Background—Congenital heart disease represents the most common severe birth defect, affecting 0.7% to 1% of live births, thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1,2 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1-4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affecting 0.2 per 1000 live births,1–4,5 thereby representing the most frequent cyanotic congenital heart disease, affect...
hormone receptor–associated protein (TRAP) 240,15 which is disrupted by a translocation breakpoint in a patient with dTGA and mental retardation. The gene was therefore termed PROSIT240 (protein similar to TRAP240). Defects in TRAP function have been previously shown to affect nuclear receptor signaling, resulting in severe defects during embryonic development. Ablation of murine TRAP220, for example, has been shown to result in impaired heart and nervous system development.16 *Drosophila* homologues of TRAP240 and TRAP230 are required for proper eye-antennal disc development.17 Thus, TRAP family members do interfere with important processes in early embryonic patterning, making the PROSIT240 gene a good candidate to be involved in the phenotype of the patient. Mutational screening of 97 patients with dTGA revealed several sequence variants, including 3 missense mutations, which were not detected in controls.

Methods

**Case Report**

The proband was a 7-year-old girl born as the first child to a healthy, 30-year-old mother and a healthy, 31-year-old father. The parents were not related, and the family history was unremarkable. The patient had a healthy, 4-year-old brother. The uneventful pregnancy ended in a spontaneous delivery in the 38th week of pregnancy (Appgar score 9/10/10). Birth length was 52 cm (75th percentile), birth weight was 2650 g (10th percentile), and head circumference (Apgar score 9/10/10). Birth length was 52 cm (75th percentile), and head circumference was 32 cm (10th percentile). Echocardiography revealed dTGA, a perimembranous ventricular septal defect, and an open foramen ovale, as well as mild coarctation of the aorta. The TGA was operated on at the age of 14 months, and the remaining ventricular septal defect was corrected at the age of 4 years.

Postnatal microcephaly developed, and at the age of 2 months, a magnetic resonance imaging scan of the brain was performed. It showed no structural abnormalities, and myelination was rather advanced. Motor development was mildly delayed. Discrete ataxia was present, and the sense of balance was impaired. Mental retardation became more obvious with age. Speech is nearly absent. The result of routine blood and urine examinations did not indicate metabolic disturbances. A deletion of chromosomal region 22q11 (CATCH22) was excluded by fluorescence in situ hybridization (FISH).

**Human Subjects and Genomic DNA**

Peripheral blood samples were taken from individuals after informed consent was obtained, after approval by the review board of ethics of the respective institutions (Medical Department, University of Heidelberg, Germany; Newcastle and North Tyneside Health Authority Joint Ethics Committee, England; and Children’s Hospital of Philadelphia, Pa).

**Genomic Clones and Breakpoint Mapping**

Yeast artificial chromosome (YAC) clones were purchased from the German Resource Center (RZPD), Berlin. PI-derived artificial chromosome (PAC) and bacterial artificial chromosome (BAC) clones were obtained from BACPAC Resources, Oakland, Calif (RPCI-1,4 and RPCI-11) or from Research Genetics, Huntsville, Ala (HCIT, CTD). Isolation of metaphase chromosomes and FISH were performed as described elsewhere.18

**Expression Studies**

Human multiple-tissue Northern blots were purchased from Clontech Laboratories. Fragment Ex1/2, covering exons 1 and 2 (bp 91 to 283; FG33289 reverse, gactacagcgccatatac; FG493822 forward, aggc-cctgaggaggtcact), was used as a gene-specific probe, and a β-actin or a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe was used as a control. Labeling was performed with α-32PdCTP (Amersham Bioscience). Probes were purified by using a purification kit (Qiajax, Qiagen) and hybridized to the filter overnight at 65°C, as recommended by the manufacturer. The membranes were washed with 2× sodium chloride sodium citrate at room temperature and with 0.2× sodium chloride sodium citrate at 50°C and exposed at −80°C for 2 hours to 4 days, depending on signal intensity. Fetal cDNA panels were purchased from Clontech Laboratories.

**Mutation Screening**

Mutation screening was performed by denaturing high-performance liquid chromatography (DHPLC). A WAVE DNA fragment analysis system (Transgenomic Inc) was used. Untranslated regions were not analyzed; exons 10 and 17 had to be subdivided because the fragments were too large. (Primer sequences and polymerase chain reaction [PCR] conditions are available on request.)

**Sequencing**

Sequencing was performed on a MegaBACE sequencer (Amersham Bioscience) and with use of a DYEnamic ET terminator cycle sequencing kit, following the manufacturer’s protocol. Sequencing reactions were performed on both DNA strands. Sequences were analyzed with the CLUSTAL program (German Cancer Research Center, Biocomputing Facility HUSAR).

**Accession Numbers**

During preparation of this manuscript, our sequence (accession No. AF515599) was confirmed on April 28, 2003, by an update of clone KIAA1025 (XM_034056), now also including exons 4 and 3 and part of exon 2.

**Results**

**Physical Mapping and Characterization of Translocation Breakpoints**

Routine cytogenetic analysis had revealed a de novo balanced chromosomal translocation 46,XX,t(12;17) (q24.1;q21). To map the breakpoints, FISH of YACs and PACs was used for gross mapping (data not shown). By using BAC and cosmid clones, we could isolate clones spanning the breakpoints, thus narrowing the critical interval (Figure 2A and 2B). Subsequent Southern blot analysis showed aberrant products in the
patient’s DNA but not in controls (Figure 2C). Cloning and sequencing of the junction fragments confirmed the location of the breakpoint and revealed a microdeletion of 16 nucleotides (data not shown).

On chromosome 17, no known gene was interrupted by the breakpoint, and no novel transcript could be isolated within this interval. The breakpoint on chromosome 12 resided in the overlapping region of RP11-101P14 and RP11-973J6. Using computer-based gene prediction (Nix at http://www.hgmp.mrc.ac.uk), a novel partial transcript of 192 bp could be amplified by reverse transcription (RT)–PCR in multiple tissues.

Isolation of a Novel Gene in 12q24 Interrupted by the Breakpoint

By RT-PCR (of heart, brain, and kidney mRNA; Clontech), subsequent cloning, and sequencing, we could show that the newly identified fragment forms a transcriptional unit with the partial cDNA clone KIAA1025, isolating 5 new exons 5’ to the already known sequence. In total, the novel gene encodes 31 exons with a transcript size of 9377 bp and spans a genomic locus of 317 kb. Exon 1, harboring the start codon at position 56, is embedded into a CpG island. A stop codon in exon 31 defines an open reading frame of 6633 bp, which encodes a putative protein of 2210 amino acids. A polyadenylation signal is predicted at position 9357. Figure 3 shows the genomic structure of the gene and gives the exon and intron sizes. The breakpoint resides between exon 1 (harboring the start codon ATG) and exon 2, therefore interrupting the genomic sequence of the gene.

Sequence Comparison

Alignment of the cDNA sequence with the databases revealed a significant homology with TRAP240. The 2 large genes showed 55% homology on the nucleotide level over the total gene length according to the CLUSTAL program and 63% according to the BESTFIT program (German Cancer Research Center, Biocomputing Facility HUSAR). On the protein level, a total of 1138 amino acids are identical (51%) according to the CLUSTAL program, with 5 subregions showing identity of >70% (Figure 4). TRAP240 is evolutionarily conserved up to yeast and represents a component of the human TRAP complex, a large multisubunit coactivator. Approximately 20 different TRAP subunits have been identified so far, among which are several that have been shown to be involved in embryonic development. TRAP240, 230, 170, and 100 were shown to possess at least 2 copies of the
LXXLL domain, responsible for nuclear hormone receptor binding. Two LXXLL motifs are also present in the novel gene at amino acids 668 and 1224 (Figure 4). Because of the structural (and possible functional) homology to TRAP240, we termed the novel gene PROSIT240. Alternatively, the name THRAP2 also has been assigned by the nomenclature committee.

Expression Analysis

To examine the expression pattern of PROSIT240, we performed Northern blot analysis on human multiple tissues. Figure 5A shows that PROSIT240 is expressed in multiple tissues, with high expression in the brain, heart, skeletal muscle, kidney, placenta, and peripheral blood leukocytes. Among the fetal tissues tested, the transcript showed strongest expression in fetal brain, but it was also expressed in all other tissues tested (Figure 5B). In addition, expression in fetal skeletal muscle, fetal spleen, and fetal thymus was shown by RT-PCR on a normalized cDNA panel (data not shown).

Because PROSIT240 was identified in a patient with a severe heart defect and mental retardation, we also investigated the expression pattern in different subregions of the heart and brain. Figure 5C shows that within heart structures, the highest expression was detectable in the aorta. Among the tested brain regions, the cerebellum showed the strongest expression; however, the novel gene is expressed in all brain subregions (Figure 5D). Hence, PROSIT240 is expressed at the right place during embryonic development to be involved in the pathogenesis of the patient’s condition.

Using zoo blot analysis, we could show that PROSIT240 is conserved within humans, mice, cattle, and chickens (data not shown). Database searches across species revealed a possible partially cloned mouse orthologue (XM_132318) residing in the syntenic region, with 88% homology on the nucleotide level and 92% homology on the protein level.

Mutational Analysis of Patients With dTGA

On the basis of the fact that the novel gene is interrupted in a patient with heart and neuronal defects, the correlating expression patterns of PROSIT240, and the high impact of related TRAP components in early embryonic development, we consider it an excellent candidate gene to be associated with the pathogenesis of the patient’s phenotype. To prove the involvement of PROSIT240, additional patients with similar phenotypes were screened for mutations. Because the patient with the chromosomal translocation is also suffering from mental retardation, a mutation screen in patients with mental retardation would be formally possible. However, because such a patient pool is both clinically and genetically highly heterogeneous, this approach is not very promising. To test its involvement in the clinically more specifically defined defect of heart formation, we screened 97 patients with dTGA for mutations in PROSIT240 by means of DHPLC and sequencing. In cases for which metaphase chromosomes were available (22 cases), FISH analysis was also performed to exclude large deletions. With the use of BACs RP11-973J6, RP11-392B15, and RP11-493P1 as hybridization probes, no gross deletion could be detected. The Table summarizes the results of mutation screening by DHPLC. In total, 6 intronic polymorphisms, 6 silent mutations, and 4 missense mutations were found, which were named according to the scheme of Antonarakis.24 In cases for which a polymorphism or mutation was not found in 6 control individuals, the number of controls was increased. In the case of 2 intronic polymorphisms (IVS5+11C→T and IVS19+22T→C) and 1 silent mutation (1563C→T), the variation could not be detected in the control cohort of 100 and 68 individuals.
respectively. Therefore, these cases cannot be excluded to be functionally important without further analysis. One of the missense mutations (2056A→C, Lys686Gln) was found once in a control cohort of 200 individuals, representing an allelic frequency of 0.25%. Because the mutation did not affect a conserved amino acid and we could not obtain further information about the person showing the mutation, we did not pursue further analysis on this case at the moment, despite the fact that it also could not be excluded to be of functional significance.

Most notably, we found 3 missense mutations (752A→G [Glu251Gly], 5615G→A [Arg1872His], and 6068A→G [Asp2023Gly]) that could be detected only in patients and not in any of the 200 ethnically matched control persons. Parental DNA was available for 3 of the 6 respective parents. The mutation Glu251Gly could also be detected in the patient’s mother, who does not have a clinically defined TGA. All 3 mutations affect amino acids, which are conserved between PROSIT240 and TRAP240 (Figure 4), and 2 mutations (Arg1872His and Asp2023Gly), which reside within the available mouse sequence, affect amino acids conserved between the human and murine PROSIT240 sequence. Two of the variations (Glu251Gly and Asp2023Gly) furthermore significantly change the biochemical properties of the amino acids.

Discussion

Early embryonic development and organogenesis require spatially and temporally tight coordination of gene expression. Transcriptional regulation in such complex processes therefore involves not only basic activators or repressors but also additional modifiers. The TRAP complex, composed of several TRAP components, represents such a global coactivator, influencing transcriptional regulation of nuclear hormone receptors20–22 and other activators like p53 or VP16.25 TRAPs have been shown to be essential for early embryonic development. TRAP220−/− mouse embryos, for example, die at ~11 days after conception because of severe heart prob-

Figure 4. Sequence comparison between PROSIT240 (top) and TRAP240 (lower) based on CLUSTAL program (HUSAR). Blue color indicates identical amino acids, red color indicates amino acids with strong similarity, and orange indicates amino acids with weak similarities. Boxed areas represent regions with identities >70%. Two LXXLL domains are indicated by horizontal pink lines at positions 683 and 1249. Green circles below amino acid indicate missense mutations found in conserved amino acids; turquoise circle indicates missense mutation in nonconserved amino acid.

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lems and impaired neuronal development. Also, some of the interacting partners of TRAPs have long been known to be involved in developmental processes. Thyroid hormone and its receptor TR, for example, play key roles in the development of the central nervous system. Patients with point mutations in the TRβ gene, for example, show mental retardation and emotional disturbance. The influence of nuclear receptors in heart formation has been strikingly demonstrated for RXR and RAR. RXR/RAR double mutants display outflow tract malformations and abnormalities of the great arteries. Using a positional cloning approach, we could show that PROSIT2 is interrupted in a patient with a severe heart defect (dTGA) and mental retardation.

The PROSIT2/THRAP2 gene comprises 31 exons encoding a putative protein of 2210 amino acids. On the protein level, 1138 amino acids (51%) are identical between PROSIT2 and TRAP2, with 5 subregions showing identity of >70%. PROSIT2 contains 2 LXXLL domains, which have been shown to be responsible for receptor binding. Both TRAP2 and PROSIT2 are ubiquitously expressed, with the highest expression in skeletal muscle. In contrast to TRAP2, however, PROSIT2 is also strongly expressed in the heart (aorta) and brain (cerebellum), where it could possibly substitute for TRAP2 function. The expression pattern correlates well with a possible role for PROSIT2 in the pathogenesis of the patient’s phenotype. Because of the high sequence homology to TRAP2 and the overlapping expression pattern, we suggest that the novel gene is functionally related to TRAP2. Future studies will have to show whether PROSIT2 is indeed a functional relative of TRAP2. It will be interesting to see whether and with what kind of activators (or repressors) the putative protein will interact, defining its biologic role during development. Considering the phenotype, RAR/RXR and TR would be particularly interesting candidates to check for interaction with PROSIT2.

For the pathogenesis of dTGA, the involvement of 2 genes has already been discussed. A nonsense mutation in the gene ZIC3, originally characterized in patients with laterality defects, was shown to segregate with dTGA in a Lebanese family. Surprisingly, a healthy, maternal uncle also showed the same mutation, pointing toward incomplete penetrance. Besides ZIC3, the EGF-CFC gene CFC1 represents an interesting candidate because of gene-targeting studies in mice. CFC1 mice develop laterality defects and complex
cardiac malformations reminiscent of human heterotaxy syndrome. Most interestingly, 82% of the homozygous mutant mice have malconnection of the great arteries, including TGA, as well as other cardiac malformations. Bamford et al identified 2 distinct mutations within the gene CFC1 in 3 independent patients with laterality defects and dTGA. Both the deletion and the missense mutation, however, were also found in normal controls or in a healthy parent of the patient. An additional splice-donor mutation was detected in the same gene, creating an alternative splice site, which is predicted to cause a frameshift. The influence of the chromosomal region 22q11 (CATCH22) in dTGA was also discussed controversially. Whereas 2 studies provided evidence that a deletion of 22q11 is causative for dTGA in 12% of patients, 2 other studies found no correlation between the deletion and the disease. These controversial data and the low mutation frequency of ZIC3 and CFC1 in dTGA patients underline the heterogeneity of this disease and demonstrate that only initial steps have been made so far toward an understanding of the pathogenesis of dTGA.

To clarify the involvement of PROSIT240 in heart formation, we screened 97 patients with dTGA for mutations. In total, we found 6 intronic polymorphisms, 6 silent mutations, and 4 missense mutations. Although intronic and silent mutations generally cannot be excluded to be functionally relevant, especially when not found in controls, we focused on the missense mutations at this stage. Three missense mutations (Glu251Gly, Arg1872His, and Asp2023Gly) found in the patient cohort could not be identified in 400 control chromosomes. These mutations all affect amino acids that are conserved between PROSIT240 and TRAP240 (Figure 4) and could therefore be important for protein function. Glu251Gly and Asp2023Gly represent exchanges that significantly change biochemical properties of the respective amino acid. One of the mutations (Glu251Gly) is also carried by the patient’s mother, who did not present with dTGA. Unfortunately, this mother was not available for further clinical testing to check for previously undiagnosed (subtle) heart defects. The mutation could represent a rare polymorphism, yet finding the mutation in a healthy parent can also be explained by incomplete penetrance, which has been previously reported in congenital heart disease (eg, in ZIC3; see above).

Detecting 3 missense mutations and 1 gene interruption (leading to haploinsufficiency) in dTGA patients strongly suggests a contribution of PROSIT240 to heart development. The putative relatedness of PROSIT240 to TRAP240 could point to an involvement of the TRAP complex. The Drosophila homologues of TRAP240 and TRAP230 were shown to act together to control cell affinity to establish cell boundaries, a process that might also be relevant to our observation relating PROSIT240 malfunction to dTGA. Amino acid exchanges caused by missense mutations could alter the ability of PROSIT240 to interact with target activators or repressors. Understanding more about the mechanisms leading to dTGA might also help to determine whether dTGA could be considered a manifestation of a left-right laterality defect.
concerning the heart, a point that has been raised based on data from animal models and the fact that ZIC3 and CFC1 mutations are detected both in patients with dTGA and in heterotaxy problems. In animal models, most interestingly, Pitx2\(^{-}\) and Dvl1\(^{-}\) mice present with outflow tract abnormalities, including TGA and laterality defects. Both genes are part of the Wnt/Dvl/β-catenin → Pitx2 pathway, which was shown to recruit TRAP components. It will be interesting to see whether there will be a common pathogenetic mechanism involved in causing dTGA and laterality defects. With PROSIT240, we were able to bring a novel, exciting player into the game, leading the way toward new questions that can be raised concerning the complexity of heart formation.

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Missense Mutations and Gene Interruption in PROSIT240, a Novel TRAP240-Like Gene, in Patients With Congenital Heart Defect (Transposition of the Great Arteries)

Nadja Muncke, Christine Jung, Heinz Rüdiger, Herbert Ulmer, Ralph Roeth, Annette Hubert, Elizabeth Goldmuntz, Deborah Driscoll, Judith Goodship, Karin Schön and Gudrun Rappold

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