predominantly expressed in the developing right ventricle, while Tbx5 is predominantly expressed in the left ventricle (Srivastava, 2006). Nkx2—5 null mice died early in utero and showed a downregulation of Hand1 expression (Tanaka et al., 1999). In contrast, Gata4 cardiac-specific knockout mice showed hypoplasia of the developing right ventricle and a downregulation of Hand2 expression (Zeisberg et al., 2005). The right ventricle-specific enhancer of Hand2 contains conserved GATA-binding elements suggesting that the expression of Hand2 is under the control of Gata4 in right ventricular formation (McFadden et al., 2000). The expression of Hand1 is, on the other hand, under the control of Nkx2—5 in left ventricular formation, although the cisregulatory element of Hand1 in the developing heart remains to be identified. Hand2 is also controlled by miRNA, miR1 in the posttranscriptional step. The level of Hand2 proteins was reduced in a miR1 overexpressing heart without a change in Hand2 mRNA levels. Targeted deletion of miR1 resulted in the upregulation of Hand2 during murine development. These results suggest that miR1 negatively regulates Hand2 function (Zhao et al., 2007).

3.2.2.3. Function of Hand genes during ventricular development Genetic manipulation of Hand genes revealed that both Hand genes are required for the development of cardiac ventricles. Hand2 null mice die around E10.5 due to cardiac failure resulting from hypoplasia of the right ventricle and pharyngeal arch artery, suggesting that Hand2 is essential for the formation of the right ventricle (Srivastava et al., 1997). To exclude the deteriorating effect of the pharyngeal arch artery, Hand2 cardiac-specific null mice were recently generated using a Cre–loxP system. Myocardial-specific Hand2 null embryos using cardiac troponin T-Cre lines showed hypoplasia of the right ventricle and outflow tract (Morikawa and Cserjesi, 2008). Specific deletion of Hand2 in the SHF using Isl1-Cre demonstrated a phenocopy of Hand2 null embryos and excessive apoptosis was observed in SHF-derived progenitor cells, suggesting that the function of Hand2 in SHF cells is essential for their survival and the formation of the right ventricle (Tsuchihashi et al., 2011). Specific deletion of Hand2 in an NCC lineage using Wnt1-Cre lines also resulted in defects of the outflow tract, suggesting a requirement of Hand2 in the NCC during development of the heart (Morikawa and Cserjesi, 2008).

Hand1 knockout mice showed an early embryonic demise before cardiac looping around E8.0 due to placental defects. Cardiac-specific knockout of Hand1 resulted in defects of the interventricular septum and atrioventricular valve and death after birth (McFadden et al., 2004). These cardiac phenotypes were much milder than Hand2 null mice. Crossing Hand1 cardiacspecific knockout mice with Hand2 knockout mice enabled

the analysis of the effect of the gradual reduction of Hand genes during heart development. Cardiac-specific Hand1 null; Hand2 heterozygous embryos showed two immature ventricles with a failure of ventricular ballooning. The ventricular cardiomyocyte marker, Mlc2v, was expressed in these immature ventricles, indicating that ventricular cell fate was specified. Both Hand1 null; Hand2 null embryos showed more severe cardiac phenotypes, showing only a single primitive ventricle. These results suggest that Hand genes are required for the ballooning process and Hand2 may genetically compensate Hand1 function in a dose-dependent fashion during cardiac ventricular development.

Either Nkx2–5 or Hand2 null embryos showed a single developing ventricle. Both Nkx2–5 and Hand2 knockout embryos showed only single cardiac chambers with an atrium-like appearance. This immature chamber directed atrial cardiomyocyte-specific marker, Mlc2a, suggesting that a genetic interaction between Nkx2–5 and Hand2 is essential for cardiac ventricular development (Fig. 4.5).

The overexpression of Hand1 or Hand2 throughout developing ventricles using a Mlc2v promoter resulted in a single ventricular chamber with abnormal development of the interventricular septum (Togi et al., 2006 ; Yamagishi et al., 2001). Ballooning of the developing left and right ventricles robustly expressing Hand1 and Hand2, respectively, may form the interventricular groove between these ventricles and, as a result, the interventricular septum may emerge in a passive manner by the mechanical force of blood flow into each ventricle.

To directly investigate the genetic redundancy of Hand genes, a gene replacement strategy of Hand genes was recently undertaken. The genomic Hand1 locus was replaced by a Hand2 genomic fragment using a homologous recombination approach. The targeting gene harboring a Hand2 knockin to the Hand1 locus expresses Hand2 under the control of the Hand1 endogenous regulatory region. Fifty-five percent of heterozygous
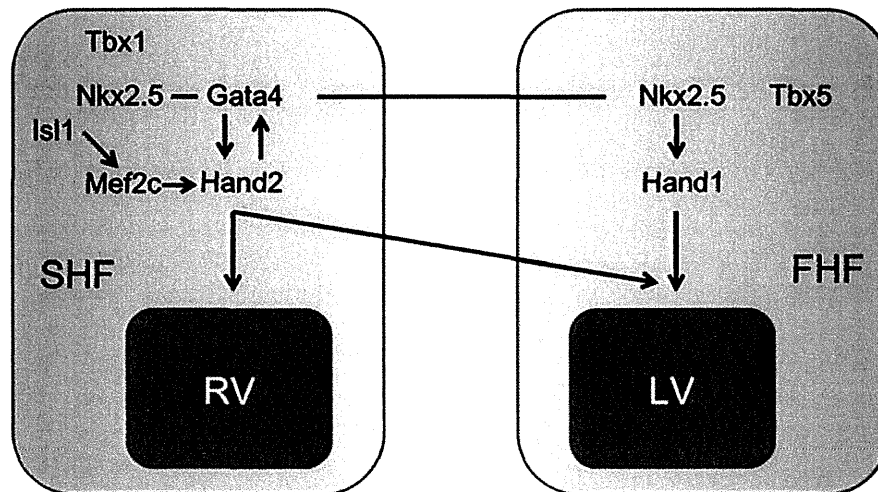
Figure 4.5 Schematic representation of the roles of Hand genes in ventricular development. The second heart field (SHF), expressing Hand2, Nkx2–5, Gata4, Isl1, and Tbx1, gives rise to the right ventricle (RV). The first heart field (FHF), expressing Hand1, Nkx2–5, and Tbx5, gives rise to the left ventricle (LV). Arrows indicates the regulatory pathway. Lines indicate the interaction between genes.

embryos for this allele were stillborn. They showed generalized edema and a complex of cardiac outflow defects, including a double outlet right ventricle, ventricular septal defect, and patent ductus arteriosus, indicating severe heart failure in utero. This result suggests that Hand proteins were not functionally redundant since the Hand1 heterozygous mutant is viable and fertile (Firulli et al., 2010). On the contrary, the heterozygous allele for the Hand1 knockin to the Hand2 locus did not show any obvious phenotype. Mice homozygous for the Hand1 knockin to the Hand2 locus survived longer than Hand2 null mice, but eventually died of heart failure before birth, suggesting that Hand1 might be able to at least partially compensate Hand2 function (Maeda, J., Yamagishi, H., and Srivastava, D, unpublished observations). The previous study also supported the idea that Hand genes share a common function and may perform redundant functions during murine development (Yamagishi et al., 2001). Further functional studies of Hand proteins are needed to explain these conflicting results.

3.2.2.4. Hand genes implicated in human congenital heart diseases
Congenital heart diseases are the most common birth defects in humans. A certain amount of congenital heart diseases are explained by defects result from specifically affecting either the left or right ventricle, such as hypoplastic left heart syndrome or tricuspid atresia, respectively. These observations are likely to reflect the genetic program that regulates the development of specific cardiac chambers together with the hemodynamic effect of blood flow in utero. Recently, genetic screening of patients with nonfamilial hypoplastic left heart syndrome revealed a common frameshift

mutation of the loop region of the bHLH domain in HAND1. The truncated HAND1 protein failed to promote transcription in vitro, suggesting that this heterozygous HAND1 mutation may result in a loss in the function of the HAND1 protein essential for heart development, and has been implicated in hypoplastic left heart syndrome in humans (ReamonBuettner et al., 2008). Moreover, a heterozygous deletion of HAND2 was detected in terminal deletions of chromosome 4q in humans. 4q syndrome is commonly associated with right ventricular outflow tract defects, such as pulmonary valve stenosis, although cytogenetic analysis failed to confirm an association between the Hand2 haploinsufficiency and cardiac phenotype in this syndrome (Huang et al., 2002).

3.2.3. A role of Tbx genes in cardiac development

3.2.3.1. Tbx genes essential for cardiac development Members of the

Tbx gene family are essential for normal development, and mutations in some human TBX genes cause congenital malformations. During development of the heart, Tbx genes play important roles in early cardiac lineage determination, chamber specification, valvuloseptal development, and diversification of the specialized conduction system (Greulich et al., 2011 ; Naiche et al., 2005; Plageman and Yutzey, 2005). The vertebrate genome has at least 18 different Tbx genes in five subfamilies with diverse regulatory functions in development and disease, and some Tbx genes are expressed in specific chambers or regions of the developing vertebrate heart, including Tbx1, Tbx2, Tbx3, Tbx5, Tbx18, and Tbx20 that are categorized in Tbx1 and Tbx2 subfamilies (Fig. 4.6; Papaioannou, 2001). Despite their overlapping expression patterns, these six genes have their own unique developmental functions. The identification of cardiac transcriptional binding partners with differential affinities for individual Tbx factors has contributed to understanding how the combined regulatory influences of multiple, related, and coexpressed genes generate unique downstream target gene expression patterns during organogenesis. Of the six Tbx genes essential for cardiac development, Tbx1 and Tbx18 ensure elongation of the heart tube at the arterial and venous pole, respectively. Tbx1 acts in the pharyngeal mesoderm to maintain proliferation of mesenchymal precursor cells for formation of a myocardialized and septated outflow tract. Tbx18 is expressed in the sinus venosus region and is required for myocardialization of the caval veins and the sinoatrial node. Tbx5 and Tbx20 function in the early heart tube and independently activate the chamber myocardial gene
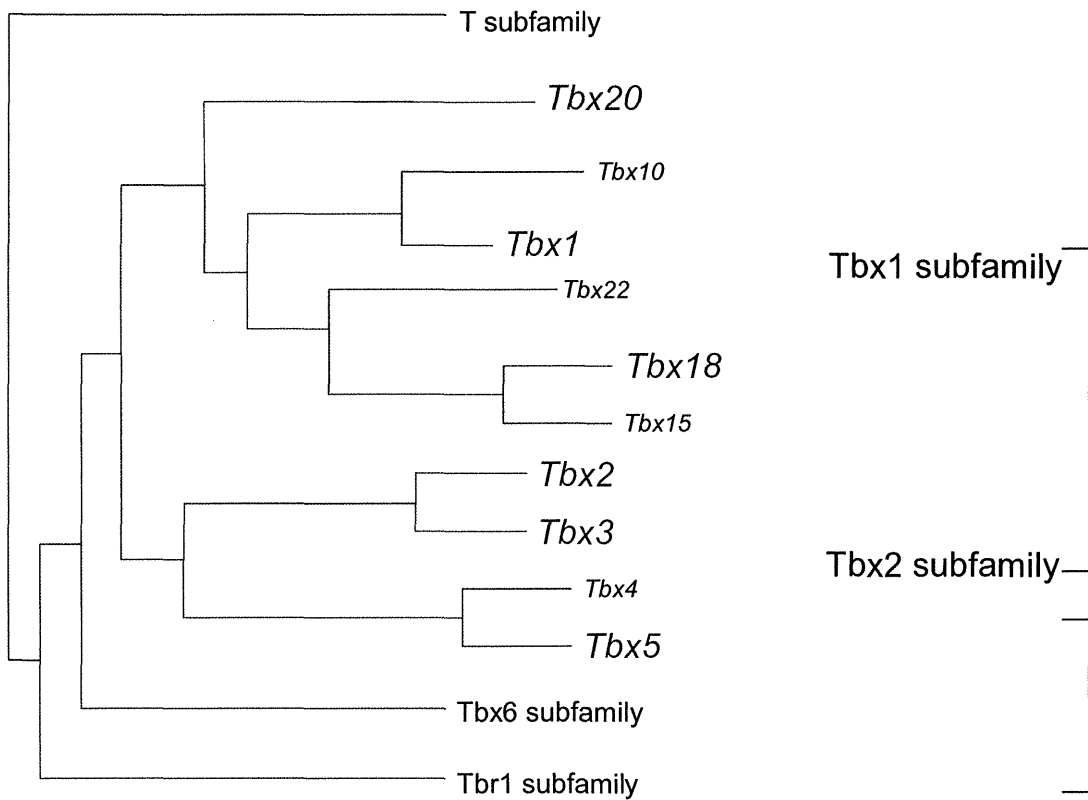
Figure 4.6 The schematic phylogenetic tree of the T-box gene family. Six T-box genes that are expressed in the heart are shown in red. These six genes are included in the Tbx1 or Tbx2 subfamily.
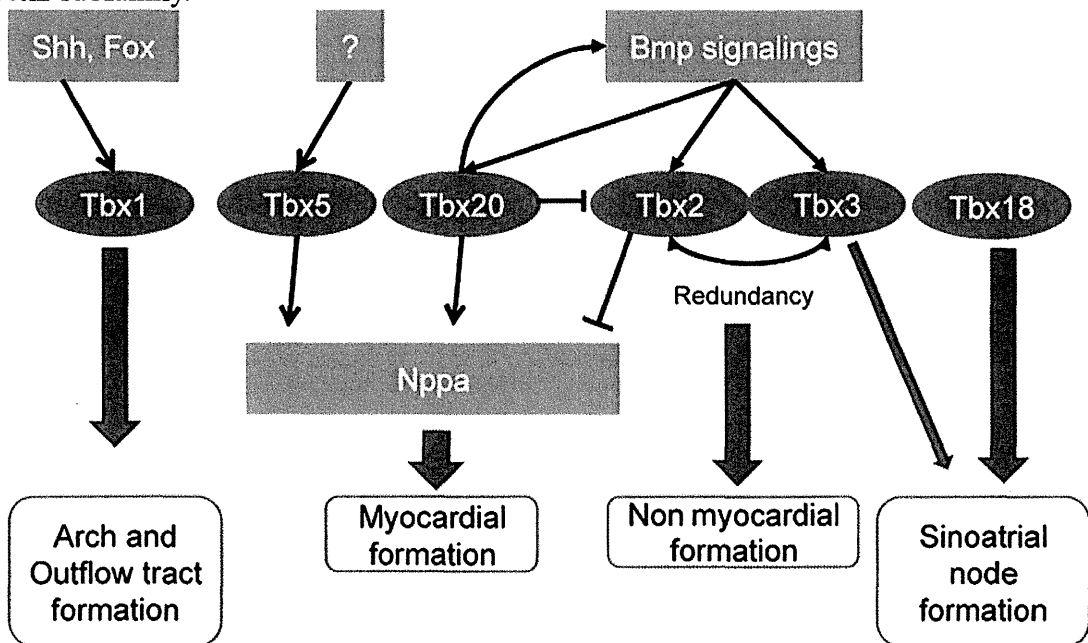

Figure 4.7 Model for regulation of heart development by T-box genes. A network of T-box genes plays roles in development of various regions of the heart.

program, whereas Tbx2 and Tbx3 locally repress this program and promote valvuloseptal formation and development of the cardiac conduction system (Fig. 4.7).

3.2.3.2. Tbx1 in cardiac development TBX1 is a major genetic determinant of the 22q11.2 deletion syndrome in humans that is highly associated with outflow tract defects, such as persistent truncus arteriosus and tetralogy of Fallot (Yagi et al., 2003; Yamagishi, 2002; Yamagishi and Srivastava, 2003). Tbx1 is expressed in the SHF, but not in the cardiac NCC (Garg et al., 2001 ; Yamagishi et al., 2003). Pharyngeal expression of Tbx1 is maintained by Shh. Shh is produced in the pharyngeal endoderm and, in the adjacent mesoderm, induces the expression of forkhead class transcription factors, FoxA2, FoxC1, and FoxC2 that directly act on the Tbx1 promoter (Fig. 4.7). Tbx1- Cre transgenic mice using the Fox-binding element of the Tbx1 promoter demonstrated that Tbx1 plays an important role for the SHF that eventually gives rise to the right ventricle and the outflow tract (Maeda et al., 2006). Tbx1 null mutant mice phenocopy the human phenotype of the 22q11.2 deletion syndrome (Jerome and Papaioannou, 2001). Mice with Tbx1 hypomorphic alleles display cardiovascular defects, but no cleft palate, a phenotype milder than Tbx1 null mutant mice (Hu et al., 2004). A gradual Tbx1 dosage reduction from 100% to 2% using two hypomorphic mouse models demonstrated a variety of outflow tract defects including persistent truncus arteriosus, tetralogy of Fallot, and double outlet right ventricle (Zhang and Baldini, 2008). Although little is known about the molecular mechanisms underlying the interaction between the SHF and cardiac NCC, Tbx1 may play a key role in its signal transduction (see previous section). Formation of pharyngeal arch arteries requires the pharyngeal surface ectoderm as a signaling center from which Gbx2, in response to Tbx1 from the SHF, triggers essential directions for the migration of the cardiac NCC to the caudal pharyngeal arches (Calmont et al., 2009).

3.2.3.3. Tbx18 in cardiac development Tbx18 is a member of the Tbx1 subfamily that is associated with the cardiac venous pole (Fig. 4.7). The venous pole of the developing heart is a particularly complex area since it contributes to the outer tissue layer of the heart, the cardiac pacemaker, as well as to the myocardium of the sinus venosus. Tbx18 is expressed in the PE, epicardium, and both in mesenchymal progenitors and the differentiated myocardium of the sinus venosus region (Kraus et al.; 2001). Genetic lineage analysis showed that Tbx18-expressing cells form the sinus horn and the sinoatrial node myocardium, but do not contribute to the atrial and pulmonary vein myocardium that instead are descendents of FHF and SHF progenitors. Tbx18 null mutant mice die perinatally and display a range of rib and vertebrae defects, along with specific defects of the systemic venous return. Caval veins are thin and show an abnormal lateral position in the pleuropericardial membrane; their myocardial differentiation is severely delayed. The sinoatrial node is dramatically

reduced, and the border between the sinoatrial node and atrial myocardium seems fuzzy (Wiese et al., 2009). In contrast, a phenotypic requirement during development of the epicardium and left ventricular myocardium has not yet been presented, although Tbx18 is also expressed in these layers. Together, these findings argue for a unique function of Tbx18 in proliferation, differentiation, and selective recruitment of precursor cells of a distinct posterior pool during formation of the systemic venous return. To date, a mutation of TBX18 in humans has not been identified.

3.2.3.4. Tbx2 and Tbx3 in cardiac development Tbx2 and Tbx3 are two closely related Tbx transcription factor genes, the expression of which is exclusively associated with the nonchamber myocardium of the atrioventricular canal and the outflow tract during cardiac development, suggesting a functional role in setting up this early nonmyocardial area (Fig. 4.7; Christoffels et al., 2004). Tbx3 is additionally found in the epithelia and NCC in the pharyngeal region, in the sinoatrial node primordium, and the central conduction system (Hoogaars et al., 2004). In humans, mutations in TBX3 is associated with ulnar mammary syndrome (Bamshad et al., 1997), which causes defects in posterior limb formation, but a mutation has not been identified for TBX2. Given the biochemical similarity and the overlapping expression domains, both genes are likely to act redundantly in the atrioventricular canal and show unique requirements in the other expression domains.

Tbx2 null mice exhibit septal defects in the outflow tract and a partial expansion of chamber-specific gene expression into the atrioventricular canal (Harrelson et al., 2004). Tbx3 null mice have a normal atrioventricular canal, but the development of the outflow tract is disturbed. This is probably secondary to an impaired contribution of the cardiac NCC to the arterial pole of the heart (Mesbah et al., 2008). The sinoatrial node is formed normally, but features an atrial, instead of a sinoatrial, node type myocardium in this mutant mouse, and the atrioventricular conduction system develops aberrantly. Tbx2, in vitro,interactswithNkx2–5andGata4andbindstoconsensusT-halfsiteson the Nppa promoter, resulting in the effective inhibition of Tbx5-mediated transactivation (Habets et al., 2002). Tbx2 and Tbx3 can repress target genes, they do not merely behave as competitors that occupy binding sites of the strong activator, Tbx5 or Tbx20. Competition for DNA-binding sites in the promoter of the N-myc1 gene that encodes a pro-proliferative transcription factor may follow a similar theme and may contribute to decreased proliferation of the nonchamber myocardium (Cai et al., 2005). Since chamber myocardial patterning appears to be achieved by localized expression of the transcriptional repressors Tbx2 and Tbx3, the cellular and molecular

mechanisms that activate and restrict these factors in the linear and looping heart are of great relevance.

3.2.3.5. Tbx5 in cardiac development Tbx5 plays an important role for myogenesis in the FHF (Fig. 4.7). The early embryonic expression of Tbx5 in the cardiac primordia and posterior region of the heart tube in mice is consistent with functions in early atrial cardiomyocyte development (Bruneau et al., 1999). In humans, a number of different mutations in the TBX5 gene are associated with Holt–Oram Syndrome, a disease associated with severe limb and congenital heart diseases including septal defects, as well as conduction disturbance. Heterozygous Tbx5 mutant mice remarkably resemble the phenotype of Holt–Oram syndrome, confirming the dose-sensitive role of Tbx5 to induce and maintain the posterior chamber and septal myocardium. Biochemical analyses suggest that Tbx5 acts as a transcriptional activator that is associated with different cofactors to achieve differential activation of genes essential for the chamber myocardium. Analysis of the murine Nppa promoter revealed two T-half sites that are bound by Tbx5, and a site for the NK-type homeobox transcription factor Nkx2–5, in close proximity. Nkx2–5 is an evolutionary conserved cardiac transcription factor that is expressed in the FHF and SHF, and is also required for chamber formation together with Tbx5 and Hands (Bruneau et al., 2001). Tbx5 null mutant mice result in an embryonic lethality at E9.5 as a result of severe hypoplasia of posterior cardiac structures originating from the FHF. As Tbx5 heterozygous mutants exhibit a reduction in Nppa expression that is intermediate between wild-type and homozygous mutant embryos, there may be a correlation between gene dosage and target gene expression levels. Heartwide overexpression of Tbx5 in mice resulted in the expansion of left ventricular identity and defects of the ventricular septum and atrioventricular canal.

3.2.3.6. Tbx20 in cardiac development Tbx20 is a member of the Tbx1 subfamily of Tbx transcription factors that is expressed in the FHF and SHF throughout cardiac development. Mutations of TBX20 have been reported to be associated with diverse cardiac defects including ventricular septal defects, aberrant valvulogenesis, tetralogy of Fallot, and cardiomyopathy (Liu et al., 2008a,b). Tbx20 activates Nmyc1 and suppresses Tbx2 expression to promote proliferation and specification of the atrial and ventricular chamber myocardium (Fig. 4.7). Tbx20 also indirectly promotes Tbx2 expression through Bmp2 (Fig. 4.7). The spatiotemporal expression of Tbx2 in the atrioventricular canal appears to be balanced between these two opposing signals mediated by Tbx20 (Cai et al., 2011). Mice lacking Tbx20 fail to form atrioventricular canal constriction and severely disturb the EMT for the atrioventricular cushion.

Downregulation of Tbx20 in the mouse embryonic heart results in the widespread upregulation of Tbx2. There are two poorly developed chamber-like structures and a reduction of Nppa expression, suggesting that Tbx20 plays a role in chamber cardiomyocyte differentiation and the maintenance of atrioventricular canal formation in the early heart tube.

3.3. Signal transduction in cardiac development and diseases

3.3.1. Ca^{2b} signaling during cardiac development and diseases 3.3.1.1. Ca^{2b}

changes in cardiac physiology Ca^{2b} is one of the most versatile molecules in various kinds of cells (Berridge et al., 2003). In the heart, Ca^{2b} is essential for two major processes, contraction and signaling. Each contraction and subsequent relaxation of the cardiac muscle requires a cycling change in cytosolic free Ca^{2b} concentration. An individual cardiac contraction depends on a transient increase in the cytosolic free Ca^{2b} concentration that is referred to as the "Ca^{2b} transient," or "contractile Ca^{2b}" (Cartwright et al., 2011). Cardiac relaxation, in turn, depends on a decrease in the cytosolic free Ca^{2b} concentration. Several changes in the frequency and amplitude of the Ca^{2b} transient induce alterations in contractility achieved by physiological stimuli, such as in response to exercise, or by pathophysiologic stimuli, in cardiac hypertrophy or heart failure (Cartwright et al., 2011).

Regulation of contractile Ca^{2b} in cardiomyocytes involves a highly specialized system of ion channels, pumps, exchangers, and microdomains (Bers and Guo, 2005). Contraction is initiated by depolarization of the plasma membrane, which enables Ca^{2b} to enter through voltage-gated L-type Ca^{2b} channels in transverse (T) tubules, causing Ca^{2b} entry in the submembrane microdomain close to the junctional sarcoplasmic reticulum (SR). This, in turn, stimulates the release of Ca^{2b} from the SR through ryanodine receptors (RyRs), and a rise in bulk $[Ca^{2b}]i$, generating contraction. This process is referred to as excitation–contraction (E–C) coupling. Relaxation occurs when Ca^{2b} is taken back up into the SR through the action of two molecules, SR Ca^{2b} ATPase (SERCA) taking Ca^{2b} back up into the SR and the sarcolemmal Na^{b}/Ca^{2b} exchanger excluding Ca^{2b} from the cell.

3.3.1.2. Contractile Ca^{2b} and signaling Ca^{2b} in the heart Cycling changes in contractile Ca^{2b} are thought to coordinate the activity of various Ca^{2b}-dependent signaling pathways, including protein kinase C (PKC), Ca^{2b}-calmodulin-dependent protein kinase (CaMK), and the Ca^{2b}activated protein phosphatase calcineurin. The Ca^{2b} regulating these Ca^{2b}dependent molecules is called "signaling Ca^{2b}" (Cartwright et al., 2011). It remains a

mystery, exactly how cardiac cells use Ca^{2b} for signaling against the background of very large swings in $[Ca^{2b}]i$ during E–C coupling cycle. Essentially, two hypotheses have been brought forward (Molkentin, 2006). The first one postulates the existence of specialized cellular microdomains in which Ca^{2b} concentration is locally regulated and sensed by macromolecular signaling complexes housed in such regions. In this hypothesis, the Ca^{2b} transient has no effect on hypertrophic signaling pathways. The second one suggests that contractile Ca^{2b} also carries signaling information. In this hypothesis, contractile and signaling Ca^{2b} are generated from the same Ca^{2b} pools. There are several molecules that support the view that signaling Ca^{2b} may be constrained to cellular microdomains. There are also several pieces of evidence to suggest that the Ca^{2b} activation requirements for calcineurin, CaMK, and PKC are not always consistent with changes in contractile Ca^{2b}.

3.3.1.3. Ca^{2b} signaling in cardiac hypertrophy and heart failure Cardiac hypertrophy, either compensated or decompensated, affects Ca^{2b} homeostasis (Roderick et al., 2007). In addition to increases in cell size, adaptive hypertrophy is characterized by a high Ca^{2b} capacity; the amplitude of each Ca^{2b} transient is increased and its rate of recovery is also increased to maximize the cardiac output. These modifications are brought about by changes in the expression and/or activity of Ca^{2b}-handling proteins, resulting in an increased SR store (Carvalho et al., 2006) and decreased Na^{b}/Ca^{2b} exchanger activity (Fowler et al., 2005). On the other hand, in the heart failure stage, Ca^{2b} capacity is low with decreased Ca^{2b} release from the SR (Wickenden et al., 1998). The phenotype of SR depletion can be induced by changes in the expression of several proteins. A change in the ratio of the SERCA pump to its negative regulator phospholamban results in less pump activity and lower SR Ca^{2b} storage. Increased activity of the Na^{b}/Ca^{2b} exchanger and an increased leak through RyR, which can be brought about by phosphorylation and interaction with FKBP12.6, also induce the depletion of the SR (Marx et al., 2000).

In response to a greater cardiovascular demand, cardiac output is enhanced through increased Ca^{2b} fluxes. Long-term greater cardiac output induces cardiomyocyte hypertrophy. Nuclear Factor of Activated T cells (NFAT) functions as a downstream effector of the Ca^{2b}-dependent signaling pathway involving cardiomyocyte hypertrophy. Calcineurin is responsible for dephosphorylation of NFAT, and NFAT translocates into the nucleus in an unphosphorylated state, forming a transcription complex with other transcription factors to activate gene expression (Wu et al., 2007). There are many molecules that are believed to influence local Ca^{2b} signaling during cardiac hypertrophy and failure, including plasma

membrane calcium ATPase 4 (PMCA$_4$), Cav3.2 voltage-dependent T-type Ca^{2b} channels, some of the transient receptor potential (TRP) protein Ca^{2b} entry channels, and inositol 1,4,5-trisphosphate receptors (IP$_3$R) (Fig. 4.8). PMCA$_4$ is a member of a family of Ca^{2b} extrusion pumps localized to caveolae in the plasma membrane and serves to compartmentalize signaling Ca^{2b} and downstream signaling events (Oceandy et al., 2007). PMCA$_4$ is known to interact with nNOS as well as with calcineurin, an inducer for cardiac hypertrophy and heart failure. Overexpression of PMCA$_4$ protects mice from pressure-overload or phenylephrine/angiotensin II induced hypertrophy (Wu et al., 2009). Cav3.2, voltage-gated T-type Ca^{2b} channels (T-channels) are normally expressed during development, but not in the normal adult heart. Ca3.2 T-type Ca^{2b} channels are upregulated in hypertrophic and failing heart tissues, and it was demonstrated that they were essential for induction of calcineurin/NFAT signaling during progression of cardiac hypertrophy using Ca3.2 knockout mice (Chiang et al., 2009). TRP is a large superfamily of proteins, some of which regulate Ca^{2b} entry into the cell in response to a variety of signals, including storeoperated Ca^{2b} entry, which leads to nuclear translocation of NFAT. TRPC1, TRPC3, and TRPC6 regulate Ca^{2b} in sarcolemmal microdomains and activate calcineurin–NFAT signaling in cardiomyocytes, upregulated in hypertrophic conditions (Kuwahara et al., 2006; Rosenberg et al., 2004; Vindis et al., 2010). IP$_3$Rs are intracellular Ca^{2b} release channels on the endoplasmic reticulum (ER)/SR and are activated by binding of IP$_3$ generated through hydrolysis of phosphatidylinositol 4 ,5-bisphosphate (PIP$_2$) by phospholipase C (PLC), which may be activated by G-proteincoupled receptors, PLC$_b$, and receptor tyrosine kinases, PLC$_g$ (Berridge et al., 2003). In atrial myocytes, IP$_3$Rs are involved in E–C coupling, the inotropic effect, and arrhythmia (Zima and Blatter, 2004). Although the role of these receptors in the ventricle remains unknown, IP$_3$R has recently been shown to have a profound effect on localized Ca^{2b} signaling with
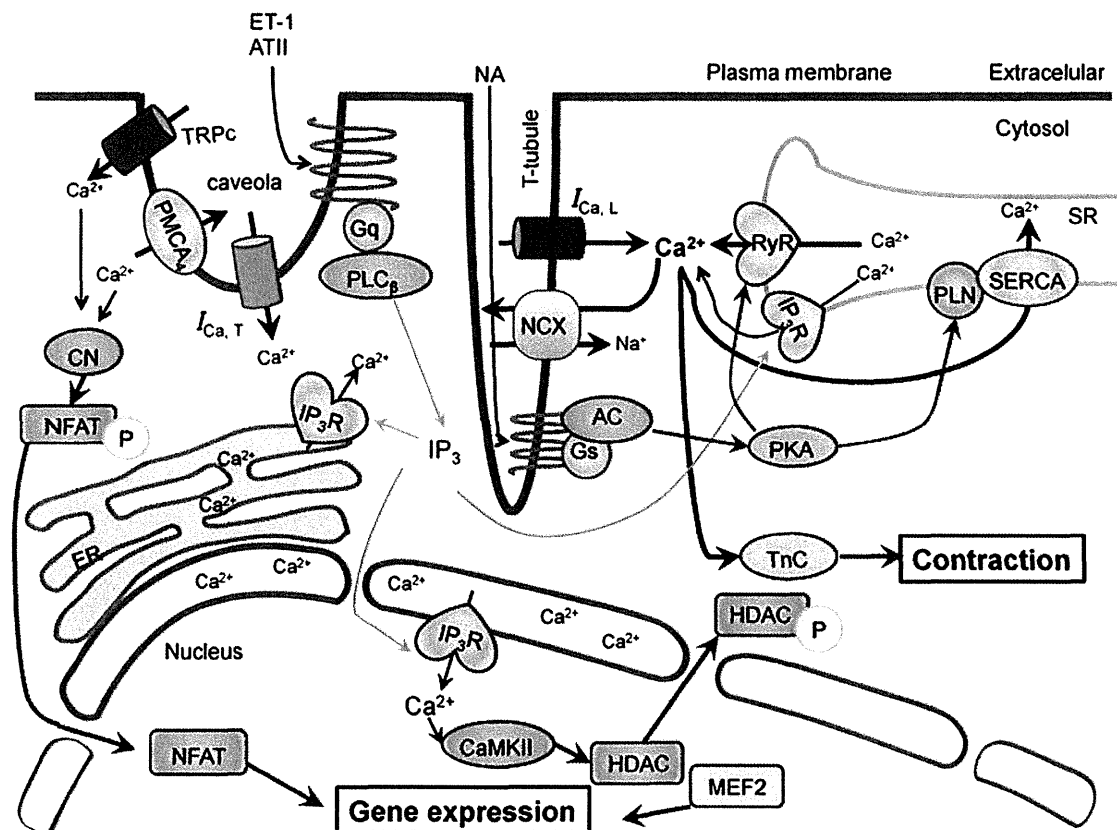
Figure 4.8 Contractile-Ca^{2b} and signaling-Ca^{2b} to cardiac hypertrophy. The figure shows potential Ca^{2b} sources that be specialized to regulate signaling pathways that are activated during process of cardiac hypertrophy and heart failure: (I) T-type Ca^{2b} channels (I_{Ca}, T) are upregulated in hypertrophic states. They could provide Ca^{2b} signaling in microenvironments. (II) Capacitative or store-operated Ca^{2b} entry through transient receptor potential channels isoform c (TRPc) could also provide a highly localized Ca^{2b} pool for controlling reactive signaling pathways in cardiomyocytes. (III) Plasma membrane calcium ATPase isoform 4 ($PMCA_4$) could regulate local C^{2b} concentration. TRPc and $PMCA_4$ regulate the Ca^{2b}-dependent phosphatase calcineurin (CN)/nuclear factor of activated T cells (NFAT) signaling. (IV) Extracellular stimuli, such as endothelin-I and angiotensin-II, bind to G-protein-coupled receptors (Gq/Gs) and then activate phospholipase C (PLC) and generate inositol 1,4,5trisphosphate (IP_3), causing IP_3 receptor (IP_3R)-mediated Ca^{2b}-release from the ER/ nuclear envelope that could also provide a localized Ca^{2b} pool for controlling reactive signaling pathways. IP_3R-mediate Ca^{2b}-release at the nuclear envelope causes a perinuclear Ca^{2b} signal through IP_3R, resulting in CaMKII activation and histone deacetylase 5 (HDAC5) nuclear export leading to activation hypertrophic genetic program. PLN, phospholamban; NCX, Na^b/Ca^{2b} exchanger; RyR, ryanodine receptor; CaMK, Ca^{2b}-calmodulin-dependent kinase; SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; SERCA, SR/ER Ca^{2b}-ATPase; ET, endothelin; AT, angiotensin; NA, noradrenaline; Tn, troponin; AC, adenylyl cyclase; PKA, protein kinase A; and MEF, myocyte enhancer factor.

evidence showing that they are expressed around the nucleus (Wu et al., 2006). Activation of IP_3R on the nuclear envelope leads to local Ca^{2b} release, resulting in activation of CaMKII. Activated CaMKII phosphorylates histone deacetylase 5 (HDAC5), and phosphorylated

HDAC5 translocates from the nucleus to the cytosol, leading to a release of the repression of MEF2 transcription factor (Fig. 4.8). A recent study has shown that transgenic mice overexpressing type 2 IP_3R, which is the isoform predominantly expressed in the heart, displayed enhanced cardiac hypertrophy, suggesting that, at least in part, IP_3R functions upstream of calcineurin–NFAT signaling in the hypertrophic response (Nakayama et al., 2010).

3.3.1.4. Ca^{2b} signaling during heart development The heart is the first definitive organ to be formed in embryos and is essential for vital circulation of embryos. While the function of Ca^{2b} in cardiomyocytes for contraction and relaxation has long been recognized, the role of intracellular Ca^{2b} signaling in differentiation of cardiomyocytes and heart development is just emerging. During embryogenesis, the expression of Ca^{2b}- handling molecules is changed developmentally. An investigation of their expression pattern in mice from E9.5 to 18.5 and adulthood indicated that type 2 RyR, SERCA2, and phospholamban were increased and that the Na^b/Ca^{2b} exchanger (NCX1) was decreased (Liu et al., 2002). Calsequestrin, a major Ca^{2b}-binding protein in the SR, was detected later in the E8.0 heart; however, calreticulin (Crt), a major ER Ca^{2b}-binding protein is expressed much earlier in the embryonic heart and is barely detected in the heart of adult mice (Mesaeli et al., 1999). Another study showed that the type 2 RyR, Ca^{2b} release channel during systole, was not abundant until E8.5 or later and IP_3Rs were expressed in the inner cell mass of embryos at E5.5 and were clearly expressed in the developing heart tube at E8.5 (Rosemblit et al., 1999).

There has been some evidence that Ca^{2b} might regulate the process of heart development (Porter et al., 2003). The whole embryo culture system with two inhibitors of voltage-gated Ca^{2b} channels resulted in the decreased expression of Gata4 and myosin light chain 2, without affecting heart rate and blood flow. Crt deficiency in mice showed an embryonic lethality at E13.5 as a result of a perturbation in cardiac development (Mesaeli et al., 1999), presenting a severely undeveloped ventricular wall. In $Crt^{/}$ cells, IP_3R signaling processes are compromised and the lethality of $Crt^{/}$ embryos can be rescued by cardiac-specific expression of activated calcineurin (Guo et al., 2002). The nuclear localization of Mef2c, one of the master regulators in heart development and most potent activators of the Crt gene, is compromised in $Crt^{/}$ mice (Lynch et al., 2005). These findings suggest that Crt may be an upstream regulator of the calcineurin/NFAT cascade together with MEF2C in the embryonic heart.

The calcineurin/NFAT cascade plays a role not only in cardiac hypertrophy but also in heart development. Deletion of Nfatc1 in mice caused heart valve and septal defects which led to embryonic lethality by

E13.5 (de la Pompa et al., 1998; Ranger et al., 1998). Double knockout of Nfatc3 and c4 in mice resulted in defects of the formation of myocardial wall and vasculature, and mice died around E10.5 (Graef et al., 2001). Mouse lacking three subtypes (Nfatc2, c3, and c4) of NFATc exhibited an endocardial cushion defect (Chang et al., 2004).

Three subtypes of IP_3R (IP_3R1, 2, and 3) have been identified and are expressed in many organs during embryogenesis and the postnatal period. We have recently found that the expression of IP_3R1 and 2 overlap during atrioventricular development, and that of IP_3R1 and 3 overlap during outflow tract development. Consistent with these findings, IP_3R1, 2 double knockout mouse ($IP_3R1'2'$) embryos and $IP_3R1'3'$ embryos died around E11, demonstrating an atrioventricular cushion defect and hypoplasia of the outflow tract, respectively (Fig. 4.9). The former defect was likely to be as a result of a disturbance in the EMT process. This defect could be recovered by the expression of a constitutively active form of calcineurin in the tissue culture of $IP_3R1'2'$ atrioventricular canals, suggesting that the signaling Ca^{2b} activating calcineurin–NFAT pathway essential for cardiogenesis may be provided by Ca^{2b} release through IP_3Rs (Uchida et al., 2010). $IP_3R1'2'$ also showed a thin myocardial layer in the developing ventricles, although the molecular insight underlying this phenotype remains to be studied. In $IP_3R1'3'$ embryos, the enhancement of apoptosis in mesodermal cells in the SHF were detected, consistent with hypoplasia of the outflow tract and right ventricle in these mutant embryos (Nakazawa et al., 2011). These results suggest that intracellular Ca^{2b} redundantly mediated by the three subtypes of IP_3R may play crucial roles in the development of each specific module of the heart (Fig. 4.9).

3.3.2. RAS/MAPK signaling for the cardiomyocyte and cardiac diseases

3.3.2.1. The RAS/MAPK pathway The RAS/mitogen-activated protein kinase (MAPK) pathway plays an essential role in cell proliferation, differentiation, survival, and cell death for numerous cell types including the cardiomyocyte. RAS proteins (HRAS, NRAS, and KRAS) are small guanosine-binding proteins acting as signal switch molecules that integrate extracellular input and activate downstream effectors. They are activated through binding of growth factors to receptor tyrosine kinases (RTK), Gprotein-coupled receptors, cytokine receptors, and extracellular matrix receptors. Cell stimulation promotes cycling between inactive GDPbound (RAS-GDP) to active GTP-bound conformations (RAS-GTP). SOS proteins are guanosine nucleotide exchange factors (GEF) that increase the RAS nucleotide exchange rate of GDP for GTP, resulting in an increase in RAS in the active GTP-bound form. The RAF–MEK–ERK cascade is one of several important downstream cascades of RAS. There are three
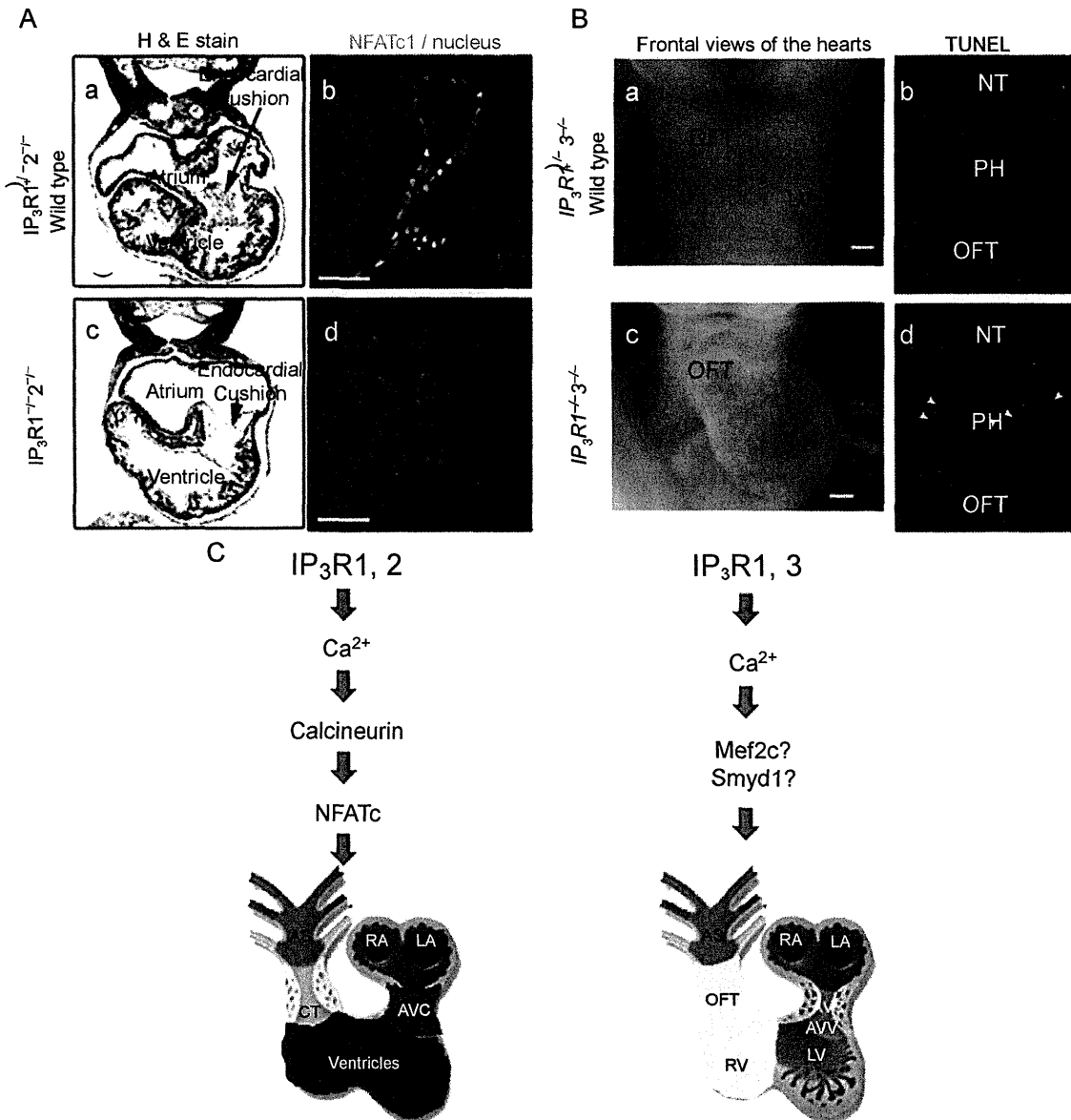
Figure 4.9 Cardiac defects in $IP3R1^{-/-}2^{-/-}$ and $IP3R1^{-/-}3^{-/-}$ mice. A. Transverse sections of hearts of $IP_3R1^{+/-}2^{-/-}$ (a,b) and $IP3R1^{-/-}2^{-/-}$ (c,d) at E9.75. The hypocellularity of the endocardial cusion in the $IP3R1^{-/-}2^{-/-}$ heart is noted (arrow in c). Immunohistochemical analysis showed that NFATc1(green signals) failed to translocate into the nuclei (red signals) of the endocardial cells in the $IP3R1^{-/-}2^{-/-}$ (d). B. The morphologies at E9.5 and the apoptosis at E9.0 of the $IP3R1^{+/-}3^{-/-}$ (a,b) and $IP3R1^{-/-}3^{-/-}$ hearts (c, d). The hypoplasty of the right ventricle (RV) and outflow tract (OFT) of the $IP3R1^{-/-}3^{-/-}$ hearts is observed (c). TUNEL analysis revealed the upregulation of the apoptotic activities (green signals with yellow arrowheads) in the $IP3R1^{-/-}3^{-/-}$ embryos (d). LV, left ventricle; NT, neural tube; PH, pharynx. C. IP3R1 and 2-mediated Ca2+ signal may regulate calcineurin/NFATc pathways during the morphogenesis of the atrioventricular canal (AVC) and ventricles. IP3R1 and 3 may be crucial, partly through Mef2c/Smyd1 pathway, for the development of the OFT and RV.

RAF serine/threonine kinases, ARAF, BRAF, and RAF1, that activate the MEK–ERK kinase cascade. ERK kinase can phosphorylate both cytosolic and nuclear substrates, which include transcription factors that control the cell cycle.

3.3.2.2. Mutations of the RAS/MAPK pathway in cardiac diseases In recent years, germline mutations in genes that encode the protein components of the RAS/MAPK pathway were shown to be involved in the pathogenesis of Noonan syndrome (NS) and of three rare syndromes with NS-overlapping features: LEOPARD syndrome, Costello syndrome, and Cardio-facio-cutaneus syndrome (Fig. 4.10). Cardiovascular abnormalities are important features of these RAS/MAPK pathway syndromes, or "Rasopathes" and present in approximately 70–90% of patients. Although pulmonary valve stenosis and hypertrophic cardiomyopathy are most prominent in these syndromes, the diversity of cardiac defects is different according to the mutated genes (Table 4.4). The molecular mechanisms by which dysregulation of RAS/MAPK signaling causes valve malformation or cardiac hypertrophy have gradually been elucidated.

3.3.2.3. PTPN11 PTPN11 encodes an SH2-containing tyrosine phosphatase SHP-2. SHP-2 regulates signaling for several RTKs, such as EGFR and FGFR, through the activation of the RAS/MAPK cascade. SHP-2 contains N-terminal and C-terminal SH2 domains and a catalytic protein tyrosine phosphatase (PTP) domain. The catalytic function of the protein is autoinhibited through a blocking interaction between the N-SH2 and PTP domains. Once the N-SH2 domain binds the phosphotyrosine peptide, conformational changes result in the active state. Germline PTPN11 mutations have been identified in approximately 50% of patients with NS and 85% with LEOPARD syndrome. The distribution of PTPN11 mutations and cardiac defects between NS and LEOPARD syndrome is different. Most mutations identified in NS were missense mutations clustered in exons 3, 4, 7, 8, 12, and 13. Y63C in exon3, Q79R in exon3, and N308D in exon 8 were identified in approximately 40% of NS patients with PTPN11 mutations. Pulmonary valve stenosis, often associated with an atrial septal defect, is the most common and is related to exon 3 and 8 mutation hot spots with NS. Specific mutations of Y279C in exon 7 and T468M in exon 12 have been identified in LEOPARD syndrome and hypertrophic cardiomyopathy is predominant in these patients. Most mutations in NS were within or around the interacting face of the N-SH2 and PTP domains. Functional analyses of SHP2 with PTPN11 mutations associated with NS have showed gain-of-function effects, primarily impairing the activation/inactivation molecular switch of SHP2, resulting in increased ERK1/2 activation. A recent study of knockin mouse bearing the Ptpn11 with D61G mutation, which is associated with NS, showed that this mutation causes
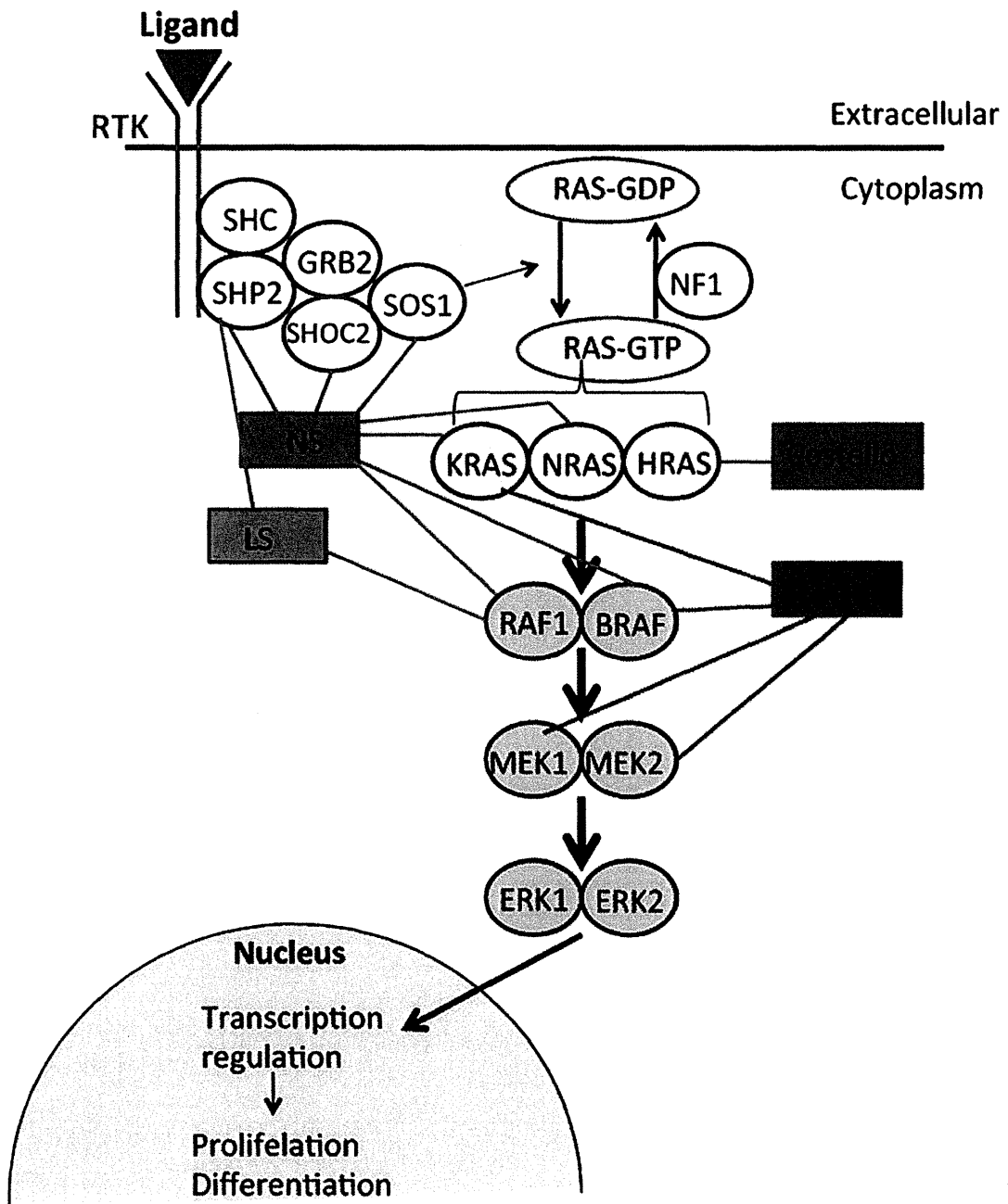
Figure 4.10 The RAS/MAPK signaling pathway and associated developmental syndromes. The RAS/MAPK pathway is activated upon ligand binding to the receptor tyrosine kinases (RTK). Germline mutations of genes encoding respective proteins in the pathway are associated with Noonan (NS), LEOPARD (LS), Costello, and cardiofacio-cutaneous (CFC) syndromes.

cardiac valve defects by increasing ERK–MAPK activation, probably downstream of ErbB family receptor tyrosine kinases, extending the interval during which cardiac endocardial cells undergo EMT (Araki et al., 2009). In contrast, mutations associated with LEOPARD syndrome were supposed to have dominant-negative effects in vitro and in vivo (Kontaridis et al., 2006 ; Marin et al., 2011).

Table 4.4   Mutations in the RAS/MAPK pathway and cardiac defects

Gene symbols	Clinical syndromes	Major cardiac abnormalities	Other cardiac problems
PTPN11 (SHP2)	NS (50%) LS (85%)	PS ASD> HCM HCM PS	AVSD VSD; MV AVSD VSD MVP; Conduction block
SHOC2	NS-like syndrome with loose anagen hair	PS¼ ASD¼ MV> HCM	
SOS1	NS (10–15%)	PS ASD> HCM	
KRAS	NS (5%), CFC (rare)	HCM > PS> ASD	
RAF1	NS (3–5%),LS (rare)	HCM PS¼ ASD	MV TOF
BRAF	CFC (50%) NS (rare)	PS¼ HCM ¼ ASD	MV
HRAS	CS (almost 100%)	HCM PS¼ ASD	Arrhythmia

NS, Noonan syndrome; LS, LEOPARD syndrome; CS, Costello syndrome; CFC, cardio-facio cutaneous syndromes; PS, pulmonary stenosis; ASD, atrial septal defect; HCM, hypertrophic cardiomyopathy; MV, mitral valve defect, MVP, mitral valve prolapse; AVSD, atrioventricular septal defect, VSD; ventricular septal defect; TOF, Tetralogy of Fallot.

3.3.2.4. RAF1 Germline mutations in RAF1 account for approximately 3–5% of cases of NS and are rare in LEOPARD syndrome. Unlike other NS alleles, RAF1 mutations are highly associated with patients with hypertrophic cardiomyopathy, and approximately 80–95% of patients with RAF1 mutations showed this disorder. This suggests that increased kinase activity as a result of RAF1 mutations may cause cardiomyocyte hypertrophy. Our laboratory of the International Research and Educational Institute for Integrated Medical Sciences (IREIIMS) identified and reported five different mutations of RAF1 associated with 10 individuals with NS for the first time in 2007. Four of these mutations, which caused changes in the CR2 domain of RAF1, were associated with hypertrophic cardiomyopathy, whereas affected individuals with mutations leading to changes in the CR3 domain were not. Cells transfected with constructs containing NS-associated RAF1 mutations showed increased in vitro kinase and ERK activation. Further, zebrafish embryos with a morpholino knockdown of raf1 demonstrated the requirement of raf1 during development of normal myocardial structure and function. Taken together, our findings explored the implication of RAF1 gain-of-function mutations as a causative agent in human cardiac diseases, representing a genetic mechanism for the activation of the RAS/MAPK signaling pathway.

3.3.2.5. SHOC2 Mutations in **SHOC2** have recently been found in a group of patients with a Noonan-like syndrome with loose anagen hair. These individuals appear to have prominent ectodermal features, including easily plucked hair with characteristic histology and a hypernasal voice. Cardiac anomalies were observed in the majority of the subjects, with mitral valve insufficiency and septal defects overrepresented compared to the general population of patients with NS.

4. Genes Involved in Skeletal Muscle Development

4.1. Myogenic regulatory factors (MRFs)

Early research on skeletal myogenesis benefited from a clonal cell line, C3H10T1/2, derived from 14- to 17-day-old C3H whole mouse embryos (Reznikoff et al., 1973). The clone maintained a fibroblast-like morphology, and converted to myofibers, adipocytes, or chondrocytes after brief treatment with 5-azacytidine, a DNA demethylating agent (Constantinides et al., 1977; Taylor and Jones, 1979). MyoD was isolated as the first myogenic regulatory gene by subtraction hybridization of cDNA from these 5-azacytidine treated cells (Davis et al., 1987). Subsequently, myogenin, myf-5, and Mrf4 were isolated having high sequence homology with MyoD (Braun et al., 1989; Wright et al., 1989). These MRFs share homology within a 70-amino acid segment that encompasses a region rich in basic residues followed by a motif predicted to form a helix–loop–helix (HLH) conformation (Olson, 1990; Weintraub et al., 1991). Dimerization is a prerequisite for specific binding of HLH-containing proteins to DNA. HLH homo- and heterodimers bind to a consensus sequence referred to as an E box (CANNTG), which has subsequently been found in the regulatory regions of most, but not all, muscle-specific genes (Buskin and Hauschka, 1989; Murre et al., 1989; Olson, 1993; Olson and Klein, 1994).

MRFs were identified as the dominant regulators of myogenic progenitors bytheiruniquepropertyofconvertingmanynonmyogeniccelltypesintocells expressing skeletal muscle markers. In vivo, they show distinct, partially overlapping, expression patterns (Buckingham, 1992). Cells that migrate from the somite do not express MRF, and begin to express MRFs only after they reach the limb (Tajbakhsh and Buckingham, 1994). Myf5 is the first MRF to be expressed before MyoD (Buckingham, 1992). MyoD, Myf5, and Mrf4 are expressed in myoblasts while myogenin is expressed in myocytes. MyoD, Mrf4, and myogenin are all expressed in the myonuclei of differentiated myofibers.

Gene knockout experiments established the involvement of MRFs in skeletal muscle differentiation. Mice homozygous for a targeted mutation in the myogenin gene are lethal perinatally owing to the failure of muscle differentiation (Hasty et al., 1993; Nabeshima et al., 1993). Therefore, myogenin is essential for terminal differentiation of functional skeletal muscle. Mutant mice for related myogenic factors Myf5 or MyoD showed no muscle defects (Hasty et al., 1993; Rudnicki et al., 1992), but mice lacking both MyoD and Myf-5 showed a complete absence of skeletal muscle and died soon after birth (Rudnicki et al., 1993). Therefore, it is believed that Myf-5 and MyoD can compensate for each other and together act in a genetic pathway upstream of myogenin (Pownall et al., 2002). Knockout mutant mice for the Mrf4 gene showed different results in three different laboratories giving viable mice, low survival, or death at birth (Olson et al., 1996). Three constructs for these knockouts deleted different portions of the Mrf4 genome, and subsequent studies revealed a complex picture where the linked Mrf4–Myf5 loci and its vicinity contained many interdigitated elements controlling the activation and maintenance of gene expression at different developmental stages and anatomical locations during development (Carvajal et al., 2001, 2008).

MRFs form heterodimers with members of the E-protein bHLH subfamily (E2A, E2-2, and HEB), and bind to a consensus E-box sequence (CANNTG) found in regulatory regions of most, but not all, muscle-specific genes (Buskin and Hauschka, 1989; Murre et al., 1989; Olson, 1993). To identify MyoD-binding sites, genome wide, high-throughput ChIP-Seq analysis was performed in differentiating myoblast C2C12 cells (Aziz et al., 2010; Cao et al., 2010). The C2C12 cell line was originally obtained from the thigh muscle of C3H mice (Yaffe and Saxel, 1977), and is capable of growing as myoblasts or being manipulated to differentiate into skeletal muscle cells. By ChIP-Seq analysis, MyoD was shown to bind to 25,956 sites throughout the genome, while only 1953 genes demonstrated modified expression in response to differentiation of C2C12. The MyoD:E-protein heterodimer showed binding to the consensus VCASCTGT sequence (V represents A, C, G, not T, and S represents G or C) (Aziz et al., 2010 ; Cao et al., 2010). Once associated with the promoter region, MyoD recruits acetyltransferases p300, leading to acetylation of histones H3 and H4, and pCAF, which acetylates MyoD (Puri et al., 1997; Sartorelli et al., 1999). Cao et al. (2010) postulated that MyoD not only binds specifically to regulate muscle gene expression but also binds genome-wide to broadly alter the epigenome in myoblasts and myotubes by acetylation of histones.