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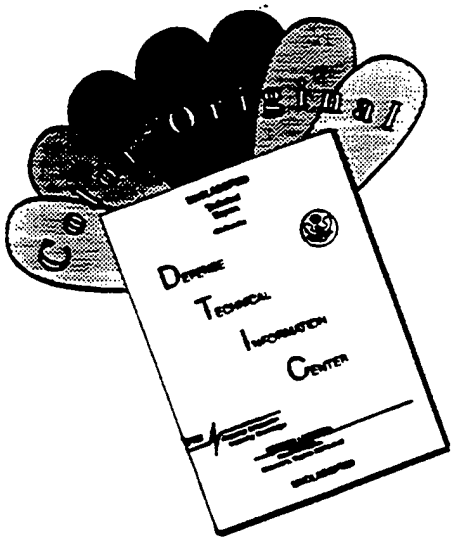

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Neurofibromatosis type 2 (NF2) is a hereditary disorder featuring the development of nervous system tumors, particularly schwannomas. The tumor suppressor responsible for NF2 is a member of a class of cytoskeletal-associated proteins, and therefore represents a new type of tumor suppressor. In an effort to develop an animal model for NF2 and investigate the function of the NF2 gene product *merlin*, we have employed gene targeting to create a mouse that is mutant at the *Nf2* locus. *Nf2* heterozygous mice are predisposed to developing a broad spectrum of tumor types which show LOH at the *Nf2* locus. Our results also demonstrate strong cooperation between mutations at the *Nf2* and *p53* or *Nf1* loci in the development of specific tumor types.

We have also determined that *Nf2* is required at multiple stages of development. *Nf2* homozygous mutant embryos fail to initiate gastrulation, apparently due to a defect in the extraembryonic lineage. The generation of chimeric embryos partially composed of *Nf2* homozygous mutant cells that are marked by the *lacZ* transgene reveals several additional stages of development during which merlin function is essential Together studies of the consequences of merlin loss during both development and tumorigenesis in the mouse will greatly enhance our understanding of the consequences of merlin loss in human disease.

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Introduction

The neurofibromatoses are heritable forms of cancer involving the development of nervous system tumors. Neurofibromatosis type II (NF2) patients develop tumors which derive from cells that support the nervous system, including schwannomas, meningiomas and ependymomas (1). The hallmark of NF2 is the development of schwannomas on or around both eighth cranial nerves; accordingly, the first sign of NF2 is often loss of hearing. In addition, NF2 patients often develop multiple schwannomas, usually within the cranium or immediately adjacent to the spinal cord. Although these tumor types generally retain a benign growth status, their placement renders surgical removal difficult and often debilitating.

The features of NF2 are consistent with a model in which the genetic lesion resides in a tumor suppressor gene, acting in a dominant fashion to predispose an individual to the development of particular tumors (2). Ultimately, a second mutation occurs in one or a few cells, which alters the remaining copy of the gene and releases the cell from growth regulation conferred by the tumor suppressor gene product (3). Accordingly, loss of heterozygosity (LOH) studies originally mapped the *NF2* gene to human chromosome 22; ultimately its identity was obtained by positional cloning (4-6). In further support of this model, recent studies have identified somatic mutations in both copies of the *NF2* coding region in most sporadically occuring schwannomas and at least a subset of meningiomas (rev. in 6). In addition to these benign tumor types, *NF2* mutations have also been documented in a high percentage of malignant mesotheliomas of the lung (7).

The NF2 gene product, *merlin*, is a member of a family of proteins that couple the cell membrane to the cytoskeleton and are thought to be involved in active cytoskeletal reorganization (rev. in 8). Merlin therefore is the defining member of a new category of tumor suppressor genes, affording the possibility of studying the role of the cytoskeleton in cell growth control, a process potentially relevant to all forms of cancer.

In an effort to initiate an extensive investigation of the function of merlin, we have utilized gene targeting to create a mouse that is heterozygous for a mutation at the murine *Nf2* locus, and therefore is the genetic analogue of a human NF2 patient. There are three questions which we initially sought to address through the generation and study of these mice: 1) Do *Nf2* heterozygous mutant mice represent an animal model of NF2? 2) Is merlin function required during normal mouse development, and if so, what can its developmental role tell us about the ramifications of merlin loss during tumorigenesis? 3) Can we exploit this system to generate various cell types which specifically lack merlin function? Such mutant cells can be used to address specific cell biological and biochemical questions that derive from our observations *in vivo*.

Through gene targeting in ES cells, we have succeeded in generating mice that are heterozygous for a mutation at the mouse *Nf2* locus. Surprisingly, these mice develop a broad spectrum of malignant tumor types, particularly several types of sarcoma. In addition, we have observed marked cooperativity between the *Nf2* gene and other tumor suppressor genes in the development of specific subsets of these tumors. While *Nf2* heterozygous mice do not represent an accurate model of NF2, they are a valuable model for studying the consequences of merlin loss in tumorigenesis in general and for ultimately therapeutically targeting the pathway(s) in which *NF2* tumor suppressor function has been lost. Moreover, we have noted that the tumors that develop in all of these mice exhibit a very high rate of metastasis, a rare feature of tumors that arise endogenously in the mouse (9). This raises the exciting possibility that merlin loss may contribute to the process of metastasis, which ultimately accounts for the death of nearly all human cancer patients.

We have also identified several different developmental contexts during which merlin function is essential. First, by intercrossing our mice that are heterozygous for a mutation at the *Nf2* locus, we have determined that the *Nf2* gene is absolutely required for proper development of the mouse embryo. Homozygous mutant embryos fail at a very early, critical time during embryogenesis, apparently failing to initiate the process of

gastrulation, which begins with the induction of mesodermal cells and results in the organization of an embryonic axis (rev. in 10). By examining the expression pattern of a number of cell type specific markers of gastrulation in the mutant embryos, we have determined that the mutant embryos never produce mesoderm, apparently due to a defect in the extraembryonic portion of the embryo.

To investigate further the function(s) of merlin during development, we created *Nf2* homozygous mutant ES cells marked by a *lacZ* transgene and used them to generate chimeric embryos partially composed of wild type cells and partially composed of marked *NF2*-deficient cells. Initially, we exploited this system to investigate the function of merlin at gastrulation; we then examined older chimeric embryos and found several subsequent developmental contexts during which merlin function is essential, including in the developing heart.

Much of our present effort is devoted to tallying the histological data from our *Nf2* heterozygous mice, analyzing the defects associated with the loss of merlin function in postgastrulation chimeric embryos and adults, and complementing these *in vivo* observations by studying the properties of a number of different cell lines we have developed that specifically lack merlin function. This work provides a solid foundation toward understanding the normal function of the *NF2*-encoded protein and how the loss of that function leads to the development of tumors in human NF2 patients. Moreover, the study of merlin may be paradigmatic for the study of the membrane-cytoskeletal interface in the development of cancer in general.

Body

Evaluation of the Nf2 mutant allele

We have previously described the construction of the *Nf2* targeting vector and the generation of *Nf2* heterozygous mutant ES cells (see Progress Report: 1/25/95). In summary, our strategy was to replace the 3' half of exon 2, all of exon 3 and intervening intronic sequences with the neomycin resistance gene (*neo*), oriented in a direction opposite to that of *Nf2* transcription. This strategy was based upon the identification of missense mutations in exons 2 and 3 and of splicing mutations that would affect the removal of exon(s) 2 and/or 3 from the human NF2 coding sequence (rev. in 6). Although this indicates that in humans, exons 2 and 3 are required for the tumor suppressor function of merlin, it is important to establish that our mutation creates a null allele. Because *Nf2* homozygous mutant embryos fail so early during development, it was impossible to obtain enough homozygous mutant tissue for evaluation of merlin protein levels from the embryos themselves. We therefore used homozygous mutant ES cell lines (see below) as a source of homozygous mutant material. Northern blot analysis of mRNA extracted from homozygous mutant ES cells revealed the presence of some *Nf2* mRNA of roughly normal size (data not shown). Extensive RT-PCR analysis revealed that the mRNA species transcribed from the mutant allele is spliced from exon 1 to exon 5. However, we failed to detect an *Nf2*-encoded protein of any size upon western blot analysis of protein extracts from homozygous mutant ES cells using two N-terminal and one C-terminal merlin antibody, indicating that we have indeed created a null allele (13; Figure 1).

Characterization of heterozygous mutant animals

Mice heterozygous for a mutation at the *Nf2* locus exhibit significantly decreased survival when compared to wild-type animals (Figure 2). Surprisingly, these mice predominantly develop osteosarcomas, as well as fibro-, and rhabdomyosarcomas. All of these tumors display loss of heterozygosity (LOH) at the *Nf2* locus, indicating that the loss of merlin function is a critical step in their development. In addition, *Nf2* heterozygotes develop a number of other tumor types at a lower frequency; we are currently evaluating the status of the *Nf2* locus in these tumors.

We have also crossed our *Nf2* mutation onto genetic backgrounds containing mutations in other tumor suppressor genes, notably the *p53* and *Nf1* genes (12,13). All three of these loci reside on mouse chromosome 11; *p53* and *Nf1* are tightly linked to each other distal to the *Nf2* locus. Since it had previously been demonstrated that the loss of an entire chromosome is a common mechanism of mutation during tumorigenesis in the mouse (14), we sought to generate mice carrying the mutations on the same chromosome 11 (in *cis*) and on opposite chromosomes 11 (in *trans*). Creation of the in *trans* configuration was straightforward, involving standard crosses between *Nf2* heterozygotes with either *p53* or *Nf1* heterozygotes. In such a cross, 25% of the offspring are expected to be heterozygous for both mutations (which are carried on opposite chromosomes 11). To generate the in *cis* configuration, these doubly heterozygous mice were then bred to wild-type mice; any progeny from such a cross that are heterozygous for both the *Nf2* and *p53* or *Nf1* mutations result from meiotic recombination that has occured between the loci, placing the mutations on the same chromosome. Interestingly, both *Nf2/p53* and *Nf2/Nf1* *cis* mice exhibited dramatically decreased survival compared to mice with any of the three individual heterozygous mutations, or any two mutations in *trans*. In both cases, the mice developed a subset of the tumor types that *Nf2* heterozygotes develop. Thus, *Nf2/p53 cis* mice develop multiple osteosarcomas and do not survive past five months of age, while

Nf2/Nf1 cis mice develop fibro- and rhabdomyosarcomas and survive somewhat longer (Figure 2). These tumors always exhibit LOH at both the *Nf2* and *p53* or *Nf1* loci, strongly indicating that the entire wild-type chromosome has been lost.

We have also observed a very high rate of metastasis associated with tumorigenesis in all of these mice, a rare feature of endogenously arising mouse tumors. For example, osteosarcomas and fibro-/rhabdomyosarcomas metastasize nearly 100% of the time in *Nf2* heterozygotes. We have crossed our *Nf2* mutation to other cancer prone mice in order to determine whether *Nf2* loss can confer metastatic potential to other mouse cancer models, including the *min* mouse model of intestinal polyp formation and the *wnt* transgenic model of breast cancer (15,16).

Characterization of homozygous mutant embryos

Our initial characterization of the *Nf2* homozygous mutant phenotype has been described previously (see Progress Report: 1/25/95). Briefly, mutant embryos are superficially distinguishable from their wild type or heterozygous littermates beginning at embryonic day 7.0 (E7.0), largely due to the lack of a distinct boundary between the embryo proper and the extraembryonic membranes (Figure 3a,b). By E7.5, *Nf2* mutant embryos were obviously smaller and had not completed gastrulation as their littermates had. Both whole mount and *in situ* hybridization revealed *Nf2* expression at low levels throughout the E6.5 embryo and at much higher levels throughout the E7.5 embryo (not shown; Figure 3f). Histologically, these embryos exhibited a disorganized extraembryonic region and showed no evidence of mesoderm formation, despite the continued growth of the embryo proper (Figure 3c-e). This observation was supported by the absence of *brachyury* or *HNF3*β expression in nearly all *Nf2* mutant embryos by *in situ* hybridization. *Brachyury* is expressed in early mesoderm and in the region of mesoderm induction known as the primitive streak, while *HNF3*β is expressed in later, more mature mesoderm (17,18). The presence of a small patch of *brachyury* expression in a thickened region of

embryonic ectoderm in at least one mutant embryo suggests that primitive streak formation occurs at least in part, without subsequent mesoderm induction (Figure 3h).

Similar analyses suggest that the lack of a distinct boundary between the embryo proper and the extraembryonic derivatives in the mutant embryos may be due specifically to the absence of extraembryonic ectoderm, a derivative of the trophectoderm (19). We are currently investigating this possibility using additional markers of extraembryonic lineages.

Generation of homozygous mutant ES cells

In order to investigate merlin function(s) during development further, we sought to create *Nf2* homozygous mutant ES cell lines that also carry a *lacZ* transgene and are therefore marked. We crossed the *Nf2* mutation into the ROSA26 strain of mice which carries a ubiquitously expressed *lacZ* transgene (20). E3.5 blastocyst-stage embryos were collected from *Nf2/ROSA+* heterozygous intercrosses and ES cell lines were established from individual blastocysts as has been described (21). In this manner, we established two *Nf2* homozygous mutant ES cell lines, one of which also carried the *lacZ* transgene.

Creation of chimeras using homozygous mutant ES cells

Nf2 homozygous mutant ES cells were injected into wild-type blastocysts, implanted into pseudopregnant females and allowed to develop *in utero* (22). We first isolated chimeric embryos at gastrulation, fixed and stained them with X-gal in order to identify the *lacZ*-expressing cells. We found that chimeric embryos could gastrulate normally despite receiving extensive contribution from the homozygous mutant cells to all three embryonic lineages: ectoderm, endoderm and mesoderm, indicating that merlin is not required cell autonomously for mesoderm formation (Figure 4a-c). This also supports the idea that the primary defect actually resides in the trophectoderm lineage, which is already committed at the blastocyst stage, and therefore does not receive contribution from the input mutant ES cells (23).

Characterization of postgastrulation chimeric embryos

Given that gastrulation was easily rescued in chimeric embryos receiving high levels of homozygous mutant contribution, we next investigated the fate of these embryos during later stages of development. Collectively, depending on both the overall level and location of the homozygous mutant contribution, chimeric embryos displayed a spectrum of overlapping phenotypic abnormalities, extending from E8.0 through late stages of gestation. Embryos receiving the highest levels of homozygous mutant contribution reproducibly exhibited a shortened anteroposterior axis and a kinked neural tube, did not initiate the turning process and ceased to develop (Figure 4d,e). Varying degrees of chimerism also led to two additional defects: a failure of chorioallantoic fusion and a greatly inflated pericardial sac associated with cardiac developmental abnormalities (Figure 4a-h). All of these defects were seen in chimeras generated from both homozygous mutant ES cell lines.

Between E8.5 and E9.0, the mesodermally-derived allantois extends up from the posterior portion of the embryo, makes contact with and fuses to the chorion (rev. in $24). A failure of chorioallantoic fusion leads to compaction of the allantoic tissue and the formation of a tight ball-like structure. Although *Nf2* mRNA is highly expressed in the allantois and to a lesser extent in the chorion (Figure 3f), the compacted allantois ball was always derived from *Nf2*-deficient cells, suggesting that the failed fusion was caused by *Nf2* deficiency in the allantois itself. This was not a secondary defect associated with early embryonic failure, as lower contribution chimeras that proceeded to later stages of development exhibited failed chorioallantoic fusion in the absence of other obvious abnormalities (Figure 5g). Given that there was no obvious abnormality in allantois or chorion formation, this defect is likely to result from impaired cell adhesion between the two.

The mouse embryonic heart develops from a pair of mesodermally-derived primordia that fuse at E8.0 and form the primitive heart tube (25). By E8.5-E9.0, primitive cardiac myoblasts differentiate into elongated myocytes forming specialized intercellular junctions and the heart tube makes its first contractions. Chimeric embryos carrying a high contribution of *Nf2* mutant cells in the cardiac region displayed an inflated pericardial sac as early as E8.0; embryos with lower levels of contribution to the heart often developed to more advanced stages of gestation, with varying degrees of pericardial ballooning (Figure 5d,e). Histological analysis of the heart tube in these chimeras revealed that areas of the myocardium composed of merlin-deficient cells were thickened and composed of multiple disorganized layers of large round cells, as opposed to the flattened one- or two-cell layered wall of the myocardium (Figure 5b,c,e,f).

Strong selection against *Nf2*-mutant cell contribution to the heart was often apparent in late-stage chimeras (Figure 5h). In fact, any *Nf2*-mutant cell contribution to the heart in older chimeric embryos was in the form of hyperplastic lesions in the myocardial wall that are probably derived from cells that failed to differentiate properly during cardiac development and continued to proliferate (Figure 5d-f). We have also observed focal regions of blue-staining cells in the head region which appear to be hyperplastic (Figure i,j). We are currently using immunohistochemical techniques to determine the cell type and differentiation status of cells that compose both of these types of lesions.

The creation and study of various Nf2 homozygous mutant cells

We have generated a number of different cell lines which specifically lack merlin function from *Nf2* heterozygous and homozygous mutant animals or embryos. *Nf2*-deficient ES cells or other embryonically-derived cells can be studied in direct comparison to wild-type and heterozygous mutant counterparts. For example, we are currently comparing the capacity of *Nf2* homozygous mutant ES cells to differentiate into different lineages under defined conditions to that of wild-type and heterozygous mutant ES cells.

We can also isolate and culture homozygous mutant cells from chimeric embryos by selecting the cells in culture with the drug G418; only the mutant cells, which carry the neomycin resistance gene, will survive. In this manner, we have already generated *Nf2*-deficient mouse embryo fibroblasts (MEFs). These will be a particularly valuable reagent, as their properties can be compared to those of many available MEF lines derived from other gene targeting experiments. This protocol will also be used to obtain and characterize *Nf2*-deficient fetal cardiac cells in order to further study the cardiac developmental defect that we have described.

In addition, tumor cells of several histologic origins have been cultured from *Nf2* heterozygotes and from *Nf2/p53* and *Nf2/Nf1 cis* animals. Reintroduction of merlin expression into these cells should allow us to detect any phenotypic differences associated specifically with the presence or absence of merlin expression. This system will also allow us to address more directly the role of merlin function in tumor suppression and metastatic potential *in vivo* by evaluating the ability of tumor cells before and after reintroduction of merlin expression to form tumors (and metastasize) in immunocompromised mice.

Conclusions

We are pleased that we have surpassed the research goals described in our grant proposal, and we are now pursuing a number of avenues of inquiry that stem from these studies. Our results demonstrate a role for merlin in tumor suppression in the mouse and identify multiple requirements for merlin function during development, underscoring the role of tumor suppressor genes in normal developmental processes. The requirement for merlin in so many developmental contexts was unexpected given both the potential for functional redundancy among merlin family members, and given the relatively limited spectrum of tumors which develop in NF2 patients. Each of these contexts represents an important line of inquiry into the normal function of merlin. Moreover, the study of merlin

function provides an avenue toward investigating poorly understood developmental processes such as gastrulation and early heart development.

Together our results suggest that there may be two mechanisms by which *Nf2* loss leads to tumorigenesis. Mice that are constitutively heterozygous for an *Nf2* mutation develop a broad spectrum of malignant tumor types which exhibit LOH at the *Nf2* locus, and which exhibit a very high metastatic potential. This may be analagous to the mechanism by which *Nf2* loss contributes to the development of malignant mesotheliomas in humans. By contrast, *Nf2*-deficient cells in the myocardium and head region of developing embryos apparently fail to differentiate properly, and continue to slowly divide, perhaps modeling the benign, slow growing schwann cell tumors which develop in human NF2 patients. The observation that schwann cell tumors do not develop in *Nf2* heterozygous mutant mice may indicate that the loss of the wild type allele is rate limiting in the mouse schwann cell. In order to address this question, we are creating adult chimeric animals that are partly composed of *Nf2* homozygous mutant cells. Contribution of the *Nf2*-deficient cells to the schwann cell lineage may remove the need to lose the wild type allele and result in the formation of a tumor.

In sum, our observations may suggest a mechanism for merlin function: merlin loss may lead to impaired cell adhesion between certain cell populations in the developing embryo or in a developing tumor. Many of the defects in our chimeric embryos that are partly composed of Nf2-deficient cells are also features of embryos lacking specific cell adhesion molecules. For example, impaired chorioallantoic fusion is a characteristic of embryos lacking either α^4 integrin or VCAM-1, transmembrane proteins which bind to each other from the surfaces of opposing cells, as well as the kinase *csk*, which negatively regulates the *src* oncoprotein, also important for integrin based signaling (26-29). Two other features of *Nf2*-mutant chimeras, a kinked neural tube and posterior truncation, are also exhibited by csk-deficient embryos and by embryos lacking either α^5 integrin or fibronectin proteins which mediate cell:extracellular matrix adhesion (30,31). Consistent

with our findings, it has recently been reported that *NF2* antisense oligonucleotides can impair cell adhesion in some cell lines in culture (32). This may also provide an explanation for tumor formation: loss of merlin function in certain cell types may lead to a loss of cell adhesion, disregulation of contact inhibited cell growth and/or failure of proper differentiation, and continued proliferation. We are currently addressing these mechanistic questions directly using Nf2-deficient cells derived from our mice.

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Appendix

Figure 1: Western blot analysis of cell lysates from wild-type, heterozygous or homozygous mutant ES cells. A) Western blot probed with polyclonal antibody N21 (ref. 20), directed against the human merlin N-terminus, which also detects ezrin and moesin. Positions of merlin, ezrin and moesin bands are shown at left. Lane 1: human fetal fibroblasts; lane 2: NIH3T3 cells; lane 3: +/+ ES cells; lane 4: +/- ES cells; lane 5: -/- ES cells. B) Western blot was probed with polyclonal antibody SC331 (Santa Cruz). Lanes 1 and 2: +/+ ES cells; lane 3; +/- ES cells; lane 4: -/- ES cells. 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.

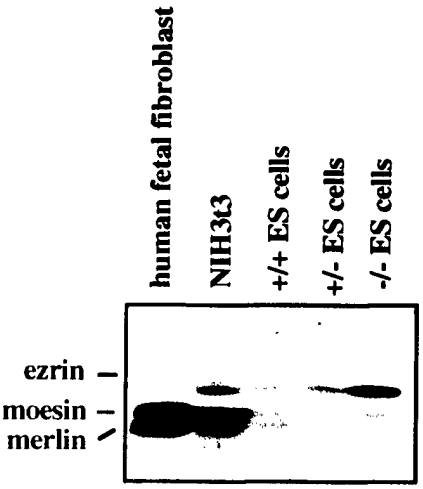
Figure 2: Relative survival of *Nf2, Nf2/p53, Nf2/Nf1, p53*, and *Nf1* heterozygous mice by comparison to that of wild type mice.

Figure 3: *Nf2* homozygous mutant embryos. Morphology of E7.25 wild type (A) and homozygous mutant (B) embryos. Arrow in (A) depicts the embryonic:extraembryonic boundary. C) Saggital section through a wild type E7.5 embryo. D-E) Saggital sections through E7.5 *Nf2* homozygous mutant embryos. Arrow in (D) indicates the thickening in the region of the primitive streak with no distinct mesodermal cell population. F) *Nf2* expression is seen throughout a wild type E7.5 embryo, including the allantois (arrowhead) and chorion (small arrows). G) *Brachyury* expression in a wild type E7.5 embryo. Expression is seen in the nodal region at the bottom of the embryo; the primitive streak is perpendicular to the plane of the photograph. *Brachyury* expression is not detected in most mutant embryos (not shown). H) A small patch of *brachyury* expression is detected in the homozygous mutant littermate shown in (E), although the positive cells are ectodermal in nature.

Figure 4: Features of *Nf2* homozygous mutant chimeric embryos. A) Gastrulating E7.25 chimeric embryo with high contribution to the embryo proper. The arrow marks the boundary between the embryonic and extraembryonic portions of the embryo. B) Transverse section of the embryo shown in (A), through a plane slightly above the embryonic:extraembryonic boundary; blue-staining cells are contributing to the developing amnion (small arrows). C) Chimeric embryo at E8.0 which has developed well beyond gastrulation with *Nf2* mutant contribution to the ectoderm, endoderm and mesoderm cell lineages. *Nf2* -/- (blue staining) contribution to the myocardium (arrow), represents the earliest stage at which merlin-deficient myocardium appears normal. D) A pair of E8.0 chimeric embryos. The lower contribution embryo (left) displays a normal anterior-posterios axis and reduced contribution to the heart. The higher contribution embryo (right) has a shortened posterior (arrows point to junctions between posterior and allantois). E) Neural tube defects in a chimeric embryo generated using a mutant ES cell line that does not carry the *lacZ* transgene, therefore *Nf2*-deficient cells are not marked.

Figure 5: Cardiac and caudal lesions associated with *Nf2*-deficiency. A) Pericardial ballooning in a highly chimeric E8.75 embryo. B,C) Section through a similar embryo depicting the overgrowth of *Nf2*-deficient cells in the myocardial wall (B,40X,C,100X). *Nf2*-deficient myocardium is composed of multiple layers of rounded cells (large arrows in C), in contrast to the small regions of thin, normal (pink) myocardium (small arrows). The large arrow in (B) indicates compacted allantoic tissue to the left of a point of partial chorioallantoic fusion. D) Older, (~E11.0) lower contribution chimeric embryo. *Nf2*-deficient populations of cells are apparent as blue 'discs' on the surface of the myocardium. E,F) Section through a similarly staged embryo depicting the appearance of *Nf2*-deficient lesions in the myocardial wall (E,40X;F,100X). G) Prominent mass of compacted, *Nf2*-

deficient allantoic tissue (arrow), reflecting a failure of chorioallantoic fusion in a ~E11.5 chimera with low overall homozygous mutant contribution. H) Marked selection against *Nf2* homozygous mutant contribution to the heart in an otherwise highly chimeric embryo. I,J) Chimeric embryo with a focal lesion in the head region (large arrow). Also note the concomitant hypertrophy of the heart (I,40X;J,100X).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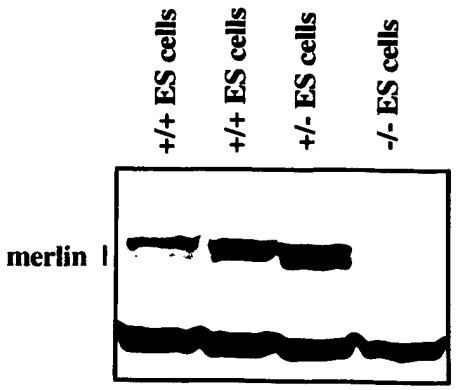
NIH3t3
+/+ ES cells
+/- ES cells
-/- ES cells
ezrin
moesin
merlin

A)


+/+ ES cells
+/+ ES cells
+/- ES cells
-/- ES cells
merlin

B)

Figure 1.

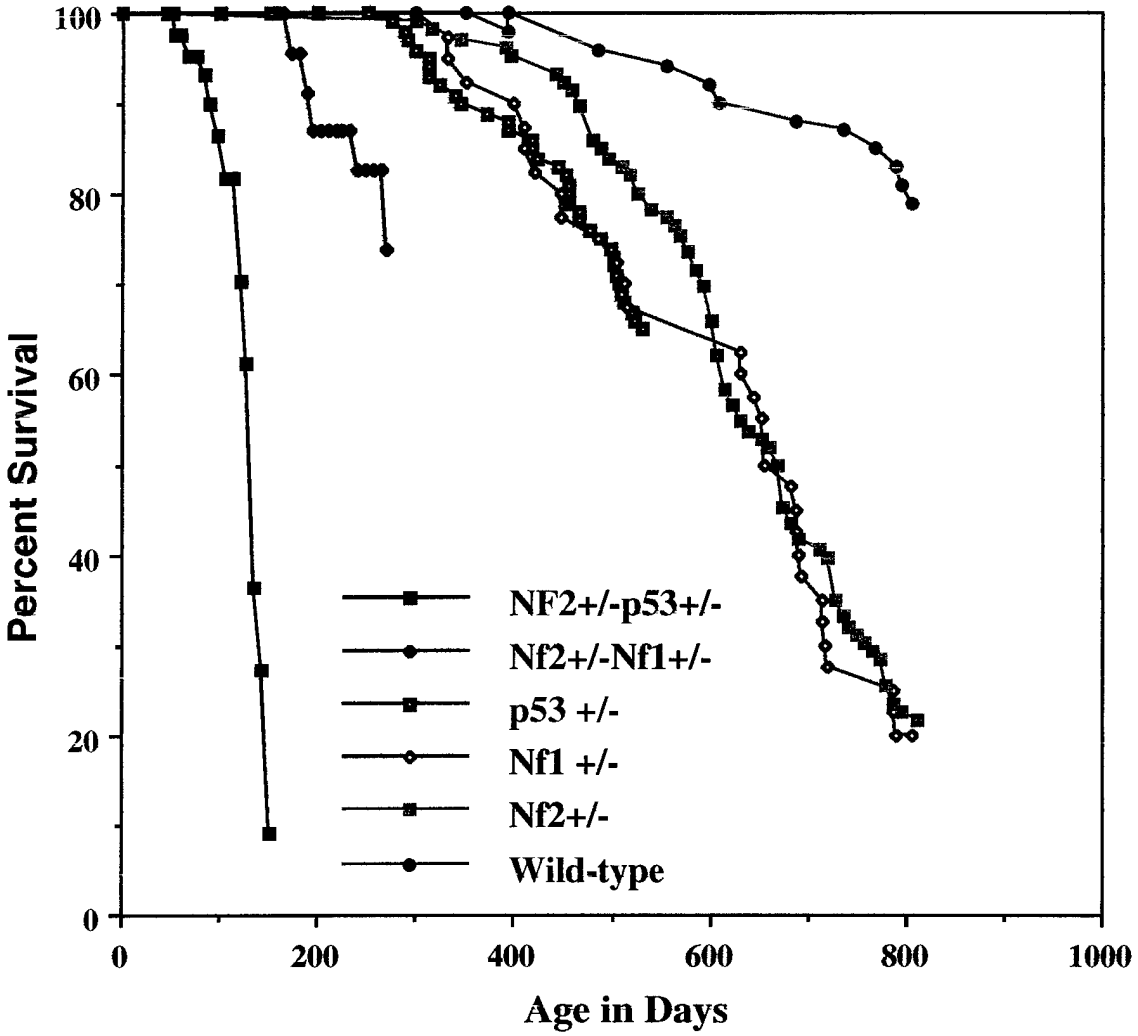

100
80
60
40
20
0
Percent Survival
0
200
400
600
800
1000
Age in Days
NF2+/-p53+/-
Nf2+/-Nf1+/-
p53 +/-
Nf1 +/-
Nf2+/-
Wild-type

Figure 2.

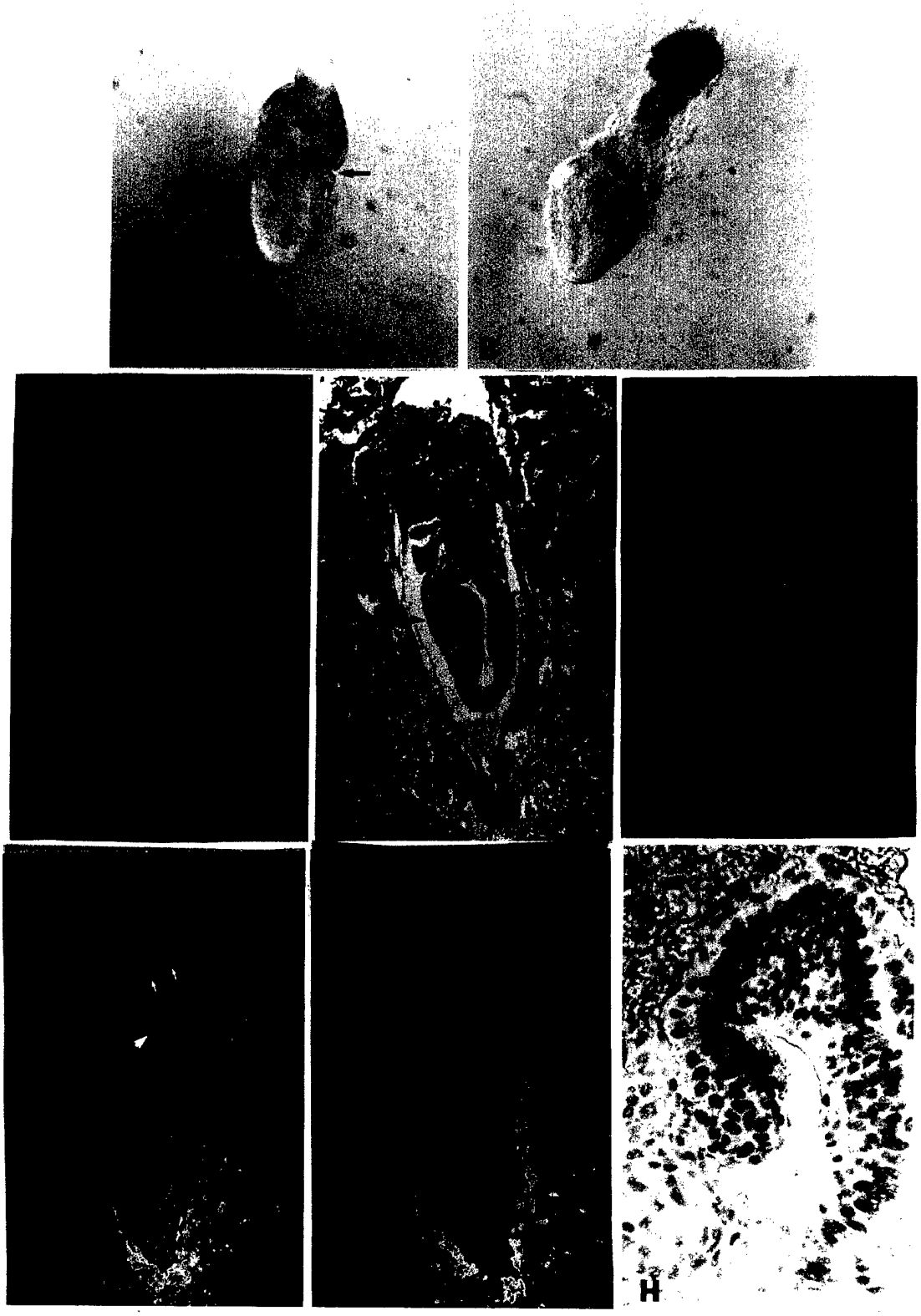


Figure 3.

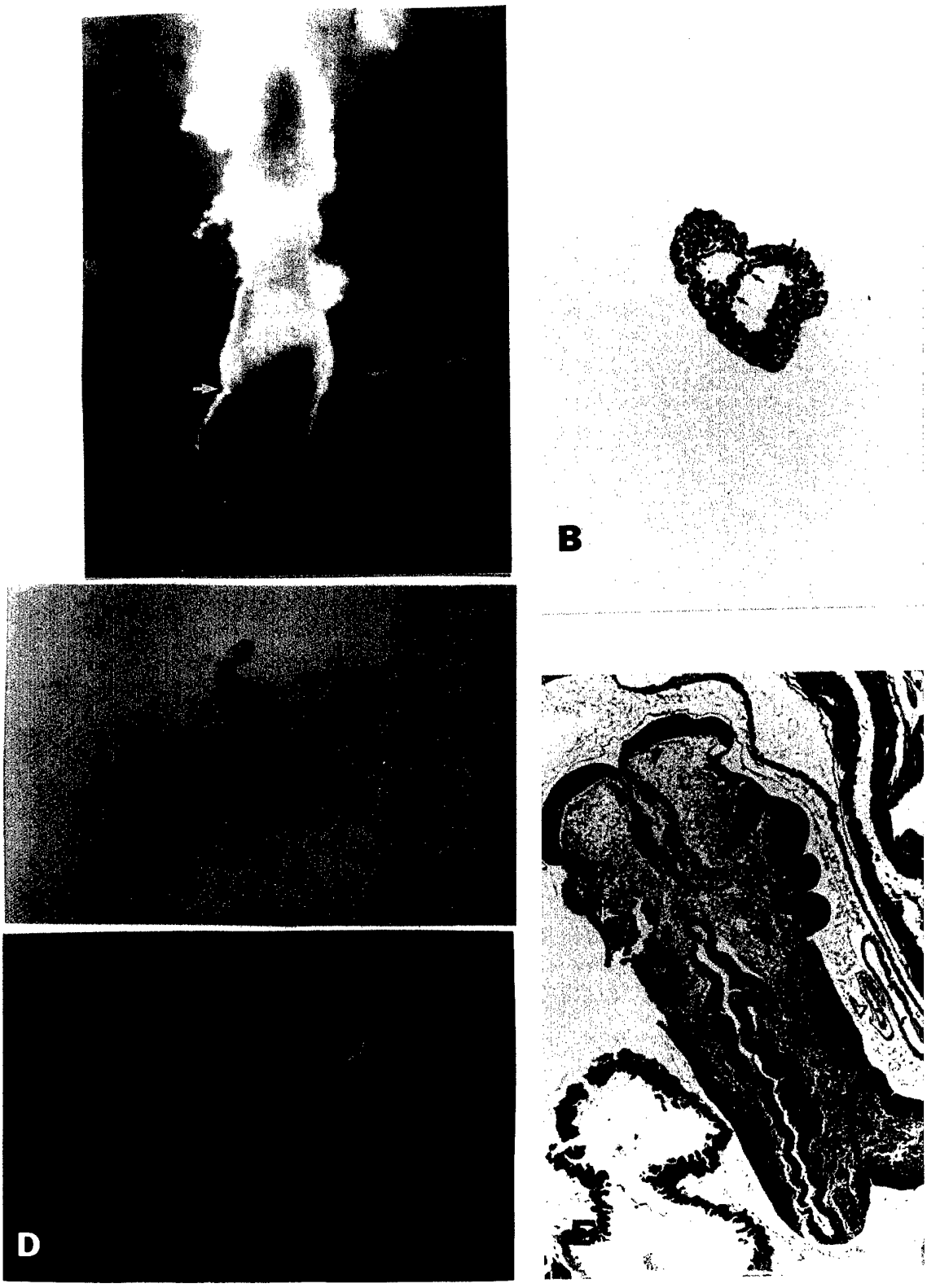


Figure 4.

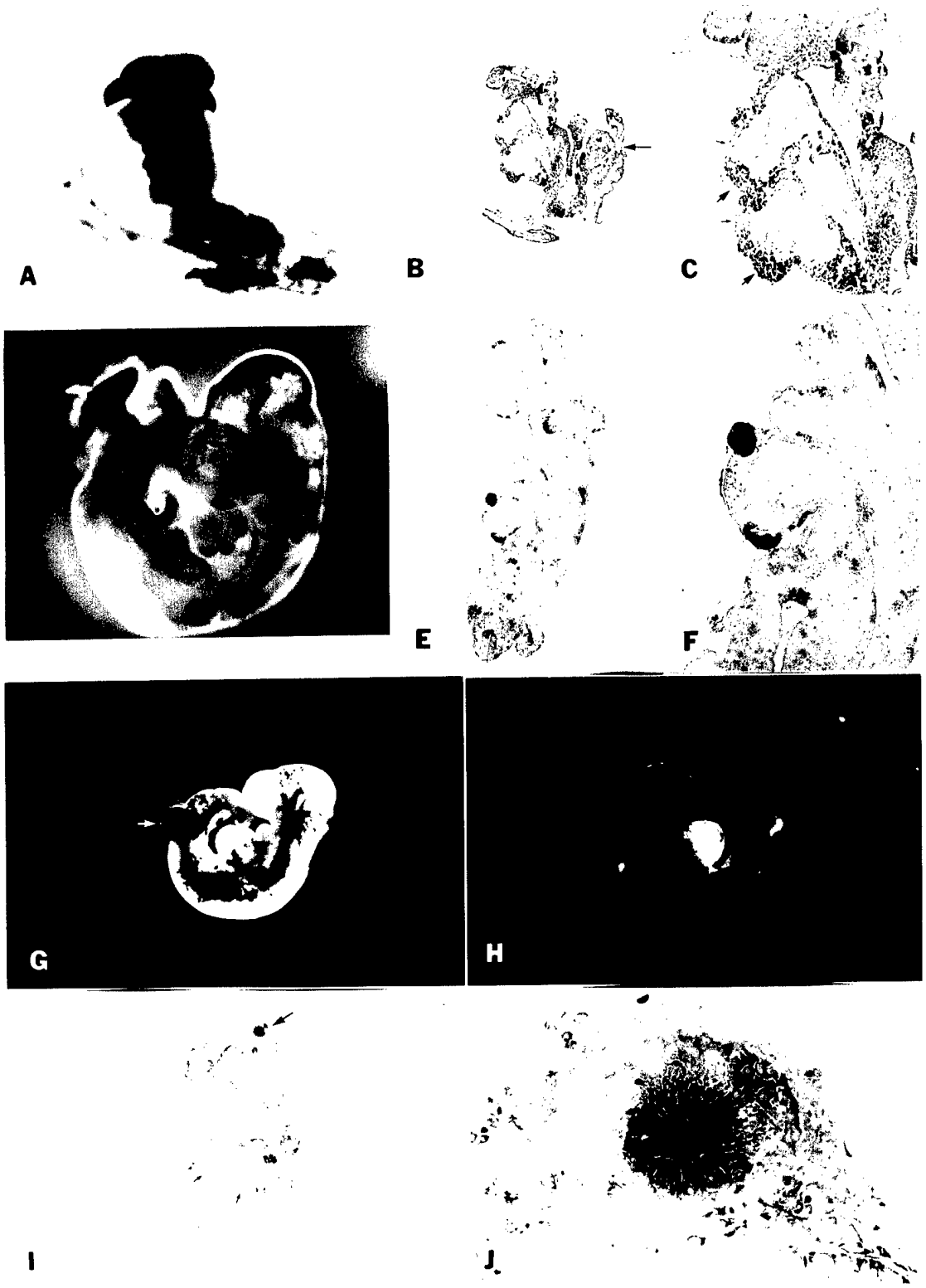


Figure 5.