ARTICLE

Defective chromosome segregation, microtubule bundling and nuclear bridging in inner centromere protein gene (Incenp)-disrupted mice


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INTRODUCTION

The centromere is a highly specialized chromosomal structure that is functionally conserved amongst eukaryotes and is essential for accurate meiotic and mitotic segregation of chromosomes. In mammalian cells, two broad groups of centromere-interacting proteins have been described: constitutively binding centromere proteins and passenger or transiently interacting proteins (reviewed in ref. 1). The constitutive proteins include: centromere protein A (CENP-A), which is a histone H3-like structural protein that may function to distinguish centromeric nucleosomes from other chromosomal nucleosomes (2,3); centromere protein B (CENP-B), which interacts directly with α-satellite DNA (4,5); and centromere protein C (CENP-C), which is a component of the inner kinetochore plate that shares homology with the yeast chromosome segregation protein Mif2 (6,7) and appears to be an important and integral structural component of the kinetochore (8).

The term ‘passenger proteins’ encompasses a broad collection of proteins that localize to the centromere during specific stages of the cell cycle (9). This association may relate to a direct role of the proteins at the centromere or, alternatively, it may simply reflect the role of the centromere as a delivery organelle from which the passenger proteins are distributed to the various final cellular sites of action. Some examples of passenger proteins are: CENP-E, MCAK, Kid and cytoplasmic dynein, which are intimately involved in kinetochore motor function (10–13); CENP-F/mitosin, which associates transiently with the kinetochore, playing an apparent role in kinetochore maturation and signalling pathways for cell division (14–16); and CLIPs, which localize to the inner surface of sister chromatids between kinetochores and are implicated in sister chromatid pairing (17).

The inner centromere proteins (INCENPs) were the founder members of the passenger group of proteins (18). These proteins display a broad localization along chromosomes in the early stages of mitosis but gradually become concentrated at centromeres as the cell cycle progresses into mid metaphase (19). During the metaphase–anaphase transition, INCENPs remain confined to the metaphase plate, associating with stem body material which coats the overlapping antiparallel microtubules of the central spindle, while sister chromatids migrate to the poles. In mid anaphase, a portion of the INCENPs also appears at the cell cortex where the cleavage furrow will later form. During telophase, the proteins are located within the...
midbody in the intercellular bridge, where they are discarded after cytokinesis (18,19).

The INCENP proteins were originally identified in chicken cells as a doublet consisting of a shorter 96 kDa INCENP and a 101 kDa INCENP polypeptide containing a 38 amino acid insertion that arises through differential splicing of a single primary RNA transcript (20,21). Homologs of INCENP have been isolated in Xenopus (22) and mouse (23,24). Analysis of these and the chicken INCENPs (20,21) have revealed complex protein structures containing multiple putative targets for Cdc2 kinase, MAP kinase, N-glycosylation, nuclear localization signals and numerous other potential phosphorylation sites. A chicken INCENP cDNA, when expressed in mammalian cells, shows an identical cell cycle distribution to the endogenous protein, suggesting conservation of functional domains between interclass species (21). More recently, overexpression of a truncation mutant of chicken INCENP and a chimeric CENP-B:INCENP protein in mammalian cell culture resulted in dominant-negative characteristics, suggestive of interference with both prometaphase chromosome alignment and completion of cytokinesis (25,26). In the present study, we have investigated the biological importance and functional role of Incenp in mouse. Using homologous recombination, we have specifically disrupted the Incenp gene. We describe here the phenotype of such a gene disruption, which provides the first reported knock-out of a mammalian chromosomal passenger protein.

RESULTS

Generation of Incenp null embryonic stem (ES) cells and mice

The genomic copy number of the mouse Incenp gene was determined previously using Southern blot analysis and mouse Incenp probes (24). The results indicated a single copy gene with no closely related homologs. Further analysis of Incenp mRNA, either by RT–PCR across the differentially spliced region observed in chicken INCENP<sub>1</sub> and INCENP<sub>2</sub> or by northern blot analysis of total RNA from ES cells and a wide range of mouse tissues, has failed to detect alternative forms of Incenp mRNA (24). These results suggested that heterozygous gene knockout could be achieved in a single homologous recombination event with our construct. In addition, in separate experiments, we have studied the chromosomal localization of the mouse Incenp protein in 129/1 mouse ES cells by immunocytochemistry using a well-characterized anti-chicken INCENP antibody provided by Dr W.C. Earnshaw (Institute of Cell and Molecular Biology, University of Edinburgh, UK) and obtained a pattern similar to that described previously for chicken cells (data not shown).

For gene targeting, a promoterless IRES/neo<sup>R</sup> element was incorporated into the targeting vector to enhance both targeting frequency (by selecting for homologous recombination downstream of the endogenous Incenp promoter) and translation of the neomycin gene product (via the internal ribosomal entry site) (27). The construct was further designed to disrupt Incenp mRNA translation at amino acid 47, which effectively removed critical downstream regions containing nuclear localizing signals and the chromosome- and microtubule-binding domains (Fig. 1a) (26). That the 47 amino acid N-terminus truncated peptide neither elicited a dominant-negative effect nor was directly functional was clearly demonstrated by the observed phenotypes of the heterozygous and homozygous mice generated (see below).

Upon transfection of the linearized construct, 163 G418-resistant 129/1 ES colonies were analysed. Of these, six demonstrated the correct targeting event (Fig. 1b), corresponding to a frequency of 3.7%. When the construct was electroporated into W9.5 ES cells, 87 G418-resistant colonies were screened, of which four were correctly targeted, yielding a frequency of 4.6%. These targeting frequencies were low compared with the 70–86% seen at the Oct4 locus (27) and 74% achieved for Cenpc gene disruption (28) using a similar strategy. However, the frequencies compared favourably with those obtained with the Cenpb (29) and cytokine DIA/LIF loci (27). The lower frequency in this case may be due to a lower rate of transcription at the Incenp locus, lower accessibility of the chromatin structure at this locus to homologous recombination mechanisms and/or omission of a 600 bp stretch of homology in our targeting vector.

The heterozygous cell lines exhibited normal morphology and growth rates. Injection of these cell lines into C57 BL/6 blastocysts resulted in two germline chimeras originating from the 129/1-derived ES cells. On mating the chimeras with C57 BL/6 mice, heterozygous mice were obtained. Intercrossing these heterozygotes produced 102 phenotypically indistinguishable progeny, of which 73 were found to be heterozygotes and 29 were wild-type (Fig. 1c). The absence of −/− animals indicated embryonic lethality. This was further indicated by the smaller litter size (7.5 ± 1) observed for the +/−/+− crosses compared with those for +/+ × +/+ (9.6 ± 2.4) and +/+ × −− (9.5 ± 1.7).

Embryonic lethality occurs before day 8.5

Females from −/− × −/− crosses were killed at day 8.5 post-virginal plug formation and embryos were removed from the uterine implantation sites for genotyping. A total of 23 embryos were obtained, 16 of which were −/− and seven were +/+. This result suggested that lethality of −/− embryos occurred earlier than 8.5 days.

We further investigated the ability of −/− embryos to develop under in vitro conditions. Embryos were harvested from the uteri of mothers of +/− × −/− crosses at day 3.5 and placed individually into tissue culture wells. These were observed for 6 days, after which they were harvested for PCR genotyping. Of 35 samples monitored, 31 hatched from the zona pellucida, attached to the culture wells, established two distinct cell types after 3 days of culture (Fig. 2a) and remained healthy throughout the study. Genotyping of these 31 hatched embryos indicated eight to be +/+ and 23 to be −/−. Of the four remaining embryos, three were unable to attach after 3 days of culture; one of these embryos was obviously dead, displaying a dense debris of degraded material, whereas the other two embryos failed to hatch out of the zona pellucida and showed spatial contraction and signs of degeneration within the zona (Fig. 2b). The last embryo (Fig. 2c) had attached after 3 days of culture but was unable to thrive or form the two distinct cell types in comparison with the well-advanced healthy embryos at this stage. These four embryos represented the presumed −/− genotype (see below) and constituted 11% of total embryos. When the experiment was repeated by harvesting embryos from the oviduct as well as the uterus, this frequency increased to 24% (5 of 21), which was closer to the expected...
Figure 1. Mouse Incenp mapping, targeting construct and screening strategies. (a) (i) Mouse Incenp locus showing the first 4 exons and the ATG start codon within exon 2. The approximate amino acid positions of the various functional domains and putative nuclear localization signals (NLS) of the chicken INCENP are shown (20,21,26). The stippled box indicates an internal 600 bp HindIII genomic fragment within intron 2 that has been deleted from the targeting construct. Restriction sites are: A, AccI; K, KspI; E, EcoRI; H, HindIII; X, XhoI. (ii) Gene targeting construct in which exon 3 has been disrupted by insertion of the IRES/neoR cassette at the EcoRI site. This cloning step required the removal of an EcoRI site, shown in parentheses. (iii) Correctly targeted Incenp allele showing the expected AccI fragment sizes for targeted and wild-type alleles detected by the 5' probe. IN1, IN2 and IN3 are PCR primers. (b) Southern screening of cell lines. Wild-type and targeted bands are 5.1 and 8 kb, respectively [refer to a(iii)]. (c) PCR screening of mouse tails. The wild-type (532 bp) and targeted (616 bp) alleles are detected by primers IN1/IN2 and IN2/IN3, respectively. M, 1 kb Plus DNA ladder (Gibco BRL).
value, suggesting that affected embryos displayed an increased residence time in the oviduct. In control +/+ × +/+ or +/– × +/+ crosses, of 44 day 3.5 embryos examined, all attached and developed well in culture, although after 6 days some sign of reduced proliferation was observed in five samples. However, as this occurred at a much later stage and clearly differed morphologically from that of presumed –/– embryos, it was assumed to be due to a culturing effect unrelated to the phenotype observed with the presumed –/– embryos.

Cell morphology of day 3.5 embryos

To further ascertain and correlate the phenotype and genotype of day 3.5 –/– embryos, samples were harvested from the uteri and oviducts of heterozygous females from +/– × +/– crosses, individually photographed and subjected to PCR. Due to the low amounts of DNA in these embryos, a modified PCR strategy (Fig. 3a) involving a random preamplification step of the whole genome was employed to enhance the level of template DNA in a linear unbiased manner (30), followed by allele-specific PCR to determine genotype. From a total of 39 embryos, 22 were +/– (56%), nine were +/+ (23%) and eight were –/– (21%) (Fig. 3b). When these genotypes were correlated with the photographic results, the –/– embryos were noticeably the only ones that exhibited abnormal morphology. These embryos consisted of large and non-uniformly sized cells which had failed to develop into inner cell masses and blastocoel cavities (Fig. 2e) compared with their healthy +/+ and +/– counterparts (Fig. 2d). These results clearly establish the presence of –/– embryos at day 3.5 as well as provide evidence for cellular abnormality at this early developmental stage.

Nuclear and chromosomal morphologies of day 3.5 and day 2.5 embryos

To examine the nuclear and chromosomal morphologies of affected embryos, samples from heterozygous crosses were fixed on slides, stained with Giemsa and analysed. Initial studies concentrated on day 3.5 embryos. At this stage, the normal embryos developed into blastocysts, each containing an average of 40–50 cells (inferred from the number of nuclei stained by Giemsa) that exhibited relatively uniform size and healthy morphology. Interphase nuclei were generally also uniform in size and contained 1–3 nucleoli/nucleus (Fig. 4a). Abnormal looking embryos, presumed to be Incenp-disrupted, contained nuclei that were both smaller in number (averaging seven per embryo) and much bigger in size (Fig. 4b–d). These embryos contained occasional micronuclei (e.g. Fig. 4d), but the majority of nuclei were significantly (5–10 times) larger than normal. These giant nuclei were often irregular and lobular in morphology and individually carried up to 20 nucleoli. The abnormal embryos comprised 14% (4 of 28) of the embryos studied, which was lower than the expected value since only mouse uteri were flushed for these experiments (see above). When the mitotic index (i.e. percentage of total cells in mitosis) of the affected embryos was determined, this was shown to be 20%, which, as a group, was significantly higher than the 3.9% value obtained for the unaffected embryos, suggesting that mitosis was severely delayed or arrested in the affected embryos.

The mitotic chromosomes of the affected embryos also showed a number of distinct abnormalities (Fig. 4b–d). Most of the chromosomes were highly condensed compared with metaphases of control embryos, although some appeared to be undergoing decondensation or deterioration, as evident from their elongated and/or fragmented morphology (Fig. 4c). A dis-
A distinct characteristic of these mitotic chromosomes was their highly disorganized or scattered nature, a feature so predominant that no specific stages of the normal mitosis could be ascribed to most of the affected mitoses. Another outstanding feature was the high prevalence of cells with giant chromosome complements several times that of the normal diploid mouse genome.

The above results indicate that a severe manifestation of Incenp deficiency has occurred in the day 3.5 embryos. To investigate earlier events related to this deficiency, day 2.5 embryos were harvested from heterozygote crosses and analysed. The results clearly indicate expression of the Incenp gene-disrupted phenotype in these earlier embryos. Normal embryos contained an average of 16 uniformly sized nuclei (Fig. 5a). Examination of affected embryos, which comprised 21% (9 of 43) of the total embryos analysed, indicated a smaller number of cells per embryo and the presence of nuclei of varying sizes (Fig. 5b–f), including micronuclei, apparently normal sized nuclei and greatly enlarged (up to 10× normal) nuclei that also contained a drastically increased number of nucleoli (see Fig. 5b for some representative examples of each of these events). An unusual feature of these nuclei was the appearance of ‘nuclear bridges’ in a substantial proportion of the cells (arrows in Fig. 5). Such bridges ranged from a thin band to a much broader region connecting two nuclei, corresponding presumably to different degrees of manifestation of an abnormal process of nuclear reformation. No nuclear bridges were ever observed in the normal embryos, where complete segregation of sister chromatids to the two poles has ensured resolute nuclear reformation around each of the two fully separated sister chromatid complements. As with the day 3.5 embryos, the mitotic chromosomes of day 2.5 embryos were often more condensed, disorganized and present in numbers greater than the usual diploid complement (Fig. 5e).

**Tubulin immunocytochemistry of day 2.5 and day 3.5 embryos**

Figure 6a shows results for the immunostaining of a normal day 2.5 embryo with anti-β-tubulin antibody. The eight cell embryo contained regular midbodies reminiscent of completed mitotic divisions. In contrast, such midbodies were undetected in nine of nine similarly stained day 2.5 morphologically abnormal,
the presence of the endogenous INCENP protein whose effect cannot be unequivocally dissociated from the range of phenotypes observed. Other studies involving microinjection of anti-centromere antibodies into cultured cells (31) suffer similar limitations in that the specificity of antibodies and potential steric effects of the antibody–antigen complex may be difficult to ascertain. To minimize these difficulties, we have employed homologous recombination to create an Incenp gene knockout in a mouse. Our knockout strategy was designed to produce premature termination of the Incenp protein which, because of the removal of all the putative nuclear localization motifs and essential domains for chromosome and microtubule binding (26), should neither be able to enter the nucleus to interact with the chromosomes and centromeres nor evoke any significant Incenp function. The apparently normal phenotype seen in the heterozygous ES cell lines and mice demonstrates directly that the targeted mutation does not exert a dominant-negative effect. We infer that the severe phenotype seen in the homozygous knockouts must arise from null mutations specifically related to the depletion of Incenp proteins.

**Cellular effect of Incenp mutation**

Analysis of the nuclear details of Incenp null embryos enables the likely progression of events underlying the phenotype to be formulated. Incenp deficiency manifests initially in the day 2.5 embryos as a mitotic missegregation problem that results in the lagging of a small number of chromosomes (leading to a few micronuclei), with the bulk of the chromosomes still able to migrate to the poles in anaphase, although aberrant migration will begin to cause an uneven distribution of chromatin at the poles and result in nuclei of varying sizes. During these early events, mitosis proceeds essentially to completion, albeit imperfectly, and results in unambiguous nuclear membrane reformation around each of the two fully separated chromatin masses. Subsequently, cytokinetic furrow formation may be initiated into the extended nuclei or nuclear bridges. Such an attempt becomes increasingly unsuccessful (evident from the giant chromosome complement; discussed below) and leads to large cells with greatly increased nuclear contents.

Two possible explanations may account for a failure in cytokinesis in the Incenp-disrupted embryos: the first implicates an underlying direct biochemical cause, while the second involves a physical barrier that prevents cytokinesis. The first possibility
was suggested by Cook et al. (19), who proposed a direct involvement of INCENP in cytokinesis. These investigators have demonstrated that the protein is one of the earliest known polypeptides to be present in the presumptive cleavage furrow and that, in addition to the stem body matrix, the protein is closely associated with the cytoplasmic face of the plasma membrane within the cleavage furrow (18). Molecular analysis has demonstrated that INCENP associates with the centromere during metaphase and with the central spindle during anaphase, while overexpressed INCENP binds cytoplasmic microtubules (21). Overexpression of an INCENP truncation mutant that targets to centromeres but lacks the microtubule association region and other C-terminal elements interferes with both prometaphase chromosome alignment and the completion of cytokinesis (26). In addition, cells expressing an artificial chimeric protein in which a truncated INCENP containing the spindle midzone targeting and microtubule-binding domains is tethered to the centromere through a fusion with the centromere-binding motif of the centromere protein CENP-B results in a block in cytokinesis (25). Interestingly, cells expressing such a chimeric protein show no evidence of prometaphase disruption or lagging chromosomes.

In contrast to these studies, our data, unencumbered by the persistence of endogenous normal INCENP or any side-effects that overexpression of mutant or artificial heterologous proteins may have, point to an over-riding primary role of Incenp in proper chromosome segregation. We have demonstrated that deficiency of this protein leads promptly to segregation errors that manifest initially in chromosome lagging (micronuclei formation) but rapidly deteriorates into a catastrophic breakdown in the polar movement of chromatin. This leads to the tethering or stalling of chromatin at the spindle midzone, followed by untimely reformation of an extended nuclear membrane around the full complement of non-separated or poorly separated chromatin, resulting in enlarged nuclei. We propose that these enlarged nuclei and the excessive chromatin material that accumulate across the presumptive cleavage furrow create a physical barrier preventing cytokinesis from proceeding to completion. Using a careful light and electron microscopy study, Mullins and Bieseke (32) have reported that where lagging chromatin is trapped in the midbody in the intercellular bridge, cytokinesis proceeds normally until it encounters the chromatin-containing midbody, at which point cytokinesis fails, followed by regres-
sion of the furrow and the persistence of the midbody. Our observation of a high prevalence of nuclear bridges is consistent with failed and regressed cytokinesis. Furthermore, it appears likely that in our Incenp-disrupted cells, a combination of the vast amount of chromatin tethering at the spindle midzone and the reformation of a highly unusual intercellular nuclear structure will constitute a significantly greater barrier to block cytokinesis than that described by Mullins and Biesele (32).

Comparison with Cenpc knockout

It is of interest to compare and contrast the phenotype observed for the Incenp knockout with that previously described for the embryonically lethal Cenpc knockout (28). This comparison highlights a more severe phenotype for the Incenp gene knockout, since mitotic problems are already well advanced by day 2.5, while similar staged Cenpc knockout embryos still appear relatively normal. Such a phenotypic difference may in part be due to the fact that in addition to the maternal mRNA pool provided by the egg cytoplasm, both the maternal and paternal kinetochores are also likely to contribute to the recycling of the constitutive Cenpc protein through the different cell division cycles. In contrast, the chromosomal passenger Incenp protein is known to be discarded in the cell midbody at the end of cytokinesis during each division cycle (19) and would therefore be rapidly depleted from either the maternal and paternal kinetochores as well as during successive embryonic cell divisions.

In both Incenp and Cenpc gene disruptions, early mitotic problems result in the formation of micronuclei and provide evidence for errors in anaphase chromosomal segregation in which missegregated or lagging chromosomes become encap-
sulated during the nuclear reformation step in telophase, prior to cytokinesis. The increased mitotic indices in the day 3.5 embryos of Incenp and Cenpc gene disruptions compared with those of normal embryos indicate that mitotic progression has been delayed or arrested. Again, the delay/arrest phenotype is significantly more severe for Incenp deficiency (mitotic index 20% compared with 3.9% in unaffected embryos) than for a defect in Cenpc (mitotic index 6.9% compared with 3.6% in normal embryos; 28). Mitotic delay or arrest can result from a metaphase checkpoint that serves to delay anaphase onset by monitoring a phosphorylation-dependent signal-generating mechanism at the kinetochore for proper bipolar microtubule attachment and metaphase congression (33). The observed higher mitotic index suggests that Incenp-disrupted cells may have a greater difficulty in overcoming this checkpoint control compared with the Cenpc mutants and/or that additional mitotic blocks may be in place. Whilst we cannot rule out the possibility of an extended checkpoint arrest in our Incenp-disrupted cells, the observed appearance of a high prevalence of cells with large chromosome complements, giant nuclei and highly abnormal nuclear bridges that are not seen in the Cenpc knockouts provides a compelling suggestion that additional mitotic blocks may operate (see below). It is also interesting that a noticeably lower prevalence of micronuclei is seen in the Incenp-disrupted cells compared with the Cenpc-disrupted cells, suggesting that missegregation that results in the lagging of only a small number of chromosomes occurs less frequently in the Incenp knockout cells. In addition, the Incenp-disrupted embryos show relatively more micronuclei at day 2.5 compared with day 3.5, with the day 3.5 embryos giving a disproportionately higher representation of macronuclei and cells with larger than normal chromosome complements.

Implications for Incenp function

It is useful to speculate on the mechanisms responsible for the observed segregation failure in the Incenp-disrupted mice. Early manifestation of the mutation in the form of chromosome lagging can result from a possible defect in the kinetochore complex or spindle integrity and function. The former will likely be the case for the Cenpc-disrupted mice, since this protein is a constitutive component of the kinetochore, a defect in which is expected to lead to defective microtubule capture or binding, causing improper chromosomal segregation, chromosome lagging and micronuclei formation. The observed distinct differences in the subsequent manifestation of the Incenp knockout, on the other hand, suggest that an Incenp defect could specifically affect microtubule dynamics rather than the centromere itself. This suggestion is consistent with the absence of spindle midbody structures and, in particular, the highly aberrant bundling of spindle fibres into gigantic spindle ‘cords’ in a significant proportion of the severely affected embryos (Fig. 7c and d). Previous studies have also demonstrated the spindle microtubule-binding property of this protein and the relocation of the protein from the centromere onto microtubules at the metaphase plate where spindle fibres overlap during late metaphase/early anaphase (18,19). A defect in microtubule function would not only have an immediate effect on prometaphase movements as proposed previously (26), but could lead to impaired chromatid separation and polar migration of chromosomes during anaphase A. Such a defect could have an even more severe effect on the subsequent step of spindle elongation in anaphase B, during which the distance between the poles is increased. Finally, in addition to our proposed primary role of Incenp in modulating spindle dynamics and chromosomal segregation, the possibility that the protein may have a further downstream biochemical effect on cytokinesis as suggested by earlier dominant-negative mutant studies cannot be ruled out. Alternatively, the observed final relocation of INCENP to the presumptive cleavage furrow structures and the midbody may not reflect any active functional role of INCENP in cytokinesis but rather simply reflect a mode of delivery to a destination for destruction or clearance at the end of each cell cycle. Further studies using our knockout system and other approaches should shed more light on these possibilities.

MATERIALS AND METHODS

Restriction mapping of mouse Incenp and construction of targeting vector

A chicken INCENP cDNA sequence (GenBank accession no. Z25420) was used to identify a mouse INCENP cDNA clone (GenBank accession no. AA014535) (23). A fragment from this clone was used to screen a 129/Sv mouse genomic phage library from which a 14 kb clone designated PIN06 was isolated. Mapping revealed that the clone contained four exons corresponding to the mouse cDNA sequence with the start site for protein translation located within exon 2 [Fig. 1a(i)]. For the construction of a gene targeting vector, a 2.5 kb Ksp–HindIII fragment containing a portion of exon 1 was cloned into Bluescript II KS (+). A 3.8 kb HindIII–Xho fragment containing exons 2–4 was further cloned into the HindIII site of this construct to form a genomic contig of 6.3 kb. An internal 600 bp HindIII fragment within intron 2 was deleted from the construct to facilitate subsequent screening. An IRES/neo element (obtained from pGT1.8Zin; a gift of Peter Mountford, Monash Medical Centre, Melbourne, Australia (27)) was cloned into the EcoRI site present within exon 3 [Fig. 1a(ii)] at a site that was expected to cause a premature termination of Incenp protein synthesis at amino acid 47 following replacement of the wild-type allele.

Generation of targeted ES cells and mice

The targeting vector was linearized at the 5’ end by restriction digestion with KspI. The mouse ES cell lines 129/1 and W9.5 were used to generate homologous recombination events. Approximately 10⁶ cells were electroporated with 50 μg of linearized construct in each transfection experiment using a single pulse from a Bio-Rad (Hercules, CA) Gene Pulse at 0.8 kV, 3 μFD, 20 μF. Cells were plated onto mitomycin C-inactivated STO-neo® feeder cells (34) in the presence of 10³ U/ml LIF (AMRAD-Pharmacia, Melbourne, Australia) and selected 24 h later in G418 (Gibco BRL, Gaithersburg, MD) at an active concentration of 300 or 250 μg/ml for 129/1 and W9.5 cells, respectively. Neo® colonies were grown for 5–8 days before genomic DNA was extracted and digested with AccI. A 1.1 kb EcoRI–KspI genomic DNA fragment 5’ of the targeting region [Fig. 1a(i)] was used as a probe in Southern blot hybridization to identify correct targeting events [Fig. 1a(ii)].

For chimeric mouse production, W9.5- and 129/1-derived targeted cell lines were microinjected into day 3.5 host...
C57BL/6 blastocysts and transferred into pseudopregnant mice following standard methods. Chimeric mice were identified by their coat color and were crossed with C57BL/6 mice to test for germline transmission by heterozygote production. Heterozygous mice were intercrossed to produce homozygous Incp- disruptions.

Genotyping of mouse tail DNA

Mouse tail biopsies were taken from 3-week-old animals and lysed overnight in lysis buffer containing 100 mM NaCl, 50 mM Tris, pH 7.5, 10 mM EDTA, 0.5% SDS and 0.2 mg/ml proteinase K. Insoluble materials such as hair and bone were pelleted and the supernatant subjected to one phenol extraction and one chloroform extraction before the DNA was ethanol precipitated and resuspended in TE buffer. An aliquot of 1 µl of this DNA solution was used in a semiduplex PCR strategy as presented in Figure 1a (i and iii), using 1 µM each of the following three primers: IN1 (5'-CTCTGGACCTTGTCTGCAATGT), IN2 (5'-TGTATTACACCGCGCTTCTTTC) and IN3 (5'-CTCTCGCTGTTCATCAGGTATC). Primers IN1 and IN2 gave an expected product of 532 bp for the wild-type allele, while IN2 and IN3 gave an expected 616 bp product for the targeted allele. PCR was performed using Perkin Elmer (Foster City, CA) PCR buffer, 0.05 U/µl Perkin Elmer Taq polymerase, 200 µM dNTPs (Boehringer Mannheim, Mannheim, Germany), 2 mM MgCl2, using 30 cycles at 94°C for 1 min, 58°C for 2 min and 72°C for 2 min.

Genotyping of day 8.5 embryos

Embryos from heterozygote matings were dissected out of their uterine implantation sites into phosphate-buffered saline (PBS) at day 8.5 of gestation. The embryos were rinsed in M2 medium (Sigma, St Louis, MO) and three changes of PBS, incubated in mouse tail lysis buffer at 55°C for 4 h and extracted for DNA as described above. Glycogen was used as an inert carrier in ethanol precipitation to maximize DNA recovery. The DNA was resuspended in 20 µl of TE and 5 µl of this solution was used in the semiduplex PCR strategy described for tail DNA.

Genotyping of day 3.5 and cultured embryos

Heterozygous mouse breeding pairs were examined daily for vaginal plugs. The day of plug formation was defined as day 0.5 of embryonic development. At 3.5 days, female mice were killed and their uterus and oviducts were dissected and flushed with M2 medium. A subset of the embryos was individually placed in thin-walled 0.2 ml PCR tubes (Perkin Elmer) and 5 µl of TE was added. Lysis and whole genome preamplification was carried out in the same reaction tube.

Targeted allele-specific PCR was performed according to the strategy presented in Figure 3a, using 2 µl of preamplification product and the primer pair neo1 (5'-GAGGATCTCTCCTGCTATCTC) and neo2 (5'-GATCATCTGTATCGACAA-GACC), which gave a product of 175 bp. The PCR conditions were 94°C for 1 min, 59°C for 2 min and 72°C for 2 min for 30 cycles. Concentrations of reagents were the same as in the strategy in Figure 1a, except the final concentration of MgCl2 was 1.5 mM. Presence of the wild-type allele was investigated using the primer pair 1n8 (5'-GCTGATTCACA-GAGCTTGG, derived from intron 2 sequence) and 1n9 (5'-CATCAGCCTGCTGCTATTGC, derived from exon 3 at nucleotide position 260 of the mouse cDNA (GenBank accession no. AA014553)), which gave a 168 bp product. PCR was performed as described for the neo1/neo2 primer pair except that MgCl2 was present at 2 mM, annealing was at 60°C and 35 cycles were performed.

Embryo morphology studies

Embryos were harvested at day 2.5 or 3.5 as described above, photographed, washed in 0.9% trisodium citrate and transferred to fresh trisodium citrate. After incubation for at least 4 min, embryos were transferred by micropipettes onto clean slides in a minimal volume. Embryos were fixed and spread onto the slides using three floods of methanol:acetic acid (3:1) fixative. Slides were stained in Giemsa solution for 15 min and rinsed for 1 min in PBS. After drying, slides were mounted in DEPEX (Crown Scientific, Melbourne, Australia) and viewed under ×400 magnification under a standard light microscope.

Immunocytochemistry of embryos

Embryos were harvested at day 2.5, rinsed in M2 medium and the zona pellucida was removed using acid Tyrode’s solution. Embryos were rinsed again and transferred to ~10 µl of M2 medium in the wells of a Terasaki dish, after which the embryos were transferred to wells containing microtubule stabilizing buffer (buffer M) comprising 25% glycerol, 50 mM imidazole HCl, pH 6.8, 50 mM KCl, 0.5 mM MgCl2, 0.1 mM EDTA, 1 mM EGTA, 1 mM β-mercaptoethanol, 1% Triton X-100 and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) (35) for 10 min. They were then removed in a minimal volume of buffer M and placed onto polylysine-coated slides. Attachment of each embryo to a slide was facilitated by gently sweeping the micropipette into the solution and across the surface of the slide to reduce the volume of liquid around the embryo. Embryos were overlaid with a modified buffer M (containing no β-mercaptoethanol, Triton X-100 or PMSF) and were fixed for 10 min at room temperature by the gentle addition of ice-cold methanol. Slides were rinsed in PBS containing 0.1%
Triton X-100 (PBS-TX) and blocked using PBS-TX containing 3 mg/ml BSA (PBS-TX-BSA) for 90 min at 37°C. Mouse monoclonal anti-β-tubulin (Boehringer Mannheim), diluted 1:25 in PBS containing 3 mg/ml BSA (PBS-BSA), was applied and slides incubated at 37°C for 90 min. Slides were then washed three times in PBS-TX-BSA. Texas red-conjugated goat anti-mouse IgG (Jackson Laboratories, West Grove, PA) was applied and slides were incubated and washed as described above. DNA was stained by mounting in Vectashield antifade containing 0.2 µg/µl 4,6-diamidino-2-phenylindole. Images were captured by a cooled CCD camera fitted to a Zeiss Axioskop fluorescence microscope using ×63 and ×100 objectives and IPLab software.

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