Chapter 3

The role of CHAP during heart development: *in vivo* and *in vitro* knockdown in mouse and chick

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Abstract

We have previously shown that Cytoskeletal Heart-enriched Actin-associated Protein (CHAP) plays an important role during skeletal and heart muscle development in zebrafish by a gene knockdown approach. In order to study the role of CHAP more specifically during muscle development and disease we targeted the CHAP genomic locus in mouse embryonic stem cells (mESC) for creating both straight and conditional knockout mice for CHAP. High percentage chimeric mice were obtained for both approaches and were further bred for germline transmission. At the present time, no germ knockout lines were generated for both targeted constructs. Besides the opportunity to study the loss of CHAP during development and disease at specific times and organs, another advantage is to study the role of CHAP during cardiomyocyte differentiation in these targeted ESC lines.

To investigate the cardiac differentiation potential of the heterozygous CHAP LacZ mESC in vitro, we differentiated these cells to cardiomyocytes by formation of embryoid bodies (EBs). qPCR analysis showed that *ChapB* was significantly downregulated in these EBs in comparison to wild type cells. Although, expression levels of *ChapA* were not significantly different, a trend towards reduced mRNA levels in the heterozygous cells could be observed. Despite decreased levels of CHAPb, both sarcomeric organization and beating frequency were not affected in CHAP heterozygous mESC lines, indicating that mutation of one single copy of CHAP does not lead to haploinsufficiency based on cellular and functional in vitro readouts.

Since CHAP is also expressed during muscle development in chick, which only has one isoform of CHAP and does not contain a duplicated genome (as in the zebrafish), we knocked down *CHAP* expression in chick during embryonic development. With increasing concentrations of *CHAP* morpholino's a higher percentage of embryo's with an aberrant cardiac looping phenotype was observed, although this was not statistically significant. These results from both mouse and chicken experiments show that reduced levels of CHAP are sufficient to maintain cardiomyocyte integrity and functionality with minimal effects on cardiac development.

Introduction

Cytoskeletal Heart-enriched Actin-associated Protein (CHAP) is highly conserved in different species such as human, mouse, zebrafish and chicken. Whereas two isoforms of CHAP exist in human and mouse, only one isoform is present in zebrafish and chicken. In previous experiments in mice we identified that the shorter isoform CHAPb is expressed during embryonic development, whereas the longer isoform CHAPa is expressed in adult heart and skeletal muscle. The longer isoform consists of a PDZ domain, important for protein-protein interactions and a nuclear localization signal (NLS), whereas the shorter isoform lacks the PDZ domain. In both zebrafish and chick only one isoform can be identified with highest homology to the longer isoform CHAPa. We have previously shown by antisense morpholino knockdown of CHAP in zebrafish resulted in cardiac defects and impaired muscle formation, suggesting an important role for CHAP during development[1].

To elucidate the function of CHAP in specific organs and at specific times during mouse development and disease we developed different strategies for generating CHAP knockout lines in mouse embryonic stem cells (mESC). First, a CHAP LacZ knockin (CHAP $LacZ^{+/-}$) mouse embryonic stem cell line was generated, which replaces the complete CHAP gene for a LacZ gene. This line was used to study the effect of CHAP knockdown on the differentiation of mESC-derived cardiomyocytes *in vitro*. Second, a conditional knockout mESC line was generated in which the CHAP exon 5 was flanked by loxP sites. Both lines can be used to generate CHAP knockout ($CHAP^{f/+}$) lines, which can be used to study the effect of CHAP knockdown in mouse *in vivo*.

In addition, we investigated the effects of *CHAP* knockdown on heart development in chick embryos. In contrast to human and mice, the chick genome contains only one *CHAP* isoform (see chapter 2) and in contrast to the zebrafish, there is no duplication of the genome. Furthermore, the four-chambered chick heart represents a convenient model for studying human heart development[2, 3]. Comparable to the zebrafish it is possible to achieve knockdown of the gene of interest. Following an antisense morpholino-mediated knockdown in chick embryos we studied the role of *CHAP* on heart development.

Materials and methods

Cell culture

Wild type (IB10) and targeted CHAP LacZ$^{+/-}$ and CHAP$^{f/+}$ mESC were grown on feeders in complete medium (CM: 1x GMEM (Invitrogen), 2 mM L-glutamin (Invitrogen), 1 mM Sodium Pyruvate (Invitrogen), non-essential amino acids (1:100, Invitrogen)), 8.8% fetal calf serum (FCS; Sigma-Aldrich Chemie). To keep the cells undifferentiated β-mercaptoethanol (1000x) and leukemia inhibitory factor (10^6 units/ml LIF, ESGRO, Millipore) were added to the CM (CM^{++}). For long-term feeder free growth of mESCs, cells were plated on gelatine coated dishes and grown in 60% BRL medium with β-mercaptoethanol and LIF added. 100% BRL medium was made by conditioning CM on Buffalo Rat Liver (BRL) cells. 60% BRL medium was made by mixing 100% BRL and CM to a 60/40 ratio.

Generation of CHAP lacZ knockin and CHAP conditional mESC lines

For generating CHAP LacZ$^{+/-}$ mESC the pNeo-LacZ-TK2 targeting vector (kindly provided by Dr. E. Olson) was used. A 5.3 kb long arm (primers: 5'- GTCGACTCAGTTTTTT-GAGACTGGAT-3' and 5'- GTCGACCGCACAGCTTAGCCTGTGG-3') was designed, amplified by PCR from a BAC-clone, subcloned into pGEM-T easy (Promega) and sequencing. The long arm was designed as such that the ATG starting codon of the CHAP gene was replaced by the coding sequence of LacZ gene (starting with ATG). A 2.5 kb short arm (primers: 5'-GGTACCCTCCTCAGCTCTCTGCTGGT-3' and 5'- ATGGTTGC-AGGTCAACACAA-3') was amplified and subcloned in a similar way as described for the long arm and was positioned at the 5' end of LacZ (see figure 1A).

For generating CHAP conditional knockout (CHAP$^{f/+}$) mESC line the pl451 targeting vector (kindly provided by J. van Es) was used. The following products were amplified and subcloned in pGEM-T easy vector and verified by sequence analysis: CHAP exon 5 (primers: 5'-CGGGATCCGCCAGCCTGGTCTACATAGC-3' and 5'-CGGGATCCAGCCTTG-TTCCTGGCTTTTG-3'), a 5.8 kb long arm (primers: 5'-CCATCGATCTTTCAAT-GACCGTGGCAAA-3' and 5'-CCATCGATGTCGGCCTTGAACTCACAGA-3') and a 2.5 kb short arm (same primers were used as for the CHAP LacZ$^{+/-}$). The products were subcloned into the pl451 targeting vector: the CHAP exon 5 product was flanked by loxP sites and the long arm and short arm were upstream and downstream of exon 5, respectively. Following correct targeting by homologous recombination exon 5 of CHAP will be flanked by LoxP sites (see figure 2A). Both targeting vectors contained a neomycin cassette for positive selection and a thymidine kinase (TK) cassette for negative selection.

The targeting constructs were linearized, purified and dissolved in sterile PBS (Invitrogen). 75-100 ug DNA was electroporated in mESC (IB10) with a 129Sv background. Cells were plated on gelatin-coated dishes and put overnight in the incubator. After 1 day medium was replaced by medium containing G418 and gancyclovir for positive and negative selection, respectively. The medium was replaced every other day. After approximately one week colonies were picked and transferred to 96 well plates on feeders. These plates were split into three other 96 well plates (two with feeders, one feeder-free). The two plates on feeders were frozen by dissolving cells in freezing medium (44.3% CM^{++}, 44.3% FCS and 11.4% DMSO) and stored at -80 ^{o}C. The feeder-free plate was used to isolate DNA to screen resistant colonies for correct homologous recombination by Southern blot (see below). Correct recombined colonies were thawed, scaled-up and frozen again in multiple aliquots in liquid nitrogen.

Southern blot
Genomic DNA of resistant ES cell colonies was extracted by adding lysisbuffer (10 mM Tris pH 7.5, 10 mM EDTA, 10mM NaCl, 0.5% sarkosyl and 2 mg/ml protK) at 60 °C overnight. DNA was precipitated and digested by BamHI (5'targeting of $CHAP^{f/+}$), NcoI (5'targeting of CHAP $LacZ^{+/-}$) or KpnI (3'targeting of $CHAP^{f/+}$and CHAP $LacZ^{+/-}$) overnight and run on a 1% v/w agarose gel. Three probes were generated using the following primers: BamHI (329 bp) 5'-GGAGAGCCAGATGCTTTTTG-3' and 5'- CCACTCTTCTGCCCAGCTAC-3', NcoI (1 kb) 5'-GTCCTTCCAAACGCTAAACG-3' and 5'-CTACCGTAGGGAC-GCAAATC-3', KpnI (416 bp) 5'-CCACTATCTGCTCCCTCTCG-3' and 5'-CTCAGAA-GAGTGGGCTCCAG-3. The PCR products were cloned into pCRII (Invitrogen) and products were excised. The products were then radioactively labelled using α-[p32]dATP (PerkinElmer) by random priming (RadPrime, Invitrogen). DNA blots (Hybond-N+, GE Healthcare) were hybridized with the radioactive probe in ExpressHyb Hybridization buffer (Clontech) and visualized by using phosphor-imager (location of probes are indicated in schematic drawings of figure 1A and 2A).

Generation of chimeric mice
Recombinant ESC clones were injected in blastocysts obtained from C57BL6 (conditional knockout) or CD1 females (LacZ knockin). Chimeric male mice were crossed with C57BL6 or CD1 females to derive germline F1 progeny.

Differentiation assays
Wild type (IB10) and CHAP $LacZ^{+/-}$ mESC were differentiated using the hanging drop method. Before the procedure, cells were plated on gelatine-coated dishes for feeder depletion and grown in CM medium with β-mercaptoethanol and LIF. Cells were then washed with PBS, trypsinised and counted. 20 µl drops containing 1500 cells in differentiation medium (Iscove's Modified Dulbecco's Medium, Invitrogen), 15% FCS (Greiner), 5% Protein free Hybridoma medium (PFHMII, Invitrogen), 2 mM L-glutamine (Invitrogen), 50 µg/ml asorbic acid, 0.3% α-MTG and 0.5% Pen/Strep (Invitrogen)) were put on the lids of bacterial dishes with PBS and left for 7 days. Then embryoid bodies (EBs) were plated on gelatine-coated dishes in differentiation medium. After 7 days cells we either fixed EBs for 30 minutes in 2% paraformaldehyde (PFA) or dissolved them in Trizol (Invitrogen) and stored at -80 °C.

RNA isolation and quantitative PCR
EBs were dissolved in Trizol (Invitrogen) and RNA was isolated. RNA was treated with DNase (DNA-free, Ambion) and cDNA was made with the iScript kit (BioRad). qPCR was performed using the CFX96 Real-Time PCR detection system (Bio-Rad). The following primers were used *ChapA* (sense: 5'-GAGGAGGTGCAGGTCACATT-3'; antisense: 5'-CTGAAGAGCCTGGGAAACAG-3'), *ChapB* (sense: 5'-CCGCCGCTTCTTAAACATAA-3 antisense: 5'-GGCTTTAAAGGGCCTTGG-3') and as reference gene *Gapdh* (sense: 5'-GTTTGTGATGGGTGTGAACCAC-3', antisense: 5'-CTGGTCCTCAGTGTAGCCCAA-3). Data were analyzed with Bio-Rad CFX Manager.

Immunofluorescence
EBs were grown on gelatine-coated coverslips and fixed in 2% PFA for 30 minutes. Cells were permeabilized in 0.1% Triton-x-100 in PBS for 8 minutes. Subsequently, cells were incubated

with primary antibodies (CHAP 1:50 (rabbit, custommade by Eurogentec), myomesin (mouse, kindly provided by E. Ehler) 1:50 and α-actinin (mouse, Sigma) 1:800) in 4% normal goat serum overnight at 4 °C. Secondary antibodies used were goat-anti-mouse cy3 (1:250, Jackson Immuno research) and goat-anti-rabbit Alexa Fluor 488 (1:200, Invitrogen) for 1 hour at room temperature. Cells were counterstained by DAPI. Stainings were analysed with SP5 confocal microscope (Leica).

Morpholino knockdown of CHAP in chick embryos

Morpholino knockdown of *CHAP* was done as described by Cing et al[2]. In brief White Leghorn eggs were incubated at 38 °C. At Hamburger and Hamilton stage 11 a window was created and the extra-embryonic membranes overlying the heart were removed. Embryos were treated with control morpholino's (mutated human β-globin; sequence: 5'-CCTCTTACCTCAGTTACAATTTATA-3'), two concentrations of *CHAP*-specific morpholino's (sequence: 5'-TGAGCATTTCTTCTTCGGCTCCCAT-3') or left untreated (wild type). After 29 hours (stage 20) embryos were harvested, washed in PBS and fixated in 4% paraformaldehyde. Embryos were dehydrated by ethanol and xylene series and embedded in paraffin. Embryos (E12.5) were sectioned (5 μM) and mounted on starfrost slides (Knittel). Antigen retrieval was performed by microwave heating of tissue sections in citrate buffer (pH 6). Endogenous peroxidase was blocked by incubating the slides in 0.3% H_2O_2 in PBS. Sections were incubated overnight with Troponin I antibody (1:400; TnI; Santa Cruz) at room temperature. Biotin-conjugated goat anti-rabbit (BA-1000, Vector Labs) was used as secondary antibody. Subsequently, the sections were incubated with Vectastain ABC staining kit (PK-6100, Vector Labs) for 45 min. Slides were rinsed in PBS and Tris/Maleate (pH 7.6). 3-3diaminobenzidine tetrahydrochloride (D5637, Sigma-Aldrich) was used as chromogen and Mayer's hematoxylin as counterstaining. Finally, all slides were dehydrated and mounted with Entellan (Merck).

Reconstructions were made as follows: a picture was taken of every TnI positive stained slide, which were adapted in Photoshop (Adobe) and the program Amira 5.3 was used to make the reconstructions.

In situ hybridization

CHAP in situ hybridization were performed as described before (van Eldik et al.; Chapter 2) and was performed on *CHAP* morpholino treated, control morpholino treated and wild type embryo's.

Statistical analysis

Data were analysed with GraphPad Prism. All data are expressed as mean + the standard error of the mean (SEM). Statistical analysis was performed using students unpaired t-test. $P < 0.05$ was considered to be statistically different.

Results

Generation of CHAP knockout mouse ESC lines

To investigate the consequences of CHAP knockdown *in vivo*, we knocked out CHAP in mESC. We used two approaches for this, a straight knockdown (CHAP LacZ$^{+/-}$), and a conditional knockdown (CHAP$^{f/+}$). In figure 1A a schematic overview for the CHAP LacZ$^{+/-}$ targeting is shown. The targeting construct was linearized and electroporated in mESC (IB10) with a 129Sv background. Screening of 200 colonies yielded two correctly recombined lines (Figure 1B). These lines were used to generate chimeric mice, by injecting mESC in blastocysts with CD1 background. Chimeric mice were obtained (Figure 1C) and high-percentage chimeric mice were crossed with CD1 mice in order to achieve germline transmission. CD1 mice have a white coat colour, whereas mice with a 129Sv background have an agouti coat colour. Germline transmission is achieved when the agouti coat colour of the 129Sv background is observed in progeny. Unfortunately, after several rounds of offspring, no germline transmission was achieved.

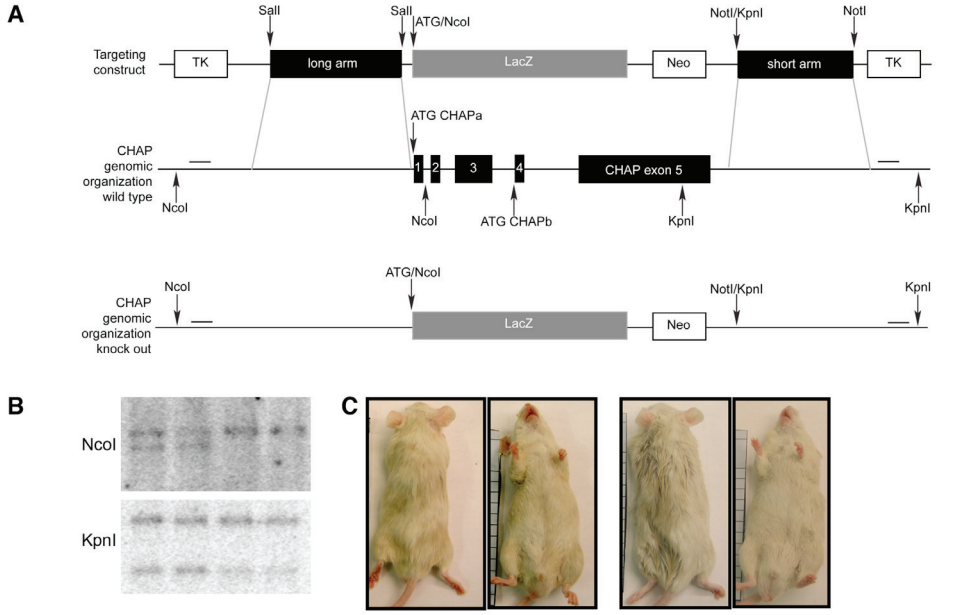

Figure 1: Targeting strategy for CHAP LacZ mouse embryonic stem cells. A) Schematic overview of CHAP LacZ targeting. First panel shows the targeting construct. The construct replaces the wild type CHAP gene (second panel) for a LacZ gene starting at the ATG site of the CHAPa gene (third panel). Neomycine and thymidine kinase cassettes can be used for positve and negative selection respectively. Probes which can be used for testing for recombination are shown (lines in second and third panel). For 5' targeting NcoI gives a 8.3 kB band (wild type) and 7 kB band (targeted). For 3' targeting KpnI gives a 5.5 kB band (wild type) and 3.9 kB band (targeted). B) Southern blot showing the results of the targeting. Two positive clones were obtained which showed recombination of both 5' and '3 arm. C) Chimeric mice obtained after blastocyst injections.

In figure 2A a schematic overview for the CHAP$^{f/+}$ is shown. In order to avoid interference of the neomycine-casette with endogenous expression of CHAP, the CHAP$^{f/+}$ line can be crossed with a mouse line expressing FLPe, leading to excision of this cassette (Figure 2A panel 3). The construct was linearized and electroporated in mESC (IB10) with a 129Sv background. Screening of 100 colonies yielded 7 correctly recombined lines (figure 2B). Two lines were used to generate chimeric mice, by injecting mESC in blastocysts with C57BL6 background. Male chimeric mice were crossed back to mice with a C57BL6 background. We obtained progeny with the agouti coat colour of the 129Sv background from one chimeric mouse, however after PCR and southern blot analysis (data not shown) these were found to be all wild type.

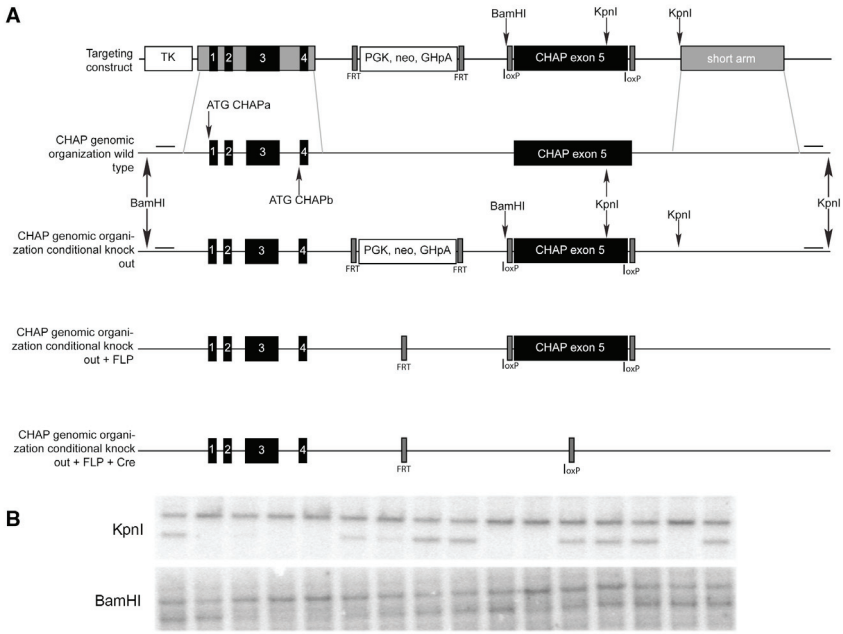

Figure 2: Targeting strategy for CHAP$^{f/+}$ mouse embryonic stem cells. A) Schematic overview of CHAP$^{f/+}$ targeting. First panel shows the targeting construct. The construct replaces the wild type CHAP gene (second panel) for a CHAP gene with a LoxP flanked exon 5 (third panel). Neomycine and thymidine kinase cassettes can be used for positve and negative selection respectively. Probes which can be used for testing for recombination are shown (lines in second and third panel). For 5' targeting BamHI gives a 15 kB band (wild type) and 11.7 kB band (targeted). For 3' targeting KpnI gives a 5.5 kB band (wild type) and 3.9 kB band (targeted). The neomycine cassette is flanked by FRT sites and can be removed by crossing CHAP$^{f/+}$ mouse with a mouse that expresses FLPe (fourth panel). A heart specific CHAP knockout mouse can be obtained by crossing this mouse with a mouse that expresses Cre B) Southern blot showing the results of the targeting. Seven positive clones were obtained which showed recombination of both 5' and '3 arm.

Cardiac differentiation of wild type and CHAP LacZ$^{+/-}$ mESC

To investigate the effect of CHAP knockdown in mESC *in vitro*, we differentiated wild type IB10 and CHAP LacZ$^{+/-}$ mESC to cardiomyocytes by using the hanging drop method. First, we investigated the expression of *ChapA* and *ChapB* in differentiated EBs. As expected qPCR analysis showed that expression of *ChapA* and *ChapB* in CHAP LacZ$^{+/-}$ EBs was approximately half of expression of wild type EBs, although this was not statistically significant for *ChapA* in three independent experiments (Figure 3).

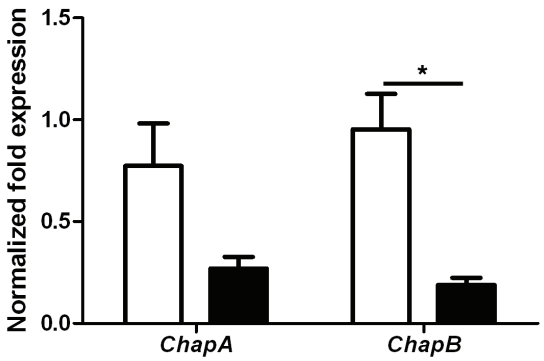

Figure 3: qPCR analysis of expression of *ChapA* and *ChapB* in wild type (white bars) and CHAP LacZ$^{+/-}$ (black bars) embryoid bodies.

Next, we investigated the functional properties of wild type and CHAP LacZ$^{+/-}$ EBs . For this we investigated if the number of beating EBs and the beating frequency (beats per minute) was different for wild type and CHAP LacZ$^{+/-}$ EBs. We did not observe a difference in the cardiac differentiation potential indicated by a similar percentage of beating EBs (Figure 4A), although we cannot exclude that the percentage of cardiomyocytes per EB is different between groups. Similarly, beating frequency also did not differ between groups (Figure 4B). In order to determine whether structural properties of wild type and CHAP LacZ$^{+/-}$ EBs were affected, we performed immunofluorescent analysis. Wild type and CHAP LacZ$^{+/-}$ EBs were co-stained for CHAP and α-actinin (Figure 5A), a Z-disc marker, and CHAP and myomesin (Figure 5B), an m-band marker. Staining of wild type and CHAP LacZ$^{+/-}$ EBs showed that there was no difference in sarcomeric organization and subcellular localization for α-actinin and myomesin between wild type and CHAP LacZ$^{+/-}$ EBs.

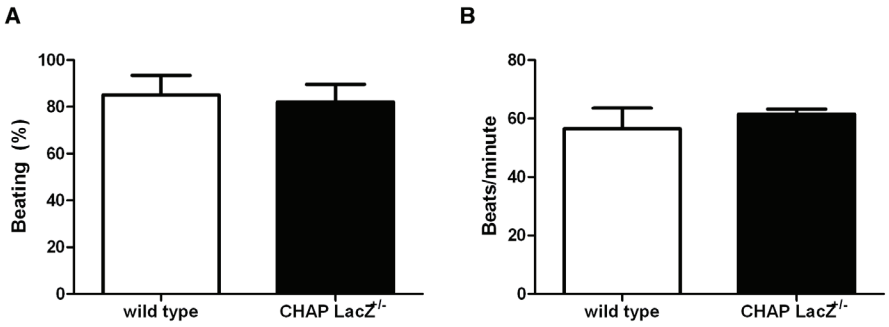

Figure 4: Differentiation potential of wild type and CHAP LacZ$^{+/-}$ mouse embryonic stem cells (mESCs). A) Percentage beating areas obtained after differentiation of wild type (white bars) and CHAP LacZ$^{+/-}$ (black bars) mESCs. B) Beating frequency of wild type (white bars) and CHAP LacZ$^{+/-}$ (black bars).

Knockdown of CHAP in developing chick embryos

In addition to analysing the function of CHAP by knockdown in mice, we also investigated the function of CHAP during chick heart development. In chapter 2 we showed that in chick only one CHAP isoform (homologous to human and mouse CHAPa) is expressed and that this isoform is expressed from the cardiac crescent stage onwards, and in later stages in somites

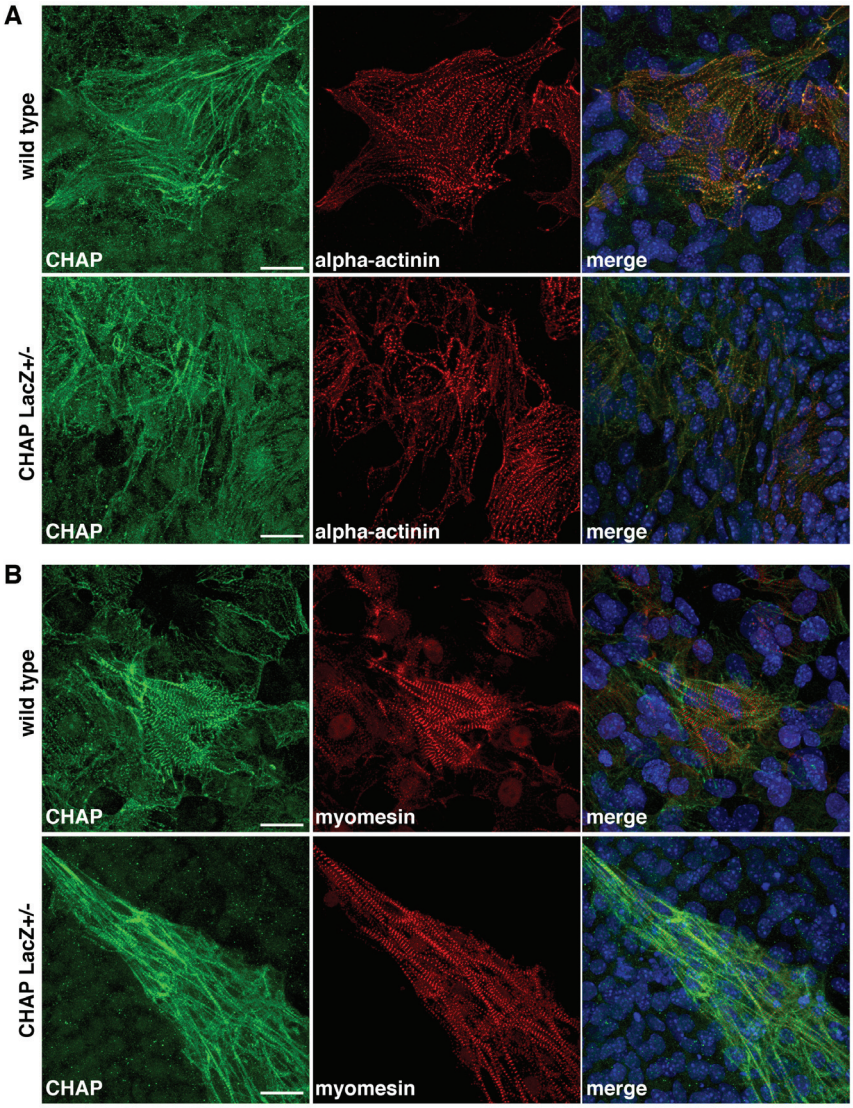

Figure 5: sarcomeric organization of wild type and CHAP LacZ$^{+/-}$ embryoid bodies. A) Wild type and CHAP LacZ$^{+/-}$ EBs stained for CHAP (green) and α-actinin (red). Merge images are shown. B) Wild type and CHAP LacZ$^{+/-}$ EBs stained for CHAP (green) and myomesin (red). Merge images are shown. Scale bars: 20 µM.

and developing muscles as well. To knockdown chick *CHAP* during development we treated embryos of stage 11 with two concentrations *CHAP* morpholinos, control morpholinos or untreated (wild type). Embryos were harvested at stage 20. Although cardiac defects, such as a cardiac looping defect, could occasionally be observed in the *CHAP* morpholino group, no consistent significant cardiac malformations were evident (Figure 6). Next, we investigated the expression of *CHAP* by in situ hybridisation. No difference was detected between the different groups (data not shown). This is not unexpected since morpholino antisense oligonucleotides block translation of RNA to protein. In addition, we also used our custommade antibody

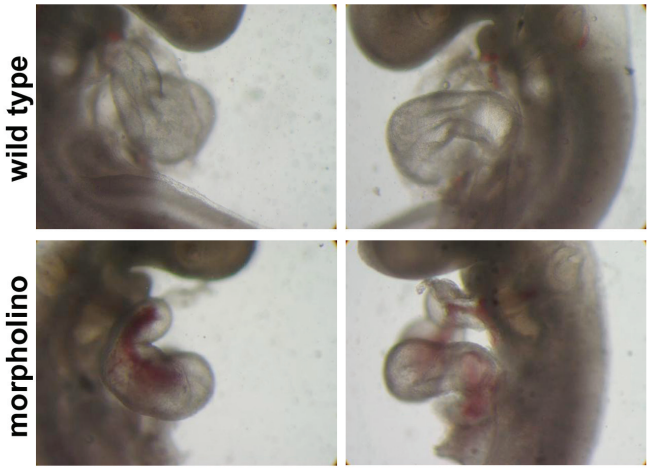

Figure 6: *CHAP* morpholino knockdown in chick embryos. Examples of wild type and *CHAP*-morpholino treated chick embryos of stage HH19 are shown. The *CHAP* morpholino treated embryo shows a looping defect.

against mouse CHAP, but unfortunately did not reveal specific staining.
To study changes in cardiac development in detail we decided to perform immunohistochemical stainings for TnI, which marks cardiomyocytes, for cardiac reconstructions. Because we found impaired looping in zebrafish embryos after *chap* morpholino treatment[1], we investigated the cardiac looping in these embryos as well. Cardiac looping varied among all embryos in the different groups, including in the control groups and therefore we cannot conclude at the present time that chick CHAP affects cardiac looping (Figure 7). To reach a possible statistical significant finding, these experiments should be extended (see discussion).

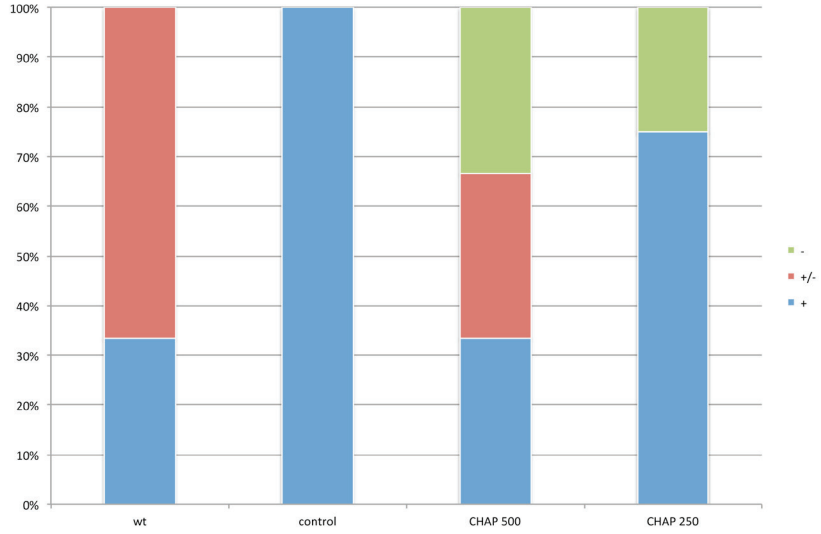

Figure 7: Summary of cardiac looping (classified as not looped -, not completely looped +/- and looped +) of wild type (wt), control morpholino and *CHAP* morpholino (concentrations 500 and 250)

Discussion and future directions

Here, we generated CHAP LacZ$^{+/-}$ and CHAP$^{f/+}$ mESC lines. Control experiments show successful targeting leading to high-percentage chimeric mice. However, several attempts (more than 6 offspring litters for each chimeric mouse) did not yield germline transmission at the present time. In addition, we used the CHAP LacZ$^{+/-}$ mESC to study the effect of CHAP knockdown on cardiac differentiation *in vitro*. Although we found downregulation of *ChapA* and *ChapB*, no effect on differentiation was found. We found no difference in the number of beating areas, beating frequency and sarcomeric organization. From this we conclude that mutation of one allele of CHAP does not lead to haploinsufficiency. Alternatively, *in vitro* experiments using CHAP siRNA in hESC-derived cardiomyocytes could be used to investigate the effect on sarcomere structure and cardiomyocyte function. Preliminary data show that siRNA knockdown of CHAP results in disruption of the sarcomeric structure. However, we were not able to investigate down regulation of CHAP protein expression. Additional future experiments using proper controls (control siRNA) and investigating the effect on RNA and protein expression will give more information about the function of CHAP in human cardiomyocytes.

New techniques for efficient generation of knockout mice have been developed. Chimeric mice with more than 90% ES cell contribution can still be inefficient germ line transmitters. Therefore, a new technique in generating chimeric mice was developed. In this approach 8 cell embryos are laser injected with mESCs. Where in the conventional blastocyst injection method the injected ESC compete with host to create the inner cell mass (ICM), which results in F0 chimeric mice, in the 8 cell stage injected ESC seem to have an advantage over the host cells, which results in an ICM that consist completely of injected cells and thus fully ES cell derived F0 mice[4]. Using this technique it would be possible to obtain CHAP LacZ$^{+/-}$ and CHAP$^{f/+}$ mice.

Morpholino mediated knockdown of *chap* in zebrafish has shown that CHAP is essential for cardiac looping[1]. In vertebrates defects in cardiac looping result in cardiac malformations like double-outlet right ventricle, double-inlet left ventricle and transpositions of the great arteries[5]. If replacement of CHAP by a LacZ gene leads to embryonic lethality, a conditional knockdown approach may be used to overcome this problem. For example, crossing the CHAP$^{f/f}$ with a mouse that expresses Cre in the cardiac (α-MHC or Nkx2.5) lineage or skeletal muscle (MyoD) will elucidate more about the specific function of CHAP in these cells. Furthermore, using a tamoxifen inducible Cre approach, CHAP can be knocked down at a specific moment in adult mice.

In addition to investigating the role of CHAP during development, the knockout models can be used to investigate the role of CHAP in disease. For example, we have shown *in vitro* that CHAP mediates calcineurin signalling. Crossing a mouse with a constitutively active calcineurin in the heart[6] with CHAP knockout mice will give more evidence about the role of CHAP in calcineurin signalling *in vivo*.

In addition to using mouse as a model organism for heart development, chick embryos can also be used as model organism. The chick has the advantage to manipulate embryos in ovo by morpholino treatment[2, 3, 7] or RNAi electroporation[8-11]. In this experiment we chose to knockdown chick *CHAP* by morpholino treatment. Unfortunately, lack of CHAP-specific chick antibody prevented us to study CHAP protein expression in these embryos. We investigated the cardiac looping in these embryos, but found no consistent abnormality

in *CHAP* morpholino treated embryos compared to controls. These experiments need to be repeated in order to make solid conclusions on whether *CHAP* affects cardiac development in chick. In summary, future experiments including knockdown of CHAP in different species (mouse, chick and human) *in vivo* (mouse and chick) or *in vitro* (human, mouse, chick) will reveal more about the putative function of CHAP isoforms during cardiac and skeletal muscle development and disease.

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