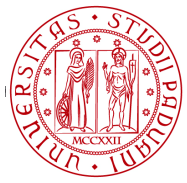

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CICLO XXV

Role of the scaffolding protein Homer 1a in cardiac hypertrophy

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Abbreviations

ANF	Atrial Natriuretic Factor
Ang-II	Angiotensin II
AP	Alkaline Phosphatase
APS	Ammonium Persulfate
AR	Adrenergic Receptor
BCA	Bicinchoninic Acid
cAMP	Cyclic Adenosine Monophosphate
CaN	Calcineurin
DAG	Diacylglycerol
DMSO	Dimethylsulfoxide
EDTA	Ethylene Diamine Triacetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
ERK/p-ERK	Extracellular Signal-Regulated Kinase /Phosphorylated Extracellular Signal-Regulated Kinase
ET-1	Endothelin-1
EVH1	Enabled/VASP (Vasodilator-Stimulated Phosphoprotein) Homology 1
FCS	Fetal Calf Serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GPCR	G-Protein-Coupled Receptor
HBSS	Hank's Balanced Salt Solution
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish Peroxidase
IEG	Immediate Early Gene
IP3	Inositol 1,4,5-Trisphosphate
IP3R	Inositol 1,4,5-Trisphosphate Receptor
ISO	Isoproterenol
LV	Left Ventricle
MAPK	Mitogen-Activated Protein Kinase
MCT	Monocrotaline
mGLUR	Metabotropic Glutamate Receptor
MHC	Myosin Heavy Chain
NE	Norepinephrine
NFAT	Nuclear Factor of Activated T Cell
PBS	Phosphate Buffered Saline
PE	Phenylephrine
PFA	Paraformaldehyde
PI3K	Phoshoinositide 3-Kinase
PKA	Protein Kinase A

PKC	Protein Kinase C
PLC	Phospholipase C
PMSF	Phenylmethanesulfonyl Fluoride
PRA	Prazosin
pRL-TK	*Renilla*- Thymidine Kinase plasmid
PRO	Propranolol
RV	Right Ventricle
RyR	Ryanodine receptor
SDS	Sodium Dodecyl Sulfate
SR/ER	Sarco/Endoplasmic Reticulum
TAC	Transverse Aortic Constriction
TBSt	Tris Buffered Saline with Tween
TEMED	Tetramethylethylenediamine
TRPC	Transient Receptor Potential Canonical (protein)

Summary

Homer proteins are a family of scaffolding proteins involved in many intracellular signaling pathways, in both excitable and non-excitable cells. These proteins participate in the assembly and regulation of functional signaling complexes, facilitating the cross-talk between surface membrane receptors and channels in the membranes of intracellular compartments (Worley PF. *et al.*, 2007). Homer proteins are constitutively expressed in the brain, where their scaffolding function is important for a variety of neuronal processes, such as intracellular Ca^{2+} homeostasis, synaptic plasticity associated with learning and memory in the mature brain, and neuronal development of the embryonic brain (Xiao B. *et al.*, 1998; Worley PF. *et al.*, 2007; Foa L. *et al.*, 2009). Among the Homer splice variants, Homer 1a isoform acts as a natural dominant-negative by disassembling signalling complexes mediated by other Homer isoforms. The Homer 1a gene is transcribed as an immediate early gene (IEG), in neuronal cells its expression is low under normal conditions and increases rapidly after neuronal activation (Brakeman PR. *et al.*, 1997). Homers proteins are also expressed in cardiac muscle, but their regulation and function remain still poorly understood. Despite their important role as regulators of multimeric signalling complex in nervous system, few reports have focused on the role of Homers in the heart. It has been reported that mRNA coding for Homer 1a rapidly and transiently increases in neonatal cardiomyocytes upon stimulation with either endothelin-1 (ET1) or other hypertrophic agonists (Kawamoto T. *et al.*, 2006). The Homer 1a protein levels are also up-regulated following AngII-induced hypertrophy in neonatal cardiomyocytes (Guo WG. *et al.*, 2010). Recently, it has been demonstrated that the variant Homer 1b/c positively regulates α_1-adrenergic dependent hypertrophy, whereas Homer 1a is able to antagonize such effect (Grubb DR. *et al.*, 2011).

This study investigated the role of Homer 1a in the cardiac hypertrophic program. Our working hypothesis is that Homer 1a may be one of the molecular modulators of cardiac hypertrophy. For this purpose, we studied the presence, sub-cellular distribution and function of Homer1a in cardiac muscle. Under resting conditions we found that Homer 1a is constitutively expressed in cardiac muscle of both mouse and rat and in HL-1 cells (a specific cardiac cell line). In addition, using immunofluorescence confocal microscopy of adult rat heart sections, we showed that Homer 1a displays a peculiar localization: it is sarcomeric and peri-nuclear.

We also analyzed Homer 1a expression under hypertrophic conditions. For this purpose, we used rat neonatal cardiomyocytes stimulated with the adrenergic agonist norepinephrine (NE). A significant increase in both Homer1a mRNA and protein was found after NE stimulation, whereas Homer 1b/c (a different Homer 1 isoform) expression remained unchanged. In this hypertrophic cellular model, we studied the adrenergic pathways involved in NE-inducted Homer 1a up-regulation by using specific α_1- and β- adrenergic receptor blockers (prazosin and propranolol, respectively). The results showed that prazosin - but not propranolol - drastically reduced NE-induced up-regulation of Homer 1a mRNA, demonstrating that the α_1-adrenergic pathway is involved. The effect of hypertrophic stimulation on Homer 1a expression was also confirmed in NE-stimulated HL-1 cardiomyocytes. In this cell line we found that 1 hour after NE stimulation Homer 1a content increased by a factor of 2.5. Overall, these results confirm our working hypothesis and demonstrate the involvement of Homer 1a in the α_1-adrenergic pathway leading to cardiac hypertrophy.

In the second part of the study we analyzed the effects of Homer 1a over-expression monitoring different hypertrophic markers, such as MAPK/ERK1/2 phosphorylation, NFAT nuclear translocation, ANF-promoter activity and increase in cell size. The results showed that during NE stimulation Homer 1a modulated many of them (except for NFAT nuclear translocation that did not appear to be affected by Homer 1a over-expression), whereas under resting conditions Homer 1a over-expression *per sè* was ineffective. In particular, we found that, in NE-stimulated HL-1 cells, over-expressed Homer 1a significantly reduced phosphorylation levels of ERK1/2 by about 40%, negatively modulating MAPK pathway. As regards the ANF promoter activity, this activity was significantly reduced by about 20% in NE-stimulated Homer 1a over-expressing cells. In order to verify the specificity of the Homer 1a effect on ANF, we performed the same experiment over-expressing Homer 1c and we found that, unlike Homer 1a, Homer 1c did not modulate the activity of ANF promoter in NE-stimulated HL-1 cells. Subsequently, we assessed the effect of Homer 1a over-expression on increase in cell size. The results obtained showed that Homer 1a counteracted the increase in NE-stimulated cell size.

Finally, a preliminary analysis, *in vivo*, of Homer 1a expression was performed in three hypertrophic models, i.e. mice with chronic transverse aortic constriction, transgenic mice over-expressing Gαq and rats treated with monocrotaline. At variance with results observed in cellular models *in vitro*, in these models Homer 1a expression did not result

affected by hypertrophic conditions, at least in the time span under investigation. However, for this approach *in vivo*, a broad time-course is needed and, therefore, further analyses are required.

In summary, our data on Homer 1a presence and sub-cellular localization in cardiac tissue demonstrate that Homer 1a is constitutively expressed and displays a sarcomeric and peri-nuclear distribution. In our cellular models *in vitro*, Homer 1a up-regulation is an early event of the NE-induced hypertrophy and, as inferred from gain-of function studies, Homer 1a isoform antagonizes initiation and development of NE-induced events leading to α_1-adrenergic-dependent hypertrophy.

In conclusion, our results *in vitro* indicate that Homer 1a is inserted into a negative feedback mechanism in which acts as negative molecular modulator, counteracting early steps of hypertrophy. However, further studies are needed to elucidate the mechanisms underlying this process.

Sommario

Le proteine Homer sono una famiglia di proteine coinvolte in molte vie di trasduzione del segnale intracellulare, in cellule eccitabili e non eccitabili. Queste proteine partecipano nell'assemblaggio e nella regolazione di complessi funzionali di 'signalling', facilitando il 'cross-talk' tra recettori della membrana plasmatica e canali posti sulle membrane dei compartimenti intracellulari (Worley PF. *et al.*, 2007). Le proteine Homer sono costitutivamente espresse nel cervello, dove svolgono la funzione di 'scaffold' in molti processi neuronali, quali ad esempio l'omeostasi del calcio intracellulare, la plasticità sinaptica associata all'apprendimento ed alla memoria nel cervello maturo, lo sviluppo embrionale del cervello (Xiao B. *et al.*, 1998; Worley PF. *et al.*, 2007; Foa L. *et al.*, 2009). Tra le diverse varianti di splicing alternativo, l'isoforma Homer 1a agisce da dominante negativo disassemblando i complessi di 'signalling' formati dalle altre isoforme Homer. Il gene Homer 1a è trascritto come gene immediato precoce, la sua espressione nelle cellule neuronali è bassa in condizioni basali ed aumenta rapidamente in seguito ad attivazione neuronale (Brakeman PR. *et al.*, 1997). Le proteine Homer sono espresse anche nel muscolo cardiaco, ma la loro regolazione e la loro funzione è ancora poco conosciuta. Nonostante l'importanza degli Homer come proteine regolatrici di complessi coinvolti nelle vie di trasduzione del segnale, pochi studi si sono focalizzati sul loro ruolo nel cuore. A tal riguardo, è stato riportato che l'mRNA codificante per Homer 1a aumenta rapidamente e transientemente in colture di cardiomiociti neonatali in seguito a stimolazione con endotelina-1 ed con altri agonisti ipertrofici (Kawamoto T. *et al.*, 2006). Un successivo lavoro ha evidenziato che, in condizioni di ipertrofia indotta da angiotensina II, anche i livelli di espressione della proteina Homer 1a risultano up-regolati in colture di cardiomiociti neonatali (Guo WG. *et al.*, 2010). Un recente studio ha, invece, dimostrato che l'isoforma Homer 1b/c regola positivamente l'ipertrofia dovuta a stimolazione α-adrenergica, mentre l'isoforma Homer 1a antagonizza tale effetto (Grubb DR. *et al.*, 2011).

In questo studio abbiamo esaminato il ruolo della proteina Homer 1a nell'ipertrofia cardiaca. La nostra ipotesi di lavoro è che la proteina Homer 1a sia un modulatore molecolare dell'ipertrofia. A tal fine, abbiamo studiato la presenza, la localizzazione sub-cellulare e la funzione di Homer 1a nel muscolo cardiaco.

Analizzando l'espressione di Homer1a in condizioni normali è emerso che la proteina Homer 1a è espressa costitutivamente nel muscolo cardiaco di topo e ratto e nelle cellule HL-1 (una specifica linea cellulare cardiaca). Mediante immunofluorescenze su sezioni di cuore di ratto adulto (analizzate utilizzando il microscopio confocale) abbiamo esaminato la localizzazione sub-cellulare di Homer 1a che risulta essere sarcomerica e perinucleare. Successivamente, abbiamo analizzato l'espressione di Homer 1a in condizioni ipertrofiche; per questa analisi sono stati utilizzati cardiomiociti neonatali di ratto stimolati con l'agonista adrenergico norepinefrina (NE). In questo sistema sperimentale, abbiamo riscontrato un aumento significativo sia dell'mRNA che della proteina Homer 1a in seguito alla stimolazione con NE, mentre non abbiamo rilevato nessuna variazione sull'espressione della proteina Homer 1b/c (una diversa isoforma degli Homer). In cardiomiociti in coltura stimolati con NE, sono state, inoltre, analizzate le vie di trasduzione del segnale adrenergico coinvolte nell'up-regolazione di Homer 1a indotta da NE, usando specifici inibitori dei recettori α_1- and β- adrenergici (prazosin e propanololo, rispettivamente). I risultati ottenuti hanno evidenziato che il prazosin, ma non il propranololo, drasticamente riduce l'up-regolazione dell'mRNA di Homer 1a indotta da NE, dimostrando che la via di trasduzione del segnale α_1-adrenergico è coinvolta. L'effetto della stimolazione ipertrofica sull'espressione di Homer 1a è stato confermato anche su cellule HL-1 stimolata con NE. In questa linea cellulare abbiamo osservato che un'ora dopo la stimolazione con NE la proteina Homer 1a aumenta di un fattore 2,5. Complessivamente, questi risultati confermano la nostra ipotesi di lavoro e dimostrano il coinvolgimento della proteina Homer 1a nella trasduzione del segnale α_1-adrenergico che induce ipertrofia cardiaca.

Nella seconda parte di questo studio abbiamo esaminato gli effetti dell'over-espressione di Homer 1a monitorando diversi markers ipertrofici, quali la fosforilazione delle proteine MAPK/ERK1/2, la traslocazione nucleare di NFAT, l'attivazione del promotore di ANF e l'aumento delle dimensioni cellulari. I risultati hanno dimostrato che durante la stimolazione con NE Homer 1a modula la maggior parte di questi (eccezion fatta per la traslocazione nucleare di NFAT che non risulta essere variata dall'over-espressione di Homer 1a), al contrario in condizioni basali (senza stimolazione con NE) l'over-espressione di Homer 1a di per sé non ha alcun effetto. Nello specifico, i risultati ottenuti hanno rilevato che in cellule HL-1 stimolate con NE la proteina Homer 1a over-espressa significativamente riduce i livelli di fosforilazione delle proteine ERK1/2 di circa il 40%, modulando negativamente la via di trasduzione del segnale MAPK/ERK1/2.

Per quanto concerne l'attività promotoriale di ANF, questa attività è significativamente ridotta di circa il 20% nelle cellule HL-1 over-esprimenti Homer 1a e stimolate con NE. Al fine di verificare la specificità di questo effetto sul promotore ANF, abbiamo condotto lo stesso esperimento over-esprimendo l'isoforma Homer 1c ed abbiamo riscontrato che, diversamente da Homer 1a, la proteina Homer 1c non ha alcun effetto sull'attività del promotore ANF in cellule HL-1 stimolate con NE. Successivamente, abbiamo analizzato l'effetto dell'over-espressione di Homer 1a sull'aumento delle dimensioni cellulari durante stimolazione con NE. I risultati ottenuti hanno dimostrato che la proteina Homer 1a è in grado di bloccare significativamente l'aumento delle dimensioni delle cellule HL-1 stimolate con NE.

Nell'ultima parte di questo lavoro, abbiamo condotto un'analisi preliminare, *in vivo*, dell'espressione della proteina Homer 1a in tre modelli di ipertrofia, quali topi con costrizione trasversale dell'aorta, topi transgenici over-esprimenti Gαq e ratti trattati con monocrotalina. Diversamente da quanto ottenuto nel modello cellulare *in vitro*, in questi modelli l'espressione della proteina Homer 1a non risulta alterata dalle condizioni ipertrofiche, almeno nell'intervallo di tempo considerato. Tuttavia, per quanto riguarda questo approccio *in vivo*, sarà necessario analizzare l'espressione della proteina Homer 1a in un intervallo di tempo più ampio e, di conseguenza, ulteriori analisi sono richieste.

In sintesi, dai nostri risultati relativi alla presenza ed alla localizzazione sub-cellulare di Homer 1a nel tessuto cardiaco è emerso che la proteina Homer 1a è costitutivamente espressa e mostra una localizzazione sarcomerica e peri-nucleare. Nei nostri modelli cellulari *in vitro*, l'up-regolazione di Homer 1a è un evento precoce dell'ipertrofia indotta da NE e, come dimostrato dagli studi di gain-of fuction, la proteina Homer 1a è in grado di antagonizzare l'avvio e lo sviluppo degli eventi che portano all'ipertrofia α_1- adrenergica dipendente.

Concludendo, i nostri dati *in vitro* indicano che Homer 1a è inserito in un meccanismo di feedback negativo in cui agisce come modulatore negativo, bloccando gli steps precoci dell'ipertrofia cardiaca. Tuttavia, ulteriori studi sono necessari per definire il meccanismo alla base di questo processo.

1. Introduction

1.1 The Homer proteins family

Homer proteins, also known as Vesl (VASP/Ena-related gene up-regulated during seizure and long term potentiation), are a family of scaffolding proteins which are involved in many intracellular signaling pathways, in both excitable and non-excitable cells (Worley PF. *et al.*, 2007). These proteins participate in the assembly and regulation of functional signaling complexes allowing cross-talk between surface membrane receptors and channels in the membranes of intracellular compartments (Fagni L. *et al.*, 2002).

In the late 1990s, the first Homer cDNA (Homer 1a) was isolated in hippocampal and cortical neurons by Brakeman et al. Homer 1a variant was initially identified after the induction of excitatory synaptic activity, as well as during both neuronal long-lasting plasticity and development (Brakeman PR. *et al.*, 1997, Kato A. *et al.*, 1997). The Homer 1a isoform, also called 'short', is a 186 amino acids long protein which presents a short carboxy-terminal (C-terminal) extension. The Homer 1a gene is transcribed as an immediate early gene (IEG): its expression in neuronal cells is low under normal conditions and increases rapidly after their stimulation (Brakeman PR. *et al.*, 1997).

Subsequent molecular cloning and sequence studies have revealed that there are three Homer genes - *Homer 1*, *Homer 2* and *Homer 3* - in mammals, each of which encodes for several transcripts (Soloviev MM. *et al.*, 2000a, Kato A. *et al.*, 1998, Xiao B. *et al.*, 1998). Table 1 summarizes all isoforms known and their relative molecular weights. Apart from the short Homer 1a, all other Homer proteins (called 'long' isoforms) present a well-characterized C-terminal domain (Xiao B. *et al.*, 1998). The long variants are constitutively expressed in the brain where their scaffolding function plays an important role in a variety of neuronal processes, such as intracellular Ca^{2+} homeostasis, synaptic plasticity associated with learning and memory in the mature brain, and neuronal development of the embryonic brain (Xiao B. *et al.*, 1998; Worley PF. *et al.*, 2007; Foa L. *et al.*, 2009).

Even though the functional importance of Homers as scaffolding in the nervous system has been well described, the precise function of Homer proteins in other tissues is still poorly understood. Besides the brain, Homer proteins are predominantly expressed in muscle tissues, heart and skeletal muscle (Xiao B. *et al.*, 1998; Sandonà D. *et al.*, 2000). Considering its important function in neuronal cells, it is plausible to hypothesize that

Homer 1 isoforms can also have a fundamental role in many of the signaling pathways in these muscle tissues. However, as regards the heart, few reports have discussed the function of Homers and its specific role in molecular events, both in cardiac physiology and pathophysiology, is still unclear.

Homer gene	Homer isoforms	Amino acids	Molecular Weight (kDa)	References
Homer 1	1a	186	28	*Brakemann PR et al., 1997*
	1b	354	47	*Xiao B et al., 1998*
	1c	366	47	*Xiao B et al., 1998*
	1d	370	48	*Saito H et al., 2002*
	1e	224	26	*Bottai D. et al., 2002*
	1f	180	21	*Klugmann M et al., 2005*
	1g	192	22	*Klugmann M et al., 2005*
	1h	238	20	*Klugmann M et al., 2005*
Homer 2	2a	343	47	*Xiao B et al., 1998*
	2b	354	47	*Xiao B et al., 1998*
	2c	171	29	*Soloviev MM et al., 2000*
	2d	182	29	*Soloviev MM et al., 2000*
Homer 3	3a00	352	48	*Soloviev MM et al., 2000*
	3a01	355	48	*Soloviev MM et al., 2000*
	3a10	355	48	*Soloviev MM et al., 2000*
	3a11	358	48	*Soloviev MM et al., 2000*
	3b00	316	45	*Soloviev MM et al., 2000*
	3b01	319	45	*Soloviev MM et al., 2000*
	3b10	319	45	*Soloviev MM et al., 2000*
	3b11	322	45	*Soloviev MM et al., 2000*
	3c	145	16	*Soloviev MM et al., 2000*
	3d	121	14	*Soloviev MM et al., 2000*

Table 1: Homer Isoforms and Molecular Weight.

1.2 The structure of Homer proteins

Homer family proteins present two main structural features: the conserved amino-terminal domain (N-terminal) and the Homer-specific carboxy-terminal domain (C-terminal) (Tu JC. *et al*., 1998; Kato A. *et al*., 1998) (fig. 1).

All Homer isoforms possess a highly conserved N-terminal domain, with a similarity of 60-70% between amino acid sequences of the different isoforms (Soloviev MM. *et al.*, 2000a).

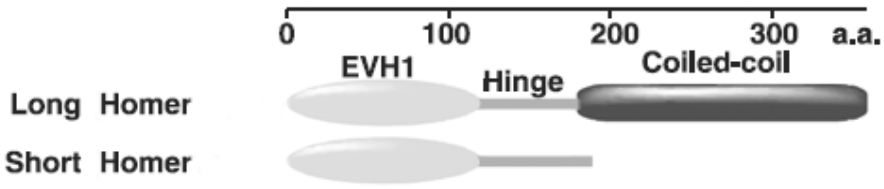


Figure 1. Homer structure. The N-terminal EVH1 domain is present in both the long and short Homer variants, whereas the C-terminal coiled coil domain is present only in long Homer isoforms. Modified from Hayashi MK *et al* 2006.

The N-terminal domain is homologous (26-30% identical amino acids) to the EVH1/WH1 domain (Ena/VASP homology 1/Wiskott-Aldrich syndrome protein homology 1), which is present in the Ena/vasodilator-stimulated phosphoprotein family (Kato A. *et al.*, 1997). Homer/EVH1 domain is a protein-protein binding module that specifically recognizes proline-rich motifs. The Homer ligand consensus motif is PPxxF, where x is any amino acid (Tu JC. *et al.*, 1998). The PPxxF ligand consensus sequence is present in many signaling molecules, including metabotropic glutamate receptor (mGluR) (Kato A. *et al.*, 1998), inositol tri-phosphate receptor (IP3R) (Yuan JP. *et al.*, 2003), ryanodine receptors (RyRs) (Feng W. *et al.*, 2002), Shank proteins (Tu JC. *et al.*, 1999), transient receptor potential canonical channels (TRPC) (Yuan JP. *et al.*, 2003), L-Type Ca-channels (Huang G. *et al.*, 2007) and various transcription factors (Cooper ST. *et al.*, 2005; Huang GN. *et al.*, 2008). Through the EVH1 domain, Homer proteins bind directly to proline-rich motifs of other scaffolding proteins or of signaling transduction molecules, thereby cross-linking and modulating their activity (fig. 2). For example, the long Homers are able to form a link between mGluR1/5, located in the plasma membrane, and the downstream effector IP3R located in the endoplasmic reticulum (Tu *et al.*, 1998). Crystallographic analysis of the Homer/EVH1 domain, both alone and complexed with a bound peptide, has been demonstrated that this domain binds ligands in a unique manner that distinguishes it from other EVH1 domains; in this way, the Homer-ligand binding minimizes potential cross-reactions with non-permissive proline-rich sequences (Beneken J. *et al.*, 2000).

The C-terminal domain of Homer proteins has only about 20% sequence identity among the different isoforms, and contains a coiled-coil (CC) domain followed by two leucine zipper motifs (Zip A consists of 34 amino acids and Zip B of 28 amino acids) (Soloviev MM. *et al.*, 2000a; Xiao B. *et al.*, 1998; Takodoro S. *et al.*, 1999). This domain mediates homophilic or heterophilic interactions within the Homer family (Beneken J. *et*

al., 2000). Crystallographic analysis of Homer 1 isoforms has revealed that Homer proteins form dimers via leucine zipper motifs at the C-terminal coiled-coil domain. Two dimers can then intercalate in a tail-to-tail fashion to form a tetramer. The tetramer is only formed by the 'long' Homer isoforms that possess the CC-domain. This tetramerization plays an important role in allowing four EVH1 domains to be exposed in an optimized configuration for ligand binding (Hayashi MK. *et al.*, 2006). The short Homers (Homer 1a and Ania-3), in contrast, are monomers - lacking the CC-domain, they contain the EVH1 domain alone, and do not self-associate (Foa L. *et al.*, 2009) (fig. 2). As a consequence of their distinct structural features, the long and short Homer isoforms participate in the formation and regulation of signaling transduction complexes in different ways.

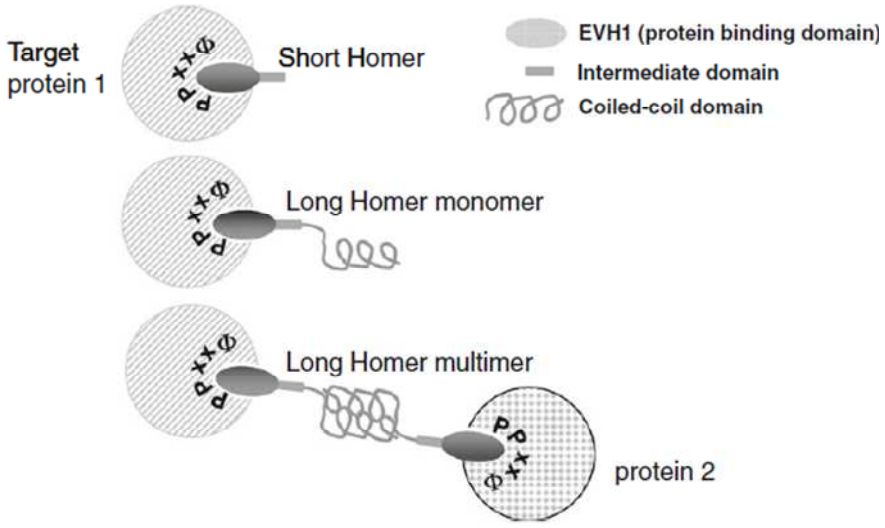


Figure 2. Distinct structural features between long and short Homers. Short Homer variant can bind to the same target protein 1 via EVH1-domain as the long isoform, but does not self-multimerize. Long Homer isoforms are able to multimerize via coiled-coil domain and bind to two different target proteins. From Pouliquin P. *et al.* 2009a.

For example, the long Homer 1 isoforms are able to multimerize via its C-terminal domain and bind to two different target proteins through their EVH1 domain. Conversely, short Homer 1 isoform (Homer 1a) can bind to the same target proteins via its conserved N-terminal domain as the long isoform, but does not multimerize and, thus, avoids the linkage of two target proteins (Fig. 2). Homer 1a therefore acts as a dominant-negative protein, interfering with Homer-multimerization and disassembling Homer-mediated complexes (Tu JC. *et al.*, 1998; Kammermeier PJ. *et al.*, 2007).

1.3 The tissue distribution and sub-cellular localization of Homer proteins

The Homer proteins family is highly conserved among different species -human, mouse, rat, *Xenopus*, *Drosophila*, zebrafish (Xiao B. *et al.*, 1998; Foa L. *et al.*, 2001; Shiraishi-Yamaguchi Y. *et al*., 2007). In mammals, all Homer isoforms are predominantly expressed in the nervous system and are widely localized at postsynaptic density, where they act as adaptor proteins for many postsynaptic density proteins (Hayashi MK. *et al.*, 2009; Foa L. *et al.*, 2009).

The long isoforms are constitutively expressed in most of the brain regions (Xiao B. *et al.*, 1998), whereas the expression of short Homer 1a isoform is transiently induced during development or in response to external stimuli (e.g. light, traumatic injury, epileptic stimulus or drugs administration) (Kato A. *et al.*, 1997; Park HT. *et al*., 1997; Huang WD. *et al.*, 2005; Li Y. *et al.*, 2012, Zhang GC. *et al.*, 2007).

Homer mRNA and proteins have also been detected both in cardiac and skeletal muscle (Sandonà D. *et al.*, 2000). The relative quantitative expression levels of Homer 1, -2 and -3 mRNAs in muscle tissues are the same as in the brain (Soloviev MM. *et al.*, 2000b). In skeletal muscle, the short inducible isoform presents a particular pattern of expression depending on conditions: i) under resting conditions it appears to be constitutively expressed, ii) during muscle regeneration it appears to be up-regulated (Bortoloso E. *et al.*, 2006). In relation to Homer sub-cellular localization in skeletal muscle, the long and short isoforms have different and distinct patterns. In C2C12 myotubes, Homer 1c (long isoform) displays a reticular-like pattern in the cytosol with punctuate labeling around the nuclei, whereas Homer 1a (short isoform) is localized homogenously in the cytoplasm (Volpe P. *et al.*, 2004). Moreover, in skeletal muscle, where the long and short Homers interact with both RyR1 (Feng W. *et al.*, 2002) and IP3R (Yuan PJ. *et al.*, 2003) (both key elements for Ca^{2+} signaling in muscle cells), immunofluorescence analysis in adult skeletal muscle has demonstrated that Homer proteins only co-localize with IP3R, and not co-localize with the RyR1 isoform (Salanova M. *et al.*, 2002; Volpe P. *et al.*, 2004).

As regards the expression of Homer isoforms in cardiac muscle, different studies *in vitro* using neonatal rat cardiomyocytes have demonstrated that the expression of the long and short Homer 1 isoforms (Homer 1c and Homer 1a, respectively) appears to be modulated following stimulation with different hypertrophic agents such as endothelin-1 (ET-1) (Kawamoto T. *et al.*, 2006), angiotensin II (Ang-II) (Guo WG. *et al.*, 2010) and

phenylephrine (PE) (Grubb DR. *et al.*, 2011). Little is known about sub-cellular distribution of Homer isoforms in cardiac tissue and, also in this case, the most significant studies regard the Homer 1 isoforms. Indeed, immunofluorescence study on adult cardiomyocytes demonstrated that Homer 1 have a striated pattern which corresponds to the Z-band, where it partially co-localizes with RyR (Kawaguchi S. *et al.*, 2007).

It should be noted that Homer isoforms are also expressed in peripheral tissues - transcripts and proteins from the three Homer genes were identified in many tissues, including the lung, liver, kidney, thymus, spleen, testes, and intestines (Shiraishi Y. *et al.*, 2004; Soloviev MM. *et al.*, 2000b).

1.4 The functional interactions of Homer proteins

The Homer/EVH1 domain is responsible for functional interactions between Homers and different target proteins, which contain the proline-rich consensus sequence (Duncan RS. *et al.*, 2005).

Functional studies have demonstrated that, in mammalian brain, all Homer proteins bind to the C-terminal intracellular tails of mGluR (mGluR1α and mGluR5) via the Homer/EVH1 domain (Xiao B. *et al.*, 1998; Tu JC. *et al.*, 1998). The mGluRs are a family of seven membrane-spanning G protein-coupled receptors (GPCRs) that allow the extracellular signals to be transducted to the inside of the cell by activating G proteins (Niswender CM. *et al*., 2010). In both neuronal and non-neuronal cells, Homer proteins are able to regulate mGluRs by modulating their expression and clustering, their activity and their coupling to signaling complexes (Thomas U. *et al*., 2002). In cortical neurons from Homer 1a-specific knockout mice it has been demonstrated a significantly increase of surface mGluRs, that was reduced with reintroduction of Homer 1a gene (Hu JH. *et al.*, 2010). In HeLa and cerebellar granule cells, it has been found that exogenous Homer 1b blocked the cell-surface targeting of mGluR5 promoting its retention in the endoplasmic reticulum, whereas exogenous Homer 1a reversed this effects enhancing surface clustering of mGluR5 (Roche KW. *et al.*, 1999; Ango F. *et al.*, 2002). Different effect has been found in cultured hippocampal neurons, where Homer 1b reduced retention in the endoplasmic reticulum of mGluR5 and increased its expression on cell surface (Serge A. *et al.*, 2002). This discrepant effects of Homer proteins on mGluR are probably due to the different distribution of Homer variants in each cell line. However, data present in literature indicate that, although both the long and short Homer proteins modulate the

expression and cell-surface clustering of mGluR, they acts in different and opposite ways (Shiraishi-Yamaguchi Y. *et al.*, 2007; Luo P. *et al.*, 2012c).

Functional studies have suggested that the binding of long Homers to mGluR1α and mGluR5 is also important to maintain the receptor in an inactive state in the absence of an agonist, whereas the binding of short isoform (Homer 1a) reverts this effect promoting constitutive activation of the receptor (Fagni L. *et al.*, 2002; Ango F. *et al.*, 2001). In this case, again, short Homer 1a isoform exerts its function negatively modulating the interactions mediated by other long Homer isoforms, interfering with the assembly of the Homer-complex.

The Homer binding to mGluRs plays also a fundamental role facilitating the cross-talk between surface mGluR and intracellular target proteins, and enhancing signal transduction. Tu et al. demonstrated that, in rat cerebellum, Homer 1 proteins mediated the linkage between mGluR1α and IP3R (Tu JC. *et al.*, 1998). IP3R is an important channel responsible for Ca^{2+} release from endoplasmic reticulum (Patterson RL. *et al.*, 2004) and contains the Homer ligand motif. This mGluR-Homer-IP3R association is involved in the post-synaptic mGluR-dependent signal transduction. Indeed, it has been reported that the expression of Homer 1a, which lacks the ability to form cross-links, disrupted the mGluR-Homer-IP3R complex altering mGluR-induced intracellular Ca^{2+} release (Tu JC. *et al.*, 1998).

Homer proteins allow also the crosstalk between several intracellular proteins, many of which belong to the Ca^{2+} signaling pathway such as the IP3R (described above) and the RyR. RyRs are the major intracellular Ca^{2+} channels localized in the plasma membrane of intracellular Ca^{2+} stores, mainly in the endoplasmic/sarcoplasmic reticulum (ER/SR). In both cardiac and skeletal muscle, RyRs are responsible for the release of Ca^{2+} from the SR during excitation-contraction (Van Petegem F. 2012). In skeletal muscle, long and short Homer isoforms bind RyR1 through the Homer EVH1 N-terminal domain. Long isoforms (Homer 1b, Homer 1c and Homer 2) modulate skeletal muscle RyR1 activity, increasing ryanodine binding, Ca^{2+} release from SR, intracellular Ca^{2+} transients in C2C12 cells, and the frequency of Ca^{2+} sparks in permeabilized skeletal muscle fibres (Feng W. *et al.*, 2002; Hwang SY. *et al.*, 2003; Pouliquin P. *et al.*, 2009a, Pouliquin P. *et al.*, 2009b; Ward CW. *et al.*, 2004), whereas short Homer 1a dose-dependently decreases the effects of long Homer 1c on RyR1 by competing for the RyR binding site (Hwang SY. *et al.*, 2003). However, the effect of the Homer binding to RyR1 is still unclear. Other studies have reported different results demonstrating that both long and short

Homer 1 variants regulate RyR1 in a similar and additive way, activating Ca^{2+} release via RyR in skeletal muscle, and modulating ryanodine binding to membranes enriched with RyR (Ward CW. *et al.*, 2004; Feng W. *et al.*, 2008).

In the heart, Homer 1 isoforms bind RyR2 and, although the functional effects of this interaction can have important consequences in the cardiac Ca^{2+}-dependent signaling, data reported require further clarification. On the one hand, it has been demonstrated that long Homer isoforms reduce RyR2 activity following agonist-dependent activation. Short Homer 1a alone has no effect on RyR2 activity, but when co-expressed with long Homers inhibits the effect of long Homers by competing for the binding sites on RyR2 (Westhoff JH. *et al.*, 2003). On the other hand, a different study demonstrated that both long Homer 1b and short Homer 1a are able to modulate RyR2 activity in a similar way by the simple binding through its EVH1 domain to RyR binding sites (Pouliquin P. *et al.*,2009b).

Homer proteins interact also with another family of Ca^{2+} channels called TRPC (canonical-type Transient Receptor Potential Cations). TRPC channels are non selective Ca^{2+} permeable cation channels that are involved in receptor-stimulated Ca^{2+} influxes (Vennekens R. *et al.*, 2002). All TRPC channels bind Homer proteins through the proline-rich consensus sequence at their C-terminus. Homer proteins, in particular Homer 1, are able to form complex between TRPC channels andIP3R. Disruption of this TRPC1-Homer-IP3Rs complex by expression of the dominant-negative Homer 1a causes the activation of TRPC channels (Yuan JP. *et al.*, 2003). Through this interaction, long Homer 1 proteins maintain the TRPC channels in a closed state. At the basal state, TRPC channels are inactive in a complex with IP3Rs that is formed by Homer 1b/c; whereas, upon cell stimulation and Homer 1a up-regulation, the complex is dissociated promoting the activation of TRPC channels (Kim JY. *et al.*, 2006).

Homer proteins interact with other scaffolding proteins, including Shank proteins (Tu JC. *et al.*, 1999). In neuronal cells, Shank/Homer complexes play a central role in the morphogenesis of dendritic spines (Sala C. *et al.*, 2001). In cardiomyocytes, instead, scaffolding protein Shank3 forms a complex with a splice variant of phospho-lipase Cβ1 (PLCβ1, important for initiating hypertrophic signaling responses) and Homer 1 proteins (in particular Homer 1c and Homer 1a). These interactions cause different functional effects on the PLCβ1b-initated pathways, which are differentially modulated by Homer 1c and Homer 1a (Grubb DR. *et al.*, 2011).

1.5 Cardiac muscle tissue: an overview

The heart is a contractile organ composed by a complex network of cells including muscle cells (cardiomyocytes), and non-muscle cells (fibroblast, endothelial cells, mast cells, vascular smooth muscle cells). In cardiac tissue, cardiomyocytes form a branching network and are attached end-to-end with specialized regions called intercalated discs (McNutt NS. *et al.*, 1974). These intercalated discs serve to maintain a close electrical communication between two contiguous cardiomyocytes allowing propagation of the action potential from one to other cell (Fawcett DW. 1996). Cardiomyocytes are composed by bundles of myofibrils that contain myofilaments. The myofibrils are structured in repeating units called sarcomeres. The sarcomere represents the basic structural and functional unit of contraction in cardiac muscle and is formed by interlacing myosin (thick) and actin (thin) filaments bordered by Z-discs. The Z-disc (see fig. 3) is present in the middle of I band (lights bands for *i*sotropic in polarized light) that contains only thin (actin) filaments, whereas in the A band (dark band *a*nisotropic in polarized light) thick (myosin) and thin filaments are found. The A band comprises the H zone, where thick (myosin) filaments are present, and the M line, where myosin filaments are anchored (Leyton RA. *et al.*, 1971; Cooper GM. 2000).

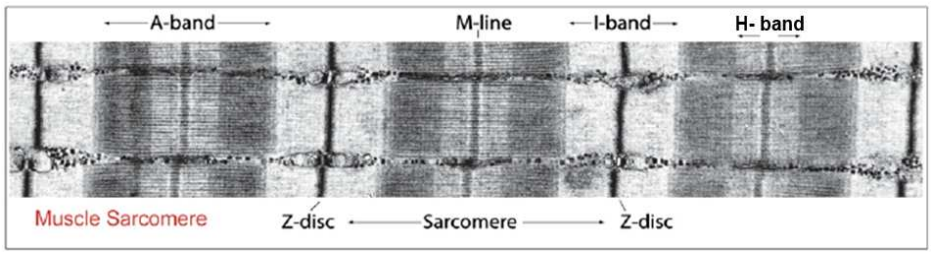


Figure 3. Sarcomeric structure. From Lee EH. *et al.*, 2007.

The main function of the heart is to pump blood throughout the body by a coordinated contraction of all cardiac four chambers. During contraction, myosin heads bind actin filaments in a physical connection (called cross-bridge) that allows the actin filament to slide past the myosin filament, causing the sarcomere shortening and, thus, the contraction. The muscle relaxation, in turn, occurs with the dissociation of the cross-bridges between myosin and actin. Propagation of electrical depolarization through the sarcolemma (the cardiac cell membrane) and the t-tubule (specialized invagination of the sarcolemma that cross the cell at the Z-line) is the initial step of the excitation–contraction coupling, a process in which the electrical excitation of cardiomyocytes is converted into

a mechanical response and induces muscle contraction (Greenstein JL. *et al.*, 2011). In this process, the second messenger Ca^{2+} is fundamental to trigger contraction of the cardiomyocytes. Indeed, the initial membrane depolarization causes Ca^{2+} influx through the voltage-dependent L-type Ca^{2+} channels. This Ca^{2+} influx induces further Ca^{2+} release from the intracellular calcium. In this way, the intracellular Ca^{2+} concentration increases and Ca^{2+} binds to the troponin C (a small regulatory protein of muscle contraction), which then allows the interaction between myosin and actin. During relaxation, the intracellular Ca^{2+} concentration decreases through several pathways involving SR Ca^{2+}-ATPase, sarcolemmal Na+/Ca^{2+} exchange, sarcolemmal Ca^{2+}-ATPase or mitochondrial Ca^{2+} uniport. The decrease of intracellular Ca^{2+} induces the dissociation of Ca^{2+} from troponin, which in a complex with tropomyosin (another regulatory protein of muscle contraction) blocks the myosin binding sites on actin and determine a relaxation of the contractile muscle fibers (Bers DM. 2002).

1.6 Cardiac hypertrophy

Cardiac hypertrophy is an increase in heart muscle mass that occurs predominantly through cellular enlargement without any proliferation (Frey N. *et al.*, 2003; Roderick HL. *et al.*, 2007). The hypertrophic process arises as an adaptive response to environmental demands and to a variety of other different stimuli (Hill JA. *et al.*, 2008). Normally, this process occurs after birth when cardiac myocytes lose the ability to proliferate and the subsequent growth of the heart occurs only by increasing the myocyte size (Olson EN. *et al.*, 2003). This growth process is called ‘physiological hypertrophy’ (Fig. 4). In adulthood, physiological conditions - such as chronic exercise training or pregnancy - also promote morphological and physiological growth of the heart. In contrast, pathological conditions - such as hypertension, neurohumoral activation, aortic stenosis and sarcomeric gene mutations - can cause pathological hypertrophic growth that, unlike physiological growth, results in a predisposition towards heart failure (fig. 4) (Bernardo BC. *et al.*, 2010).

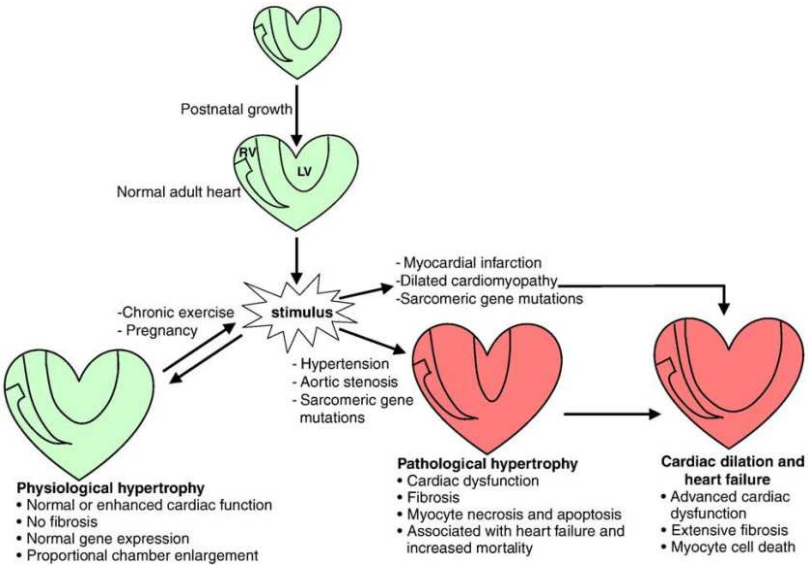


Figure 4. Differences between physiological and pathological hypertrophy. From Bernardo BC. *et al.* , 2010.

Cardiac hypertrophic growth can be classified as concentric or eccentric based on changes in heart shape. In concentric hypertrophy, the thickness of the ventricular wall increases with no changes in heart volume. The concentric remodelling is characterized by an increase in cardiac myocyte greater in width rather than in length, in which the sarcomeres are added in a parallel way. In contrast, eccentric hypertrophy is characterized by dilation and thinning of the heart wall that cause an enlargement of heart volume. In the eccentric sarcomeres are added in series (Fig. 5) (Heineke J. *et al.*, 2006).

In response to various stimuli (in particular to pathological stimuli), initial hypertrophy occurs as a compensatory mechanism required to normalize wall stress and to sustain normal cardiac function. In the long term, however, a prolonged cardiac hypertrophy may decompensate and progress to heart failure independently from the hypertrophic causes. Heart failure is one of the major causes of death in the Western society, and the risk of heart failure increases with age (Levy D. *et al.*, 2002). At present, there is no definitive therapy for heart failure, but there is a great interest to characterize the intracellular signaling pathways implicated in pathological cardiac growth in order to identify therapeutic strategies for prevention of heart failure.

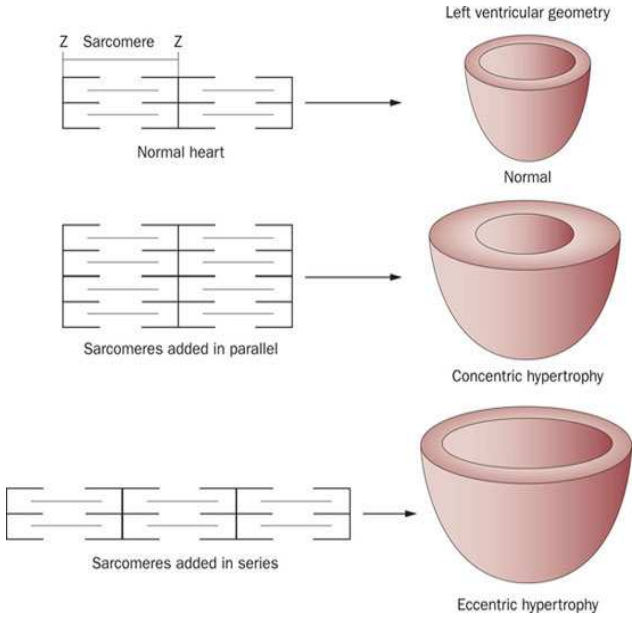


Figure 5. Differences between concentric and eccentric hypertrophy. In concentric hypertrophy sarcomeres are added in a parallel way, whereas in eccentric hypertrophy sarcomeres are added in series. From Gjesdal O. *et al.*, 2011.

1.7 Distinct features in physiological and pathological cardiac hypertrophy

Physiological and pathological cardiac hypertrophy are both defined as an enlargement of the heart characterized by an increase in cell size; it is important to remark that the physiological hypertrophy occurs without significant clinical consequences. At structural level, during physiological hypertrophy muscle thickness increases in a proportional way in respect to the chamber size of the heart (Chen QM. *et al.*, 2001) and the fibrillar collagen network that surrounds the cardiomyocytes provides structural support preserving the normal cardiac function (Bernardo BC. *et al.*, 2010).

On the contrary, the pathological hypertrophy can be divided into two stages: an early and a late stage. During early stage, the changes in cardiac structure compensate the increased stress on the heart, meanwhile in late stage the heart becomes decompensated and is unable to pump sufficient blood to maintain a cardiac output adequate to body's oxygen demand (Czubryt MP. *et al.*, 2004). At the morphological level, the pathological hypertrophy develops in an uncoordinated manner with loss of cardiomyocytes that are replaced by fibrous tissue; this causes stiffness of the ventricles, which in turn impairs cardiac function (Feng QZ. *et al.*, 2008; McMullen JR. *et al.*, 2007). At the gene expression level, pathological hypertrophy is characterized by the re-expression of fetal genes - genes that are normally expressed only in the heart development and are

repressed in the adult heart (Schaub MC. *et al.*, 1997). The re-activation of fetal genes, such as β-myosin heavy chain, α-skeletal actin and atrial natriuretic factor (ANF), does not occur in physiological hypertrophy (Barry SP. *et al.*, 2008; Bishopric NH. *et al.*, 1991).

1.8 Signaling pathways in cardiac hypertrophy

Cardiac hypertrophy occurs in response to an initial stimulus that acts on cell membrane and induces activation of intracellular signaling pathways. The process of cardiomyocyte hypertrophy can be divided into three well-defined stages (Glennon PE. *et al.*, 1995):

I. the initial binding of extracellular hypertrophic agonists on membrane receptors;
II. the subsequent activation of intracellular signaling pathways;
III. the final activation of nuclear events leading to hypertrophic phenotype.

As shown in fig. 6, a wide array of extracellular factors can stimulate several receptors on the plasma membrane and trigger different intracellular signaling pathways that ultimately affect nuclear factors.

In this way, activation of the hypertrophic cellular program culminates at the nucleus level in an alteration of gene expression (e.g. re-expression of fetal gene) and, at the cytoplasmic level, in an increase in protein translation and a decrease in protein degradation (Heineke J. *et al.*, 2006). Many studies conducted both *in vitro* and *in vivo* have identified important signaling pathways activated during cardiac hypertrophy, that involve increases in cytoplasmic Ca^{2+} (Wilkins BJ. *et al.*, 2004; Molkentin JD 2006**)**, and include activation of G-proteins (D'Angelo DD. *et al.*, 1997), of MAPK (mitogen-activated protein kinase) pathway (Clerk A. *et al.*, 1999), and of PI3K (phoshoinositide 3-kinase) pathway (Luo J. *et al.*, 2005).

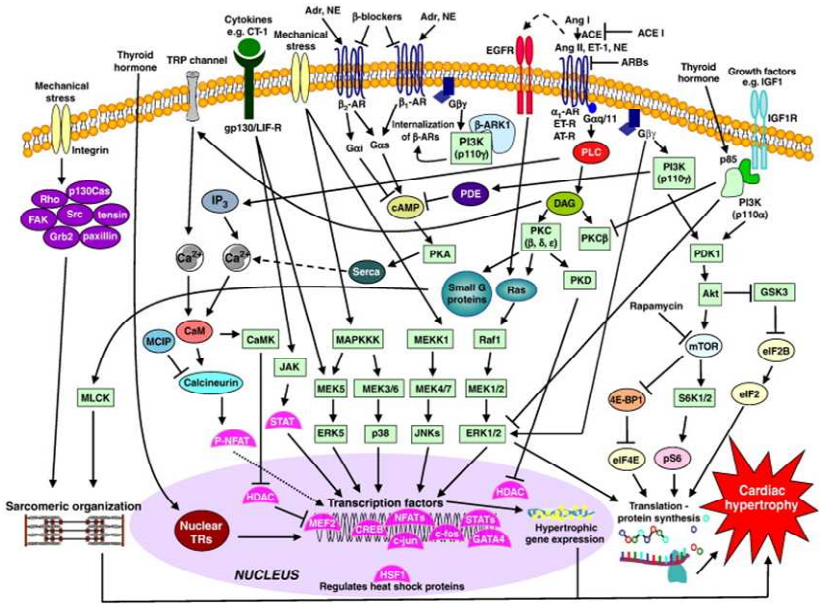


Figure 6. Intracellular signaling pathways that regulate cardiac hypertrophic response. From Bernardo BC. *et al.* , 2010.

In cardiomyocytes, the most important cell-surface receptors involved in the hypertrophic signaling cascades are classified as:

I. **G-protein coupled receptors** for catecholamines that induce the mobilization of Ca^{2+} from intracellular stores and the activation of both MAPK pathways and calcineurin-NFAT pathways (Molkentin JD. *et al.*, 2001);

II. **Tyrosine kinase receptors** for insulin growth factor, fibroblast growth factor and transforming growth factor, that activate downstream PI3K/Akt pathways (McMullen JR. *et al.*, 2007);

III. **Cytokine receptors** for cardiotrophin-1 that mediate the activation of JAK/STAT pathways (Barry SP. *et al.*, 2008).

The G protein coupled receptors (GPCR) are a group of seven-transmembrane-spanning receptors coupled to heterotrimeric G proteins that play a major role in response to hypertrophic stimuli. There are three principal classes of heterotrimeric G protein - Gs, Gq/G11, and Gi - which transduce the extracellular signal towards intracellular effectors (Rockman HA. *et al.*, 2002). All heterotrimeric G-proteins presents two subunits: Gα and Gβγ; when the receptor is activated these subunits dissociate and induce the activation of signaling pathways interacting with downstream effector molecules (Rohini A. *et al.*, 2010). Studies in genetically modified mouse models have demonstrated that over-expression of wild-type Gαq subunit induces cardiac hypertrophy associated with a depressed cardiac function, whereas deletion of both Gαq and Gα11 subunits in

transgenic mice with pressure-overload induced by aortic constriction does not cause ventricular hypertrophy (D'Angelo DD. *et al.*, 1997; Wettschureck N. *et al.*, 2001). Thus, these data indicate that the Gαq/G11-mediated pathway is essential for the cardiac hypertrophic process.

Of all the members of the GPCR superfamily, adrenergic receptors (ARs) are the most important in the heart and they are classified into three major subfamilies: α_1-AR, α_2-AR and β-AR. ARs are activated by catecholamines (such as noradrenaline and adrenaline) and play an important role in the control of cardiac function, myocyte growth and cell death. Depending on adrenergic subtypes, ARs are coupled to Gαq, Gαs or Gαi (Rockmann HA. *et al.*, 2002). The binding of adrenergic agonists to ARs induces activation of effector molecules, such as adenylyl cyclases, phospholipases and ion channels (in particular, Ca^{2+} channels) and these downstream effectors, in turn, activate important hypertrophic signaling, such as MAPKs, calcineurin-dependent, and PI3K-dependent pathways (Xiao L. *et al.*, 2001; Zou Y. *et al.*, 1999; Molkentin. JD. *et al.*, 2001; Zhang W. *et al.*, 2011).

Cathecolamines, ET-1 and Ang-II are well-characterized neurohumoral and endocrine factors that are released in response to a pathological stimulus, such as pressure overload, and are able to induce cardiomyocytes hypertrophy through both β- and α- ARs coupled to either Gαs or the Gαq/Gα11 subclass (Yamazaki T. *et al.*, 1997).

Activation of β-Adrenergic receptors coupled to Gαs induces adenylyl cyclase activity that causes production of cAMP and, then, activation of protein kinase A (PKA) (see Figure 7). PKA in turn promotes the cardiomyocytes contractility by activating proteins involved in cardiac contraction (L-type calcium channels, phospholamban and troponin) and also activates signaling pathways involved in cell growth (MAPK pathway) (Marian AJ. 2006; Yamazaki T. *et al.*, 1997).

α-Adrenergic receptors coupled to Gq are able to activate pospholipase C (PLC), inducing the generation of inositol 1,4,5-triphosphate (IP3) and DAG. In this cascade of process, DAG in turn activates protein kinase C (PKC), whereas IP3 binds to the IP3R causing the release of Ca^{2+} (see Figure 7). Both these downstream events (activation of PKC and increases in cytosolic Ca^{2+}) are potential triggers for the activation of pro-hypertrophic transcription factors to nucleus (Heineke J. *et al.*, 2006; Berridge MJ. 2006; Palaniyandi SS. *et al.*, 2009).

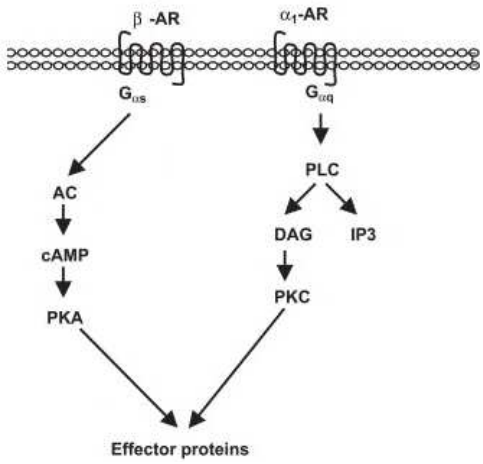


Figure 7. Schematic illustration of intracellular signaling pathways activated upon NE-stimulation of α_1- and β- adrenergic receptors. Modified from Wang QD *et al* 2004.

As mentioned before, the adrenergic receptor stimulation induces activation of many intracellular signaling pathways, that include MAPK and calcineurin-NFAT pathway.

MAPKs pathway represents one of the principal cascades involved in cardiomyocyte hypertrophic responses. The MAPK superfamily consists of three main members: the extracellular signal-regulated kinases (ERKs), the *c-Jun* N-terminal kinases (JNK), and the p38 kinases (Kehat I. *et al*., 2010). ERK1/2 have been reported to play an important role in hypertrophy, both *in vitro* and *in vivo*, as important mediator of cardiac responses (Glennon PE. *et al.*, 1996, Xiao L. *et al.*, 2001; Izumi Y. *et al.*, 1998). ERK1/2 are protein kinases that induce reprogramming of gene expression by phosphorylating various cytosolic and nuclear substrates. When ERK1/2 are activated, they translocate to the nucleus and directly phosphorylate transcription factors such as Elk-1, c-Fos, and GATA4, which are involved in growth and proliferation. Constitutive ERK1/2 activation in the heart is sufficient to evoke a cardiac hypertrophic phenotype (Bueno OF. *et al.*, 2000; Kehat I. *et al.*, 2010; Lorenz L. *et al.*, 2009).

Calcineurin-NFAT pathway represents an important Ca^{2+}-dependent signaling pathway involved in cardiac hypertrophy (Colella M. *et al.*, 2008). Calcineurin (CaN) is a serine-threonine phosphatase that is activated by increases in cytosolic Ca^{2+} level. Once activated, CaN directly dephosphorylates members of the NFAT family, promoting their translocation into the nucleus and the activation of pro-hypertrophic gene expression (Molkentin JD. 2006). Studies using both *in vitro* and *in vivo* models have extensively demonstrated that CaN–NFAT signaling plays a role in mediating pathological

hypertrophy. In particular, it has been demonstrated that constitutive activated form of CaN in heart promotes hypertrophy, ultimately leading to cardiac failure (Molkentin JD. *et al.*, 1998).

1.9 Homer 1a and cardiac hypertrophy

Although Homer proteins can be regarded as important regulators of multimeric complexes involved in signal transduction, little is known about the role that Homer proteins play in signalling pathways of cardiac muscle and, in particular, in cardiac hypertrophic pathways.

With respect to the short Homer 1a isoform, the presence of its mRNA transcripts was identified in cardiac muscle under resting conditions in a previous study by our group (Sandonà D. *et al.*, 2000), but their function in the heart remains poorly understood. Data present in literature support the hypothesis of an involvement of the short isoform Homer 1a in activation of cardiomyocyte hypertrophy. Kawamoto et al. were the first who investigate Homer 1a expression in stimulated cultured neonatal cardiomyocytes. In this study, it has been demonstrated that mRNA coding for Homer 1a was rapidly and transiently increased in neonatal cardiomyocytes upon stimulation with several hypertrophic agonists, including PE, isoprotenerol (ISO), Ang-II and ET-1 (the latter most markedly induced Homer 1a expression) (Kawamoto T *et al.*, 2006). These findings provide the first evidence of the association between Homer 1a up-regulation and cardiac hypertrophic activation. In the same way, Guo et al. reported that Homer 1a protein levels were also up-regulated following Ang-II induced hypertrophy in H9C2 cells and neonatal rat cardiomyocytes (Guo WG. *et al.*, 2010). Contrary to previously reported, a later study conducted by Grubb et al. indicated that long isoform Homer 1c increased during stimulation with an hypertrophic agent (such as PE) and, in absence of other effectors, induced cardiomyocyte hypertrophy. In this model, Homer 1a did not cause cardiomyocyte hypertrophy by itself, but was able to inhibit hypertrophy induced by PE, although its expression was unchanged during hypertrophic stimulation (Grubb DR. *et al.*, 2011).

Although all these studies demonstrate that hypertrophic stimulation modulates expression of Homer 1 isoforms, data appear controversial in particular about the modulation of Homer 1a expression (it is not up-regulated in the later study). Further investigations are required to identify which isoforms (Homer 1a or Homer 1c) are really

involved during hypertrophic stimulation and to clarify their specific role in cardiac hypertrophy.

2. Aims of the study

The aim of this study was to investigate the role of the scaffolding protein Homer 1a in cardiac function and hypertrophy. Our working hypothesis was that Homer 1a may be one of the molecular modulators of cardiac hypertrophy. To test this, we investigated the presence, sub-cellular localization and function of Homer 1a in cardiac tissue.

We developed our study as follows:

1) First, we studied Homer 1a expression and its sub-cellular localization in cardiac tissue under resting conditions. Although many studies have reported that in the nervous system Homer proteins are constitutively expressed and are found at the postsynaptic density, few reports have focussed on Homer 1a in the heart, in particular, on its expression and sub-cellular localization.
2) Next, in order to establish whether Homer 1a takes place in the hypertrophic program of cardiomyocytes, we monitored Homer 1a expression (both at mRNA and protein level) in *in vitro* models under conditions mimicking hypertrophic stimulation.
3) Finally, to determine the effect of Homer 1a, we used gain-of-function approaches for Homer 1a in a model of cardiac hypertrophy *in vitro*. The goal of this analysis was to identify the intracellular hypertrophic pathways modulated by Homer 1a and, consequently, the role of Homer 1a in cardiac hypertrophy.

In the last part of this study, we performed preliminary analyses to evaluate Homer 1a expression in three different models of cardiac hypertrophy *in vivo*.

3. Materials and Methods

3.1 Tissue sources

To perform Western blot and immunofluorescence analysis on cardiac tissue, adult Wistar rats (~250 g of body weight) and CD1 mice (~45 g of body weight) were used. Following animal sacrifice, the heart was removed and frozen in liquid nitrogen. Experimental protocols have been approved by the University of Padua Review Board.

3.2 Treatment of monocrotaline in adult rats

Male Wistar rats (*n*=18, 125-150 g) were housed in a standard environment with a 12-h light/12-h dark cycle and free access to food and water. The rats were treated with a single intraperitoneal injection of monocrotaline (MCT, 30 mg/kg dissolved in 0.9% NaCl) or vehicle. Monocrotaline is a toxic pyrrolizidine alkaloid of plant origin; administration of MCT produces hypertension followed by right ventricular (RV) failure (Dalla Libera L. *et al*., 2004). At 1, 2 and 4 weeks after the MCT injection, the rats were killed and their hearts quickly removed and weighed; heart weights and body weights were recorded. Hearts were divided into the left ventricular wall and the right ventricular wall and tissue lysates were then prepared by homogenization in RIPA buffer as described below. The experiments were approved by the University of Padua Biological Ethical Committee.

3.3 Mice with transverse aortic constriction

Heart lysates from mice that had undergone transverse aortic constriction (TAC) were kindly provided by Dr. N. Kaludercic (CNR, Padua, Italy). TAC was induced by controlled constriction of the transverse aortic arch; sham-operated mice, which had undergone a similar surgical procedure without aortic constriction, were used as control. The mice hearts were harvested at 1 (n=4), 3 (n=3), 6 (n=3) and 9 (n=3) weeks after TAC, and homogenized for Western blotting analysis. Homer 1a protein content was determined as described below.

3.4 Gαq over-expressing mice

Heart lysates from Gαq over-expressing mice were kindly provided by Dr. N. Kaludercic (CNR, Padua, Italy). Hearts from 18-week old mice (n=4) were harvested and

homogenized for Western blotting analysis. Homer 1a protein content was determined as described below.

3.5 Homogenates from rat and mouse hearts

Hearts homogenates were obtained as follows: frozen tissues were triturated in a mortar, then homogenated with Polytron for 10 sec at 18,000 g in 10 volumes of 3% SDS, 1 mM EGTA, 0.2 mM phenylmethanesulphonyl fluoride (PMSF) and 0.8 mM benzamidine. They were then boiled for 5 min and centrifuged at 18,000 g for 30 min in order to remove the debris. The protein content of homogenates was determined by using a bicinchoninic acid protein assay system (Pierce).

3.6 Cell cultures:

3.6.1 Preparation of neonatal rat cardiomyocytes

Primary cultures of cardiomyocytes were prepared from 1- 2 day-old Wistar rats. The hearts were dissected and the ventricles were removed aseptically and washed with ADS buffer (106 mM NaCl, 5.3 mM KCl, 0.8 mM NaH_2PO_4, 0.4 mM $MgSO_4$, 20 mM HEPES, 5 mM glucose, pH 7.4). Using micro-dissecting scissors, the hearts were minced until the pieces were approximately 1 mm^3 and treated with 10 ml of collagenase A (0.45 mg/ml; Roche) and pancreatin (1.25 mg/ml; Sigma) for 15 min at 37°C. The supernatant was then removed and the tissue was treated with fresh collagenase/pancreatin solution for an additional 15 min. Cells in the supernatant were transferred to a tube containing 100% fetal calf serum (FCS). The tubes were centrifuged at 250 g for 5 min at room temperature and the cell pellet was resuspended in the appropriate volume of cell culture medium. The above procedures was repeated 5–6 times until little tissue was left. Cell suspensions were collected and incubated in a 100 mm dish for 1-2 h to reduce fibroblast contamination. The resulting purified cardiomyocytes were collected, counted using Trypan Blue solution (Sigma) and plated at 8×10^5 cells per well on 0,1% gelatin (Sigma) coated 6-well plates containing 65% DMEM, 17% M199 medium supplemented with 10% horse serum, 5% FCS, 2 mM L-glutamine, 100 µM penicillin and streptomycin. To prevent growth of non-myocytes and to inhibit fibroblast proliferation, the medium was also supplemented with 20 µM cytosine-D-arabinofuranoside (AraC) 24 h after plating. Cardiomyocytes were grown at 37°C in 5% CO_2 and 95% air.

3.6.2 HL-1 cardiomyocytes

HL-1 cells are currently the only cardiomyocyte cell line available that continuously divides and spontaneously contracts while maintaining a differentiated cardiac phenotype (Claycomb WC. *et al.*, 1998). HL-1 cells also express many of the cardiac-specific genes, possess intercalated discs, maintain contractile activity, retain basic electro-physiological characteristics and display the pharmacological properties of primary cardiac myocytes (White SM. *et al.*, 2004). HL-1 cells express functional α and β-adrenergic receptors and respond to inotropic and chronotropic agonists, allowing the study of intracellular pathways (McWhinney CD. *et al.*, 2000).

HL-1 cells were cultured as reported, except for the lack of NE in the medium prior to experiments (Claycomb WC. *et al.*, 1998). Cells were maintained in Claycomb Medium (Sigma) supplemented with 4 mM L-glutamin, 100 μM penicillin and streptomycin, 50 μM NE and 10% FCS. The medium was changed approximately every 48 h. When the cells reached 80% confluence, they were washed with Hanks' Balance salt solution (HBSS) and treated with 0.05% p/v trypsin-EDTA in order to detach them from the flasks. Then, cells were centrifuged at 250 g for 5 min and counted. 5 x 10^5 cells/well were plated in 6-well plates pre-coated with 0.02% gelatin and 25 μg/ml fibronectin (Sigma). Cells were grown at 37°C in 5% CO_2 and 95% air. HL-1 cells were switched to a medium without NE for 5 days prior to experimentation.

To store the HL-1 cells in liquid nitrogen, the cell pellet was resuspended in 5% sterile DMSO with 95% FBS, and put into criovials; the cells were frozen slowly and then placed in a -80°C freezer for one day before permanent storage in liquid nitrogen.

3.7 Cell stimulation assay in HL-1 cells and neonatal rat cardiomyocytes

After 5 days of incubation in a medium lacking NE, HL-1 cells were washed with HBSS and incubated in the presence or absence of 75 μM NE. After treatment, cells were washed with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% DOC, 1% NP-40 and 2 mM EDTA).

In neonatal rat cardiomyocyte culture, the medium was changed 24 h after plating. Cardiomyocytes were washed with HBSS and serum-starved 1 day before the experiments. The experiments were carried out in DMEM/M199 medium supplemented with 2 mM L-glutamine, 100 μM penicillin and streptomycin. The cardiomyocytes were

then preincubated with 10 μM prazosin or 10 μM propranolol to block α and β-adrenergic receptors respectively, for 30 min before stimulation with 10 μM NE. For western blotting, cardiomyocytes were lysed as described above. For real-time PCR analysis, cells were processed as described in the *mRNA* section.

3.8 mRNA extraction

Total RNA of neonatal rat cardiomyocytes was extracted using TRIzol® (Invitrogen), in accordance with the manufacturer's instructions. Cells were washed with PBS and 500 μl of TRIzol® reagent were added in each well of a 6-well plate. Cells were disrupted and homogenated by passing them through a pipette several times. The samples were left for 5 min at room temperature and 100 μl chloroform were added, samples were vortexed for 15 sec and again left at room temperature for 5 min. Then, samples were centrifuged at 12,000 g for 15 min at 4°C to separate the phases. After centrifugation, the upper aqueous phase with RNA was collected and the RNA was precipitated with 250 μl isopropranol and incubated for 10 min at room temperature. Total RNA was centrifuged at 12,000 g for 10 min at 4°C and the pellet was washed with 75% ethanol and centrifuged again at 7500 g for 5 min at 4°C. The dried RNA was suspended in RNase-free water stored at -80°C until use. Finally, the RNA concentration and purity were measured by Nanodrop (Thermo Scientific).

3.9 cDNA synthesis

Synthesis of cDNA was carried out by reverse transcription (RT-PCR). 400 ng of RNA were transcripted in cDNA using random hexanucleotides primers and SuperScript® VILO™ reverse transcriptase (Invitrogen), according to the manufacturer's instructions. RT-PCR was performed in a thermal cycler (Applied Biosystems, Foster City, CA): 25°C for 10 min, 42°C for 90 min, 85°C for 5 min, and 4°C for 5 min. All RNA samples were converted to cDNA at the same time, in order to minimize technical variability.

3.10 Primer design

Specific primers used were either designed (*) using Primer3 software (http://frodo.wi.mit.edu/, Whitehead Institute for Biomedical Research) or were already published (see ref). Their thermodynamic specificity was determined using BLAST

sequence alignment (NCBI) and vector NTI® software (Invitrogen) software. All primers were purchased from Sigma-Aldrich.

The sequences of primers used for real-time PCR were as follows:

Homer1a (Grubb DR. *et al.*, 2012)	Fw: CCAGAAAGTATCAATGGGACAGATG
	Rv: TGCTGAATTGAATGTGTACCTATGTG
Homer1bc (*)	Fw: GTGAAGCAGTGGAAGCAACA
	Rv: CAGCTCCTGCACTGTCTGAC
TBP1 (Rossi AC. *et al.*, 2012)	Fw: TCAAACCCAGAATTGTTCTCC
	Rv: AACTATGTGGTCTTCCTGAATCC
Hypoxanthine guanine phosphoribosyl transferase (HPRT1,*)	
	Fw: CTCATGGACTGATTATGGACAGGAC
	Rv: GCAGGTCAGCAAAGAACTTATAGCC

3.11 Quantitative real time-PCR (qPCR)

qPCR was performed in duplicate in a 96-wells IQ5 Thermal Cycler (Bio-Rad) using SYBR Green chemistry. The reaction mix consisted of 10 µl of 2x iQ SYBR® Green Supermix (Bio-Rad), 0.3 pmol/ µl primers, 10 ng of cDNA and DNase/RNase free water up to 20 µl. The PCR parameters were initial denaturation at 95°C for 30 sec followed by 40 cycles of 10 sec at 95°C and 30 sec at the corresponding annealing temperature (53-57 °C) in order to acquire the fluorescence signal. In addition, a melting curve was generated by the iQ5 software following the end of the final cycle for each sample, by continuous monitoring of the SYBR Green fluorescence throughout the temperature ramp from 65°C to 99°C in 0.5 sec increments, in order to confirm the specificity of the amplified product. TBP1 and HPRT genes were tested as candidate reference genes being the latter the most stable to normalize Ct values. All samples were run together with negative controls (no RNA or no reverse transcriptase enzyme). Normalization was performed with the deltaCT method using HPRT as the reference gene.

3.12 Transfection of HL-1 cells

For transient transfection and co-transfection experiments, HL-1 cells were seeded at a suitable cell density for obtaining 50–60% confluence at the moment of transfection. After 18 h, cells were transfected using FuGENE®HD (Promega) transfection reagent in accordance with the manufacturer's instructions. A ratio of 3:1 between FuGENE® HD Transfection Reagent and DNA was used for all transfections. For each well of the 6-well plate, 6 µg DNA were diluted to 300 µl of serum-free and antibiotic-free Claycomb medium and vortexed gently. 18 µl of Fugene HD were added to medium with the DNA; FuGENE/DNA mixture was mixed gently and incubated for 15 min at room temperature. The transfection mixture was added to each well of cell culture. Cells were typically assayed 24-48 h after transfection, depending on the type of experiment.

3.13 Plasmids

Plasmids encoding either Homer 1a-HA1, Homer 1c-HA1 or pcDNA3 (empty vector) were cloned as previously described (Sandonà D. *et al.*, 2000). Plasmids encoding prom-ANF luciferase (pANFluc) and *Renilla* luciferase (pRL-TK) were kindly provided by Dr. CC. Glembotski (San Diego State University, San Diego, CA, USA) and Dr. F. Zorzato (University of Ferrara, Ferrara, Italy), respectively. Plasmid encoding NFATc1-GFP were provided by Prof. S. Schiaffino (University of Padua, Padua, Italy).

3.14 Plasmid DNA amplification and purification

The plasmid DNA was amplified in bacterial cultures grown in presence of a selective antibiotic. In order to transform bacterial cells to assume the plasmid/foreign DNA, *E. Coli* XL1-blue competent for electroporation were used. 50 -100 ng of DNA were used for the electroporation; the electric shock was used at 1800 V. The bacteria were rapidly resuspended with SOC medium (20 mM glucose, 10 mM $MgCl_2$, 10 mM $MgSO_4$) in LB medium (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl) and incubated for 1 h at 37°C under rotation. Then, bacteria were plated on LB-agar plates (LB with 1.5% agar) with the selective antibiotic (Ampicillin 100 µg/ml or Kanamycin 30 µg/ml, both in sterile H_2O) and were grown overnight at 37°C. A well-formed and isolated colony on the agar plate was selected, placed in LB medium with appropriate antibiotics and incubated at 37°C for 8 h with shaking. After this time, culture was transferred into

750 ml LB medium with the antibiotic and incubated for 12-16 h at 37° C with sharing. After 12-16 h, bacterial cells were harvested by centrifugation at 6000 g for 15 min at 4°C. The plasmid DNA from bacteria was extracted and purified using QIAprep Maxiprep Kit (QIAGEN). The QIAGEN Kit protocol are based on modified a alkaline lysis procedure, followed by binding of plasmid DNA to anion-exchange resin under appropriate low-salt and pH conditions. RNA, protein and impurities were removed by a medium-salt wash. Finally, plasmid DNA was dissolved in sterile H_2O and quantified using the spectrophotometer NanoDrop (Thermo Scientific). The purity of the DNA was evaluated considering the ratios 260/280 (ideal between 1.8-2) and 260/230 (ideal between 2 and 2.2). For each bacterial preparation, a stock of transformed bacteria was performed by adding 20% (v/v) sterile glycerol in bacterial cultures, and freezing them directly at -80°C.

3.15 Protein extraction and quantification

After treatment, either HL-1 cells or cardiomyocytes were washed three times with PBS and lysed with RIPA buffer containing 0.2 mM PMSF, 0.8 mM benzamidine and 10 mM sodium fluoride. Cells were scraped, incubated at 4°C for 30 min with gentle agitation and centrifuged at 14,000 g for 10 min at 4°C. The supernatants were harvested and used for protein quantification. To determine the concentration of protein in cell lysate, the bicinchoninic acid assay (BCA assay, Pierce-Thermo Scientific) was used in accordance with the manufacturer's instructions. The BCA assay is a highly sensitive colorimetric method, based on the principle that protein can reduce Cu^{2+} to Cu^{1+} in an alkaline solution (the biuret reaction). The amount of reduction is proportional to protein content. BCA forms a blue complex with Cu^{1+} that absorbs light at a wavelength of 562 nm, thus allowing to monitor the reduction of Cu^{2+} by proteins using a spectrometer and comparing protein solutions with known concentrations.

3.16 SDS-Polyacrylamide Gel Electrophoresis

Sodium Dodecyl Sulfate -Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to separate proteins according to their size. To avoid that different proteins with similar molecular weights may migrate differently due to their differences in secondary, tertiary or quaternary structure, SDS is used in SDS-PAGE to reduce proteins to their primary structure and coat them with negative charges. After protein quantification,

samples were mixed with Laemmli Sample Buffer (10% (w/v) glycerol, 5% (w/v) β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, 0.03% (w/v) bromophenol blue, pH 6.8) and loaded into a polyacrylamide gel. Polyacrilammide gels, composed of a 4% stacking gel and a 10% running gel, were cast between a pair of glass plates with 1.5 mm spacers. The solutions used to prepared the stacking gel were: 40% solution of acrylamide:bisacrylamide 29:1 (Amresco), 0.5 M Tris-HCl pH 6.8 and 0.4% w/v SDS in H_2O. The solutions used to prepared the running gel were: 40% solution of acrylamide:bisacrylamide 29:1, 1.5 M Tris-HCl pH 8.8 and 0.4% w/v SDS in H_2O. The polymerization of gel is induced by both ammonium persulfate (APS) and TEMED, added to promote polymerization. In each SDS-PAGE a pre-stained protein marker (prestained standard-Low range, Bio-Rad) was loaded in order to estimate the protein molecular weight. Samples were loaded onto the wells and gel was run in running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS) with a voltage of 70 V in the stacking gel and 150 V in the running gel. After SDS-PAGE separation, samples were transferred from the gel onto a 0.45 μm nitrocellulose filter (Bio-Rad) for detection. In order to remove the excess of SDS, the gel was washed in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) and the transfer "sandwich" was assembled in the following order: a fiber pad, a 3 MM Whatmann paper impregnated with transfer buffer, nitrocellulose, the gel, and another impregnated paper and fiber pad. Air bubbles were gently removed, then transfer cassette was firmly closed and put with the membrane facing the positive pole of the transfer tank (Hoefer Scientific, Newcastle, Staffs., U.K.) with transfer buffer. A constant current of 100 mA was applied overnight. After transferring the proteins from SDS-PAGE onto the membrane, it was immersed in sufficient Ponceau S Staining Solution (0.2% (w/v) Ponceau S, 3% (w/v) Trichloroacetic acid) for 2 min. Ponceau Solution is used for the detection of proteins on nitrocellulose membranes; this staining technique is reversible, and can be removed with water.

3.17 Immunoblotting

The nitrocellulose membranes were incubated for 1 h with 10% (v/v) milk in TBS (50mM Tris-HCl, pH 7.5; 150 mM NaCl) with 0.1% Tween 20 (TBSt) in order to block residual protein binding sites. Blocked membranes were incubated overnight at 4°C under gently shaking with the appropriate primary antibody diluted in TBSt. Then, primary antibody was removed and blots were washed 3 times, for 10 min each time, with 2% milk in TBSt. In order to detect primary antibody, the blots were incubated with the

appropriate secondary antibody for 1 h. Secondary antibodies (anti-goat, anti-mouse and anti-rabbit) were labeled with either Alkaline Phosphatase (AP) or Horse Radish Peroxidase (HRP), and diluted 1:10000 in TBSt with 2% milk. After incubation, secondary antibody was removed and the blots were washed 3 times for 10 min with 2% milk in TBSt. Immunodetection was carried out using two different systems depending the secondary antibody used. To detect the signal from AP, blots were washed with AP Buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) twice for 5 min so as to raise the pH. Immunoreactive bands were detected using a ready-to-use, precipitating substrate system for alkaline phosphatase (BCIP/NBT; Sigma). To detect the signal from HRP, blots were washed twice with TBSt and incubated for 1 min with a mixture of the Chemilumiscence substrates of the LiteAblot Plus kit (Euroclone) and exposed to photographic films. For phospho-ERK, the same membrane was stripped at room temperature for 2 h in stripping solution (62.5 mM Tris-HCl pH 7.5, 2% SDS, 0.1 M 2-mercaptoethanol) and re-probed with anti-total ERK1/2 antibody for internal control. Densitometric analysis of the immunoblot signal was performed with Scion Image for Windows, version Beta 4.0.2 (Scion Corp., www.scioncorp.com).

3.18 Antibodies for Western blotting

The following primary antibodies were used:

- anti-Homer 1a (goat, Santa Cruz), dilution 1:300;
- anti-Homer 1b/c (mouse, Santa Cruz), dilution 1:200;
- anti-βtubulin (mouse, Developmental Studies Hybridoma Bank), dilution 1:2000;
- anti-βactin (mouse, Sigma), dilution 1:1000;
- anti-phospho ERK1/2 (rabbit, Sigma), dilution 1:1000;
- anti-ERK1/2 (rabbit, Santa Cruz), dilution 1:1000;
- anti-HA (rabbit, Santa Cruz), dilution 1:1000;
- anti-GAPDH (mouse, Chemicon), dilution 1:200;
- anti-ANF (rabbit, Peninsula), dilution 1:1000.

The following secondary antibodies were used for Western blot:

- anti-mouse IgG (Sigma) conjugated to alkaline phosphatase, dilution 1:10000;
- anti-goat IgG (Sigma) conjugated to alkaline phosphatase, dilution 1:10000;
- anti-rabbit IgG (Sigma) conjugated to alkaline phosphatase, dilution 1:10000;
- anti-mouse IgG (Sigma) conjugated to peroxidase, dilution 1:10000;

- anti-goat IgG (DAKO) conjugated to peroxidase, dilution 1:10000;
- anti-goat IgG (Sigma) conjugated to peroxidase, dilution 1:10000;
- anti-rabbit IgG (Sigma) conjugated to peroxidase, dilution 1:10000;

3.19 Immunofluorescence

To perform immunofluorescence staining of cardiac tissue sections, hearts were frozen in liquid nitrogen. Four µm cryostat sections were fixed in 4% paraformaldehyde (PFA) and washed three times with PBS; this procedure was performed in order to preserve cellular morphology. Sections were permeabilized with blocking solution (1% swine serum, 0.1% Tween-20 in PBS) for 30 min at room temperature and incubated with specific antibodies in PBS, 1% swine serum and 0.1% Tween-20 for either 60 min at room temperature or overnight at 4°C, depending on the primary antibody used. Sections were rinsed 3 times in PBS to remove the excess of the primary antibody and incubated for 45 min at room temperature with appropriate secondary antibodies in PBS with 1% swine serum. After this incubation, the sections were rinsed again in PBS and mounted with Mounting Medium (Sigma). When indicated, sections were treated with Hoechst (Sigma) before mounting. Immunofluorescence staining of cardiac tissue sections was performed by Dr. E. Bortoloso (University of Padua, Padua, Italy). To perform immunofluorescence staining of HL-1 cells, cells were grown on gelatin and fibronectin pre-coated cover-slips and immunostained as described above. Images were obtained with either a Leica DMRB microscope or a DMIRE Leica confocal microscope.

3.20 Antibodies for immunofluorescence staining

The following primary antibodies were used:

- anti- Homer 1a (goat, Santa Cruz), dilution 1:100;
- anti- β-tubulin (mouse, Developmental Studies Hybridoma Bank), dilution 1:300;
- anti-myosin heavy chain (MHC mouse, Developmental Studies Hybridoma Bank);
- anti- α-actinin (mouse, Sigma);
- anti-HA (rabbit, Sigma) dilution 1:100.

The following secondary antibodies were used:

- anti-goat IgG (Jackson ImmunoResearch) conjugated to Cy3, dilution 1:500;
- anti-rabbit IgG (Jackson ImmunoResearch) conjugated to Cy2, dilution 1:300;

- anti-mouse IgG (Jackson ImmunoResearch) conjugated to Cy2, dilution 1:300.

Cy2 conjugates have maximum adsorption/excitation around 492 nm and emission at 510 nm; Cy3 conjugates is excited at 550 nm, with peak emission at 570 nm.

3.21 Analysis of NFAT nuclear translocation

To assess the nuclear translocation of NFAT upon NE-stimulation, HL-1 cells were analyzed by immunofluorescence microscopy. HL-1 cells were co-transfected with either pHomer1a-HA1 and N-terminally GFP-tagged NFATc1 plasmid (pNFATc1-GFP), or with pcDNA3 (empty vector) and pNFATc1-GFP, and cultured in serum antibiotic-free conditions. 24 h after transfection, cells were stimulated with 75 µM NE for 12 h. The effective co-transfection was assessed by staining the cells with anti-HA antibodies; cells were therefore fixed with 4% PFA for 10 min, and immunostained using the affinity-purified anti-HA monoclonal antibody (1:100 dilution). In over-expressing Homer 1a-HA cells, GFP subcellular localization was analyzed in HA-positive cells in either the presence or absence of NE. The quantification of NFAT nuclear translocation was determined as follows: (i) GFP-positive HL-1 cells were selected; (ii) in the case of Homer 1a-HA over-expressing cells, the GFP subcellular localization was only analyzed in cells which were HA-positive; (iii) the GFP fluorescence was considered nuclear if its intensity in the nuclear area was greater than in the cytoplasm. At least 30 HL-1 cells for each assay condition were analyzed in three independent experiments. The ratio NFATc1nuclear/NFATc1cytoplasmic was used to analyze data. Following the same criteria, NFAT nuclear translocation was also quantified in non-transfected HL-1 cells, in either the absence or presence of NE treatment, in order to characterize the hypertrophic cellular model.

3.22 Measurement of cell size

HL-1 cells were analyzed by immunofluorescence microscopy in order to monitor the increase in cell area upon NE-stimulation. For this reason, cells were transfected with either pHomer1a-HA1, pHomer1c-HA1 or pcDNA3, and cultured in serum-free conditions. 24 h after transfection, cells were stimulated with 75 µM NE for 48 h, then fixed with 4% paraformaldehyde for 10 min. Double staining was performed using the anti-HA monoclonal antibody (1:100 dilution) and the anti-β tubulin polyclonal antibody (1:200 dilution). The surface area in HA staining-positive HL-1 cells was compared with

that in HA-negative cells. Acquired confocal fluorescence images were analyzed and compared using Scion Image software. The surface area of at least 30 HL-1 cells for each assay condition was measured in three independent experiments. This method was also used to analyze non-transfected HL-1 cells in either the absence or presence of NE treatment, in order to characterize the hypertrophic cellular model.

3.23 Dual luciferase assay

The gene expression of the ANF at promotorial level was measured with the Dual Luciferase reporter assay system (DLR assay, Promega), a efficient method used to study gene expression. In the DLR™ Assay, the activities of firefly (Photinus pyralis) and *Renilla* (Renilla reniformis, also known as sea pansy) luciferases were analyzed sequentially from a single sample.

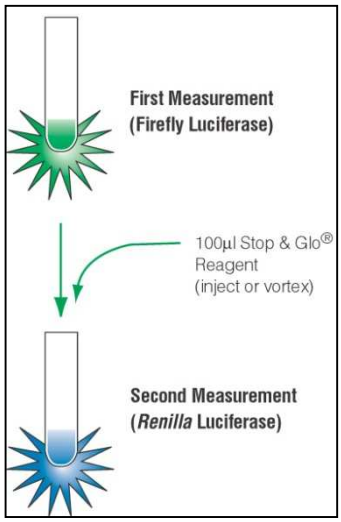


Figure 8. Format of the DLR™ Assay using a manual luminometer. From Promega Corporation.

First, firefly luciferase was measured by adding specific substrates; after quantification, the reaction was quenched, and the *Renilla* luciferase reaction was initiated by simultaneously adding specific reagent to the same tube (fig. 8). Relative luciferase units were calculated by determining the ratio of the intensity of the light produced by firefly luciferase reporter plasmid to that produced by *Renilla* luciferase pRL-TK plasmid (used as internal control).

During the experiment, the HL-1 cells were co-transfected with 3 µg of prom-ANF-firefly luciferase reporter plasmid, 0.06 µg of pRL-TK vector, and 3 µg of either

Homer 1a-HA1 or Homer 1c-HA1 plasmid. 24 h after transfection, cells were cultured in either the presence or absence of 75 µM NE. At the end of NE-stimulation, cells were rinsed with PBS and lysed with the Passive Lysis Buffer (PLB, Promega). The luciferase activities were determined with an analytical luminometer, used in accordance with the manufacturer's instructions.

3.24 Statistical analysis

All values are means ± SE. Individual means were compared using either a paired 2-tailed *t*-test or one-way ANOVA using Origin®8 software. The statistical differences were considered significant at the 0.05 level of confidence.

4. Results

4.1 Presence and sub-cellular localization of Homer 1a in the heart

4.1.1 Analysis of Homer 1a expression in cardiac tissue under resting conditions

Our research focused on the role of Homer 1a in the heart. Since little is known about the presence and sub-cellular localization of Homer 1 isoforms in the heart, we began our research by analyzing the expression pattern and sub-cellular distribution of Homer 1a in cardiac tissue under resting conditions.

For this purpose, the expression of both long and short Homer 1 isoforms (Homer 1b/c and Homer 1a, respectively) was analyzed in cardiac muscle (H) from both adult rat and mouse, and cardiac HL-1 cells. Western blot analysis of total homogenates (Fig. 9) was carried out with specific antibodies for either Homer 1b/c or Homer 1a (upper and lower lanes, respectively). Homer 1b/c was detected in all lanes as a specific band of 45 kDa and was found to be virtually identical in all samples. Anti-Homer 1a antibodies clearly recognized a band of ~27 kDa in all samples.

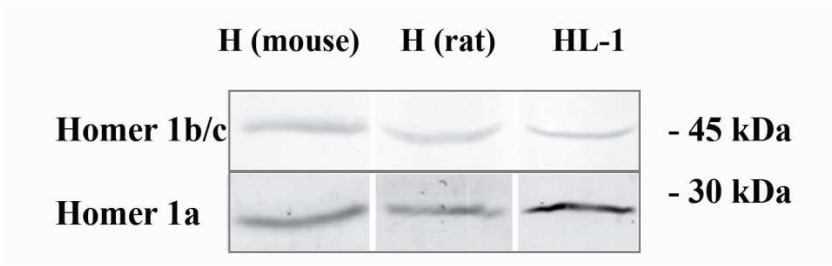


Figure 9. Homer 1 isoforms in cardiac (H) muscle of rat and mouse and in HL-1 cells. Homogenates (200µg/lane) were analyzed by Western blot using specific antibodies for either Homer 1a or Homer 1b/c as described in the Materials and Methods section. Immunoblots are representative of three experiments.

These results show that not only the long isoform Homer 1b/c but also the short Homer 1a is constitutively expressed, independently from the species. The constitutive expression of Homer 1a protein in cardiac muscle, as shown here, seems to agree with previous findings concerning the presence of mRNA coding for Homer 1a in skeletal and cardiac muscles (Sandonà D. *et al.*, 2000). Since Homer 1a is not only an IEG product, but is also constitutively expressed, the characterization of Homer 1a gene in neuronal tissue as an IEG (Xiao B. *et al.*, 1998) becomes less rigorous, supporting the hypothesis

that there are two pools of Homer 1a: inducible and constitutive (Bortoloso E. *et al.*, 2006).

4.1.2 Sub-cellular localization of Homer 1a in rat heart

After analysing Homer 1a protein expression in the heart under resting conditions, we carried out further analysis on the sub-cellular distribution of Homer 1a by confocal microscopy. Homer 1a sub-cellular distribution was analyzed in adult rat heart using specific antibodies.

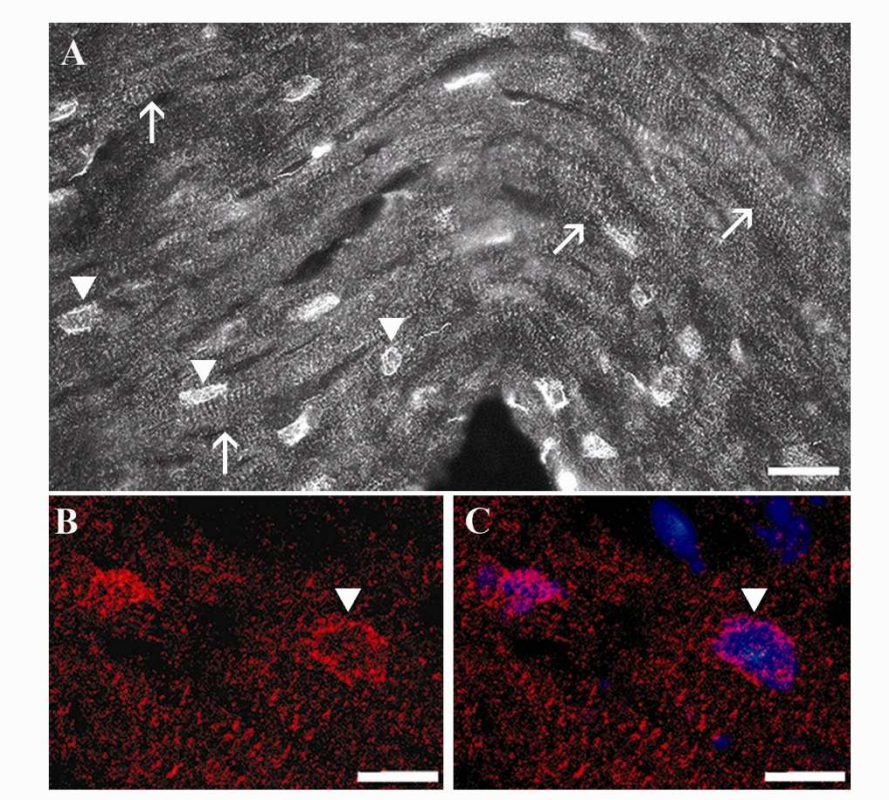


Figure 10. Subcellular distribution of Homer 1a in adult rat heart. Immunofluorescence microscopy of heart sections labelled with specific antibody for Homer 1a (A-C). (A) Low-magnification field showing sarcomeric (arrows) and nuclear (arrowheads) localization of Homer 1a. (C) Higher magnification field showing Hoechst-positive (blue labelled) nuclei. The Homer 1a signal is clearly associated with the nuclear domain (arrowheads in both pictures). Immunofluorescence of the heart section labelled with the secondary antibody alone was used as negative control for each experiment (data not shown). Bar: 40 µm in A, 25 µm in B, C.

Fig. 10 A-C shows that Homer 1a had a peculiar localization: it was sarcomeric (arrows) and perinuclear (arrowheads), as judged by immunostaining around Hoechst-positive nuclei (C).

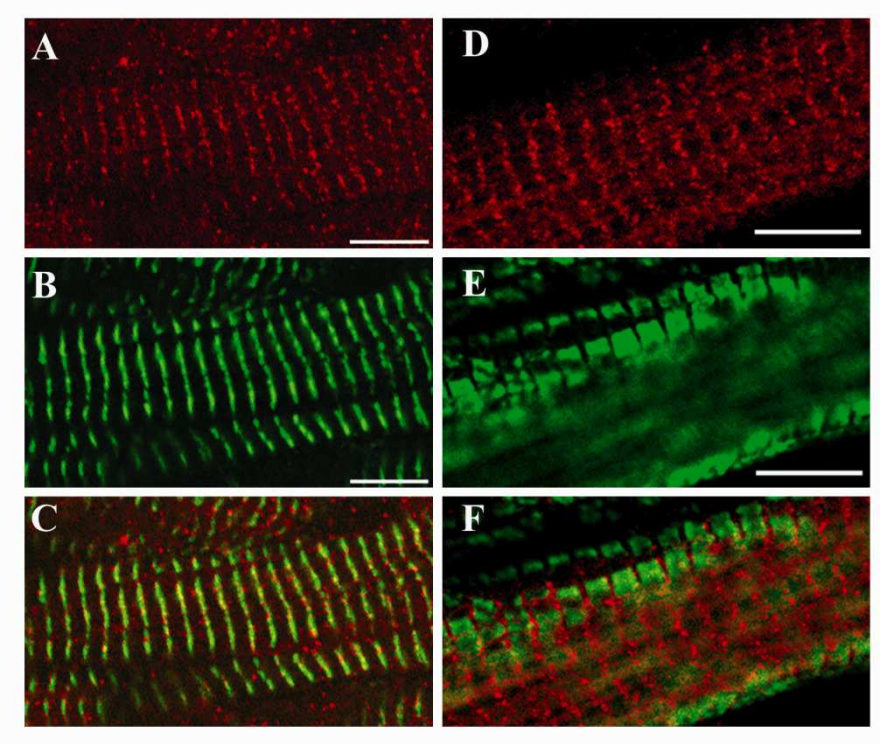


Figure 11. Subcellular distribution of Homer 1a, α-actinin and MHC in adult rat heart. Immunofluorescence microscopy of heart sections labelled with specific antibodies for either Homer 1a and α-actinin (A-C) or Homer 1a and MHC (D-F). A-C: Homer 1a (red labelling in A) and α-actinin (green labelling in B). Merge image indicates partial co-localization between Homer 1a and α-actinin (C). D-F: Homer 1a (red labelling in D) and MHC (green labelling in E). Merge image indicates no co-localization between Homer 1a and MHC (F). Immunofluorescence of heart section labelled with secondary antibodies alone was completely negative. Bar: 10 µm in A-F.

Homer 1a sarcomeric localization was investigated in relation to two distinct sarcomeric proteins: α-actinin, that is localized to the sarcomeric Z-discs, and myosin heavy chain (MHC), which is present at the A-band. Double immunofluorescence labelling (anti-Homer 1a/anti α-actinin and anti-Homer 1a/anti-MHC) was performed in sections of adult rat heart. As shown in fig. 11, sarcomeric localization at the Z-line level of Homer 1a was clearly revealed by double labelling with antibodies for α-actinin (Fig. 11 panels A-C), whereas at the A-band level the lack of co-localization of Homer 1a with MHC is evident (panels D-F).

These results show that, despite being a soluble protein, Homer 1a displayed a sarcomeric localization in the heart at the Z-disc level, and was present in close proximity of the nuclear envelope. This peculiar localization might provide the structural basis for understanding the role of Homer 1a in cardiac tissue.

4.2 Homer 1a expression under cardiac hypertrophic conditions

4.2.1 Analysis of Homer 1a expression in two hypertrophic models in vitro

Few studies have focused on Homer 1a in the heart under hypertrophic conditions. Furthermore, *in vitro* reports present conflicting data: on the one hand, both mRNA and protein of the short Homer 1 isoform (Homer 1a) were upregulated following hypertrophy induced by several hypertrophic agents (Kawamoto T. *et al.*, 1998; Guo WG. *et al.*, 2010); on the other hand, a recent study has demonstrated that only the long isoform Homer 1c increased after PE stimulation, whereas Homer 1a remained unchanged (Grubb DR. *et al.*, 2011).

In order to better understand the modulation of Homer 1 isoforms expression in the hypertrophic pathways, we started our study by analyzing the expression of Homer 1c and Homer 1a in two *in vitro* cell cultures (i.e., primary neonatal rat cardiomyocytes and HL-1 cell line), both stimulated with the hypertrophic adrenergic agonist NE.

4.2.2 Homer 1a expression in neonatal rat cardiomyocytes following NE stimulation

Initially, both Homer 1 mRNA and protein were monitored in culture of neonatal cardiomyocytes following stimulation with 10 μM NE. Primary neonatal rat cardiomyocytes were prepared as reported in Materials and Methods.

The abundance in cardiomyocytes (despite fibroblast) was evaluated in each experiment by immunostaining the cells with α-actinin (used as a specific marker of cardiomyocytes). As reported in Fig. 12 A-B, α-actinin staining showed an enrichment in cardiomyocytes of about 80%.

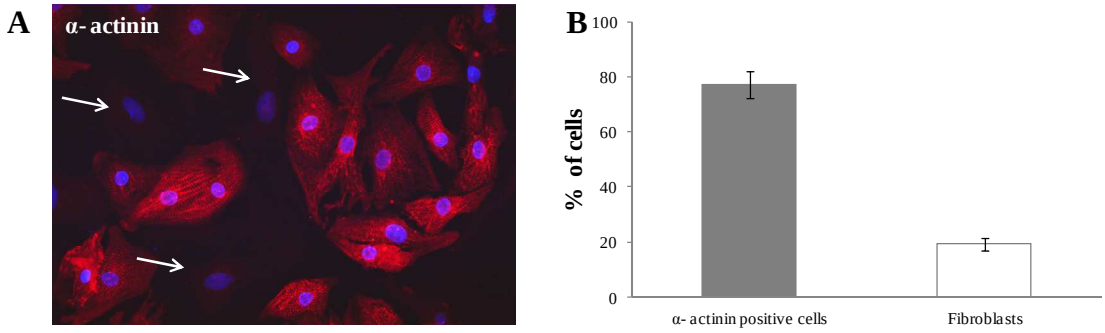


Figure 12. Enrichment of neonatal rat cardiomyocytes in primary culture. Panel A shows a representative immunofluorescence image of cultured cells labelled with specific antibody for α-actinin (red labelling in A) and with Hoescht for the nuclei (blue labelled in A). For each experiment, we determined the

number of positive and negative (fibroblasts) α-actinin cells. As shown in panel B, the percentage of α-actinin positive cells was about 80%.

Homer 1a mRNA was assessed by qPCR at the indicated time points. Fig. 13 (panel A) shows that Homer 1a transcript was found to be significantly increased after 1 h and remained constant at 2 h. As shown in fig. 13 (panel B), the Homer 1a-Homer 1b/c ratio also increased in the same time span, indicating that only transcription of Homer 1a is up-regulated during stimulation with NE.

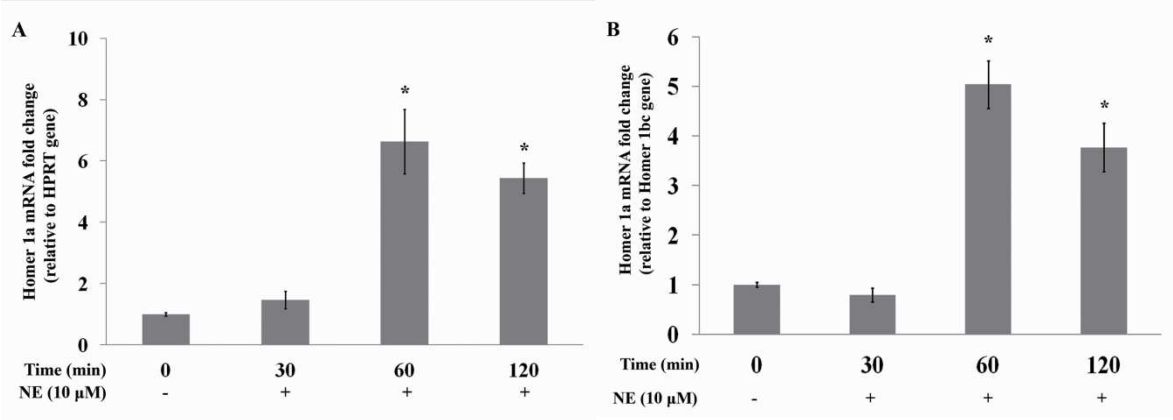


Figure 13. Homer 1a and Homer 1b/c upon NE-induction in rat cardiomyocytes: qPCR analysis. (A) Time course of Homer 1a mRNA upon induction of cardiomyocytes with 10 µM NE. (B) The Homer 1a-Homer 1b/c ratio was significantly higher after 60 min and 120 min of NE stimulation compared with that of non-stimulated cells (0 min). In A and B, data are shown as fold induction of non-stimulated cells. Values are means ± SE; n = 3. *P < 0.05, 120 min and 60 min *versus* 0 min in panels A and B.

Many studies have indicated that NE is able to induce cardiac hypertrophy by activating PKA and PKC through α_1- and β- adrenoceptors (ARs), respectively (Yamazaki T. *et al.*, 1997). In the cellular model used in our experiments, the adrenergic pathways involved in NE-induced Homer 1a up-regulation were assessed using specific α_1 and β inhibitors: prazosin and propranolol, respectively. As shown in Fig. 14, the α_1-blocker prazosin, but not the β-blocker propranolol, significantly reduced the up-regulation of Homer 1a mRNA during NE-induction, as compared to control. These results indicate that NE induces Homer 1a transcription through the α_1-ARs.

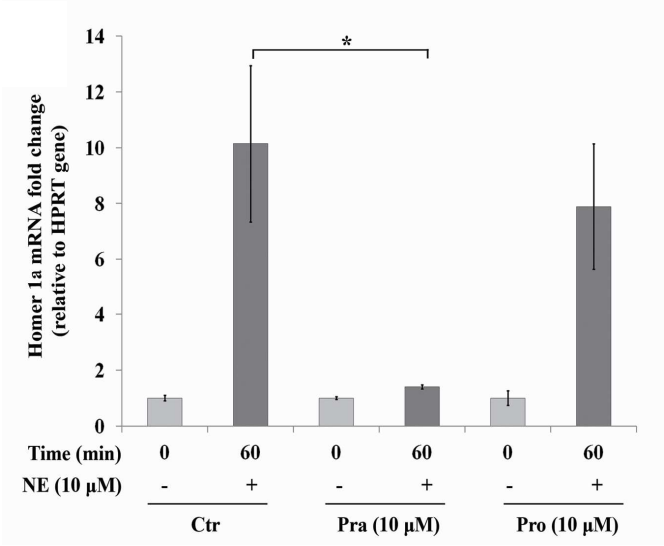


Figure 14. The effect of specific adrenergic inhibitors on the up-regulation of Homer 1a. After 30 min pre-treatment with either 10 µM prazosin (PRA) or 10 µM propranolol (PRO), cardiomyocytes were stimulated with 10 µM NE for 60 min. Data are given as fold change of relative non-stimulated cells, and are means ± SE of two independent experiments performed in triplicate. *P < 0.05, 60 min control (Ctr, +NE) *versus* 60 min Pra (+NE).

Next, the expression of Homer 1a protein was monitored in neonatal cardiomyocytes following incubation with 10 µM NE by Western blot. As shown in Fig. 15, the content of Homer 1a protein was doubled 2 h after stimulation (panels A, B).

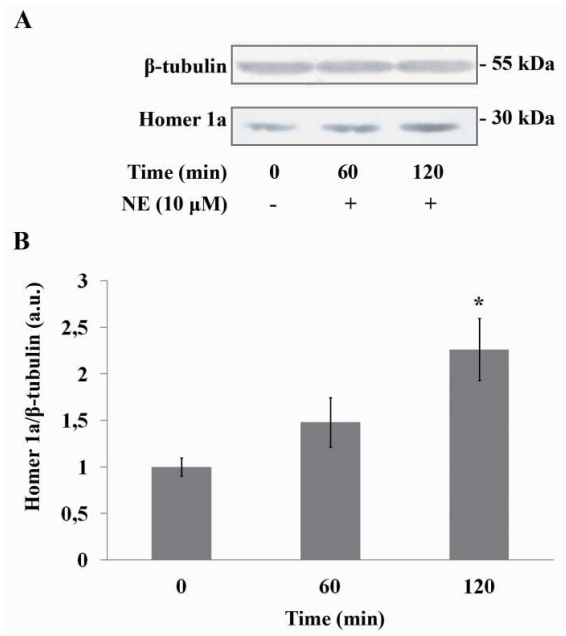


Figure 15. Homer 1a upon NE-induction in neonatal rat cardiomyocytes: Western blot analysis. (A) Representative Western blot of Homer 1a content in neonatal rat cardiomyocytes treated with 10 µM NE at the indicated time points. (B) Densitometric analysis. Values were normalized to β-tubulin and are means ± SE; n = 3. *P < 0.05 120 min *versus* 0 min (non-stimulated cells).

We also investigated the effect of NE on the expression of the long isoform Homer 1b/c. Fig. 16 shows that Homer 1b/c, unlike Homer 1a, was unchanged during adrenergic stimulation (panels A, B).

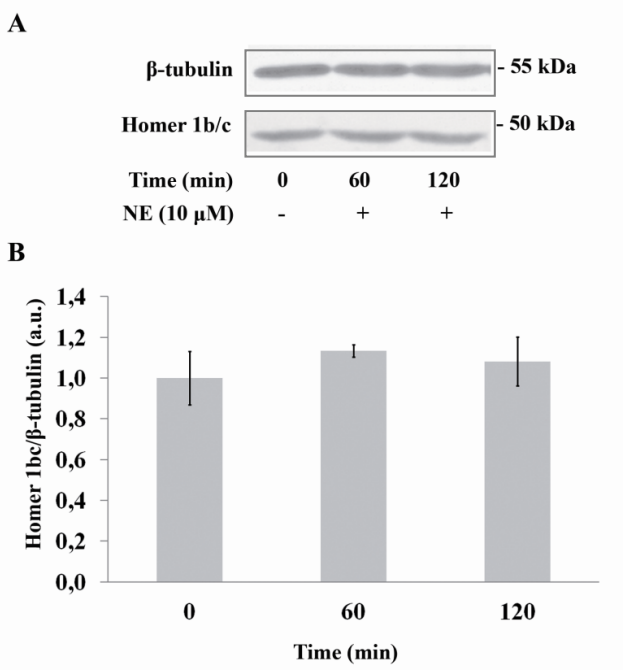


Figure 16. Homer 1b/c upon NE-induction in rat cardiomyocytes: Western blot analysis. (A and B) Representative Western blot and densitometric analysis of Western blot obtained with specific antibodies for Homer 1b/c. Values were normalized to β-tubulin and are means ± SE; n = 3.

Collectively, these results demonstrate that *in vitro* hypertrophic stimulation affects the short isoform Homer 1a, but not the long Homer 1b/c, and support the hypothesis of an involvement of Homer 1a in the α_1-adrenergic pathway leading to cardiac hypertrophy.

4.2.3 Homer 1a expression in HL-1 cells following NE stimulation

As the next step, we tried to verify these findings using a different *in vitro* cellular model. For this purpose, we used the HL-1 cell line. HL-1 cardiomyocytes are a stabilized cell line of cardiac myocytes derived from murine tumor atrial cells, and exhibit an adult cardiomyocyte-like gene expression profile (Claycomb WC. *et al*., 1998). HL-1 cells are an experimental model which are useful in studying the intracellular signalling pathways activated during cardiac hypertrophy (Chandrasekar B. *et al.*, 2005; Brunt KR. *et al.*, 2009; Wang W. *et al.*, 2008; Piñeiro R. *et al.*, 2005; López-Andrés N. *et al.*, 2008). As described in Materials and Methods, the experiments were carried out by stimulating HL-1 cells with 75 µM NE. Fig. 17 shows that treatment of HL-1 cardiomyocytes with NE

significantly increased Homer 1a within 1 h. Homer 1a content (panel A) was up-regulated by a factor of 2.5, as determined by densitometry (panel B).

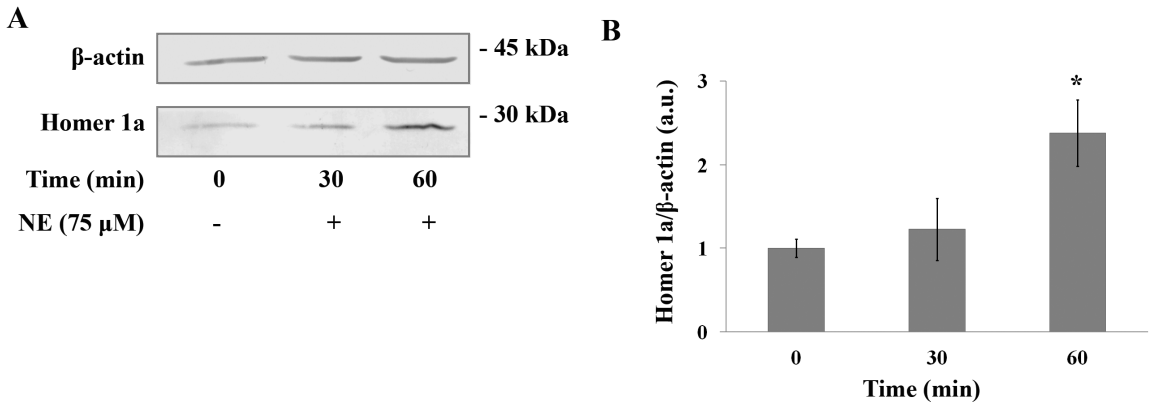


Figure 17. NE-induced change in the expression of Homer 1a in HL-1 cells. (A) Cell lysates were probed with antibodies specific for Homer 1a; (B) Densitometric data of Western blot. Values are means ± SE; n = 5. *P < 0.05 60 min *versus* 0 min (non stimulated cells).

Under the same experimental conditions, we analyzed Homer 1b/c over the same time span, and we found the level of Homer 1b/c protein expression to be unchanged (Fig. 18).

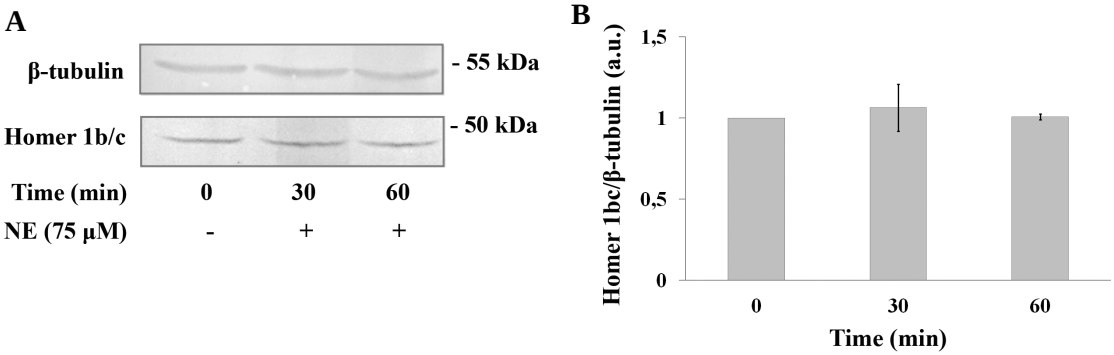


Figure 18. Homer 1c expression in NE-induced HL-1 cells. (A) Cell lysates were probed with antibodies specific for Homer 1b/c; (B) Densitometric data of Western blot. Values are means ± SE; n = 5.

These data validate the results obtained in neonatal cardiomyocytes, strengthening the evidence of an involvement of Homer 1a, but not Homer 1b/c, in cardiac hypertrophy.

4.3 The effects of NE on HL-1 cells

4.3.1 Characterization of hypertrophic responses in HL-1 upon NE stimulation

For our study, HL-1 cell line was used as a cellular model to study the effect of Homer 1a on NE-activated hypertrophic pathways. We had previously carried out a preliminary characterization of the HL-1 hypertrophic phenotype following stimulation with NE. For this purpose, the hypertrophic phenotype was detected by monitoring: i) activation of intracellular signalling pathways such as MAPK/ERK and NFAT, ii) activation of fetal genes such as ANF, and iii) increase in cell size. All of these are well-known events leading to cardiac hypertrophy.

MAPK pathways, such as ERK1/2, are thought to play an important role in the activation of hypertrophic responses. We examined the changes in MAPK pathways, in particular ERK1/2 phosphorylation, at different time points following NE treatment. Both NE-treated and untreated HL-1 cells were collected and analyzed by Western blot. As shown by densitometric analysis, there was a peak in activity (Fig. 19, panel A) with a four-fold increase of p-ERK1/2 within 5 min (Fig. 19, panel B).

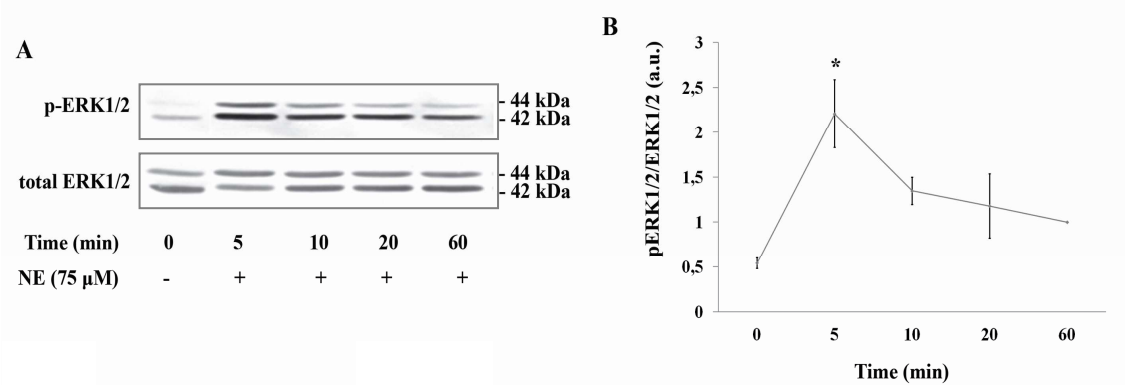


Figure 19. NE-dependent activation of MAPK/ERK1/2 in HL-1 cells (A, B). Phosphorylated ERK was determined on cell lysates by Western blot using phospho-specific ERK1/2 antibodies. The membrane was then stripped and re-probed with a pan (total)-ERK antibody. The phosphorylated ERK1/2-total-ERK1/2 ratio was obtained at specified time points following the application of 75 µM NE. Values were obtained by densitometric analysis (in B panel), and are expressed as means ± SE, n = 3; *P < 0.05, 5 min *versus* 0 min.

Over the next 60 min, levels of p-ERK activation decreased to two-fold higher than basal levels (Fig. 19).

Next, we examined if NE affects NFAT pathways. NFAT is a Ca^{2+}-sensitive transcription factor that is activated by calcineurin, resulting in translocation to the nucleus and activation of gene transcription. Four different NFAT isoforms are expressed

in the heart (NFATc1, NFATc2, NFATc3 and NFATc4). It is not fully understood if there is a common mechanism in cardiac cells that induces the activation of all isoforms, or if there are isoform-specific or tissue-specific differences (Rinne A. *et al.*, 2010). However, in our cellular model, we previously investigated the effect of NE on two NFAT isoforms, NFATc1 and NFATc4. In our experimental system, only the NFATc1 isoform was found to be affected by NE stimulation (data not shown) so, following preliminary studies, only that isoform was examined. NFATc1-GFP was therefore expressed in HL-1 cells via transfection and the transfected cells were then stimulated with NE. NFATc1-GFP sub-cellular distribution was monitored using confocal microscopy at two well-defined periods – 5 h and 12 h - after treatment with NE. As shown in the bar graph, NFATc1-GFP nuclear translocation increased after 5 h of NE-treatment and a significant nuclear translocation was observed after 12 h.

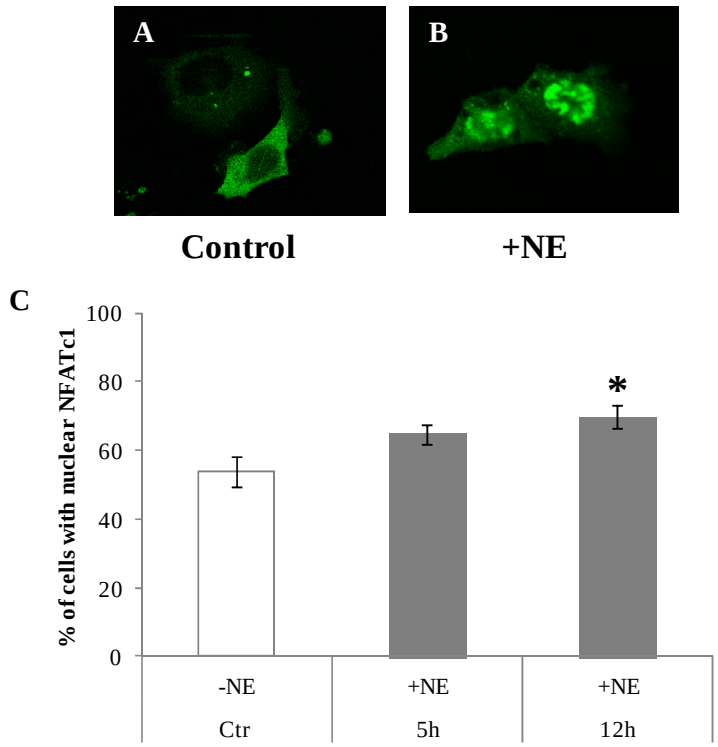


Figure 20. NFATc1-GFP nuclear translocation following NE stimulation. HL-1 expressing NFATc1-GFP in the absence of NE (fig. A) or the presence of 75µM NE (fig. B) are shown. NFATc1 nuclear translocation was measured as described in Materials and Methods. Bar graph in C shows the percentage of HL-1 cells with nuclear predominant fluorescence observed under normal conditions or NE-stimulation after 5 h and 12 h. NFAT nuclear translocation significantly increased after 12 h of NE-stimulation as compared to control. Data are given as percentage relative to the ratio of the number of cells with nuclear fluorescence to that of cells with cytosolic fluorescence, and are expressed as means ± SE; *P < 0.05, +NE 12 h *versus* control (Ctr, -NE).

At gene expression level, up-regulation of fetal genes is a peculiar feature of cardiac hypertrophy; in particular, the re-expression of ANF is most commonly used as molecular marker of hypertrophy. The expression of ANF was therefore monitored at the promoter level in both NE-treated and untreated HL-1 cells. All samples were analyzed by the dual luciferase assay.

The results infer that NE stimulation caused a two-fold increase in ANF promoter activity (Fig. 21), as was to be expected from the knowledge that pathological hypertrophy is accompanied by re-activation of the fetal gene program (Schaub MC. *et al.*, 1997).

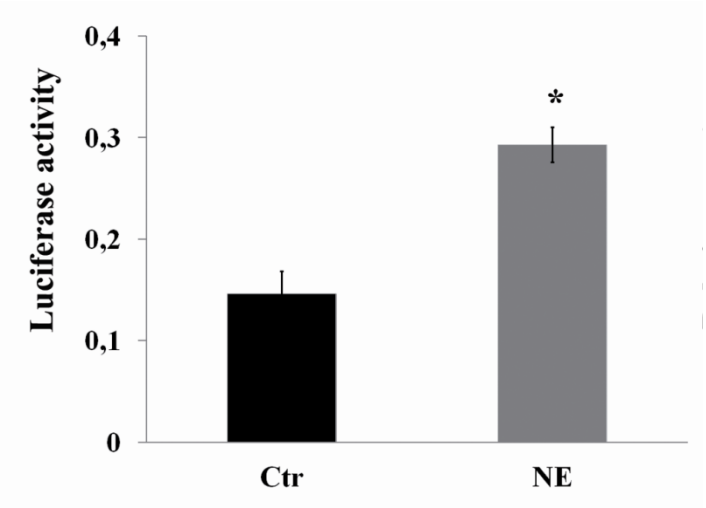


Figure 21. NE-dependent activation of ANF-promoter activity in HL-1 cells. ANF-promoter activity of HL-1 cells was measured by the luciferase assay, as detailed in Materials and Methods, in both the presence and absence of 75 µM NE applied for 24 h. Values are expressed as means ± SE. *P < 0.05, NE *versus* control (Ctr).

At morphological level, cardiac hypertrophy is mainly characterized by a marked increase in cell size. Therefore, we verified if the HL-1 stimulated with NE underwent any changes at this level. After both 24 h and 48 h of incubation with NE, the size of HL-1 cells was measured using confocal microscopy. The bar graph in fig. 22-panel C shows the relative increases in cell size. The results indicate that NE stimulation positively affected cell size, causing a 35% increase in cellular area after 48 h, as indicated by morphometry (Fig. 22). In Fig. 22 panels A and B, representative HL-1 cells before and after NE-induction show the average increase in cell size.

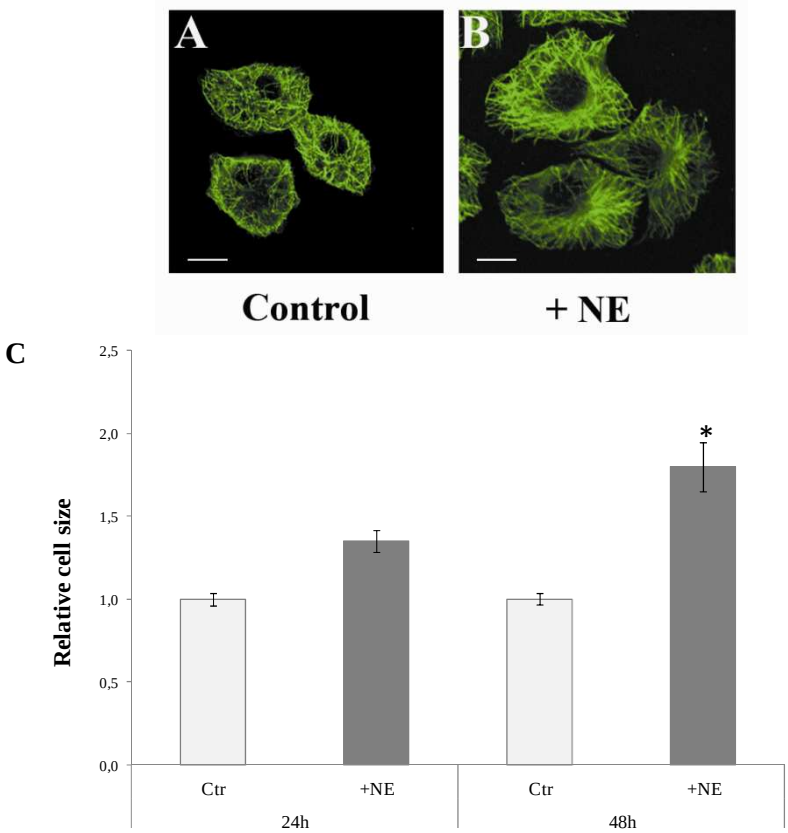


Figure 22. NE-dependent increases in the size of HL-1 cells. A and B show individual and representative HL-1 cells immunostained with β-tubulin in the absence and presence of 75 µM NE for 48 h, respectively. (C) The size of HL-1 cells was measured by morphometry, as described in Materials and Methods, in both the presence and absence of 75 µM NE applied for 24h and 48 h. Cells were selected randomly, and at least 30 cells were examined in each group. Data are given as fold change relative to average surface area of untreated cells (control, Ctr), and are expressed as means ± SE; *P < 0.05, 48 h +NE *versus* control (Ctr). Bar: 200µm.

Taken together, these results clearly demonstrate that the HL-1 cells exhibited an hypertrophic phenotype as a consequence of the NE stimulation, and prompted us to use this *in vitro* hypertrophic model to study the effect of Homer 1a using gain-of-function approaches.

4.4 The role of Homer 1a in cardiomyocyte hypertrophy

4.4.1 The effects of Homer 1a over-expression on NE-induced hypertrophy in HL-1 cells

The experiments described above suggest that NE stimulation markedly induces up-regulation of Homer 1a and that it is able to activate important events leading to hypertrophy. Consequently, we decided to assess the effects of over-expression of Homer 1a in HL-1 cells stimulated with NE. To this end, HL-1 cells were transfected with Homer 1a plasmid (pHomer 1a) for 24 h and then stimulated with NE.

First, in order to verify the efficiency of transfection, HL-1 cells transfected with pHomer 1a-HA were analyzed by Western blotting using antibodies against HA, the tag of exogenous Homer 1a. As reported in Fig. 23, Western blot analysis shows that anti-HA antibodies selectively detected a band of ~ 27 kDa, corresponding to Homer 1a protein.

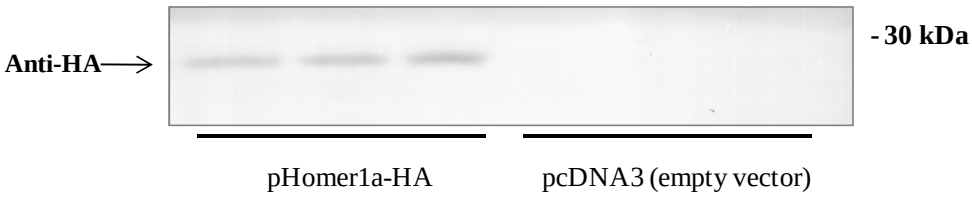


Figure 23. HL-1 cells transfected with Homer 1a-HA plasmid. Cells were transiently transfected with either Homer1a-HA plasmid or pcDNA3 plasmid (empty vector used as control). 24 h after transfection, the cells were lysed in RIPA buffer. Cell lysates were resolved by SDS-PAGE and immunoblotted with anti-HA antibody. A single band was detected at the expected size (~30 kDa) in HA-tagged samples alone. Three replicates for each condition were reported.

Next, we analyzed the same hypertrophic events which had been considered previously during NE stimulation - i.e. ERK phosphorylation level, NFAT nuclear translocation, ANF promoter activity and increase in cell size - using Homer 1a over-expressing HL-1 cells.

It should be noted that, in the absence of NE addition, Homer 1a over-expression did not affect the hypertrophic events investigated, suggesting that Homer 1a isoform *per se* did not promote cardiomyocytes hypertrophy (cfr. Time 0 in Fig. 24, -NE in Figs 25, 26 and 28).

With respect to MAPK/ERK activation, levels of phosphorylated ERK1/2 (p-ERK1/2) were monitored in NE-inducted HL-1 cells transfected with either pcDNA3 (used as control-empty vector) or pHomer1a. Both Western blot analysis (panel A) and densitometry (panel B) are reported in Fig. 24. Densitometric analysis infers that

exogenous Homer 1a significantly reduced peak levels of p-ERK1/2 by about 40%, and negatively modulated MAPK pathway along the entire experimental time span (panel B).

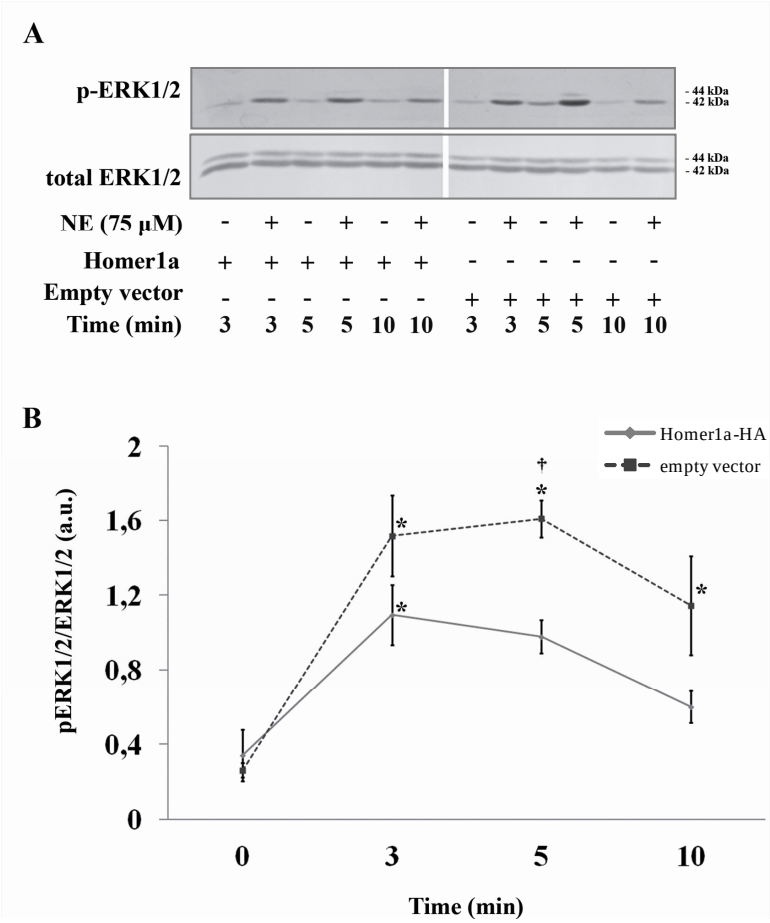


Figure 24. NE-dependent activation of MAPK/ERK1/2 in HL-1 cells: the effects of plasmid-driven over-expression of Homer 1a. Experiments were carried out as described in Materials and Methods and in the notes for Fig. 19, using HL-1 cells transfected either with pcDNA3 (empty vector) or pHomer 1a. (A) Representative Western blot analysis; (B) densitometric data showing the relative levels of p-ERK1/2. Data are given as means ± SE, n = 5-10. *P < 0.05; 10, 5, 3 min, *versus* 0 min. †P< 0.05; 5 min Homer 1a *versus* 5 min empty vector.

Next, we studied the effects of Homer 1a on NFAT translocation. In this set of experiments, HL-1 cells were co-transfected with both Homer1a-HA and NFATc1-GFP plasmids; effective co-transfection was assessed by staining with anti-HA antibodies. By immunofluorescence analysis, GFP sub-cellular localization was compared in HA-positive cells in both the absence and presence of NE after 5 h and 12 h of NE-treatment. HL-1 cells transfected with both pcDNA3 and NFATc1-GFP were used as control.

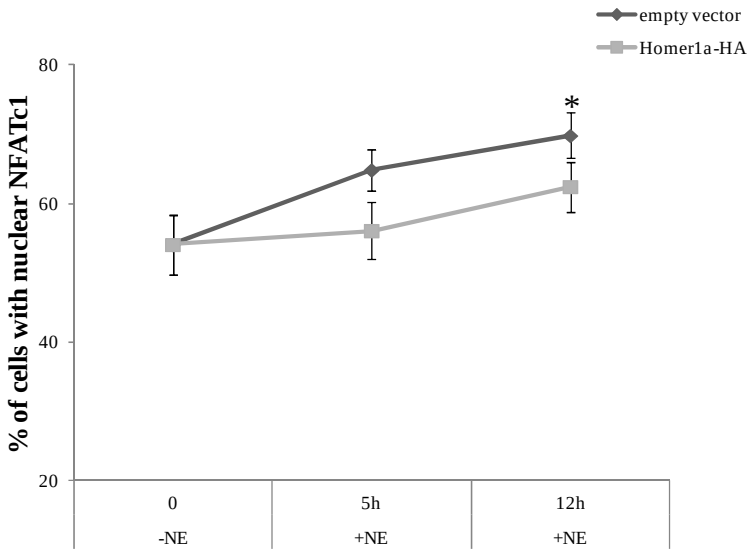


Figure 25. NFATc1-GFP nuclear translocation in Homer 1a over-expressing HL-1 cells following NE stimulation. GFP sub-cellular localization was analyzed in HA-positive cells in either the presence or absence of NE. The quantification of NFAT nuclear translocation was determined as reported in Materials and Methods. At least 30 HL-1 cells for each condition were analyzed in three independent experiments. NFAT nuclear translocation in Homer 1a over-expressing cells was lower after 5 h and 12 h of NE-stimulation than that in the control, but there was no statistically significant difference as compared to control. Data are given as percentage relative to ratio of the number of cells with nuclear fluorescence to that of cells with cytosolic fluorescence, and are expressed as means ± SE. * $P<0.05$ Empty vector 12 h+NE *versus* empty vector –NE.

Different observations were gathered from this analysis. Although the percentage of fluorescence nuclei in Homer 1a over-expressing cells was lower than that of the control during the total time span, we did not find a statistically significant difference between Homer-1a over-expressing cells and control. The results therefore indicate that, after NE-induction, the percentage of fluorescent nuclei in Homer 1a over-expressing cells was similar to that of the control, likely indicating an inability of Homer 1a to affect this pathway. However, since NFAT activation is controlled by CaN-dependent de-phosphorylation, these results can be confirmed with further approaches, such as considering CaN enzymatic activity.

As regards the ANF promoter activity, the previous experiments demonstrate that NE is able to induce re-expression of ANF in HL-1 cells. We therefore decided to verify if the effect of NE on ANF promoter activity is affected by Homer 1a over-expression conditions.

HL-1 cells were co-transfected with either Homer1a plasmid or empty vector (pcDNA3), ANFprom-Luciferase plasmid and *Renilla* luciferase vector. After 24 h of

treatment, cell lysates were analyzed for firefly and *Renilla* luciferase activities in both the absence and presence of NE. As shown in fig. 26, NE-stimulated increase of ANF promoter activity was reduced in Homer 1a over-expressing cells. In fact, data indicate that exogenous Homer 1a significantly reduced ANF promoter activity by about 20%.

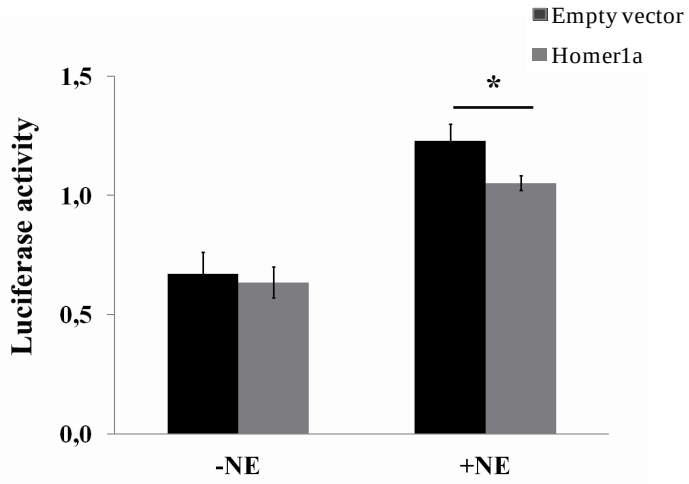


Figure 26. NE-dependent activation of ANF-promoter activity in HL-1 cells: the effect of exogenous Homer 1a. Experiments were carried out as described in Materials and Methods and in the notes for Fig. 21, using HL-1 cells transfected either with pcDNA3 (empty vector, black histograms) or pHomer 1a (gray histograms), in either the absence (-NE) or presence (+NE) of 75 µM NE. ANF promoter activity is the average of four experiments for Homer 1a. Data are given as mean ±SE. * $P<0.05$ Homer 1a +NE *versus* empty vector +NE.

In order to verify the specificity of Homer 1a effect on this hypertrophic process, we performed the same experiment over-expressing Homer1c-HA, one of the long Homer isoforms. The luciferase activity of ANF-promoter was measured under the same experimental conditions, as previously described for Homer 1a.

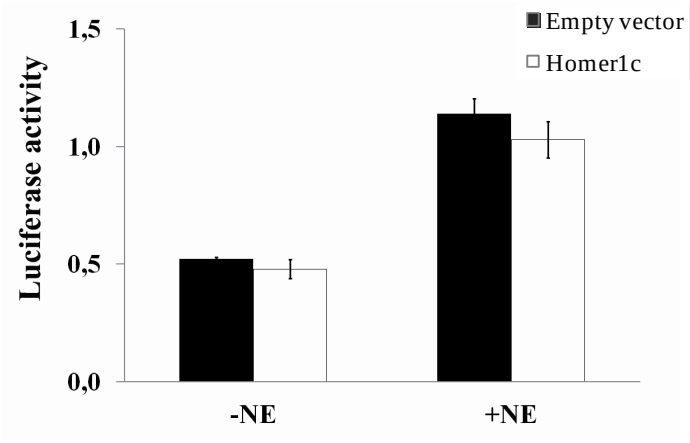
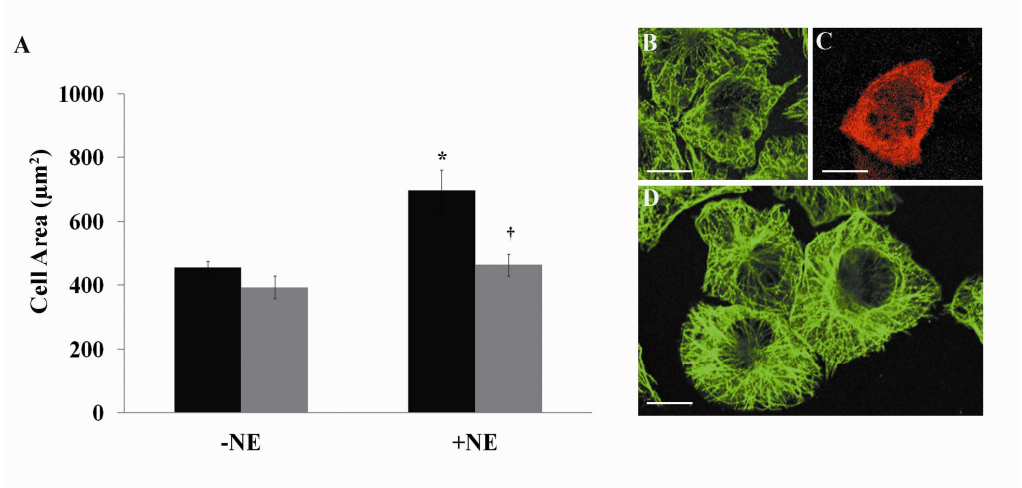


Figure 27. NE-dependent activation of ANF-promoter activity in HL-1 cells: the effect of exogenous Homer 1c. Experiments were carried out as described in Materials and Methods and in the notes to Fig. 21, using HL-1 cells transfected either with pcDNA3 (empty vector, black histograms) or pHomer 1c (white histograms), in either the absence (-NE) or presence (+NE) of 75 µM NE. ANF promoter activity is the average of three experiments for Homer 1c. Data are given as mean ±SE.

As shown in fig. 27, exogenous Homer 1c (unlike Homer 1a) did not affect the activity of ANF-promoter in NE-stimulated HL-1 cells. However, it should be noted that the over-expression of Homer 1c *per se* - as for Homer 1a - did not change the ANF promoter activity in the absence of NE stimulation (cfr. -NE in Figs. 27).

Previously, we found that NE induced an hypertrophic phenotype which promotes an increase in the size of HL-1 cells. Considering this result, we assessed the effect of Homer 1a over-expression in this morphological response during NE-treatment. HL-1 cells were transfected with either Homer1a-HA plasmid or empty vector (used as control). The surface area of HA staining-positive HL-1 cells was compared with that of control cells, in both the absence and presence of NE treatment. As inferred by morphometric analysis (Fig. 28), the increase in NE-stimulated cell size was significantly counteracted by exogenous, over-expressed Homer 1a.



E

	Cell size	
	- NE	+NE
Empty vector	1 ± 0.04	1.530 ± 0.14 *
Homer1a-HA	1 ± 0.15	1.073 ± 0.12
Homer1c-HA	1 ± 0.04	1.333 ± 0.04 *

Figure 28. NE-dependent increase in cell size of HL-1 cells: the effect of exogenous Homer 1a and exogenous Homer 1c. (A) Experiments were carried out as described in Materials and Methods and in the notes to Fig. 22, using HL-1 cells transfected either with pcDNA3 (empty vector, black histograms) or pHomer 1a-HA1 (gray histograms), in either absence (-NE) or presence (+NE) of 75 µM NE. Data are given as mean ±SE, * P<0.05 empty vector +NE *versus* empty vector –NE; † P<0.05 Homer1a +NE *versus* empty vector +NE. B-D show individual and representative HL-1 cells stimulated with 75 µM NE, transfected with either pHomer 1a and labelled with antibodies for β-tubulin (B), for epitope HA (C), or pcDNA3 and labelled with antibodies for β-tubulin (D). E summarizes the effect of Homer 1a and Homer 1c on cell size. Data are given as fold change relative to the average surface area of untreated cells (-NE). In E, * P<0.05 empty vector +NE *versus* empty vector –NE and Homer 1c-HA+NE *versus* Homer 1c-HA-NE.

Panels B-D show the inhibitor effect of Homer 1a on the hypertrophic phenotype in HL-1 cells. Again, we confirmed the specificity of this effect by over-expressing the long Homer 1 isoform (Homer 1c-HA) and we found that, under the same experimental conditions, the over-expression of Homer 1c did not exert significant effects on cell size. As reported in table E, cell size significantly increased upon NE stimulation only in Homer 1c over-expressing conditions and in pcDNA3-trasnfected cells, whereas with Homer 1a there was no increase in cell size in the presence of NE.

However, it should be pointed out that our results - as opposed to those of Grubb et al. (Grubb DR. *et al.*, 2011) who indicated that Homer 1c causes an increase in cardiomyocyte size in the absence of other effectors - did not demonstrate any pro-hypertrophic effect of Homer 1c over-expression.

In summary, our data indicate that NE-induction leading to hypertrophy is associated to up-regulation of Homer 1a but not Homer 1c. In addition, the over-

expression of Homer 1a for 24 h did not induce hypertrophy, but antagonized the initiation and development of hypertrophic responses triggered by NE; over-expression of Homer 1c, instead, was ineffective. The cogent interpretation of these results is therefore that Homer 1a is able to negatively modulate molecular mechanisms leading to pathological hypertrophy.

4.5 From *in vitro* to *in vivo*: Homer 1a expression in three different *in vivo* hypertrophic models

Since data obtained in our *in vitro* model suggest a role of Homer 1a in cardiac hypertrophy, we decided that the next step was to analyze the expression of Homer 1a in three different *in vivo* models. For this section of our study, we analyzed expression levels of Homer 1a protein in lysates obtained from the following *in vivo* cardiac hypertrophic models: i) Gαq over-expressing mice, ii) mice with transverse aortic constriction (TAC), and iii) rats treated with MCT.

4.5.1 Homer 1a protein expression in Gαq over-expressing mice

As mentioned in the introduction, Gαq protein is a subtype of the G protein that displays a pivotal role in the activation of signaling pathways leading to hypertrophic responses. Reports from different laboratories indicate that over-expression of the wild-type Gαq (or an activated form of Gαq) in the heart is sufficient to induce pathological cardiac hypertrophy resulting in heart failure and death (Mende U. *et al.*, 1998; Adams JW. *et al.*, 1998). In mouse heart, cardiac hypertrophy induced by over-expression of Gαq is characterized by an activated program of fetal gene expression, an increased heart weight in relation to body weight, and an increase in cardiomyocyte size (D'Angelo D. *et al.*, 1997).

For our study, mouse heart lysates, derived from hearts of 18-week-old transgenic mice over-expressing wild type Gαq, were kindly provided by Dr. N. Kaludercic, and were analyzed for their Homer 1a content by Western blotting. As reported in fig. 29, the amount of Homer1a did not increase, as compared to control (at least at the time indicated).

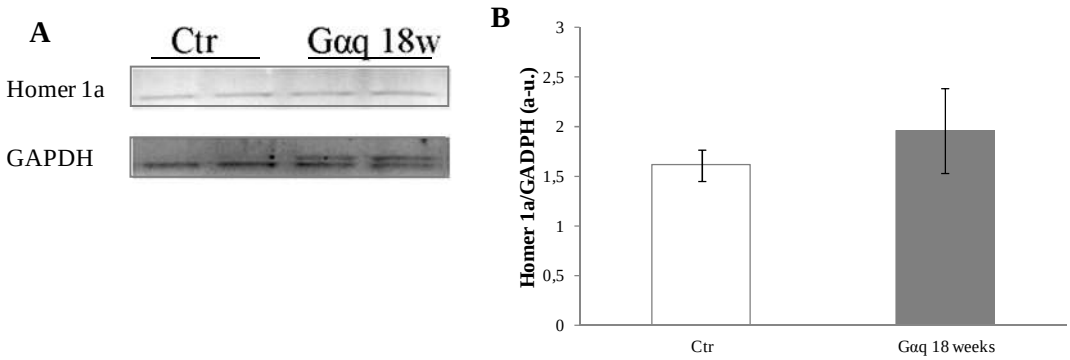


Figure 29. Homer 1a expression in Gαq over-expressing mice: Western blot analysis. (A) Representative Western blot of Homer 1a content in heart lysates from 18-week-old Gαq over-expressing mice. (B) Densitometric analysis. Values were normalized to GAPDH and are means ± SE; n = 4 for both conditions (Ctr and Gαq mice).

A possible explanation of this result is that we analyzed mice in a phase of cardiac hypertrophy too late, without considering the initial phases. This preliminary analysis therefore suggests that a time-course analysis of Homer 1a expression will be necessary, including the early phases of hypertrophy development up to progression to heart failure.

4.5.2 Homer 1a protein expression in mice with TAC

Transverse aortic constriction (TAC) in mice is a commonly-used microsurgical technique for pressure overload-induced cardiac hypertrophy and heart failure. This *in vivo* model is an useful tool for investigating the signalling processes involved in cardiac hypertrophic response and heart failure (deAlmeida AC *et al.*, 2010). In this experimental model, mice with TAC initially develop a compensated hypertrophy of the heart; however, over time, the response to chronic pressure overload becomes maladaptive, causing cardiac dysfunction and heart failure (Rockman HA *et al*. 1991).

In our study, we analyzed Homer 1a protein expression by Western blotting using mouse heart lysates collected at 1, 3, 6 and 9 weeks after TAC. The samples were kindly provided by Dr. N. Kaludercic. Densitometric analysis reported in fig. 30 shows that, at least at the indicated time points, Homer 1a protein did not increase, as compared to control. We observed, instead, an unexpected Homer 1a down-regulation after 1 week of TAC. Since there was not the analysis of Homer 1a in the first days after TAC, the interpretation of this expression pattern for Homer 1a remains incomplete. However, these results prompt us to investigate in the future Homer 1a expression (both at mRNA and protein level) at the very early phase after TAC, for example considering a time span that includes the immediate subsequent hours after TAC up to the first week.

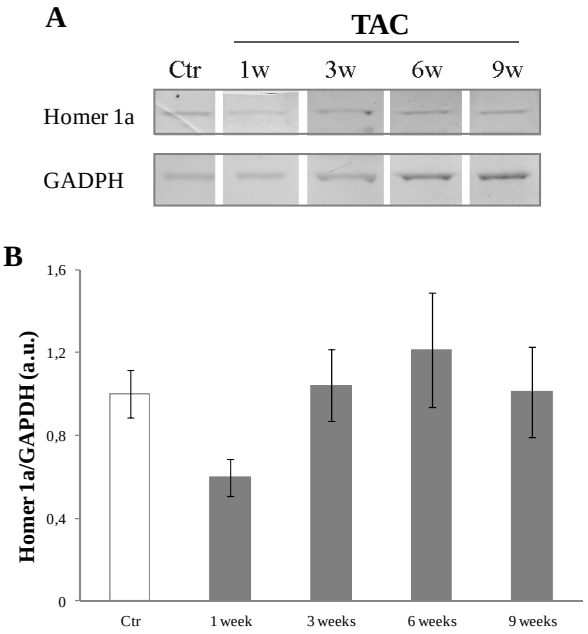


Figure 30. Homer 1a expression in mice with TAC: Western blot analysis. (A) Representative Western blot of Homer 1a content in heart lysates from mice with TAC. (B) Densitometric analysis. Values were normalized to GAPDH and are means ± SE; 1-week TAC n = 4, 3-week TAC n = 3, 6-week TAC n = 3, 9-week TAC n = 3, Ctr n=11.

4.5.3 Homer 1a protein expression in rats treated with MCT

MCT is a toxic pyrrolizidine alkaloid found in the plant species Crotalia spectabilis (Monnet E. *et al.*, 2005). Administration of small doses of MCT, or its active metabolite monocrotaline pyrrole, to rats causes progressive lung injury characterized by pulmonary hypertension (Schulze AE. *et al.*, 1998). MCT-induced pulmonary hypertension is associated to development of right-ventricle hypertrophy progressing to failure within weeks (Kőgler H. *et al.*, 2003).

We used this *in vivo* model to monitor the expression of Homer 1a during the development of right ventricular hypertrophy. Rats were sacrificed weekly to obtain a time course of hypertrophy induction. Changes in body and heart weight were registered at 1, 2 and 4 weeks. Heart to body weight ratio was used as an indicator of hypertrophy: as shown in the bar graph below (Fig. 31), it was significantly higher in the 4th week after MCT administration than that of both control group and of MCT-rats at the 1st and 2nd week after MCT administration.

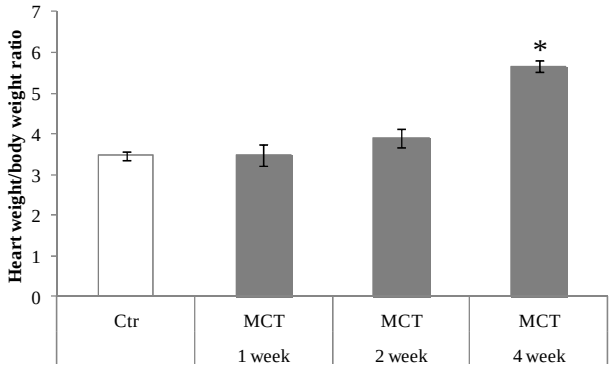


Figure 31. Time-course of heart progression of MCT-induced hypertrophy: analysis of heart weight/body weight ratio. Hearts from MCT-treated rats were harvested at three points in time: 1, 2 and 4 weeks after MCT induction. The increase in heart weight/body weight ratio in rats treated with MCT was significantly increased 4 weeks after MCT administration. Data are given as mean ±SE, Ctr n=6, rat-MCT (1 week) n=3, rat-MCT (2 week) n=3, rat-MCT (4 week) n=3, * P<0.05 Rat-MCT at 4 week *versus* CTR, Rat-MCT at 1 and 2 week.

We also measured the expression of both ANF and Homer 1a proteins by Western blot. As reported in the graphs (fig. 32 and fig. 33), we analyzed both proteins in tissue lysates from both left and right ventricles (LV and RV, respectively). Level of ANF was found to be significantly increased at the 4th week of MCT administration in MCT-treated rats.

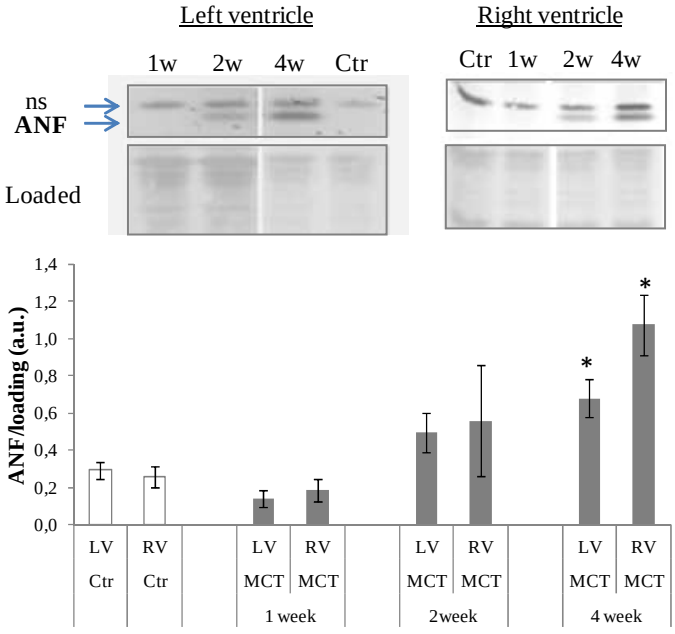


Figure 32. Time-course of ANF re-expression in MCT-treated rats: Western blot analysis. ANF re-expression was measured in both left and right ventricles at three points in time: 1, 2 and 4 weeks after MCT induction. The ANF content in both left and right ventricles was significantly increased four weeks after MCT administration. Data are given as mean ±SE, Ctr n=6, rat-MCT (1 week) n=3, rat-MCT (2 week) n=3, rat-MCT (4 week) n=3, * P<0.05 Rat-MCT at 4th week *versus* CTR, Rat-MCT at 1st and 2nd week. ns=non specific.

Fig. 33 shows the time-course of Homer 1a expression in both left and the right ventricle of MCT-rat. As reported in the graphs (panels C and D), Homer 1a protein was unchanged at the indicated time points, as compared to control.

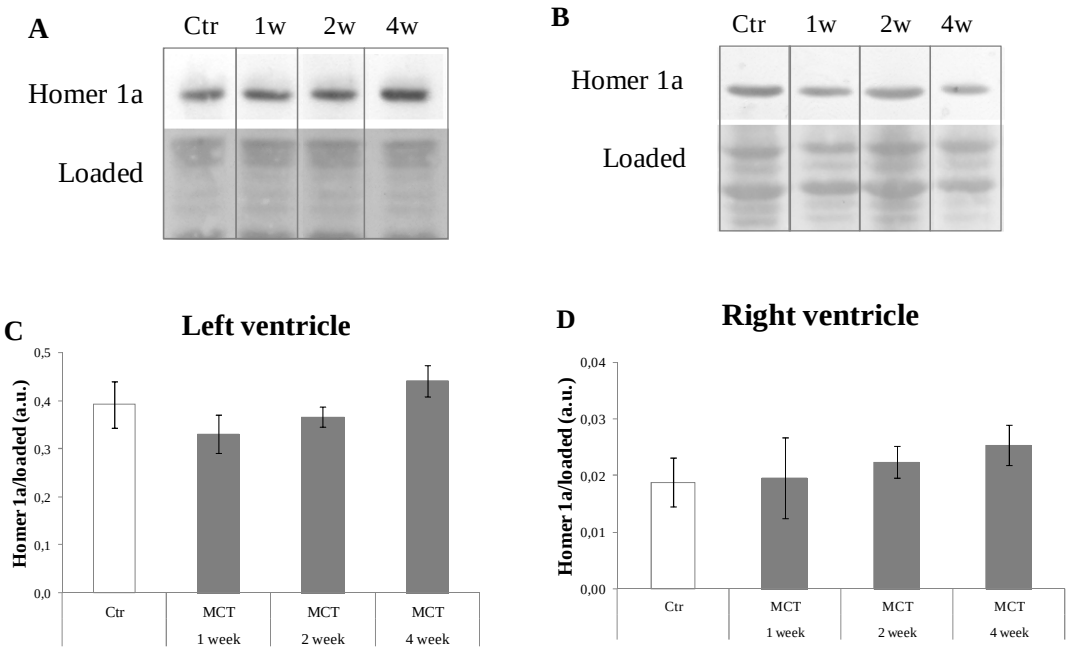


Figure 33. Homer 1a expression in MCT-treated rats: Western blot analysis. (A) Representative Western blot of Homer 1a content in heart lysates from the left ventricle of rats at 1, 2 and 4 weeks after MCT administration. (B) Representative Western blot of Homer 1a content in heart lysates from the right ventricle of rats at 1, 2 and 4 weeks after MCT administration. (C) and (D) Densitometric analysis. Values are means ± SE; Ctr n=6, rat-MCT (1 week) n=3, rat-MCT (2 week) n=3, rat-MCT (4 week) n=3.

In this latter, *in vivo* hypertrophic model, like in Gαq and TAC models, the expression of Homer 1a protein did not result affected by hypertrophic conditions.

That being so, there is a discrepancy between our *in vitro* and *in vivo* results for Homer 1a expression. However, it must be considered that up-regulation of inducible Homer 1a, in our *in vitro* model as well as in other neuronal models, is a response that occurs rapidly and transiently following stimulation. Therefore, since Homer 1a content, in the *in vivo* models, was not measured immediately after hypertrophic activation (for example, a few hours after TAC or MCT administration) or in the early stage of progression of hypertrophy (for Gαq), this *in vivo* analysis results incomplete, lacking the initial hypertrophic phase. This might likely be the explanation of our conflicting results. Further investigation is in order.

5. Conclusions

This study provides the structural and functional basis to understand the role of the scaffolding protein Homer 1a in cardiomyocyte hypertrophy. Here, we investigated the presence, sub-cellular localization of Homer 1 isoforms and their putative role in modulating cardiac hypertrophy.

Our results demonstrated that, like the long Homer isoforms, the short isoform Homer 1a is constitutively expressed in cardiac tissue. The Homer 1a constitutive expression, described also in the brain and in the skeletal muscle (Shiraishi Y. *et al.*, 2004; Bortoloso E. *et al.*, 2006), demonstrates that Homer 1a behaves not only as an IEG product; therefore, as discussed in the Results section, the assumption that there are two pools of Homer 1a: one constitutively expressed and one inducible (stimulus-dependent) (Bortoloso E. *et al.*, 2006), becomes more persuasive and is confirmed from our results.

In the nervous system, being scaffolding/modulatory proteins, Homers are localized into specific and restricted signalling microdomains, e.g., at the neuronal postsynaptic density (Hayashi MK. *et al.*, 2009; Worley PF. *et al.*, 2007). In heart, we demonstrated that, despite its nature of soluble protein (Pouliquin P. *et al.*, 2009), Homer 1a displays a sarcomeric localization at the level of the Z-disc, consistent with the model in which Homer 1a may regulate upstream intracellular signaling pathways, among which one might be the α_1-adrenergic signaling pathway. On the other hand, Homer 1a localization in close proximity of the nuclear envelope might also be suggestive of additional role on downstream signaling (either related or unrelated to hypertrophy), e.g., on activation of signaling pathways via IP3R, known to be localized on the nuclear membrane (Wu X. *et al.*, 2006). The latter is at present a mere speculation amenable, however, of further direct experimentation.

In both neuronal and non-neuronal cells, it has been demonstrated that various stimuli induce expression of two Homer 1 isoforms, Homer 1a and Homer 1b/c (Huang WD. *et al.*, 2005; Dietrich JB. *et al.*, 2007). Here, in *in vitro* cardiomyocyte models, we found that hypertrophic stimulation with NE increases the expression of Homer 1a mRNA and protein, but does not affect mRNA and protein levels of Homer 1b/c. In particular, we observed that NE-treatment leading to hypertrophy is associated with a rapid, α_1-adrenergic dependent up-regulation of Homer 1a. These results confirmed those of Kawamoto et al. in which mRNA for Homer 1a is increased in neonatal

cardiomyocytes upon stimulation with ET-1 and other adrenergic agonists (Kawamoto T. *et al.*, 2006) and those of Guo who indicate an increase in Homer 1a protein level following stimulation with Ang-II (Guo WG. *et al.*, 2010). Conversely, our findings are strikingly different from those of Grubb et al. who indicate that Homer 1a mRNA is not increased during 24 h of PE treatment (Grubb DR. *et al.*,2011). This discrepancy is probably due to different experimental approach: Grubb et al. missed early changes of Homer 1 isoforms, since the analysis were performed in cardiomyocytes 24 h after induction. Overall, these data support our initial hypothesis of an involvement of Homer 1a in cardiac hypertrophy and demonstrate that Homer 1a up-regulation is associated with the activation of hypertrophic signalling pathways.

Next, in an effort to better understand the role of Homer 1a in cardiac hypertrophy, different hypertrophic features were monitored, from cell size to ANF promoter activity. Using a gain-of function approach, we found that Homer 1a affects many of them, except for NFAT nuclear translocation. In the latter case, since NFAT activation pathway is regulated by the Ca^{2+}/calmodulin-dependent phosphatase calcineurin and Homer 1 isoforms modulate *in vitro* various players controlling intracellular calcium homeostasis, the result is inconsistent. Therefore, further studies will be necessary to clarify the effect of Homer 1a in this Ca^{2+}-dependent pathway. Nonetheless, we found that exogenous, over-expressed Homer 1a is able to modulate other events of the hypertrophy program, antagonizing important hypertrophic responses triggered by NE. In particular, Homer 1a attenuates NE-induced ERK1/2 activation and ANF promoter activity, and counteracts the increase in cell size. In this case, our observations are consistent with those of Grubb et al. showing an inhibitory effect of Homer 1a against hypertrophy and are mechanistically compatible with data obtained by Tappe et al. on spinal cord neurons – Homer 1a attenuates glutamate-induced MAP kinase activation and reduces synaptic contacts on neurons integrating pain inputs (Tappe A. *et al.*, 2006) - and by Luo et al. on PC12 cells - pERK is reduced by LV-Homer 1a and is increased by si-Homer 1a (Luo P. *et al.*, 2012a and Luo P. *et al.*, 2012b). Thus, not only in cardiomyocytes (present work) but also in spinal cord neurons and PC12 cells, it appears clear that Homer 1a antagonizes stimulation-dependent effects, i.e., chronic pain (Tappe A. *et al.*, 2006), apoptosis (Luo P. *et al.*, 2012a) and oxidative stress (Luo P. *et al.*, 2012b).

In conclusion, our *in vitro* results indicate that Homer 1a up-regulation is associated to early stages of cardiac hypertrophy and Homer 1a appears to play a role as a

negative molecular modulator, counteracting early steps leading to α_1-adrenergic-dependent hypertrophy. Considering its role as dominant-negative, Homer 1a would be inserted into a negative feedback mechanism that modulates pathological hypertrophy disassembling signaling complexes (yet to be identified) mediated by other Homer isoforms. However, further studies are needed to elucidate the mechanism underlying this process.

With respect to the *in vivo* approaches, our study presents some limitations. Although *in vitro* we observed the same expression pattern of Homer 1a in both neonatal rat cardiomyocytes and HL-1 cells under hypertrophic conditions, the results obtained in *in vivo* models are inconsistent. Preliminary studies addressing Homer 1a expression in mice with TAC, transgenic mice over-expressing Gαq and rats treated with MCT, demonstrate that Homer 1a did not change, at least in the time span under investigation. This discrepancy is likely due to an incomplete time-course analysis (due to unavailability of additional samples). Indeed, since *in vitro* Homer 1a is rapidly up-regulated following NE-stimulation and unveils its negative feedback on initiation and development of hypertrophic responses, a further, detailed *in vivo* analysis of early hypertrophic phase is in order.

6. References

- **Adams JW, Sakata Y, Davis MG, Sah VP, Wang Y, Liggett SB, Chien KR, Brown JH, Dorn GW 2nd.** Enhanced Galphaq signaling: a common pathway mediates cardiac hypertrophy and apoptotic heart failure. Proc Natl Acad Sci U S A 95(17):10140-10145, 1998.
- **Ango F, Prézeau L, Muller T, Tu JC, Xiao B, Worley PF, Pin JP, Bockaert J, Fagni L.** Agonist-independent activation of metabotropic glutamate receptors by the intracellular protein Homer. Nature 411(6840):962-965, 2001.
- **Ango F, Robbe D, Tu JC, Xiao B, Worley PF, Pin JP, Bockaert J, Fagni L.** Homer-dependent cell surface expression of metabotropic glutamate receptor type 5 in neurons. Mol Cell Neurosci 20:323-329, 2002.
- **Barry SP, Davidson SM, Townsend PA.** Molecular regulation of cardiac hypertrophy. Int J Biochem Cell Biol 40(10):2023-2039, 2008.
- **Beneken J, Tu JC, Xiao B, Nuriya M, Yuan JP, Worley PF, Leahy DJ.** Structure of the Homer EVH1 domain-peptide complex reveals a new twist in polyproline recognition. Neuron 26(1):143-154, 2000.
- **Bernardo BC, Weeks KL, Pretorius L, McMullen JR**. Molecular distinction between physiological and pathological cardiac hypertrophy: experimental findings and therapeutic strategies. Pharmacol Ther 128:191–227, 2010.
- **Berridge MJ.** Remodelling Ca^{2+} signalling systems and cardiac hypertrophy. Biochem Soc Trans 34(Pt 2):228-231, 2006.
- **Bers DM.** Cardiac excitation-contraction coupling. Nature 415(6868):198-205, 2002.
- **Bishopric NH, Kedes L.** Adrenergic regulation of the skeletal alpha-actin gene promoter during myocardial cell hypertrophy. Proc Natl Acad Sci U S A 88(6):2132-6, 1991.
- **Bortoloso E, Pilati N, Megighian A, Tibaldo E, Sandonà D, Volpe P.** Transition of Homer isoforms during skeletal muscle regeneration. Am J Physiol Cell Physiol 290(3):C711-8, 2006.

- **Bottai D, Guzowski JF, Schwarz MK, Kang SH, Xiao B, Lanahan A, Worley PF, Seeburg PH.** Synaptic activity-induced conversion of intronic to exonic sequence in Homer 1 immediate early gene expression. J Neurosci 22(1):167-75, 2002.
- **Brakeman PR, Lanahan AA, O'Brien R, Roche K, Barnes CA, Huganir RL, Worley PF.** Homer: a protein that selectively binds metabotropic glutamate receptors. Nature 386: 284-288, 1997.
- **Brunt KR, Tsuji MR, Lai JH, Kinobe RT, Durante W, Claycomb WC, Ward CA, Melo LG.** Heme oxygenase-1 inhibits pro-oxidant induced hypertrophy in HL-1 cardiomyocytes. Exp Biol Med 23:582-594, 2009.
- **Bueno OF, De Windt LJ, Tymitz KM, Witt SA, Kimball TR, Klevitsky R, Hewett TE, Jones SP, Lefer DJ, Peng CF, Kitsis RN, Molkentin JD.** The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. EMBO J 19:6341–6350, 2000.
- **Chandrasekar B, Mummidi S, Claycomb WC, Mestril R, Nemer M.** Interleukin-18 is a pro-hypertrophic cytokine that acts through a phosphatidylinositol 3-kinase-phosphoinositide-dependent kinase-1-Akt-GATA4 signaling pathway in cardiomyocytes. J Biol Chem 280:4553–4567, 2005.
- **Chen QM, Tu VC, Purdon S, Wood J, Dilley T.** Molecular mechanisms of cardiac hypertrophy induced by toxicants. Cardiovasc Toxicol 1(4):267-83, 2001.
- **Claycomb WC, Lanson NA, Stallworth BS, Egeland D, Delcarpio J, Bahinski A, Izzo NJ Jr.** HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypical characteristics of adult cardiomyocytes. Proc Natl Acad Sci USA 95(6):2979–2984, 1998.
- **Clerk A, Sugden PH**. Activation of protein kinase cascades in the heart by hypertrophic G protein-coupled receptor agonists. Am J Cardiol 83(12A):64H-69H, 1999.
- **Colella M, Grisan F, Robert V, Turner JD, Thomas AP, Pozzan T.** Ca^{2+} oscillation frequency decoding in cardiac cell hypertrophy: role of calcineurin/NFAT as Ca^{2+} signal integrators. Proc Natl Acad Sci U S A 105(8):2859-64, 2008.
- **Cooper GM.** The Cell. A Molecular Approach. 2nd edition. 2000

- **Cooper ST, Hanson IM.** A screen for proteins that interact with PAX6: C-terminal mutations disrupt interaction with HOMER3, DNCL1 and TRIM11. BMC Genet 12;6:43, 2005.
- **Czubryt MP, Olson EN.** Balancing contractility and energy production: the role of myocyte enhancer factor 2 (MEF2) in cardiac hypertrophy. Recent Prog Horm Res 59:105-124, 2004.
- **Dalla Libera L, Ravara B, Volterrani M, Gobbo V, Della Barbera M, Angelini A, Danieli Betto D, Germinario E, Vescovo G.** Beneficial effects of GH/IGF-1 on skeletal muscle atrophy and function in experimental heart failure. Am J Physiol Cell Physiol 286(1):C138-44, 2004.
- **D'Angelo DD, Sakata Y, Lorenz JN, Boivin GP, Walsh RA, Liggett SB, Dorn GW 2nd.** Transgenic Galphaq overexpression induces cardiac contractile failure in mice. Proc Natl Acad Sci U S A 94(15):8121-6, 1997.
- **deAlmeida AC, van Oort RJ, Wehrens XH.** Transverse aortic constriction in mice. J Vis Exp 21;(38), 2010.
- **Dietrich JB, Arpin-Bott MP, Kao D, Dirrig-Grosch S, Aunis D, Zwiller J.** Cocaine induces the expression of homer 1b/c, homer 3a/b, and hsp 27 proteins in rat cerebellum. Synapse 61(8):587-594, 2007.
- **Duncan RS, Hwang SY, Koulen P.** Effects of Vesl/Homer proteins on intracellular signaling. Exp Biol Med 230(8):527-35, 2005.
- **Fagni, L, Worley, PF, Ango, F.** Homer as both a scaffold and transduction molecule. *Sci STKE* 137:re8, 2002.
- **Fawcett DW.** Bloom & Fawcett- A textbook of Histology. 12th edition, 1996.
- **Feng QZ, Zhao YS, Abdelwahid E.** The role of Fas in the progression of ischemic heart failure: prohypertrophy or proapoptosis. Coron Artery Dis 19(7):527-534, 2008.
- **Feng W, Tu J, Pouliquin P, Cabrales E, Shen X, Dulhunty A, Worley PF. Allen PD, Pessah IN.** Dynamic regulation of ryanodine receptor type 1 (RyR1) channel activity by Homer 1. Cell Calcium 43(3):307-14, 2008.
- **Feng W, Tu J, Yang T, Vernon PS, Allen PD, Worley PF, Pessah IN.** Homer regulates gain of ryanodine receptor type 1 channel complex. J Biol Chem 277(47):44722-44730, 2002.
- **Foa L, Gasperini R.** Developmental roles for Homer: more than just a pretty scaffold. J Neurochem 108(1):1-10, 2009.

- **Foa L, Rajan I, Haas K, Wu GY, Brakeman P, Worley P, Cline H.** The scaffold protein, Homer1b/c, regulates axon pathfinding in the central nervous system in vivo. Nat Neurosci 4(5):499-506, 2001.
- **Frey N, Olson EN.** Cardiac hypertrophy: the good, the bad, and the ugly. Annu Rev Physiol 65:45-79, 2003.
- **Gjesdal O, Bluemke DA, Lima JA.** Cardiac remodeling at the population level--risk factors, screening, and outcomes. Nat Rev Cardiol 8(12):673-685, 2011.
- **Glennon PE, Kaddoura S, Sale EM, Sale GJ, Fuller SJ, Sugden PH.** Depletion of mitogen-activated protein kinase using an antisense oligodeoxynucleotide approach downregulates the phenylephrine-induced hypertrophic response in rat cardiac myocytes. Circ Res 78(6):954-61, 1996.
- **Glennon PE, Sugden PH, Poole-Wilson PA.** Cellular mechanisms of cardiac hypertrophy. Br Heart J 73(6):496-9, 1995.
- **Greenstein JL, Winslow RL.** Integrative systems models of cardiac excitation-contraction coupling. Circ Res 108(1):98-112, 2011.
- **Grubb DR, Luo J, Yu YL, Woodcock EA.** Scaffolding protein Homer 1c mediates hypertrophic responses downstream of Gq in cardiomyocytes. FASEB J 26:596-603, 2011.
- **Guo WG, Su FF, Yuan LJ, Yang GD, Shi XQ, Li RY, Shu Q, Liu XT, Lu ZF, Zheng QS.** Simvastatin inhibits angiotensin II-induced cardiac cell hypertrophy: role of Homer 1a. Clin Exp Pharmacol Physiol 37(1):40-45, 2010.
- **Hayashi MK, Ames HM, Hayashi Y.** Tetrameric hub structure of postsynaptic scaffolding protein homer. J Neurosci 26(33):8492-8501, 2006.
- **Hayashi MK, Tang C, Verpelli C, Narayanan R, Stearns MH, Xu RM, Li H, Sala C, Hayashi Y.** The postsynaptic density proteins Homer and Shank form a polymeric network structure. Cell 137(1):159-171, 2009.
- **Heineke J, Molkentin JD**. Regulation of cardiac hypertrophy by intracellular signalling pathways. Nat Rev Mol Cell Biol 7(8):589-600, 2006.
- **Hill JA, Olson EN.** Cardiac plasticity. N Engl J Med 358(13):1370-1880, 2008.
- **Hu JH, Park JM, Park S, Xiao B, Dehoff MH, Kim S, Hayashi T, Schwarz MK, Huganir RL, Seeburg PH, Linden DJ, Worley PF.** Homeostatic

scaling requires group I mGluR activation mediated by Homer1a. Neuron 68:1128– 1142, 2010.

- **Huang G, Kim JY, Dehoff M, Mizuno Y, Kamm KE, Worley PF, Muallem S, Zeng W.** Ca^{2+} signaling in microdomains: Homer1 mediates the interaction between RyR2 and Cav1.2 to regulate excitation-contraction coupling. J Biol Chem 282(19):14283-14290, 2007.
- **Huang GN, Huso DL, Bouyain S, Tu J, McCorkell KA, May MJ, Zhu Y, Lutz M, Collins S, Dehoff M, Kang S, Whartenby K, Powell J, Leahy D, Worley PF.** NFAT binding and regulation of T cell activation by the cytoplasmic scaffolding Homer proteins. Science 319(5862):476-481, 2008.
- **Huang WD, Fei Z, Zhang X.** Traumatic injury induced homer-1a gene expression in cultured cortical neurons of rat. Neurosci Lett 389(1):46-50, 2005.
- **Hwang SY, Wei J, Westhoff JH, Duncan RS, Ozawa F, Volpe P, Inokuchi K, Koulen P.** Differential functional interaction of two Vesl/Homer protein isoforms with ryanodine receptor type 1: a novel mechanism for control of intracellular calcium signaling. Cell Calcium 34(2):177-84, 2003.
- **Izumi Y, Kim S, Murakami T, Yamanaka S, Iwao H.** Cardiac mitogen-activated protein kinase activities are chronically increased in stroke-prone hypertensive rats. Hypertension 31(1):50-6, 1998
- **Kammermeier PJ, Worley PF.** Homer 1a uncouples metabotropic glutamate receptor 5 from postsynaptic effectors. Proc Natl Acad Sci U S A 104(14):6055-60, 2007.
- **Kato A, Ozawa F, Saitoh Y, Fukazawa Y, Sugiyama H, Inokuchi K.** Novel members of the Vesl/Homer family of PDZ proteins that bind metabotropic glutamate receptors. J Biol Chem 273(37):23969-23975, 1998.
- **Kato A, Ozawa F, Saitoh Y, Hirai K, Inokuchi K.** Vesl, a gene encoding VASP/Ena family related protein, is upregulated during seizure, long-term potentiation and synaptogenesis. FEBS Lett. 412:183-189, 1997.
- **Kawaguchi S, Shoji S, Sunamori M, Furuichi T, Kawano S.** The fundamental properties of Homer 1 in association with cardiac ryanodine receptor in mouse heart. J Med Dent Sci 4(1):103-8, 2007.

- **Kawamoto T, Togi K, Yamauchi R, Yoshida Y, Nakashima Y, Kita T, Tanaka M.** Endothelin-1 activates Homer 1alpha expression via mitogen-activated protein kinase in cardiac myocytes. Int J Mol Med 18:193-196, 2006.
- **Kehat I, Molkentin JD.** Extracellular signal-regulated kinase 1/2 (ERK1/2) signaling in cardiac hypertrophy. Ann N Y Acad Sci 1188:96-102, 2010.
- **Kim JY, Zeng W, Kiselyov K, Yuan JP, Dehoff MH, Mikoshiba K, Worley PF, Muallem S.** Homer 1 mediates store- and inositol 1,4,5-trisphosphate receptor-dependent translocation and retrieval of TRPC3 to the plasma membrane. J Biol Chem 281(43):32540-9, 2006.
- **Klugmann M, Symes CW, Leichtlein CB, Klaussner BK, Dunning J, Fong D, Young D, During MJ.** AAV-mediated hippocampal expression of short and long Homer 1 proteins differentially affect cognition and seizure activity in adult rats. Mol Cell Neurosci 28(2):347-60, 2005.
- **Kögler H, Hartmann O, Leineweber K, Nguyen van P, Schott P, Brodde OE, Hasenfuss G.** Mechanical load-dependent regulation of gene expression in monocrotaline-induced right ventricular hypertrophy in the rat. Circ Res 93(3):230-237, 2003.
- **Lee EH, Hsin J, Mayans O, Schulten K.** Secondary and tertiary structure elasticity of titin Z1Z2 and a titin chain model. Biophys J 93(5):1719-1735, 2007.
- **Levy D, Kenchaiah S, Larson MG, Benjamin EJ, Kupka MJ, Ho KK, Murabito JM, Vasan RS**. Long-term trends in the incidence of and survival with heart failure. N Engl J Med 347(18):1397-13402, 2002.
- **Leyton RA, Sonnenblick EH.** The sarcomere as the basis of Starling's Law of the heart in the left and right ventricles. Meth Exp Path 5:22-59, 1971.
- **Li Y, Krogh KA, Thayer SA.** Epileptic stimulus increases Homer 1a expression to modulate endocannabinoid signaling in cultured hippocampal neurons. Neuropharmacology 63(6):1140-9, 2012.
- **López-Andrés N, Iñigo C, Gallego I, Díez J, Fortuño MA.** Aldosterone induces cardiotrophin-1 expression in HL-1 adult cardiomyocytes. Endocrinology 149:4970–4978, 2008.
- **Lorenz K, Schmitt JP, Vidal M, Lohse MJ.** Cardiac hypertrophy: targeting Raf/MEK/ERK1/2-signaling. Int J Biochem Cell Biol 41(12):2351-235, 2009.

- **Luo J, McMullen JR, Sobkiw CL, Zhang L, Dorfman AL, Sherwood MC, Logsdon MN, Horner JW, DePinho RA, Izumo S, Cantley LC.** Class IA phosphoinositide 3-kinase regulates heart size and physiological cardiac hypertrophy. Mol Cell Biol 25(21):9491-9502, 2005.
- **Luo P, Zhao Y, Li D, Chen T, Li S, Chao X, Liu W, Zhang L, Qu Y, Jiang X, Lu G, Poon W, Fei Z.** Protective effect of Homer 1a on tumor necrosis factor-α with cycloheximide-induced apoptosis is mediated by mitogen-activated protein kinase pathways. Apoptosis 17(9):975-988, 2012a.
- **Luo P, Chen T, Zhao Y, Xu H, Huo K, Zhao M, Yang Y, Fei Z.** Protective effect of Homer 1a against hydrogen peroxide-induced oxidative stress in PC12 cells. Free Radic Res 46(6):766-776, 2012b.
- **Luo P, Li X, Fei Z, Poon W.** Scaffold protein Homer 1: implications for neurological diseases. Neurochem Int 61(5):731-738, 2012c.
- **Marian AJ.** β-adrenergic receptors signaling and heart failure in mice, rabbits and humans. J Mol Cell Cardiol 41(1):11-13, 2006.
- **McMullen JR, Jennings GL.** Differences between pathological and physiological cardiac hypertrophy: novel therapeutic strategies to treat heart failure. Clin Exp Pharmacol Physiol 34(4):255-62, 2007.
- **McNutt NS, Fawcett DW.** Myocardial ultrastructure in 'The mammalian myocardium'. Chapter one, 1974.
- **McWhinney CD, Hansen C, Robishaw JD.** Alpha-1 adrenergic signaling in a cardiac murine atrial myocyte (HL-1) cell line. Mol Cell Biochem 214(1-2):111-119, 2000.
- **Mende U, Kagen A, Cohen A, Aramburu J, Schoen FJ, Neer EJ.** Transient cardiac expression of constitutively active Galphaq leads to hypertrophy and dilated cardiomyopathy by calcineurin-dependent and independent pathways. Proc Natl Acad Sci U S A 95(23):13893-13898, 1998.
- **Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN.** A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 93(2):215-28, 1998.
- **Molkentin JD.** Dichotomy of Ca^{2+} in the heart: contraction versus intracellular signaling. J Clin Invest 116(3):623-6, 2006.
- **Molkentin, JD, Dorn GW II.** Cytoplasmic signaling pathways that regulate cardiac hypertrophy. Annu Rev Physiol 63:391-426, 2001.

- **Monnet E, Chachques JC.** Animal models of heart failure: what is new? Ann Thorac Surg 79(4):1445-1453, 2005.
- **Niswender CM, Conn PJ.** Metabotropic glutamate receptors: physiology, pharmacology, and disease. Annu Rev Pharmacol Toxicol 50:295-322, 2010.
- **Olson EN, Schneider MD.** Sizing up the heart: development redux in disease. Genes Dev 17(16):1937-1956, 2003.
- **Palaniyandi SS, Sun L, Ferreira JC, Mochly-Rosen D.** Protein kinase C in heart failure: a therapeutic target? Cardiovasc Res 82(2):229-239, 2009.
- **Park HT, Kang EK, Bae KW.** Light regulates Homer mRNA expression in the rat suprachiasmatic nucleus. Brain Res Mol Brain Res 52(2):318-22, 1997.
- **Patterson RL, Boehning D, Snyder SH.** Inositol 1,4,5-trisphosphate receptors as signal integrators. Annu Rev Biochem. 73:437-465, 2004.
- **Piñeiro R, Iglesias JM, Eiras S.** Leptin does not Induce Hypertrophy, Cell Cycle Alterations, or Production of MCP-1 in Cultured Rat and Mouse Cardiomyocytes. Endocrine Research 31:375–386, 2005.
- **Pouliquin P, Dulhunty AF.** Homer and the ryanodine receptor. Eur Biophys J 39(1):91-102, 2009a.
- **Pouliquin P, Pace SM, Dulhunty AF**. In vitro modulation of the cardiac ryanodine receptor activity by Homer1. Pflugers Arch 458(4):723-32, 2009b.
- **Rinne A, Kapur N, Molkentin JD, Pogwizd SM, Bers DM, Banach K, Blatter LA.** Isoform- and tissue-specific regulation of the $Ca(^{2+})$-sensitive transcription factor NFAT in cardiac myocytes and heart failure. Am J Physiol Heart Circ Physiol 298(6):H2001-H2009, 2010.
- **Roche KW, Tu JC, Petralia RS, Xiao B, Wenthold RJ, Worley PF.** Homer 1b regulates the trafficking of group I metabotropic glutamate receptors. J Biol Chem, 274:25953-25957,1999.
- **Rockman HA, Koch WJ, Lefkowitz RJ.** Seven-transmembrane-spanning receptors and heart function. Nature 415(6868):206-212, 2002.
- **Rockman HA, Ross RS, Harris AN, Knowlton KU, Steinhelper ME, Field LJ, Ross J Jr, Chien KR.** Segregation of atrial-specific and inducible expression of an atrial natriuretic factor transgene in an in vivo murine model of cardiac hypertrophy. Proc Natl Acad Sci U S A 88(18):8277-8281, 1991.

- **Roderick HL, Higazi DR, Smyrnias I, Fearnley C, Harzheim D, Bootman MD.** Calcium in the heart: when it's good, it's very very good, but when it's bad, it's horrid. Biochem Soc Trans 35(Pt 5):957-61, 2007.
- **Rohini A, Agrawal N, Koyani CN, Singh R.** Molecular targets and regulators of cardiac hypertrophy. Pharmacol Res 61(4):269-80, 2010.
- **Rossi AC, Mammucari C, Argentini C, Reggiani C, Schiaffino S.** Two novel/ancient myosins in mammalian skeletal muscles: MYH14/7b and MYH15 are expressed in extraocular muscles and muscle spindles. J Physiol 588(Pt2):353-364, 2010.
- **Saito H, Kimura M, Inanobe A, Ohe T, Kurachi Y.** An N-terminal sequence specific for a novel Homer1 isoform controls trafficking of group I metabotropic glutamate receptor in mammalian cells. Biochem Biophys Res Commun 296(3):523-529, 2002.
- **Sala C, Piech V, Wilson NR, Passafaro M, Liu G, Sheng M.** Regulation of dendritic spine morphology and synaptic function by Shank and Homer. Neuron 31:115–130., 2001.
- **Salanova M, Priori G, Barone V, Intravaia E, Flucher B, Ciruela F, McIlhinney RA, Parys JB, Mikoshiba K, Sorrentino V.** Homer proteins and InsP(3) receptors co-localise in the longitudinal sarcoplasmic reticulum of skeletal muscle fibres. Cell Calcium 32(4):193-200, 2002.
- **Sandonà D, Tibaldo E, Volpe P.** Evidence for the presence of two Homer 1 transcripts in skeletal and cardiac muscles. Biochem Biophys Res Commun 279: 348-353, 2000.
- **Schaub MC, Hefti MA, Harder BA, Eppenberger HM.** Various hypertrophic stimuli induce distinct phenotypes in cardiomyocytes. J Mol Med (Berl) 75(11-12):901-920, 1997.
- **Schultze AE, Roth RA.** Chronic pulmonary hypertension--the monocrotaline model and involvement of the hemostatic system. J Toxicol Environ Health B Crit Rev 1(4):271-346, 1998.
- **Serge A, Fourgeaud L, Hemar A, Choquet D.** Receptor activation and homer differentially control the lateral mobility of metabotropic glutamate receptor 5 in the neuronal mem- brane. J Neurosci 22:3910-3920, 2002.

- **Shiraishi Y, Mizutani A, Yuasa S, Mikoshiba K, Furuichi T.** Differential expression of Homer family proteins in the developing mouse brain. J Comp Neurol 473:582-599, 2004.
- **Shiraishi-Yamaguchi Y, Furuichi T.** The Homer family proteins. Genome Biol 8(2):206, 2007.
- **Soloviev MM, Ciruela F, Chan WY, McIlhinney RA.** Molecular characterisation of two structurally distinct groups of human homers, generated by extensive alternative splicing. J Mol Biol 295:185-200, 2000a.
- **Soloviev MM, Ciruela F, Chan WY, McIlhinney RA.** Mouse brain and muscle tissues constitutively express high levels of Homer proteins. Eur J Biochem 267(3):634-9, 2000b.
- **Tadokoro S, Tachibana T, Imanaka T, Nishida W, Sobue K.** Involvement of unique leucine-zipper motif of PSD-Zip45 (Homer 1c/vesl-1L) in group 1 metabotropic glutamate receptor clustering. Proc Natl Acad Sci U S A 96(24):13801-13806, 1999.
- **Tappe A, Klugmann M, Luo C, Hirlinger D, Agarwal N, Benrath J, Ehrengruber MU, During MJ, Kuner R.** Synaptic scaffolding protein Homer1a protects against chronic inflammatory pain. Nat Med 12(6):677-681, 2006.
- **Thomas U.** Modulation of synaptic signalling complexes by Homer proteins. J Neurochem 81:407-413, 2002.
- **Tu JC, Xiao B, Naisbitt S, Yuan JP, Petralia RS, Brakeman P, Doan A, Aakalu VK, Lanahan AA, Sheng M, Worley PF.** Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. Neuron 23(3):583-592, 1999.
- **Tu JC, Xiao B, Yuan JP, Lanahan AA, Leoffert K, Li M, Linden DJ, Worley PF.** Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. Neuron 21(4):717-726, 1998.
- **Van Petegem F.** Ryanodine receptors: structure and function. J Biol Chem 287(38):31624-31632, 2012.
- **Vennekens R, Voets T, Bindels RJ, Droogmans G, Nilius B.** Current understanding of mammalian TRP homologues. Cell Calcium 31(6):253-64, 2002.

- **Volpe P, Sandri C, Bortoloso E, Valle G, Nori A.** Topology of Homer 1c and Homer 1a in C2C12 myotubes and transgenic skeletal muscle fibers. Biochem Biophys Res Commun 316(3):884-92, 2004.
- **Wang QD, Bohlooly-Y M, Sjöquist PO.** Murine models for the study of congestive heart failure: Implications for understanding molecular mechanisms and for drug discovery. J Pharmacol Toxicol Methods 50(3):163-174, 2004.
- **Wang W, Liao X, Fukuda K, Knappe S, Wu F, Dries DL, Qin J, Wu Q.** Corin variant associated with hypertension and cardiac hypertrophy exhibits impaired zymogen activation and natriuretic peptide processing activity. Circ Res 103:502–508, 2008.
- **Ward CW, Feng W, Tu J, Pessah IN, Worley PK.** Schneider MF. Homer protein increases activation of Ca^{2+} sparks in permeabilized skeletal muscle. J Biol Chem 279(7):5781-7, 2004.
- **Westhoff JH, Hwang SY, Duncan RS, Ozawa F, Volpe P, Inokuchi K, Koulen P.** Vesl/Homer proteins regulate ryanodine receptor type 2 function and intracellular calcium signaling. Cell Calcium 34:261-269, 2003.
- **Wettschureck N, Rütten H, Zywietz A, Gehring D, Wilkie TM, Chen J, Chien KR, Offermanns S.** Absence of pressure overload induced myocardial hypertrophy after conditional inactivation of Galphaq/Galpha11 in cardiomyocytes. Nat Med 7(11):1236-1240, 2001.
- **White SM, Constantin PE, Claycomb WC.** Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function. Am J Physiol Heart Circ Physiol 286(3):H823-829, 2004.
- **Wilkins BJ, Molkentin JD.** Calcium-calcineurin signaling in the regulation of cardiac hypertrophy. Calcium-calcineurin signaling in the regulation of cardiac hypertrophy. Biochem Biophys Res Commun 322(4):1178-91, 2004.
- **Worley PF, Zeng W, Huang G, Kim JY, Shin DM, Kim MS, Yuan JP, Kiselyov K, Muallem S.** Homer proteins in Ca^{2+} signaling by excitable and non-excitable cells. *Cell Calcium* 42: 363-371, 2007.
- **Wu X, Zhang T, Bossuyt J, Li X, McKinsey TA, Dedman JR, Olson EN, Chen J, Brown JH, Bers DM.** Local InsP3-dependent perinuclear Ca^{2+}

signaling in cardiac myocyte excitation-transcription coupling. J Clin Invest 116(3):675-682, 2006.

- **Xiao B, Tu JC, Petralia RS, Yuan JP, Doan A, Breder CD, Ruggiero A, Lanahan AA, Wenthold RJ, Worley PF.** Homer regulates the association of group 1 metabotropic glutamate receptors with multivalent complexes of homer-related, synaptic proteins. Neuron 21(4):707-716, 1998.
- **Xiao L, Pimental DR, Amin JK, Singh K, Sawyer DB, Colucci WS.** MEK1/2-ERK1/2 mediates alpha1-adrenergic receptor-stimulated hypertrophy in adult rat ventricular myocytes. J Mol Cell Cardiol 33(4):779-787, 2001.
- **Yamazaki T, Komuro I, Zou Y, Kudoh S, Shiojima I, Hiroi Y, Mizuno T, Aikawa R, Takano H, Yazaki Y.** Norepinephrine induces the raf-1 kinase/mitogen-activated protein kinase cascade through both alpha 1- and beta-adrenoceptors. Circulation 95(5):1260-1268, 1997.
- **Yuan JP, Kiselyov K, Shin DM, Chen J, Shcheynikov N, Kang SH, Dehoff MH, Schwarz MK, Seeburg PH, Muallem S, Worley PF.** Homer binds TRPC family channels and is required for gating of TRPC1 by IP3 receptors. Cell 114(6):777-789, 2003.
- **Zhang GC, Mao LM, Liu XY, Parelkar NK, Arora A, Yang L, Hains M, Fibuch EE, Wang JQ.** In vivo regulation of Homer1a expression in the striatum by cocaine. Mol Pharmacol 71(4):1148-1158, 2007.
- **Zhang W, Yano N, Deng M, Mao Q, Shaw SK, Tseng YT.** β-Adrenergic receptor-PI3K signaling crosstalk in mouse heart: elucidation of immediate downstream signaling cascades. PLoS One 6(10):e26581, 2011.
- **Zou Y, Komuro I, Yamazaki T, Kudoh S, Uozumi H, Kadowaki T, Yazaki Y.** Both Gs and Gi proteins are critically involved in isoproterenol-induced cardiomyocyte hypertrophy. J Biol Chem 274(14):9760-9770, 1999.

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