A Molecular Pathway Revealing a Genetic Basis for Human Cardiac and Craniofacial Defects

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Microdeletions of chromosome 22q11 are the most common genetic defects associated with cardiac and craniofacial anomalies in humans. A screen for mouse genes dependent on dHAND, a transcription factor implicated in neural crest development, identified Ufd1, which maps to human 22q11 and encodes a protein involved in degradation of ubiquitinated proteins. Mouse Ufd1 was specifically expressed in most tissues affected in patients with 22q11 deletion syndrome. The human UFD1L gene was deleted in all 182 patients studied with 22q11 deletion, and a smaller deletion of approximately 20 kilobases that removed exons 1 to 3 of UFD1L was found in one individual with features typical of 22q11 deletion syndrome. These data suggest that UFD1L haploinsufficiency contributes to the congenital heart and craniofacial defects seen in 22q11 deletion.

Congenital heart defects (CHDs) are the most common of all human birth defects and are responsible for the 22q11 deletion syndrome (4). CHDs involving the outflow tract of the aortic arch, 30% with persistent truncus arteriosus (failure of septation of aorta and pulmonary arteries), and 15% with tetralogy of Fallot (malalignment of aorta and pulmonary artery with ventricles) (5). Such cardiac defects are common after neural crest ablation in chick embryos (6), suggesting that one or more genes regulating neural crest cells may map to 22q11. A region 2.0 megabases in length is most commonly deleted and is called the DiGeorge Critical Region (DGCR). Extensive mapping, positional cloning, and sequencing of the human and syntenic mouse DGCR have been performed (7, 8); however, mutation analyses of candidate genes in humans and deletion studies in mice have failed to identify any genes responsible for the 22q11 deletion syndrome (7).

The basic helix-loop-helix transcription factor dHAND is required for survival of cells in the neural crest–derived branchial and aortic arch arteries and the right ventricle (9–11). Mice lacking endothelin-1 (ET-1) have cardiac and cranial neural crest defects typical of 22q11 deletion syndrome and display down-regulation of dHAND (11, 12), suggesting that a molecular pathway involving dHAND may be disrupted in this syndrome. The genes for dHAND, ET-1, or the ET-1 receptor, however, do not map to 22q11 in humans (11, 12).

To investigate the potential mechanisms through which dHAND might function, we identified dHAND-dependent genes by suppressive-subtractive hybridization (13), a procedure that yielded genes expressed in wild-type but not dHAND mutant embryos at embryonic day 9.5 (E9.5). One of the dHAND-dependent genes (Ufd1) was the mouse homolog of a yeast gene involved in degradation of ubiquitinated proteins (14). Ufd1 is essential for cell survival in yeast (14) and is highly conserved from yeast to humans (15). Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) (16) confirmed that Ufd1 was down-regulated in dHAND-null hearts at E9.5 (Fig. 1A).

This finding that Ufd1 was down-regulated in dHAND mutants was intriguing because human UFD1L is located in the DGCR (Fig. 1B) and was shown to be deleted in 13 patients with 22q11 deletion (15). To determine the frequency of UFD1L deletion, we studied 182 patients with 22q11 deletion, as detected by fluorescence in situ hybridization (FISH) (17). Our FISH analysis revealed UFD1L...
deletion in 182 of 182 patients (Fig. 1, C and D).

To determine if Ufd1, like dHAND, might play a role in cardiac and craniofacial development, we examined the embryonic expression pattern of mouse Ufd1 (18). Ufd1 was expressed in the first through fourth branchial arches, abnormalities of which are the basis of much of 22q11 deletion syndrome, with enrichment in the tips of the branchial arches, similar to dHAND expression (Fig. 2, A, B, and G). Ufd1 expression in the palatal precursors and frontonasal region was prominent (Fig. 2, B, D, and G), an important finding because cleft palate and facial anomalies are common features of 22q11 deletion syndrome. The developing ear (otocyst) as previously reported (Fig. 2E). A role for Ufd1 in the developing ear (otocyst) as previously reported (Fig. 2E). A role for Ufd1 in the hippocampus, which is involved in long-term memory, would be consistent with the learning impairment that often accompanies 22q11 deletion.

Within the heart, neural crest–derived cells are required for septation of the cardiac outflow tract into the aorta and pulmonary artery (19) and for remodeling of the bilaterally symmetric aortic arch arteries that form the mature aortic arch and proximal pulmonary arteries (20). Ufd1 transcripts were detected in the conotruncus (cardiac outflow tract) just as neural crest cells condensed and underwent ectomesenchymal transformation (Fig. 2I). At E9.5 to E10.0, Ufd1 expression was most evident in the fourth aortic arch artery (Fig. 2, B and I), which is responsible for formation of the segment of the aortic arch that lies between the left carotid and subclavian arteries (Fig. 3E). This segment does not form in interrupted aortic arch type B, one of the most common cardiac defects associated with 22q11 deletion (Fig. 3F). Vascular mesenchymal cells surrounding the proximal aorta also express Ufd1 (Fig. 2H), similar to dHAND. In dHAND-null embryos, Ufd1 was down-regulated in the branchial arches and conotruncus, but was detected at lower levels in the limb bud and was not affected in the developing ear (Fig. 2C). The expression of Ufd1 in numerous tissues affected in 22q11 deletion syndrome and its involvement in a molecular pathway regulating neural crest development suggest that Ufd1 may play a role in many features of this disease.

To determine if UFD1L haploinsufficiency is responsible for part of the 22q11 deletion phenotype, we searched for UFD1L deletions in individuals with cardiac and craniofacial defects who did not have detectable 22q11 deletions (21). Southern analysis of genomic DNA hybridized with a UFD1L cDNA probe revealed one individual (JF) with monoallelic deletion of exons 1 to 3 of UFD1L (Fig. 3, A and D), leaving exons 4 to 12 intact. Deletion of exons 1 to 3 was not detected in the parents, both phenotypically normal, suggesting that the UFD1L deletion in JF occurred de novo. Deletion of UFD1L was not detected in 100 normal unrelated individuals. As expected, expression of UFD1L mRNA in the thymus of patient JF (22) was diminished compared to controls without cardiac neural crest defects (Fig. 3B), confirming haploinsufficiency associated with this deletion.

The phenotype of patient JF encompassed nearly all features commonly associated with the 2-Mb 22q11 deletion. Four days after birth, she was diagnosed with interrupted aortic arch (Fig. 3F), persistent truncus arteriosus, cleft palate, small mouth, low-set ears, broad nasal bridge, neonatal hypocalcemia, T
lymphocyte deficiency, and syndactyly of her toes. Although other chromosome deletions can also result in a similar phenotype (23), the genotype of JF and the UFD1L expression pattern suggest that UFD1L haploinsufficiency can contribute to many features observed in 22q11 deletion syndrome.

There is substantial variability in the phenotype associated with 22q11 deletions. Thus, it is possible that other genes in this region, distant modifier genes, or environmental factors could contribute to distinct features of 22q11 deletion syndrome. HirA, the human homolog of a yeast histone regulatory gene (24), is expressed in the cardiac neural crest and maps 50 kb centromeric of UFD1L. However, HirA was not deleted and was expressed normally in the thymus of patient JF (25). CDC45, the human homolog of a yeast cell cycle protein, is immediately telomeric to UFD1L (26) and was used to define the 3' breakpoint of the deletion in patient JF (21, 25) to the region between exons 5 and 6 of Cdc45 (Fig. 3D). Although the ubiquitous nature of Cdc45 expression (27) and its normal expression in dHAND mutants (Fig. 3C) (16) make it an unlikely candidate gene for the 22q11 deletion syndrome, it is conceivable that the deletion of CDC45 may act as a modifier of patient JF's phenotype.

Our results support a role for ubiquitin-mediated mechanisms in controlling neural crest development. Ubiquitin-specific proteases are essential for regulating numerous critical cellular pathways, including those involving p53-related cell survival and NF-kB (nuclear factor-kB) activity (28). In vitro overexpression or inhibition of ubiquitin-specific proteases results in programmed cell death, indicating that their activity is dose-dependent (29). Yeast lacking Ufd1 exhibit a cell survival defect that is incompletely rescued by one allele of Ufd1 (14), consistent with the notion that haploinsufficiency of UFD1L may contribute to the phenotype seen in 22q11 deletion syndrome. We speculate that UFD1L haploinsufficiency leads to accumulation of certain proteins and defective survival of cardiac and cranial neural crest cells, resulting in premature thymic apoptosis and loss of cells that contribute to the transverse aortic arch, palate, and craniofacial structures. Further mutation analysis of UFD1L in humans and elucidation of the cellular pathways regulated by UFD1L may provide new directions in understanding basic mechanisms of neural crest development and congenital cardiac and craniofacial defects.

References and Notes

Regulation of Chamber-Specific Gene Expression in the Developing Heart by Irx4

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The vertebrate heart consists of two types of chambers, the atria and the ventricles, which differ in their contractile and electrophysiological properties. Little is known of the molecular mechanisms by which these chambers are specified during embryogenesis. Here a chicken iroquois-related homeobox gene, Irx4, was identified that has a ventricle-restricted expression pattern at all stages of heart development. Irx4 protein was shown to regulate the chamber-specific expression of myosin isoforms by activating the expression of the ventricle myosin heavy chain-1 (VMHC1) and suppressing the expression of the atrial myosin heavy chain-1 (AMHC1) in the ventricles. Thus, Irx4 may play a critical role in establishing chamber-specific gene expression in the developing heart.

During embryonic development, the ventricles and atria of the heart arise from a single tubular structure (1, 2). Mature atria and ventricles differ in their contractile and electrophysiological characteristics and express distinct sets of genes (3, 4). Most of the known chamber-specific genes encode isoforms of contractile proteins, including the myosin heavy chains and light chains (4). The correct expression of these myosin isoforms is essential for embryonic survival and proper function of the mature heart (5). The mechanisms involved in regulation of these chamber-specific patterns in the developing heart are unknown. Here we show that in chick hearts, this process requires the proper regulation of the iroquois-related homeobox gene Irx4.

The Irx4 gene was identified by a low-stringency hybridization screening of a chick embryonic day 6 to 8 (E6–E8) retinal cDNA library, with probes derived from mouse and human EST clones that span the Iroquois homeodomains (6). The predicted open reading frame contains a homeodomain highly homologous to those in the Drosophila Iroquois proteins (7, 8), and it is most closely related to the human IRX4 (83% amino acid identity overall and 70% amino acid identity outside of the homeodomain) (Fig. 1, and supplementary data available at www.sciencemag.org/feature/data/985642.shl). We have also identified a mouse IRX4 with 71% overall amino acid homology to chick Irx4 (9).

By in situ hybridization (10), we detected Irx4 expression in the retina, a subset of nuclei in the hindbrain, the developing feather buds, and the heart (Fig. 1, B to D) (11). Irx4 was highly expressed in the ventricular myocardium, but expression was absent from the atria or the distal outflow tract in the developing heart. Low levels of expression were observed in the proximal outflow tract. The ventricle-specific expression pattern was observed as stage 10 in the prospective ventricular region and persisted in all developmental stages examined (Fig. 1, B to D, and Fig. 2). The expression of the mouse Irx4 gene was similarly restricted to the ventricles in all stages of developing heart and adult heart (9).

We next compared Irx4 with other genes known to have a chamber-restricted expression pattern in the developing chick heart. Irx4 expression was first observed at Hamburger-Hamilton (HH) stage 10, when the developing heart is a linear tube. At stage 10, Irx4 expression was already restricted to the middle portion of the heart tube, which corresponds to the prospective ventricles (Fig. 2B). The ventricle-restricted pattern persisted to later stages (Fig. 2, E and H). In contrast to Irx4, ventricle myosin heavy chain-1 (VMHC1) gene expression was detected earlier and in regions of the heart tube destined to become both atria and ventricles (Fig. 2, A and D) (12). As with VMHC1, early atrial myosin heavy chain-1 (AMHC1) gene expression also was not restricted (Fig.