

Editorial

A single mutation causes a spectrum of cardiovascular defects: the potential role of genetic modifiers, epigenetic influences, and stochastic events in phenotypic variability

Keywords: CHF1; Hey2; Gridlock; Hrt2; Hesr2; Sox10; Tbx1; Tbx5; Epigenetic; Genetic modifiers; Ventricular septal defect; VSD; Outflow tract; Mouse; Developmental noise; Stochastic; BALB/c; C57BL/6; Hirschsprung; DiGeorge; Holt–Oram

How can an identical mutation result in distinct phenotypes in different individuals? This simple question underscores the importance of genetic background in disease severity and progression and highlights one of the challenges in understanding complex diseases. The role of genetic modifier genes in phenotypic variability and disease progression has long been appreciated, but the identification of modifiers has been difficult particularly in the highly variable human population [1]. There are numerous examples of variability in disease progression in humans, even in cases where identical causative mutations are present, indicating a strong influence of familial history and genetic background [1].

1. Hirschsprung disease: a congenital disorder with incomplete penetrance and phenotypic variability

A classic example of a variable human congenital disease phenotype is seen in the genetics of Hirschsprung disease. Hirschsprung disease is a disorder affecting the developing neural crest and is characterized by congenital intestinal aganglionosis with associated pigmentation defects and hearing loss in some cases [2]. Hirschsprung disease is clearly a genetic disorder with mutations in the genes encoding the RET receptor or its ligands or in the gene encoding the transcription factor Sox10 making up the majority of human cases [2,3]. Interestingly, however, for each of the Hirschsprung disease genes identified to date, incomplete penetrance and variable phenotypes have been observed, presumably due to modifier loci [2,3]. The variability ranges from no phenotype to severe aganglionosis in the presence of the same causative mutation [3]. However, the identification of these modifiers has been difficult. In this regard, the development of mouse models of human congenital anomalies has proven valuable. The ability to mutate genes in mice using reverse genetic strategies, combined with the availability of inbred strains, has allowed investigators to reduce phenotypic variability due to genetic background effects within a given strain and then to

use differences in phenotype in different strains to map modifier loci. In the case of Hirschsprung disease, several modifiers of Sox10 have been identified in mice, including Sox8 and the endothelin receptor gene *Ednrb* [4,5]. Several additional loci have been identified as modifiers of Sox10 by examining differences in the severity of the megacolon phenotype in two different inbred mouse strains by linkage analysis [6].

2. Several congenital cardiac disorders display phenotypic variability

Several examples of mutations that affect cardiovascular development and display significant variation in phenotype or disease severity have also been described, and a number of these mutations occur in genes encoding cardiac restricted transcription factors. A well-studied example is the microdeletion of human chromosome 22q11 or DiGeorge syndrome (DGS), which causes craniofacial and cardiovascular defects as well as several other abnormalities [7]. The most common deletion results in the loss of approximately 3 Mb and about 30 genes in the region [8]. DGS may be a contiguous gene deletion syndrome where multiple genes in the region contribute to the disease phenotype, or it may result primarily from the loss of a single gene *TBX1* [8]. Indeed, Yagi et al. [9] identified patients with mutations in *TBX1* that exhibited phenotypes characteristic of the complete syndrome, suggesting that mutations in *TBX1* may be the primary lesion in DGS. Interestingly, family members carrying the same *TBX1* mutation exhibited a highly variable phenotype ranging from mild to severe, implicating an important role for modifier genes [9]. Work in mice has suggested that *Crkl*, a gene commonly deleted in the DGS critical region on 22q11, may be responsible for some of the symptoms of DGS, which supports the idea of DGS being a contiguous gene deletion syndrome [10]. In addition, other work in mice has shown that deletion of *Fgf8*, which encodes a signaling molecule involved in pharyngeal arch development, results in a phenocopy of DGS

[11]. *Fgf8* is not present in the DGS critical region, and it appears to be downstream of *Tbx1* in the mouse, which suggests the strong possibility that *Fgf8* may be a modifier of *Tbx1* in mice and possibly in DGS in humans [11–13]. Additional work in mouse models will be important to define the role of other genes that are linked and unlinked to the DGS critical region for their role as modifiers of the DGS phenotype.

Mutations in the *TBX5* gene, which encodes another T box transcription factor related to *Tbx1*, cause Holt–Oram syndrome [14,15]. Holt–Oram syndrome is a haploinsufficiency disorder that results in congenital heart and forelimb defects and is characterized by a high degree of phenotypic variability, which is thought to be due in large part to the influence of genetic modifiers [15]. Again, *Tbx5* heterozygosity in mice recapitulates the majority of the Holt–Oram phenotype and provides the opportunity to identify modifier genes that influence the phenotype in mice and probably in humans as well [16]. Heterozygous mutations in the homeodomain transcription factor gene *NKX2-5* are associated with familial cases of atrial septal defects in humans [17]. In still another example, mice with a targeted null mutation in *Nkx2-5* also exhibit defects in the atrial septum, and the severity of these defects is dependent on genetic background and the likely effects of modifier genes [18].

3. Deletion of *CHF1/Hey2* in mice results in variable cardiac defects depending on genetic background

Another excellent example of genetic background influencing phenotype in mice harboring a transcription factor gene mutation is observed with the *cardiovascular basic helix–loop–helix factor 1 (CHF1)* gene. *CHF1* (also known as *Hrt2*, *Hey2*, and *hesr2*) encodes a basic helix–loop–helix (bHLH) transcription factor related to *Drosophila* Hairy and functions as a downstream effector of Notch signaling [19–21]. *CHF1* is expressed in the developing ventricular myocardium from early in mouse development and appears to function as a transcriptional repressor via the recruitment of corepressors and histone deacetylases [20]. The *CHF1* ortholog in the zebrafish, *gridlock*, was shown to be essential for the initial formation of the aorta and suggested that the bHLH transcription factor encoded by *gridlock* was important for arterial endothelial cell specification [19,22]. Interestingly, several independent *CHF1* knockouts have been generated in mice and none displayed obvious vascular defects of the type seen in zebrafish with the *gridlock* mutation [23–26]. Furthermore, the null phenotype of each of the different *CHF1* knockouts was different. Each had a cardiac phenotype, but the range, type, and severity of the defects differed among the different knockouts. In one case, the primary defect was a fatal cardiomyopathy [25], while another group reported cardiomyopathy and an associated ventricular septal defect [23]. In another case, knockout mice exhibited a broader array of defects grossly resembling Tetralogy of Fallot, including pul-

monic stenosis, right ventricular hypertrophy, and ventricular septal defects, as well as tricuspid atresia [24]. Finally, a more recent study has suggested that the primary defect in the absence of *CHF1* is in atrioventricular valve development [26].

As is the case for many mutations, variability in *CHF1* null phenotypes observed in mice has been explained as the likely influence of modifier genes due to variation in genetic background [20,27,28]. In this issue of the *Journal of Molecular and Cellular Cardiology*, Sakata et al. [27] present the first systematic study designed to determine the role of genetic background in the phenotypic variation observed in the absence of *CHF1* in mice by examining the loss of this gene in two different inbred strains of mice. The authors of the present study observe significant differences when *CHF1* is deleted in BALB/c compared to C57BL/6 backgrounds, and the phenotypes observed in each of these backgrounds are different from those observed in previous studies [23–27]. Mice lacking *CHF1* in either the BALB/c or the C57BL/6 background are viable at birth, indicating that *CHF1* is not required for development. However, by weaning, nearly all the homozygous null animals were dead, regardless of background, indicating a requirement for postnatal viability. Sakata et al. observe a spectrum of phenotypes resulting from loss of *CHF1* in an inbred C57BL/6 background, including valve defects, large ventricular septal defects, and ventricular wall thinning. A spectrum of defects were also observed when *CHF1* was deleted in the BALB/c background, but the defects were different than those observed in the C57BL/6 background. *CHF1* deletion in the BALB/c background resulted in a moderate incidence of dysmorphic ventricular septum, small ventricular septal defects, valve defects, and occasional overriding aorta and immature right ventricle development. Ventricular septal defects were present in the absence of *CHF1* in both backgrounds, but the penetrance and severity differed with 100% penetrance and severe ventricular septal defects observed in the C57BL/6 background and much milder and only partially penetrant ventricular septal defects observed in the BALB/c background. Furthermore, ventricular wall thinning was only observed in the C57BL/6 background, and overriding aorta was only observed in the BALB/c background.

Mutation of the *CHF1* ortholog, *gridlock*, in the zebrafish results in defective outflow tract development, and those studies have suggested that the primary defects in the zebrafish mutants are vascular in origin [19,22]. Surprisingly, none of the previous studies of *CHF1* mutant mice has reported anatomical vascular defects [23–26]. Importantly, in their study, Sakata et al. [27] observed defective outflow tract development in the absence of *CHF1* in a C57BL/6 background. In that background, both the aorta and pulmonary artery have thinner walls, and this phenotype is probably due to decreased vascular smooth muscle proliferation [29]. The defects in vessel wall thickness indicate a role for *CHF1* in vascular development in the mouse and reinforce the conservation of this transcription factor's function across divergent classes of vertebrates [27].

The differences in the phenotypes observed in the absence of *CHF1* in different genetic backgrounds is not unexpected since it agrees with the variability in *CHF1* null phenotypes observed in previous studies [23–26], and it has been hypothesized that background likely had a profound effect on the observed phenotypes [20,28]. The present studies clearly indicate that modifiers of *CHF1* are important for its function in vivo, and that allelic variation in these genetic modifiers has significant impact on the severity, penetrance, and types of defects seen in the absence of *CHF1* function. The most likely explanation for the difference in *CHF1* null phenotypes observed in the different genetic backgrounds is that genes that encode proteins that function in the same pathway with *CHF1* differ in each of the examined strains. Thus, a given modifier gene may be mutated in C57BL/6 mice, for example, and that mutation in combination with mutation of *CHF1*, results in severe ventricular septal defects. Alternatively, the differences observed in the severity of ventricular septal defects in *CHF1* mutant mice in the BALB/c and C57BL/6 backgrounds could be due to more subtle variations in modifier gene alleles. A normal allelic variant of a modifier gene that only slightly impacted its function in vivo and would result in no phenotype on its own may have a dramatic impact on cardiac development in combination with loss of *CHF1* function.

4. Future challenges

The systematic identification of distinct *CHF1* phenotypes in different mouse strains now provides essential genetic tools to assist in the identification of those modifier genes. By intercrossing BALB/c and C57BL/6 mice that are each heterozygous for *CHF1* to generate F1 hybrids at every allele except for *CHF1* and then intercrossing the F1 offspring, it will be possible to begin to map loci responsible for influencing the *CHF1* null phenotype in mice using microsatellite markers, radiation hybrid mapping, and other genetic approaches [30,31]. Identification of the modifier genes that influence the *CHF1* null phenotype in mice will be important for our understanding of the function of the CHF/Hey family of transcription factors and may also identify additional key regulators of cardiovascular development in mice and humans.

In their study, Sakata et al. [27] also observe significant phenotypic variability in the absence of *CHF1* in the BALB/c inbred background. In the BALB/c background, about 20% of the animals exhibit a ventricular septal defect, while another 30% display a dysmorphic ventricular septum but without ventricular septal defect. Additionally, 20% of the *CHF1* mutants in the BALB/c background had outflow tract defects characterized by an overriding aorta. While the variability observed in the absence of *CHF1* function in different genetic backgrounds is not surprising and is explained by the probable existence of modifier genes, the variability observed within the inbred background is more intriguing. Because all loci are theoretically homozygous in an inbred genetic back-

ground, such as BALB/c or C57BL/6, the striking variability seen for the *CHF1* ventricular septal defect phenotype in the BALB/c background cannot be easily explained by the function of genetic modifiers. Rather, these observations suggest an epigenetic or environmental influence on heart development, which is revealed in the absence of *CHF1*.

A challenging problem in understanding the development of disease is how nongenetic influences, including epigenetic changes, environmental influences, and stochastic processes interact with genes to ultimately determine phenotype. An intriguing example of stochastic events influencing the epigenetic state of chromatin and subsequently influencing phenotype is the case of agouti coat color in mice [32]. Genetically identical mice that harbor the agouti viable yellow allele (A^{vy}) display a range of coat colors despite the fact that they have an identical A^{vy} allele on an inbred background [32]. Interestingly, the differential coat color phenotype is heritable in this background, at least in part, indicating that the epigenetic marks within the *agouti* locus can be inherited [33]. The epigenetic influence at the *agouti* allele is probably due to DNA methylation, which is stochastically determined but can be partly inherited [32].

Several examples of epigenetic or environmental influences on cardiovascular development have also been described in mice. For example, in the case of mice heterozygous for *Nkx2.5* on an inbred background, only a small percentage of the animals displayed septal dysmorphogenesis in spite of the fact that all animals are, at least in principle, genetically identical [18]. Similarly, epigenetic or stochastic modifiers may also influence DGS phenotypes in the *Df1/+* deletion in mice, where incomplete phenotypic penetrance was observed in several different inbred backgrounds [34]. In the case of the variability in *Nkx2.5* and *Df1* heterozygosity, genetic modifiers clearly had a strong influence on phenotype, but the entire range of variability cannot be explained solely by genetic influences [18,34]. Likewise, mutation of *CHF1* in mice results in variable phenotypes that are strongly influenced by genetic and nongenetic modifiers, which may include epigenetic, stochastic, or environmental influences [27]. Ultimately, it will be essential to identify the genetic modifiers of *CHF1* function in order to determine how those genes interact with *CHF1* to influence cardiovascular development. It will also be important to determine the nature and inheritance of stochastic and epigenetic influences on *CHF1* and other cardiovascular transcription factor genes to gain insight into the phenotypic variability frequently observed in human congenital cardiovascular disease.

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