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Mouse *gridlock*: No Aortic Coarctation or Deficiency, but Fatal Cardiac Defects in *Hey2* −/− Mice

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Summary

***Gridlock* (*grl*) is one of the first mutations characterized from the large zebrafish mutagenesis screens, and it results in an arterial (aortic) maturation defect, which was proposed to resemble aortic coarctation, a clinically important human malformation [1–3]. While the *grl* mutation appears to be a hypomorph, *grl* knockdown experiments have shown even stronger effects on arterial development [4]. We have generated a knockout of the murine *Hey2* (*gridlock*) gene to analyze the mammalian phenotype. Surprisingly, *Hey2* loss does not affect aortic development, but it instead leads to a massive postnatal cardiac hypertrophy with high lethality during the first 10 days of life. This cardiomyopathy is ameliorated with time in surviving animals that do not appear to be manifestly impaired during adult life. These differences in phenotypes suggest that changes in expression or function of genes during evolution may lead to quite different pathological phenotypes, if impaired.**

Results and Discussion

Mouse *Hey2* has been identified as part of a subfamily of three *hairy/Enhancer-of-split*-related basic helix-loop-helix transcription factors [5–8]. We detected three *Hey*-related genes in zebrafish, and molecular phylogenetic analysis clearly indicated that *Hey2* is the ortholog of the zebrafish *gridlock* gene (Figure 1; C. Winkler and M.G., unpublished data). This is supported by chromosomal synteny of zebrafish *grl* (linkage group 20, http://www.zfin.org) and mouse or human *Hey2* (chromosome 10 or 6q21–22) [9], and this synteny is shared by the flanking loci *GJA1* or *PEX7* in all three species. None of the other mapped genes from linkage group 20 in zebrafish are located on the corresponding mouse chromosome or human chromosome arm where *Hey1* or *HeyL*, respectively, have been placed.

Furthermore, all major expression sites in the embryo are shared between *grl* and *Hey2*. Both genes are expressed in arterial blood vessels during development in both species [1, 5]. In the mouse, there is strong expression in the embryonic heart ventricle that is gradually lost during postnatal development. In zebrafish, a similar transient expression in the embryonic heart is seen [1] (C. Winkler and M.G., unpublished data). Finally, expression in the dorsal root ganglia and the mid-hindbrain boundary is also shared between mouse and zebrafish embryos. This strong overlap in expression sites, which is different from that seen with *Hey1* or *HeyL*, further strengthens the notion that mouse *Hey2* and zebrafish *gridlock* are orthologs.

Recently, it has been shown that the zebrafish *gridlock* mutation likely represents a hypomorphic allele, with *grl* involved in the allocation of angioblast precursors to the arterial versus venous lineages [4]. The anteriormost part of the zebrafish aorta, the bifurcation, may represent the most vulnerable site that is affected in *grl* mutants. Further diminution of *grl* with antisense oligonucleotides led to disruption of increasingly larger segments of the aorta. The intriguing question of whether the exciting zebrafish phenotype can be translated to mammals and in fact represents a phenocopy of human aortic coarctation, remained open, however.

We have generated a complete knockout of the *Hey2* gene in the mouse by *lacZ* insertion in exon 2 to compare phenotypes in both species (Figure 2A). Heterozygous mice are phenotypically normal and show strong cardiac and arterial expression of the *Hey2-lacZ* allele during development, and these findings are in agreement with previous mRNA in situ hybridization [5] (Figures 2B and 2C). Homozygous *Hey2* −/− mice are born at Mendelian ratios, but the majority shows failure to thrive and dies within the first 10 days of life. Only about 20% of −/− mice survive this critical period. They then gradually catch up and reach normal size by 2–3 months of age, with no excess morbidity or lethality later on.

There are no apparent defects of embryonic vessel formation in Hey2 −/− mice. LacZ staining patterns reflecting endogenous Hey2 expression in +/− and −/− embryos only showed quantitative differences, which are expected to occur due to different gene copy numbers. Importantly, angiography at 3, 9, and 28 days of age revealed regular aortic development with no evidence of the functional stenosis or collateral formation that would be expected to occur in coarctation (Figures 3A and 3B). Thus, either *Hey2* is not essential to build the aorta as in zebrafish, or its loss can be compensated for by other genes.

Necropsy of *Hey2* −/− mice revealed a massive increase in heart size, with heart versus body weight ratios increased by 30% and up to 300%, depending on age and severity (Figures 3C and 3D). Both ventricles appeared greatly enlarged and had thickened muscular

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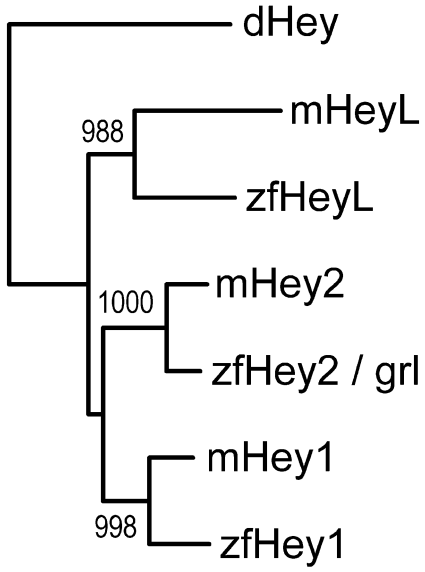

Figure 1. Phylogeny of *Hey* Genes

A phylogenetic tree of mouse (m) and zebrafish (zf) *Hey* genes and the *Drosophila dHey* ancestor was generated from a multiple alignment of protein sequences with the ClustalX program. The numbers represent bootstrap values at critical nodes that strongly support this tree.

walls and expanded lumina. Histological examination revealed large, activated cardiomyocytes with abnormal mitotic figures, accompanied by fiber disarray and a mild degree of fibrosis (Figures 3E–3G). The myofibrillar disorganization may well lead to the massive compensatory cardiac enlargement that is prone to failure. As expected, there is a strong induction of classical markers of hypertrophic gene expression, like *ANF* (atrial natriuretic factor) and *CARP* (cardiac ankyrin repeat protein), in early postnatal *Hey2* −/− heart ventricles (Figure 4). In situ hybridization with several markers of earlier cardiac development, like *Gata4*, *Irx4*, *Tbx5*, *or Nkx2.5* (not shown), did not reveal differences between knockout and control hearts at different stages. This is also in line with the absence of morphological changes before birth, suggesting that initial organ patterning is not disturbed (see Note Added in Proof).

Interestingly, the dramatic cardiac changes that are seen in young *Hey2* −/− mice are not progressive. While overall heart shapes remained altered in older *Hey2* −/− mice that had survived, there was no longer a consistent and strong increase in heart versus body weight. Histopathological changes were also limited to a slightly increased fibrosis, and there was no unexplained morbidity or mortality in these mice. This suggests that loss of *Hey2* in the mouse results in a critical cardiomyocyte defect that becomes acerbated during the transition from fetal to postnatal function, a period of increased stress to the heart. Decompensation of cardiomyopathy during this fragile period will be lethal in most cases. It will be very interesting to uncover the molecular correlates of *Hey2* loss in the mouse and to determine if induced cardiac stress during later life can again tip the balance in these genetically compromised hearts.

It remains undisputed that zebrafish mutants have provided us with an enormous wealth of new leads in human and mouse development and disease, but, as shown here, care has to be taken in translating a phenotypic description from fish to the murine or human situation. Future research will show if these differences in phenotypes result from evolutionary changes and neofunctionalization within the *Hey* gene family.

Experimental Procedures

Analysis of Zebrafish *Hey* Genes

Degenerate primers were used to amplify *Hey*-related sequences from zebrafish embryo cDNA of various stages, as described for

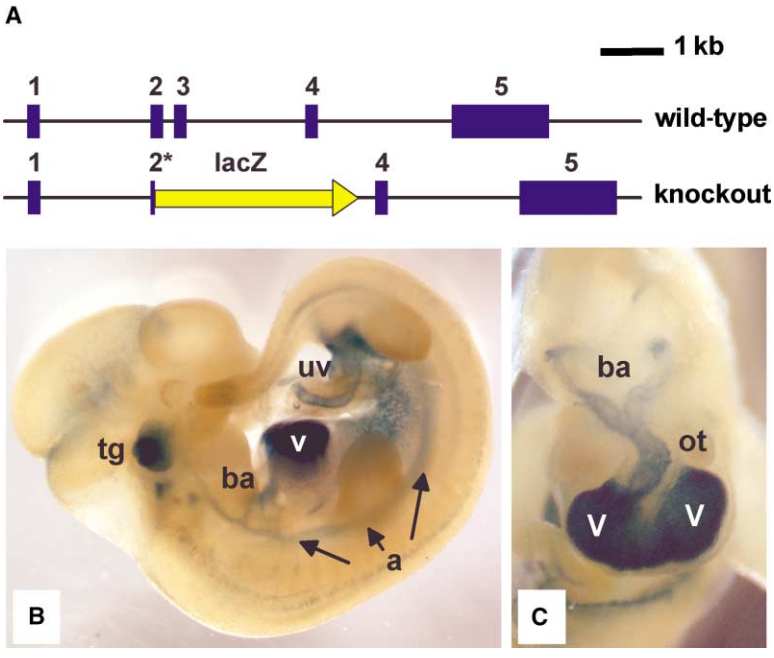

Figure 2. Knockout of *Hey2* in the Mouse

(A) The knockout allele generates an in-frame *Hey2-lacZ* fusion protein that contains only 30 amino acids derived from Hey2, preceding the basic helix-loop-helix region. Most of exon 2 (remainder labeled 2*) and exon 3 were deleted. Details of the genomic locus have been published before [9]. There is no expression of the remaining exon 4–5 region, as tested by mRNA in situ hybridization.

(B and C) A lateral and cranial view of an E10.5 *lacZ*-stained *Hey2* +/− embryo reveals strong expression in the heart ventricle (v), outflow tract (ot), branchial arch arteries (ba), aorta (a), and umbilical vessels (uv). The strong signal in the head identifies the trigeminal ganglion (tg).

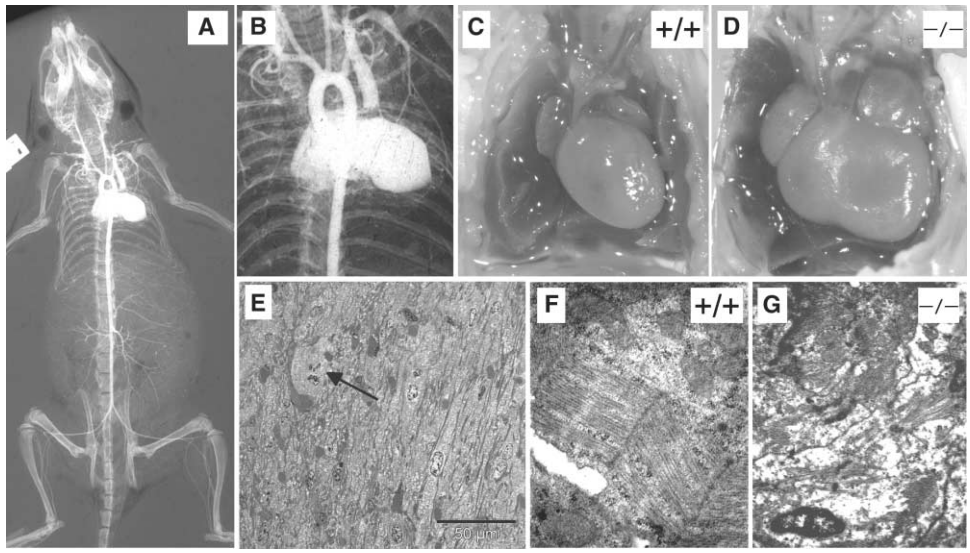

Figure 3. Cardiomyopathy of *Hey2* −/− Mice

(A and B) Angiography of a 4-week-old *Hey2* −/− mouse. The heart shape is altered, consistent with severe biventricular enlargement. The entire aorta and its main branches have normal diameters, and there is no evidence of stenosis, atresia, or the formation of collateral vessels.
(C and D) A massively enlarged heart in a 3-day-old *Hey2* −/− mouse compared to a wild-type +/+ littermate (same magnification). Atria are not consistently enlarged in other knockout mice.
(E) A semi-thin section of the −/− ventricle showing marked hypertrophy of cardiomyocytes and a significant increase in mitotic activity (arrow).
(F and G) Electron micrographs document drastic fiber loss and disarray in *Hey2* −/− hearts compared to controls (+/+).

chick *Hey* genes before [10]. Full-length cDNA clones were generated by RACE-PCR or by using partial GenBank sequences. Detailed sequence comparison with orthologs from other fish species will be described elsewhere (C. Winkler and M.G., unpublished data).

Knockout of Mouse *Hey2*

The *lacZ* insertion vector that was used to transfect E14.1 ES cells contained a floxed neo cassette and homology regions of 1.6 kb (5′) and 5.8 kb (3′). Three independent mouse lines were generated from 12/288 correctly targeted ES cells, and the neo selection marker was eliminated by mating with CMV-cre transgenic mice. There were no phenotypic or expression differences between the three mouse lines. All genotyping was done by PCR with a combination of three primers: Hey2-ko-test-5′ (common, 5′-GCTGTCTCAA GGCCTCAACAGCATTG-3′), Hey2-ko-test-3′ (only wild-type allele, 5′-TCGGTGAATTGGACCTCATCACTGAGC-3′), and M13 (specific for the *lacZ* allele, 5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) generated fragments of 253 and 370 base pairs from wild-type and knockout alleles, respectively. Most analyses were done on mice that had been crossed into a C57BL/6 background for several generations.

Angiography

The thoracic cavity was opened under CO_2 narcosis, and a contrast agent containing 5% gelatin and 20% bismuth oxychloride was injected at a temperature of about 35°C into the left ventricle. The mice were immediately chilled in ice water and fixed in cold 4% paraformaldehyde. X-ray angiograms were produced by using a Machlett-Balteau X-ray source set at 20 kV and 8 mA for 2.5 min to produce "soft" X-rays with the animals immersed in ice water.

Histology and Gene Expression Analysis

For histology and electron microscopy, standard protocols were used on glutaraldehyde-fixed tissues. Northern Blot analysis was done as described in [11]. For mRNA in situ hybridization, paraffin

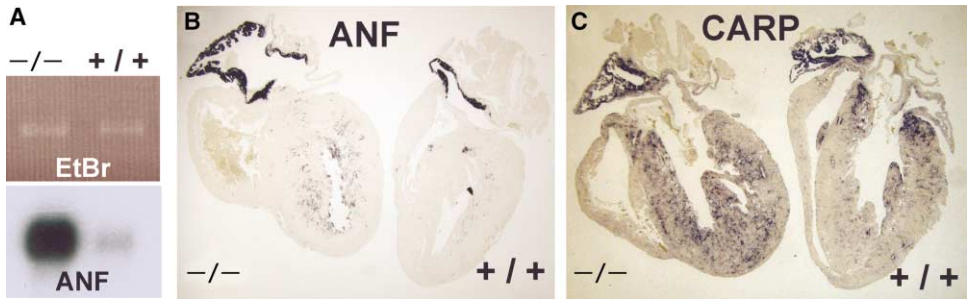

Figure 4. Expression of Hypertrophy Markers in Hey2 Knockout Hearts

(A) Total ventricular RNA (2 μg) from 2-day-old −/− and +/+ littermates was used for Northern Blot analysis. Ethidium bromide (EtBr) staining shows equal loading, but hybridization with an *ANF* probe documents strong induction in the *Hey2* −/− ventricle.
(B) *ANF* in situ hybridization on heart sections of 10-day-old littermates shows strong atrial expression in both cases but enhanced ventricular expression only in the *Hey2* −/− heart.
(C) *CARP* mRNA expression is similarly increased in the entire ventricle of the knockout mouse.

sections were hybridized with digoxigenin-labeled riboprobes as detailed in [11]. Riboprobes were generated from PCR-amplified cDNA fragments of approximately 600 base pairs cloned into pCS2 vectors. All primer sequences are available upon request. β-galactosidase staining was done on fixed embryos with X-gal substrate according to a protocol published by C. Lobe (http://www.cancerbiology.org/protocols.html).

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Note Added in Proof

An extended analysis of additional *Hey2*−/− mice from higher C57Bl/6 backcross generations revealed that some of our *Hey2* knockout mice have large ventricular and atrial septum defects, although these defects were clearly absent in the initial series of histopathological analyses upon reexamination. A similar knockout of the *Hey2* gene by M.T. Chin and colleagues (personal communication) likewise resulted in a very high incidence of ventricular septum defects, especially on a 129SV mouse background. It remains to be seen how frequent septal defects or other structural malformations occur in *Hey2* knockout mice on different mouse backgrounds. The most parsimonious explanation is that lack of *Hey2* function results in a critical cardiomyocyte developmental defect with pleiotropic effects that may either manifest as septal defects or as a globally insufficient working myocardium.