hesx1* expression is detected in the anterior visceral endoderm, but not in the epiblast. **m**, Chimaera with high contribution of mutant ES cells in a diploid host at 7.5 d.p.c., *LacZ* stain. **n**, Transverse section as indicated in **m**. Mesoderm wings are predominantly composed of wild-type cells, whereas mutant cells expressing *Eo^{LacZ}* persist in the embryonic ectoderm. *X-gal*/Safranin stain. **o**, Teratomas derived from homozygous mutant ES cells show extensive differentiation into mesodermal cell types. HE stain. sm, striated muscle; bo, bone; ps, primitive streak; EMT, epithelial-to-mesenchymal transition; n, node; mw, mesoderm wings. All embryos are shown in lateral view, anterior facing to the left. Scalebars, 200 μ m.

trophoblast differentiation and gastrulation might be the control of migratory and invasive properties. □

Methods

Mutant ES cells and mice

Complementary DNAs and genomic clones were isolated as described²⁸. In the targeting vector, parts of exon 2 and intron 2 were replaced with an IRES-*LacZ*-MCneo cassette (Fig. 2b). Germline transmission and homozygous mutant ES cell lines were obtained from two independent ES cell clones. Preimplantation embryos and blastocyst outgrowths were genotyped by multiplex polymerase chain reaction. Embryo culture, *LacZ* staining, immunofluorescence and *in situ* hybridization were performed according to standard protocols²⁹.

Generation of chimaeric embryos and teratomas

Tetraploid host embryos were generated by electrofusion²⁹. Inner cell masses were isolated by immunosurgery from blastocysts derived from heterozygote intercrosses and injected into tetraploid host blastocysts. Homozygous mutant ES cells were aggregated with tetraploid host morulae. Similar results were obtained with homozygous ES cell lines and ICMS. Teratomas were generated by subcutaneous injection of 2×10^6 heterozygous or homozygous mutant ES cells into syngenic hosts and dissected for histology after 6–8 weeks.

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p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours

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p73 (ref. 1) has high homology with the tumour suppressor p53 (refs 2–4), as well as with p63, a gene implicated in the maintenance of epithelial stem cells^{5–7}. Despite the localization of the p73 gene to chromosome 1p36.3, a region of frequent aberration in a wide range of human cancers¹, and the ability of p73 to transactivate p53 target genes¹, it is unclear whether p73 functions as a tumour suppressor. Here we show that mice functionally deficient for all p73 isoforms exhibit profound defects, including hippocampal dysgenesis, hydrocephalus, chronic infections and inflammation, as well as abnormalities in pheromone sensory pathways. In contrast to p53-deficient mice, however, those lacking p73 show no increased susceptibility to spontaneous tumorigenesis. We report the mechanistic basis of the hippocampal dysgenesis and the loss of pheromone responses, and show that new, potentially dominant-negative, p73 variants are the predominant expression products of this gene in developing and adult tissues. Our data suggest that there is a marked divergence in

the physiological functions of the p53 family members, and reveal unique roles for p73 in neurogenesis, sensory pathways and homeostatic control.

Our analysis of murine p73 complementary DNAs revealed transcripts encoding p73 proteins that lack an amino-terminal transactivation domain (ΔN-p73), in addition to those that show the carboxy-terminal diversity reported for human p73 (Fig. 1a)^{1,8}. Like the transcripts encoding ΔN-p63 (ref. 5), the ΔN-p73 messages are derived from an alternative promoter located in intron 3. ΔN-p73 failed to activate transcription from a p53-reporter gene (data not shown) but suppressed the transactivation activity of p73α by hetero-oligomerization and competition for DNA binding (Fig. 1b, c). *In situ* hybridization experiments on mouse embryos failed to detect p73β, γ or δ transcripts, whereas p73α messenger RNA was highly expressed in a number of epithelial and neural structures, including nasal epithelium, the vomeronasal organ, the hippocampus and the hypothalamus (Fig. 1d; and data not shown). Additional probes revealed that ΔN-p73 transcripts appeared to be the predominant p73 gene product in adults (data not shown) and during embryogenesis (Fig. 1e).

Mice with disrupted p73 alleles were produced using homologous recombination in embryonic stem (ES) cells to replace exons 5 and 6, which encode the DNA-binding domain, with the neomycin-resistance (NEO^R) gene^{9,10} (Fig. 1f). The offspring of p73 heterozygous mice showed a mendelian distribution of genotypes indicating that p73, like p53, is not required for embryogenesis^{11,12} (Fig. 1g). Polymerase chain reaction with reverse transcriptase (RT-PCR) and sequence analysis of RNA from murine embryonic fibroblasts (MEFs) of known p73 genotypes confirmed the disruption of the p73 gene (Fig. 1g).

p73^{-/-} pups showed a runting phenotype and high rates of mortality (Fig. 2a, b). Most commonly, death followed massive gastrointestinal haemorrhages, although intracranial bleeding was apparent in ~15% of the mortalities. Histological examinations of the gastrointestinal tract of the p73^{-/-} postnatal day 7 (P7) mice revealed numerous abnormalities including erosion marked by a loss of enterocytes and excessive mucosecretions in the duodenum, ileum and cecum (Fig. 2c; and data not shown), which may underlie the wasting syndrome and the intestinal haemorrhaging in these mice.

A striking feature of the p73^{-/-} mice was severe rhinitis and purulent otitis media (Fig. 2d; and data not shown). Massive neutrophil infiltrates of these sites were obvious at the earliest ages examined (P2 pups) and persisted through adulthood, when greater than 80% of p73^{-/-} mice exhibited chronic, bilateral rhinitis, otitis, periorbital oedema and conjunctivitis. Microbiological analysis of affected sites from p73^{-/-} weanlings (P21) revealed the presence of *Escherichia coli*, *Pasteurella aerogenes* and micrococcal species. Despite these indications of inflammation and infection, no obvious deficiencies in lymphoid or granulocyte populations were detected in the p73^{-/-} mice, indicating that there might be defects in other components of the natural immune system. In support of this notion, p73 was highly expressed in the epithelia bordering the sites of infections in P21 mice (Fig. 2e). These epithelia function, in part, as barriers to external pathogens through secretion of mucus, cytokines and antimicrobial factors¹³. Despite the massive sinus inflammation marked by mucoserous secretions, neutrophil infiltration and goblet cell hyperplasia of the surrounding epithelia in the P2 pups (data not shown), these pups also lacked obvious Gram-staining pathogens. Together, these data indicate that the infections seen in the older pups may be, as with cystic fibrosis^{14,15}, the result of inappropriate or hyperactive epithelial responses, which in turn render these sinuses habitable by microorganisms. These observations raise the possibilities that the infections in the p73^{-/-} mice are secondary to constitutive inflammatory signals promoted by the loss of p73, and may be linked to the gastrointestinal phenotype as a generalized pan-mucositis.