The Functional Diversity of Essential Genes Required for Mammalian Cardiac Development

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Summary: Genes required for an organism to develop to maturity (for which no other gene can compensate) are considered essential. The continuing functional annotation of the mouse genome has enabled the identification of many essential genes required for specific developmental processes including cardiac development. Patterns are now emerging regarding the functional nature of genes required at specific points throughout gestation. Essential genes required for development beyond cardiac progenitor cell migration and induction include a small and functionally homogenous group encoding transcription factors, ligands and receptors. Actions of core cardiogenic transcription factors from the Gata, Nkx, Mef, Hand, and Tbx families trigger a marked expansion in the functional diversity of essential genes from midgestation onwards. As the embryo grows in size and complexity, genes required to maintain a functional heartbeat and to provide muscular strength and regulate blood flow are well represented. These essential genes regulate further specialization and polarization of cell types along with proliferative, migratory, adhesive, contractile, and structural processes. The identification of patterns regarding the functional nature of essential genes across numerous developmental systems may aid prediction of further essential genes and those important to development and/or progression of disease. genesis 52:713–737, 2014.

INTRODUCTION

Alteration or deletion of genes is a valuable methodology to determine gene function. The tools available to modern molecular biologists have rendered the removal of genes a systematic process as reflected by the current availability of increasing numbers of targeted deletions and conditional alleles (Dolgin, 2011; Skarnes et al., 2011). Additionally, chemical mutagenesis particularly in combination with modern sequencing approaches continues to provide an invaluable resource in attempts to complete the functional annotation of the genome (Arnold et al., 2011; Brown et al., 2013; Gondo et al., 2010; Kile et al., 2003; Oliver and Davies, 2012; Probst and Justice, 2010). The case of performing genetic manipulations in the mouse and the early availability of its genomic sequence led to its emergence as the mammalian model of choice for functional studies (Waterston et al., 2002). As functional data accumulates, it is now possible to identify which individual genes and biological functions are indispensable for the progression of specific processes. Genes absolutely required for an organism to develop to maturity and for which there is no compensation for critical aspects of their function are considered essential. The proportion of all mouse genes that are essential for embryonic...
development as well as their genomic distribution/density and the roles of their human orthologs in human diseases are among questions that are being actively pursued (Dickerson et al., 2011; Georgi et al., 2013; Hentges et al., 2007; Wilson et al., 2005). Less consideration has been paid to the functional diversity of genes that are essential to specific developmental processes and the temporal requirements for differential functions. We argue that at different timepoints in cardiac specification, the functional diversity of essential genes changes, with different functions becoming more or less represented in cardiac essential genes.

Many genes involved in mammalian cardiac development are essential, due to the requirement for cardiac function in utero early during gestation. Cardiac development involves the spatially and temporally coordinated actions of individual cells, tissues, and regulated gene expression for cell recruitment, differentiation, and organ morphogenesis (Bruneau, 2002; Buckingham et al., 2005; Conway et al., 2003; DeRuiter et al., 1992; Dunwoodie, 2007; Vincent and Buckingham, 2010; Wagner and Siddiqui, 2007a, 2007b; Zaffran and Frasch, 2002). Embryonic lethality has been shown to result from the altered function of a wide range of genes contributing to cardiac development. In this review, cardiac development will be analyzed from a temporal and structural perspective. Examples of genes essential to cardiac development at different stages or in specific processes will be detailed, and their roles analyzed to present a composite of the functional diversity of cardiac essential genes. Some of the processes required in cardiogenesis are needed in embryogenesis in general, so cardiac essential genes may also affect the development of the early embryo or other organ systems. This review predominantly covers genes and genetic pathways specific to cardiogenesis.

**CARDIAC INDUCTION AND MIGRATION**

During the first stages of cardiac development, after gastrulation, at embryonic day (E) 6.5–7.5, cardiac progenitors from the anterior mesodermal primitive streak migrate anteriorly and laterally on either side of the embryonic midline (Fig. 1A) (Tam and Behringer, 1997; Tam et al., 1997); primary heart field (PHF) cells are derived from these cells. The PHF cells then migrate medially, form the epithelial cardiac crescent (Fig. 1B) and begin to differentiate in situ before fusing to form the linear heart tube (Fig. 1C) (Bruneau, 2002; Buckingham et al., 2005; Dunwoodie, 2007; Wagner and Siddiqui, 2007b; Zaffran and Frasch, 2002). In addition to intrinsic signals from within the primitive streak,
cardiac cell migration and induction depends on signaling from the anterior lateral mesoderm, anterior endoderm and nonneural ectoderm during gastrulation and crescent formation. These signals regulate cardiac induction while defining the mediolateral borders of the heart-forming region (Arai et al., 1997; Audaboucher et al., 2000; Dunwoodie, 2007).

During gastrulation, anterior primitive streak cells transiently express the basic helix-loop-helix family (bHLH) transcription factor Mesp1, recognized as the first detectable cardiac marker (David et al., 2008; Kitajima et al., 2000; Saga, 1998; Saga et al., 2000; Saga et al., 1999). Mesp1 expressing cells are incorporated into all mature cardiac layers (i.e., endothelem, endocardium, myocardium and epicardium) (Saga et al., 2000). Mesp1 knockout mice display lethality by E10.5 due to cardiac bifida through failure of the linear heart tube to fuse (Saga et al., 1999). Mesp1 and Mesp2 double knockouts, however, display complete migratory block of cardiac precursors and do not develop mesoderm-derived structures including the heart, somites or gut (Kitajima et al., 2000). This indicates that Mesp1 and Mesp2 may be able to compensate for the lack of each other to some extent. Mesp1 may also be critical in promoting differentiation of cells that contribute to the cardiovascular system including cardiomyocytes, endothelial, and smooth muscle cells (Bondue et al., 2008; Bondue et al., 2011; David et al., 2008; Lindsley et al., 2008).

Bone morphogenetic proteins (BMPs) predominantly promote cardiac specification in the PHF. The specifics of BMP signal transduction have been considered in greater detail elsewhere (van Wijk et al., 2007; Wang et al., 2011). However, several ligands from this family are essential to cardiac development. Bmp2 null mutant mice experience embryonic lethality between E7.0 and E10.5 with severely delayed, ectopic or absence of cardiac development (Zhang and Bradley, 1996). Bmp4 null mice similarly demonstrate lethality from E6.5 to E9.5 where the majority of null embryos lack mesoderm-derived structures; those that do achieve the initiation of the heartbeat die soon thereafter with widespread and severe developmental delays (Winnier et al., 1995). Unsurprisingly, BMP receptor null mice such as Alk3, Alk2, and Bmpr2 mutants mirror these phenotypes with lethality occurring by E9.5 due to absence of mesoderm-derived structures (Beppu et al., 2000; Gu et al., 1999; Mishina et al., 1995).

Fibroblast growth factor (Fgf) function is also required for cardiac development. Fgfr1 knockout mice demonstrate failure of mesodermal cell migration from the primitive streak during gastrulation at ~E7.0 and lack mesoderm-derived and endoderm-derived tissues, including the heart, despite cells undergoing epithelial-to-mesenchymal transition (EMT) (Sun et al., 1999). The cardiac abnormalities found in Fgfr1 mutants are mirrored by Fgfr1 null mice (Deng et al., 1994). There is considerable crosstalk between the BMP and FGF signaling pathways in chick models; FGF signaling maintains a pool of undifferentiated stem cells. For cardiomyocyte induction to occur, BMP signaling must downregulate the FGF pathway (Hutson et al., 2010; Tirosh-Finkel et al., 2010). It is not unreasonable to propose that a similar crosstalk mechanism occurs in mice.

HEART FIELDS AND CARDIAC PROGENITOR CONTRIBUTIONS

The mutant phenotypes described above are associated with the absence of PHF cell contributions and failure of PHF cell programming towards a cardiogenic fate. After its formation, the linear heart tube grows through cellular contributions at the arterial and venous poles (Buckingham et al., 2005; Vincent and Buckingham, 2010; Viragh and Challice, 1973). This was originally thought to be entirely from the PHF; however, LacZ transgene expression under Fgf10 promoter control in mice revealed contributions of a second heart field (SHF) to the outflow tract (OFT) myocardium, with transgene expression originating in the pharyngeal mesoderm from E7.5 (Kelly et al., 2001). The SHF was found to lie anteriorly and dorsally to the PHF before migrating caudally and medially to the cardiac crescent and then dorsally to the linear heart tube (Fig. 1B). DiI labeling and retrospective clonal analysis later demonstrated SHF contributions to the OFT, the majority of the right ventricle and parts of the atri (PHF cells were found to contribute to the entire left ventricle, the majority of both atria and parts of the right ventricle (Meilhac et al., 2004; Zaffran et al., 2004). Later contributions of cardiac progenitor cells come from the proepicardial organ (PEO), which will be discussed in more detail below.

Contributions of the SHF to myocardial and endocardial cell populations have been shown in vivo and SHF progenitor cells demonstrate differentiation into myocardial, endocardial and smooth muscle cells in vitro (Moretti et al., 2006; Verzi et al., 2005). The LIM- and homeodomain-containing transcription factor Islet1 (Isl1) is vital to SHF migration, survival, and differentiation. Mice null for Isl1 display absence of SHF derived structures (OFT and right ventricle) and have severely reduced atrial tissue (Cai et al., 2003; Lin et al., 2007); they experience developmental arrest by ~E9.5 and lethality around E10.5. Isl1 activity in the SHF is at least partly mediated by forkhead transcription factors such as Foxc1 and Foxc2 that bind SHF-specific enhancer sequences within the Isl1 locus to direct Isl1 expression (Kang et al., 2009). Mice null for Foxc1 or Foxc2 and heterozygous null/wildtype for the other gene experience lethality from E12.0 to E12.5; the Foxc2 null mice particularly had a shortened OFT and smaller right ventricle (Seo and Kume, 2006). However, double
knockout mice exhibit lethality by E9.5 with a complete absence of the OFT and right ventricle, indicating that Foxc1 and Foxc2 are partially functionally redundant (Seo and Kume, 2006). Similar phenotypes were observed in Foxb1 null embryos (von Both et al., 2004). As the SHF has not yet been genetically defined, additional genes essential for and specific to SHF development likely await discovery.

CHARACTERISTICS OF ESSENTIAL GENES IN EARLY CARDIAC DEVELOPMENT

Essential genes that cause lethality early in cardiac development, disrupting the processes of induction, migration of cardiac progenitors to the cardiac crescent, and heart field formation, represent a small and functionally homogeneous group with respect to their biochemical function. This group of genes consists predominantly of transcription factors, ligands and receptors. The lack of functional diversity at this stage of heart development is unsurprising given that the early-gestational embryonic heart represents a relatively homogenous cell population. Conversely, some genes essential to cardiac development are essential to other earlier developmental processes. Fgf and Bmp signaling pathway members both frequently demonstrate multiple developmental requirements, with null alleles exhibiting lethality during gastrulation, although these genes have later cardiac functions (Arman et al., 1998; Marguerie et al., 2006; Qi et al., 2007; Sirard et al., 1998). Furthermore, the presence or absence of functional redundancy can obscure the role of some genes in early-gestational cardiac development. For example, Wnt signaling ligands possess high protein sequence homology, overlapping expression domains and overlapping functions during early cardiac development which compensates for the absence of each other (Cohen et al., 2008), making the dissection of their specific functions challenging. Some processes occurring during early cardiac development may only have later functional consequences. For example, cardiac looping begins at ~E8.5 but problems resulting from loss of concordance between the heart and other organs may not cause lethality until later in development (Kim, 2011). The limited numbers of genes with essential functions specific to early cardiac developmental processes suggest a relatively restricted genetic programme required for cardiac specification and heart field formation.

CARDIAC CHAMBER FORMATION

Positional identity is established within cardiac progenitors early during cardiac development, prior to the formation of the linear heart tube. However, regions of force-producing chamber and primary nonchamber myocardium are only formed on the onset of cardiac looping (Fig. 1D). Differentiation into atrial and ventricular chamber myocardium occurs locally on the outer curvature of the looping heart simultaneously with rapid proliferation of differentiated cells to form the chambers. During this process, cell-type specific transcriptional programmes that pattern the chambers along the left–right, anteroposterior, and dorsoventral axes also initiate the specialization of cells within the chambers. This collective process of differentiation, proliferation, and specialization is known as the ballooning model of chamber morphogenesis (Christoffels et al., 2004a; Christoffels et al., 2000; Delorme et al., 1997; Moorman et al., 2010). Cardiac cell terminal differentiation and chamber morphogenesis are governed by a core set of essential transcription factors, particularly those from the Nkx, Gata, Mef, Hand, and Tbx families. These transcription factors act as a point of convergence for earlier, upstream pathways and act combinatorially with each other, as well as with cardiac-specific and ubiquitous transcription factors to target the expression of genes involved in later events in cardiac development (Olson, 2006). Understandably, genes from these families are indispensable for the progression of cardiac development beyond midgestation.

In cardiac development, Gata4 has been the most extensively studied family member of Gata zinc finger-containing transcription factors. Gata4 is expressed in the precardiac mesoderm from E7.0 before expanding to the endocardium and myocardium throughout heart tube formation and persisting through adult life (Heikinheimo et al., 1994). Homozygous Gata4 deficient mice suffer embryonic lethality between E7.0 and E9.5 and fail to form the linear heart tube due to inappropriate lateral to ventral embryonic folding and extraembryonic defects (Kuo et al., 1997; Molkentin et al., 1997). Embryo-specific deletion of Gata4 in mice also results in a range of cardiac defects including incorrect looping morphogenesis, thin myocardium with altered cytoarchitecture, reduced trabeculation, absence of the atrioventricular canal (AVC) and bulboventricular groove, absence of endocardial cushions (ECCs) and absence of the proepicardium (Watt et al., 2004). The range in phenotypes caused by disruption of GATA4 function is partly due to its involvement in the expression of sarcomeric genes including Myb6, Myl1, and Tnni3 (Di Lisi et al., 1998; McGrew et al., 1996; Molkentin et al., 1994). Gata4 has additional roles in cardiogenesis; it interacts with Fog2 and a Cx30.2 enhancer to promote ECC formation and the atrioventricular conduction system development, respectively (Flagg et al., 2007; Munshi et al., 2009). Furthermore, chamber expansion appears reliant on Gata family proteins as Gata4 interacts with core cell cycle machinery and cooperates with Gata5 to regulate cardiomyocyte proliferation (Rojas et al., 2008; Singh et al., 2010; Trivedi et al., 2010). Thus, Gata4 acts at both transcriptional
and posttranscriptional levels with several other transcription factors and DNA regulatory elements in a cardiac cell-type specific manner (Brown et al., 2004; Lien et al., 1999; Lozano-Velasco et al., 2011; Maitra et al., 2009; Munshi et al., 2009; Sepulveda et al., 1998; Sepulveda et al., 2002; Singh et al., 2010).

The homeodomain-containing transcription factor Nkx2-5 is another key regulator and essential gene during cardiac development. Nkx2-5 forms complex regulatory loops with Gata4 and lies both upstream and downstream of Gata4 in different systems, although its expression is more spatially restricted than Gata4 within the heart (Brown et al., 2004; Riazi et al., 2009). Nkx2-5 is expressed in cardiac progenitors within the mesoderm during mouse development from E7.5 before being present in myocardial cells throughout cardiac development and adult life (Kasahara et al., 1998; Lints et al., 1993). Nkx2-5 null mice experience lethality between E9.5 and E11.5 due to subsets of abnormalities including cardiac looping defects, abnormal OFT development, absent ECCs, reduced trabeculation, shortened AVC, absence of one ventricle and lack of cardiomyocyte differentiation (Lyons et al., 1995; Tanaka et al., 1999). In addition to cardiomyocyte differentiation, Nkx2-5 appears essential for differentiation of endocardium, components of the conduction system, epicardium and formation of the AVC (Ferdous et al., 2009; Habets et al., 2002; Jamali et al., 2001; Moskowitz et al., 2007; Prall et al., 2007; Zhou et al., 2008b). Again, this is unsurprising given that Nkx2-5 acts upstream of numerous critical genes expressed during cardiac development such as Npr1, Ankrd1, Cx40, Actc1, and Myocd (Bruneau et al., 2001; Chen and Schwartz, 1996; Shiojima et al., 1999; Ueyama et al., 2003; Zou et al., 1997). Additionally, Nkx2-5 is central to pathways controlling cardiac cell proliferation (Prall et al., 2007; Qi et al., 2007; Zhang et al., 2010b). These cellular and morphological events are collectively mediated through differential interactions with cardiac-specific transcription factors and DNA regulatory elements in a cell type-specific manner (Chen and Schwartz, 1996; Puskaric et al., 2010; Sepulveda et al., 2002; Shiojima et al., 1999; Zou et al., 1997). Given the influence of Gata4 and Nkx2-5, many midgestational lethal cardiac phenotypes demonstrate aberrant signaling pathways associated with Gata4 and Nkx2-5.

The MADS-box transcription factor Mef2c acts immediately downstream of and cooperates with Nkx2-5 and Gata4 in cardiac development (Dodou et al., 2004; Skerjanc et al., 1998; Vincentz et al., 2008). Mef2c expression is first detected in the precardiac mesoderm at E7.5, then in the common atrium and ventricle of the heart tube and throughout the myocardium of all chambers during midgestation before declining to lower level expression in late-gestation to adult life (Edmondson et al., 1994; Naya et al., 1999). Mef2c null mice suffer lethality between E9.5 and E10.5 due to inability to undergo cardiac looping, absence of the right ventricle, atrial and ventricular hypoplasia, reduced trabeculation and shortened AVC with absent ECCs. Like Gata4 and Nkx2-5, Mef2c appears to be essential for cardiomyocyte differentiation in vivo (Karamboulas et al., 2006). Several genes have also been identified whose cardiac expression is dependent on upstream signaling of Mef2c including Cad, Tmnt3k, Ctnnα, Champ, and Smyd1 (Liu et al., 2001; Phan et al., 2005; Qiu and Michalak, 2009; Vanpoucke et al., 2004; Wang et al., 2008). Lineage analyses have demonstrated contributions of Mef2c expressing cells to the ventricles and atrioventricular bundle and a role for Mef2c in allocating cells of PHF origin to ventricular or sinoatrial node fate (Anhaanen et al., 2010; Vong et al., 2006). Mef2c has also been suggested to have specific SHF roles downstream of Isl1 and Gata4 (Dodou et al., 2004).

Cardiac chamber formation is achieved by dividing the developing heart into different functional compartments (Fig. 1E). The heart and neural crest derivatives expressed transcript (Hand) genes are among the earliest differentially expressed genes during cardiac development. The Hand1 and Hand2 bHLH family transcription factors are both uniformly expressed in mice at E7.75 during the cardiac crescent stage of heart development before becoming restricted to left and right ventricles, respectively, during and after cardiac looping (Srivastava et al., 1997; Thomas et al., 1998). Hand2 null mouse embryos suffer lethality around E11.0 due to absence of a right ventricle and lack of aortic arch arteries (Srivastava et al., 1997). Rescue of extraembryonic defects by tetraploid aggregation in Hand1 null mice (to circumvent peri-implantation lethality) demonstrated cardiac defects including defective looping and failure to establish distinct atria and ventricles and lack of trabeculation (Riley et al., 1998). The critical roles of Hand1 are in maintenance of proliferation of cardiomyocytes. Removal of Hand1 and 2 in a dose-dependent manner demonstrate their cooperative regulation of ventricular chamber growth and ventricular hypoplasia (McFadden et al., 2005). Furthermore, Hand1 has been suggested to regulate the balance between cardiomyocyte differentiation and proliferation. Mouse embryos overexpressing Hand1 exhibit overexpansion of the linear heart tube whilst embryonic stem cells overexpressing Hand1 upregulate cell cycle gene expression of cyclin D2 (Ccnid2) and cyclin-dependent kinase 4 (Cdk4), which prevents cell cycle exit (Risebro et al., 2006). Ventricular growth mediated by Hand proteins depends on the regulation of myocardial gene expression, which is mostly achieved cooperatively with upstream signals from Gata4, Nkx2-5, and Mef2c (Bruneau et al., 2000; Dai
The T-box (Tbx) transcription factors represent a group of at least 18 transcription factors that have diverse roles in cardiac development. Regarding chamber development, Tbx5 plays important roles in regulating an atrial-specific transcriptional program. During development, Tbx5 is expressed from cardiac crescent to looping heart tube stage hearts in areas destined to become the atria and sinus venosus. Tbx5 expression then gradually expands to the left ventricle and right ventricular trabeculae, atrial septum, left aspect of the ventricular septum, and atrioventricular valves as the heart matures (Bruneau et al., 1999). Tbx5 null mice suffer lethality by E10.5 due to looping abnormalities, failure to form two atria and hypoplasia of the single atrium and left ventricle (Bruneau et al., 2001).

Tbx2 is essential during cardiac development with respect to nonchamber structures. Tbx2 is expressed in the AVC and atrioventricular cushions, OFT and inflow tract in the linear and looping heart before gradually decreasing to background levels by E15.0. In these regions, Tbx2 is thought to repress chamber-specific gene expression such as Nppa, Smpx, Cx40, and Slit3 allowing these areas to remain as primary myocardium for purposes of chamber alignment and to form specialist structures of ECCs, valves, septa, and conduction system components (Aanhaanen et al., 2011; Christoffels et al., 2004b; Medioni et al., 2010). Tbx2 null mice suffer lethality by E14.5 demonstrating abnormal AVC morphology, severely retarded ECC growth, OFT septation defects and ectopic expression of chamber-specific genes Nppa and Smpx (Harrelson et al., 2004). Ventricular defects have also been demonstrated in Tbx2 deficient embryos due to reduced contributions of cells from the primary myocardium that typically turn off Tbx2 expression before contributing to the expanding chambers and septum (Aanhaanen et al., 2009). Tbx2 expression is directly repressed by Tbx20; overexpression of Tbx2 in the myocardium and loss of Tbx20 generates similar cardiac phenotypes (Cai et al., 2005; Christoffels et al., 2004b). Evidence suggests that spatial regulation of primary myocardium-specific gene expression is as equally important as initiation of chamber-specific expression during chamber development.

CHAMBER EXPANSION

After chamber identity is fixed, the myocardium expands to ensure it can pump blood around the growing embryo, while the heart responds to signaling cues directing chamber separation and orientation (Fig. 1F). While the muscle mass is growing the myocardium begins compaction from E11.5 (most markedly between E13 and E14) which contributes to formation of the interventricular septum, papillary muscle, and conduction system and maximizes the amount of muscle that can contribute to the heart. The concurrent actions of ventricular myocardial growth and compaction of the muscle itself allows for production of greater contractile force (Dunwoodie, 2007; Risebro and Riley, 2006; Wagner and Siddiqui, 2007b). Several genes that maintain cardiac chamber expansion and morphogenesis following the actions of the core cardiogenic transcription factors are essential for cardiac development. Transcription factors are important in regulating growth of the cardiac chambers, as well as their specification. The MADS-box transcription factor Srf acts with Gata4 and Nkx2-5 to regulate chamber myocardium growth (Sepulveda et al., 1998). Global knockouts of Srf are early lethal (Arsenian et al., 1998), but cardiomyocyte, smooth muscle, and endothelial-specific knockouts of Srf all exhibit midgestation lethality with a panoply of cardiac defects caused by defective chamber specific differentiation (Holtz and Misra, 2008; Miano et al., 2004; Niu et al., 2005; Parlakian et al., 2004). Tef1, a downstream effector of Srf1, has important roles in muscle-specific gene expression and recognizes regulatory sequences present in the cardiac-specific genes Myb7, Myocd, and Acta2 (Gan et al., 2007; Gupta et al., 2001; Yoshida, 2008). Retroviral gene-trap of Tef1 in mice results in embryonic lethality in homozygous mutants at E11.5–E12.5 with mutants exhibiting thin myocardium and decreased trabeculation (Chen et al., 1994).

The maintenance of mechanotransductive signals is required to support cardiac chamber morphogenesis and expansion. By midgestation genes that sense mechanical stress also become essential for cardiogenesis. G-protein coupled receptors (GPCRs) initiate cascades in response to mechanical force, controlling heart rate, and force of contraction. This response can be desensitized by agonists binding to GPCRs resulting in their subsequent phosphorylation by GPCR kinases (Hata and Koch, 2003). A fine balance must be achieved between desensitization and cascade initiation during cardiac development. Disruption of adrenergic receptor kinase Adrbk1 in mice causes embryonic lethality due to heart failure in homozygous mutants between E9.0 and E15.5 caused by hypoplasia of the ventricular septum and all cardiac chambers, resulting in poor ejection fraction and heart failure (Jaber et al., 1996). Myocardial-specific ablation of Fak, which is needed for stretch induced Myf2 activation (Nadruz et al., 2005), induces defects in cardiomyocyte proliferation and ventricular septal defects (Peng et al., 2008). Other proteins involved in mechanotransduction, such as Erk1, Erk2, and Pxn, also display cardiac morphological defects which suggest links between blood flow, cardiac contraction, and cardiomyocyte proliferation (Granados-Riveron and Brook, 2012).
CHARACTERISTICS OF GENES ESSENTIAL IN CHAMBER FORMATION AND EXPANSION

During the processes of cardiac chamber formation and expansion there is a great increase in the number of transcription factors required, as compared to earlier stages of cardiac development. Members of several different transcription factor families, such as Nkx2, Gata, Mef2, Tbx, and Hand all have been shown to be essential during chamber formation. The functions of these transcription factors ultimately serve to establish chamber identity, so downstream effectors direct appropriate cell differentiation linked to the anatomical position within the heart. Cardiac chamber expansion also requires proteins that drive cell proliferation, which include both transcription factors (Holtz and Misra, 2008; Miano et al., 2004; Niu et al., 2005; Parlakian et al., 2004), as well as mechanotransducers (Granados-Riveron and Brook, 2012). It is not surprising that there are continued requirements for transcription factors during the chamber formation and expansion stages, given the essential role of transcriptional networks in directing cell fate. However, links between mechanotransduction and chamber formation are perhaps more surprising, and underscore the developmental need for cardiac function to propagate the continued cardiac developmental programme.

TRABECULATION

Interactions between the endocardium and myocardium are critical during cardiac development to form essential specialized structures such as ECCs, valves, and trabeculae (Fig. 2). Trabeculation begins around E9.0 in mice with the migration and recruitment of ventricular myocardial cells into the cardiac jelly between the myocardium and endocardium (Samsa et al., 2013). Cells then proliferate and differentiate (earlier than in compact myocardium) forming long thin projections into the endocardium which increase surface area available for oxygen uptake, help to prevent inappropriate blood flow between cardiac chambers and provide the contractile force to support the growing mouse heart from E9.5 to E14.5 (Samsa et al., 2013). During compaction, these trabeculae collapse leaving shorter thicker projections to support cardiac structure and contraction during development and through adult life (Risbro and Riley, 2006).

Null mutations of Nrg1, ErbB2, and ErbB4 all display similar phenotypes of poorly trabeculated ventricles and lethality between E10.5 and E11.5 in mice (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995). Endocardial Nrg1 signaling mediates ligand-dependent heterodimerization of ErbB2 and ErbB4 within the heart myocardium. These dimers become phosphorylated and enable docking of cytoplasmic proteins involved in signal transduction (Negro et al., 2004). Mice null for Efnb2, encoding EphrinB2, and its specific receptor EpbB4 both display failure to form ventricular trabeculae and lethality between E10.5 and E11.0 due to defective signaling between endocardial cells (Gerety et al., 1999; Wang et al., 1998). These examples suggest that intercellular signaling is important in trabeculation.

Signaling within the trabeculae themselves also regulates their growth. Bmp10 is transiently expressed within the trabecular myocardium between E9.0 and E13.5. Bmp10 null mice have thin hypoplastic ventricular walls and demonstrate arrested growth of trabeculae and lethality around E10.5 (Chen et al., 2004). This phenotype may be attributed to essential interactions between Bmp10, cardiogenic transcription factors Nkx2–5, Mef2c and Tbx20, and the trabeculae-specific cell cycle regulator Cdkn1c (Chen et al., 2004; Kochilas...
et al., 1999; Zhang et al., 2011). It has been elegantly shown that Bmp10, Nrg1, and EphrinB2 signaling act downstream of Notch signaling during trabeculation (Grego-Bessa et al., 2007). However, the lethality of Notch pathway mutants has yet to be linked to trabeculation defects.

The formation of large specialized structures such as ventricular trabeculae concomitantly requires genes encoding proteins with specialized function beyond signaling and cell programming. For example, the cardiac jelly must contain a permissive microenvironment to allow the passage of signaling molecules and which trabeculae can physically negotiate. Adamts1 is a matrix metalloproteinase that degrades cardiac jelly components including Versican (Kern et al., 2006; Kuno et al., 2000; Rodriguez-Manzaneque et al., 2002). Although not fully penetrant, deletion of Adamts1 in mice results in ~50% embryonic lethality likely due to uncontrolled trabecular growth (Stankunas et al., 2008). The requirement for genetic functions beyond cell signaling and programming is a characteristic shared with ECC formation.

**ECC FORMATION**

The mature adult heart contains pulmonary and aortic valves in its arterial pole and mitral and tricuspid valves separating atria from the ventricles in the left and right sides, respectively. Valvulogenesis begins with the formation of ECCs during cardiac looping, which persist through midgestation (Fig. 2). ECC formation takes place in the OFT and AVC beginning with localized production of extracellular matrix (ECM) by myocardium forming the cardiac jelly (Miquerol and Kelly, 2013). These ECM proteoglycans are hydrophillic and the volume of matrix produced causes tissue swelling, forming cushions to prevent the inappropriate backflow of blood. Myocardial and endocardial signals then induce activation of endocardial cells, allowing these cells to break interaction with the neighboring endocardium and invade the cardiac jelly. Migrating cells then undergo EMT to populate ECCs with mesenchymal cells (Combs and Yutzey, 2009; Person et al., 2005; Schroeder et al., 2003). The ECCs then undergo ECM remodeling and elongate to form the heart valve leaflets (Chakraborty et al., 2009).

Similarly to trabeculation, a permissive microenvironment must be present to allow ECC formation. Cushions are absent in Has2 and Vcan null mice due to absence of Hyaluronan and Versican, respectively, in both cases causing lethality from E9.5 to E11.5 (Camenisch et al., 2000; Mjaatvedt et al., 1998; Yamamura et al., 1997). Hyaluronan and Versican interact with fellow ECM components and initiate signaling cascades (Asberg et al., 1999; Binette et al., 1994; Hirose et al., 2001; Kawashima et al., 2000; Kern et al., 2006; LeBaron et al., 1992; Lionetti et al., 2010; Maioli et al., 2010; Zhang et al., 1998). If Hyaluronon and Versican act as links between the ECM and intracellular signaling cascades, this could explain why they are so essential for ECC formation. Hyaluronan has also been associated with cell migration which precedes EMT since Has2 null AVC explants could be rescued by Hyaluronan treatment, which induces phosphorylation of ErbB2 and ErbB3, thus rescuing cushion mesenchyme formation (Camenisch et al., 2002).

In valvulogenesis genes that control cell proliferation become especially important during valve elongation, particularly in endocardial derived ECC cells. The transcription factor Nfatc1 is highly expressed in valve endocardial cells and is essential for valve and septum formation; in mice with nonfunctional Nfatc1 cardiac valves remained immature (de la Pompa et al., 1998; Ranger et al., 1998). Nfatc1 supports valvulogenesis by promoting valve endocardial proliferation and simultaneously inhibiting EMT, ensuring a large enough endocardial population for proper valve growth (Wu et al., 2011). Endocardial expression of Tbx20 promotes cell proliferation in valvulogenesis, as well as Wnt/β-catenin signaling, but is dispensable for EMT (Cai et al., 2013).

Classical intercellular signaling is important in ECC formation. All Notch receptors and ligands are expressed in mouse endocardium during the onset of ECC formation (Timmerman et al., 2004). Deletion of Notch pathway components Rbpjk and Notch1 result in collapse of ECCs due to failure of cells to undergo EMT and exhibit lethality at E10.5. The majority of evidence supports ECC explant culture experiments suggesting that Rbpjk and Notch1 signal via Snai1. Snai1 in turn represses VE-Cadherin expression, allowing endocardial cells to destabilize adherens junction cell contacts, delaminate, and invade the cardiac jelly to undergo EMT (Saad et al., 2010; Timmerman et al., 2004). However, Notch1 also appears to act downstream of and converge with Bmp2 signaling during cushion cell invasion and EMT, forming a complex regulatory web between the myocardium and endocardium. Myocardial Bmp2 upregulates endocardial Notch, which in turn downregulates endocardial, but not myocardial, Bmp2 (Fischer et al., 2007; Kokubo et al., 2005; Luna-Zurita et al., 2010).

**TRABECULATION AND ECC DEVELOPMENT DISPLAY OVERLAPPING ESSENTIAL FUNCTIONS**

Genes with essential functions during trabeculation and ECC formation reveal an overlapping requirement for ECM and basement membrane proteins (Costell et al., 2002; Sasse et al., 2008) during these processes. The increasing importance of the extracellular environment as cardiac development progresses is apparent, as animal mutants with perturbations in the production or function of molecules needed for the ECM resemble human...
congenital heart defects (Lockhart et al., 2011). Although ECM is found within all the structures of the developing heart, and is present in the form of cardiac jelly in the early heart tube (Markwald et al., 1977), the ECM is particularly important for the formation of endocardial cushions (Lockhart et al., 2011) and trabeculation. Mutants lacking various matrix molecules or enzymes needed for matrix protein production display thin myocardial walls that lack trabeculae in addition to ECC defects (Camenisch et al., 2002; Camenisch et al., 2000; Mjaatvedt et al., 1998; Yamamura et al., 1997), highlighting the similar processes needed to create these disparate structures. Despite this, the diversity in the signaling cascades required in the processes of ECC formation and trabeculation provides for distinctions between these two events during cardiac development.

EPICARDIAL DEVELOPMENT

In mice, the PEO originates from the pericardial mesothelium covering the pericardial surface of the septum transversum below the heart near the sinus venosus, where a villous structure protruding toward the looped heart is formed (reaching maximal size around E9.5; Fig. 3). In mammals, cells from this structure are thought to undergo vesicular budding into the pericardial space followed by adherence to the myocardium (E9.5–10). However, in rats and mice the PEO and dorsal surface of the heart interact directly, allowing cells to adhere to myocardium via ECM or adhesive protein tissue bridges (Nesbitt et al., 2006; Rodgers et al., 2008). Once the cells have reached the myocardial surface they proliferate and migrate laterally to eventually envelop the heart (completed by E11). The PEO then ceases to exist and PEO-derived cells become differentiated to form the epicardium. The epicardium makes cellular contributions to multiple lineages of the myocardium via delamination, EMT and myocardial invasion. The epicardium is also the main source of cardiac fibroblasts (reviewed in (Souders et al., 2009), prior to EMT the whole epicardium expresses Tcf21, which is necessary but not sufficient for fibroblast fate specification (Acharya et al., 2012). In addition to cellular contributions, the epicardium is also a potent source of trophic factors that stimulate myocardial growth, differentiation, and coronary artery formation (Cai et al., 2008; Lie-Venema et al., 2007; Smart et al., 2007; Sucov et al., 2009; Zhou et al., 2008a).

Migration of epicardial progenitors to and along the myocardium is an essential requirement for cardiac development, and correct cell polarity is required for this process. Par3 null mouse embryos suffer lethality from E10.5 to E12.5 because a loss of cell polarity results in a failure to form the vesicles that bud from the PEO, causing subsequent absence of epicardium in mouse hearts. These defects were identified through aberrant intracellular localization of Pard6β, Prkci, and Ezrin (Hirose et al., 2006). Additionally, Pdpn null mice demonstrate lethality during midgestation due to failure to break cell–cell contact and subsequent decreased delamination of cells from the PEO associated with inability to remove E-Cadherin (Mahtab et al., 2008).

Once cells have migrated from the PEO, they must adhere to the myocardium. Mice null for the myocardially expressed cell adhesion molecule Vcam1 display lethality around E11.5–E12.5 exhibiting absent epicardium with subsequent thinner compact myocardium accompanied by pericardial haemorrhage (Kwee et al., 2011).
1995). Mutations in Itga4, coding for α4 integrin, mirror these defects as αβ3 integrin, along with its two major ligands Vcam1 and Fibronectin, are essential for epicardial adhesion both initially and long-term (Sengbusch et al., 2002; Yang et al., 1995). The proepicardium marker gene Wilms tumor suppressor 1 (Wt1) regulates expression of Itga4; embryos deficient in α4 integrin resemble Wt1 knockout embryos (Kirschner et al., 2006).

Once attached, epicardial cells direct myocardial cell proliferation, maturation, mechanical/electrical coupling, and cellular alignment to maximize contractile force (Weeke-Klimp et al., 2010). Wt1, which in the heart is specific to the epicardium, controls synthesis of the highly potent morphogen retinoic acid (RA) through Raldh2 expression (Guadix et al., 2011; Kirschner et al., 2006). Raldh2 null mice demonstrate lethality by E10.5 with defective chamber development, myofilament gene expression, cardiomyocyte differentiation and mechanical looping defects (Niederreither et al., 2001), suggesting that epicardially synthesized RA is needed for myocardial development. Within the epicardium, RA signaling appears reliant on retinoid receptor Rxrα. Rxrβ null mice exhibit lethality from E13.5 to E17.5 due to ventricular hypoplasia and reduced trabeculation that appears to stem from failure in epicardial adhesion to the myocardium (Sucov et al., 1994). Subsequent studies on Rxrα null mice have showed delayed cellular migration from the PEO, elevated apoptosis of PEO cells, increased myocardial fibronectin and decreased epicardial Vcam1 causing increased area of subepicardial spaces between epicardium and myocardium (Hoover et al., 2008; Jenkins et al., 2005). Nonlocal sources of RA are also essential; RA signaling in the liver controls Epo expression, whose product travels to the heart and stimulates myocardial cell proliferation (Brade et al., 2011). Expression of its receptor, Epor, is found in the epicardium while expression Epo itself is absent from the heart; both are essential genes (Koury et al., 1988; Wu et al., 1999).

Perhaps the transcription factor that has been most characterized with regards to its function in the epicardium is Wt1. Wt1 has been shown to promote epicardial EMT via transcriptional regulation of Snai1 and E-cadberin (Martinez-Estrada et al., 2010) and regulate RA signaling through transcriptional activation of Raldh2 (Guadix et al., 2011). Subsequent to epicardial EMT, epicardial-derived cells contribute to the developing coronary vasculature, which is critical for lategestation cardiac function (Dong et al., 2008; Perez-Pomares and de la Pompa, 2011).

ESSENTIAL GENES FOR EPICARDIAL FUNCTION

The development of the epicardium from the PEO coincides with an expansion in the complexity of the developing heart, and concomitantly, an increase in the number of essential genes. As the epicardium migrates onto the heart surface during development to envelop the developing myocardium, cellular interactions between the epicardium and myocardium are formed. Genes mediating these interactions are essential for cardiac development (Kwee et al., 1995; Sengbusch et al., 2002; Yang et al., 1995). Yet the epicardium is also a source of signals to promote ventricular development, and, therefore, a diverse array of molecules is essential for epicardial function. Many mutants with epicardial defects display a thin ventricular myocardium, indicating a link between epicardial function and myocardial cell proliferation or compaction (Brade et al., 2011; Mahtab et al., 2008; Niederreither et al., 2001; Sucov et al., 2009; Weeke-Klimp et al., 2010). There are both signaling molecules and transcription factors that interconnect epicardial and myocardial development. Following epicardial EMT, epicardial derived cells (EPDCs) contribute to the forming coronary vasculature (Perez-Pomares and de la Pompa, 2011; Riley and Smart, 2011; Ruiz-Villalba and Perez-Pomares, 2012; von Gise and Pu, 2012). As many processes in epicardial formation are conserved and essential throughout embryogenesis, loss of these genes often results in early lethality. Embryos that survive to midgestation exhibit lethality because defects in epicardial cell function will cause defects in coronary vessel (CV) formation.

CARDIAC NEURAL CREST CELLS AND PHARYNGEAL ARCH ARTERY FORMATION

Neural crest cells (NCCs) migrate from the dorsal neural tube between the midotic placode and the caudal boundary of the third somite into the pharyngeal arches and OFT where they eventually form part of the OFT septum (Jiang et al., 2000). The pharyngeal arch arteries (PAAs) emerge proximally from the aortic sac and distally from the descending aorta to form continuous arteries during early to midgestation. The PAAs are numbered 1–6, although the fifth artery is thought to be rudimentary or absent, and form sequentially. The first PAA emerges by E9.0, the second by E9.5, and the third, fourth, and sixth between E9.5 and E10.0. The PAAs are initially formed from mesoderm-derived endothelial cells and rely on cranial and cardiac (from rhombomeres 1, 2, and 4, and 6–8, respectively) NCC contributions for their maintenance and remodelling. Specifically, cranial NCCs contribute to the first and second pharyngeal arches including the first and second PAAs, while the cardiac NCCs contribute to the third, fourth, and sixth pharyngeal arches including the third, fourth, and sixth PAAs. Beginning at ~E11.0, the right-sided PAAs regress and are lost, leaving the left-sided PAAs to be remodelled and become integral components of the vascular system. For example, the fourth
PAA becomes the arch of the aorta from which the left subclavian and common carotid arteries emerge. Unsurprisingly, the precise spatiotemporal regulation required for PAA development provides ample opportunity for defects to manifest themselves when pathway components are altered or absent (Graham, 2003; Hiruma et al., 2002; Snider et al., 2007).

Similar cardiac inductive and migratory processes are required in mouse NCCs as in PHF and SHF as shown by mutations of members of the Bmp, Fgf, and Wnt pathways (Kubota and Ito, 2000; Nie et al., 2008; Schleiffarth et al., 2007; Song et al., 2010; Tang et al., 2010; Zhang et al., 2010a). Genes specific to NCCs (in the context of cardiogenesis) include Sox9, Snai2, and Foxd3, with the former two influencing EMT and differentiation and the latter influencing NCC induction (Cheung et al., 2005). Interactions between the SHF and NCCs appear to be mutually important in regulating EMT and NCC migration; this is revealed in mutants with loss of function of Pax3 or components of Fgf signaling (Bradshaw et al., 2009; Park et al., 2008; Zhang et al., 2008). Foxd3 and Pax3 interact genetically to allow cell survival in cardiac NCC progenitors, although the nature of this interaction is unknown (Nelms et al., 2011). Unsurprisingly, the majority of defects observed in the cardiac NCCs affect OFT septation and alignment, as observed in embryos with Pax3 loss of function or NCC-specific deletion of N-cadherin (Bradhaw et al., 2009; Luo et al., 2006). These defects arise from wide ranging aspects of NCC migration, specification, proliferation, and EMT (Snider et al., 2007; Vincent and Buckingham, 2010).

Tgfβ signaling also appears to be an integral component of PAA development, suggesting similarities between PAA and normal vascular development. Tgfβ2 null mice experience inappropriate apoptosis of the fourth PAA, which contributes to the perinatal lethality observed in these mice (Molin et al., 2002; Molin et al., 2004). Similarly, NCC-specific deletion of Tgfβ receptor Alk5 results in abnormal PAA remodelling due to aberrant apoptosis of neural crest derived cells (Wang et al., 2006). Function of the transcription factor Mrtfb, which is needed for vascular smooth muscle cell (VSMC) differentiation, is mediated by SMAD2 (Li et al., 2005; Xie et al., 2013). Mice null for transcription factor Mrtfb experience lethality by E14.5, displaying widespread haemorrhaging as well as defects in PAA remodeling and VSMC differentiation (Oh et al., 2005).

Bmp signaling is critical for the induction and/or EMT of NCCs that contribute to PAAs; mice with NCC-specific deletion of Smad4 are lethal by E12 with reduced expression of NCC markers including Tfailpa2a, Sox9, and Msx1 and 2. This causes apoptosis of both neural crest and nonneural crest derived cells within the developing pharyngeal arches (Nie et al., 2008). Similarly, NCC-specific conditional mutants of Bmpr1a display hypoplasia of OFT cushions, inappropriate backflow of blood and lethality (Nomura-Kitabayashi et al., 2009).

### ESSENTIAL GENES FOR CARDIAC NCC FUNCTION AND PAA FORMATION

Cardiac NCC function, migration and the subsequent formation of the PAAs shares many of the same processes as cardiac chamber or epicardium formation and maturation. In all these events cellular differentiation, migration, and adhesion are indispensable, as are the genes that control these processes. As a result, it is not surprising that familiar pathways, such as the Fgf, Bmp, and Tgfβ signaling pathways, are needed in cardiac NCC and PAA development. Where genes specific to cardiac NCC and PAA fit into established pathways is less well characterized, but it is clear that other signaling pathways and transcription factors are also essential, and these may be poorly understood. For instance, mice null for genes needed to produce endothelin 1, such as Edn1 and Ece1, exhibit inappropriate persistence and/or regression of the PAAs during remodelling (Yanagisawa et al., 1998). It is likely that many genes and pathways which contribute to defects in PAA remodelling are yet to be discovered, given the complexity and lack of complete understanding of this developmental process.

### SARCOMERE FORMATION

As the heart begins to circulate blood, structural genes, and the genes that control them, become essential. Contraction of muscle fibers including those of the heart is achieved through the actions of the sarcomere, the functional unit of contractility, which contains contractile, structural and regulatory proteins. Fully formed sarcomeres are visible from E9.0 in mice and are large multiprotein complexes (Craig and Padron, 2004), which retain their structural integrity despite having to constantly and rapidly contract/relax and switch on/off to meet the demands of the heart. These structures allow transduction of force throughout the cell to the ECM and sense mechanical stretch (Boateng and Goldspink, 2008). In contrast to the transcriptional and signaling pathways that direct many of the events of cardiac development, the functions encoded by genes lethally disrupting sarcomere function do not show much overlap with other groups of cardiac essential genes.

The structure of the sarcomere is understood in terms of its longitudinal appearance via electron microscopy in relaxed state (Fig. 4). The sarcomere is bordered at each end by the dark Z-disc of ~0.1 μm length. The Z-disc bisects the I-band, which is ~1 μm, is shared between adjacent sarcomeres and is
made up of thin filaments (10 nm diameter). The A-band lies between I-bands and is predominantly made up of thick filaments (15 nm diameter) with a slight overlap of thin filaments and is \( \approx 1.6 \, \mu m \) length. At the centre of the A-band is the higher density H-zone appearing lighter with the M-line in turn at its centre. In cross section, where thick and thin filaments overlap, thin filaments are hexagonally arranged around thick filaments (Craig and Padron, 2004).

Several structural sarcomeric proteins perhaps unsurprisingly have proven to be essential for cardiac development. At up to 3.7 MDa, Titin is the largest known protein and spans from the Z-disc to the M-line within the sarcomere (LeWinter et al., 2007). Due to the vast size and volume of interacting partners, Titin has been studied on a modular basis. Evidence has suggested Titin regulates sarcomeric length, plays important roles in cardiac stress responses and contributes to diastolic properties of the heart (Kontrogianni-Konstantopoulos et al., 2009; Linke, 2008). A truncation mutant of Ttn (which encodes Titin) thought to cause degradation of Titin causes embryonic lethality at \( \approx \)E9.0 due to defective sarcomeric formation (Gramlich et al., 2009). An M-line deficient mutant of Titin led to developmental delay by \( \approx \)E11.0 followed by embryonic lethality due to lateral growth defects in sarcomeres and their consequent disassembly (Weinert et al., 2006).

The thick filaments of sarcomeres are made up of members of the Myosin II superfamily, as well as other nonmyosin proteins. Each Myosin II molecule comprises two myosin heavy chains which form an insoluble coil via their tail domains and two pairs of myosin light chains which attach to the neck domain of each heavy chain, respectively, leaving the head domains in close proximity (Craig and Padron, 2004; Craig and Woodhead, 2006). When in a high Ca\(^{2+}\) environment the head domains interact with actin and ATP, providing the energy for contraction, which occurs through the slippage of cross bridges between myosin and actin (Huxley and Hanson, 1954). Given the important structural and enzymatic properties of myosin heavy chains, it is unsurprising that they are essential genes for cardiogenesis. For instance, deletion of Myosin II member Myb6 causes embryonic lethality from E11.0 to E12.0.
(Jones et al., 1996). Deletion of atrial or ventricle-specific Myosin light chains Myl7 or Myl2 results in embryonic lethality at E11.5 and E12.5, respectively, due to sarcomeric disorganization accompanied by morphological cardiac defects (Chen et al., 1998; Huang et al., 2003).

The thin filaments of sarcomeres consist of a double helix of two F-Actin polymeric chains, along which are bound two parallel Troponin complexes and periodic Troponin complexes (reviewed in Craig and Padron, 2004). In a Ca$^{2+}$ rich environment, inhibition of contraction by Troponin is lifted, allowing Actin-Myosin interaction, ATPase activity and movement of the thin filaments along the thick filament towards the M-line (Galinska et al., 2010; Galinska-Rakoczy et al., 2008; Huxley and Hanson, 1954; Lehman et al., 2009; Solaro, 2010). Tnnt2 encodes the cardiac-specific isofrom of Troponin subunit TnT of which null mice demonstrate lethality at ~E10.0 with absent sarcomeres and absent heartbeat (Nishii et al., 2008). Tropomodulin prevents Actin depolymerization and elongation at the end of appropriately sized thin filaments and also interacts with Troponyosin. Unsurprisingly, Tmod1 null mice exhibit lethality by ~E9.5, with an absence of sarcomeres and defective cardiac looping (McKeown et al., 2008). Mice null for cardiac Actin Actc1 also experience 56% embryonic lethality rate in late gestation due to sarcomeric disorganization and presumptive compromised cardiac function (Kumar et al., 1997).

A number of nonstructural genes are essential for sarcomere development; these are genes needed to induce proper sarcomere formation or regulatory pathway components enabling sarcomeres to respond to stress, developmental stimuli or to maintain sarcomeric function. These genes by necessity have more diverse functions than structural sarcomeric functions. Cardiac-specific light chain kinase, which phosphorylates Myosin regulatory light chain 2 ventricular/cardiac isoform (Mlc2v), is essential for sarcomere assembly (Seguchi et al., 2007) and appears to be under the control of Nkx2–5 (Chan et al., 2008). Calreticulin is an endoplasmic reticulum protein which binds Ca$^{2+}$ and regulates its intracellular homeostasis (Michalak et al., 1999). Calr null mice demonstrate embryonic lethality from mid to late gestation with ventricular hypoplasia, random sarcomeric orientation, sarcomeric waviness, and thinning (Lozyk et al., 2006; Mesaeli et al., 1999).

**SARCOMERE FUNCTIONAL ESSENTIALITY IS DISTINCT**

In contrast to the transcriptional and signaling pathways that direct many of the events of cardiac development, the functions encoded by genes lethally and exclusively disrupting sarcomere function do not show much overlap with other groups of cardiac essential genes. From midgestation, the mammalian embryo requires cardiac contractility to pump blood throughout the cardiovascular system, delivering nutrients to embryonic cells. Impaired cardiac function as a result of disordered or functionally incomplete contractile apparatus assembly leads to embryonic lethality. Structural proteins that allow myofilament assembly and function are required within the sarcomere to form the contractile units of the developing heart. The specific mechanical and structural role of sarcomere proteins makes it unsurprising that there is a functionally distinct group of genes displaying essentiality during sarcomere assembly, which are not involved in other aspects of cardiac development.

**CONCLUSIONS**

Complete functional annotation of mammalian genomes is beginning to appear as a realistic possibility (Dolgin, 2011; Skarnes et al., 2011; White et al., 2013). The increasingly systematic nature of random mutagenesis and mutation identification, targeted mutagenesis/cloning and identification of epigenetic modifications along with increasing computational power and variety of bioinformatic approaches are facilitating these efforts. Systems biological approaches can be used to model metabolic pathways, dynamics of specific cell behavior under varying stimuli, disease causing aberrations in functional networks and in screening for potential therapeutic agents (Chen et al., 2012; Lambrou et al., 2012; Nagasaki et al., 2011; Tan et al., 2013). These approaches can also demonstrate the consequences of nonsynonymous DNA polymorphisms on protein interactions and identify mutations leading to development of diseases (Reimand and Bader, 2013; Yates and Sternberg, 2013a). Perhaps most relevantly, analysis of the characteristics of genes that confer essentiality or disease-susceptibility will lead to insights into the genetic networks required for cellular functions (Yates and Sternberg, 2013b). As systems biological data accumulates it may be possible in the near future to scale these networks up to construct a hierarchy for the functional importance of genes or domains at the whole mammalian genome scale.

Efforts to functionally annotate the mammalian genome have increased the identification of essential genes. Recent experimental studies support the prediction that approximately 40% of mammalian genes are essential (White et al., 2013). Interlinked with the identification of essential genes is the discovery of new developmental functions, as essential genes inherently are required during development. The in utero demand for a functional cardiovascular system further underscores the integration of gene essentiality and cardiac development. During the process of heart formation genes with diverging functions become essential. An
analysis of the functional diversity of cardiac essential genes throughout development therefore reveals the changing cellular and biochemical demands of the developing heart. As more essential genes are annotated, links between gene function and specific cardiac developmental events will expand our knowledge of genetic factors that may contribute to cardiac developmental defects including congenital heart disease.

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