Department of Physics, Chemistry and Biology

Introductory essay

Governors of Growth and Regulators of Remodeling

The Genetics Determining Heart Size

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Contents

1 How does it know – to grow fast or grow slow?

How body and organ size, and thus also proportionality of the full grown body, is genetically regulated during development from zygote to adult is one of the least known matters in developmental biology. The size of an animal organ depends on the number of cells, the size of the cells and the amount of extracellular fluid and other extracellular components. Cell number, in turn, depends on cell proliferation and apoptosis and cell size depends on cell growth. Cell number and cell size have been shown to work very tightly in concert to achieve a body composition and organ size that is proportional. For example, the cells of a tetraploid salamander are twice the size of diploid salamander cells, yet the salamanders and their individual body parts are the same size because cell number is halved in the tetraploid to compensate for the larger size of individual cells (Conlon and Raff 1999). A similar pattern has been shown in the heart were a heart that has overgrown through hyperproliferation at the time of birth heart attains normal size in adulthood by means of decreased cell size (Xin et al. 2011). Thus both the number and size of cells can be adjusted to achieve proper final size. What that proper size is, obviously differs between different organisms and species. A larger animal needs a larger heart than a small animal to meet the needs of tissue oxygenation. But it is not as simple as to say that larger animals have larger hearts. Heart size in proportion to body size, or heart weight/body weight (HW/BW) is an often used measure that corrects for the impact of body size on the size of the heart. When comparing HW/BW ratios, between species, but also between different developmental stages in one species, one finds large differences (Lee et al. 1975) which suggests that there are genetic differences governing proper adult size of the heart.

Organ growth depends both on extrinsic and intrinsic cues. Among the most important extrinsic cues is information on nutritional status, most predominantly through insulin signaling (Crickmore and Mann 2008). But for some organs, including the heart, the most influential information on final size determination seems to be found in the organ itself. Transplantation of multiple fetal thymus glands into a developing mouse lets each of the transplanted thymuses grow to normal final size and fetal rat hearts attain proper adult size when transplanted into adult rats (Dittmer et al. 1974; Conlon and Raff 1999). This shows that the heart adopts proper final size even if taken out of its' developmental context, and thus depleted of the extrinsic cues that are involved in developmental growth.

The heart, during embryonic development, grows mainly by means of hyperplasia. Once the animal is born, cardiac growth through hypertrophy

becomes more predominant and cell growth is responsible for the heart attaining proper adult size (Soonpaa and Field 1998). The results from Dittmer et al. (1974) and Xin et al. (2011)suggest that the size of the heart is determined by intrinsic cues at the level of final organ size, rather than rate of proliferation in development, number of progenitor cells, final cell size or extrinsic input. However the genes and genetic pathways governing this well orchestrated growth are still mostly unknown. In this essay, the growth of the heart, from fetal life to adult size, will be divided into two subparts: "Prenatal hyperplasia" and "Cardiomyocyte maturation and growth hypertrophy". The terminology is in agreement with Zak (1974).

1.1 Prenatal hyperplasia

During fetal development the heart grows by cell proliferation, or hyperplasia. Thus, the rate of cell division can be seen as the master regulator of how large the heart grows during fetal life. Very little is known about which genes that are involved in the regulation of proliferation rate but one key player has been suggested in recent years; the Hippo pathway, previously known as an important regulator of organ size in *Drosophila*. The Hippo pathway is so far the sole known actor in the play of genetic size determination in prenatal heart growth.

1.1.1 The Hippo pathway

The regulatory pathway Hippo, was found in Drosophila and has been shown to control organ size in the fly by controlling both cell proliferation and apoptosis rates (Edgar 2006; Pan 2007). The Hippo pathway is evolutionarily conserved in vertebrates and in mammalian systems the Hippo homologs have been shown to play important roles in regulation of cell contact inhibition and cancer development as well as a leading role in organ size control (Camargo et al. 2007; Dong et al. 2007; Zhao et al. 2007). The core of the pathway (figure 1) in *Drosophila* is defined by a kinase cascade where, Hippo (Hpo), in complex with Salvador (Sav) activates a kinase complex consisting of Warts (Wts) and Mats (Mts) which allows Wts to phosphorylate the most downstream effector of the core kinase cascade, the transcriptional coactivator Yorkie (Yki). Phosphorylation of Yki on Ser111, Ser168 and Ser250 creates a 14-3-3 binding site which signals for binding to a 14-3-3 protein located in the cell membrane, resulting in cytoplamsic retention of Yki and subsequent downregulation of the pro-growth and anti-apoptotic Yki target genes (Dong et al. 2007; Yin and Zhang 2011). Activated Yki, located in the nucleus has several described binding partners with DNA binding ability and target genes include several genes that play roles in

cell proliferation and survival. Thus Yki induces growth by enhancing proliferation and inhibiting apoptosis, and by inhibiting Yki the Hippo pathway effectively works as a brake to slow the growth down. Upstream of the Hippo pathway a number of regulatory components and cascades have been described. One of them is the Fat (Ft) branch of regulatory input on Hippo signaling. Ft is a transmembrane receptor which is mediated by the ligand Dachsous (Ds). Binding of Ds to Ft is promoted by Four-jointed (Fj). Ft signaling activates Dachs which functions as a negative regulator transducing Ft signaling to the core of the Hippo pathway by inhibiting Wts. Another branch of upstream regulation of Hippo signaling consists of the Expanded/Merlin/Kibra complex (Ex/Mer/Kibra). The mechanisms of Ex/Mer/Kibra regulation of Hippo are still rather unclear but it is believed that the complex might recruit core Hippo pathway proteins to the apical membrane for activation. It has been shown that Ex binds Hpo, Mer binds Sav and Kibra binds Sav and Wts but the details on how they regulate Hippo signaling on the molecular level are not yet known. Hippo is also regulated by cell density information in the developing tissue through the actions of apico-basal cell polarity proteins and by cell tension and mechanotransduction through F-actin and E-cadherin (Yin and Zhang 2011).

The mammalian homologs of the *Drosophila* Hippo genes are summarized in table 1. The core kinase cascade in vertebrates and mammals consist of the Hpo homologs MST1-2 which bind to Sav1/WW45, the scaffold homologs of Sav. LATS1-2 and Mob1-2 are vertebrate homologs of Wts and Mts respectively and work in a conserved mane r to phosphorylate YAP/TAZ, the mammalian homologs of Yki and thus suppressing the activity of these two transcriptional cofactors by introducing a 14-3-3 binding motif, very similar to that created by the Hippo cascade on Yki, signaling for cystoplasmic retention. The fact that the overall role and mechanisms of the Hippo pathway are highly conserved from fly to mammal is well elucidated by the finding that expression of some of the corresponding human homologs can rescue the tissue overgrowth phenotypes in flies resulting from knock out of *Drosophila* Hippo genes (Wu et al. 2003; Lai et al. 2005). Also upstream regulators of the Hippo pathway are evolutionarily conserved to mammals, even though their exact functions and mechanisms have not been fully investigated. Mammalian homologs of Ft, Ds and Fj and to Ex/Mer/Kibra have been described as summarized in table 1 (Xin et al. 2011).

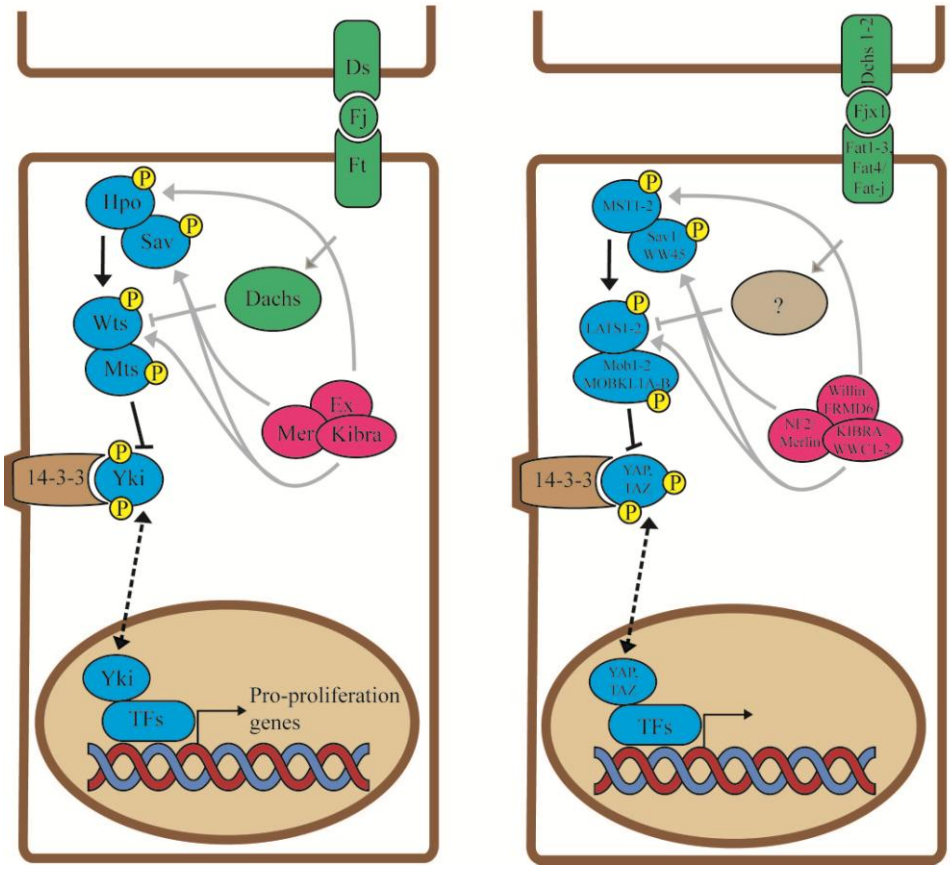

Figure 1. The Hippo pathway with upstream regulators. Left panel depicts Drosophila *genes and right panel shows mammalian homologs. Grey arrows picture putative interactions.*

Table 1. Components and regulators of the core kinase cascade of the Hippo pathway in Drosophila *and mammals.*

Drosophila	**Mammals**	**Conserved domain/motifs**
Hippo (Hpo)	MST1-2	Ste20 Ser/Thr, SARAH domains
Salvador (Sav)	Sav1/WW45	WW and SARAH domains
Warts (Wts)	LATS1-2	NDR Ser/Thr kinase domain and PPxY motif
Mats (Mts)	Mob1-2, MOBKL1A-B	Mob1/phoein domain
Yorkie (Yki)	YAP, TAZ	WW and TEAD-binding domain
Fat (Ft)	Fat1-3, Fat4/Fat-j	Calcium-binding EGF-like domain, Laminin G domain and Cadherin repeat domain
Dachsous (Ds)	Dchs1-2	Cadherin repeat domain
Four-jointed (Fj)	Fjx1	Golgi Ser/Thr kinase domain
Dachs	?	Myosin motor domain
Expanded	Willin/FRMD6	FERM N-terminal domain and Pleckstrin homology-like domain
Merlin	NF2/Merlin	FERM domain
Kibra	KIBRA/WWC1-2	WW and C2 domain

1.1.2 The Hippo pathway in cardiogenesis

Very recently, the role of the Hippo pathway in cardiogenesis and heart growth in mammals has started to be unraveled. Heallen et al. (2011) showed that the Hippo signaling pathway represses proliferation in the developing mouse heart and that cardiac specific knock out of *Sav1,* the scaffold protein that allows MST1-2 to phosphorylate LATS1-2 led to a decreased phosphorylation (inhibition) of YAP. The phenotypic effects were enlarged hearts or cardiomegaly with elevated proliferative acitivity of cardiomyocytes. Atrioventricular connections and arrangements of chambers and valves of the heart as well as cardiomyocyte size were unaffected by knockout of *Sav1.* The authors further showed that the increased proliferation in *Sav1 CKO* mice hearts was mediated by an up regulation of canonical Wnt signaling resulting in a robust increase of the pro-growth transcription cofactor β-catenin in nuclei and up regulation of genes implicated in cardiac repair, cell reprogramming, cell growth and anti-apoptosis. Decreased expression dosage of β-catenin suppressed the cardiac overgrowth phenotype of the *Sav1 CKO* mice. This indicates cross-talk between the Hippo and Wnt signaling pathways and a putative role for Wnt signaling in prenatal heart growth.

The Hippo pathway has also been connected to the insulin-like growth factor (IGF) pathway in developing myocardium. The most downstream effectors in the Hippo pathway, YAP and TAZ, activate IGF signaling, leading to inactivation of the glycogen synthase kinase 3β (GSK-3β). Since GSK-3β is a part of a β-catenin degrading complex its' inactivation leads to increased β-catenin (Xin et al. 2011). The latter study also showed that genetic deletion of YAP was lethal in mice at embryonic day 10.5 (E10.5) due to reduced cardiomyocyte proliferation, decreased heart size and insufficient contractility while cardiac-specific overexpression of active (phosphorylated)YAP promoted cardiomyocyte proliferation and increased heart size (Xin et al. 2011).

The results from the studies by Heallen et al. (2011) and Xin et al. (2011) both show that YAP and β-catenin, the most downstream effectors of the Hippo and Wnt pathways respectively, play important and tightly connected roles in regulation of cardiac growth. Indeed Heallen et al. (2011) showed that nuclear, nonphosphorylated YAP forms a complex with β-catenin and that they in combination potentiate the transcriptional activity of target genes. Since the targets for both YAP and β-catenin transcriptional cofactors have been shown to be pro-growth and pro-proliferative genes (Wu et al. 2003; Huang et al. 2005; Gessert and Kuhl 2010) this reveals a mechanism for antagonistic control over heart growth

by the canonical Wnt and the Hippo patways, since canonical Wnt signaling activates β-catenin while Hippo signaling inhibits as well YAP as β-catenin.

Results by Mao et al. (2011) establish a role also for the upstream regulation of the Hippo pathway by Fat4 and Dchs1 in cardiac development. By constructing gene targeted mutations in *Dchs1* and comparing results to already established *Fat4* mutants the authors showed that both mutations on their own and in combination resulted in mice with hearts of normal size but with defects in atrial septation.

In the chicken *(Gallus gallus)* most homologs of the mammalian hippo genes are annotated (see table 2 for summary). Annotations are also highly similar to those of mammalian homologs but very little work concerning the function of the Hippo genes in chicken has been done to date. Interesting avenues for elucidation of the Hippo mechanisms in chicken with special respect to the devolpment of the heart would be to investigate expression patterns of the Hippo genes in chick cardiogenesis over time. In combination with studies of cardiomyocyte proliferation rates this would allow investigation of how Hippo signaling and activity correlate with cardiomyocyte proliferation rates in different developmental stages. Isolated proliferative cardiomyocytes could be used to 1)investigate how experimental regulation of Hippo signaling, by disrupting core components by for example RNAi, affects proliferation and/or 2) investigate how extrinsic developmental cues, such as hormones that are known to affect proliferative ability of cardiomyocytes affect, and possibly act through, Hippo signaling. In this case chicken cardiomyocytes might be especially interesting because of their ability to proliferate for several weeks or even months after hatching compared to mammalian cardiomyocyte that rapidly lose their proliferative ability after birth (Beinlich et al. 1995; Li et al. 1996; Li et al. 1997; Leu et al. 2001). This opens up the possibility to study the putative role of the proliferation regulator Hippo also in a postnatal proliferative heart and perhaps to experimentally alter the proliferation rates of the post-hatching cardiomyocytes.

Table 2. Mammalian Hippo genes and homologs in Gallus gallus.

Mammalian gene	**Gallus gallus gene or locus**	**Chr.**	**Entrez#**	**Ensembl#**
MST1	MST1	12	NC_006099.2	ENSGALG00000002722
MST2	LOC776975 similar to MST2	?	NW_001485207.1	-
	LOC431237 similar to MST2	?	NW_001472432.1	-
Sav1/WW45	Sav1	5	NC_006092.2	ENSGALG00000012341
LATS1	LATS1	3	NC_006090.2	ENSGALG00000006659
LATS2	LATS2	1	NC_006088.2	ENSGALG00000017130
Mob1	Mob1 (MOBKL1A)	4	NC_006091.2	ENSGALG00000011573
Mob2	Mob2	5	NC_006092.2	ENSGALG00000006659
YAP	YAP1	1	NC_006088.2	ENSGALG00000017188
TAZ	WWTR1	9	-	ENSGALG00000010412
Fat1	Fat1	4	NC_006091.2	ENSGALG00000013579
Fat2	Fat2	13	NC_006100.2	ENSGALG00000004320
Fat3	Fat3	1	NC_006088.2	ENSGALG00000017229
Fat4/Fat-j	Fat4	4	NC_006091.2	ENSGALG00000011823
Dchs1	Dchs1	1	NC_006088.2	ENSGALG00000017334
Dchs2	Dchs2	4	-	ENSGALG00000009245
Fjx1	?	-	-	-
Willin/FRMD6	FRMD6	5	NC_006092.2	ENSGALG00000012374
NF2/Merlin	NF2	15	NC_006102.2	ENSGALG00000008073
KIBRA/WWC1	WWC1	13	NC_006100.2	ENSGALG00000001826
KIBRA/WWC2	WWC2	4	NC_006091.2	ENSGALG00000010668

1.2 Cardiomyocyte maturation and growth hypertrophy

Before or shortly after birth, mammalian cardiomyocytes mature, meaning that they lose their ability to proliferate (Beinlich et al. 1995; Li et al. 1996; Leu et al. 2001), and after that the growth of the heart occurs mainly through hypertrophy, or cell enlargement. The maturation is coupled to mitotic arrest and a binucleation event, where the cell goes through nuclear division without cell division, and after that the level of DNA synthesis in mammalian cardiomyocytes is exceedingly low (Soonpaa and Field 1998). The same does not apply for the chicken heart though, where cardiomyocytes keep their proliferative ability at least 42 days post hatching (Li et al. 1997). This shows that cardiomyocytes are different between mammals and birds when it comes to myocyte maturation and mitotic arrest.

The maturation of mammalian cardiomyocytes occurs simultaneously with a peak in circulating thyroid hormone, which has lead to the belief that thyroid hormone (T_3) is driving the maturation process. Indeed it has been shown in sheep that a premature onset of high levels of T_3 leads to binucleation, surpression of proliferation and increased cell size (Chattergoon et al. 2012). But yet again the chicken heart differs from the mammalian heart. In the chicken T_3 levels around the time of hatching show a pattern highly similar to the levels in sheep around the time of full term, though in chicken T_3 does not evoke maturation of cardiomyocytes (Thommes and Hylka 1977; Christensen et al. 1995). In mammals maturation of cardiac myocytes is also connected to a switch in the expression of myosin heavy chain mRNA in the heart; from the β-isoform which is predominant during embryogenesis to the α-isoform which becomes the most common isoform around the time of maturation and keeps its predominance throughout the life of the animal. The switch is thought to be driven by thyroid hormone (Ng et al. 1991). In chicken there seems to be no switch and a single MHC is expressed through both development and adulthood (Zhang et al. 1986).

How cardiomyocyte maturation, mitotic arrest and binucleation are regulated is to the large part still a mystery, even if the results mentioned represent some pieces of the puzzle that have been found. Interesting avenues are further investigation of the special features of cardiomyocytes of post-hatch chicks; when do they mature and as a response to what? Can mitotic, post-hatching chicken cardiomyocytes be influenced by factors shown to induce proliferation and would such a hyperproliferative heart be beneficial or detrimental for the animal in the setting of extreme growth that is indicative of today's chicken industry? Since it is known that cell size is decreased to compensate for an increased cell number in order to obtain an appropriately sized heart, it would be interesting to pursue how the efficiency of the heart as a pump would be affected by having larger or smaller cells. Much is known about how the size of the organ and its main parts (atria and ventricles) affect efficiency and function but what of cell size?

The heart is performing its function of pumping blood already during development, but after birth an increased work load is imposed on the left ventricle (LV) because of the hemodynamic changes that occur at birth. These circulatory changes are met by a rapid growth of the heart, primarily of the LV in the first few days after birth (Camacho et al. 1990; Beinlich et al. 1995). During the first 10 days of life the left ventricular free wall of the piglet heart grows approximately three times faster than the left ventricular free wall (Peterson et al. 1989). This rapid growth of

the left ventricle is a common phenomenon across species but the regime of growth differs. In the sheep, mitotic arrest of cardiomyocytes occurs before birth, and the rapid postnatal growth is due to hypertrophy only (Smolich et al. 1989; Beinlich et al. 1995). In rats however, mitotic arrest is not as early and Clubb and Bishop (1984) show that the rapid postnatal cardiac growth happens through hyperplasia. Whether there are mammalian species in which the rapid postnatal growth of the left ventricle occurs through hyperplasia and hypertrophy combined is not yet known. The latter has been shown to be true in the chicken where it has been observed that increased cell volume does not account for the total increase in heart size in postnatal growth and that post hatching cardiomyocytes do undergo mitosis. Chicken cardiomyocytes are proliferative for at least 42 days post hatching (Li et al. 1997). Thus, the chicken heart grows by hyperplasia and hypertrophy in combination whereas the mammalian heart seems to switch more abruptly between the two. It lies close at hand to suspect that there might be strain specific differences in the regime of post-hatching cardiac growth within the chicken species, since strains are so diverse in overall growth rate and many strains have been bred to grow extremely fast. Does a fast growing chicken with a fast growing heart have a more or less proliferative heart? And are the effects of factors that affect proliferation/hypertrophy comparable between fast and slow growing chickens?

When this delayed cardiomyocyte maturation evolved in birds, or was lost in mammals, is to my knowledge not clear to date as too little work has been done on the proliferative ability of vertebrate cardiomyocytes in vivo. Literature shows that adult amphibian cardiomyocytes can proliferate in vitro and upon cardiac damage in vivo (Oberpriller and Oberpriller 1974). The latter is also true for zebrafish (*Danio rerio*), which has been shown to have the ability of cardiac regeneration and repair upon myocardial infarction (Chablais et al. 2011) and partial myocardial resection (Poss et al. 2002; Jopling et al. 2010). Cardiac repair through cardiomyocyte proliferation upon resection is also possible in mammals, but only up to the point of cardiomyocyte maturation and binucleation and thus not in adult mammals (Porrello et al. 2011). One possibility is that delayed cardiomyocyte maturation was a common trait in the ancestors of today's mammals, birds and fish but that the trait has been lost in mammals during evolution.

The following sections will give an overview of what is known about regulation of the postnatal growth hypertrophy that follows cardiomyocyte maturation and mitotic arrest. No studies using chicken as the model system have been done and all results are obtained in

mammals. Most studies have been done in mice which have been genetically engineered to have altered expression of the putative hypertrophy regulator. Several of the studies using overexpression techniques use the α-myosin heavy chain gene promoter to drive the overexpression. Given the postnatal switch in expression pattern from β to α-myosin heavy chain this is an efficient way to look at the role of the gene exclusively in postnatal growth, since the technique excludes potential effects on prenatal hyperplasia. The summary of findings that follows sheds light on an intricate relationship between pro- and anti-hypertrophic signaling pathways that work in concert in the healthy heart to result in normal growth hypertrophy and appropriate heart size in the adult animal. These pathways are in several cases involved in cross-talk with each other and the paths from upstream regulators to downstream effectors are far from linear. Figure 2 provides a schematic summary of the pathways and their interactions.

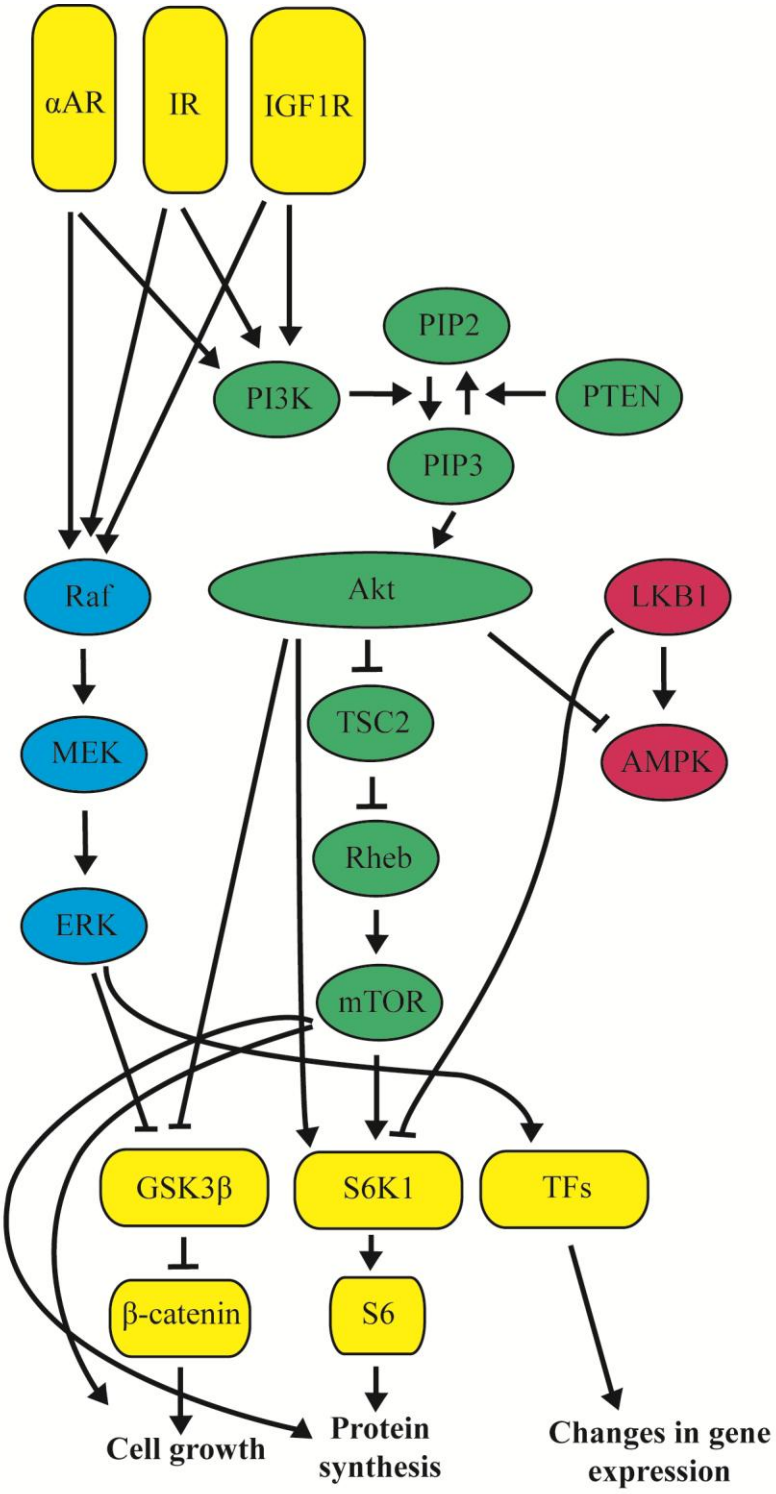

Figure 2. Summary of the PI3K, MAPK and AMPK signaling pathways with upstream input receptors and downstream effectors, their roles in growth control and relationships to each other.

1.2.1 Intracellular signaling pathways

1.2.1.1 The phosphoinositide 3-kinase pathway

The phosphoinositide 3-kinase (PI3K) pathway lies downstream of many receptors including the α-adrenergic receptors (αARs), the insulin receptor (IR) and the IGF-1 receptor (IGF1R). Upon activation of the respective receptors, PI3K is activated through phosphorylation. The active PI3K in turn phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5 trisphosphate (PIP3) which accumulates in the cell membrane. Here PIP3 recruits the serine/threonine kinase Akt (also known as protein kinase B) leading to the activation of Akt, which is one of the major and most thoroughly described downstream effectors of PI3K known to affect for example protein synthesis and cell size control. Akt in turn can phoshorylate and thereby inactivate the tuberosclerosis complex 2 (TSC2) gene product, tuberin. This reduces the ability of tuberin to stimulate the conversion of Rheb from active to inactive form. Active Rheb activates mammalian target of rapamycin (mTOR) which conveys the PI3K-Akt signal to cell growth and protein synthesis enhancers (Shioi et al. 2000; Matsui et al. 2002; Shiojima et al. 2002; Luo et al. 2005). Akt also has several targets besides tuberin, for example inhibition of the β-catenin degrader GSK-3β and activation of S6K1 which is a physiological kinase for the ribosomal S6 protein. Since increased β-catenin promotes pro-growth transcription and phosphorylation of S6 increases the rate of translational initiation all the mentioned effects of Akt activity leads to promotion of cell growth (Shioi et al. 2002). S6K1 is also a target of mTOR.

The PI3K pathway has been coupled tightly to postnatal cardiac growth and the specific roles of several of the included genes have been investigated. Heart specific expression of constitutively active PI3K (caPI3K) results in mice with larger hearts (increased HW/BW), consisting of larger cardiomyocytes but with normal ventricular/atrial/septal/wall thickness proportions compared to control (Shioi et al. 2000; Rigor et al. 2009). Expression of dominant negative PI3K (dnPI3K) on the other hand results in mice with smaller hearts (decreased HW/BW) with smaller cardiomyocytes (Shioi et al. 2000; Rigor et al. 2009). Both phenotypes remain after >1 year of observation without signs of heart failure and without alterations in echocardiographic parameters, suggesting that the hypertrophic mechanisms mediated by PI3K signaling at the level of PI3K were purely phyisiological (Shioi et al. 2000; Rigor et al. 2009). PTEN is a PI3K antagonist which acts by dephosphorylating PIP3. By knocking out PTEN, the hypertrophic effect

of PI3K signaling has been further proven. PTEN knockout mice develop larger hearts and higher HW/BW ratios, but have normal body sizes, normal myocyte shape and size and a normal proportionality of the cardiac components. The phenotype is reversed by expressing a dnPI3K isoform (Crackower et al. 2002).

There are three classes of PI3Ks, but the focus in this text will be on class I PI3Ks which are responsible for the production of PIP3 and involved in the signaling cascade described. This class in turn has two subclasses: Class I_A PI3Ks and I_B PI3Ks. Class I_A PI3Ks are activated by both tyrosine kinase receptors (eg. IR and IGF1R) and G-protein coupled receptors (eg. αARs) and consist of a p85 regulatory subunit and a p110 catalytic subunit. GPCRs can also activate class I_B PI3Ks, which consist of regulatory subunit p101 and the catalytic subunit p110γ. In cardiac growth and remodeling it seems like class I_B PI3Ks are involved in pathological hypertrophy (Luo et al. 2005) while class I_A PI3Ks, which are sensitive to growth hormone and nutritional status input, regulate physiological hypertrophy. The latter is supported by the results of Luo et al. (2005) that show that deletion of the regulatory p85 subunit in the heart results in reduced heart size as a consequence of reduced myocyte size.

The role of Akt as the putative mediator of the hypertrophic PI3K signaling has also been investigated specifically in the heart. Indeed the work by Shioi et al. (2000) showed that expression of caPI3K and dnPI3K altered the activity of Akt significantly, increasing it and decreasing it respectively. But the role of Akt in cardiac growth has also been determined more closely by targeted mutation. Cardiac specific expression of constitutively active Akt results in mice with large heart phenotypes. The viable lines of caAkt mice develop cardiac phenotypes well in line with the PI3K results described above; larger hearts, with larger cells but with normal proportions and preserved systolic function (Matsui et al. 2002; Shioi et al. 2002). Further Shioi et al. (2000) showed that expression of kinase deficient Akt (kdAkt) did not result in a smaller heart, but did attenuate the large heart phenotype of caPI3K mice, just as the caAkt mutant circumvented the retarded growth phenotype of the dnPI3K mouse heart. These results together, indeed indicate that Akt is one of the effectors mediating PI3K signaling induced hypertrophy, but that active Akt is not required for normal cardiac growth.

The overgrowth phenotype of caAkt was attenuated by administration of rapamycin suggesting that mTOR or its effectors are involved in mediating the Akt-induced hypertrophy (Shioi et al. 2002). Conflicting results have been found by Shen et al. (2008) who showed that cardiac

specific overexpression of kinase dead mTOR (kd-mTOR) did not result in an altered cardiac phenotype. Thus neither kdAkt nor kd-mTOR resulted in the hypothesized small heart phenotype, while PI3K itself is needed for normal heart growth. This suggests that PI3K signaling can work via other mediators in parallel with Akt and/or mTOR, possibly in a redundant manner, in the heart. This suggests roles for, for example GSK-3β and S6K1 as downstream effectors of PI3K signaling as previously mentioned.

1.2.1.2 MEK-ERK signaling

The MAPK superfamily consists of three subfamilies and one of them, the extracellularely responsive kinases (ERKs), are intimately connected to cell growth. The ERKs are responsive to insulin signaling as well as signaling through other polypeptide growth hormones. Also GPCR signaling activates ERK and all four subfamilies of G proteins have been implicated in the activation of ERK signaling (Sugden and Clerk 1997). Once activated, ERK phosphorylates numerous intracellular targets, including many transcription factors, which results in cardiac gene expression changes. Upstream of ERK is MEK and Raf. Raf is activated at the cell membrane and in turn activates MEK either at the membrane or in the cytoplasm. MEK in turn activates ERK. Studies of high copy number MEK1 transgenic mice have shown that activation of the ERK pathway leads to increased HW/BW ratio and concentric cardiac hypertrophy at 3 weeks. Further, adenoviral transfection of cultured cardiomyocytes with MEK1 induces a hypertrophic response (Bueno et al. 2000). The hypertrophy of the described transgenic mice was characterized as compensatory and without any pathological hallmarks.

The ERK and PI3K pathways co-regulate many targets, for example S6 and GSK-3β (Mendoza et al. 2011) but Shioi et al. (2000) showed in their caPI3K and dnPI3K mice that PI3K activity level did not have an impact on ERK activity. Thus it seems like PI3K and ERK signaling work in a parallel and possibly partially redundant manner conveying signaling from the same receptors to the same downstream effectors in the same direction but without interfering with each other.

1.2.1.3 LKB1-AMPK signaling

The serine/threonine kinase LKB1 is ubiquitously expressed and gives upstream input to the members of the AMP-activated protein kinase (AMPK) superfamily. LKB1 is known as a growth suppressor and increased cardiomyocyte activity of LKB1 inhibits the protein synthesis involved in hypertrophy by decreasing the pro-translational

phosphorylation of the S6 ribosomal protein in vitro (Noga et al. 2007). Cardiac specific deletion of LKB1 in mice leads to hypertrophy with atrial enlargement by 4 weeks and increased HW/BW by 12 weeks. The phenotype is pathological with interstitial fibrosis and impaired cardiac function (Ikeda et al. 2009).

Interestingly knock out of either of the two AMPK α-subunit isoforms, α_1 or α_2, does not result in any heart phenotype different from wild type (Dolinsky and Dyck 2006) suggesting that LKB1 control of heart size might signal through another downstream effector than AMPK or that the two AMPK α-subunit isoforms have overlapping functions.

Activation of Akt results in phosphorylation and inhibition of of cardiac AMPK (Kovacic et al. 2003) showing cross-talk between the AMPK and PI3K pathways of signaling, and possibly revealing the function by which insulin inhibits AMPK. The relationship between AMPK and PI3K signaling is further proven by the finding that pharmacological activation of AMPK inhibits Akt-induced hypertrophy (Chan et al. 2004), clearly suggesting an antagonistic relationship between the pro-hypertrophic effects of PI3K and the growth inhibitory effects of LKB1-AMPK signaling.

1.2.2 Extracellular receptors

1.2.2.1 α-adrenergic signaling

The family of α-adrenergic receptors comprises six subtypes; the G_q coupled α_1-ARs, $\alpha_{1A/C}$, α_{1B} and α_{1D} and the G_i coupled α_2ARs, α_{2A}, α_{2B} and α_{2C}. All six are present and annotated in the chicken. All three α_1 subtypes are transcribed in the heart but only $\alpha_{1A/C}$- and α_{1B}-ARs are expressed as functional proteins in mammalian hearts (Lin et al. 2001; O'Connell et al. 2003). The roles of $\alpha_{1A/C}$ and α_{1B} respectively in growth hypertrophy of the heart have been studied by cardiac specific over expression (using the αMHC promoter) and knock out in mice. Neither overexpression nor KO of $\alpha_{1A/C}$ or α_{1B} alone resulted in any heart phenotype different from wild type (Akhter et al. 1997; Cavalli et al. 1997; Lin et al. 2001; Rokosh and Simpson 2002). Surprisingly though, expression of a constitutively active mutant form of α_{1B} induced cardiac hypertrophy in 10 week old mice, shown by increased HW/BW and increased myocyte cross sectional area (Milano et al. 1994). Combined disruption of $\alpha_{1A/C}$ and α_{1B} simultaneously resulted in reduced HW/BW , left ventricular wall thickness, end systolic chamber size and reduced

myocyte cross sectional area in male mice while in female mice the heart phenotype was no different from WT. This indicates that males, but not females, require α_1-AR signaling for normal growth hypertrophy, but that the functions of the two subtypes found in the heart overlap to make signaling through one subtype sufficient for normal growth (O'Connell et al. 2003).

α_2ARs and their function in developmental hypertrophy have been studied by generating mouse KOs of the three subtypes (α_{2A}, α_{2B} and α_{2C}) respectively and in combinations of two KO genes at a time. No one α_2AR KO alone resulted in any altered heart phenotype. Double KO of α_{2A} and α_{2C} produced the only viable combinatory KO strain. These mice developed cardiac hypertrophy by four months of age (Hein et al. 1999). Thus mice develop normally devoid of any one of the α_2ARs but need at least two functional subtypes for survival and/or normal growth hypertrophy.

Thus, α_1-AR signaling regulates hypertrophy in a positive manner (in males) while α_2AR signaling regulates hypertrophy negatively. This is not surprising given that they signal through different types of G-proteins (G_q and G_i respectively).

1.2.2.2 Insulin and Insulin-like growth factor signaling

Insulin and Insulin-like growth factor (IGF) and their respective receptors are upstream of the PI3K and MEK-ERK signaling cascades, but may also affect AMPK signaling. The role of these molecules in growth hypertrophy has been investigated through generation of heart specific knock-outs of the insulin and IGF receptors. The insulin receptor (IR) is knocked out in MIRKO mice, the IGF1 receptor (IGF1R) is knocked out in MIGF1RKO and both receptors are knocked-out in MI^{2}RKO mice. MIRKO and MI^{2}RKO mice developed smaller hearts than control littermates, both in absolute and relative measures (Laustsen et al. 2007). MIGF1RKO mice on the other hand developed slightly heavier hearts than control littermates. MI^{2}RKO hearts consisted of larger cells than control while MIRKO and MIGF1RKO hearts had normally sized cells. MI^{2}RKO mice further developed cardiomyopathy and heart failure, while mice with either of the receptors functional had altered cardiac function but did not die from heart failure (Laustsen et al. 2007). Since insulin/IGF signaling is upstream of PI3K the activation of Akt was also studied in the KO mice. It was shown that insulin could activate Akt in MIRKO hearts and IGF activated Akt in MIGF1RKO hearts, suggesting cross-reactivity between the substrates and receptors. All data was obtained at or before 20 days of age (Laustsen et al. 2007). Overexpression of

IGF1R in the hearts of mice lead to cardiac hypertrophy with increased systolic function by 3 months of age in mice, the enhanced cardiac function was maintained at 12 months of age. The effect was solely relayed by the PI3K-Akt pathway since expression of dnPI3K abolished the hypertrophic effect of IGF1R overexpression (McMullen et al. 2004). Downstream of the IR and the IGF1R are the IRS proteins. There are four members of the IRS family which have all been subjected to targeted deletion in mouse models. Only deletion of IRS-1 gave an abnormal cardiac phenotype with significantly reduced HW/BW (Pete et al. 1999). Taken together these data suggests that insulin/IGF signaling is required for normal growth hypertrophy and that the hypertrophy is physiological, relayed by PI3K-Akt and regulated in a positive direction by insulin/IGF signaling in mammals.

Studies using overexpression of IGF1 in mice have given surprising results. Systemic overexpression of the human IGF-1B gene under a rat α-MHC promoter in mice resulted in increased heart size by 45 days of age; interestingly the cardiomegaly was due to hyperplasia rather than hypertrophy since cardiomyocyte number was increased and cell size maintained in the transgenic animals (Reiss et al. 1996). Expression of human IGF-1 under the muscle specific α-skeletal actin promoter resulted in mice with increased heart size by 10 weeks of age (Delaughter et al. 1999). In the latter study cardiomyocyte size or number was not investigated, thus it is not known if local IGF-1 expression promotes proliferation in the same way as increased circulating IGF-1 seems to do. Even though the study by Reiss et al. (1996) does not investigate the proliferation pattern during growth in detail, they do show that isolated cardiomyocytes from the transgenic line are proliferative at 75 days (~0,8% BrdU positive cells). This proliferative ability is far beyond what is normal in mice as previously discussed. At 10 weeks the mice in both studies had normal cardiac function and no pathological hallmarks.

Very recent results from studies on isolated chicken skeletal myoblasts and in vivo studies suggest that insulin signaling in might be important for myocyte differentiation and maturation in avians. Treatment with insulin inhibited myoblast differentitation and enhanced proliferation in vitro and administration of the pharmacological insulin stimulator tolbutamide improved chicken growth performance post hatching (Sato et al. 2012). The effect of the insulin signaling was shown to be mediated by PI3K-Akt. Though this work has been done in skeletal muscle cells the finding of this species specific mode of insulin signaling is an interesting finding and unraveling whether the effects are comparable in myocardium is worth pursuing. Insulin signaling is highly different between birds and

mammals. Chickens and mammals have comparable concentrations of circulating insulin but chickens are hyperglyceamic despite the presence of insulin. Chickens also show a resistance to the effects of insulin; a high dose is required to induce hypoglycemia (Dupont et al. 2009). In summary, insulin signaling in birds and mammals are quite different but share common features and investigation of how these differences and features reflect on the growth of the chicken heart is one way to further reveal how insulin signaling works in birds.

2 Cardiac remodeling – when balance is shifted

The growth of the heart in postnatal life, adulthood establishes and maintains the size of the heart in a species specific proportion to the body weight. This, as has been pictured above, occurs through signaling pathways working in a delicate balance and the maintenance of that balance is vital for the size and shape of the heart also in adult life. The task of the heart is to supply the tissues with oxygenated blood containing the nutrients they need, and that task is the same in a newborn as in an elder. Though, the cardiac output that the body requires and the workload posed on the heart to satisfy the needs of the tissues changes through life and thus the heart must adapt in order to carry out its mission. The changes can have both physiological and pathological causes. Physiological causes may be physical exercise which increases the demand of oxygen and nutrient transport. Pathological causes are overloads on the heart in a setting of illness; hypertension for example results in pressure overload on the heart as it need to develop an increased pressure to overcome the increased peripheral resistance. Another example is regurgitation which causes volume overload as the heart needs to increase stroke volume to supply the body with sufficient output. As the causes for the changed demands on the heart vary, the strategies and mechanisms of the adaptation of the heart vary as well.

2.1 Remodeling, hypertrophy or a bit of both?

Cardiac remodeling is an often used umbrella-term for the structural changes that occur in the heart while adapting to changed demands. *Hypertrophy*, a word used frequently in previous chapters of this essay, is another term often used. Once, the two words had distinct definitions; hypertrophy usually referred to an increase in the mass of the heart irrespective of geometry of the organ and remodeling implied changes in geometry regardless of heart mass (Carabello 2006). Apart from growth hypertrophy, where the heart increases in mass without changes in geometry and which has already been thoroughly discussed, changes in mass and changes in geometry are hardly ever isolated events; which is why the term *cardiac remodeling* will be used for all structural changes that alter the HW/BW ratio and/or geometry from what is established by normal growth hypertrophy. Remodeling resulting from physiological cues will be referred to as *physiologic remodeling* and analogously *pathologic remodeling* will be the term used for remodeling which results from pathological cues.

Hypertrophy will, just as in previous chapters, be used to describe organ growth through cell enlargement. Hypertrophy is a often occurring part of a remodeling response. Nonproliferative myocytes grow by addition of sarcomeres. If sarcomeres are added in series, myocytes become elongated and the chamber they surround becomes larger in diameter; this is called eccentric hypertrophy. If sarcomeres are added in parallel, myocytes get increased cross sectional area and the chamber wall grows thicker; this is called concentric hypertrophy. Eccentric and concentric hypertrophies can, and often do, occur in parallel (Weeks and McMullen 2011). This is the case in growth hypertrophy where chamber dilation and wall thickening are both required for the heart to grow with maintained proportions.

2.2 Pathological or physiological remodeling?

Pathological remodeling starts as a compensatory remodeling of the myocardium to balance out the overload on the heart caused by a pathological state. In volume overload, the remodeling response consists mainly in eccentric hypertrophy. Chamber dilation allows the heart to pump a larger volume to overcome the anemic state caused by, for example, aortic regurgitation (Carabello 2006). In pathological eccentric hypertrophy, proportional wall thickness is often decreased. Pressure overload results in concentric hypertrophy; thickening of the chamber wall decreases the wall stress caused by the increased pressure demand (Bernardo et al. 2010). These types of remodeling are per se not pathological; rather they represent an ability of the heart to rise to the challenges caused by pathology. In states of compensatory remodeling cardiac function is still normal but if the pathologic stressor on the heart persists the compensatory remodeling will develop into pathologic remodeling. The shift from compensatory to pathological remodeling is an area of intense investigation since it is not known what triggers it. The criteria for a state of remodeling to be called pathologic is cardiac dysfunction (systolic, diastolic or both). Systolic impairment is characterized by reduced ejection fraction while diastolic impairment presents as impaired left ventricular filling. Pathologic remodeling is further associated with cell death, increased interstitial fibrosis and increased risk of heart failure (Carabello 2006; Bernardo et al. 2010). Since cardiac function in compensatory remodeling is normal, one question arises; what separates compensatory from physiological remodeling? Perrino et al. (2006) showed that even in mild hypertrophies, with maintained cardiac function that are caused by overload due to pathology, there can be interstitial fibrosis and reduction in capillary density. These are both pathological hallmarks that do not arise in

physiological remodeling and thus compensatory remodeling should be distinguished from the physiological counterpart based on the nature of the overload.

Physiological remodeling occurs in response to intense exercise and pregnancy and is associated with normal cardiac structure and normal, or enhanced, cardiac function. Physiological remodeling is reversible. Just as pathological remodeling, physiological remodeling can also present itself as concentric and/or eccentric hypertrophy. Aerobic exercise/endurance training and pregnancy increase venous return and thus results in a volume overload which is compensated by eccentric hypertrophy. Unlike the eccentric hypertrophy in pathological remodeling, the chamber enlargement in physiological eccentric hypertrophy is accompanied by a proportional increase in wall thickness. Strength training on the other hand results in concentric hypertrophy in response to pressure load on the heart (Carabello 2006; Bernardo et al. 2010). Many cases of physiological remodeling present as combinations of eccentric and concentric hypertrophy since load is not purely volume or pressure and the human heart has a high capacity for physiological remodeling; in highly trained athletes the left ventricle mass can be increased by 50% from sedentary level (D'Andrea et al. 2002).

To examine the genetic regulation and the respective roles of genes involved in cardiac remodeling in experimental settings, remodeling is often induced. Physiological cardiac remodeling in vivo is most often induced by subjecting model animals to exercise training whereas pathologic remodeling is induced by surgical production of volume or pressure overload. These regimes are often used in transgenic animals, over- or underexpressing the gene of interest. In the chicken model there is very little work done in this field and genetic manipulation is more difficult and far less explored in the chicken than in the conventional rodent models (Mozdziak and Petitte 2004). Nevertheless, the chicken poses a highly interesting model for studying development of cardiac remodeling, much thanks to the delayed cardiomyocyte maturation described above. The fact that the chicken has proliferative cardiomyocytes for at least 42 days post hatching (Li et al. 1997) would make it possible to study the role of proliferative capacity of the heart in a far more grown model than the rodent models when subjected to treatments that induces cardiac remodeling; physiologic or pathologic. Does the chicken heart respond differently to exercise training or pressure overload? Is the proliferative ability a part of that response? And is it able to repair itself from ischemic injury through proliferation? To gain a better understanding of what regulates these responses in mammalian

models and to provide inspiration for the development of the chicken model in this field, the chapters below summarize some of the current knowledge on the genetic regulation of cardiac remodeling.

2.3 Genes regulating physiologic remodeling

The genetic regulation of physiologic cardiac remodeling is not nearly as well investigated as the pathologic counterpart, and many mechanisms remain elusive. Often the genetic basis of physiologic remodeling in response to exercise is studied as a parallel treatment in models generated to study pathological remodeling. Just as in growth hypertrophy, one of the best understood regulators of physiologic remodeling is the PI3K pathway with IGF-1 as upstream input and Akt as downstream effector. IGF-1 expression has been shown to be upregulated in the heart of animals subjected to physical exercise (Neri Serneri et al. 2001; Scheinowitz et al. 2003). At the level of Akt, generation of $akt1^{-/-}$ mice has shown that Akt deficient mice had normal heart phenotypes at baseline but that their hearts were impaired in the physiologic remodeling response to exercise. Isolation of transgenic myocytes and subsequent stimulation with IGF-1, a treatment known to induce a hypertrophic response in cardiomyocytes, showed that the impaired growth of the heart was due to reduced protein synthesis (DeBosch et al. 2006). This suggests that signaling through Akt is required for induction of physiologic remodeling in response to exercise.

Class I_A PI3Ks (PI3Kα) are involved in physiologic hypertrophy and their role in exercise induced remodeling has been evaluated in several transgenic models. dnPI3K transgenic mice were used by McMullen et al. (2003) and subjected to swim training. Mice expressing dominant negative mutation of PI3K (dnPI3K) mice had a significantly attenuated cardiac growth response. Mice devoid of the PI3K regulatory subunit p85 also show attenuated growth in response to exercise (Luo et al. 2005). Interestingly, in the latter study, it was shown that the transgenic mice had higher basal and post exercise levels of activated Akt. The authors hypothesize that adrenergic signaling through Class I_B PI3Ks (PI3Kγ) is elevated when the PI3Kα pathway is not functional and that this is responsible for the increased Akt activity. This is to some extent supported by the finding that insulin treatment, which signals through PI3Kα, did not elevate Akt activity in the transgenic mice. The importance of IGF-1 signaling in physiologic remodeling has also been evaluated at the level of the IGF-1 receptor (IGF-1R). Knock out of IGF-1R resulted in severely attenuated heart growth response to swim exercise (Kim et al. 2008). Akt phosphorylation was increased post exercise in both WT and IGF-1RKO mice which is in line with the results by Luo et

al. (2005), but the question arises why the Akt activity was not sufficient to induce physiologic remodeling. IGF-1R - PI3Kα signaling must have another important role than activation of Akt in the setting of heart growth regulation, since presence and activity of Akt is required but not sufficient to induce physiologic remodeling in response to exercise.

Physiological remodeling also arises during pregnancy because of the prolonged volume overload that accompanies pregnancy. Little work has been put into investigating the signaling and genetic mechanisms underlying pregnancy induced physiological remodeling but very recently Chung et al. (2012) showed that both PI3Kα and ERK1/2 signaling is mediating remodeling in pregnancy. The ERK1/2 activity was induced by physiological levels of progesterone in cardiomyocytes in vitro. The progesterone treated cardiomyocytes increased in size and in protein content without expressing the pathological fetal gene pattern which suggests that progesterone acts through ERK1/2 signaling to induce physiological cardiac remodeling during pregnancy.

2.4 Genes regulating pathologic remodeling

Cardiac pathologies and heart failure are among the major health problems in the developed world and the genetic regulation of pathologic remodeling has been, and still is, an area under intense investigation (Blinderman et al. 2008; Bernardo et al. 2010). Many genes and genetic pathways have been identified as players in the setting of pathologic remodeling and hypertrophy but since most of the networks found are not unique to the regulation of heart size and geometry but rather have important functions in many other tissues and settings in the body, using the knowledge gained to cure cardiac disease has proven to be far from straightforward. The genes and genetic pathways described below, are all part of the response of the heart to pathological insults such as volume or pressure overload. The pathways are divided in protective and inductive regulators. Protective regulators have been found to act by delaying or counteracting pathologic remodeling or the transition from compensatory to pathologic remodeling and heart failure, whereas inductive regulators have been shown to have active roles in inducing the pathological remodeling responses.

2.4.1 Protective pathways

2.4.1.1 MAPK signaling

Mitogen-activated protein kinases (MAPKs) are a superfamily of kinases which constitute several intermediate signal transduction pathways for multiple stimuli. The three most well investigated subfamilies are the extracellular responsive kinases (ERKs) which have already been covered as a mediator of growth hypertrophy, c-Jun N-terminal kinases (JNKs) and the p38-mitogen activated protein kinases (p38-MAPKs). All three of these subfamilies have been implicated in pathologic remodeling. The results from studies on MAPK signaling are conflicting on means and extension of their effects but they all seem to have protective roles in pathologic remodeling.

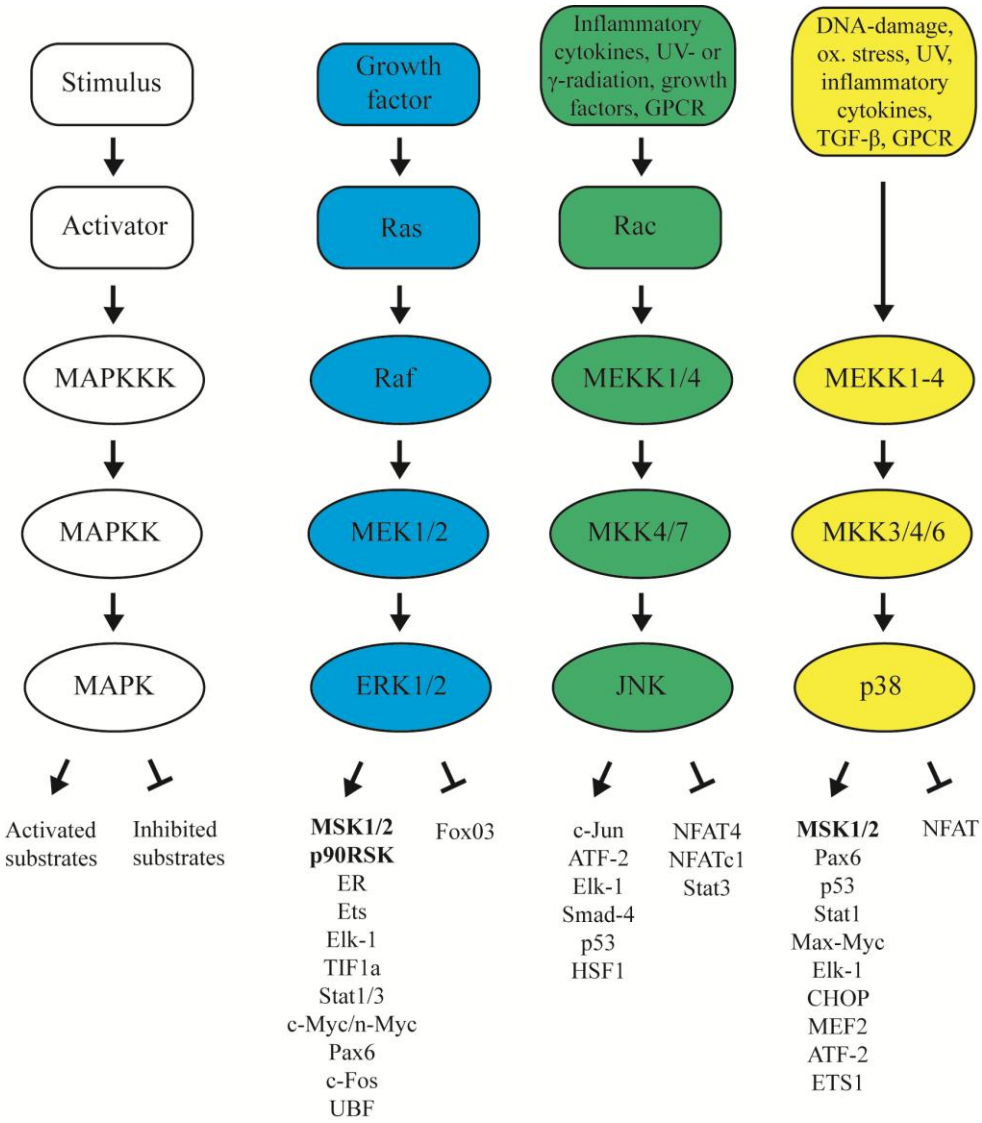

Figure 3. MAPK subfamilies ERK1/2, JNK and p38 with upstream regulators and downstream effectors.

In mammals three genes are coding for JNKs; Jnk1 or MAPK8, Jnk2 or MAPK9 and Jnk3 or MAPK10. All three are also present and annotated in the chicken. JNKs are activated in response to stress and membrane receptor signaling which activates the MAPK kinase kinases MEKK1 and MEKK4 through Rac. MEKK1/4 in turn activate the MAPK kinases MKK4 and MKK7 which in their turn activate JNK (see figure 3). When activated, JNK phosphorylates several regulatory targets including transcriptional and apoptotic regulators, which regulate transcription and apoptosis both directly and indirectly. JNK has been shown to be upregulated in human heart failure (Cook et al. 1999) and in rats following myocardial infarction (Li et al. 1998). Disturbance of JNK signaling has been achieved through targeting of several genes of the pathway in mice. Pressure overload has then been induced in the hearts of the transgenics by transverse aortic constriction (TAC). KO of JNK1, JNK2 and JNK3 with subsequent TAC showed that JNK2 and JNK3 respectively either play no role in the heart or that redundancy between the isoforms rescues the heart from any deleterious effect of their respective KO (Tachibana et al. 2006). KO of JNK1 on the other hand resulted in hearts with hypertrophy comparable to wild type after TAC but with increased apoptosis, fibrosis and decreased cardiac function, suggesting that JNK1 is protecting the myocytes from apoptosis and the tissue from pathological rearrangement upon pathological insults. However it is not involved in hypertrophic growth. Expression of dnJNK1/2 and targeted mutation of JNK1/2 followed by TAC on the other hand resulted in enhanced hypertrophic growth without pathological signs (Liang et al. 2003), suggesting that JNK signaling antagonizes hypertrophy triggered by pressure overload. Perturbations in the pathway upstream of JNK has also provided interesting results. Mice deficient in MEKK1 showed a response very similar to that of JNK KO mice after TAC; hypertrophy comparable to wild type but with a higher degree of apoptosis, lesions, fibrosis and cardiac dysfunction (Sadoshima et al. 2002). Cardiac specific deletion of MEKK4 in combination with TAC on the other hand, resulted in enhanced hypertrophy compared to wild type but was also accompanied by decreased contractility and interstitial fibrosis (Liu et al. 2009). MEKK1 and MEKK4 both cross over between the JNK and p38-MAPK pathways, making it possible that effects seen at perturbation of them might not be relayed solely by JNK signaling. Liu et al. (2009) controlled for this when studying MEKK4 by showing that deletion of MEKK4 had no effect on p38 signaling. Since perturbations at different levels in the JNK have given differing results the overall effects of JNK signaling on cardiac remodeling and hypertrophy upon

pathological insult are not quite clear. Most studies have in common that disruptions of the pathway lead to increased apoptosis, lesions and impaired cardiac function which thus indeed suggests that JNK signaling protects the heart from pathologic insults. This is further underscored by the finding that JNK signaling acts to inhibit nuclear translocation of NFAT and thus downregulates NFAT transcriptional activity (Liang et al. 2003; Liu et al. 2009). NFAT has been tightly coupled to induction of pathologic remodeling (see subchapter on Calcium signaling below) and thus its inhibition should be considered a protective activity.

The mammalian genome has four subfamilies of p38-MAPKs; α, β, γ and δ, of which p38α is the most common. p38 has been shown to be activated upon hypertrophic stimulation in cardiomyocytes in vitro, in mouse hearts subjected to pressure overload and in failing human hearts (Cook et al. 1999; Nishida et al. 2004). Studies on p38 function have generated similar results to the studies on JNK signaling. Expression of dnp38α as well as dominant negative forms of MKK3 and MKK6 (specific activators of p38) resulted in enhanced hypertrophy and cardiomyopathy in response to TAC (Braz et al. 2003) while in cardiac specific knockouts of p38α hypertrophy was comparable to wild type after TAC but hearts were dilated with increased fibrosis and apoptosis. Also in p38 signaling a connection has been tied to NFAT, showing that p38 directly phosphorylates NFAT and that targeted inhibition of p38 enhances NFAT transcriptional activity (Braz et al. 2003). Thus p38 should be considered a protective signaling pathway by the same rationale as for JNK.

The role of ERK signaling has also been investigated in pressure overload setting. Expression of dnRaf revealed that hearts devoid of ERK signaling were sensitized to pressure overload as pathologies became severe and mortality was high following TAC. As dnRaf hearts were surprisingly almost totally resistant to hypertrophy, these results suggest that ERK signaling is protecting the myocardium from apoptosis, fibrosis and stiffness and is required for the induction of hypertrophic growth. Since the hypertrophy is not pathologic per se, but rather can work in a compensatory manner, and mortality was elevated in these model mice, also ERK signaling must be considered to be protecting the heart from the deleterious effects of pathologic remodeling.

2.4.1.2 Class I_A PI3K signaling

Since class I_A PI3K plays a vital role in physiologic remodeling and in growth hypertrophy its possible role in pathologic remodeling has been of clear interest and it has been hypothesized that class I_A PI3K signaling might be protective against pathologic remodeling. Indeed McMullen et al. (2007) showed that upregulation of cardiac PI3K (p110α) activity prolonged the survival of a dilated cardiomyopathy (DCM) model by 15-20% and that downregulation shortened the lifespan of the same model with about 50%. The authors further showed that an increase in PI3K activity in the heart had a protective role after TAC as PI3K activity reduced fibrosis and attenuated pathologic remodeling. Interestingly, it was shown that exercise (swim training) had the same effect on the DCM model as genetically increased PI3K activity, suggesting that exercise may be very beneficial in a setting of pathologic remodeling. Expression of dnPI3K in combination with TAC results in hearts with hypertrophy comparable to WT but with impaired cardiac function (McMullen et al. 2003), suggesting that PI3K is not essential for the hypertrophy but either protective against deleterious effects of the remodeling or that PI3K mediated growth is the growth response of choice when subjected to a pathologic stimuli and that the second choice, which comes into play to produce compensatory growth when PI3K is not available, produces pathologies faster.

Results from Akt deficient mice subjected to pressure overload through TAC are in line with PI3K results. Akt1 deficient mice did not produce a significantly greater hypertrophy in response to TAC but did develop cardiac dysfunction (DeBosch et al. 2006). Since Akt is the mediator of PI3K signaling this is not surprising and further underscores the protective role of class I_A PI3K signaling.

Downstream of PI3K-Akt, but also of MAPK is GSK-3. The β-isoform of GSK-3 has been presented previously as a negative regulator of β-catenin activity in the nucleus, and thus a negative regulator of growth in the proliferative stage of heart development. In the setting of pathologic remodeling GSK-3β and its sister isoform, GSK-3α have different and antagonistic functions. Phosphorylation by upstream kinases such as Akt or ERK leads to inactivation of GSK-3. By inhibiting the phosphorylation of GSK-3, Matsuda et al. (2008) showed that the increased activity of GSK-3α exacerbates hypertrophy and development of pathologies in response to TAC, while increased activity of GSK-3β inhibits any hypertrophy or remodeling. This indicates that phosphorylated, inactive GSK-3α is protective, while nonphosphorylated, active GSK-3β is protective in a setting of pressure overload. Cardiac specific

overexpression of GSK-3α has produced similar results with increased pathologic remodeling, fibrosis and apoptosis in response to TAC (Zhai et al. 2007). In this model hypertrophy was less severe in the transgenic model mice than in WT which contradicts the results by Matsuda et al. (2008). The studies do agree on the role of GSK-3α inhibition as an important protective mechanism. It was further shown that GSK-3α reduces hypertrophy in pressure overload by inhibiting ERK via MEK1 (Zhai et al. 2007), which is yet another example of how intricate the relationships between these genetic regulators are.

2.4.1.3 α_1-adrenergic receptor signaling

α_1ARs have a role in postnatal heart growth as regulators of heart size as described earlier. $\alpha_{1A/c}$ and α_{1B} double KO (ABKO) mice were vital for the generation of those results and have also been used in search for a potential role for α_1AR signaling in pathologic remodeling. ABKO mice develop severely pathological heart phenotypes with ventricular dilation, apoptosis and fibrosis and reduced cardiac function in response to pressure overload and mortality is dramatically elevated one week after TAC. This suggests that α_1AR signaling is an important protective mechanism in response to pathologic insult to the heart. But α_1AR signaling does not seem to regulate the hypertrophy of the response to overload, as shown by the fact that hypertrophic response to TAC is not different between ABKO and WT mice (O'Connell et al. 2003; O'Connell et al. 2006). In vitro results lead to the hypothesis that the deleterious effect of α_1AR-signaling deprivation was due to changes in βAR-signaling. Indeed it has been shown that hearts from ABKO mice were markedly desensitized to βAR activation by Isoproterenol (O'Connell et al. 2006).

2.4.1.4 Jak-Stat signaling

Jak-Stat signaling conveys input from outside the cell via transmembrane receptors and an activated kinase cascade to the nucleus where changes in gene expression constitute the response of the cell to receptor activation. There are several receptors that convey Jak-Stat signals, just as there are several members of the Jak and Stat families. Over the years Stat3 has received attention in cardiac remodeling Stat3 activity is increased following myocardial infarction (Negoro et al. 2000) and in pressure overload in rat (Pan et al. 1997). Stat3 is activated by Jak upon activation of the interleukin-6 (IL-6) receptor by a cytokine of the IL-6 family. Once Stat3 is active it translocates to the nucleus where it functions as a transcriptional regulator. Stat3 signaling is also involved in crosstalk with both the PI3K and ERK signaling pathways; Jak directly activates PI3K

and indirectly activates ERK. ERK in turn inhibits Stat3 which means that there is a feedback loop on Stat3 signaling via ERK. There is also an inhibitory feedback on Jak from the transcriptionally regulated targets through SOCS (see figure 4). Experiments on transgenic mice overexpressing Stat3 specifically in the heart suggests a substantial role for Stat3 signaling as a protector against pathologic insults in the heart. The use of the antitumor drug Deoxorubicin (Dox) results in cardiomyopathy over time but mice overexpressing Stat3 were significantly protected from the Dox induced cardiac pathologies with lower mortality from congestive heart failure (Kunisada et al. 2000). A key component of the IL-6R is the subunit gp130. Stat3 signaling has been perturbed at the level of gp130 through KO and expression of a dominant negative form. Following pressure overload induction by TAC both models developed impairments in the cardiac function, dilation, apoptosis and fibrosis (Hirota et al. 1999; Uozumi et al. 2001), suggesting that that functional gp130 and thus Stat3 signaling indeed is protective upon overload insult. The dngp130 model developed less marked hypertrophy after TAC than the wild type controls, suggesting that Stat3 is also involved in the hypertrophic response.

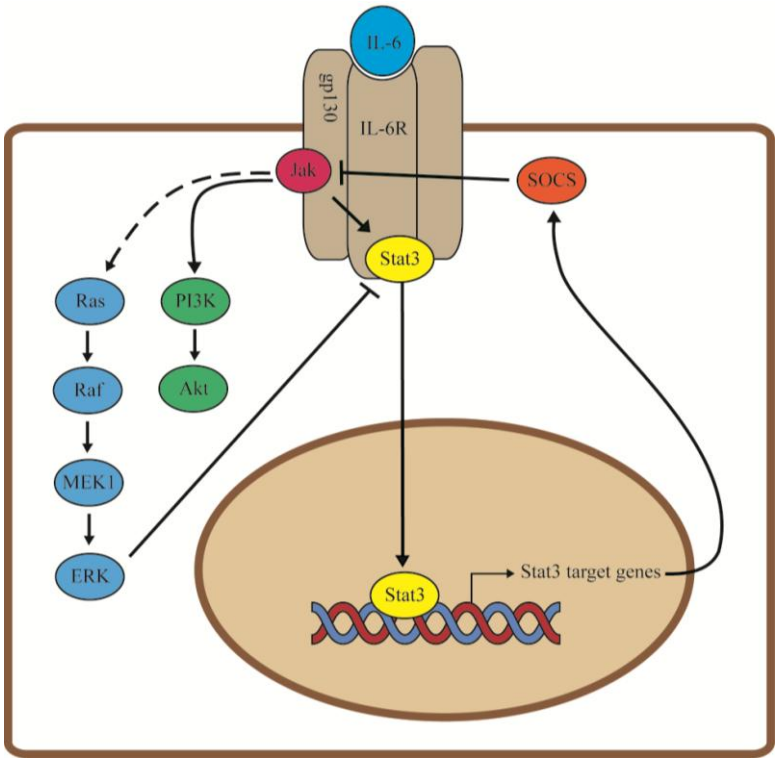

Figure 4. Jak-Stat signaling pathway. Dashed line indicates indirect regulation.

2.4.2 Inductive pathways

2.4.2.1 Calcium signaling

Calcium (Ca^{2+}) plays a vital role in cardiac physiology as it mediates the process of excitation-contraction coupling. Therefore its intracellular handling and balance are of great importance for the heart to maintain normal function and in the setting of pathologic remodeling and heart failure Ca^{2+} is altered from normal. Failing human myocardium displays prolonged contractions and Ca^{2+} transients as well as diminished capacity to restore low resting Ca^{2+} during diastole (Gwathmey et al. 1987) and hearts from a rat model of pathologic hypertrophy show slowing of both Ca^{2+} mobilization and removal (Seki et al. 2003). Angiotensin II (AngII), phenylephrine (PE) and endothelin-1 (ET-1) are all humoral factors which are known to induce hypertrophic responses in cardiomyocytes. They also share the common ability to elevate intracellular Ca^{2+} concentrations, suggesting that Ca^{2+}-signaling might be involved in the hypertrophic response.

Besides inducing contraction by opening the RyR2 channels in cardiomyocytes, Ca^{2+} also works as an important second messenger. For example Ca^{2+} binds and activates the intermediate messenger protein calmodulin (CaM). CaM activation in turn leads to activation of the protein phosphatase calcineurin (CN) which, when active, activates the transcription factor NFAT by dephosphorylation. Dephosphorylated, active NFAT translocates to the nucleus where it induces changes in gene expression. CaM can also act through activation of Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII). CaMKII is a protein kinase with a broad spectrum of substrates. Among those substrates are both the RyR2 channel and the L-type calcium channel (LTCC), both involved in Ca^{2+} flux in the cardiomyocytes. Phosphorylation of RyR2 by CaMKII leads to increased channel sensitivity and open probability (Wehrens et al. 2004) and CaMKII phosphorylation of LTCC result in longer open times of the channel (Dzhura et al. 2000). Both these effects result in larger/longer influx of Ca^{2+} in to the myocyte, which in turn leads to an increased acitivity of CaM/CaMKII – thus this signaling, unless perturbed at any point, exerts a positive feedback on itself.

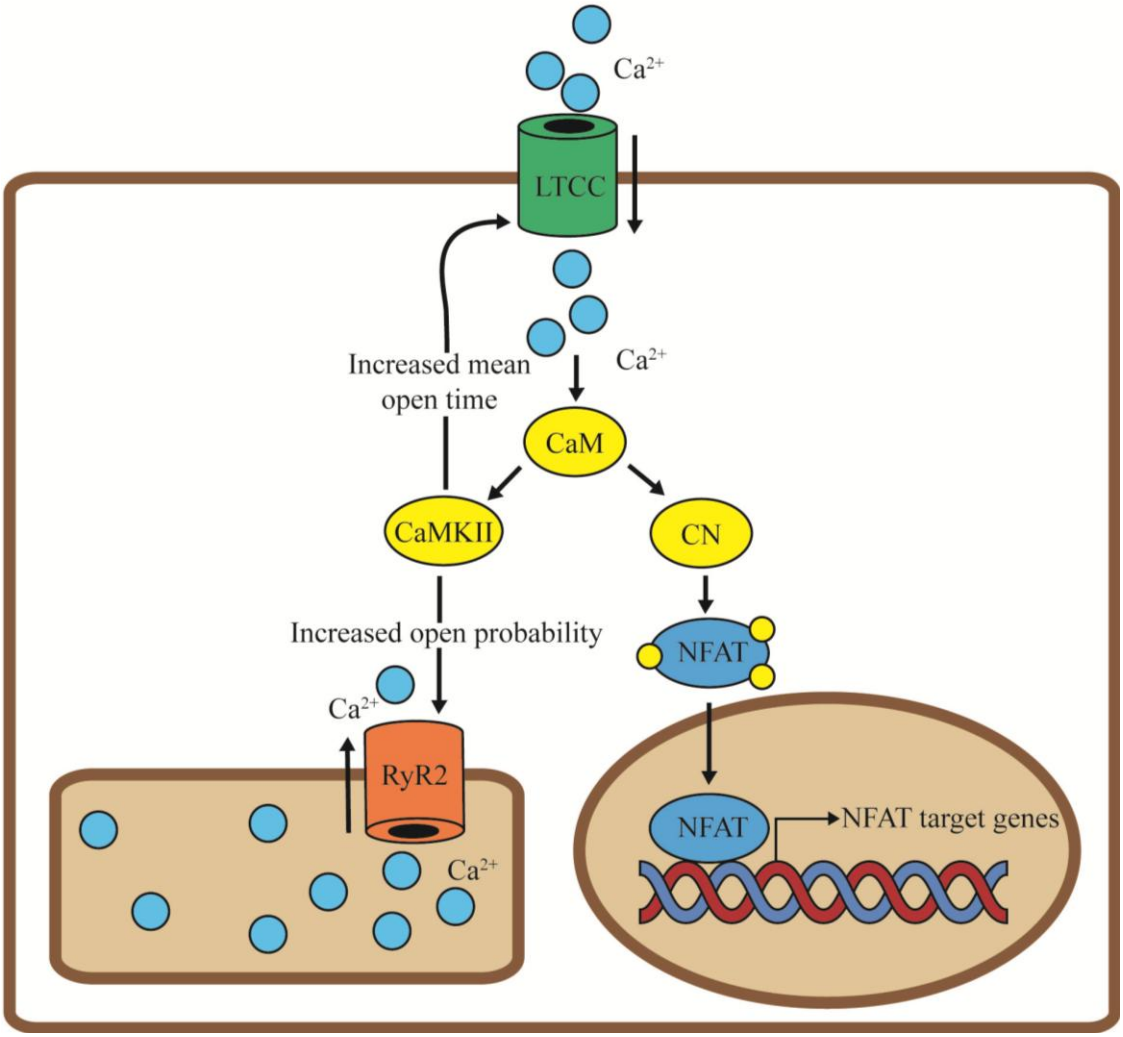

Figure 5. Role of Ca^{2+}-signaling in cardiac remodeling.

Both the Ca^{2+}-CaM-CN-NFAT and Ca^{2+}-CaM-CAMKII pathways play inductive roles in the setting of pathologic cardiac remodeling. Pressure overload produced through constriction of the abdominal aorta in rat increases the activity of CN in the heart and induces hypertrophy with fibrosis. Treatment with the CN inhibitor FK506 inhibits the activation of CN and prevents the hypertrophic and fibrotic response to the pressure overload insult (Shimoyama et al. 1999). Inhibition of CN in vitro through transfection prevents the hypertrophic response of cardiomyocytes to AngII and PE (Taigen et al. 2000). Targeted disruption of CN suppresses the hypertrophic and fibrotic response to both pressure overload insult and hypertrophy inducing agonists in mice in vivo ((Zou et al. 2001; Bueno et al. 2002). This suggests that signaling through CN is necessary in the induction of pathologic remodeling. Interestingly Zou et al. (2001) showed that depletion of CN signaling attenuated pressure overload-induced ERK activity in response to TAC, indicating crosstalk

between the Ca^{2+} and ERK signaling pathways. Since ERK is a protective signaling pathway in pathologic remodeling this suggests a rather interesting balance where the inductive CN-signaling activates a counteracting protective pathway.

Wilkins et al. (2004) has investigated the role of NFAT in pathologic cardiac remodeling in different experimental models. Pressure overload through TAC increased NFAT activity in the nucleus by 2-3 fold, though the activation of NFAT took 48 h to take place. Myocardial infarct (MI) through left anterior descending coronary artery (LAD) occlusion results in a model of heart failure with significantly higher NFAT activity than sham operated control animals. Remodeled non-failing hearts did not have as up-regulated NFAT activity as the failing hearts, suggesting that the magnitude of CN-NFAT signaling correlates with the severity of the pathology. Expression of a constitutively active form of NFAT is enough to induce spontaneous hypertrophy with fibrosis and cellular disarray. This pathology is relieved by administration of the CN inhibitor cyclosporine A (Molkentin et al. 1998). The results above taken together clearly suggests that increased signaling through CN-NFAT is enough to cause pathological remodeling even without other insult and that CN-NFAT signaling is necessary for the induction of pathologic remodeling.

There are four isioforms of CaMKII (α, β, δ and γ)which are encoded by different genes. CaMKIIδ is the predominant isoform in the heart (Backs et al. 2009). Knock out of CaMKIIδ combined with pressure overload through TAC has generated differing but not conflicting results. In a study by (Ling et al. 2009) TAC on CaMKIIδ-null mice resulted in a hypertrophy comparable to WT TAC but with fewer apoptotic cells and less fibrosis. Interestingly the hearts of KO mice in this study were also rescued from transition to heart failure and death due to cardiac pathologies after TAC were significantly fewer in KO mice than WT. On the other hand, (Backs et al. 2009) showed that CaMKIIδ developed a markedly lower level of hypertrophy in response to TAC. Cardiac fibrosis, which was extensive in WT was also abolished in KO mice. Even though these results are to some extent conflicting it is obvious that CaMKIIδ activity in the heart is inducing pathologic remodeling and it seems to be required for the remodeling response a pressure overload insult to be pathologic and for heart failure to develop. Thus both legs of the Ca^{2+}-signaling pathway described here (CaM-CN-NFAT and CaM-CaMKII) are required for induction of pathologic cardiac remodeling.

As suggested in the chapter on the protective role of MAPK signaling above, signals that act to inhibit NFAT nuclear translocation protect the heart from pathologic remodeling. Oliveira et al. (2009) has shown in a

mouse model of pathologic remodeling that aerobic training can have just that effect. Exercise increased the cardiac function of the model from their baseline exercise intolerance to WT exercise capacity. Eight weeks of exercise had a significant effect reversing the pathologic remodeling of the model, reducing heart weight and cardiomyocyte size. Exercise also decreased the nuclear localization of NFAT in the model to WT levels. It was further shown that Akt activity was not elevated by exercise in this study, which could be due to the moderation of the exercise intensity applied due to the impaired baseline cardiac function of the model. It has previously been shown by (Wilkins et al. 2004) that IGF-Akt signaling does not regulate NFAT activity, so even if PI3K-Akt signaling is the predominant mediator of physiological hypertrophy in an exercise setting it does not seem to have a role here. This protective/reversing effect of exercise on the pathologically remodeled heart is likely mediated by another signaling pathway. Close at hand are JNK and p38 signaling which have both previously been described as protective and both been shown to inhibit NFAT nuclear translocation and activity (Braz et al. 2003; Liang et al. 2003; Liu et al. 2009).

2.4.2.2 Class I_B PI3K signaling

Class I_B PI3Ks (PI3Kγ) lie downstream of GPCRs and receive signals through binding to the βγ subunit complex of the trimeric G-protein. Activated PI3Kγ has a broad range of substrates and downstream signaling effects of which Akt with its downstream effectors is one, but PI3Kγ also directly phosphorylate MEKs and induces ERK activation (Patrucco et al. 2004). As has already been covered above, genetic disruption of PI3Kγ does not affect heart size or development at baseline (Crackower et al. 2002) but plays an important role in pathologic remodeling in response to overload or agonist induced remodeling. Mice genetically deficient of PI3Kγ (PI3KγKO) subjected to isoproterenol (ISO) infusion developed attenuated hypertrophy with significantly less fibrosis compared to wild type mice subjected to the same treatment (Oudit et al. 2003). When subjecting the same PI3KγKO mice to TAC the result was a more dramatic phenotype with a similar attenuation in hypertrophy but with severe compromises in structure and function with ventricular dilation, apoptosis and fibrosis (Patrucco et al. 2004). Interestingly subjection of a kinase dead PI3Kγ (PI3KγKD) mutant mouse strain to TAC resulted in a phenotype much more similar to the phenotype of PI3KγKO in response to ISO infusion; reduced hypertrophy and preserved function with lower mortality and without apoptosis or fibrosis (Nienaber et al. 2003; Patrucco et al. 2004). The difference in

remodeling in response to TAC between the PI3KγKO and PI3KγKD mice is intruiging. Patrucco et al. (2004) showed that this difference is most likely due to the effect of PI3Kγ on cAMP-levels, increased in PI3KγKO compared to control (Crackower et al. 2002). Indeed, TAC resulted in elevated cAMP levels in all three strains (PI3KγKO, PI3KγKD and WT) which is to be expected, but elevation was significantly greater in PI3KγKO mice, suggesting that the kinase activity of PI3Kγ is not necessary for its effect on cAMP while the presence of the protein is vital, and that the differences in phenotype in response to TAC might be due to this differential effect on the second messenger cAMP.

The effect of PI3Kγ in pathologic remodeling is thought to be mediated mainly by β-adrenergic receptor (βAR) signaling activity and therefore the effects of genetic perturbation have also been investigated at the receptor level. Cardiac specific overexpression of the β_2AR in combination with TAC results in a more severe type of remodeling with a greater degree of dysfunction and pathological hallmarks in response to TAC compared to WT mice (Du et al. 2000). In line with those results, knockout of β_1- and β_2-ARs in mice attenuates the hypertrophic and fibrotic response to TAC (Kiriazis et al. 2008), clearly suggesting that β-AR activity is inducing pathologic remodeling.

PI3Kγ activity is increased in WT mice when subjected to TAC and also in failing human hearts (Naga Prasad et al. 2002; Perrino et al. 2007). PI3Kγ activity has also been shown to correlate with β-AR trafficking and internalization in the pathologic human heart (Perrino et al. 2007) and has been shown to play an important role in a setting of chronic β-AR stimulation by favoring β-AR internalization and thus β-AR-signaling desensitization (Naga Prasad et al. 2002). It turns out that efficient internalization of β-ARs requires the recruitment of PI3K to the agonist stimulated β-AR. This recruitment is done by the β-AR kinase, βARK1 which binds PI3K at the phosphoinositode kinase (PIK) domain (Perrino et al. 2007). Thus PI3Kγ is mediating a negative feedback effect of the β-AR on itself. β-ARs are significantly downregulated at the plasma membrane in the myocyte of the failing human heart, and this correlates with the upregulation of PI3Kγ activity. Interestingly, it has been shown that mechanical unloading of the failing left ventricle promotes redistribution of the internalized β-ARs back to the plasma membrane and that this in turn correlates with a downregulation of PI3Kγ-activity (Perrino et al. 2007). Further, by actively displacing PI3Kγ from βARK1 by overexpressing a peptide containing only the PIK domain, desensitization of the β-ARs in the failing human cardiomyocytes and β-AR internalization in response to ISO infusion in mice were both

reversed and β-AR responsiveness and contractile function in isolated cardiomyocytes from the failing pig heart was restored (Perrino et al. 2005; Perrino et al. 2007). It is thus shown that activity of PI3Kγ is not only important for the desensitization of β-ARs but that its downregulation can restore β-AR function in a failing heart; and this may be a potent avenue for research on therapeutic strategies in heart failure.

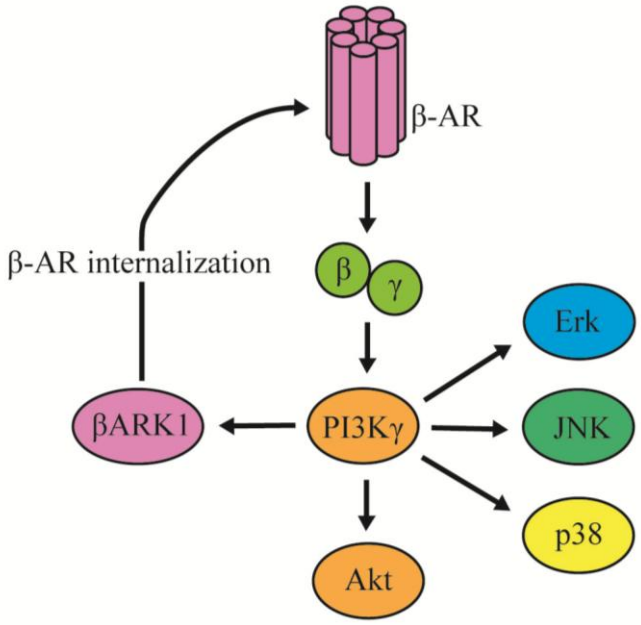

Figure 6. Role of PI3Kγ in cardiac remodeling and β-AR desensitization.

3 Summary

The genetic regulation of organ size is one of the most puzzling and least known areas in developmental biology. The size of an organ is determined by cell number and cell size, and both parameters can be regulated in order to change the size of the organ. The heart is formed early in embryonic development and the task of supplying the tissues in the body with oxygen and nutrients is the same throughout life and across species. Naturally a larger animal needs a larger heart to fulfill that mission, but even when comparing heart size corrected for body weight there are large differences in heart size between species suggesting that there are genetic differences governing the size of the heart. During embryonic development, the heart grows by hyperplasia and at around the time of birth there is a shift to hypertrophic growth. The prenatal hyperplasia and the postnatal hypertrophy are regulated through distinct

signaling pathways. The Hippo pathway, discovered in *Drosophila* and conserved to mammals and other vertebrates, is so far the only pathway described to regulate hyperplastic growth of the prenatal heart. Through a kinase cascade it works an efficient brake to slow the rate of proliferation and animal models devoid of this breaking signal develop enlarged, hyperproliferative hearts.

Once cardiomyocytes mature (at or around the time of birth in mammals) they lose their ability to proliferate and from that point heart growth occurs through hypertrophy. Growth hypertrophy up to adulthood establishes and maintains the size of the heart in a species specific proportion to body weight. But as the demands on the heart changes through life the heart must adapt to fulfill its mission, through changing its size and/or shape from what has been established through growth hypertrophy. The changes in demand can have physiological causes, such as exercise or pregnancy, or pathological causes, such as hypertension or leaky valves. All of these changes are met by remodeling responses, where the whole heart or parts of it hypertrophy to compensate for the extra load. In physiological remodeling cardiac structure is maintained and cardiac function is normal or enhanced. In pathological remodeling on the other hand, cardiac structure is compromised by fibrosis and apoptotic lesions and cardiac function is compromised. Growth hypertrophy and physiological remodeling are to large parts regulated through the same pathways while other pathways regulate pathological remodeling (see figure 7 for a summary).

This introductory essay outlines some of what is known to date about the genes that are regulating heart size and remodeling responses, but plenty of unexplored avenues remain and the big question is still unanswered – what determines heart size?

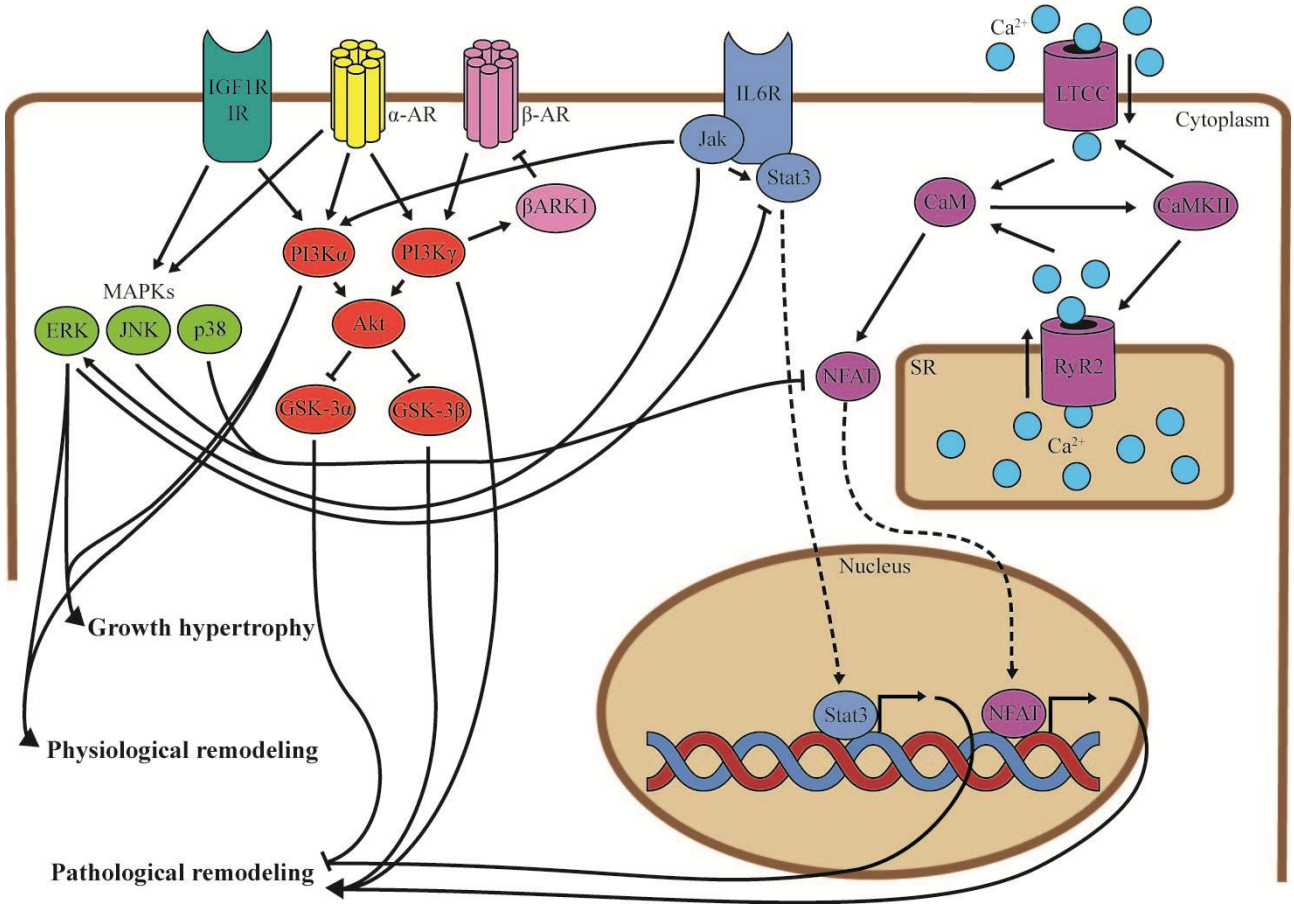

Figure 7. Schematic summary of the signaling pathways involved in growth hypertrophy, physiological remodeling and pathological remodeling. Dashed lines indicate nuclear translocation.

References

Akhter, S. A., C. A. Milano, et al. (1997). "Transgenic mice with cardiac overexpression of alpha1B-adrenergic receptors. In vivo alpha1-adrenergic receptor-mediated regulation of beta-adrenergic signaling." J Biol Chem **272**(34): 21253-21259.

Backs, J., T. Backs, et al. (2009). "The δ isoform of CaM kinase II is required for pathological cardiac hypertrophy and remodeling after pressure overload." Proceedings of the National Academy of Sciences **106**(7): 2342-2347.

Beinlich, C. J., C. J. Rissinger, et al. (1995). "Mechanisms of rapid growth in the neonatal pig heart." J Mol Cell Cardiol **27**(1): 273-281.

Bernardo, B. C., K. L. Weeks, et al. (2010). "Molecular distinction between physiological and pathological cardiac hypertrophy: Experimental findings and therapeutic strategies." Pharmacology & Therapeutics **128**(1): 191-227.

Blinderman, C. D., P. Homel, et al. (2008). "Symptom Distress and Quality of Life in Patients with Advanced Congestive Heart Failure." Journal of Pain and Symptom Management **35**(6): 594-603.

Braz, J. C., O. F. Bueno, et al. (2003). "Targeted inhibition of p38 MAPK promotes hypertrophic cardiomyopathy through upregulation of calcineurin-NFAT signaling." The Journal of Clinical Investigation **111**(10): 1475-1486.

Bueno, O. F., L. J. De Windt, et al. (2000). "The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice." EMBO J **19**(23): 6341-6350.

Bueno, O. F., B. J. Wilkins, et al. (2002). "Impaired cardiac hypertrophic response in Calcineurin Aβ-deficient mice." Proceedings of the National Academy of Sciences **99**(7): 4586-4591.

Camacho, J. A., C. J. Peterson, et al. (1990). "Accelerated ribosome formation and growth in neonatal pig hearts." American Journal of Physiology - Cell Physiology **258**(1): C86-C91.

Camargo, F. D., S. Gokhale, et al. (2007). "YAP1 increases organ size and expands undifferentiated progenitor cells." Curr Biol **17**(23): 2054-2060.

Carabello, B. A. (2006). Ventricular Remodeling in Pressure vs. Volume Overload. Cardiac Remodeling, Mechanisms and Treatment. B. Greenberg. New York, Taylor & Francis Group.

Cavalli, A., A. L. Lattion, et al. (1997). "Decreased blood pressure response in mice deficient of the alpha1b-adrenergic receptor." Proc Natl Acad Sci U S A **94**(21): 11589-11594.

Chablais, F., J. Veit, et al. (2011). "The zebrafish heart regenerates after cryoinjury-induced myocardial infarction." BMC Developmental Biology **11**(1): 21.

Chan, A. Y. M., C.-L. M. Soltys, et al. (2004). "Activation of AMP-activated Protein Kinase Inhibits Protein Synthesis Associated with Hypertrophy in the Cardiac Myocyte." J Biol Chem **279**(31): 32771-32779.

Chattergoon, N. N., G. D. Giraud, et al. (2012). "Thyroid hormone drives fetal cardiomyocyte maturation." Faseb J **26**(1): 397-408.

Christensen, V. L., G. B. Havenstein, et al. (1995). "Egg characteristics, carbohydrate metabolism, and thyroid hormones in late chick embryos from different genetic lines." Poult Sci **74**(3): 551-562.

Chung, E., F. Yeung, et al. (2012). "Akt and MAPK signaling mediate pregnancy-induced cardiac adaptation." Journal of Applied Physiology **112**(9): 1564-1575.

Clubb, F. J., Jr. and S. P. Bishop (1984). "Formation of binucleated myocardial cells in the neonatal rat. An index for growth hypertrophy." Lab Invest **50**(5): 571-577.

Conlon, I. and M. Raff (1999). "Size Control in Animal Development." Cell **96**(2): 235-244.

Cook, S. A., P. H. Sugden, et al. (1999). "Activation of c-Jun N-Terminal Kinases and p38-Mitogen-activated Protein Kinases in Human Heart Failure Secondary to Ischaemic Heart Disease." J Mol Cell Cardiol **31**(8): 1429-1434.
Crackower, M. A., G. Y. Oudit, et al. (2002). "Regulation of myocardial contractility and cell size by distinct PI3K-PTEN signaling pathways." Cell **110**(6): 737-749.
Crickmore, M. A. and R. S. Mann (2008). "The control of size in animals: insights from selector genes." BioEssays **30**(9): 843-853.
D'Andrea, A., G. Limongelli, et al. (2002). "Association between left ventricular structure and cardiac performance during effort in two morphological forms of athlete's heart." International Journal of Cardiology **86**(2–3): 177-184.
DeBosch, B., I. Treskov, et al. (2006). "Akt1 Is Required for Physiological Cardiac Growth." Circulation **113**(17): 2097-2104.
Delaughter, M. C., G. E. Taffet, et al. (1999). "Local insulin-like growth factor I expression induces physiologic, then pathologic, cardiac hypertrophy in transgenic mice." Faseb J **13**(14): 1923-1929.
Dittmer, J. E., R. J. Goss, et al. (1974). "The growth of infant hearts grafted to young and adult rats." Am J Anat **141**(1): 155-160.
Dolinsky, V. W. and J. R. B. Dyck (2006). "Role of AMP-activated protein kinase in healthy and diseased hearts." Am J Physiol Heart Circ Physiol **291**(6): H2557-H2569.
Dong, J., G. Feldmann, et al. (2007). "Elucidation of a universal size-control mechanism in Drosophila and mammals." Cell **130**(6): 1120-1133.
Du, X.-J., D. J. Autelitano, et al. (2000). "ß2-Adrenergic Receptor Overexpression Exacerbates Development of Heart Failure After Aortic Stenosis." Circulation **101**(1): 71-77.
Dupont, J., S. Tesseraud, et al. (2009). "Insulin signaling in chicken liver and muscle." General and Comparative Endocrinology **163**(1–2): 52-57.
Dzhura, I., Y. Wu, et al. (2000). "Calmodulin kinase determines calcium-dependent facilitation of L-type calcium channels." Nat Cell Biol **2**(3): 173-177.
Edgar, B. A. (2006). "From cell structure to transcription: Hippo forges a new path." Cell **124**(2): 267-273.
Gessert, S. and M. Kuhl (2010). "The multiple phases and faces of wnt signaling during cardiac differentiation and development." Circ Res **107**(2): 186-199.
Gwathmey, J., L. Copelas, et al. (1987). "Abnormal intracellular calcium handling in myocardium from patients with end-stage heart failure." Circ Res **61**(1): 70-76.
Heallen, T., M. Zhang, et al. (2011). "Hippo pathway inhibits Wnt signaling to restrain cardiomyocyte proliferation and heart size." Science **332**(6028): 458-461.
Hein, L., J. D. Altman, et al. (1999). "Two functionally distinct alpha2-adrenergic receptors regulate sympathetic neurotransmission." Nature **402**(6758): 181-184.
Hirota, H., J. Chen, et al. (1999). "Loss of a gp130 Cardiac Muscle Cell Survival Pathway Is a Critical Event in the Onset of Heart Failure during Biomechanical Stress." Cell **97**(2): 189-198.
Huang, J., S. Wu, et al. (2005). "The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP." Cell **122**(3): 421-434.
Ikeda, Y., K. Sato, et al. (2009). Cardiac-specific Deletion of LKB1 Leads to Hypertrophy and Dysfunction. The Journal of Biological Chemistry. **284:** 35839-35849.
Jopling, C., E. Sleep, et al. (2010). "Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation." Nature **464**(7288): 606-609.
Kim, J., A. R. Wende, et al. (2008). "Insulin-Like Growth Factor I Receptor Signaling Is Required for Exercise-Induced Cardiac Hypertrophy." Molecular Endocrinology **22**(11): 2531-2543.

Kiriazis, H., K. Wang, et al. (2008). "Knockout of β1- and β2-adrenoceptors attenuates pressure overload-induced cardiac hypertrophy and fibrosis." British Journal of Pharmacology **153**(4): 684-692.
Kovacic, S., C.-L. M. Soltys, et al. (2003). "Akt Activity Negatively Regulates Phosphorylation of AMP-activated Protein Kinase in the Heart." J Biol Chem **278**(41): 39422-39427.
Kunisada, K., S. Negoro, et al. (2000). "Signal transducer and activator of transcription 3 in the heart transduces not only a hypertrophic signal but a protective signal against doxorubicin-induced cardiomyopathy." Proceedings of the National Academy of Sciences **97**(1): 315-319.
Lai, Z.-C., X. Wei, et al. (2005). "Control of Cell Proliferation and Apoptosis by Mob as Tumor Suppressor, Mats." Cell **120**(5): 675-685.
Laustsen, P. G., S. J. Russell, et al. (2007). "Essential role of insulin and insulin-like growth factor 1 receptor signaling in cardiac development and function." Mol Cell Biol **27**(5): 1649-1664.
Lee, J. C., F. N. Taylor, et al. (1975). "A comparison of ventricular weights and geometry in newborn, young, and adult mammals." J Appl Physiol **38**(1): 147-150.
Leu, M., E. Ehler, et al. (2001). "Characterisation of postnatal growth of the murine heart." Anatomy & Embryology **204**(3): 217-224.
Li, F., M. R. McNelis, et al. (1997). "Hyperplasia and hypertrophy of chicken cardiac myocytes during posthatching development." Am J Physiol **273**(2 Pt 2): R518-526.
Li, F., X. Wang, et al. (1996). "Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development." J Mol Cell Cardiol **28**(8): 1737-1746.
Li, W. G., A. Zaheer, et al. (1998). "Activation of JNK in the Remote Myocardium after Large Myocardial Infarction in Rats." Biochemical and Biophysical Research Communications **246**(3): 816-820.
Liang, Q., O. F. Bueno, et al. (2003). "c-Jun N-terminal kinases (JNK) antagonize cardiac growth through cross-talk with calcineurin-NFAT signaling." Embo J **22**(19): 5079-5089.
Lin, F., W. A. Owens, et al. (2001). "Targeted alpha(1A)-adrenergic receptor overexpression induces enhanced cardiac contractility but not hypertrophy." Circ Res **89**(4): 343-350.
Ling, H., T. Zhang, et al. (2009). "Requirement for Ca2+/calmodulin–dependent kinase II in the transition from pressure overload–induced cardiac hypertrophy to heart failure in mice." The Journal of Clinical Investigation **119**(5): 1230-1240.
Liu, W., M. Zi, et al. (2009). "Cardiac-Specific Deletion of Mkk4 Reveals Its Role in Pathological Hypertrophic Remodeling but Not in Physiological Cardiac Growth." Circ Res **104**(7): 905-914.
Luo, J., J. R. McMullen, et al. (2005). "Class IA phosphoinositide 3-kinase regulates heart size and physiological cardiac hypertrophy." Mol Cell Biol **25**(21): 9491-9502.
Mao, Y., J. Mulvaney, et al. (2011). "Characterization of a Dchs1 mutant mouse reveals requirements for Dchs1-Fat4 signaling during mammalian development." Development **138**(5): 947-957.
Matsuda, T., P. Zhai, et al. (2008). "Distinct roles of GSK-3α and GSK-3β phosphorylation in the heart under pressure overload." Proceedings of the National Academy of Sciences **105**(52): 20900-20905.
Matsui, T., L. Li, et al. (2002). "Phenotypic spectrum caused by transgenic overexpression of activated Akt in the heart." J Biol Chem **277**(25): 22896-22901.
McMullen, J. R., F. Amirahmadi, et al. (2007). "Protective effects of exercise and phosphoinositide 3-kinase(p110α) signaling in dilated and hypertrophic

cardiomyopathy." Proceedings of the National Academy of Sciences **104**(2): 612-617.
McMullen, J. R., T. Shioi, et al. (2004). "The insulin-like growth factor 1 receptor induces physiological heart growth via the phosphoinositide 3-kinase(p110alpha) pathway." J Biol Chem **279**(6): 4782-4793.
McMullen, J. R., T. Shioi, et al. (2003). "Phosphoinositide 3-kinase(p110α) plays a critical role for the induction of physiological, but not pathological, cardiac hypertrophy." Proceedings of the National Academy of Sciences **100**(21): 12355-12360.
Mendoza, M. C., E. E. Er, et al. (2011). "The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation." Trends in Biochemical Sciences **36**(6): 320-328.
Milano, C. A., P. C. Dolber, et al. (1994). "Myocardial expression of a constitutively active alpha 1B-adrenergic receptor in transgenic mice induces cardiac hypertrophy." Proc Natl Acad Sci U S A **91**(21): 10109-10113.
Molkentin, J. D., J.-R. Lu, et al. (1998). "A Calcineurin-Dependent Transcriptional Pathway for Cardiac Hypertrophy." Cell **93**(2): 215-228.
Mozdziak, P. E. and J. N. Petitte (2004). "Status of transgenic chicken models for developmental biology." Developmental Dynamics **229**(3): 414-421.
Naga Prasad, S. V., S. A. Laporte, et al. (2002). "Phosphoinositide 3-kinase regulates β2-adrenergic receptor endocytosis by AP-2 recruitment to the receptor/β-arrestin complex." J Cell Biol **158**(3): 563-575.
Negoro, S., K. Kunisada, et al. (2000). "Activation of JAK/STAT pathway transduces cytoprotective signal in rat acute myocardial infarction." Cardiovascular Research **47**(4): 797-805.
Neri Serneri, G. G., M. Boddi, et al. (2001). "Increased Cardiac Sympathetic Activity and Insulin-Like Growth Factor-I Formation Are Associated With Physiological Hypertrophy in Athletes." Circ Res **89**(11): 977-982.
Ng, W. A., I. L. Grupp, et al. (1991). "Cardiac myosin heavy chain mRNA expression and myocardial function in the mouse heart." Circ Res **68**(6): 1742-1750.
Nienaber, J. J., H. Tachibana, et al. (2003). "Inhibition of receptor-localized PI3K preserves cardiac β-adrenergic receptor function and ameliorates pressure overload heart failure." The Journal of Clinical Investigation **112**(7): 1067-1079.
Nishida, K., O. Yamaguchi, et al. (2004). "p38α Mitogen-Activated Protein Kinase Plays a Critical Role in Cardiomyocyte Survival but Not in Cardiac Hypertrophic Growth in Response to Pressure Overload." Mol Cell Biol **24**(24): 10611-10620.
Noga, A. A., C.-L. M. Soltys, et al. (2007). Expression of an active LKB1 complex in cardiac myocytes results in decreased protein synthesis associated with phenylephrine-induced hypertrophy. Am J Physiol Heart Circ Physiol. **292:** H1460-H1469.
O'Connell, T. D., S. Ishizaka, et al. (2003). "The α1A/C- and α1B-adrenergic receptors are required for physiological cardiac hypertrophy in the double-knockout mouse." The Journal of Clinical Investigation **111**(11): 1783-1791.
O'Connell, T. D., P. M. Swigart, et al. (2006). "α1-Adrenergic receptors prevent a maladaptive cardiac response to pressure overload." The Journal of Clinical Investigation **116**(4): 1005-1015.
Oberpriller, J. O. and J. C. Oberpriller (1974). "Response of the adult newt ventricle to injury." J Exp Zool **187**(2): 249-253.
Oliveira, R. S. F., J. C. B. Ferreira, et al. (2009). "Cardiac anti-remodelling effect of aerobic training is associated with a reduction in the calcineurin/NFAT signalling pathway in heart failure mice." The Journal of Physiology **587**(15): 3899-3910.
Oudit, G. Y., M. A. Crackower, et al. (2003). "Phosphoinositide 3-Kinase γ–Deficient Mice Are Protected From Isoproterenol-Induced Heart Failure." Circulation **108**(17): 2147-2152.
Pan, D. (2007). "Hippo signaling in organ size control." Genes Dev **21**(8): 886-897.

Pan, J., K. Fukuda, et al. (1997). "Role of Angiotensin II in Activation of the JAK/STAT Pathway Induced by Acute Pressure Overload in the Rat Heart." Circ Res **81**(4): 611-617.
Patrucco, E., A. Notte, et al. (2004). "PI3Kγ Modulates the Cardiac Response to Chronic Pressure Overload by Distinct Kinase-Dependent and -Independent Effects." Cell **118**(3): 375-387.
Perrino, C., S. V. Naga Prasad, et al. (2005). "Restoration of β-Adrenergic Receptor Signaling and Contractile Function in Heart Failure by Disruption of the βARK1/Phosphoinositide 3-Kinase Complex." Circulation **111**(20): 2579-2587.
Perrino, C., S. V. N. Prasad, et al. (2006). "Intermittent pressure overload triggers hypertrophy-independent cardiac dysfunction and vascular rarefaction." The Journal of Clinical Investigation **116**(6): 1547-1560.
Perrino, C., J. N. Schroder, et al. (2007). "Dynamic Regulation of Phosphoinositide 3-Kinase-γ Activity and β-Adrenergic Receptor Trafficking in End-Stage Human Heart Failure." Circulation **116**(22): 2571-2579.
Pete, G., C. R. Fuller, et al. (1999). "Postnatal growth responses to insulin-like growth factor I in insulin receptor substrate-1-deficient mice." Endocrinology **140**(12): 5478-5487.
Peterson, C., V. Whitman, et al. (1989). "Mechanisms of differential growth of heart ventricles in newborn pigs." Circ Res **64**(2): 360-369.
Porrello, E. R., A. I. Mahmoud, et al. (2011). "Transient Regenerative Potential of the Neonatal Mouse Heart." Science **331**(6020): 1078-1080.
Poss, K. D., L. G. Wilson, et al. (2002). "Heart Regeneration in Zebrafish." Science **298**(5601): 2188-2190.
Reiss, K., W. Cheng, et al. (1996). "Overexpression of insulin-like growth factor-1 in the heart is coupled with myocyte proliferation in transgenic mice." Proc Natl Acad Sci U S A **93**(16): 8630-8635.
Rigor, D. L., N. Bodyak, et al. (2009). "Phosphoinositide 3-kinase Akt signaling pathway interacts with protein kinase Cbeta2 in the regulation of physiologic developmental hypertrophy and heart function." Am J Physiol Heart Circ Physiol **296**(3): H566-572.
Rokosh, D. G. and P. C. Simpson (2002). "Knockout of the alpha 1A/C-adrenergic receptor subtype: the alpha 1A/C is expressed in resistance arteries and is required to maintain arterial blood pressure." Proc Natl Acad Sci U S A **99**(14): 9474-9479.
Sadoshima, J., O. Montagne, et al. (2002). "The MEKK1-JNK pathway plays a protective role in pressure overload but does not mediate cardiac hypertrophy." The Journal of Clinical Investigation **110**(2): 271-279.
Sato, K., M. Aoki, et al. (2012). "Administration of insulin to newly hatched chicks improves growth performance via impairment of MyoD gene expression and enhancement of cell proliferation in chicken myoblasts." General and Comparative Endocrinology **175**(3): 457-463.
Scheinowitz, M., G. Kessler-Icekson, et al. (2003). "Short- and long-term swimming exercise training increases myocardial insulin-like growth factor-I gene expression." Growth Hormone & IGF Research **13**(1): 19-25.
Seki, S., M. Nagai, et al. (2003). "Impaired Ca2+ Handling in Perfused Hypertrophic Hearts from Dahl Salt-Sensitive Rats." Hypertension Research **26**(8): 643-653.
Shen, W. H., Z. Chen, et al. (2008). "Cardiac restricted overexpression of kinase-dead mammalian target of rapamycin (mTOR) mutant impairs the mTOR-mediated signaling and cardiac function." J Biol Chem **283**(20): 13842-13849.
Shimoyama, M., D. Hayashi, et al. (1999). "Calcineurin Plays a Critical Role in Pressure Overload–Induced Cardiac Hypertrophy." Circulation **100**(24): 2449-2454.
Shioi, T., P. M. Kang, et al. (2000). "The conserved phosphoinositide 3-kinase pathway determines heart size in mice." Embo J **19**(11): 2537-2548.

Shioi, T., J. R. McMullen, et al. (2002). "Akt/protein kinase B promotes organ growth in transgenic mice." Mol Cell Biol **22**(8): 2799-2809.
Shiojima, I., M. Yefremashvili, et al. (2002). "Akt signaling mediates postnatal heart growth in response to insulin and nutritional status." J Biol Chem **277**(40): 37670-37677.
Smolich, J. J., A. M. Walker, et al. (1989). "Left and right ventricular myocardial morphometry in fetal, neonatal, and adult sheep." Am J Physiol **257**(1 Pt 2): H1-9.
Soonpaa, M. H. and L. J. Field (1998). "Survey of studies examining mammalian cardiomyocyte DNA synthesis." Circ Res **83**(1): 15-26.
Sugden, P. H. and A. Clerk (1997). "Regulation of the ERK Subgroup of MAP Kinase Cascades Through G Protein-Coupled Receptors." Cellular Signalling **9**(5): 337-351.
Tachibana, H., C. Perrino, et al. (2006). "JNK1 is required to preserve cardiac function in the early response to pressure overload." Biochemical and Biophysical Research Communications **343**(4): 1060-1066.
Taigen, T., L. J. De Windt, et al. (2000). "Targeted inhibition of calcineurin prevents agonist-induced cardiomyocyte hypertrophy." Proceedings of the National Academy of Sciences **97**(3): 1196-1201.
Thommes, R. C. and V. W. Hylka (1977). "Plasma iodothyronines in the embryonic and immediate post-hatch chick." Gen Comp Endocrinol **32**(4): 417-422.
Uozumi, H., Y. Hiroi, et al. (2001). "gp130 Plays a Critical Role in Pressure Overload-induced Cardiac Hypertrophy." Journal of Biological Chemistry **276**(25): 23115-23119.
Weeks, K. L. and J. R. McMullen (2011). "The Athlete's Heart vs. the Failing Heart: Can Signaling Explain the Two Distinct Outcomes?" Physiology **26**(2): 97-105.
Wehrens, X. H., S. E. Lehnart, et al. (2004). "Ca2+/calmodulin-dependent protein kinase II phosphorylation regulates the cardiac ryanodine receptor." Circ Res **94**(6): e61-70.
Wilkins, B. J., Y.-S. Dai, et al. (2004). "Calcineurin/NFAT Coupling Participates in Pathological, but not Physiological, Cardiac Hypertrophy." Circ Res **94**(1): 110-118.
Wu, S., J. Huang, et al. (2003). "hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts." Cell **114**(4): 445-456.
Xin, M., Y. Kim, et al. (2011). "Regulation of insulin-like growth factor signaling by Yap governs cardiomyocyte proliferation and embryonic heart size." Sci Signal **4**(196): ra70.
Yin, M. and L. Zhang (2011). "Hippo signaling: A hub of growth control, tumor suppression and pluripotency maintenance." Journal of Genetics and Genomics **38**(10): 471-481.
Zak, R. (1974). "Development and proliferative capacity of cardiac muscle cells." Circ Res **35**(2): suppl II:17-26.
Zhai, P., S. Gao, et al. (2007). "Glycogen Synthase Kinase-3α Reduces Cardiac Growth and Pressure Overload-induced Cardiac Hypertrophy by Inhibition of Extracellular Signal-regulated Kinases." Journal of Biological Chemistry **282**(45): 33181-33191.
Zhang, Y., S. A. Shafiq, et al. (1986). "Detection of a ventricular-specific myosin heavy chain in adult and developing chicken heart." J Cell Biol **102**(4): 1480-1484.
Zhao, B., X. Wei, et al. (2007). "Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control." Genes Dev **21**(21): 2747-2761.
Zou, Y., Y. Hiroi, et al. (2001). "Calcineurin Plays a Critical Role in the Development of Pressure Overload–Induced Cardiac Hypertrophy." Circulation **104**(1): 97-101.