Chapter 7
General discussion
In this thesis the function of a novel Z-disc protein Cytoskeletal Heart-enriched Actin-associated Protein (CHAP) is described. CHAP was identified previously from a genome-wide transcriptome study in human embryonic stem cells differentiating to cardiomyocytes\(^1\). In mouse and human genomes two isoforms of CHAP were identified; a longer isoform CHAPa containing a N-terminal PDZ-domain and nuclear localization signal (NLS) and a shorter isoform CHAPb, which lacks the PDZ-domain. Interestingly, mouse CHAP isoforms have a distinct expression pattern; whereas CHAPb is predominantly expressed in heart and skeletal muscle during embryonic development, CHAPa is clearly higher expressed in adult heart and skeletal muscle. We have recently demonstrated that zebrafish *chap* is essential for skeletal muscle and heart development\(^2\). In this thesis I further explored the function of CHAP by several *in vivo* and *in vitro* approaches. And discuss here the potential role(s) of CHAP during development, adult stages and disease.

**Function of CHAP during heart and skeletal muscle development**

The first clues on CHAP function came from its expression pattern during embryonic development. CHAP is expressed in the developing heart and somites (that give rise to skeletal muscle), and this is conserved between species (zebrafish, mouse and chick; previously studied\(^2\) and chapter 2). We showed in chapter 2 that in chick embryos CHAP is expressed in the linear heart tube from Hamburger and Hamilton (HH) stage 8 onwards and at older stages, found expression in somites as well as the heart. As mentioned before, CHAPb is the predominant isoform during mouse embryonic development. However, both in chick and zebrafish only one isoform of CHAP exists, containing the CHAPa characteristic PDZ domain, suggesting that during embryonic development in chick and zebrafish, CHAP may have a similar role as CHAPb. Alternatively, other proteins that resemble CHAP may substitute for the lack of embryonic CHAPb. In this regard, CHAP belongs to a family of actin-bundling proteins, with synaptopodin being the first described member and myopodin the second member of this family. Whereas synaptopodin is expressed in brain and kidney\(^3\), myopodin is expressed in heart, skeletal muscle and smooth muscle\(^4\) and is thus the most likely candidate protein to partially substitute CHAP functionality during muscle development in chick and zebrafish.

We have demonstrated previously that morpholino-mediated knockdown of *chap* in zebrafish led to impaired heart looping and disturbed muscle development\(^5\), indicating the importance of *chap* during muscle development. However, the role of CHAP in heart and skeletal muscle in higher vertebrates still needed to be investigated, especially with respect to the functions of the different CHAP isoforms. To investigate the role of CHAP in higher vertebrates, we used CHAP specific morpholino’s to knockdown CHAP in developing chick embryos and followed different to strategies generate CHAP knockout mice (conditional and LacZ knock-in; chapter 3). Although knockdown of CHAP in chick embryos led to abnormalities during cardiac development, such as cardiac looping, these results were variable and not statistically significant. Therefore, to study the role of CHAP during heart development it is essential to generate CHAP (conditional) knockout mice. However, in first attempts we were unable to achieve germline transmission for CHAP mutant embryonic stem cells, which resulted in the study of CHAP in alternative animal models as a first priority: overexpression of CHAP in mice or in cells/cardiomyocytes *in vitro*.

**The role of CHAP in actin signaling**

What could be the mechanism by which CHAP affects cardiac development, or more
specifically, cardiomyocyte function? One possible role of CHAP involves interference with the actin cytoskeleton. This has been evidenced by previous studies on other family members of CHAP. Synaptopodin has been shown to regulate the actin-bundling activity of α-actinin and also regulates actin-bundling via RhoA. In line with these findings, myopodin also has been shown to have actin-bundling activity and binds directly to actin. Furthermore, we observed co-localization of CHAP and actin, suggesting a role for CHAP in actin signaling.

The actin cytoskeleton plays an important role in formation of sarcomeres in muscle cells. It has been postulated that formation of actin bundles may serve as a scaffold for the formation of I-Z-I complexes (referring to the I-bands and Z-discs in sarcomeres). These complexes are composed of Z-bodies containing α-actinin and titin which are linked to the actin bundles and are associated with the membrane. In the next stage, myosin thick filaments are organized with the I-Z-I complexes and dissociate from the membrane, forming immature sarcomeres that already show contraction. In the last stage thin filaments form and sarcomeres mature. RhoA and its down-stream effector Rho-associated kinase (ROCK) have been shown to be involved in the formation of actin fibers and in this way play an important role sarcomere formation.

We have shown in chapter 3 that CHAP knockdown and deletion of one allele did not affect sarcomere formation in vitro. However, this did not result in a complete loss of CHAP and therefore remaining levels of CHAP may be sufficient for sarcomere formation. Knockdown of chap in zebrafish resulted in sarcomeric disorganization, suggesting that chap knockdown was more efficient in zebrafish or that the timing of interference is crucial. In the future, complete removal of CHAP in vivo and in vitro will tell us more about the possible function of CHAP in sarcomere formation.

In chapter 4 we showed that cardiac-specific overexpression of CHAPb in mice (CHAPb transgenic [Tg]) induced cardiomyopathy which was accompanied by activation of actin signaling, indicated by the formation of stress fibers. These stained for α-actinin and CHAP, and increased expression of actin, the small GTPase molecule RhoA, Ezrin/Radixin/Moesin (ERM) and actin binding protein cofillin was observed. Furthermore, we showed that sarcomeric expression of RhoA was reduced, whereas expression of RhoA at the membrane was increased. To investigate if these observed effects in the Tg mice were a direct effect of CHAPb we generated CHAPA- and b adenoviruses to express CHAP in embryonic day 17.5 (E17.5) cardiomyocytes in culture and in C2C12 cells, a mouse skeletal myoblast cell line. Overexpression of CHAP in these cells did not result in increased expression of actin, RhoA, cofillin or ERM (chapter 5), suggesting that CHAP does not directly regulate expression of members of the actin-signaling pathway and that secondary effects, timing, or additional factors, that are not present in vitro cultures, must be involved in vivo.

**CHAP and transcriptional regulation**

Another possible role for CHAP could be on transcriptional regulation. The first indication is provided by the localization of CHAP in the nucleus of undifferentiated myoblast cells, where it co-localizes with RhoA. Both proteins are translocated to the cytoplasm upon muscle differentiation (figure 1). It has been suggested that RhoA is directly involved in maintaining skeletal myoblast cells in an undifferentiated state by activating SRF and suppressing transcription factor MyoD, a key factor for skeletal muscle differentiation. Furthermore, RhoA inhibits M-cadherin mediated muscle cell fusion, suggesting that RhoA activity needs to be suppressed for muscle differentiation. On the other hand, RhoA may regulate Serum Response Factor (SRF), a transcription factor, which plays an important role in skeletal
muscle growth and maturation\textsuperscript{13}. Thus, the role of RhoA/SRF in skeletal muscle development is rather complex. CHAP expression levels increase during muscle differentiation. However, exogenous expression of CHAP\textsubscript{a} or CHAP\textsubscript{b} in C2C12 myoblasts cells prevent muscle fusion (chapter 5), similar to overexpression of a dominant positive isoform of RhoA\textsuperscript{12}. Whether CHAP plays a role in muscle differentiation by affecting subcellular localization and/or expression levels of RhoA and SRF or any other mechanism needs to be determined. In CHAP\textsubscript{b} Tg mice we showed an increase in the expression of transcription factors SRF and Myocyte Enhancer Factor 2 (MEF2). However, adenoviral overexpression of CHAP\textsubscript{a} and CHAP\textsubscript{b} in fetal cardiomyocytes did not lead to an upregulation of these transcription factors, suggesting that CHAP may not directly influence transcriptional regulation.

In addition to the RhoA-SRF pathway, CHAP might also influence transcription via the calcineurin-NFAT (nuclear factor of activated T-cells) pathway, a key pathway in cardiac hypertrophy and disease (see below). In CHAP transfected cells the cardiac fetal genes and hypertrophic markers ANF, BNP and beta-MHC were downregulated, which was correlated with translocation of NFATc2 from the nucleus to the cytosol. Furthermore, we recently found that CHAP interacts with the phosphatase calcineurin and with calcineurin-interacting

Figure 1: Localization of CHAP (green) and RhoA (red) in undifferentiated, 3 days differentiated and 6 days differentiated C2C12 cells. Merge images are shown.
proteins, such as calsarcins (Beqqali personal communication), suggesting a function for CHAP in calcineurin-mediated hypertrophy. Therefore, it would be interesting to investigate the function of CHAP in this pathway by generating CHAPa- and b knockout models and by analyzing CHAP in different hypertrophy models, such as calcineurin Tg and calsarcin and cypher knockout mice. Furthermore, CHAP might have a direct effect on transcription, because we have observed nuclear localization of CHAP in cardiomyocytes (chapter 5 and unpublished data of A. Beqqali). However, \textit{in vivo} we never observed nuclear localization of CHAP.

Role of CHAP in sarcomere integrity, cardiac hypertrophy and disease

We have shown previously that CHAP is expressed at the Z-disc of adult hearts and that it interacts with α-actinin-2. Several Z-disc components have been identified that interact with α-actinin-2 and act as a stretch-sensor. The phosphatase calcineurin is a well-described key regulator of hypertrophy. During cardiac hypertrophy, which occurs in response to various pathophysiological conditions or events, such as myocardial infarction, increased levels of \( \text{Ca}^{2+} \) lead to activation of calcineurin. Subsequently, activated calcineurin dephosphorylates nuclear factor of activated T-cells (NFAT), leading to nuclear translocation of NFAT and activation of a fetal gene expression program\textsuperscript{14, 15}. Calsarcins represent a new family of calcineurin-interacting proteins, bind to other sarcomeric proteins such as α-actinin-2, are expressed in heart and skeletal muscle\textsuperscript{16} and protect the heart against calcineurin-induced hypertrophy\textsuperscript{17, 18}. Cypher (Oracle) is another α-actinin-2-interacting protein and is expressed in the heart and skeletal muscle\textsuperscript{19, 20} and cypher knockout mice displayed decreased calsarcin expression\textsuperscript{21} and development of a dilated cardiomyopathy phenotype in mice\textsuperscript{21} and zebrafish\textsuperscript{22}. Both deletion of calsarcin and cypher leads to disruption of the Z-disc\textsuperscript{17, 21}. In addition to calsarcin and cypher, CHAP might regulate sarcomeric integrity and cardiac hypertrophy via calcineurin-NFAT and/or SRF. SRF, which was upregulated in CHAPb Tg hearts (chapter 4), is also involved in maintaining Z-disc integrity in adult skeletal muscle by regulation of transcription of sarcomeric proteins\textsuperscript{23}.

In order to investigate the role of CHAP \textit{in vivo}, we generated transgenic mice by heart-specific overexpression of both isoforms of CHAP. We did not observe any abnormalities in hearts of CHAPa Tg mice. Although, we found upregulation of \textit{ChapA} mRNA expression by qPCR experiments and CHAP protein by western analysis in CHAPa Tg mice, we could not detect the transgenic protein with a specific FLAG antibody (chapter 4) and therefore cannot exclude the possibility that CHAPa protein was partially degraded and functionally inactive. In CHAPb Tg mice both mRNA and protein levels of CHAPb were stably expressed. We observed mild hypertrophy and interstitial fibrosis in hearts of CHAPb Tg mice at three months of age. This was more severe at six months of age and was associated with activation of the hypertrophic gene program and expression of collagens. Furthermore, we observed conduction disturbances in CHAPb Tg mice, which coincided with a remarkable suppression of atrial connexins (Cx40 and Cx43), crucial for electrical coupling of cardiomyocytes. Besides electrical dysfunction, CHAPb Tg mice showed diastolic dysfunction \textit{in vivo} and in isolated cardiomyocytes \textit{in vitro}. These features are comparable to the cardiac abnormalities seen in patients with hypertrophic cardiomyopathy (HCM), i.e. cardiac hypertrophy, diastolic dysfunction and increased occurrence of arrhythmia (in particular atrial fibrillation). In order to identify a possible working mechanism for the observed phenotype, we further studied
CHAPb Tg hearts at the cellular level. We observed stress fiber-like structures in the hearts of CHAPb Tg mice, that stained for both CHAP and α-actinin. In addition, we showed that the actin signaling pathway was upregulated in CHAPb Tg hearts, as discussed previously. These observations led us to conclude that the fetal isoform CHAPb can be involved in the onset and progression of cardiac disease, strongly resembling characteristics of cardiac hypertrophy and HCM. This is further corroborated by recent findings, in which we found that expression of CHAPb is upregulated in several models of hypertrophy (personal communication A. Beqqali). These data suggest a role for CHAPb during hypertrophic events and it would be interesting to investigate the possible working mechanisms further. It would also be interesting to analyze the function of CHAP in human disease by sequence analysis of CHAP in HCM and in dilated cardiomyopathy (DCM). In HCM and DCM sarcomeric proteins are mutated and most mutated proteins are beta-MHC, myosin-binding protein C and troponin T. However, several mutations in other Z-disc components were also identified and these mutations interfered with interaction between Z-disc components. For example, a mutation found in cypher (D626N) in DCM patients increased the interaction with protein kinase C, a key regulator of contractility and growth of cardiomyocytes. Mutations in Tcap result in HCM (T137I and R153H) or DCM (E132Q) and resulted in increased interaction with titin and calsarcin-1 or decreased interaction with muscle LIM protein, calsarcin-1 and titin, respectively. Mutations in Z-disc protein nexilin (G650del, Y652C and P611T) leads to disruption of the Z-disc, although the exact mechanism was not identified.

Thus, in vivo experiments in mice and sequence analysis in HCM and DCM patients may reveal more information about the putative function of CHAP in Z-disc integrity and cardiac disease.

**CHAP in skeletal muscle**

In skeletal muscle we found expression of both CHAPa and CHAPb (chapter 2 and chapter 6), with expression of CHAPa approximately 10 fold higher than CHAPb. CHAPa overexpression in skeletal muscle cells (C2C12) resulted in disruption of the Z-disc, whereas CHAPb overexpression induces stress fibers (chapter 5). As in cardiomyocytes, CHAPa could function in maintaining Z-disc integrity in skeletal muscle cells. Mutations in cypher have been described in muscular dystrophy and these resulted in disintegration of the Z-disc. Therefore, it would be interesting to search for CHAPa mutations in muscular dystrophy patients. We also found low expression of CHAPb in skeletal muscle cells. In contrast to cardiomyocytes, skeletal muscle cells have the ability to regenerate in adult mice. The function of CHAPb here could be, as suggested by its role in cardiac development, to induce actin polymerization and serve as a scaffold for new Z-disc formation.

In addition to Z-disc integrity CHAP could also be involved in determining the slow muscle fiber phenotype in adult animals. We find highest CHAPa expression in soleus muscle, which is predominantly composed of slow muscle fibers. Calcineurin, NFAT and MEF2 are important regulators of the slow muscle fibers. In slow muscle fibers there is a tonic motor nerve activity that leads keeps Ca\(^{2+}\) levels constant and calcineurin-NFAT signaling active. In fast muscle fibers, on the other hand, there is phasic firing of the motor nerve, leading to high amplitude Ca\(^{2+}\) transients that are insufficient to activate the calcineurin-NFAT pathway. All NFAT isoforms are important for determining the slow/fast muscle switch. A specific combination of NFAT isoforms determines the slow or fast muscle gene expression. MEF2 is also regulated by calcineurin to induce slow muscle gene expression and the combined
binding of MEF2 and NFAT to slow muscle fiber gene promoters leads to the subsequent gene expression. Calsarcins have been implicated in determining the fast skeletal muscle phenotype. Both calsarcin-2 and -3 are expressed specifically in fast skeletal muscle and knockdown of calsarcin-2 leads to a switch to slow skeletal muscle fibers phenotype by activation of the calcineurin-NFAT pathway. Our results suggest that CHAPb regulates MEF2 levels in vivo (chapter 4) and both CHAPa and –b are involved in calcineurin-NFAT signaling (chapter 5 and unpublished results). Therefore, it would be interesting to investigate the function of the CHAP isoforms in determination of skeletal muscle fiber phenotype. This could be achieved by generating tissue-specific (inducible) knockout mice for both isoforms or by overexpression or knockdown of CHAP isoforms in vitro in muscle progenitor cells.

Possible role of CHAP in smooth muscle cells
Besides expression in adult heart and skeletal muscle, we also found CHAP to be expressed in smooth muscle cells (chapter 2). Additional western blot analysis showed that CHAPa was expressed in these cells (data not shown). During embryonic development we showed expression of CHAP in cardiomyocytes adjacent to the vascular smooth muscle cells (VSMCs) of the vena cava at embryonic day 17.5 (chapter 2). Although CHAPb is the predominant form in striated muscle cells during embryonic development, we cannot exclude CHAPa being expressed in these cells. However, CHAP expression was not detected in smooth muscle cells during development, suggesting that CHAP has no role during VSMC development. In smooth muscle cells contraction is regulated by so-called dense bodies, co-staining with α-actinin will indicate whether CHAP is expressed in dense bodies. During pathophysiological events it is possible that CHAPb, like in hypertrophic cardiomyocytes, is upregulated. VSMCs respond to hypertension or other stimuli by a phenotypic transition from a contractile state to a synthetic state, leading to increased synthesis of extracellular matrix and migration of VSMCs to the subendothelial layer, where they proliferate to form an atherosclerotic lesion. Cytoskeletal proteins have been implicated in the migration of VSMCs. Phosphorylation of RhoA by cyclic AMP dependent protein kinase leads to migration of VSMCs in vitro. In addition, interleukin-19 inhibits platelet derived growth factor induced VSMC migration by decreasing myosin light chain, RhoA activation and coflin dephosphorylation. Also CHAPa could function the pathophysiology of VSMCs since contractile proteins have indeed been implicated. For example, SM22α restricts plaque growth by inhibiting the contractile/synthetic phenotypical switch. Furthermore, a role for contractile proteins has also been implicated in determining the vascular stiffness. Both CHAPa and CHAPb could be involved here. Additional experiments in mouse models for atherosclerosis (e.g. ApoE knock out mice) could address this issue.

In summary CHAPa is expressed in adult heart, skeletal and smooth muscle cells, but CHAPb is expressed in embryonic muscle cells only (previous section) and upregulated in pathophysiological circumstances (A. Beqqali, personal communication).

Additional functions of CHAPb
Besides expression during heart and skeletal muscle development, we showed that CHAPb is expressed in the small intestine and kidney. In these organs, CHAPb expression is adjacent to microvilli that can be recognized by staining with phalloidin for filamentous actin (chapter 6). Actin dynamics is involved in many cellular processes like cell movement, cell division and cell shape changes. Therefore, CHAPb could also be involved in several other cellular...
processes. In chapter 4 we showed that CHAPb is involved in signaling via RhoA-SRF. SRF and RhoA are implicated in several other cellular processes, such as cell polarity, adhesion, cell movement, that are important during development and in pathophysiological situations as cancer\textsuperscript{44-46}. Moreover, the CHAP homolog myopodin has been implicated as a tumor suppressor in prostate cancer\textsuperscript{47, 48}. Therefore, it would be interesting to investigate the role of CHAPb in these cellular processes by \textit{in vitro} and \textit{in vivo} experiments.

Conclusions and future directions
In this thesis, the distribution and function of a novel Z-disc protein CHAP was described. We showed that CHAPa is expressed in adult muscles and has a function in integrity of the Z-disc \textit{in vitro}. CHAPb, on the other hand, is predominantly expressed during development and has a putative function in cardiac and skeletal muscle development. We showed that CHAPb is involved in actin signaling by overexpression \textit{in vivo} and \textit{in vitro}. Moreover, CHAPb is expressed in adult organs and has a putative actin-bundling function. To further explore the function of CHAPa and CHAPb \textit{in vivo} it will be necessary to generate (conditional) CHAP knockout models in mice. Furthermore, investigating the function of CHAP in other transgenic/knockout cardiac development and disease models should reveal more about the putative function of CHAP. The role of CHAP in human development and disease can be unraveled by making use of DNA databases of congenital heart disease patients or HCM/DCM patients, such as the CONCOR database, established ten years ago at the Amsterdam Medical Centre with support of ICIN funding.
References


