The *Nf2* tumor suppressor gene product is essential for extraembryonic development immediately prior to gastrulation

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The neurofibromatosis type II (NF2) tumor suppressor encodes a putative cytoskeletal associated protein, the loss of which leads to the development of Schwann cell tumors associated with NF2 in humans. The NF2 protein merlin belongs to the band 4.1 family of proteins that link membrane proteins to the cytoskeleton and are thought to be involved in dynamic cytoskeletal reorganization. Beyond its membership in this family, however, the function of merlin remains poorly understood. In order to analyze the function of merlin during embryogenesis and to develop a system to study merlin function in detail, we have disrupted the mouse *Nf2* gene by homologous recombination in embryonic stem cells. Most embryos homozygous for a mutation at the *Nf2* locus fail between embryonic days 6.5 and 7.0, exhibiting a collapsed extraembryonic region and the absence of organized extraembryonic ectoderm. The embryo proper continues to develop, but fails to initiate gastrulation. These observations are supported by the expression patterns of markers of the extraembryonic lineage and the lack of expression of mesodermal markers in the mutant embryos. Mosaic studies demonstrate that merlin function is not required cell autonomously in mesoderm, and support the proposition that merlin function is essential for the development of extraembryonic structures during early mouse development.

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The study of the molecular changes underlying the development of human cancer has largely focused on perturbations in normal cellular pathways that converge on the nucleus of the cell. By contrast, very little is understood about the interface between the proliferative state of the cell and the cytoskeleton, which must reorganize during the processes of cell division and differentiation, as well as during the transformation and invasion steps of tumorigenesis. In this context, we have focused on the neurofibromatosis type II (NF2) tumor suppressor gene, which encodes a putative cytoskeletal-associated protein. NF2 is a hereditary disorder featuring the development of benign nervous system tumors including schwannomas, meningiomas, and ependymomas (Huson 1994). The hallmark of NF2 is the development of Schwann cell tumors on both eighth cranial (auditory) nerves. Mutations and associated loss of heterozygosity (LOH) have been detected at the *NF2* locus in tumors from NF2 patients and in sporadically occurring tumors

of the same types, indicating that *NF2* behaves as a classic tumor suppressor gene (for review, see Gusella et al. 1996). In addition to the benign tumors that develop in NF2 patients, mutations in the *NF2* gene have been identified in malignant mesotheliomas of the lung (Sekido et al. 1995).

Through positional cloning and LOH studies, the *NF2* gene was cloned and its protein product identified by homology as a member of the band 4.1 family of proteins, some of which have been demonstrated to link transmembrane proteins to cytoskeletal or cytoskeletal-associated proteins (Seizinger et al. 1986, 1987a,b; Rouleau et al. 1993; Trofatter et al. 1993). The NF2 protein is most closely related to a subset of this family that contains three other members to date: ezrin, radixin, and moesin (the ERM family), and has therefore been dubbed *merlin* (moesin, ezrin, radixin like protein). ERM family members are localized to cortical actin structures, particularly in areas undergoing active reorganization such as membrane ruffles, neuronal growth cones, or the cleavage furrow of dividing cells, where they are similarly thought to link membrane proteins to the cytoskel-

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eton (Arpin et al. 1994). Moesin and ezrin have been reported to bind directly to actin (Turunen et al. 1994; Pestonjamas et al. 1995), and all three proteins can bind to CD44, a transmembrane receptor for hyaluronic acid in the extracellular matrix (ECM) (Tsukita et al. 1994). More recently, it has been demonstrated that ezrin function is required for aggregation of the cell adhesion molecule ICAM-2 and formation of cell extensions on target cells that are recognized by natural killer (NK) cells during immune clearance, suggesting a role for ERM proteins in cell adhesion (Helander et al. 1996).

Although nothing is known about any protein interactions involving merlin, initial studies suggest that both exogenous and endogenous merlin can localize to cortical actin structures, particularly membrane ruffles, microvilli, and the cleavage furrow (den Bakker et al. 1995; Gonzalez-Agosti et al. 1996; R.J. Shaw, A.I. McClatchey, and T. Jacks, in prep.). Therefore, merlin represents a novel type of tumor suppressor and provides the opportunity to investigate the mechanism by which a protein thought to be involved in cytoskeletal reorganization also functions to regulate cell growth and suppress tumor formation.

To understand how loss of merlin function contributes to tumorigenesis and the development of NF2 in humans, it is important to gain an understanding of the normal function of merlin in various cellular contexts. It is becoming increasingly apparent that the normal functions of tumor suppressor proteins are often manifest during development, as revealed by gene targeting experiments in mice (for review, see Jacks 1996). The study of a tumor suppressor's function during development provides a framework for understanding how loss of that function in the adult contributes to tumorigenesis. Furthermore, data from gene targeting experiments often contribute valuable information toward understanding specific developmental processes. In an effort to investigate merlin function in detail, we have generated a targeted mutation at the mouse *Nf2* locus. We have determined that a homozygous mutation at the *Nf2* locus leads to early embryonic failure at embryonic day (E)6.5–7.0, with embryos displaying poorly developed extraembryonic structures and a lack of organized extraembryonic ectoderm. These observations are supported by the expression patterns of *pem-1* protein and *L14* lectin mRNA, markers of the extraembryonic lineage, in the mutant embryos. Despite this defect, the embryonic portion of the mutant embryo continues to develop, but the embryo does not gastrulate as evidenced both morphologically and by the lack of expression of the mesodermal markers *brachyury* or *HNF3 β* . In order to investigate this defect further, we developed *Nf2* homozygous mutant embryonic stem (ES) cells that also carry a ubiquitously expressed *lacZ* transgene and generated chimeric embryos that are composed of both wild-type cells and marked merlin-deficient cells. Using this system, we found that *Nf2*-deficient cells can become mesoderm, indicating that merlin is not required cell autonomously for mesoderm formation. Furthermore, chimeras receiving high levels of *Nf2*-deficient contribution to all three

germ layers in the embryo proper gastrulate properly, strongly suggesting that the primary defect in *Nf2*-deficient embryos resides in the extraembryonic lineage.

Results

Targeting strategy

Using a probe corresponding to the entire human *NF2* cDNA, we screened a genomic library constructed from the 129/SvJ strain of mice (Stratagene). One isolated clone contained a 18-kb insert corresponding to the genomic region spanning exons 2–5 of the mouse *Nf2* gene. This clone was used subsequently to construct the targeting vector depicted in Figure 1A. A number of mutations, both in the germ line of NF2 patients and in sporadically occurring schwannomas and meningiomas in humans, have been found in the region of exons 2 and 3, including splice site mutations or deletions that would cause the removal of exon 2 or 3 or both from the *NF2* mRNA (for review, see Gusella et al. 1996). We chose to target this region of the mouse *Nf2* gene, replacing the 3' half of exon 2, all of exon 3 and intervening intronic sequences with the neomycin resistance gene (*neo^r*) oriented in a transcriptional direction opposite to that of *Nf2* (Fig. 1a).

This targeting construct was electroporated into 129/Sv D3 ES cells (Gossler et al. 1986), which were then subjected to both G418 and gancyclovir selection. A total of 231 clones surviving both types of selection were screened by Southern blotting using probes p5' and p3' (Fig. 1a,b). Four separate clones gave the correct pattern of bands with both probes, for a final targeting frequency of 4 of 231, or 1.7%. One of these clones was also found to have a second copy of the targeting sequences inserted elsewhere within the genome, and was not used further. The low targeting frequency at this locus may in part reflect a very high concentration of repetitive elements present throughout this region (see Materials and Methods). Furthermore, we identified an intronic polymorphism between the targeting vector (generated from 129/SvJ genomic DNA) and the 129/Sv D3 ES cells (see Materials and Methods). It has been observed previously that even subtle differences between input and endogenous sequences can dramatically affect targeting frequency, prompting the use of isogenic DNA in constructing targeting vectors (te Riele et al. 1992).

In order to characterize the molecular effects of the targeted mutation, we first determined whether mRNA was transcribed from the mutant allele. Northern blot analysis of total RNA isolated from +/+, +/-, and -/- ES cells (see below for derivation of -/- ES cells) revealed the presence of some *Nf2* mRNA of approximately the normal size (~4.5 kb) in -/- cells, although at lower levels than that seen in +/+ or +/- mutant cells (data not shown). A probe derived from the *neo^r* coding region detected only the 1.2-kb species expressed from the PGK-*neo* cassette (see Fig. 1; data not shown), indicating that a composite *Nf2-neo* mRNA was not generated as has been described for other targeted mutations (e.g., see

Figure 1. The generation of a targeted mutation at the mouse *Nf2* locus. (a) Genomic organization of the mouse *Nf2* locus and design of the targeting construct. Exons 2 and 3 are depicted as blackened boxes. The targeting construct contains the region surrounding exons 2 and 3; 6.5 kb of genomic DNA between a *Pst*I site located in exon 2 and a downstream *Xho*I site is replaced by the *neo^r* cassette. Homologous sequences in the targeting vector are flanked by the HSV-*tk* gene. Probes p5' and p3' and primers a, b, and c are indicated above the genomic maps. The polymorphic *Xba*I site is marked by an asterisk (see Materials and Methods). (b) Southern blot of genomic DNA digested with *Stu*I from a series of candidate ES cell clones and hybridized with probe p3' that detects a 4.3-kb fragment from the wild-type allele and a 3.9-kb fragment from the targeted allele. (x) *Xba*I; (p) *Pst*I; (s) *Stu*I.

Jacks et al. 1994). RT-PCR analysis of mRNA isolated from $-/-$ ES cells using various sets of primers revealed that the entire 3' two-thirds of the *Nf2* mRNA was produced in the mutant cells. Moreover, RT-PCR using primers in exons 1 and 9 produced a product that matched the predicted size of a message that skipped from exons 1 to 5. Cloning and sequencing of this product confirmed that a mutant *Nf2* mRNA was being transcribed and spliced, joining exon 1 to 5 and maintaining the original reading frame (data not shown).

We next determined whether a protein product was generated from this mutant mRNA by isolating protein extracts from $+/+$, $+/-$, and $-/-$ ES cells. Immunoblotting of these extracts using two different polyclonal antibodies directed against the amino terminus of the human NF2 protein (sc331 and N21; Fig. 2A,B) and one against the carboxyl terminus (sc332; not shown) failed to detect any full-length protein product in the $-/-$ cells nor any lower molecular weight species that might correspond to a shortened protein product. We have also failed to detect protein of any size in tumor cell lines derived from *Nf2* heterozygous mice that exhibited LOH at the *Nf2* locus (A.I. McClatchey, I. Saotome, K. Mercer, D. Crowley, R. Bronsen, J.F. Gusella, and T. Jacks, in prep.). Given that exons 2–4 encode amino acids 39–149, and that these antibodies were raised against fragments of merlin corresponding to amino acids 2–21 (sc-331), 1–332 (N21), and 570–587 (sc-332), it is likely that we would have detected a mutant isoform if it were produced. Together

these data suggest that if any protein is translated from this mutant *Nf2* allele, it is unstable in these cell types. Therefore, we believe that this mutation causes full loss of *Nf2* function.

Nf2 is required for early embryogenesis

All three of these properly targeted ES cell clones were injected into C57BL/6J blastocyst stage (E3.5) embryos and implanted into pseudopregnant CD1 females (Bradley 1987). Twenty-one chimeric animals were generated; at least one chimera representing each ES cell clone transmitted the *Nf2* mutation through its germ line to ~50% of its offspring when mated to wild-type C57BL/6J animals (F_1 generation). We have maintained separate lines of mice derived from these three original clones throughout the following experiments and have obtained identical results with all three. When F_1 heterozygotes were intercrossed and the resulting F_2 progeny genotyped at weaning, 16 wild-type, 25 heterozygous, and 0 homozygous mutant animals were identified from the first 10 litters, suggesting that loss of *Nf2* function causes embryonic or early postnatal lethality. We then examined the litters from these heterozygous intercrosses at various times during gestation by removing the embryos from the decidua and evaluating them morphologically, photographing them, and genotyping either the yolk sac or the entire embryo using a PCR-based strategy (Fig. 1; Table 1).

Figure 2. Western blot analysis of cell lysates from wild-type, heterozygous, or homozygous mutant ES cells. (A) Western blot probed with monoclonal antibody N21, directed against the human merlin amino terminus (amino acids 1–332), which also detects ezrin and moesin. Positions of merlin, ezrin, and moesin bands are shown on the *left*, and the positions of molecular weight standards on the *right* (Amersham). (Lane 1) Human fetal fibroblasts; (lane 2) NIH-3T3 cells; (lane 3) +/+ ES cells; (lane 4) +/- ES cells; (lane 5) -/- ES cells. (B) Western blot was probed with polyclonal antibody SC331 (raised against amino acids 2–21; Santa Cruz). (Lanes 1,2) +/+ ES cells; (lane 3) +/- ES cells; (lane 4) -/- ES cells. Merlin is often detected as a doublet because of differential phosphorylation (R. Shaw, A.I. McClatchey, T. Jacks, in prep.). The lower band detected with this antibody is a constant band seen in all mouse cells and controls for the amount of protein extract loaded. The position of a protein molecular weight standard is indicated to the right (GIBCO BRL).

We examined these litters at progressively earlier stages of gestation and were not able to identify homozygous mutant tissue until E8.0–E8.5, when an occasional embryo was identified as a mutant (Table 1). These mutants were nearly all in late stages of resorption and were much smaller than their wild-type or heterozygous mutant littermates. At E7.5 most homozygous mutant embryos were intact, but were clearly smaller than their wild-type and heterozygous mutant littermates and appeared externally not to have initiated gastrulation. Most homozygous mutant embryos were of normal size at E7.0, but were often misshaped and lacked a distinct extraembryonic–embryonic boundary (Fig. 3A,B, arrow). At E6.5, most homozygous mutant embryos were superficially indistinguishable from their wild-type or heterozygous mutant littermates (not shown); however, a few small, grossly abnormal embryos also exhibited a homozygous mutant genotype (see below).

Histological evaluation of Nf2-deficient embryos

Histological sectioning of homozygous mutant embryos in their decidua allowed a more detailed examination of their phenotype and revealed the existence of two distinct classes of mutants (Table 1). At E7.5, wild-type and heterozygous littermates had nearly completed gastrulation; a well-defined mesodermal layer had migrated throughout the embryo between the ectoderm and endoderm as well as into the extraembryonic portion of the embryo (Fig. 3C; for review, see Faust and Magnuson 1993). In contrast, a subset of the embryos were smaller, displaying a disorganized mass of extraembryonic tissue and an expanded egg cylinder composed of discrete ectodermal and endodermal layers with no evidence of mesoderm induction (Fig. 3D,E). There appeared to be an absence of extraembryonic ectoderm in these putative mutants, and the overlying ectoplacental cone was small

Table 1. Genotypes and phenotypes of embryos from Nf2 heterozygous intercrosses

A. Genotypes				
	Number of embryos			
Gestational age	+/+	+/-	-/- ^a	N.D. ^b
E6.5–E7.5	10	20	10	5
E8.5	14	23	5	5
B. Phenotypes (paraffin sections)				
	Number of embryos			
Gestational age	normal	presumptive mutant	presumptive early mutant	Empty deciduum ^c
E6.5	38	5	6	2
E7.5	118	29	13	13

^aEmbryos of the early mutant class are under-represented, as they were too small to dissect free of the surrounding deciduum.

^bEmbryonic tissue too small to genotype accurately.

^cEmpty decidua were classified by the presence of trophoblast giant cells only or by the lack of any discernible embryonic cells.

Figure 3. *Nf2* homozygous mutant embryos. Morphology of E7.25 wild-type (A) and homozygous mutant (B) embryos. Arrow in A depicts embryonic–extraembryonic boundary. (C–E) Sagittal sections through an E7.5 wild-type (C) and *Nf2* homozygous mutant (D,E) embryos exhibiting underdeveloped ectoplacental cones and a lack of organized extraembryonic ectoderm. The embryonic ectoderm in D continued to expand and folded back on itself, whereas the columnar visceral endoderm folded into a series of pleats at the extraembryonic–embryonic juncture probably because of the failed development of underlying extraembryonic structures. The embryo in E is misoriented with respect to the mesometrial–antimesometrial axis of the deciduum (mesometrium is toward the top of the figure in each case), and exhibits variable regions of squamous and columnar visceral endoderm. Note the lack of a distinct mesodermal cell population in either mutant embryo. (F,G) Wild-type E6.5 embryo (F) and *Nf2* mutant E6.5 embryo (G). The proamniotic cavity in G has formed, but neither organized extraembryonic ectoderm nor ectoplacental cavity has formed. The cell death apparent in the embryo in G is not a consistent feature of *Nf2* mutant embryos. Arrows in F and G indicate embryonic–extraembryonic juncture. (ec) Ectoplacental cone; (eed) extraembryonic ectoderm; (ed) embryonic ectoderm; (cve) columnar visceral endoderm; (sve) squamous visceral endoderm; (m) mesometrial; (a) antimesometrial.

and appeared to be collapsed. A striking feature of the mutant embryos is that they were often misoriented within the deciduum (Fig. 3E), which was obvious upon three-dimensional visualization of the embryos from serial sections. This was further emphasized by examination of the visceral endoderm in sagittal sections. By E7.5 the visceral endoderm should form a columnar epithelium surrounding the extraembryonic half of the embryo and a flattened, squamous epithelium surrounding the embryo proper (see Fig. 3C). In sagittal sections of E7.5 *Nf2* mutant embryos, the visceral endoderm often exhibited more than one region of squamous morphology, as if the primary embryonic axis (ultimately the dorsal–ventral axis) was not established properly with respect to the mesometrial–antimesometrial (MA) axis of the uterus

(Smith 1985; Fig. 3E). Similarly, sagittal sections of some mutant embryos displayed visceral endoderm that appeared to retain its columnar morphology around nearly the entire embryo; examination of later sections revealed that the distal pole of the embryo surrounded by squamous visceral endoderm was pointed away from the sagittal plane. Finally, the visceral endoderm was often folded into a series of pleats at the boundary between the embryo proper and the collapsed extraembryonic tissue (Fig. 3D) as if growth of the visceral endoderm continued in the absence of expansion of the underlying extraembryonic ectoderm. HNF4, a marker of columnar visceral endoderm, was expressed appropriately wherever the visceral endoderm was columnar in morphology (Chen et al. 1994; data not shown). Despite the underdeveloped

extraembryonic lineage in the mutant embryos, the embryonic half of the embryo continued to expand, often folding back upon itself, and sometimes displaying a thickening on one side reminiscent of primitive streak formation (Fig. 3D; data not shown). At E6.5, when extraembryonic ectoderm is normally forming, mutant embryos were the same size as their wild-type and heterozygous littermates, but exhibited underdeveloped extraembryonic structures. For example, the homozygous mutant embryo shown in Figures 3G and 5B (below) which was genotyped by in situ hybridization (see below), is distinguishable histologically by the lack of organized extraembryonic ectoderm.

Only 18% of the embryos at E7.5 were represented by this class of mutants, instead of the expected 25%, implying that some mutants were failing at an even earlier stage of development. In addition to virtually empty decidua containing only giant cells (~7%; Table 1), which are found with similar frequency in control litters, we detected a number of decidua containing only a small mass of disorganized embryonic cells surrounded by trophoblast giant cells, suggesting that there might be a second class of homozygous mutant embryos that failed shortly after implantation (Fig. 4D,E). Interestingly, both types of mutants were identified on inbred 129/Sv and highly enriched C57BL/6J (5 backcross generations; generations 3 and 4 were screened for enriched C57BL/6J

contribution by allelotyping) as well as mixed 129/Sv × C57BL/6J backgrounds, suggesting that this variation may be attributable to stochastic differences and not genetic modification. In situ hybridization using a probe corresponding to the deleted *Nf2* exons confirmed that these were indeed *Nf2*-deficient embryos (see below; Fig. 4D–F).

Nf2 expression during early mouse development

We performed in situ hybridization to determine the pattern of *Nf2* expression in early stage mouse embryos and to confirm the genotypes of putative homozygous mutant embryos. In situ hybridization using an in vitro transcribed probe corresponding to exons 2–4, which are not expressed from the mutant allele, confirmed the homozygous mutant genotype of both classes of mutants (not shown; Fig. 4D–F). Both this probe and one corresponding to a much larger portion of the mouse *Nf2* mRNA were used to examine the pattern of *Nf2* expression in wild-type embryos at E6.5 and E7.5. *Nf2* expression was found throughout the embryonic ectoderm, mesoderm, visceral endoderm, extraembryonic ectoderm, and ectoplacental cone (Fig. 4A–C). Much lower levels of expression were detected in parietal endoderm and trophoblast giant cells (Fig. 4B). Interestingly, *Nf2* expression was low throughout most of the maternally derived

Figure 4. *Nf2* mRNA expression in gastrulating embryos. In situ hybridization of E7.5 wild-type (A–C) and early mutant (D–F) embryos using probes corresponding to the 3' half of the mouse *Nf2* mRNA (A,B) and to the deleted exons 2–4 (C,E,F). In wild-type embryos, high levels of *Nf2* expression are detected with both probes throughout the embryo proper, the extraembryonic ectoderm and ectoplacental cone (A–C) and at lower levels in trophoblast giant cells and parietal endoderm (A,B). The arrowhead in A marks the extraembryonic–embryonic boundary. (D–F) Genotyping of the early mutant phenotype as seen at E7.5. The hematoxylin and eosin stained early mutant in D exhibited no hybridization to a probe corresponding to exons 2–4, which are not transcribed from the *Nf2* mutant allele (E,F), although hybridization to the angiogenic region of that deciduum was strongly positive (not shown). (ec) Ectoplacental cone; (eed) extraembryonic ectoderm; (ve) visceral endoderm; (m) mesoderm; (ed) embryonic ectoderm.

Figure 5. Expression pattern of markers of extraembryonic lineages in the *Nf2* mutant embryos. (A,B) Immunohistochemical analysis of pem-1 protein expression in wild-type E6.5 (A) and *Nf2* mutant E6.5 (B) embryos. Note the lack of elongation of mutant embryo in B and the small population of darkly staining cells underneath the ectoplacental cone, which may represent unorganized primitive extraembryonic ectoderm. The embryos in A and B are the same embryos shown in Figs. 3F,G. (C–F) *L14* lectin expression in E7.5 wild-type (C,D) and *Nf2* mutant (E,F) embryos. Panels on right correspond to boxed areas in left panels. Note the extended region of intermediate levels of *L14* expression corresponding to extraembryonic ectoderm in the wild-type embryo (D) in contrast to the abrupt transition between ectoplacental cone and embryonic ectoderm in the mutant (F). (ec) Ectoplacental cone; (eed) extraembryonic ectoderm; (ed) embryonic ectoderm; (ve) visceral endoderm; (m) mesometrial; (a) antimesometrial; (tr) trophoblast.

deciduum, but was very high in the angiogenic region of the deciduum, providing an internal control for genotyping the mutant embryos by in situ hybridization (data not shown).

Expression of markers of the extraembryonic lineage in Nf2 mutant embryos

Upon implantation of the mouse blastocyst at E4.5, the mitotically inactive trophoblast cells surrounding the blastocoel cavity invade the uterine stroma and differentiate into multinucleate trophoblast giant cells (Thieler 1989; Rugh 1990). In contrast, trophoblast cells apposed to the inner cell mass are proliferating; these cells differentiate into the ectoplacental cone, which invades the deciduum in a mesometrial direction, and into the extraembryonic ectoderm, a cuboidal epithelium that forms between the nascent ectoplacental cone and the egg cylinder (Thieler 1989; Rugh 1990). Morphologically, it appeared that little, if any, extraembryonic ectoderm formed in the *Nf2*-deficient embryos (Fig. 3D,E,G). We therefore examined the expression patterns of pem-1 protein and *L14* lectin mRNA, two markers of extraembryonic lineages in the mutant embryos. In wild-type E6.5 embryos, pem-1 protein is expressed at particularly high levels in extraembryonic ectoderm, at lower levels in giant and diploid trophoblast cells, and throughout the visceral endoderm (Fig. 5A; Lin et al. 1994). The pattern of expression is similar at E7.5, although expression in the visceral endoderm is restricted to cells of columnar morphology.

Immunohistochemical analysis of E6.5 *Nf2*-deficient embryos revealed moderate pem-1 staining throughout the visceral endoderm, in trophoblast giant cells surrounding the embryo, and in diploid trophoblasts forming an abnormally small ectoplacental cone (Fig. 5B). At the junction between the embryo proper and the ectoplacental cone, where a layer of well-organized cuboidal extraembryonic ectoderm should be, there were a few disorganized, but darkly staining cells, which may represent a primitive extraembryonic ectoderm, but no evidence of an organized epithelium (Fig. 5B). Pem-1 expres-

sion in mutant embryos at E7.5 was similar, although visceral endoderm expression became restricted to areas of columnar morphology, which were often improperly distributed around the embryo (see above; data not shown).

L14 mRNA is expressed at very high levels in both giant trophoblast cells and in the ectoplacental cone, at intermediate levels in the extraembryonic ectoderm, and

is undetectable in the embryo proper (Poirier et al. 1992; Fig. 5D). The pattern of *L14* expression in *Nf2*-mutant embryos similarly supported a lack of organized extra-embryonic ectoderm: The heavily staining ectoplacental cone was collapsed onto the embryonic ectoderm, which was completely devoid of expression (Fig. 5F).

Despite the malformation of extraembryonic structures, the mutant embryo proper continued to develop, yet failed to form a distinct mesodermal cell layer. Therefore, it was important to determine whether primitive streak formation and mesoderm induction were ever taking place. The transcription factor *brachyury* is a well-established marker of primitive streak formation and mesoderm induction in the gastrulating embryo. *brachyury* expression is first detected in the earliest population of mesodermal cells that is induced and in the immediately adjacent primitive ectoderm (Wilkinson et al. 1990). Therefore, we examined the pattern of *brachyury* expression in *Nf2* mutant embryos and in adjacent normal littermates. In multiple sections of several mutant embryos, we were unable to detect any *brachyury* expression. In a single case, a mutant displayed a small patch of cells of ectodermal morphology that expressed moderate levels of *brachyury* (not shown). This could represent ectopic expression or possibly evidence of early primitive streak formation. This result suggests that mesoderm induction fails to occur in most, if not all *Nf2* mutant embryos; however, it is possible that mesodermal cells are induced but are abnormal and unable to express all mesoderm markers. This notwithstanding, we also failed to detect expression of *HNF3 β* , a marker of later, more anterior mesoderm (Sasaki and Hogan 1993; data not shown).

Generation of *Nf2*^{-/-} *lacZ*⁺ ES cells

The lack of mesoderm induction in *Nf2* mutant embryos could be attributable to a direct or indirect requirement for merlin in the production of the inductive signal itself or to a cell-autonomous defect in the ability of *Nf2*-deficient cells to become mesoderm. To distinguish between these two possibilities, we generated *Nf2* homozygous mutant ES cells that are marked and used them to create chimeric embryos in which cell lineages arising from the merlin-deficient cells could be followed.

By crossing our *Nf2* mutation into the ROSA26 strain of mice, which carries a ubiquitously expressed *lacZ* transgene (Friedrich and Soriano 1991), we generated *Nf2* heterozygous mice that also carried *lacZ*. We bred these mice to *Nf2* heterozygotes and established ES cell lines from individual blastocysts (Robertson 1987; Materials and Methods), 50% of which were expected to be *lacZ*⁺ and 25% to be homozygous for the *Nf2* mutation. Overall, we were able to establish ES cell lines from 21% of isolated blastocysts from a hybrid C57BL/6J:129Sv background. We generated four ES cell lines that were homozygous for the *Nf2* mutation (lines 1076.7, 945.3, 2600E, and 2640D), three of which carried the *lacZ* transgene (1076.7, 2600E, and 2640D).

Nf2 homozygous mutant contribution to gastrulating embryos

The merlin-deficient ES cells were used to examine the consequences of homozygous mutant contribution to various cell types in the gastrulating embryo. Mutant cells from line 1076.7 or 2600E were injected into wild-type C57BL/6J blastocysts, which were implanted into pseudopregnant females and allowed to develop in utero (Bradley 1987). Chimeric embryos were retrieved at E8.5 (allowing one day of delay for embryo manipulation). Embryos were fixed and stained with X-gal to identify cells derived from the *Nf2*^{-/-};*lacZ*⁺ ES cells. After staining, embryos that exhibited areas of blue color (indicative of contribution from the homozygous mutant cells) were photographed and sectioned.

By visual inspection, we were able to identify apparently normal, gastrulating embryos that had received extensive contribution from either homozygous mutant cell line, such as the embryo shown in whole mount in Figure 6A. The top half of the embryo with no mutant contribution represents the extraembryonic portion of the embryo, which is largely committed at the blastocyst stage; ES cells introduced into blastocysts contribute almost exclusively to the embryo proper (Nagy et al. 1990). Blue-staining cells on the left side of the embryo extending upward past the embryonic-extraembryonic boundary (arrow) are mesodermal cells that have migrated to contribute to the formation of the amnion and nascent allantois. A section through a similar chimeric embryo is shown in Figure 6B. Sectioning of E7.5–E8.5 chimeric embryos (>20 with varying degrees of chimerism) revealed that the homozygous mutant cells were able to contribute extensively to all three germ layers including the mesoderm. Figure 6C depicts a chimeric embryo at E8.0 that has successfully completed gastrulation: Blue-staining cells are prominent in the ectoderm, including the neural folds, in the somites, and extensively throughout the mesenchyme. These results indicate that merlin is not required cell autonomously for the formation of mesoderm. Moreover, they demonstrate that embryos can gastrulate normally with extensive contribution of *Nf2* mutant cells to all three embryonic germ layers, consistent with the idea that the primary requirement for merlin in early embryogenesis resides in the extraembryonic tissues.

Discussion

The identification of the product of the *NF2* tumor suppressor gene as a member of a cytoskeletal-associated family of proteins affords the possibility of examining a link between the regulation of cytoskeletal reorganization and tumor development. However, the *NF2* gene was identified by positional cloning, and the family to which its encoded protein belongs is poorly understood. Thus, there is currently little understanding of its mechanism of action as a tumor suppressor. We have used gene targeting in the mouse to investigate the normal function of merlin and to generate tools with which

Figure 6. Features of *Nf2* homozygous mutant chimeric embryos. (A) Typical chimeric embryo undergoing gastrulation with high contribution to the embryo proper. The arrow marks the embryonic-extraembryonic boundary; *Nf2*-deficient (blue-staining) cells representing embryonic mesoderm are migrating into the extraembryonic portion of the embryo to contribute to the formation of the amnion and the allantois. (B) Saggittal section of a similar embryo, depicting *Nf2*-deficient contribution to all three germ layers in the embryo proper. (C) Chimeric embryo at E8.0 that has developed well beyond gastrulation with *Nf2* mutant contribution to the ectodermal, endodermal and mesodermal lineages. (m) Mesoderm/mesenchyme; (en) endoderm; (ec) ectoderm; (nf) neural folds; (s) somite.

to study the molecular pathway(s) in which it operates. Preliminary analysis of mice heterozygous for this mutation indicates that loss of *Nf2* function contributes to tumor formation in the mouse. However, although we have observed a broad spectrum of malignant tumors in these animals, they do not develop the tumor types characteristic of human NF2 at high frequency (A.I. McClatchey, I. Saotome, K. Mercer, D. Crowley, R. Bronsen, J.F. Gusella, and T. Jacks, in prep.).

In the present study, we have examined the developmental consequences of a homozygous mutation at the mouse *Nf2* locus and discovered that *Nf2*-deficient embryos arrest at E6.5–E7.0, associated with abnormally developed extraembryonic structures and failed gastrula-

tion. An essential role for merlin at this early stage of mouse development is surprising given the potential for redundancy among other family members. Interestingly, ERM mRNAs are also expressed during mouse gastrulation: Ezrin is expressed at low levels in both extraembryonic and embryonic ectoderm; moesin is expressed at low levels in all three germ layers of the embryo proper; whereas radixin is expressed at high levels throughout the embryonic and extraembryonic portions of the gastrulating embryo and is particularly abundant in the visceral endoderm (A.I. McClatchey, unpubl.).

Morphologically, most *Nf2*-deficient embryos display poorly developed extraembryonic structures and a lack of organized extraembryonic ectoderm. Extraembryonic ectoderm arises as a cuboidal epithelium from the polar trophoblast and has been proposed to provide a mechanical force elongating the egg cylinder into the former blastocoel cavity (Copp 1976). Given its localization to specialized regions of the actin cytoskeleton at the periphery of the cell, merlin would be expected to act in a cell-autonomous fashion. Merlin function may therefore be required for the organization of the extraembryonic ectoderm into an epithelium. Absence of the appropriate epithelial structures could cause abnormal elongation of the embryo that, in turn, could lead to a failure to establish the proper mesometrial-antimesometrial axis and the observed misorientation of the homozygous mutant embryos. Alternatively, trophoblast invasion of the deciduum in a mesometrial direction may be impaired. Perhaps mesometrial trophoblast invasion allows proper elongation of the extraembryonic ectoderm, which may collapse and fail to fully differentiate without it. In fact, it has been shown that the inhibition of metalloproteinases *in vivo*, which are known to be involved in trophoblast invasion of the deciduum, leads to the development of smaller than normal decidia containing misoriented embryos (Alexander et al. 1996). A defect in the adhesion or invasion of the trophoblast might also account for the early mutant phenotype that we observed. Compensatory adhesion events may allow trophoblast interaction with the uterus at or immediately after implantation in most but not all *Nf2* mutant embryos.

Interestingly, hyaluronic acid (HA) is found throughout the mouse uterus at the time of implantation, but subsequently is cleared dramatically from the antimesometrial side of the deciduum and redistributed to the mesometrial side in the region of trophoblast invasion during ectoplacental cone formation (Brown and Papaioannou 1993; Fenderson et al. 1993). It has been suggested that this clearance is achieved via receptor-mediated endocytosis of HA-receptor complexes and serves to direct the expansion of the trophoblast lineage in a mesometrial direction and thus orient the embryo properly (Fenderson et al. 1993). This is particularly interesting because the major receptor for HA is the transmembrane protein CD44, which binds via its cytoplasmic domain to a region in the amino terminus of ERM proteins that is conserved in merlin (Tsukita et al. 1994). Moreover, a *Drosophila* homolog of merlin has been shown to localize to endocytic vesicles, raising the possibility that mer-

lin plays a roll in that process (McCartney and Fehon 1996). Given that HA-CD44 interactions are thought to be involved in cell migration and proliferation, it is possible that loss of merlin function in the trophoblast impairs these processes through altered HA-CD44 function. Although it has been reported that CD44 expression cannot be detected immunohistochemically in mouse trophoblast (Brown and Pappaionnou 1995; A.I. McClatchey, unpubl.), it is possible nonetheless that a CD44-like protein or very low levels of CD44 are present in that cell type. Whether merlin itself binds to or somehow alters the ability of other ERM proteins to bind CD44 or a CD44-like protein remains to be determined.

The inability of *Nf2*-deficient embryos to produce mesoderm most likely results either directly or indirectly from the impaired development of the extraembryonic lineage. Considerable evidence indicates that a proliferative burst in the egg cylinder is required for mesoderm induction (Power and Tam 1993; Mishina et al. 1995; Hakem et al. 1996). However, proliferation of embryonic cells per se does not appear to be defective in the *Nf2*-deficient embryos. Moreover, *Nf2*-deficient cells can contribute extensively to the embryonic ectoderm in chimeric embryos that gastrulate successfully. Thus, the underlying defect appears unrelated to the control of proliferation in the embryo proper. Instead, the failure to produce proper extraembryonic structures and associated defects in embryonic elongation and orientation may prevent necessary cell-cell contacts required for production of the mesoderm-inducing signal from the embryo proper. Alternatively, the mesoderm-inducing signal may arise from the extraembryonic lineage itself. Indeed, the source of this signal in mouse embryos is not yet known.

In fact, very little is known about the early development and function of the extraembryonic lineage during mammalian development. Interestingly, the *Nf2* homozygous mutant phenotype strongly resembles that of *exed*, an uncloned mutation that maps to the albino deletion locus of mouse chromosome 7. *Exed* mutant embryos fail to produce extraembryonic ectoderm and also fail to gastrulate (Lewis et al. 1976; Niswander et al. 1988, 1989). Therefore it is possible that the product of the *exed* locus functions analogously or in the same pathway as merlin. In addition, homozygosity for an uncloned mutation that maps to the *short ear* locus (*se¹*) leads to embryonic failure at E7.5 with a dramatic hyperproliferation of extraembryonic ectoderm and a failure to gastrulate (Dunn 1972). The product of this locus might somehow antagonize the function of merlin or the *exed* gene product. The study of merlin function and the identity of the *exed* and *se¹* gene products will contribute valuable information toward understanding the development of extraembryonic structures and their role in the ability of the embryo to proceed through gastrulation. Conversely, the study of merlin function in the morphogenesis of the extraembryonic portion of the early mouse embryo undoubtedly will help us to understand how merlin loss contributes to tumorigenesis in adult mice and humans.

Materials and methods

Construction of targeting vector

A mouse 129/SvJ genomic library was screened with a full-length human *NF2* cDNA. Two positive clones were isolated, one of which contained exons 2–5 of the mouse *Nf2* gene. After extensive mapping of this clone, 4.2-kb *PstI*–*ApaI* and 2.6-kb *XbaI*–*XhoI* fragments were subcloned into Bluescript SK II (Stratagene) and excised with *KpnI*–*BamHI* and *NotI*–*XhoI* respectively, to acquire appropriate restriction sites. These fragments were then cloned into the targeting vector pPNT (Tybulewicz et al. 1991) such that the intervening 6.5 kb of genomic sequence, which included the 3' half of exon 2, intron 2, and all of exon 3, was deleted and replaced with the *pgk-neo* expression cassette oriented in a direction opposite to that of *Nf2* transcription. Genomic sequences were flanked on one side by the HSV-*tk* cassette (Fig. 1A). Electroporation into D3 ES cells and subsequent drug selection was performed as described (Jacks et al. 1992).

Screening of ES cell clones

DNA was isolated from aliquots of surviving ES cell clones and screened by Southern blotting. Both probes p5' and p3' were generated by subcloning 4.1-kb and 800-bp genomic fragments, respectively, sequencing all or part of them, and identifying regions devoid of repetitive elements. For probe p5' a 194-bp *PstI*–*EcoRI* restriction fragment represented a unique sequence; for probe p3', primers were generated that would amplify a 233-bp unique sequence by PCR. Southern blotting of *StuI*-digested genomic DNA was followed by hybridization to probe p3' (Fig. 1B), which detects a 4.3-kb band representing the 3' end of the wild-type allele, and a 3.9-kb band that represents the correctly targeted mutant allele. Of 231 total clones screened, 4 displayed both a wild-type and a mutant band (Fig. 1B). These four clones were further characterized by Southern blotting of *XbaI*–*BamHI*-digested DNA and hybridization to probe p5' that should detect 2.4-kb and 1.6-kb bands representing the 5' end of the wild-type and mutant alleles respectively. Careful evaluation of the resulting pattern of bands revealed that this *XbaI* site was not present in the ES cells themselves, and therefore represented a polymorphism between the 129/SvJ genomic library and the 129/Sv-derived D3 ES cells (Fig. 1A, asterisk). Furthermore, because the *XbaI* site was situated in the middle of the arm of homology, depending on the site of recombination, the *XbaI* site could have been transferred. We found that one clone acquired the *XbaI* site, two did not, and one displayed a pattern of bands indicating that one copy of the targeting vector recombined into the correct locus without the *XbaI* site, whereas a second copy of the targeting vector recombined into another site in the genome, carrying the *XbaI* site with it (not shown). This was confirmed using a probe derived from the *neo^r* coding region in the targeting vector and this clone was subsequently not used. Investigation of other restriction sites in this region indicated that the 129/SvJ clone and the ES cell DNAs were otherwise identical, and that this was likely to be a single nucleotide or microsequence polymorphism. Hybridization of probe p5' to *StuI*-digested DNA gave the expected pattern of 1.1-kb wild-type and 2.1-kb mutant bands, confirming the correct integration of the 5' targeting sequences in the three ES cell clones (not shown).

Characterization of the *Nf2* mutant allele

For analysis of mRNA transcribed from the mutant allele, total RNA was isolated from several tissues from *Nf2*^{+/–} mice using

RNAzol-B (Cinna/Bioteckx Laboratories). Ten micrograms of each was run on a formaldehyde gel, transferred to Hybond membrane (Amersham), and hybridized to a 1.7-kb *XhoI-XbaI* fragment from the pPNT targeting vector that contained the entire *neo*^r coding region. Subsequent RT was carried out on 2 µg of total RNA isolated from +/+, +/-, and -/- ES cells and two tumor cell lines from *Nf2* heterozygous mice (in preparation) using oligo(dT) as a primer. PCR was performed on the resulting cDNA using the following primer pairs: exon 1 (5'-CCGAGATGGAGTTCAACTGCG-3')-exon 9 (5'-TAACACGAAGCTTTCGAGAG-3'); exon 1-exon 5 (5'-AGAGGGGTCATAGTCGCG-3'); exon 6 (5'-GCACCGGGGCAGAGCCAG-3')-exon 10 (5'-CTTCTCTTCCCTGGCCTGGGC-3'); exon 10b (5'-CTATTATGAGGCGACGG-3')-exon 12 (5'-CTCTCTGACTCCTCAGCC-3'); exon 14 (5'-CCACCACCACTGCCTC-3')-exon 17 (5'-AGGCCACTCGGGACTTGGCGC-3'). The RT-PCR products corresponding to exons 1-9 from +/+ and -/- ES cells were purified from low-melt agarose, cloned into the TA vector pT7Blue (Novagen), and sequenced.

For Western blot analysis, 9×10^6 ES cells were trypsinized and resuspended in 250 µl of lysis buffer (2% SDS, 0.5 mM PMSF, 1 µg/ml of leupeptin, 1 µg/ml of aprotinin, 1 µg/ml pepstatin) on ice. Following protein quantification using a detergent compatible protein assay (BioRad), ~400 µg (80-100 µl) of total protein was loaded on a 10% SDS-PAGE gel and run for 3 hr at 175 V. Protein was transferred to methanol-activated PVDF membrane (Schleicher and Schuell) overnight in 5 mM Tris, 380 mM glycine, 0.1% SDS, and 20% MeOH at 20 mA (BioRad wet transfer). Membrane was then probed with the polyclonal anti-merlin antibody sc-331 or sc-332 (1 µg/ml; Santa Cruz Biotechnology) or the monoclonal anti-merlin antibody N21 (1 µg/ml; Gonzalez-Agosti et al. 1996) and visualized with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham).

Generation of *Nf2* mutant animals

All three correctly targeted ES cell clones were expanded and injected into wild-type C57BL/6J blastocyst stage embryos that were then implanted into pseudopregnant CD1 females. A total of 21 chimeric animals were generated in this manner, with varying levels of heterozygous mutant contribution (~30% to >90%) as judged by coat color analysis. These animals were bred to wild-type C57BL/6J animals; tail DNA from agouti F₁ offspring was genotyped by PCR amplification using a cocktail of primers: a (5'-GGGGCTTCGGGAAACCTGG-3'), b (5'-GTC-TGGGAAGTCTGTGGAGG-3'), and c (5'-CTATCAGGACATAGCGTTGG-3') (see Fig. 1A). Primer pair a-b amplifies a 306-bp product from the wild-type allele, and primer pair a-c amplifies a 575-bp product from the mutant allele.

Histological analysis of mutant embryos

Uteri containing F₁ intercross embryos were isolated at E6.5, E7.25, and E7.5 in phosphate buffered saline (PBS), fixed overnight in 4% paraformaldehyde, dehydrated, and paraffin-embedded. The staging of embryos was based upon both the timing of embryo isolation (E0 = midnight) and upon morphological criteria. Serial sections (4 µ) were generated, stained with hematoxylin and eosin, examined, and photographed under a Nikon Labophot-2 microscope.

In situ hybridization of histological sections

In situ hybridizations were carried out essentially according to Wilkinson (1993) with minor modifications. Unstained sections

(see above) were hybridized to ³⁵S[UTP]-labeled probes. A plasmid containing the entire *brachyury* coding sequence (a generous gift of Andreas Kispert, Harvard University, Cambridge, MA) provided the template for generation of a *brachyury* probe. Clones containing mouse *Nf2* bp 798-2251 (Haase et al. 1993) and bp 91-468 (exon 1-5; see above) served as the templates for generation of *Nf2* probes. *HNF3β*, *HNF4*, and *L14* probes were generated by RT-PCR from total adult liver cDNA using the following primers: *HNF3β*, 5'-AGAAGATGGCTTTCAGGCC-3' and 5'-CTGCAGATGTCTTGAGAAGC-3', corresponding to bp 1491-1510; *HNF4*, 5'-ACACGTCCCCATCTGAAGGTG-3' and 5'-CTTCTTCTTTCATGCCAGCCC-3', corresponding to bp 160-429; *L14*, 5'-GGGAGAGGTGGCCTCGGACGC-3' and 5'-GGCTGGCTTCACTCAAAGGCC-3', corresponding to bp 135-487. Amplified products were cloned into the TA-vector.

In vitro transcription was carried out on 1 µg of purified, linearized template as described (Wilkinson 1993); probes were not degraded. Section in situ hybridizations were performed overnight at 55°C in a humidified incubation chamber (Omni-slide, Hybaid). Following extensive washing and RNase A treatment, slides were dipped in photographic emulsion (Kodak NTB3) and developed after 10-16 days.

Immunohistochemistry

Histological sections were rehydrated, microwaved in citric acid buffer 2× for 5 min each, and rinsed with PBS. Endogenous peroxidase was removed in 30% H₂O₂ in MeOH for 30 min. Sections were then blocked with 3% normal goat serum for 30 min, incubated with a polyclonal pem-1 antibody (1:2000 in 1% NGS; Lin et al. 1994; provided by Dr. C. MacLeod and Jeff Pitman, University of California, San Diego) for 4 hr and with a biotinylated anti-rabbit IgG secondary antibody (1:500 in 1% NGS; Vectastain ABC kit) for 30 min. Hybridization was visualized with an avidin and biotinylated horseradish peroxidase complex (Vectastain ABC kit).

Homozygous mutant ES cell derivation

129/Sv *ROSA26* +/- mice (Freidrich and Soriano 1991) were bred to 129/Sv *Nf2* +/- animals to generate doubly heterozygous mice. For *ROSA26* genotyping, ear punches were rinsed in PBS, then incubated for 2-4 hr in X-gal staining solution (0.02% NP-40, 0.01% SDS, 2 mM MgCl₂, 5 mM K-ferricyanide, 5 mM K-ferrocyanide, 1 mg/ml of X-gal). 129/Sv *ROSA26*+/-/*Nf2*+/- males were then mated to mixed background (129/Sv × C57BL/6J) *Nf2* +/- females. ES cell lines from individual blastocyst-stage embryos were generated as described (Robertson 1987). Briefly, females were ovariectomized 2 days following the detection of a vaginal plug. Delayed blastocysts were collected 4 days later and placed on irradiated feeders in a 24-well tissue culture well in ES media. After 4-6 days, blastocysts were picked, disaggregated, and redispersed onto fresh feeders (1 well of a 24-well dish). Undifferentiated colonies that grew up were picked 1-2 weeks later and replated onto fresh feeders (1 well of a 24-well dish). Usually the line grew exponentially at this point and was passaged to 1 well of a 6-well dish and grown to near confluency; three-quarters of the cells were then frozen down and one-quarter used for DNA isolation and genotyping.

Generation of homozygous mutant chimeras

Ten to twelve 1076.7 or 2600E ES cells grown in the presence of irradiated feeders were injected into wild-type C57BL/6J blastocysts, which were implanted into pseudopregnant Swiss Webster females. Allowing 0.5 to 1.0 day of developmental delay

because of manipulation, embryos were isolated at various times, removed from their decidua, rinsed in 0.1 M NaPO₄ buffer (pH 7.3), fixed (0.2% glutaraldehyde, 5 mM EGTA, 2 mM MgCl₂, 94 mM NaPO₄) for 5–30 min depending on the size of the embryo, washed 3× for 5 to 30 min (2 mM MgCl₂, 0.02% NP-40, 0.01% deoxycholate, 98 mM NaPO₄), and stained overnight in X-gal (1 mg/ml of X-gal, 5 mM K-ferricyanide, 5 mM K-ferrocyanide) at room temperature. Embryos displaying homozygous (blue) contribution were then photographed, dehydrated, imbedded in paraffin, and serial, sectioned (4 μ).

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