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Chapter 1

General introduction
Development of the different muscle types

Muscles can be broadly divided into three types: heart, skeletal and smooth muscle. Their common features include contractility and the presence of sarcomeres, but they differ significantly in their developmental origin and functional characteristics. This thesis concerns a novel protein important for contractile function in all muscle cell types.

Development of the heart
The heart is the first functional organ in the embryo. In mammals and birds the cardiac crescent, the first distinct cardiac structure, is formed from cardiac precursor cells that originate from the anterior lateral plate mesoderm. The cells in the cardiac crescent are specified by multiple inhibiting and inducing signals which result in a defined area of cardiac cells, known as the first heart field. Inhibitory canonical Wnt signals (Wnt3a and Wnt8) from the ectoderm and Noggin and Chordin (bone morphogenetic protein (BMP) antagonists) from the notochord prevent formation of cardiac mesoderm. On the other hand, cardiac mesoderm is induced by signals activated by BMP2, fibroblast growth factor 8 and sonic hedgehog homolog (Ssh) secreted from the pharyngeal endoderm. Fusion of the cardiac crescent at the midline of the embryo results in the formation of a linear heart tube that shows polarity at the outset, with anterior (ventricular) and posterior (atrial) specification. In all vertebrates the linear heart undergoes rightward looping and in higher vertebrates, the looped heart subsequently undergoes septation at the level of the atrium, ventricle and arterial pole. At embryonic day 8 (E8) in the mouse embryo and Hamburger-Hamilton (HH) stage 24 in the chick embryo, new myocardium is added to the outflow tract of the heart. These cells are derived from the second heart field (SHF), which is located in the pharyngeal mesoderm (Figure 1). The first heart field (FHF) gives rise to the linear heart tube, definitive left ventricle and part of the inflow region of the heart, whereas the second heart field (SHF) gives rise to right ventricular and outflow tract myocardium and part of the inflow region.

Skeletal muscle development
In vertebrates, muscles are formed from the paraxial mesoderm, which segments into somites. The ventral part of the somite, the sclerotome, gives rise to the cartilage and bone of the vertebral column and ribs, whereas the dorsal part of the somite, the dermomyotome, give rise to skin and muscles of the trunk and the limbs. The dermomyotome can be divided into epaxial and hypaxial parts (Figure 2), which give rise to different muscle groups and
differ in the way myogenesis is induced. The epaxial part is located adjacent to the neural tube and notochord and will form the muscles of the back. The hypaxial part forms the muscles of the limbs, body wall and tongue. In the epithelium of the hypaxial dermomyotome, that is located opposite to the limb buds, muscle progenitor cells delaminate and migrate into the limb buds, forming the muscles of the limbs\(^6,8\). Myogenesis is regulated largely by the MyoD family of basic helix-loop-helix factors\(^6,8\). In the epaxial part, paired homeobox gene 3 (Pax3), myogenic factor (Myf) 5 and 6 induce myogenic differentiation 1 (MyoD1). In the hypaxial part, on the other hand, Pax3 induces MyoD indirect, via activation of Myf5 expression (see figure 2). These signaling cascades are induced by Shh from the ventral neural tube and notochord and (non-)canonical Wnt signals from the neural tube (Figure 2). Two phases of myogenesis can be distinguished. The primary myotome is formed from the dermomyotome. The epithelium of the dermomyotome is formed as a central sheet that is surrounded by four lips. During the first myogenic phase, cells of the four epithelial lips of the dermomyotome delaminate and locate between the dermomyotome and sclerotome to form the primary myotome\(^7\) (primary fibers); this occurs between E11 and E14 in the mouse\(^6\). In the second myogenic phase the central part of the dermomyotome epithelializes, resulting in a second phase of muscle formation\(^7\) (secondary fibers), which occurs between E14 and E16 in the mouse\(^6\). Generally speaking, the primary fibers tend to become slow fibers, whereas the secondary fibers become fast fibers\(^6\). In the postnatal period the muscle mass increases by hypertrophy (growth of individual cells) of existing muscle fibers\(^8\).

Figure 2: Schematic overview of myogenic induction in the mouse. In the epixial part myogenic differentiation 1 (MyoD1) is induced by synergetic action of paired box gene 3 (Pax3), myogenic factor (Myf) 5 and 6, which are induced by Sonic hedgehog and Wnt1 from the notochord and neural tube, respectively. In the hypaxial part, on the other hand, Pax3 induces Myf5 and 6, which in turn induces MyoD1.

Adult myogenesis is achieved by activation of quiescent satellite cells, which are marked by expression of Pax7, c-met proto-oncogene and M-cadherin. After muscle injury, these cells commit to a muscle-progenitor fate and activate transcription factors such as Pax3 and Myf5\(^6,7\), leading to differentiation of skeletal muscle cells. The satellite cells only have limited capacity of self-renewal, therefore skeletal muscle degenerates under pathological conditions\(^6\).
Vascular smooth muscle cell differentiation

Vascular smooth muscle cells (VSMCs) have different embryonic origins which are reflected in their distribution in different tissues and organs. The walls of the great arteries (pulmonary artery, dorsal aorta, carotid and subclavian arteries) for example are derived from progenitor cells that migrate from the dorsal surface of the neural tube (neural crest) into the pharyngeal arch complex between E8.5 and 9.5 in the mouse. VSMCs of the coronary arteries arise from the epicardium, which forms during embryonic development from pro-epicardial cells (pro-epicardial organ) and grow over the surface of the myocardium to form a single layer of epicardial cells. Between E13.5 and E14.5 some of these cells undergo epithelial-to-mesenchymal transition and form the VSMCs of the coronary arteries. A third source of VSMC progenitors is an Islet-1 positive cell population, which produces the VSMC in the walls of the pulmonary trunk, aortic root and the branching coronary arteries.

Development of VSMCs from embryonic progenitors is regulated in three phases. During the first phase, vasculogenesis occurs and angioblasts differentiate into endothelial cells that assemble in a capillary vascular network. In the second phase (E10.5 in the mouse), increased cardiac output demands a more active vascular network, which leads to the production of chemo-attractants by the endothelial cells, this in turn attracts progenitors of VSMCs, the mesenchymal cells, to surround the vessel wall. Close contact between mesenchymal cells and endothelial cells, mediated by platelet derived growth factor, initiates SMC differentiation. In the last phase the cells further mature and form the vascular extracellular matrix.

Differentiated VSMCs are defined by expression of a specific subset of cytoskeletal and contractile proteins, such as α-smooth muscle actin (ASMA), smooth muscle calponin and smooth myosin heavy chain (MHC), SM22-α, desmin and smoothelin. The function of mature differentiated VSMCs is contraction, regulation of vascular tone, important for the regulation of blood pressure and blood flow distribution. Adult VSMCs proliferate very slowly, have a very low synthetic activity (low production of extracellular matrix) and are non-migratory. However, unlike cardiac and skeletal muscle cells, adult SMCs can undergo reversible changes in phenotype between a contractile and synthetic state in response to changes in the local environment. For example, after vascular injury contractile SMCs are capable of undergoing a transient modification of their contractile phenotype to a synthetic phenotype and in this way play an essential role in the repair of the vascular injury by producing extracellular matrix.

Sarcomeres: contracting units of striated muscle and function in cardiac disease

In adult animals these three types of muscle cells can be recognized: heart, skeletal muscle and smooth muscle. In heart and skeletal muscle cells are ‘striated’, a striped pattern visible microscopically made up of sarcomeres. In these muscles sarcomeres form the basis for contraction. The function of sarcomeres in healthy and diseased muscle is discussed in the following section.

Structure of sarcomeres

Sarcomeres are multi-protein complexes responsible for the contraction of striated muscle. Contraction is regulated by two filament systems, the thin actin and the thick myosin filaments, that slide over each other. The sarcomere is a highly organized structure which can be divided
into several subcompartments. The Z-discs form the boundary of each sarcomere, the I-band (for isotropic) surrounds the Z-disc and is composed of only actin filaments, the A-band (for anisotropic) is composed of actin and myosin filaments and the M-band (middle) forms the centre of each sarcomere (Figure 3)\textsuperscript{12, 13}.

Protein composition of the sarcomere is determined by the specific muscle type. In the heart the giant protein titin is the structural backbone of the sarcomere and two of these molecules run from Z-disc to Z-disc. Titin determines the stiffness of the sarcomere and keeps the A-band in the middle of the sarcomere during contraction. Via T-cap, titin is connected to α-actinin\textsuperscript{14, 15}, the major component of the Z-disc. There are four α-actinin isoforms (1-4), of which α-actinin-2 and -3 are the only isoforms expressed in striated muscle. In cardiac tissue only α-actinin-2 is expressed. In the Z-disc, α-actinin-2 is organized as homodimers in an antiparallel manner and in this way cross-links actin\textsuperscript{16}. Furthermore, α-actinin-2 functions in binding several proteins that are involved in stretch-sensor function of the Z-disc\textsuperscript{12}. This will be discussed in more detail later in this chapter.

Actin is a ubiquitously expressed protein important in many fundamental processes, such as cell cycle regulation, cell motility and muscle contraction. In striated muscle, two actin forms are present, cardiac actin and skeletal actin. In adult cardiomyocytes cardiac actin is the major isoform present\textsuperscript{16}. Via CapZ, a protein composed of α and β subunit, actin filaments are connected to α-actinin-2, linking the thin filaments to the Z-disc\textsuperscript{12, 16}. Mutations in sarcomeric proteins can lead to the development of cardiomyopathy.

Figure 3: Schematic overview of a sarcomere. The Z-disc forms the boundary of the sarcomere and is composed of α-actinin homodimers that bind several signaling molecules (see text). The I-band is composed of only actin filaments and the A-band is composed of actin and myosin filaments. The M-band forms the middle of the sarcomere. Adapted from\textsuperscript{13}.

**Mutations in sarcomeric proteins result in cardiomyopathy**

Cardiomyopathy is a group of disorders of the heart that result in inadequate pumping of blood around the body. Hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) are mainly caused by mutations in sarcomeric proteins. HCM is characterized by left ventricular hypertrophy (resulting from increased cell size or volume), increased systolic function and decreased diastolic function. At the histological level, hypertrophy and disarray of cardiomyocytes and interstitial fibrosis is observed\textsuperscript{17, 18}. DCM, on the other hand, is characterized by ventricular dilation and contractile dysfunction of the left and/or right ventricles. Depending on the location of the mutation, HCM or DCM is induced; in HCM regions of sarcomeric proteins are mutated that are directly involved in force generation,
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whereas the regions that are mutated in DCM are involved in force transmission from the sarcomere to the extrasarcomeric cytoskeleton.

Several mouse models for HCM and DMC have been generated, which reflect the pathology of the human disease. For example in a mutant mouse with a truncation in myosin binding protein C (MyBP-C), a mild HCM phenotype was observed, while a mutation (R403Q) in α-MHC resulted in a more severe HCM phenotype. Mouse models with mutations in cardiac troponin T (cTnT) at different domains, R92Q (transgenic) and ΔK210 (knock-out), resulted in HCM and DCM, respectively.

Several mouse models for HCM and DCM have been reviewed in Beqqali et al and are summarized in table 1.

Table 1: Overview of animal models of hypertrophic and dilated cardiomyopathy. Adapted from 24.

<table>
<thead>
<tr>
<th>Disease model</th>
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<th>Mutation</th>
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The Z-disc as stretch-sensor

α-actinin-2 anchors several proteins to the Z-disc, which act as stretch sensor. Several Z-disc proteins have been associated with cardiomyopathies, for example interacting proteins of α-actinin-2 such as calcineurin, muscle-specific LIM protein (MLP), T-cap, calcarin-1, cypher and Enigma homologue protein (ENH). MLP has been found to be an essential component of the T-cap and titin complex, stabilizing the interaction between these proteins, which can then sense increase in stretch and activate downstream signals for hypertrophy.

The phosphatase calcineurin, a heterotrimer consisting of a catalytic subunit A (CnA), calcineurin B and calmodulin, is a key regulator of hypertrophy. In the calcineurin pathway hypertrophic stimuli lead to an elevation in the intracellular Ca2+ levels and subsequent activation of calcineurin. Activated calcineurin dephosphorylates nuclear factor of activated T-cells (NFAT), which then translocates to the nucleus to activate genes that are associated with stress, such as atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP), and genes that encode fetal isoforms of contractile proteins such as β-myosin heavy chain (β-MHC). Molketin et al. generated transgenic (Tg) mice that express a constitutively active form of the catalytic subunit of calcineurin in the heart, by using of the α-MHC promoter to drive the calcineurin construct. Hearts of calcineurin Tg mice show massive hypertrophy, disorganized cardiomyocytes, fibrosis and enlarged nuclei. Furthermore, with ageing calcineurin Tg mice show dilatation of the ventricles and are highly susceptible to sudden cardiac death. NFATc2 and NFATc3 are essential mediators of the calcineurin induced hypertrophy, which is reduced in NFATc2−/− or NFATc3−/− mice. However, NFATc4−/− mice do not exhibit reduced calcineurin induced hypertrophy, suggesting that NFATc4 does not play an important role in the calcineurin-dependent hypertrophy pathway.

Calsarcin, a novel target for calcineurin, binds to both calcineurin and α-actinin. Overexpression of Calsarcin-1, both in vitro and in vivo, has protective effects on hypertrophy by interfering with calcineurin-NFAT signaling, while lack of calsarcin-1 enhances this signaling pathway.

In addition to the interaction with calsarcin and calcineurin, α-actinin has also been shown to interact with several PDZ-LIM proteins. Cypher (or Oracle/ZASP) is expressed in heart and
skeletal muscle during mouse and zebrafish development\textsuperscript{44-46}, and interacts with α-actinin-2\textsuperscript{44} and calsarcin\textsuperscript{47}. Deletion of cypher in mice and zebrafish results in disruption of the Z-disc structure and leads to the development of DCM\textsuperscript{46-48}. However, in mice only deletion of the long isoform of cypher leads to development of late-onset DCM, whereas deletion of the short isoform has no effect\textsuperscript{49}. More interestingly, mutations in cypher have been identified in humans that cause DCM\textsuperscript{50}. These results indicate cypher as an essential component of the α-actinin-calcineurin-calsarcin complex to maintain cardiac structure and function.

ENH is another PDZ-LIM protein which interacts with α-actinin-2\textsuperscript{51}, calsarcin and cypher. In ENH\textsuperscript{-/-} mice expression of the short cypher isoform and calsarcin is lost, which leads to disruption of the Z-disc and development of DCM\textsuperscript{52}.

These data show an essential role for the Z-disc in the development of cardiomyopathies and targeting these components could reveal strategies for possible new therapies for the treatment of these diseases. Therefore, it will be necessary ultimately to identify all Z-disc components and in this way, increase understanding of Z-disc function in the development of cardiomyopathies. Work in this thesis has identified one new protein that is part of the Z-disc complex.

\section*{Actin signaling}

\textit{Regulation of cardiac development and disease via actin signaling pathway}

The actin cytoskeleton is important in regulation cell shape changes and gene expression during development and disease. Polymerization of monomeric globular actin (G-actin) in filamentous actin (F-actin) is regulated by several actin binding proteins\textsuperscript{53}. Rho GTPases (RhoA, Rac and Cdc42) induce actin polymerization via the Rho-associated kinase (ROCK)-LIM kinase-cofilin pathway\textsuperscript{53-56}. RhoA can be induced by phosphorylation of ezrin/radixin/moesin (ERM), proteins that function in linking the membrane to the actin cytoskeleton\textsuperscript{57}. RhoA is highly expressed in embryonic hearts, downregulated in adults and re-expressed during cardiac hypertrophy\textsuperscript{58}. Heart-specific overexpression of RhoA leads to the development of a DCM-phenotype\textsuperscript{59}, whereas inhibiting ROCK protects the heart after aorta constriction\textsuperscript{60}.

Serum response factor (SRF) is a member of the MADS (\textit{MCM1}, \textit{Agamous}, \textit{Deficiens}, SRF) family of transcription factors, which share a conserved motif of 57 amino acids mediating homodimerization, association with other factors and DNA-binding activity\textsuperscript{61}. SRF is expressed in many cell types and functions in many biological processes, including gastrulation, development and function of the cardiovascular system, T-cell and B-cell activities in the immune system and neuronal functions of the developing and adult brain. Homodimeric SRF binds to a palindromic DNA sequence (CC(A/T)\textsubscript{6}GG), which is called the CArG box\textsuperscript{53}.

Several SRF-mouse models have shown that SRF is essential for cardiac development, as well as maintaining adult cardiomyocytes. SRF is responsible for maintaining the sarcomeric integrity in cardiomyocytes\textsuperscript{62-64} and disruption of its expression results in DCM in mice\textsuperscript{65, 66}. Therefore, it is not surprising to find several sarcomeric genes, such as skeletal and cardiac actin, α-MHC and β-MHC, myosin light chain (MLC)\textsubscript{2a} and 2\textsubscript{v}, myomesin, troponin C, and titin, to be targets of SRF\textsuperscript{62}.

Myocardin and myocardin related transcription factors (MRTFs) function as co-activators of SRF resulting co-activator specific gene transcription determining skeletal and smooth muscle
differentiation. Furthermore, these factors also have distinct functions in pathophysiological remodeling of the heart\textsuperscript{61}.

Myocardin, MRTF-A and -B share a conserved so-called RPEL domain, which in MRTFs functions as G-actin binding domain. In myocardin, the RPEL domain does not bind G-actin, therefore myocardin is localized exclusively in the nucleus\textsuperscript{61}. Myocardin is essential for smooth muscle differentiation and activates expression of smooth muscle genes in promoters with two adjacent CArG boxes by binding these as homodimers\textsuperscript{67}. Tracing studies show that myocardin specifically marks cardiac, smooth and skeletal muscle lineages during embryonic development. In skeletal muscle precursors, myocardin expression leads to inhibition of differentiation, whereas in smooth muscle cell precursors it induces differentiation\textsuperscript{68}. Myocardin\textsuperscript{-/-} mice die around E10.5 and these mutant embryos show vascular abnormalities which result from impaired smooth muscle cell differentiation\textsuperscript{69, 70}. Cardiac specific deletion (α-MHC-Cre) of myocardin leads to decreased expression of sarcomeric genes (α-cardiac actin, MLC2v, and tropomyosin) and connexin 43 and desmin, which results in disruption of the structural organization of the sarcomere and intercalated discs, respectively. Furthermore, the hearts of α-MHC-Cre-myocardin\textsuperscript{+/-} showed left atrial enlargement, hypertrophy, interstitial fibrosis and a decreased cardiac function (decreased ejection fraction)\textsuperscript{71}. Myocardin also has an essential function during pathophysiological events in the heart; it is upregulated in several \textit{in vivo} and \textit{in vitro} models of hypertrophy, as well as in human patient hearts with idiopathic DCM. The hypertrophic marker ANF is a direct target of myocardin and over-expression of myocardin in cardiomyocytes \textit{in vitro} leads to a hypertrophic response, which can be abolished by expression of a dominant negative isoform lacking the transcriptional activation domain\textsuperscript{72}.

MRTF-A\textsuperscript{-/-} mice are viable and show no cardiac defects. Female MRTF-A\textsuperscript{-/-} mice are unable to nurse their offspring effectively, due to a failure in myoepithelial cell differentiation. These are cells that posses characteristics of both epithelial and SMC and surround the milk producing cells giving structural and contractile support\textsuperscript{73}. Deletion of MRTF-A in the heart has a protective effect on the heart and shows that MRTF-A has a role in controlling the expression of a fibrotic gene program and smooth muscle cell differentiation. Scar formation and fibrosis are decreased in MRTF-A\textsuperscript{-/-} mice after myocardial infarction or Angiotensin II treatment\textsuperscript{74}. MRTF-B is expressed in derivatives of the neural crest. Therefore, MRTF-B knockout mice show cardiovascular abnormalities, resulting from impaired smooth muscle cell differentiation\textsuperscript{75, 76}.

Myocyte Enhancer Factor-2 (MEF2) is another MADS-box transcription factor. Four isoforms of MEF2 exist (A-D), of which isoforms A, C and D have distinct functions in the heart. Knockout studies show that MEF2A is essential for energy metabolism and sarcomeric organization in adult hearts\textsuperscript{77}, MEF2C is involved in cardiac morphogenesis \textsuperscript{78}, and MEF2D is a mediator of stress-dependent pathological remodeling of the adult heart\textsuperscript{79}. On the other hand, overexpression of these factors induce (dilated) cardiomyopathy in mice\textsuperscript{80, 81}.

Transcriptional activity of SRF is regulated by \textit{Striated Muscle Activator of Rho Signaling} (STARS). STARS binds to actin and activates RhoA in this way promoting actin polymerization and depletion of the G-actin pool. Subsequently, MRTFs translocate to the nucleus and activate gene expression by acting as co-factor for SRF. SRF induces actin genes, in this way providing a negative feedback loop\textsuperscript{82, 83}. STARS is a direct target of MEF2, STARS expression is induced by MEF2 in several models of hypertrophy\textsuperscript{84}. Thus, the formation of actin bundles is not only necessary for cell movement and polarization, but also leads to the induction of signal-transduction pathways important for cardiac development and hypertrophy. The actin
signaling pathway is summarized in figure 4.

![Diagram](image-url)

**Figure 4: The actin signaling pathway.** Rho is activated by Striated Muscle Activator of Rho (STARS) which leads to polymerization of G-actin into F-actin and subsequent deletion of the G-actin pool. Myocardin Related Transcription Factors (MRTFs), normally inhibited by G-actin, translocate to the nucleus to act as transcriptional co-activator of Serum Response Factor (SRF). Actin is a target of SRF, in this way providing a negative feedback loop mechanism.

**The synaptopodin gene family: role in actin polymerization**

Proteins belonging to the synaptopodin gene family are involved in actin signaling. Synaptopodin is the founding member of the synaptopodin gene family. Three isoforms (of one gene) of synaptopodin exist: Synpo-short (685 aa) expressed in the brain, Synpo-long (903 aa) and Synpo-T (181 aa) which are both expressed in kidney podocytes. Both Synpo-long and Synpo-short interact with α-actinin-2 and -4 resulting in elongation and bundling of actin filaments, which is regulated by preventing proteasomal degradation of RhoA.

Synaptopodin−/− mice lack ‘spine apparatus’, an organelle present in dendritic spines, which leads to defects in behavior. The structure and function of kidney podocytes is not affected by decreased synaptopodin expression. This can be explained by the fact that in the brain of Synaptopodin−/− mice α-actinin-2 is downregulated, resulting in a reduction in actin filament formation. In contrast, kidney podocytes of Synaptopodin−/− mice upregulate Synpo-T and therefore have normal levels of α-actinin-4, resulting in rescue of actin filament formation.

Myopodin is the second member of the synaptopodin gene family, and has actin binding activity as well. It is expressed in adult heart, skeletal muscle and smooth muscle cells. Myopodin localizes at the Z-disc of skeletal muscle cells and cardiomyocytes and can translocate to the nucleus. Myopodin forms a protein complex with α-actinin, calcineurin, protein kinase A (PKA) and calmodulin-dependent kinase II (CaMKII). Activation of PKA and CaMKII or inhibition of calcineurin results in phosphorylation of myopodin and its
subsequent translocation to the nucleus\textsuperscript{90}.

**CHAP is a new member of the synaptopodin gene family**

Micro-array analysis of gene expression technique is a powerful tool to discover new genes associated with specific biological processes. Using an assay in which human embryonic stem cells (hESC) were differentiated to cardiomyocytes, we discovered a new cardiac enriched gene, which was homologous to synaptopodin and myopodin. This new gene was annotated as Synpatopodin-2-like (SYNPO2L)\textsuperscript{91} and we renamed it later Cytoskeletal Heart-enriched Actin-associated Protein (CHAP). Mouse CHAP is located on chromosome 14 and exists as two isoforms: a long isoform CHAPa (978 aa) and a shorter isoform CHAPb (749 aa), produced by an alternative ATG site at the beginning of exon 4. The CHAPa and b isoforms are almost identical, with a nuclear localization signal (NLS) and actin binding sites. However, the N-terminal of CHAPa is longer and contains a PDZ domain.

During development, *ChapB* is first expressed at the cardiac crescent stage (E7.75), later in the linear heart tube and in the looped heart. Furthermore, from E10 onward expression is also detected in the somites. Expression of *ChapB* is downregulated in adult hearts, whereas *ChapA* is expressed adult heart and skeletal muscle (Figure 5)\textsuperscript{92}.

In cardiomyocytes, CHAP is detected in the Z-disc and co-stains with α-actinin-2, whereas

![Figure 5: Whole mount in situ hybridization (A-D) showing expression of *Chap* during mouse development. *Chap* is first expressed in the cardiac crescent (cc) at E7.75 (A). In later stages *Chap* expression is observed throughout the whole heart at E8.5 (B) and E9.5 (C). At E10 (D), besides expression of *Chap* in the heart, *Chap* expression observed in the somites. E: Section of an E8.5 mouse embryo showing *Chap* expression in cardiomyocytes of the heart. F: Section of an E10.5 mouse embryo showing *Chap* is expressed in the myotome part of the somites. G: RT-PCR analysis of showing expression of *ChapA* in adult heart and skeletal muscle, and expression of *ChapB* in the heart during embryonic development. H: western blot analysis of CHAPa and b expression during embryonic development and in adult heart and skeletal muscle. Adapted from\textsuperscript{92}.](image-url)
no co-staining is seen with the M-band marker myomesin. Furthermore, CHAP can interact directly with α-actinin-2. CHAP can also translocate to the nucleus, suggesting a function in transcription as a co-factor.

Morpholino mediated knockdown of *chap* in zebrafish resulted in defects in heart looping, cardiac edema and disturbed skeletal muscle development. These initial data showed an essential function of CHAP in cardiac and skeletal muscle development. Given the homology between CHAP, synaptopodin and myopodin it is likely that CHAP also functions as an actin bundling protein and/or in the calcineurin pathway. Furthermore, the location of CHAP in the Z-disc and the binding to α-actinin-2 suggests a role for CHAP in the development of cardiomyopathy. To elucidate this, we investigated the role of CHAP during development and disease in this thesis.

**Aim and outline**

In this thesis the function of CHAP during embryonic development and in adult tissues and disease was analyzed by making use of transgenic mouse models, expression analysis and *in vitro* experiments. We show that CHAP expression during development is conserved between species, which implies an important role during cardiac and skeletal muscle development. Furthermore, we show *in vivo* and *in vitro* that CHAPa and CHAPb have distinct functions in muscle development and function.

**Chapter 2** describes the sequence and expression analysis of the chick (Gallus gallus) homolog of human and mouse CHAPa. Furthermore, detailed expression analysis of mouse CHAP in embryonic and adult skeletal and smooth muscle is given. To investigate the function of CHAP during muscle development and disease in more detail, we attempted to generate a CHAP knockout mouse. As a first step, **chapter 3** gives a detailed description of generation of CHAP targeted mouse embryonic stem cells. These cells were used to investigate the consequences of CHAP knockdown on cardiac differentiation *in vitro*. Their use to generate chimeric mice is pending. In addition, we investigated the effect of morpholino mediated knockdown of CHAP in chick embryos. In **chapter 4** we analyzed the role of both CHAP isoforms in the adult heart by generation of CHAPa- and b heart-specific transgenic mice. Whereas, in CHAPa Tg mice no phenotype is observed, CHAPb Tg mice develop cardiomyopathy with diastolic dysfunction. In addition, the CHAPb Tg mice show formation of actin stress fibers and upregulation actin signaling. To study the effect of CHAP overexpression more directly, we generated adenoviruses and in **chapter 5** used these to study the function of CHAP by *in vitro* overexpression of CHAPa- and b in a model of skeletal muscle development (C2C12 cells) and E17.5 mouse cardiomyocytes. We show that, as in the Tg mice, an actin bundling function *in vitro* for CHAPb and also show a function for CHAPa in the integrity of the Z-disc. However, unlike *in vivo* overexpression of CHAPb, the actin signaling pathway is not affected to the same extent by *in vitro* overexpression. In **chapter 6** a more detailed expression analysis of CHAP is given. In this chapter expression of CHAP in small intestine, kidney and brain is shown. CHAPb expression in these organs is related to the expression of F-actin and show again a function for this protein in actin bundling.

The results of this thesis are summarized and discussed in **chapter 7** and suggestions for future research are given.
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