# Chapter 2

## CHAP is expressed in striated and smooth muscle cells in chick and mouse during embryonic and adult stages

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# Abstract

We recently identified a new Z-disc protein, CHAP (Cytoskeletal Heart-enriched Actin-associated Protein), which is expressed in striated muscle and plays an important role during embryonic muscle development in mouse and zebrafish. Here, we confirm and further extend these findings by (i) the identification and characterization of the CHAP orthologue in chick and (ii) providing a detailed analysis of CHAP expression in mouse during embryonic and adult stages. Chick CHAP contains a PDZ domain and a nuclear localization signal, resembling the human and mouse CHAPa. *CHAP* is expressed in the developing heart and somites, as well as muscle precursors of the limb buds in mouse and chick embryos. CHAP expression in heart and skeletal muscle is maintained in adult mice, both in slow and fast muscle fibers. Moreover, besides expression in striated muscle, we demonstrate that CHAP is expressed in smooth muscle cells of aorta, carotid and coronary arteries in adult mice, but not during embryonic development.

# Introduction

The Z-disc delineates the borders of the sarcomere, the contractile unit of striated muscle and represents an anchoring plane for various proteins. In addition to their role in force transmission, Z-disc proteins may also be involved in signal transduction[1]. Recently, we indentified a novel Z-disc protein, which we named CHAP (Cytoskeletal Heart-enriched Actin-associated Protein) [2, 3]. We have shown that CHAP has two isoforms, CHAPa and CHAPb, in both humans and mice. CHAPa, the longest isoform (978 amino acids (aa)) contains an N-terminal PDZ domain, as well as a nuclear localization signal (NLS). The shorter CHAPb isoform (749 aa) lacks the PDZ domain, but still contains the NLS. CHAP interacts with α-actinin-2, another Z-disc protein, and is able to translocate to the nucleus [3]. Previously, we have shown that *ChapA* is predominantly expressed in adult heart and muscle tissues, whereas *ChapB* is expressed at higher levels in striated muscles during embryonic development. *ChapB* expression in mouse is evident from the cardiac crescent stage (E7.5) onwards. During later embryonic stages *ChapB* expression is maintained in the developing heart, but is also expressed in the somites (giving rise to skeletal muscle). In addition, we have identified the zebrafish *chap* orthologue and demonstrated by morpholino antisense oligonucleotide-mediated knockdown that *chap* is essential for zebrafish heart and muscle development. Knockdown of *chap* in the zebrafish resulted in aberrant muscle development, indicated by defects in cardiac looping, formation of pericardial oedema and disorganized sarcomeres [3].

Here, we identified the chick (Gallus gallus) orthologue of CHAP. We show that CHAP gene and protein resembles the human and mouse CHAPa isoform, with the predicted PDZ and NLS domains. A detailed analysis of *CHAP* mRNA and protein expression during development in chick and mouse embryos and in adult tissues is shown. Furthermore, we demonstrate that CHAP is not only expressed in cardiomyocytes, and slow and fast skeletal muscle, but interestingly also in smooth muscle cells of the cardiovascular lineage.

# Materials and methods

*Animals*

Swiss mice were intercrossed and females were sacrificed for collection of embryos at different time points (E12.5, 13.5 and 17.5). Fertilized eggs of White Leghorn chicken were incubated at 37 °C and 80% humidity. Chick embryos were staged according to the criteria of Hamburger and Hamilton (HH)[4]. Mouse (E12.5 and E13.5) and chick embryos (HH8-HH30) were collected and fixed in 4% paraformaldehyde (PFA; w/v) in phosphate buffered saline (PBS) overnight at 4 °C. Embryos were further processed for whole-mount or section *in situ* hybridization, or immunohistochemistry. Processing of mouse embryos for cryosections was adapted from [5]. In brief, E17.5 embryos were fixed in 0.2% PFA solution containing 4% sucrose, 0.12mM $CaCl_2.2H_2O$, 0.2M $Na_2HPO_4.2H_2O$, 0.2M $NaH_2PO_4.H_2O$ over night at 4 °C. Next, embryos were washed in the same solution without PFA during the day at 4 °C, followed by 0.24M phosphate buffer and 30% sucrose over night at 4 °C. The next day embryos were embedded in Tissue-Tek (Sakura Finetek) on dry ice and stored at -20 °C until sectioning. Organs of adult Swiss mice were isolated, rinsed in PBS and processed for RNA isolation and cryosectioning.

*RNA isolation and cDNA synthesis*

Total RNA from chick hearts of stage 19, 21 and 27 was isolated using Trizol reagent (Invitrogen), followed by chloroform extraction and ethanol precipitation. Subsequently mRNA was reverse transcribed into cDNA using Superscript II (Invitrogen) and random primers according to the suppliers' protocol.

For qPCR analysis, total RNA was isolated from gastrocnemius and soleus muscles of adult Swiss mice using Trizol reagent as describe above, followed by DNase-treatment (DNA-free, Ambion) and conversion to cDNA using iScript cDNA synthesis kit according to the suppliers' protocol (Bio-Rad). qPCR was performed using the CFX96 Real-Time PCR detection system (Bio-Rad). The following primers were used: C*hapA* (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.

*Cloning of full-length chick CHAP*

Using the mouse and human CHAP coding sequences, a chick *CHAP* orthologue (XM_421618) was identified after BLAST search. Full-length cDNA of chick *CHAP* was amplified by PCR (multiple clones) from chicken HH19-27 hearts and subsequently cloned into the pCRII-TOPO vector (Invitrogen). The full-length coding sequence of chick *CHAP* was confirmed by sequencing.

*Whole- mount and section in situ hybridization*

RNA probes were generated from PCR products cloned into pCRII-TOPO using T7 or SP6 primers. For generation of a chick *CHAP* probe template, the following primers were used: sense 5'- GGTCTCCCCTTTCTCACCTC-3' and antisense 5'-CACCACAAACTTGTCCATGC-3' (PCR product: 810 bp). Probes for detection of mouse *Chap* were generated as described previously [3]. Probes were digoxigenin-labeled according to the suppliers' protocol (Roche

Applied Science). For *in situ* hybridization 10 μm sections of chick or mouse embryos were mounted on superfrost slides (Menzel). *In situ* hybridization was performed as previously described [6]. Whole-mount *in situ* hybridization was performed as previously described [7].

*Immunofluorescence*
Mouse embryos and adult tissues were sectioned (5 μM) and mounted on starfrost slides (Knittel). Antibody staining on cryo-sections was performed as previously described [8]. Primary antibodies were as follows: anti-CHAP (rabbit, 1:50, custom made by Eurogentec), anti-myosin (mouse, 1:1000, NOQ7.5.4D, Sigma-Aldrich Chemie) and anti-α-smooth muscle actin (mouse, 1:500, 1A4, Sigma-Aldrich Chemie). Secondary antibodies were as follows: Cy-3 conjugated anti-mouse (1:250, Jackson Immunoresearch Laboratories) and Alexa488 conjugated anti-rabbit (1:200, Invitrogen). Cell nuclei were stained with DAPI (Molecular Probes)

*Immunohistochemistry*
Mouse 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 CHAP antibody (1:2000) 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). 3-3′diaminobenzidine tetrahydrochloride (D5637, Sigma-Aldrich) was used as chromogen and Mayer's hematoxylin as counterstaining. Finally, all slides were dehydrated and mounted with Entellan (Merck).

# Results and discussion

After BLAST search using full-length sequences of the previously identified *CHAP* orthologues (zebrafish, mouse, and human) [3], we identified the chick (Gallus gallus, Gg) orthologue of *CHAP* (GenBank accession no. XM_421618). For amplification, cloning and sequencing of chick *CHAP* we amplified the predicted full-length open reading frame (ORF) from hearts of chick embryos (HH19, 23 and 27), which resulted in a 3243 bp PCR product. After sequencing we found that the 1081 aa predicted chick CHAP protein (Fig. 1C) had a 48% and 47% homology with human (Hs) and mouse (Mm) CHAPa, respectively (Fig. 1A). Furthermore, there was a 43% and 40% homology with zebrafish Chap-1 (DrChap-1) and Chap-2 (DrChap-2), respectively (Fig. 1A). The NLS and PDZ domain are conserved between the different species. The chick PDZ domain of CHAP showed 63% and 61% homology with the PDZ domains of human and mouse, respectively. The *CHAP* gene is mapped on chromosome 6 and consists of four exons (Fig. 1B). Similar to zebrafish *chap*, no chick CHAPb (lacking the PDZ-domain) was identified. In addition to homology of sequence between different species, we also observed conserved syntenic organization. For instance, *myozenin-1*, another z-disc protein, is located immediately downstream of the mouse *Chap* gene [3]. A similar genomic organization was found in the chick genome.

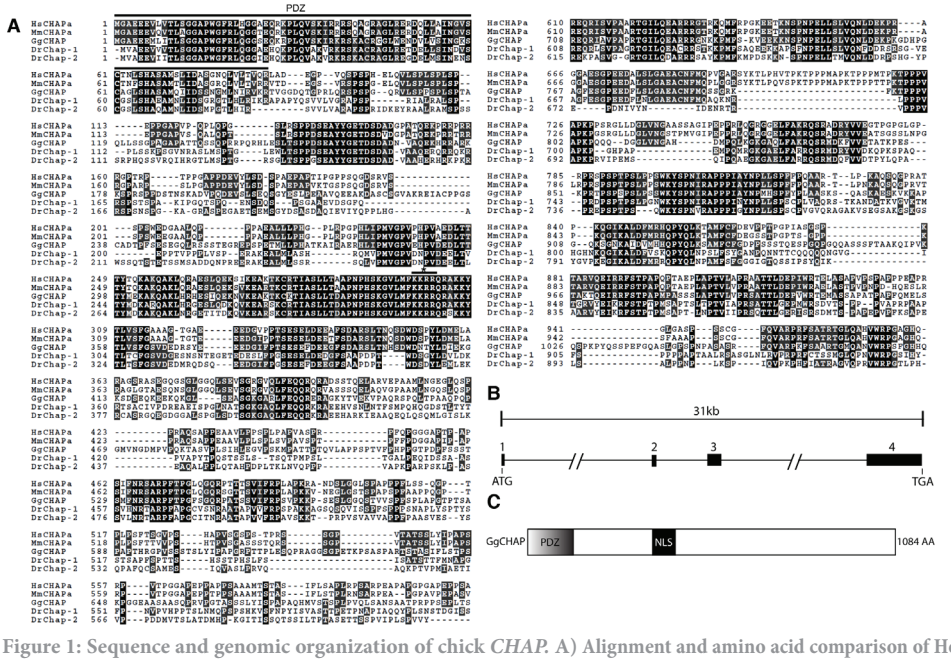


**Figure 1: Sequence and genomic organization of chick *CHAP*. A) Alignment and amino acid comparison of Homo sapiens (Hs), Mus musculus (Mm) CHAPa, Gallus gallus (Gg) and Danio rerio (Dr) CHAP. Identical amino acids are shown in black, similar amino acids are gray and gaps are represented by a dash. PDZ and NLS (*) domains are indicated. B) Genomic organization of chick CHAP gene. ATG represents start codon, TGA represents stop codon and exons (black boxes) are numbered. C) Schematic representation of chick (Gg) CHAP protein showing the location of the PDZ and NLS domains.**

*Expression of CHAP during development in chick and mouse embryos*

To investigate the expression pattern of chick *CHAP* during development we performed whole-mount *in situ* hybridization. Expression of *CHAP* could first be detected at stage HH8 in the cardiac crescent (Fig. 2A). At HH10 and HH11 *CHAP* was expressed in the linear and looping heart tube (Fig. 2B and C). *CHAP* could be detected in the ventricle and outflow tract (OFT), but not in the sinus venosus. At stage HH13, *CHAP* was strongly expressed in the looping heart (Fig. 2D). From HH15 onward *CHAP* was expressed in the heart and somites (Fig. 2E), with higher expression in the anterior somites, which are more mature than the posterior somites. At HH17 (Fig. 2F) *CHAP* was also expressed in the more posterior somites, suggesting that the expression increases as the somites mature. At stage HH17 (Fig. 2F) and stage HH21 (Fig. 2G and H) *CHAP* expression was maintained in the heart and somites. At stage HH25 *CHAP* was expressed in the muscle precursors of the limb buds, heart and somites (Fig. 2I and J). The expression of *CHAP* in muscle precursors of the limb buds of the developing legs and wings was more pronounced in embryos in stages HH28-30 (Fig. 2K, N and O). Hearts of HH30 showed a high expression of *CHAP* in both atria and ventricles, but not in the OFT (Fig. 2L and M). Comparison of expression pattern of *CHAP* during early embryonic development with that of mouse and zebrafish, demonstrate that the *CHAP* expression pattern is conserved between these species [3].

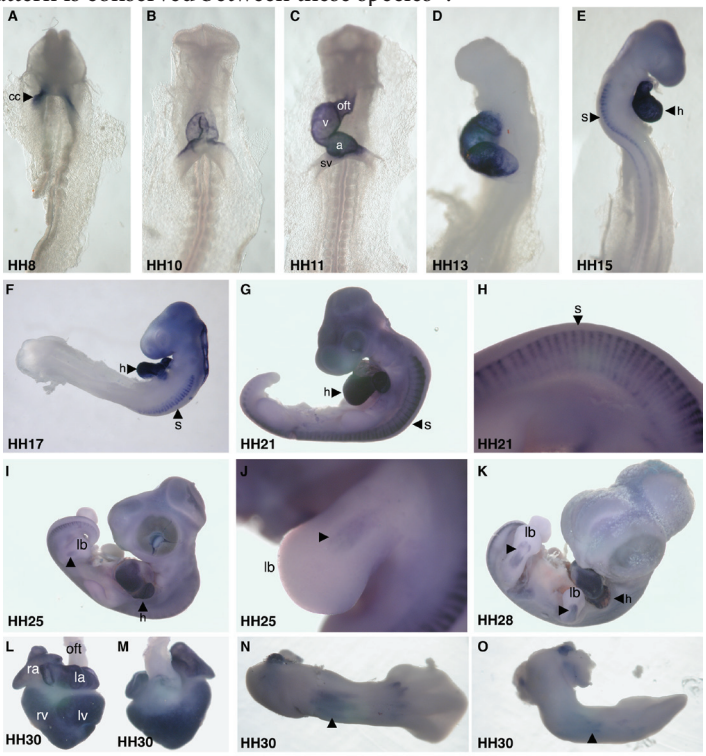


**Figure 2: Whole mount in situ hybridization of chick *CHAP* during development. A-O) Whole mount in situ hybridization with digoxigenin-UTP labeled chick *CHAP* riboprobe on different developmental stages of chick embryos: HH8 (A), HH10 (B), HH11 (C), HH13 (D), HH15 (E), HH17 (F), HH21 (G-H), HH25 (I-J), HH28 (K), HH30 heart (L-M), HH30 leg (N) and HH30 wing (O). Cardiac crescent (cc), ventricle (v), atrium (a) and outflow tract (oft), sinus venosus (sv), heart (h), somites (s), muscle precursors (arrow head) of the limb buds (lb) , left ventricle (lv), right ventricle (rv), left atrium (la), right atrium (ra).**

To study *CHAP* expression during chick development in more detail, we performed section *in situ* hybridization at different stages. At stage HH18 and HH26 *CHAP* was expressed in heart muscle cells and the myotome part of the somites (Fig. 3A-C), confirming the results of whole-mount *in situ* hybridization. Occasionally, higher levels of *CHAP* could be identified at the border with the dermatome, suggesting that *CHAP* is expressed in more mature cells of the somites, which is also in agreement with the higher expression of *CHAP* in more anterior somites. At stage HH30 expression of *CHAP* was localized in ventricles and atria, whereas endocardial cushions are negative for *CHAP* (Fig. 3D and G). Furthermore, trunk muscle masses (Fig. 3E and H), muscle groups from the limb buds of the developing wings (Fig. 3F), tongue, eyes and jaws show *CHAP* expression (Fig. 3I).

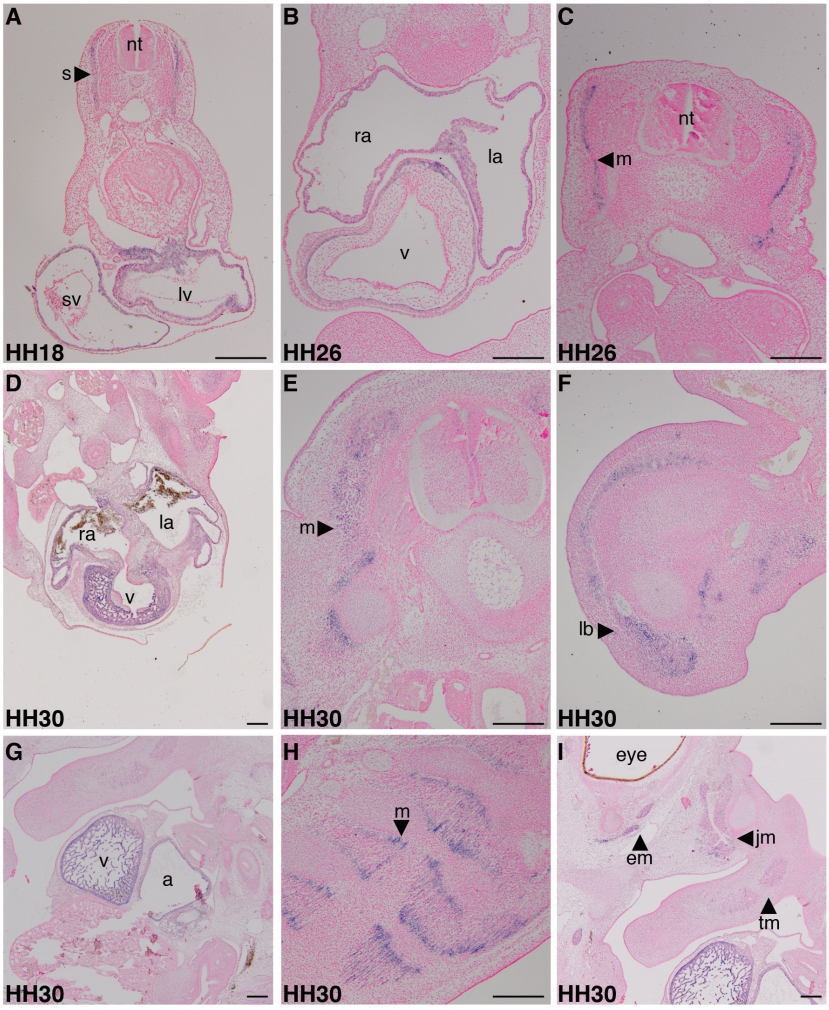


**Figure 3: In situ hybridization of *CHAP* chick on sections. A-I: In situ hybridization with digoxigenin-UTP labeled *CHAP* riboprobe was performed on sections of chick embryos on stages HH18 (A), HH26 (B-C) and HH30 (D-I). Somites (s), myotome (m), limb buds (lb), eye muscles (em), jaw muscles (jm) and tongue muscles (tm), sinus venosus (sv), left ventricle (lv), ventricle (v), left atrium (la), right atrium (ra), atrium (a), neural tube (nt). Scale bars: 250 µm.**

To compare CHAP expression in chick and mouse embryos, we performed *in situ* hybridization on sagittal sections of E13.5 mouse embryos, using a probe that recognizes both *ChapA* and *ChapB* isoforms. As expected, expression of *Chap* was pronounced in the ventricles and atria of the heart (Fig. 4A and B). Furthermore *Chap* was also expressed in the developing muscles of the tongue, jaw, limb buds and tail (Fig. 4A). Detection of *Chap* was considered to be expression of *ChapB*, since we have previously demonstrated that *ChapB* is the predominant isoform in embryonic and fetal stages [3]. In order to determine whether *Chap* mRNA expression was comparable with protein expression, we performed immunohistochemistry with an antibody that specifically recognizes CHAP (both a and b isoforms). In E12.5 embryos, CHAP protein was detected in heart, trunk muscle masses and muscles of the tongue, eyes and jaws, which was comparable to *Chap* mRNA expression (Fig. 4C and D). Furthermore, a striated expression pattern for CHAP, as expected for Z-disc proteins, was observed in muscle cells (Fig. 4E), confirming the specificity of the antibody staining.

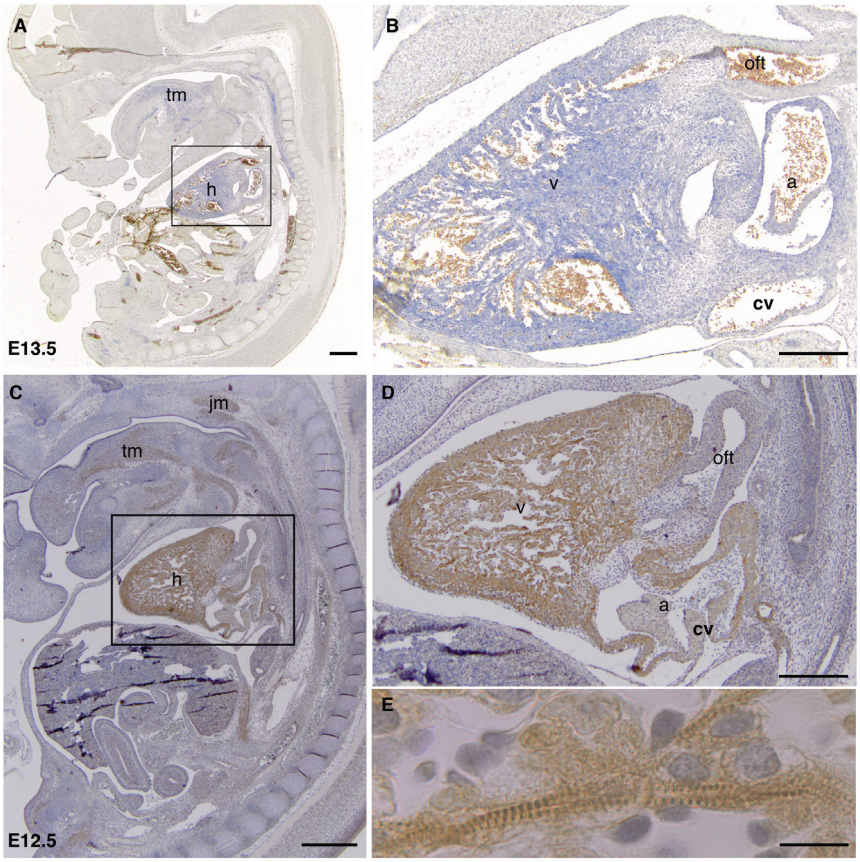


**Figure 4: Expression and localization of CHAP in mouse embryos. A-B) Section in situ hybridization with digoxigenin-UTP labeled mouse *Chap* riboprobe was performed on sagittal sections of E13.5 mouse embryos. A higher magnification of *Chap* expression in the heart (box in A) is shown in (B). C-E: Immunostaining for CHAP on sagittal sections of E12.5 mouse embryos (C). Magnification of C (box) is shown in (D). A higher magnification (E) of heart shown in (D) displays a sarcomeric pattern for CHAP. Heart (h), jaw muscle (jm), tongue muscle (tm), ventricle (v), atrium (a), cardinal vein (cv), and outflow tract (oft). Scale bars in A/C 500 µm, in B/D 250 µm and in E 10 µm.**

*Expression of CHAP in adult skeletal muscle*

We have previously shown that both CHAPa and CHAPb are expressed in adult mouse heart and skeletal muscle [3]. Here, we investigated the expression of CHAP in adult skeletal muscles in more detail. Skeletal muscle can be divided into slow (type I) and fast (type II) twitch fibers. To determine the expression levels of both *Chap* isoforms, we isolated slow (soleus) and fast (gastrocnemius) muscles of adult mice. qPCR analysis of these tissues showed that both *ChapA* (Fig. 5, black bars) and *ChapB* (Fig. 5, white bars) were expressed higher in soleus than in gastrocnemius. Expression of *ChapA* was approximately 10-fold higher than

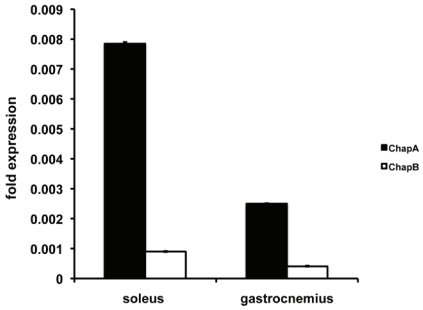


**Figure 5: ChapA and ChapB expression in adult skeletal muscle. Quantitative real-time PCR expression of *ChapA* and *ChapB* mRNA in soleus (slow) and gastrocnemius (fast) muscles. *Gapdh* used as internal control.**

*ChapB* expression in both soleus and gastrocnemius. To confirm expression at the protein level, we performed CHAP antibody staining in combination with myosin, a slow muscle fiber marker, on sections of mouse soleus and gastrocnemius. Indeed, CHAP protein was expressed in both soleus and gastrocnemius muscle cells (Fig. 6), confirming the results of the qPCR. Although *Chap* mRNA levels were approximately 3-fold higher in soleus muscle, immunofluorescence did not show obvious differences for CHAP protein expression between soleus and gastrocnemius muscles. However, more quantitative proteins assays would be necessary to confirm this.

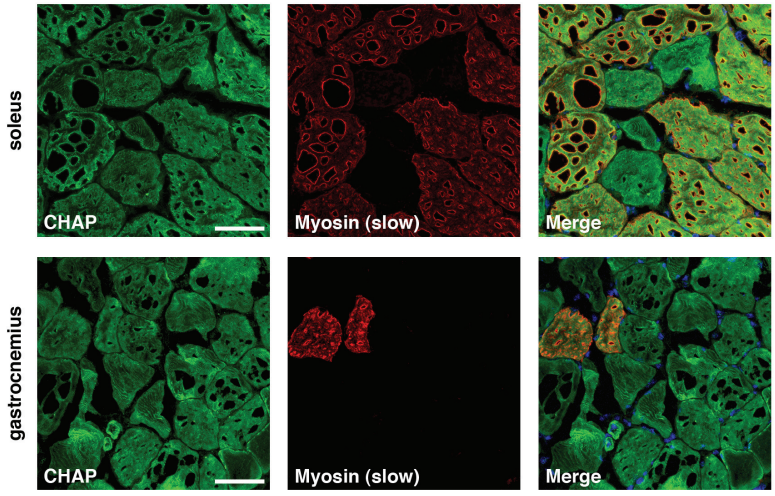


**Figure 6: Expression and localization of CHAP in adult fast and slow muscle. Immunostaining on cross-sections of mouse soleus (slow, upper panel) and gastrocnemius (fast, lower panel) using anti-CHAP (green) and anti-myosin (red) antibodies. Myosin staining (red) was used as a marker for slow muscle fibers, and DAPI was used to stain nuclei (blue). Scale bars: 50 µm.**

*CHAP is expressed in smooth muscle cells*

We have previously observed CHAP expression at the Z-disc in cardiomyocytes of adult mice. More detailed examination indicated CHAP expression in smooth muscle cells. To confirm this observation we performed immunostainings for CHAP and α-smooth muscle actin (ASMA), a smooth muscle marker (but also expressed in cardiomyocytes during early cardiac development), on sections of embryonic (E17.5), adult mouse heart, carotid arteries and aorta. We observed that besides expression in cardiomyocytes in E17.5 mouse embryos CHAP was also expressed in the vena cava, but not in the aorta (data not shown), carotid arteries (Fig. 7A) and subclavian artery (Fig. 7B and C). A higher magnification (Fig. 7B and C) showed that CHAP is expressed in a striated pattern and co-localized with ASMA-positive cells, suggesting that CHAP is expressed in cardiomyocytes from the vena cava. It has been demonstrated previously that cardiomyocytes are present in the tunica media of the vena cava during embryonic development [9, 10]. However, in adult tissues CHAP expression was also observed in smooth muscle cells of aorta, carotid arteries and coronary arteries (Fig. 8). Although CHAP and ASMA are expressed in the same cells, the subcellular localization of both proteins is clearly different.

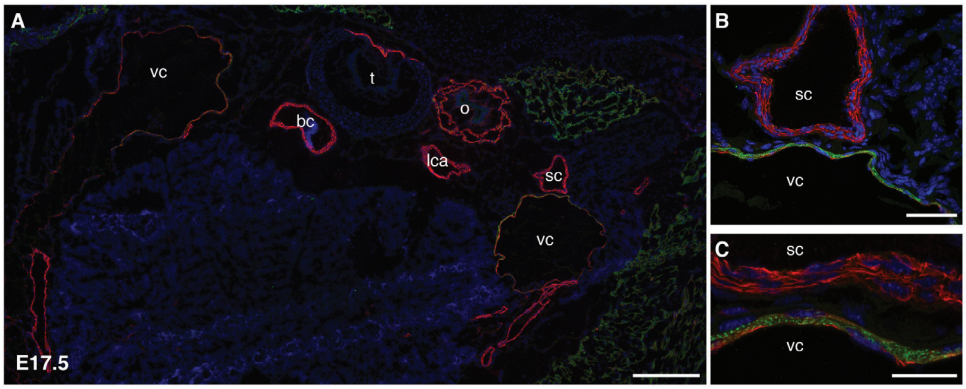


**Figure 7: Co-localization of CHAP with α-smooth muscle actin (ASMA) in cardiomyocytes of the vena cava. Immunostaining for CHAP (green) and α-smooth muscle actin (red) on cross-sections of E17.5 mouse embryos (A-C). DAPI was used to stain nuclei (blue). Vena cava (vc), brachiocephalic artery (bc), left carotid artery (lca), subclavian artery (sc) trachea (t), and oesophagus (o). Scale bars: in A 250 µm, B 50 µm and C 20 µm.**

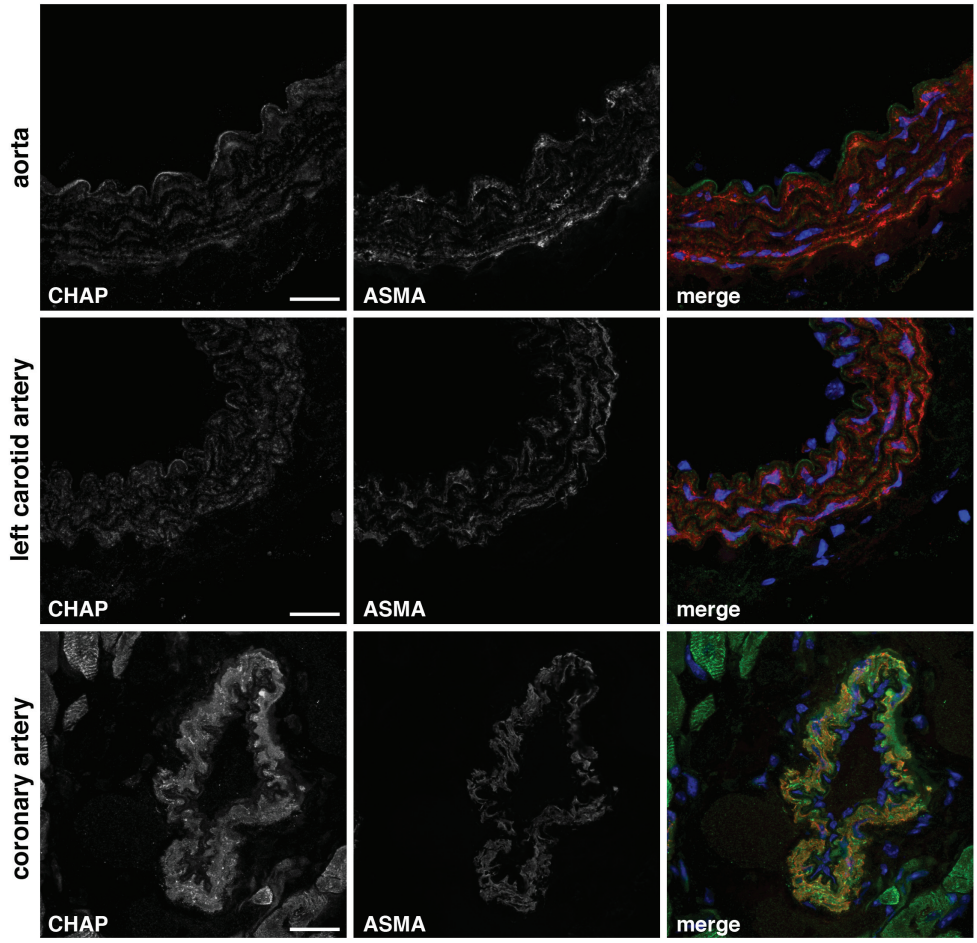


**Figure 8: Expression and localization of CHAP in adult smooth muscle cells. Immunostaining for CHAP (green) and α-smooth muscle actin (ASMA; red) on cross-sections of adult mouse aorta, left carotid artery and coronary artery. DAPI was used to stain nuclei (blue). Scale bars: 20 μm.**

*Conclusion*

In the present study we identified and characterized chick CHAP which has significant homology to the human and mouse CHAPa isoform and zebrafish Chap-1 and -2, and contains highly conserved motifs such as the N-terminal PDZ domain and the NLS. Expression of both chick *CHAP* and mouse *Chap* mRNA and protein were detected in heart and skeletal muscle (such as limbs, jaw, eye and tongue) throughout embryonic development. Furthermore, we demonstrate in the present study that CHAP is also expressed in cardiomyocytes from the vena cava during mouse embryonic development and in smooth muscle cells of aorta, and coronary and carotid arteries of adult mice. It has been shown previously that multipotent cardiovascular progenitor cells, marked by transcription factors Isl-1 and Nkx2.5, have the capacity to differentiate to vascular smooth muscle cells and cardiomyocytes. [11, 12]. Since cardiomyocytes and smooth muscle cells are derived from different origins during development, this suggests that CHAP is expressed in different cardiovascular progenitor cells.

Conserved predicted motifs, syntenic organization and developmental expression patterns, suggest that CHAP may play an important role during muscle development. Indeed, knockdown of *chap* by antisense oligonucleotides in zebrafish had shown previously decreased cardiac contractility, defects in cardiac looping and myofibrillar disarray in muscle cells during embryonic development [3]. CHAP displays homology to synaptopodin and myopodin, which have both been shown to function in actin/α-actinin binding and /or signaling [13-15]. Whereas synaptopodin is expressed in kidney and brain in an isoform specific manner [15], expression of myopodin can be found in smooth, skeletal and heart muscle cells, which is comparable to that of CHAP. Like CHAP, myopodin is localized at the Z-disc of striated muscles and is able to translocate to the nucleus [16, 17]. It has been shown for myopodin that trafficking between the Z-disc and the nucleus is dependent on PKA and CamKII kinase and calcineurin phosphatase activities [18]. Future studies will elucidate whether similar pathways control CHAP trafficking in muscle cells. Furthermore, it would be of interest to study the function and mechanism of action of CHAP in physiological and healthy and pathophysiological situations related to striated and smooth muscle cells. In humans, hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) have been associated with mutations in cytoskeletal and sarcomeric proteins [19, 20]. In addition, it has been shown in different mouse models that mutations in sarcomeric proteins such as α-myosin heavy chain [21, 22], myosin binding protein C [23, 24] and cardiac troponin T [25] can cause HCM-like phenotypes. The sarcomeric protein CHAP may represent a novel candidate gene for screening mutations in DCM and HCM patients.

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