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REVIEW ARTICLE
Miller-Dieker Syndrome: Analysis of a Human Contiguous Gene Syndrome in the Mouse

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The understanding of gene function and its relevance to human disease is one of the most important objectives in the postgenome era. The most direct avenue to uncovering this relationship is the positional cloning of human disease genes, particularly for disorders with Mendelian inheritance. Even when multiple mutational mechanisms result in the same disorder, affected individuals from different families can be ascertained, to find those individuals with specific mutations on a single gene. This provides solid evidence that mutation of a gene is causative of the disease and gives important information regarding gene function.

The relationship between gene function and phenotype is not as clearly delineated in more-complicated situations. In the case of contiguous-gene syndromes caused by heterozygous gene deletions, it may be possible to use the extent of deletions to map the relevant genes for specific phenotypes directly in the human. However, specific deletion mechanisms may make the frequency of certain deletions so high as to preclude detailed deletion analysis, as is the case for DiGeorge syndrome/velocardiofacial syndrome (DGS [MIM 188400]; VCFS [MIM 194230]). In DGS/VCFS, a heterogeneous disorder, the majority of the patients have the same 3-Mb deletion, whereas a small subset have a nested 1.5-Mb deletion, and very few patients have different deletions from the two common ones (Baldini 2002). In this situation, it may be impossible to definitively assign a phenotypic function to a gene on the basis of the extent of deletion.

As an alternative strategy, mouse models can be used to determine the contribution of regions or even individual gene mutations to a complicated phenotype in contiguous-gene syndromes. Over distances of 1–2 Mb, the order and gene content are often well conserved between syntenic segments in the human and the mouse although there are occasional inversions and loss of some genes. Knockout mice can provide clues to gene function and an entry point to modeling complex human phenotypes. In this review, we will discuss the prospects of using the mouse to model a representative contiguous-gene–deletion syndrome, Miller-Dieker syndrome (MDS).

Isolated Lissencephaly Sequence and MDS

Isolated lissencephaly sequence (ILS [MIM 607432]) and MDS (MIM 24720) are specific human malformation syndromes, both of which exhibit classical lissencephaly, literally "smooth brain." Classical lissencephaly is a heterogeneous human developmental brain disorder caused by defects in neuronal migration events that normally occur at 9–13 wk of embryonic development. Lissencephaly encompasses varying degrees of agyria or pachygyria (the absence or thickening, respectively, of the convolutions of the cerebral cortex). Grade 1 consists of complete agyria, grades 2 and 3 describe mixed agyria/pachygyria, and grade 4 consists solely of pachygyria (de Rijk-van Andel et al. 1990). Classical lissencephaly is further characterized by a thick cerebral cortex with disorganized cortical layers, dysmorphic or hypoplastic corpus callosum, generalized neuronal heterotopias, enlarged ventricles, and microcephaly (Dobyns et al. 1983).

Individuals with ILS have lissencephaly of grades 2–4 but no other major malformations, whereas patients with MDS have a more severe grade of lissencephaly and display a characteristic dysmorphic facial appearance. This includes prominent forehead with bitemporal hollowing, short nose with upturned nares, thickened upper lip with a thin vermilion upper border, widely spaced eyes, low ears, and small jaw. MDS has been associated with other abnormalities, including central hypotonia, heart malformations, omphalocele, inguinal hernia, duodenal atresia, pelvic and cystic kidneys, sacral tails, cryptorchidism, polydactyly, clinodactyly, camptodactyly, transverse palmar creases, and polyhydramnios during pregnancy (Dobyns et al. 1991). Patients with ILS or MDS exhibit mental and motor impairment, epilepsy, and a reduced life span.

All cases of MDS and most cases of ILS are found to be associated with haploinsufficiency on chromosome

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17p13.3. (Schwartz et al. 1988; Dobyns et al. 1991; Ledbetter et al. 1992). Other cases of ILS are often associated with mutations in the doublecortin gene, located on chromosome Xq22.3-q23. In effect, all patients with MDS and ∼40% of patients with ILS have visible or submicroscopic deletions on 17p13.3 that are detectable by FISH. The MDS deletions are generally larger and more distal than those in patients with ILS. The *LIS1* gene was cloned from the 17p13.3 region and was found in certain patients with ILS to have either point mutations or an intragenic deletion (Reiner et al. 1993). This demonstrated that defects in *LIS1* are responsible for classical lissencephaly seen in patients with ILS and MDS. The heterozygous deletions and mutations of *LIS1* suggest that proper neuronal migration is sensitive to *LIS1* gene dosage. Thus, haploinsufficiency is the likely pathogenic mechanism, since there is no evidence for imprinting of *LIS1*. The spectrum of agyria/pachygyria in ILS is dependent on the location and type of mutation in the *LIS1* gene. Patients with missense mutations have a milder grade, whereas truncation and deletion mutations resulted in a more severe phenotype (Cardoso et al. 2000).

The most severe form of lissencephaly (grade 1) and other major MDS abnormalities are found only with larger (>250 kb) and more-telomeric deletions on chromosome 17p13.3, including and extending beyond *LIS1* (Chong et al. 1997). This is consistent with MDS being a contiguous-gene syndrome, in which deletion of physically contiguous but functionally unrelated genes causes a complex human genetic disease. Therefore, it has been suggested that genes distal to *LIS1* may be responsible for the facial dysmorphisms and other abnormalities seen in patients with MDS. Since complete agyria is seen only in MDS and not ILS, it has also been hypothesized that a second gene located within the MDS deletion may also be required for proper neuronal migration. It is noteworthy that, for 17p13.3-linked diseases, the facial features of MDS have not yet been seen without lissencephaly, and neither ILS nor MDS has been observed without *LIS1* deletion or mutation. In addition, although deletions have been documented that extend from *LIS1* toward the centromere, there appear to be no additional phenotypic consequences to the extension of deletions beyond *LIS1* (Chong et al. 1997). These data pose an interesting question: Does deletion of individual genes telomeric to *LIS1* contribute to specific MDS abnormalities, or are deletions of *LIS1* and a number of telomeric genes necessary for the complete MDS phenotype?

In a recent study, detailed analysis using FISH was performed on samples from 30 patients with ILS, ILS with mild facial dysmorphisms (ILS+), or MDS, to determine the difference in deletion size and to identify genes that may be responsible for the MDS phenotype (Cardoso et al. 2003); Cardoso et al. showed that as the deletion size increased, patients displayed more characteristics of MDS, such as facial dysmorphisms and a more severe LIS grade. Furthermore, the facial abnormalities seemed to indicate a more severe LIS grade. Patients with ILS always had LIS grade 3 or 4; patients with ILS+ had LIS grade 2 or 3; and only those with typical MDS facial dysmorphisms had LIS grade 1. The authors defined a telomeric MDS critical region of 400 kb that is consistently deleted in patients with ILS+ and MDS. The region contains eight genes: *PRP8, RILP, SREC, PITPNa, SKIP MYO1C, CRK,* and *14-3-3ε*.

Haploinsufficiency of these genes in addition to *LIS1* constitutes a contiguous-gene–deletion syndrome that contributes to both the facial and the brain dysmorphisms seen in MDS. The study by Cardoso et al. (2003) also confirmed that the severest form of LIS (complete agyria) is seen only in patients with MDS. The authors used PCR to map deletions gene by gene in patients with MDS and complete agyria. Two genes (*CRK* and *14-3-3ε*) were specifically deleted in patients who had MDS with the most severe form of LIS, indicating that deletion of one or both of these genes in addition to loss of *LIS1* may be responsible for complete agyria in MDS. Deletion telomeric of *14-3-3ε* causes no change in phenotype; however, deletion from the telomere through the MDS critical region without mutations or deletions of *LIS1* is associated with mental retardation. These patients did not have lissencephaly, indicating the presence of a gene or genes that may be involved in or responsible for mental retardation (Cardoso et al. 2003).

Human 17p13.3 and Mouse 11

The human data led to the determination of this MDS critical region. However, it is difficult, on the basis of human data, to directly assess the contribution of each gene in the MDS critical region to the phenotype. ILS and MDS are rare, making it nearly impossible to analyze a large enough panel of patients to allow for definitive conclusions. The mouse may be the most appropriate model to dissect and understand human contiguous-gene–deletion syndromes for the following reasons: the human and mouse genomes are similar in terms of number and order of genes of 1–2 Mb; humans and mice develop in a similar manner; and it is possible to specifically modify the mouse genome by gene-targeting and transgenic technologies.

Fortuitously, the genetic organization of the MDS critical region appears to be reasonably conserved from human to mouse, in that most of the known or predicted human genes have a mouse counterpart and are present in almost the same order in both organisms. The region deleted in MDS is located on a region of ∼2.9 Mb (from

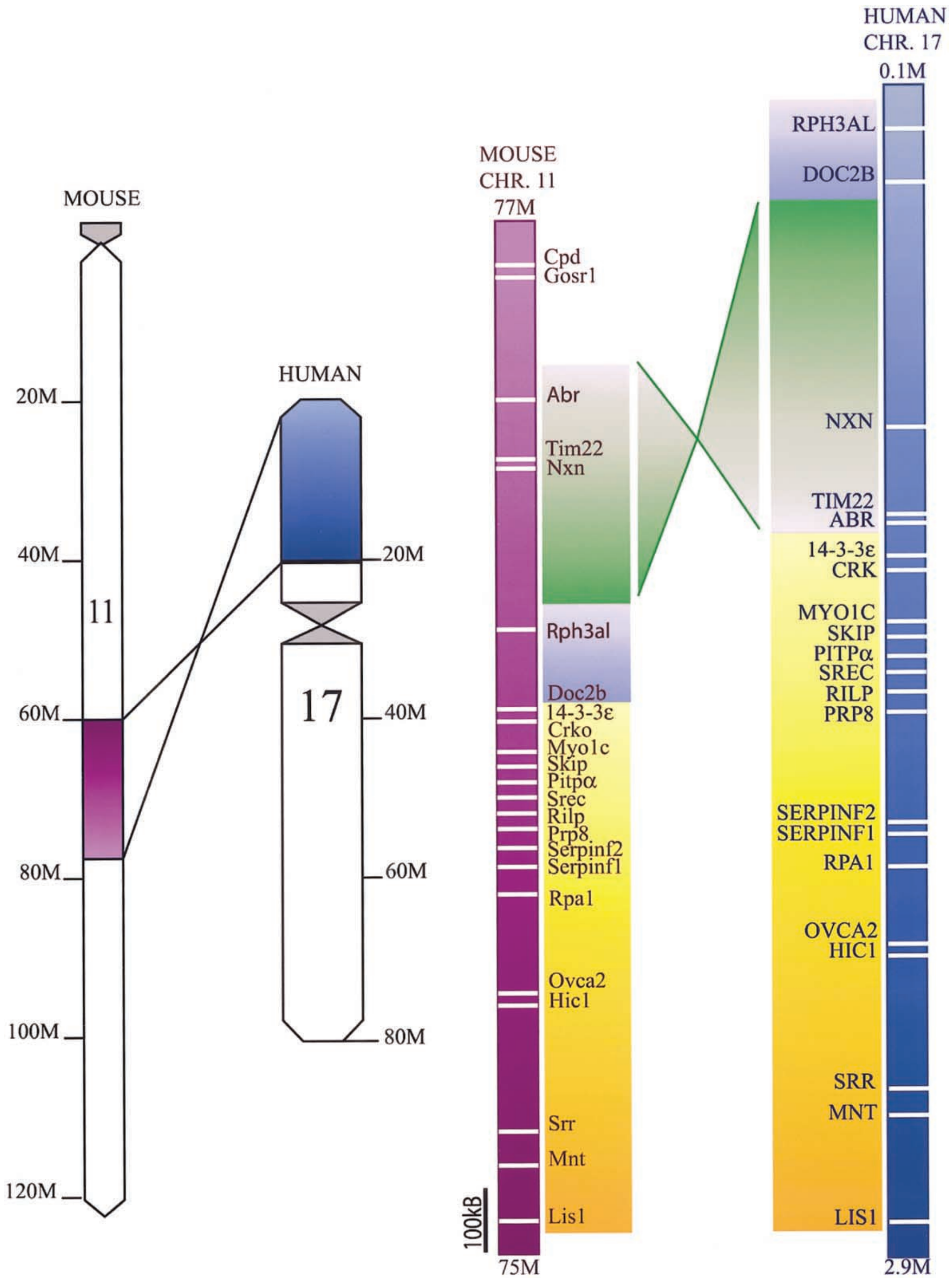

Figure 1 Schematic of the syntenic regions on human chromosome 17p13.3 and mouse chromosome 11. The distance between genes is approximated for clarity. The order and position of genes was obtained from the Human Genome Project Ensembl Database and Celera. There are some discrepancies between the two databases, and, when possible, these discrepancies were resolved by analysis of BACs (Cardoso et al. 2003).

0.1 Mb to 2.9 Mb) on chromosome 17p13.3, extending from *LIS1* to the telomere (fig. 1). Currently, 21 genes have been identified (fig. 1), and 28 genes have been proposed by Ensembl predictions for 17p13.3. Within this region, the MDS critical region appears to be located between *LIS1* and *14-3-3ε*. The short arm of human chromosome 17 corresponds to a region in the middle of mouse chromosome 11 (75.2–77.3 Mb). This region in the mouse is smaller (~1.9 Mb) than in the human, and homologues of all 21 known human genes in 17p13.3 are present. Of the 28 proposed genes in the human MDS region, mouse homologues of all but 9 have been found by Ensembl's Blast analysis. In addition, there are 11 proposed genes scattered throughout the region in both the mouse and human that have not been characterized or grouped by Ensembl predictions.

The genes from *LIS1* to *14-3-3ε,* corresponding to the MDS region, are in the same order in mice and humans. From *SERPINF2* to *PRP8* in the human genome, a potential region of duplication exists in the form of proposed genes that are similar to other genes in the region, but it is not known if these are functional genes. This duplication is not found in the mouse genome. The region of 17p13.3 adjacent to *14-3-3ε* in the human sequence, extending from *ABR* to *DOC2B,* is present in the mouse, but it is removed from the synteny block, placed centromeric to *Rph3al,* and inverted. Thus, all known genes in the MDS region are present in syntenic regions of the mouse and human genome, but there is some reorganization of adjacent genes.

Phenotypes of Mice Created with Mutations in Genes from the MDS Region

Null and conditional knockout alleles in the mouse have been generated for *Lis1* and *Mnt,* and null alleles have been produced for *Hic1* and *14-3-3ε*. For *Lis1* and *Pitpα,* hypomorphic alleles also exist. The phenotypes of these mice will be discussed in the order they occur in the MDS region.

Lis1

LIS1 is a 45-kDa protein that encodes for a noncatalytic subunit of platelet-activating factor acetylhydrolase 1B (PAFAH1B), so the formal name for *LIS1* is "*PAFAH1B1*" (Hattori et al. 1994). Although PAFAH has been shown to have a variety of neuron-specific effects, it remains unclear how PAFAH functions in neuronal migration. *LIS1* has homologues in all eukaryotes. Initial insight into the role of *LIS1* in neuronal migration came from identification of a *LIS1* homologue as the gene mutated in the nuclear distribution mutant *nudF* of the filamentous fungus *Aspergillus nidulans* (Xiang et al. 1995). NudF was shown to genetically interact with cytoplasmic dynein to properly position the nucleus within growing germlings. On the basis of these studies, it was proposed that LIS1 regulates dynein function to cause nuclear movement. Several studies have confirmed that *LIS1* homologues regulate cytoplasmic dynein in *Aspergillus, Drosophila,* and mammalian organisms (Willins et al. 1997; Liu et al. 1999; Smith et al. 2000), so it is believed that loss of this regulation may also be involved in the defects in neuronal migration that are associated with *LIS1* mutations in humans.

To further understand *LIS1* function and to evaluate its role in neuronal migration, two mutant alleles in mouse *Lis1* were generated in our laboratory. The knockout allele (*Lis1*^{ko} or *Pafah1b1-loxP*) is a complete null, and the conditional knockout allele (*Lis1*^{cko} or *Pafah1b1-neo*) is hypomorphic, because of disruption of transcription or splicing by the insertion of a phosphoglycerate kinase–neomycin cassette from the targeting vector into the second intron (Hirotsune et al. 1998). Complete loss of *Lis1* in *Lis1*^{ko} homozygotes results in early embryonic lethality. By mating mice to obtain various combinations of alleles, mice were generated with graded reduction in *Lis1* dosage. As *Lis1* dosage decreased, mice exhibited increased disorganization of the cortical layers, hippocampus, and olfactory bulb, because of migration defects (Hirotsune et al. 1998; Gambello et al. 2003). The cortex had no discernible lamination and was thinner than the wild type.

As *Lis1* dosage decreased, neuronal migration rates were also dramatically reduced both in vivo and in vitro (Hirotsune et al. 1998; Gambello et al. 2003). In vivo analysis of neuronal migration was performed using BrdU birth-dating. BrdU is incorporated into the newly synthesized DNA of S-phase cells and is often used to estimate the fraction of cells in S phase. During cortical development in mice (E14.5–E17.5), neuronal precursor cells divide in the ventricular zone and migrate through the cortex to their final position, as a function of time from birth date. A subset of proliferating neurons can be labeled with BrdU at one time point, and animals can be killed at later times to analyze the migration rate or distance traveled from the ventricular zone. The *Lis1*$^{+/ko}$ (~50% LIS1 protein) mice appear normal as adults, but BrdU birth-dating analysis in vivo shows that these neurons have reduced migration rates compared with the wild type. After a prolonged period, they reach their appropriate destination. The most severe genotype, *Lis1*$^{cko/ko}$ (~35% of LIS1 protein), exhibits a broader and more disorganized band of migrating neurons that remain closer to the ventricular zone. The migration rates were also tested in vitro by measuring the distance that cerebellar granule cells migrated along neurites from cell clusters plated on laminin. The wild-type neurons migrated four times farther than those of the *Lis1*$^{+/ko}$ mice. There was no difference in mean neurite length

(Hirotsune et al. 1998). These results confirmed that the neuronal migration phenotype seen in ILS is the result of dosage sensitivity and haploinsufficiency. Furthermore, *Lis1*$^{cko/ko}$ mice provide a model that reiterates many components of the human disease: disorganized cortical layers, generalized neuronal heterotopias, enlarged ventricles, and microcephaly (Gambello et al. 2003). Another mouse mutant was produced with a deletion of the first exon and part of the *LIS1* promoter (Cahana et al. 2001). A short LIS1 protein is produced in these mice, presumably because of initiation at the second methionine codon of *LIS1*. The protein appears to be a hypomorphic or combined hypomorphic/neomorphic allele, since heterozygotes are less severely affected than the *Lis1*^{ko} heterozygotes, but homozygotes display early embryonic lethality similar to that of *Lis1*^{ko} homozygotes.

In addition to its contribution to neuronal migration, there is evidence that LIS1 participates in cell division. In vitro cell culture studies indicate that LIS1 is localized at prometaphase kinetochores and at the cell cortex of dividing cells, both known binding sites for cytoplasmic dynein. Overexpression of LIS1 in fibroblasts results in mitotic defects, such as spindle misorientation and metaphase chromosome misalignment. When LIS1 is depleted, either by application of LIS1 antisense oligonucleotides or microinjection of anti-LIS1 antibody, mitotic defects are also observed (Faulkner et al. 2000). In the *Lis1*$^{cko/ko}$ mice, the brains are reduced in size. This may be the result of defects in neuronal proliferation or differences in survival. BrdU birth-dating analysis of *Lis1*$^{cko/ko}$ mice suggested that they have fewer proliferating neuroblasts and more apoptotic cells in the ventricular zone than do wild-type mice (Gambello et al. 2003). Both mechanisms are likely to contribute to a reduction in the number of neuronal precursors, leading to the hypothesis that, in addition to neuronal migration defects in lissencephalic brains, deficiencies in the genesis of neuronal progenitors may also be responsible for some aspects of the brain phenotype in ILS and MDS.

The histological defects in the mouse were associated with motor and cognitive impairments and increased susceptibility to seizures. *Lis1*$^{+/ko}$ mice were impaired on a rotarod test and a spatial learning version of the Morris water task (Paylor et al. 1999). The behavioral tests indicate that defects in neuronal migration and, perhaps, neurogenesis produced by *Lis1* haploinsufficiency have an impact on certain complex behavioral responses. Neuronal migration defects also affected the hippocampus of *Lis1*$^{+/ko}$ mice, which appeared disorganized, with loosely packed granule cells as well as discontinuities and splitting of the pyramidal cell layer, which included cells that are normally found in other regions. When neurons were impregnated with silver nitrate by the Golgi method, these heterotopic pyramidal cells displayed a significantly smaller dendritic arbor than was seen in wild-type or properly localized *Lis1*$^{+/ko}$ neurons. Electrophysiological studies confirmed that *Lis1*$^{+/ko}$ mice were more susceptible to seizures. Enhanced excitability and disorganized field potentials were seen in the *Lis1*$^{+/ko}$ hippocampus, in addition to hyperexcitability to electric stimulation (Fleck et al. 2000). These integrative behavioral and electrophysiological studies of *Lis1*$^{+/ko}$ mice provide a more comprehensive picture and relate the defects in neuronal migration to mental retardation and seizures seen in the patients with ILS.

Overall, the *Lis1*$^{+/ko}$ and *Lis1*$^{cko/ko}$ mice are excellent models for classical lissencephaly that reiterate many of the histological and behavioral defects, although not at the same dose reduction as in the human. In humans, a 50% reduction of *LIS1* in heterozygotes results in severe neuronal migration defects and lissencephaly. By contrast, severe defects analogous to human lissencephaly are seen only in *Lis1*$^{cko/ko}$ mutants, which have <50% of wild-type *Lis1* levels. These differences may be the result of more complicated development and longer migration in the human brain than in the mouse brain.

Mnt

The Max network consists of a group of transcription factors whose combination of interactions result in gene-specific transcriptional activation or repression that affects cell proliferation, differentiation, and death. To accomplish these tasks, Max forms heterodimers, through basic helix-loop-helix zipper motifs, with Myc and Mad protein family members, as well as the Mnt and Mga proteins. The biosynthesis of the Max-interacting proteins is strictly regulated, and they have short half-lives, whereas Max is stable and constitutively expressed (for a comprehensive review on the Myc/Max/Mad network, see Grandori et al. [2000]). The regulation and role of Max in this network depends upon Max-interacting proteins. Myc plays a central role in promoting proliferation and, consequently, in inducing neoplastic disease. Myc:Max heterodimers activate and repress transcription at certain target promoters to advance proliferation and block terminal differentiation. Mad:Max heterodimers appear to act as an opposing force to Myc:Max; Mads are transcriptional repressors with antiproliferative effects produced in a variety of cell types upon differentiation. The roles of Mnt and Mga are less clearly defined. Mnt displays similarities to Mad, in that Mnt can recruit the mSin3 corepressor complex to repress transcription in some cell types, and Mnt can prevent Myc/Ras transformation of primary cells.

Transgenic mice or mice with knockouts of Mad network members have not completely clarified the roles of these proteins in development. A null mutation in c-*Myc* or N-*Myc* is lethal in homozygous mice at E.10–E.12.

The embryos were smaller and were retarded in development, and they had abnormalities of the heart, pericardium, and neural tube (for review see Davis and Bradley [1993]). The inhibitory effects of Mad and Mnt on cell proliferation were demonstrated in vivo using mice that expressed a transgene of either *Mad* or *Mnt* under control of the β-actin promoter. Ubiquitous expression of *Mad* in transgenic mice led to early postnatal lethality and reduced body and organ weights; hematopoietic cells and embryonic fibroblasts isolated from these mice showed a decreased proliferative capacity (Queva et al. 1999). Mnt overexpression results in a developmental delay and death at ~E8.5–E10.5 (Hurlin et al. 1997).

On the basis of these data, one could postulate that knocking out *Mnt* or *Mad* might cause gigantism in mice, because these factors would not be present to oppose Myc in its promotion of cell proliferation. However, our data show that *Mnt* null mutant mice die perinatally and are smaller than wild type. Depending upon the genetic background, $Mnt^{-/-}$ mice have defects in thymic and craniofacial development, specifically skeletal abnormalities of the skull and cleft palate that are associated with a small jaw (K.T. and A.W-B., unpublished data). Patients with MDS as well as lissencephaly and cleft palate have been reported (Kerner et al. 1999). It may be that the cleft palate phenotype displayed by $Mnt^{-/-}$ mice is a model for the abnormalities in craniofacial development, including the small midface and jaw, that are seen in patients with MDS.

Hic1

HIC1 is a transcription factor and candidate tumor suppressor. *HIC1* stands for "hypermethylated in cancer," because, in many human cancers, *HIC1* is subject to gene silencing by abnormal methylation of its promoter (Issa et al. 1997; Melki et al. 1999). HIC1 is proposed to function as a transcriptional repressor that can interact with C-terminal binding protein (CtBP) and recruit histone deactylases (HDACs) (Deltour et al. 2002), but the target genes and pathways that are affected by HIC1 activity have not been determined.

HIC1 is found in all adult tissues, but its embryonic expression shows an interesting pattern. With the use of in situ hybridization, *HIC1* mRNA was detected in the precursors of certain tissues that are affected in MDS. For example, *HIC1* is expressed in the first branchial arch, which gives rise to the maxillary and mandibular components of the jaw and the face. Further *HIC1* expression is found in the mesenchyme surrounding peripheral nerves, adjacent to epithelial buddings for the nose, salivary glands, and pharyngeal pouches, and, transiently, in early corneal development. Notably, the mesenchyme that surrounds the infolding nasal epithelium was positive for *HIC1* mRNA. *HIC1* was also detected in the limb mesenchyme that surrounds the skeletal condensations and later in distinct rostral and caudal digit anlagen. Other regions of *HIC1* expression include the lateral body wall and the developing gut and kidney (Grimm et al. 1999).

Hic1 knockout mice die perinatally and exhibit a range of gross developmental defects, such as acrania, exencephaly, cleft palate, limb abnormalities, and omphalocele (Carter et al. 2000). No disorganization of the cerebral cortex or neuronal migration defects are observed. $Hic1^{-/-}$ embryos are generally smaller than wild-type embryos and are underdeveloped, but this may be secondary to the severe developmental abnormalities. Heterozygous *Hic1* mutants have no distinctive developmental phenotype, as is true of *Mnt* heterozygotes, but are predisposed to malignant tumors (Chen et al. 2003). The craniofacial defects include eye and jaw abnormalities, truncated or open secondary palate, shorter snouts, and lower-set, less developed ears. Is this the mouse version of the craniofacial defects seen in MDS (short nose with upturned nares, thickened upper lip with a vermilion upper border, widely spaced eyes, low ears, and small jaw), as argued elsewhere (Carter et al. 2000)? The evidence is ambiguous at this point, because *Hic1* heterozygotes have no distinctive facial phenotype, whereas, in humans, haploinsufficiency of either one or multiple genes is responsible for facial dysmorphisms. However, the knockout mouse does provide evidence that *Hic1* plays a role in craniofacial development.

Some $Hic1^{-/-}$ embryos have smaller and dysmorphic hind limbs and digits. Although these abnormalities do not exactly replicate the MDS phenotype (clinodactyly, camptodactyly, and polydactyly), they do suggest that *Hic1* is important for proper limb and digit development. $Hic1^{-/-}$ embryos also display ventral body wall defects, such as omphalocele, in which intestinal loops are seen outside the body cavity. In mouse development, the midgut grows in the body stalk while the abdominal cavity expands; at ~E16.5, the midgut returns to the body cavity, folding and rotating, and the ventral body wall fuses. In humans, rapid growth of the midgut around the 6th wk causes the intestine to herniate through the umbilical ring; in the 10th wk, the intestine returns to the abdominal cavity, and the umbilical ring fuses. Patients with MDS have presented with omphalocele and umbilical hernias. Abdominal wall defects result from the failure of the mesoderm to replace the body stalk. This, in turn, causes insufficient outgrowth and increased diameter of the umbilical ring. The similarities between the omphalocele in the $Hic1^{-/-}$ embryo and in patients with MDS underscore the possible role of *Hic1* in this aspect of the MDS phenotype.

Why is there no heterozygous phenotype in *Mnt* and *Hic1* mutants? It is possible that the craniofacial defects of MDS cannot occur without heterozygous deletion of

both *Hic1* and *Mnt* or, perhaps, the loss of these two genes as well as other genes. Defects in the heterozygotes might also be subtle, as are neuronal migration defects in heterozygous mouse models. Production of *Hic1*/*Mnt* double heterozygotes in the mouse would help to address these questions.

Phosphatidylinositol Transfer Protein α (PITPα)

PITPα is important for normal brain function, since reductions in PITPα mRNA levels were found in the spontaneous mouse mutant *vibrator* (*vb*). Described in 1982, *vb* carries an autosomal recessive mutation that causes an unusual rapid whole-body action tremor that begins around postnatal day 11 in homozygotes (Weimar et al. 1982). The tremor was attributed to degeneration of specific neurons in the spinal cord and, later, in the brainstem and cerebellum. It remains to be determined why only specific neurons in *vb* mice are sensitive to PITPα reduction. The *vb* neuropathology consists of progressive degenerative changes, with the appearance of dilated endoplasmic reticulum in cell bodies, dendrites, and axons. The neurons eventually develop severe intracellular vacuolation and undergo chromolysis (Weimar et al. 1982). Depending on the genetic background, the life spans of homozygous mice may vary from 30 to >160 d. The *vb* mutation was cloned using positional complementation. The cause of the *vb* mutation is a retroposon insertion in *Pitpα* that interferes with normal RNA accumulation of the gene. Reduced levels of PITPα protein to 60% and 20% of those in wild-type mice were seen in *vb* heterozygotes and homozygotes, respectively. The *vb* heterozygotes display no obvious phenotypic abnormalities (Hamilton et al. 1997).

PITPs exist in all eukaryotes, from yeast to humans. Their role in membrane trafficking is essential during regulated exocytosis, biogenesis of secretory vesicles/granules, and intra-Golgi transport. PITPs are also necessary for inositol lipid signaling via phospholipase C and phophoinositide 3 kinases (Cockcroft 2001). Reduction of PITPα protein may cause misregulation of downstream signaling pathways or a block in intracellular transport. PITPα knockout mice exhibit spinocerebellar disease, hypoglycemia, and intestinal and hepatic steatosis. Alb et al. (2003) suggest this phenotype is due to defective trafficking of lipids and fatty acids from the endoplasmic reticulum. Heterozygous PITPα mice have no distinctive phenotype.

It is unclear whether PITPα is involved in any of the neuronal defects displayed by patients with MDS. Tremors or neurodegeneration have not been described in these patients, which may be a consequence of their reduced life span although neurodegeneration in *vb* mutants appears to be related to onset of physical activity. It may also indicate that, for neurodegeneration to occur, PITPα must be reduced to levels <50% of normal, as is the case for the *vb* homozygote.

Crk

Crk was originally identified in chicken tumor samples as a viral oncogene that caused an increase in tyrosine-phosphorylated proteins. After the realization that *Crk* was a cellular gene, human and mouse *Crk* and *Crk*-like genes (*CRKL* in humans and *Crkol* in mice) were cloned. From there, a large number of studies have investigated the role of *Crk* family members in signal transduction. Crk/CRKL proteins form selective multiprotein complexes that produce different biological outcomes in response to a variety of extracellular stimuli (a review of Crk-family binding proteins and biological roles has been published elsewhere [Feller 2001]). The Crk family plays a major role in the reorganization of the cytoskeleton during cell migration, in cell-shape changes, and in the integration of growth and adhesion signals at focal adhesions. Also, Crk/CRKL are activated downstream of integrins and migration-inducing factors.

Both *CRK* and *CRKL* are located within deletion regions for syndromes with craniofacial phenotypes. Although *CRK* maps to the common deletion region for MDS, *CRKL* maps within that for DGS/VCFS, on human chromosome 22q11. The phenotype of DGS/VCFS includes cardiac anomalies, facial dysmorphisms, thymic hypoplasia, cleft palate, learning dysfunction, and hypocalcemia. DGS/VCFS is the result of developmental defects involving the third and fourth pharyngeal pouches, the result of defective migration of the neural crest cells during the 4th wk of embryogenesis.

During mouse development, *Crkol* is highly expressed in neural crest–derived tissues. The neural crest contributes to proper formation of connective tissue and skeletal structures of the head. Mice homozygous for a targeted null mutation in *Crkol* exhibit multiple defects in cranial and cardiac neural crest derivatives, including cranial ganglia, aortic arch arteries, cardiac outflow tract, thymus, parathyroid glands, and craniofacial structures (Guris et al. 2001). Less than 2% of *Crkol*$^{-/-}$ mice survive past birth. Those that do have recognizable abnormalities of facial features, such as ocular hypertelorism and a wider, shorter skull. Because migration and early expansion of neural crest cells is normal in *Crkol*$^{-/-}$ mice, a postmigratory defect in differentiation, survival, or function of specific neural crest cells is most likely affected.

The defects seen in *Crkol*$^{-/-}$ mice argue against redundant functions for *Crk* and *Crkol*. However, this does not exclude the hypothesis that Crk may be necessary for neural crest migration or may be part of other, possibly cooperative, signaling pathways for neural differentiation, survival, and function. Overexpression of

v-Crk in sensory, motor, and enteric neurons potentiates motor neuron survival and axonogenesis, even in the absence of exogenous growth factor. In this system, v-Crk also increased the volume of motor end plates and the complexity of neuromuscular junctions (Weinstein et al. 1999). This study provides important in vivo evidence of the role of *Crk* family members in developing neurons. The development of a knockout mouse will be important to the understanding of whether loss of *Crk* also affects neural crest cells and the tissues derived from them or whether loss of *Crk* has some other unpredicted role in development.

14-3-3ε

The *14-3-3* family of genes is found in all eukaryotic cells. These highly conserved regulatory molecules bind to phosphoserine/phosphothreonine motifs in a sequence-specific manner. Through this interaction, 14-3-3 proteins are known to have >100 binding partners and to be involved in modulating a multitude of cellular functions, including signal transduction, cell cycle control, apoptosis, stress responses, vesicular transport, and malignant transformation (Skoulakis and Davis 1998; Fu et al. 2000). 14-3-3 proteins can regulate their binding partners by any of the following methods: affecting the target proteins' ability to bind other partners, changing the proteins' rate of nuclear transport, altering their catalytic activity, protecting them from proteolysis or dephosphorylation, or providing an adapter/scaffold function to bridge protein interactions (Tzivion and Avruch 2002).

Separate genes encode for the seven 14-3-3 isoforms known in mammals, each named with a Greek letter (β, ε, γ, η, σ, τ, or ζ). Ten isoforms are found in plants and two each in yeast, *Drosophila,* and *Caenorhabditis elegans*. The 14-3-3 proteins can form homo- and heterodimers, and mutant forms of these proteins can act as dominant inhibitors in vivo. The dimerization state may act to cross-link two proteins, to generate specificity, or to increase the affinity for one protein (Yaffe et al. 1997). The degree of specificity and redundancy within the 14-3-3 family is not known, because the key residues in the ligand-binding domain are conserved among all the proteins. However, variable residues around the ligand-binding domain could confer specificity. In vitro, 14-3-3 proteins show variable affinities to certain target proteins, and it has been proposed that slight changes in binding affinity may have biological relevance in vivo. Furthermore, phosphorylation and protein level, both temporal and spatial, may modulate dimerization and function of 14-3-3 proteins. Since heterodimers can form, there is great potential for exquisite specificity for target binding that will require further biochemical and genetic analysis.

14-3-3 proteins were initially discovered in 1967 in a biochemical screen for brain-specific proteins (Moore and Perez 1967). With the use of starch electrophoresis, 14-3-3 proteins were easily detected because of their relative abundance (~1% of proteins) in the brain. In 1996, 14-3-3 proteins were implicated as binding partners to oncogenic proteins, and further research has focused mainly on their role in cell cycle, apoptosis, and differentiation (Jelinek et al. 1996; Liu et al. 1996). The various expression patterns of *14-3-3* isoforms in the brain during mouse embryogenesis underscore their potential significance in neuronal development (Watanabe et al. 1993; McConnell et al. 1995). The 14-3-3 proteins are also important in signaling and neuronal development in *Drosophila* (Skoulakis and Davis 1998). 14-3-3ε plays an important role in *Drosophila* eye development by increasing the efficiency of RAS1 signaling (Chang and Rubin 1997). Homozygous null mutants of *leonardo* (14-3-3ζ) die as mature embryos, and viable *leonardo* mutants show olfactory learning deficits correlated with the level of expressed protein (Skoulakis and Davis 1996; Broadie et al. 1997), supporting a role for *14-3-3* dosage in neurons. These studies also suggest that haploinsufficiency of *14-3-3*ε in MDS may have negative effects on neuronal development.

In our laboratory, a null allele of *14-3-3*ε was created. Recently, we found that mouse mutants of *14-3-3*ε display dose-dependent defects in neuronal development and migration (Toyo-oka et al. 2003). In both *14-3-3*$\varepsilon^{+/-}$ and *14-3-3*$\varepsilon^{-/-}$, the cortex and hippocampus form in the normal inside-out fashion, similar to developmental events in *Lis1*$^{+/ko}$ mice. The *14-3-3*ε heterozygotes show mild disorganization in the hippocampus and thinning of the cortex that becomes more severe with complete loss of *14-3-3*ε. Neuronal precursor proliferation in the ventricular zone that occurs between E12.5 and E15.5 is not affected in either *14-3-3*$\varepsilon^{+/-}$ or *14-3-3*$\varepsilon^{-/-}$ mice, as detected by BrdU analysis, but a threefold increase, compared with wild type, in apoptotic cells in the cortex at E12.5 of *14-3-3*$\varepsilon^{-/-}$ may account for the cortical thinning. Neuronal migration was also quantitated by BrdU birthdating analysis, which revealed that, as in *Lis1* mutant mice, the rate of neuronal migration decreases as the dose of *14-3-3*ε is reduced.

Double heterozygotes of *Lis1* and *14-3-3*ε were generated to test whether the similarity of phenotypes between *Lis1* and *14-3-3*ε mutants signified a genetic interaction necessary for proper neuronal migration. Indeed, double heterozygotes of *14-3-3*ε and *Lis1* display more-severe migration defects in both the cortex and hippocampus than do single heterozygotes, providing genetic evidence that *14-3-3*ε may be responsible for the most severe form of lissencephaly (complete agyria) seen in MDS (Toyo-oka et al. 2003).

The genetic interaction seen in vivo between *Lis1* and

14-3-3ε stimulated an investigation of how 14-3-3ε works within the LIS1 pathway/complex to affect neuronal migration (Wynshaw-Boris and Gambello 2001; Gupta et al. 2002). LIS1 binds to cytoplasmic dynein heavy chain (CDHC) and NUDEL, which is one of two identified mammalian homologues of the nuclear distribution mutant *nudE* of *A. nidulans* (Niethammer et al. 2000; Sasaki et al. 2000). Part of the regulation of this complex depends upon phosphorylation of NUDEL by CDK5, a kinase required for proper neuronal migration (Niethammer et al. 2000; Sasaki et al. 2000). CDK5 phosphorylates NUDEL in the region required for CDHC binding and is thought to regulate activity or motility of dynein in this manner (Sasaki et al. 2000). Two-hybrid and immunoprecipitation experiments in yeast demonstrated that phosphorylation of NUDEL by CDK5 is necessary for 14-3-3ε binding (Toyo-oka et al. 2003). 14-3-3ε may protect NUDEL from dephosphorylation by PP2A phosphatase. NUDEL binds specifically to 14-3-3ε but not to other 14-3-3 proteins. This interaction is important for proper localization of dynein, presumably by maintaining NUDEL in the phosphorylated state so that it can interact with dynein (Toyo-oka et al. 2003).

The study reported by Toyo-oka et al. (2003) demonstrates the power of mouse genetics to determine the contribution of each gene to a complicated phenotype by combining mouse mutants. We must await the creation of a *Crk* null allele to test whether *CRK* also plays a role in neuronal migration and can genetically interact with *LIS1* and *14-3-3ε*.

Other Genes with Functions that May Be Important for MDS

The remaining 15 known genes in the 17p13.3 region are briefly described in table 1. Some of the genes in this region code for proteins that run the gamut of cellular functions that are important for development, including proliferation, migration, cytoskeletal organization, dynein regulation, lipid signaling, membrane trafficking, and transcriptional control (see table 1 for references). Five of the eight genes identified as part of an MDS critical region remain to be discussed: *PRP8, RILP, SREC, SKIP,* and *MYO1C* (Cardoso et al. 2003). We can speculate that some of these genes may contribute to the MDS phenotype. *PRP8,* the most conserved splicing factor, regulates spliceosome activity. Neuronal migration relies on the integrity and function of the cytoskeleton, as we discussed in the *"Lis1"* section above. *CRK* and *SKIP* are proposed to modulate changes in the cytoskeleton. Deficiencies in the above genes could affect craniofacial development and neuronal migration by disrupting proliferation, migration, or cytoskeletal integrity.

Another group of genes in this region are involved in membrane trafficking and exocytosis. These genes may be important for neuronal homeostasis and transmitter release. RILP recruits dynein-dynactin motor complexes to Rab7-containing late endosomes and lysosomes. Thus, RILP could interact with the LIS1/dynein complex, thereby modulating the latter's function, although it is not known whether such interactions exist. MYO1C, a member of the myosin I motor family, localizes at regions of high actin concentration and within the Golgi apparatus. Other genes involved in membrane trafficking and exocytosis include *SREC* and *PITPα*. Haploinsufficiency of these genes may cause mislocalization of membrane components and misregulation of neurotransmitter release. This disruption in homeostasis could result in neuronal loss or, possibly, seizures.

Conclusions and Speculation

The genetic analysis of ILS and MDS in 30 patients revealed that *CRK* and *14-3-3ε* were exclusively deleted only in those patients with complete agyria, implicating one or both genes in cortical development. Furthermore, the data show that, as the deletion size increased to include the newly defined telomeric MDS critical region, the lissencephaly and facial phenotype worsened. The consensus MDS facial phenotype was also seen only with deletion of *CRK* and *14-3-3ε* (Cardoso et al. 2003). Heterozygous deletion of both *Lis1* and *14-3-3ε* in the mouse produces a greater defect in neuronal migration than do single heterozygous mutations (Toyo-oka et al. 2003). The human and mouse data indicate that the severe neuronal migration phenotype (complete agyria) seen in patients with MDS may be due to deletion of *LIS1* and *14-3-3ε* although it remains to be determined whether *CRK* or other genes in the MDS critical region play a role in cortical development.

Concerning the MDS facial dysmorphisms, it remains uncertain whether the distinctive facial phenotype is a result of lissencephaly or haploinsufficiency of multiple genes important for craniofacial development. One patient, who presented with mild mental retardation but without lissencephaly and facial dysmorphisms, had a deletion from the telomere through *CRK*, *14-3-3ε,* and other genes in the MDS critical region (Cardoso et al. 2003). However, we cannot draw meaningful conclusions without analysis of additional patients with 17p13.3 deletions. Patients with mild facial abnormalities have LIS grade 2 or 3, whereas only patients with LIS grade 1 (complete agyria) have consensus MDS facial phenotypes. The development of additional mouse knockouts of other genes in the MDS critical region, especially *Crk,* will help dissect the genetic contributions to the craniofacial defects seen in patients with MDS.

From the study of these various mouse mutants, specific directed questions can be asked about the relationship between deletion size, haploinsufficiency effect,

Table 1

Genes Located on Human Chromosome 17p13.3

Gene	Description
LIS1 (PAFAH1B)[a]	Noncatalytic subunit of platelet-activating factor acetylhydrolase; part of conserved dynein regulatory pathway; when mutated or deleted, causes classical lissencephaly (Hirotsune et al. 1998; Wynshaw-Boris and Gambello 2001)
MNT[a]	Basic-helix-loop-helix zipper transcription factor; part of the Max network affecting cell proliferation, differentiation, and death (Grandori et al. 2000)
SRR	Serine racemase; catalyzes synthesis of D-serine from L-serine; possible roles in neurotransmitter synthesis and signaling (De Miranda et al. 2000; Snyder and Kim 2000)
HIC1[a]	Transcription factor; interacts with C-terminal binding protein and recruits histone deacytelases to repress transcription; *Hic1*$^{-/-}$ mice die perinatally and exhibit a range of gross developmental defects (Carter et al. 2000; Deltour et al. 2002)
OVCA2 (DPH2L)	Candidate tumor suppressor genes; diphthamide biosynthesis protein 2–like; ubiquitously expressed but reduced mRNA levels in analyzed ovarian tumors and tumor cell lines (Schultz et al. 1996; Wiper et al. 1998; Prowse et al. 2002); inhibits proliferation, suggested to be a growth regulator (Phillips et al. 1996; Liu et al. 2000)
RPA1	Replication protein A1; ubiquitous eukaryotic gene with roles in DNA replication, repair, and recombination (Umezu et al. 1998)
PEDF (SERPINF1) (SERPINF2)	Pigment epithelium–derived factor; factor that promotes survival, differentiation, and neuroprotection in a variety of neuronal cell culture models, including cerebellar granule cells, photoreceptors, and spinal cord motor neurons (Araki et al. 1998; Houenou et al. 1999; Jablonski et al. 2001; Yabe et al. 2001)
PRP8[b]	Most highly conserved splicing factor; important for coordination of multiple processes in spliceosome activation (Kuhn et al. 2002)
RILP[b]	Rab7-interacting lysosomal protein; involved in the recruitment of functional dynein-dynactin motor complexes to Rab7-containing late endosomes and lysosomes (Jordens et al. 2001)
SREC[b]	Scavenger receptor expressed by endothelial cells; mediates endocytosis of modified lipoproteins, such as acetylated or oxidized low-density lipoprotein (Adachi and Tsujimoto 2002)
PITPα[a,b]	Important for inositol lipid signaling and membrane trafficking; gene mutated in *vibrator* mouse (Hamilton et al. 1997; Cockcroft 1999)
SKIP[b]	Skeletal muscle and kidney enriched inositol phosphatase; may negatively regulate the actin cytoskeleton through hydrolyzing inositol signaling (Ijuin et al. 2000)
MYO1C (MYO1E, MYR-3)[b]	Intestine-specific single-headed member of the myosin I motor family; localized at adherens-type intercellular junctions, regions of high actin concentration, and Golgi apparatus (Stoffler et al. 1998; Skowron and Mooseker 1999)
CRK I, CRK II[b]	SH2/SH3 adaptor molecules; isoforms produced by alternate splicing; integrates many signal transduction pathways to affect changes in the cytoskeleton during cell growth and migration (Feller 2001)
14-3-3ε[a,b]	Part of large family of proteins that are involved in many cellular functions; regulates binding partners by binding to specific phosphoserine/phosphothreonine motifs (Yaffe et al. 1997; Fu et al. 2000; Tzivion and Avruch 2002)
ABR	Active breakpoint cluster region (BCR)–related gene; may function with BCR and Rho family members in signaling; double homozygous mice (ABR$^{-/-}$/BCR$^{-/-}$) exhibit vestibular dysgenesis and glial cell abnormalities (Tan et al. 1993; Chuang et al. 1995; Kaartinen et al. 2001; Kaartinen et al. 2002)
TIMM22	Predicted gene; mitochondrial import inner membrane translocase subunit (Ensembl gene number ENSG00000108911)
NXN	Considered an ambiguous gene; Blast analysis suggests it is similar to mouse nucleoredoxin, which regulates protein activity and gene expression through thiol-mediated oxidoreduction (Hirota et al. 2000)
DOC2B	Implicated in synaptic vesicle exocytosis; homologue DOC2A is brain specific, but DOC2B is expressed ubiquitously and may have other developmental functions (Verhage et al. 1997; Korteweg et al. 2000)
RPH3AL	Rabphilin-like gene; similarities suggest involvement in endocrine exocytosis and interactions with cytoskeleton (Smith et al. 1999)

NOTE.—Genes are shown in order from *LIS1* to the telomere.

[a] Mouse models have been created.

[b] Genes identified as being within the critical region for Miller-Dieker syndrome (Cardoso et al. 2003).

and phenotype. Double heterozygotes might be informative if two genes interact in the same pathway or if haploinsufficiency of both genes compounds the phenotype. For example, *Hic1*- and *Mnt*-deficient mice each have distinctive craniofacial defects, whereas the heterozygotes do not have any discernible phenotypic abnormalities. Double heterozygotes may reveal that haploinsufficiency of both *Hic1* and *Mnt* is necessary to produce a defect in craniofacial development and would suggest that multiple genes contribute to the MDS facial phenotype. Alternatively, it is possible that responsibility is not restricted to one or two genes and that the entire region must be deleted for the MDS phenotype to develop. If this is the case, strategic deletions of varying size within the MDS critical region in the mouse will need to be generated using combinations of conditional knockout alleles. With this strategy, a mouse could be generated with a deletion of all the genes in the region except *Lis1*. This would answer a long-standing question that has not been resolved by the patient data: Is deletion of *Lis1* necessary for the craniofacial defects?

In considering the use of mouse models to analyze haploinsufficiency in human disease, we must also consider how to approach the analysis of milder phenotypes in the mouse. The absence of a phenotype in heterozygous mice may truly represent a lack of a discernibly distinct phenotype, but alternative explanations are possible. For example, it may be that examination of these mice has not been sufficiently rigorous, that certain genes are not susceptible to dosage, or that there are genetic background effects that mask the phenotype. To discern subtle changes in phenotype and to make comparisons between mice, careful phenotypic analysis must be applied. For example, the $Lis1^{+/ko}$ mice appear outwardly normal; however, thorough BrdU analysis at various time points demonstrates slower rates of neuronal migration in vivo (Hirotsune et al. 1998). In adult mice, these neurons eventually reach a position close to the proper destination. In the case of dysmorphism in mice, detection of mild craniofacial defects may require comprehensive skeletal measurements on many animals, using detailed and consistent standards that are applied across mouse models.

The existence of seemingly normal heterozygous mice also raises questions about whether mice are as sensitive as humans to haploinsufficiency. To see a significant change in phenotype, dosage may need to be manipulated to levels <50%. For example, two alleles exist for *Lis1:* a null allele ($Lis1^{ko}$) and a hypomorphic allele ($Lis1^{cko}$). *Lis1* protein levels are ~45% those of wild-type mice in the $Lis1^{+/ko}$ animals and 35% in the $Lis1^{cko/ko}$ animals. This additional 10% reduction results in dramatic neuronal migration defects and shortened life spans that appear to be more representative of the severe ILS phenotype than are $Lis1^{+/ko}$ mice (Gambello et al. 2003).

Genetic background effects can also mask distinctive phenotypes in mice. *vb* and *Mnt* knockout mice have strain-dependent phenotypes. The ability to control strain background in mice will likely be an advantage in studying the mechanisms of variability in humans, since it is known that patients from the same family with precisely the same deletion do not necessarily present with the same features. If phenotypic modifiers are mapped, they may implicate larger pathways that may provide further insight into the range of phenotypes seen in the human disease.

There is still much we need to understand about the genes responsible for complex phenotypes in contiguous-gene–deletion syndromes, such as MDS. Much of what we learn will, by necessity, require careful study of human patients. However, the utility of mouse models for refining our understanding of the relationship between gene function and phenotypes will greatly contribute to these studies and will help to provide further mechanistic insight into the developmental and genetic pathways that contribute to these complex phenotypes.

Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

Ensembl Genome Browser, http://www.ensembl.org (for order and position of genes and for *TIMM22* [accession number ENSG00000108911])
Celera, http://publication.celera.com/humanpub/index.jsp (for order and position of genes)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for DGS, ILS, MDS, and VCFS)

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