Loss of proteostasis as a substrate for atrial fibrillation

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Loss of Proteostasis as a substrate for Atrial Fibrillation

Defining novel targets for therapy

Roelien Amanda Marjolein Meijering

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General introduction and scope of the thesis

Partially adapted from:

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Loss of proteostatic control as a substrate for atrial fibrillation: a novel target for upstream therapy by heat shock proteins.

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Atrial Fibrillation

1.1 Atrial Fibrillation

Atrial fibrillation (AF) is the most common clinical tachyarrhythmia which significantly contributes to cardiovascular morbidity and mortality (Nattel, 2002;Nattel et al., 2008;Dobrev et al., 2012). During AF, the heart rate is increased and the rhythm is irregular due to a disruption of the normal electrical impulse generation and propagation (Figure 1) (Nattel, 2002;Dobrev et al., 2012). In the healthy heart, i.e. during normal sinus rhythm (SR), an electrical impulse is generated by specialized cells in the right atrium called the sinus node. At rest, the sinus node provides a regular rhythm of approximately 60-70 beats per minute (bpm), which can increase to 180-200 bpm during heavy exercise. The electrical impulses generated by the sinus node are conducted through the right and left atria, where it will induce the coordinated contraction of the atria. This will pump blood from the atria (upper heart chambers) into the ventricles (lower heart chambers). The electrical impulses subsequently reach a group of specialized cells called the atrioventricular node (AV node). Here the electrical impulses are slightly delayed, to ensure that atrial contraction fills the ventricles with blood. The electrical impulses are conducted further past the AV node, through the bundle of His and Purkinje fibers to the ventricles, where it causes the coordinated contraction of the ventricles, resulting in blood being pumped to the lungs and the rest of the body. During AF, the electrical impulses generated by the sinus node are overwhelmed by disorganized and much faster electrical pulses (400-600 bpm), originating from the atria and pulmonary veins, leading to a loss of the coordinated contraction of the atria (Nattel, 2002;Dobrev et al., 2012). Further, a part of the irregular impulses are transferred to the ventricles via the AV node, leading to an increased and irregular heart rate. As this affects the coordinated contraction of atria and ventricles, blood is pumped less efficiently to the ventricles and hence to the pulmonary and systemic circulation (Nattel, 2002;Nattel et al., 2008;Dobrev et al., 2012). The duration of these AF episodes can vary, with episodes lasting minutes to weeks, or even more prolonged periods of time of months to even years (Camm et al., 2012). AF itself has a natural tendency to progress towards longer and more frequent attacks and many patients will develop persistent AF over a period of years (Wijffels et al., 1995;Camm et al., 2012).

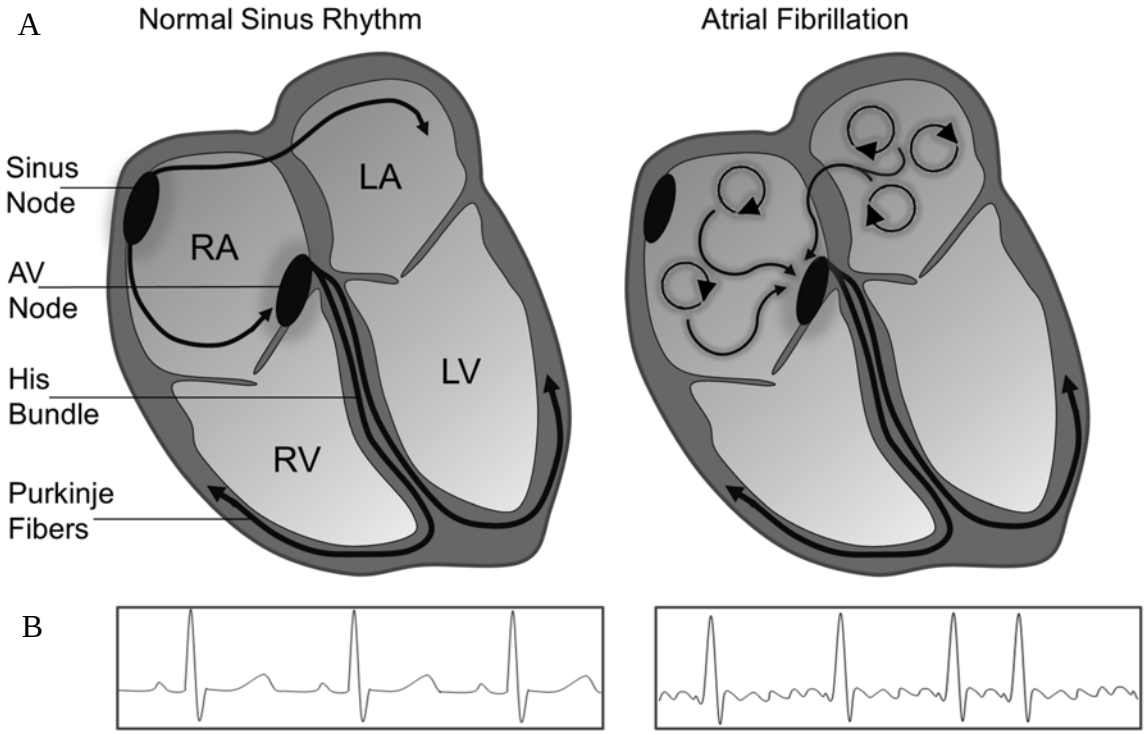

Figure 1 Electrical impulses in the normal heart and during AF.
A In the healthy heart, the sinus node generates rhythmical electrical impulses, which are conducted through the upper heart chambers (atria) and cause the synchronized contraction of the atria. The impulses are further conducted through the atrioventricular node (AV node), the bundle of His and the Purkinje fibers to the ventricles, where it causes the synchronized contraction of the ventricles. During AF, the normal electrical signals of the sinus node are overwhelmed by disorganized impulses, causing the loss of synchronized atrial contraction and an increased and irregular heartbeat, due to partial conduction of the impulses via the AV node to the ventricles.
B Electrocardiogram (ECG) tracing of normal sinus rhythm versus AF. During AF the ECG shows a characteristic sawtooth pattern and an irregular heart rhythm.

Abbreviations; RA; Right Atrium, LA; Left Atrium, RV; Right Ventricle, LV; Left Ventricle. Adapted from (Page and Roden, 2005)

1.2 Symptoms and current treatment options

In some patients AF may be asymptomatic, but often it causes symptoms related to the rapid heartbeat, including lightheadedness, fatigue, palpitations and chest discomfort (Nattel, 2002). In addition, because of atrial enlargement and stasis of blood, AF itself contributes to the development of other severe cardiovascular problems, such as chronic heart failure (CHF) and thromboembolic events resulting in stroke or myocardial infarction (Nattel, 2002;Nattel et al., 2008;Camm et al., 2010) .

In AF management, the therapeutic strategy depends on the presentation and duration of AF, as well as any underlying cardiovascular and/or pulmonary disease. AF can be present without other cardiovascular and pulmonary diseases (lone AF), or in combination with a variety of underlying primary conditions such as hypertension, cardiac surgery, pericarditis, congestive/coronary heart failure, congenital heart disease, pneumonia or other acute pulmonary disease (Kourliouros et al., 2009;Camm et al., 2010). These primary conditions are thought to create a substrate for AF initiation and maintenance (Camm et al., 2010). Based on the presentation and duration of AF, patients are classified into five groups; 1) first detected, to which all patients are initially assigned, 2) paroxysmal (self-terminating), where episodes of AF last less than or equal to 7 days (mainly less than 24 h), 3) persistent AF, with an AF duration of more than 7 days, 4) long-standing persistent AF, where AF lasted at least a year before rhythm control strategies are attempted, and lastly 5) permanent or chronic AF, where cardioversion failed or was considered unnecessary and no rhythm control strategy is applied (Camm et al., 2010). This classification in combination with AF-related symptoms (EHRA (European Heart Rhythm Association) score) determine the clinical management of AF and current guidelines are shortly summarized below (Camm et al., 2010).

In case of paroxysmal AF, in general no therapy is needed unless there are severe symptoms (hypotension, heart failure, angina pectoris or disabling symptoms of AF itself). The only pharmacological intervention are anti-coagulants administered as needed based on the CHA_2DS_2-Vasc and HAS-BLED score, to counteract the increased risk of thrombo-embolic events and stroke. In case of persistent AF, two guidelines may be applied, either accepting persistent AF with only anti-coagulants and rate control as needed, or rhythm control in addition to rate control to relieve severe AF-related symptoms. Rhythm control can be achieved via pharmacological or electrical cardioversion (acute management) or long term anti-arrhythmic drugs. Depending on the presence of underlying heart or pulmonary disease different anti-arrhythmic drugs can be applied (Camm et al., 2010;Camm et al., 2012). However, these drugs have side effects on the electrophysiology of the ventricles, in such a way that they can paradoxically cause arrhythmic disorders and increase mortality (Van Gelder et al., 2002;Camm et al., 2010;Camm et al., 2012).

In those cases where cardioversion or anti-arrhythmic drugs are not effective and patients remain symptomatic, more invasive techniques

such as ablation or a maze surgery may be indicated. Ablation and the maze surgery are aiming at the electrical isolation of regions which are the origin of the aberrant disorganized electrical impulses, such as the pulmonary vein. Ablation is recommended as a first line therapy for AF rhythm control in selected patients with paroxysmal AF and a low risk profile for procedure-associated complications (Camm et al., 2012). In contrast to paroxysmal and persistent AF, treatment options for permanent AF are far less numerous, as the longer AF persists the less effective current pharmacological and electrical cardioversion therapies are (Van Gelder et al., 1996;Van Gelder et al., 2002;Van Gelder et al., 2010). Current treatment options are mainly focused on the management of AF and although ablation preserves the sinus rhythm better than antiarrhythmic drugs, early and late recurrence of AF is still reported in about 50% of patients after a single procedure and 10-20% in patients with two or more procedures (Aliot and Ruskin, 2008;Camm et al., 2010;Camm et al., 2012). As the recurrence and progression of AF further limits the efficacy of current therapies, an increasing amount of research has been directed at elucidating the molecular mechanisms responsible for AF maintenance and progression in the hope to identify novel therapeutic (upstream) targets to prevent AF initiation, persistence and progression.

1.3 Upstream therapy

The recent change towards the identification of molecular mechanisms underlying AF substrate induction (initiation) and maintenance has led to the development of novel upstream therapies. Upstream therapy refers to the use of non-ion channel anti-arrhythmic drugs that modify the atrial substrate or target specific mechanisms of AF with the ultimate aim to prevent the occurrence (primary prevention) or recurrence (secondary prevention) of the arrhythmia (Van Gelder et al., 2010;Savelieva et al., 2011). Novel upstream therapeutic approaches include angiotensin-converting enzyme inhibitors, aldosterone antagonists, statins, polyunsaturated fatty acids, and colchicine (Imazio et al., 2011;Savelieva et al., 2011). However, the efficacy of commonly used drugs, including glucocorticoids and statins, in precluding remodeling is limited (Camm et al., 2010;Dobrev and Nattel, 2010) and (serious) adverse effects have been reported, indicating the need for more effective therapeutic agents with less adverse effects.

AF and derailment of proteostasis

2.1 AF and the proteostasis network

Although the exact molecular mechanism(s) underlying AF initiation, maintenance, and progression have not yet been completely elucidated, an important recognition was that derailment of cellular proteostasis results in many systemic diseases, including cardiovascular disorders (Balch et al., 2008). We and others recently obtained evidence that derailment of proteostasis, i.e. the homeostasis of protein production, breakdown, and function, contributes to AF induction and progression (Brundel et al., 2001;Allessie et al., 2002;Ausma et al., 2003;Cha et al., 2004;Todd et al., 2004;Brundel et al., 2006a;Dobrev et al., 2012). Proteostasis involves controlling the concentration, conformation, binding interaction, kinetics, and location of individual proteins via diverse signaling pathways and other modifiers, called the proteostasis network, of which a conceptual overview is depicted in figure 2.

The proteostasis network is responsible for generating and maintaining functional proteins in the heart and counteracts proteotoxic stress, such as during AF. The proteostasis network is composed of pathways central to proteostasis (first layer Figure 2), including components involved in protein synthesis (transcription and translation) and correct folding of proteins such as ribosomes and chaperones (heat shock proteins, HSPs) (Hartl et al., 2011). Other components central to proteostasis consists of diverse pathways involved in the degradation of damaged or unwanted proteins, which help to maintain and restore cellular proteostasis, and include the ubiquitin proteasome system (UPS), endoplasmic reticulum (ER) associated degradation (ERAD) systems, proteases, autophagic pathways, lysosomal/endosomal targeting pathways, and phagocytic pathways (Roth and Balch, 2011). Further, the levels and/or activity of the components of the proteostasis network are regulated by other (stress) signaling pathways (second layer Figure 2) through post-translational modifications, like ubiquitination, phosphorylation, acetylation and sumoylation (Karve and Cheema, 2011). Activation of these stress signaling pathways aim to restore proteostasis in response to stress, but in case restoration of proteostasis fails can also contribute to apoptosis (Ron and Walter, 2007;Morimoto, 2008). Finally, other modulating factors (outer layer Figure 2) consist of genetic and epigenetic pathways, physiological stress, and intracellular metabolites that affect

the presence and/or activity of components of the second and first layers (Balch et al., 2008;Roth and Balch, 2011). Together all these components comprise a highly dynamic proteostasis network. Interestingly, the proteostasis network and hence cellular proteostasis can be pharmacologically or genetically adjusted at the diverse layers (Roth and Balch, 2011), indicating the applicability of therapeutic (upstream) intervention strategies to prevent loss of cellular proteostasis, as described in AF (Brundel et al., 2001;Allessie et al., 2002;Ausma et al., 2003;Cha et al., 2004;Todd et al., 2004;Brundel et al., 2006a;Dobrev et al., 2012).

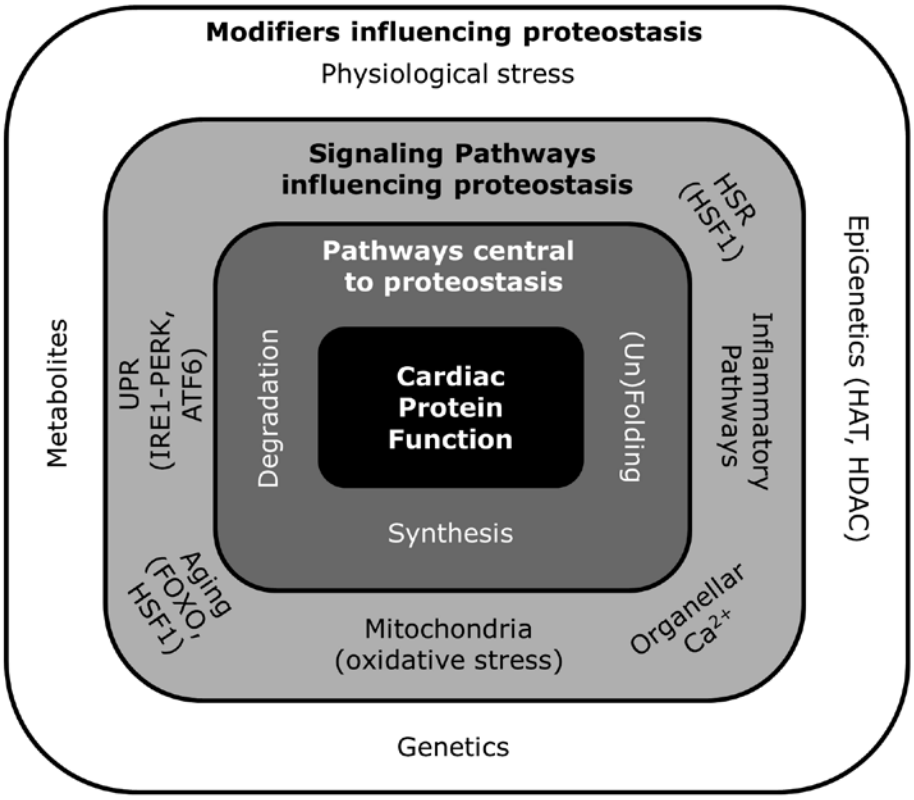

Figure 2 Proteostasis network regulating cardiac protein function. Depicted is the proteostasis network with the pathways central to proteostasis, hence pathways involved in protein degradation, synthesis and folding. Further, the second and third layer consists of modifiers and signaling pathways influencing the activity and/or levels of components in the second and first layer of the proteostasis network. Together these layers regulate cellular proteostasis and maintain functional proteins in the heart. Figure adapted from (Balch et al., 2011).

Abbreviations, HSR; Heat Shock Response, HSF1; Heat Shock Factor 1, IGF1R; Insulin-like Growth Factor 1 Receptor, FOXO; FOrkhead boX O, UPR; Unfolded Protein Response, IRE1 (ERN1); Endoplasmic Reticulum to Nucleus signaling 1, PERK; PRKR-Like Endoplasmic Reticulum Kinase, ATF6; Activating Transcription Factor 6, HAT; Histone Acetyl Transferase, HDAC; Histone DeACetylase.

2.2 AF initiation and loss of proteostatic control

An important recognition of recent research is that AF induction requires a suitable substrate as well as a trigger that acts on the substrate (Nattel et al., 2008). Various clinical conditions, e.g., several heart diseases, hypertension and cardiac surgery, are risk factors for the first-onset of AF, as they create a substrate(s) and/or trigger(s) for the initiation of AF, by inducing loss of proteostatic control (Figure 3; (Benjamin et al., 1998;Kannel et al., 1998;Chelazzi et al., 2011). Key AF-promoting factors have been identified, including inflammation, oxidative stress, active Rho-GTPase, fibrosis, and atrial muscle bundle dissociation (Allessie et al., 2010;de Groot et al., 2010), creating a substrate for AF. Subsequent triggers will act on the substrate and will induce AF (Sah et al., 1999;Dudley et al., 2005;Engelmann and Svendsen, 2005;Adam et al., 2007;Burstein and Nattel, 2008;Nattel et al., 2008), as depicted in figure 3.

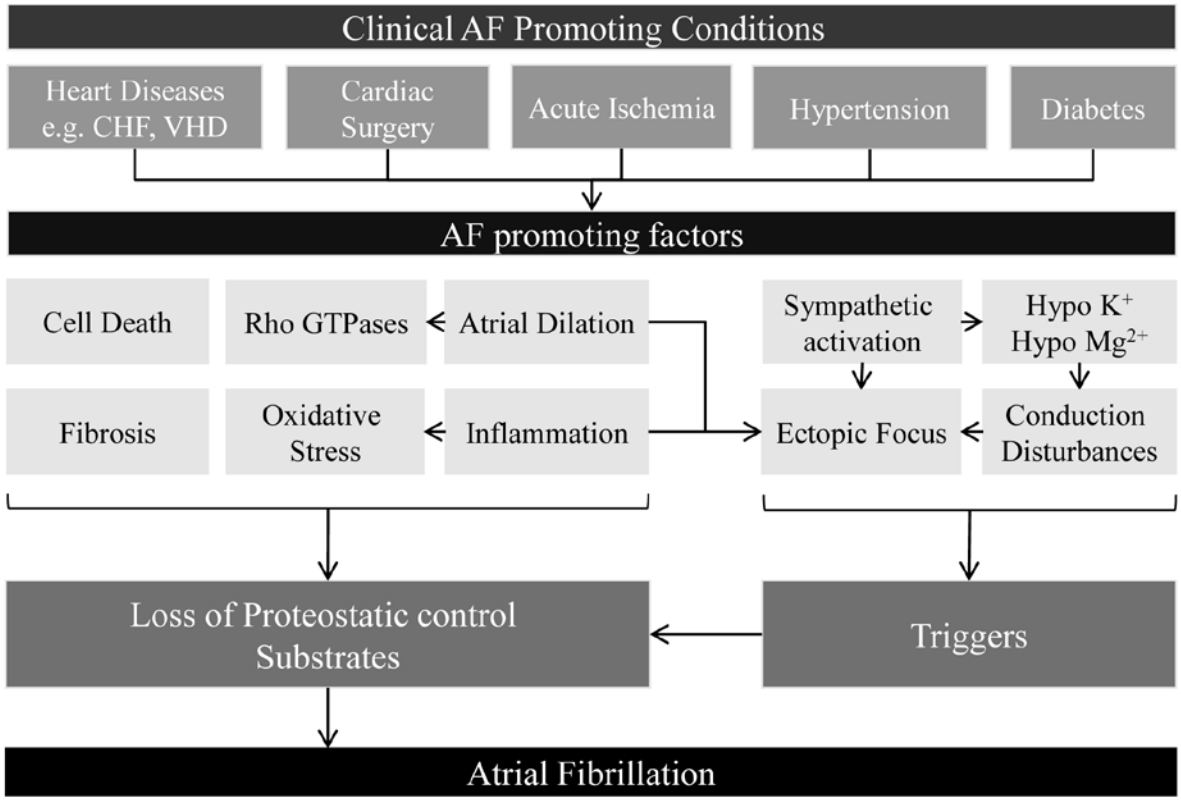

Figure 3 AF-promoting factors contributing to first-onset AF.
Various clinical conditions induce AF-promoting factors. These factors can induce triggers for AF or are responsible for the loss of proteostatic control, thereby inducing remodeling, and creating a substrate for AF. Triggers will act on the vulnerable substrate to induce first-onset AF.

2.3 Maintenance and progression of AF and derailed proteostasis

Once AF is initiated, AF itself will cause further derailment of proteostasis, hence further electrical and structural remodeling, in a manner that contributes to AF maintenance and progression (Wijffels et al., 1995). A conceptual model is depicted in the figure below (Figure 4).

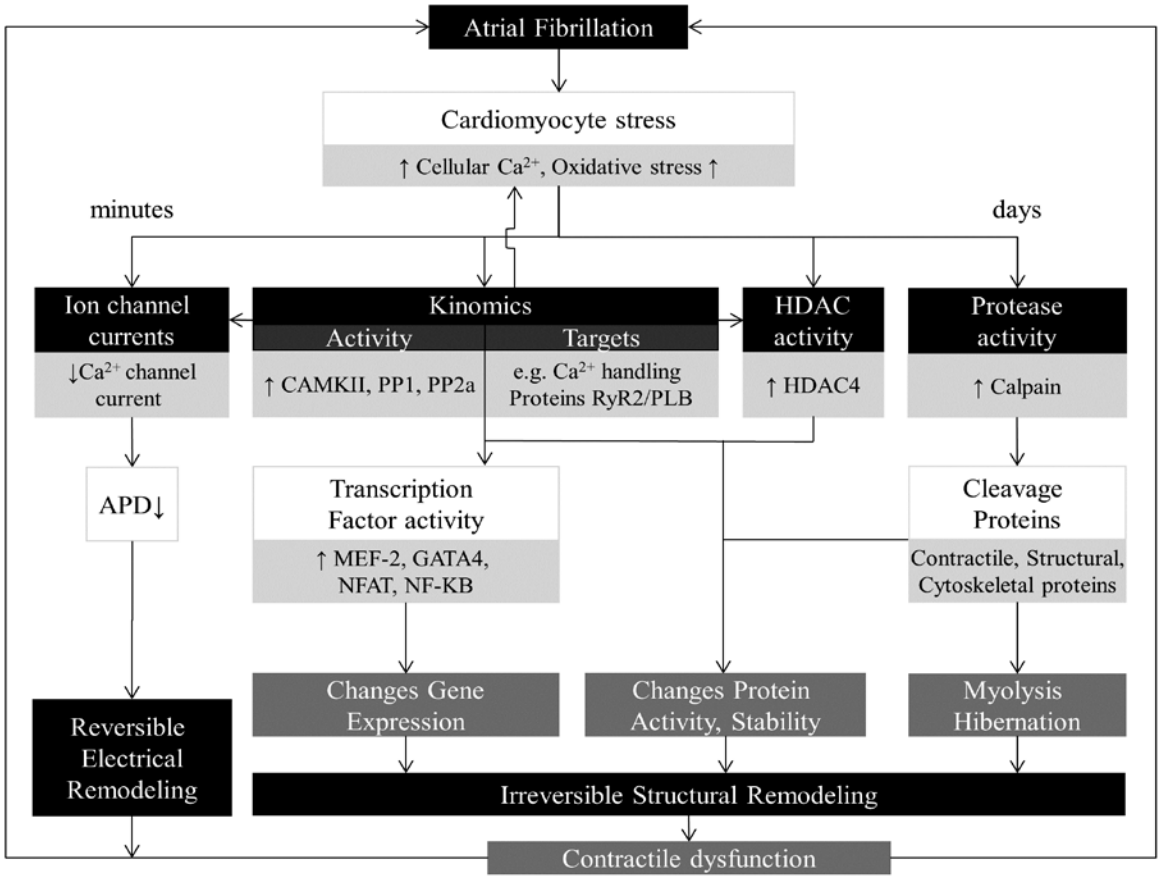

Figure 4 AF-induced derailment of cardiomyocyte proteostasis.
AF induces time-related progressive cardiomyocyte remodeling. First, AF causes cellular Ca^{2+} overload and oxidative stress, which results in a direct inhibition of the L-type Ca^{2+} channel, shortening of action potential duration (APD), and contractile dysfunction. These changes have an early onset and are reversible. The early processes protect the cardiomyocyte against Ca^{2+} overload but at the expense of creating a substrate for persistent AF. When AF persists, derailment of proteostasis occurs via activation of Calpain, kinases/phosphatases and HDACs. The key modulators can also activate each other. Derailment of proteostasis results in irreversible myolysis/hibernation, alterations in structural proteins and pathological gene expression, which are substrates for impaired contractile function and AF persistence.

Abbreviations, CAMKII; Ca^{2+}/calmodulin-dependent protein Kinase 2, PP1; Protein Phosphatase 1, PP2a; Protein Phosphatase 2A, RyR2; Ryanodine Receptor, PLB; Phospholamban, HDAC4; Histone Deacetylase 4, MEF2; Myocyte Enhancer Factor 2, GATA4; GATA binding protein 4, NFAT; Nuclear Factor of Activated T-cells, NF-KB; Nuclear Factor Kappa-light-chain-enhancer of activated B cells.

During AF, the rapid and irregular impulses cause a rapid Ca^{2+} overload and oxidative stress, resulting in progressive cardiomyocyte remodeling. Early responses include changes in signaling pathways (kinomics) and ion channel currents. A consistent finding is the decrease in ICaI, hence functional downregulation of the L-type Ca^{2+} channel, which leads to shortening of the action potential duration (APD) and contractile dysfunction, thereby providing a further substrate for AF (Figure 2; (Goette et al., 1996;Leistad et al., 1996;Van Wagoner et al., 1997;Yue et al., 1997;Ausma et al., 2000a;Brundel et al., 2001;Qi et al., 2008). The functional changes have a rapid onset following initiation of AF and are reversible (Schotten et al., 2003). In addition to the L-type Ca^{2+} channel, also other channel currents are affected, as reviewed by Dobrev and Voigt (Dobrev and Voigt, 2011). Ion channel currents, hence protein function, are regulated by expression level, phosphorylation, and redox status of the channel, all of which are altered during AF (Brundel et al., 1999;Wang, 2003;Dobrev and Voigt, 2011). In dog atrial cardiomyocytes, tachypacing induced the activation of calcineurin via the Ca^{2+}/calmodulin system, which in turn changes cardiomyocyte proteostasis by stimulating nuclear translocation of NFAT, resulting in transcriptional and translational downregulation of the L-type Ca^{2+} channel. Subsequently downregulation of the L-type Ca^{2+} channel induces atrial remodeling and AF progression (Qi et al., 2008). Phosphorylation status of ion channels are altered due to changes in activity of various kinases and phosphatases, such as enhanced CaMKII, PP1, and PP2a activity (Anderson, 2004;Dobrev and Voigt, 2011). Further, oxidative stress during AF can contribute to changes in redox status, thereby altering ion channel currents (Wang, 2003;Dobrev and Voigt, 2011). In addition to modification of ion channel currents through (de)phosphorylation, also the function of other downstream target proteins is affected by the altered activity of kinases and phosphatases (Anderson, 2004;Christ et al., 2004;El-Armouche et al., 2006;Greiser et al., 2007;Dobrev and Nattel, 2008), including calcium handling proteins, such as the ryanodine receptor (RyR2) and phospholamban (PLB) (Schwinger et al., 1999;Hagemann and Xiao, 2002;Bers, 2006;Carter et al., 2011). Modification of calcium handling protein function will contribute to calcium overload and subsequent contractile dysfunction (Dobrev and Nattel, 2008). Electrical remodeling resulting in shortening of APD, slowing of conduction, and abnormal calcium handling will all contribute to AF maintenance (Dobrev and Voigt, 2011). When AF persists, the calcium overload is thought to cause irreversible (late) changes in structural remodeling, especially cardiomyocyte hibernation (Ausma et al., 1997a;Ausma et al.,

2000b;Brundel et al., 2002). Hibernation is characterized by irreversible degradation of the myofibril structure (myolysis) and mitochondrial damage, which leads to contractile dysfunction (Vanoverschelde et al., 1993;Ausma et al., 1997a;Sherman et al., 2000;Bito et al., 2004;Ke et al., 2008). Other characteristics are redistribution of nuclear chromatin and pathological gene expression, possibly regulated via altered kinase/phosphatase and HDAC activity (Backs et al., 2006;Liu et al., 2008), causing a deficiency in healthy cardiomyocyte proteostasis (Ausma et al., 1995;Ausma et al., 1997a;Ausma et al., 1997b;Ausma et al., 1998;Allessie et al., 2002;Thijssen et al., 2004). While early electrical remodeling is reversible (Schotten et al., 2003), the derailment of proteostasis underlies irreversible structural remodeling and thereby AF progression (Allessie et al., 2002;Brundel et al., 2002;Ausma et al., 2003;Cha et al., 2004;Todd et al., 2004).

HSP induction as target for upstream therapy in AF

As derailment of proteostasis contributes to AF substrate formation, upstream therapy directed at preventing derailment or restoring cellular proteostasis might prove a valuable therapeutic approach. Since, heat shock proteins (HSPs) are known for their broad cytoprotective effects and key role in maintaining cellular proteostasis (Kampinga, 1993;Balch et al., 2008;Powers et al., 2009;Kampinga and Craig, 2010;Hoogstra-Berends et al., 2012), their pharmacological induction would be a likely candidate to help cardiomyocytes cope with stress and restore cardiac proteostasis in AF.

3.1 HSPs and their function

The heat shock proteins are a family of proteins comprised of five subgroups, including the HSPA (HSP70), HSPB (small HSP), HSPC (HSP90), HSPD/E (chaperonin families HSP60/HSP10), HSPH (HSP110) and the DNAJ (HSP40) families of proteins. Each family comprises of several family members and co-factors, which are localized in various cellular compartments and have distinct and/or overlapping functions (Vos et al., 2008;Kampinga et al., 2009). Members can be constitutively expressed or are induced upon many different forms of intrinsic and/or extrinsic stressors or during differentiation (Morimoto, 1993;Lanneau et al., 2007;Lanneau et al., 2008;Vos et al., 2008;Hageman and Kampinga, 2009). HSP proteins are mainly involved in protein quality control systems and assist the proper folding and unfolding of proteins (HSPD, HSPH,

HSPA, DNAJ). This includes binding to unfolded or misfolded proteins, thereby preventing protein aggregation and providing a time window for proper protein (re)folding (HSPA, DNAJ, HSPB) (Kampinga and Craig, 2010). Further, HSPs support protein degradation or specifically target proteins towards degradation pathways in case proper refolding failed (Kampinga and Craig, 2010). In addition to their central roles in protein folding, HSPs also convey other cytoprotective effects, as will be discussed below.

3.2 Cardioprotective HSPs

Induction of diverse HSPs has been reported beneficial in various cardiac diseases of which an overview is presented in table 1. Due to the well described cytoprotective effects of HSPs in diverse cardiac diseases, several studies investigated the cardioprotective potential of HSPs in AF (Marber et al., 1995;Plumier et al., 1995;Efthymiou et al., 2004;Kupatt et al., 2004;Brundel et al., 2006b;Hayashi et al., 2006;Inagaki et al., 2006;Kim et al., 2006;Kampinga et al., 2007;Kwon et al., 2007;Lin et al., 2007;Rajasekaran et al., 2007;Ago et al., 2008;Balch et al., 2008;Powers et al., 2009;Fan and Kranias, 2011;Ke et al., 2011).

3.2.1. HSPs in the prevention of AF initiation

HSPs protect against a variety of factors that promote the initiation of AF. Protective downstream effects of HSPs against cell death, fibrosis, oxidative stress and inflammation have been described, indicating their potential in preventing loss of proteostatic control and formation of AF substrates (Arya et al., 2007;Wakisaka et al., 2007;Jones et al., 2011;Ke et al., 2011). It is unclear if HSPs could affect the formation of triggers. However, since triggers need a vulnerable substrate to act on (Allessie et al., 2010), the prevention of AF substrate formation, may be sufficient to protect against AF initiation. Indeed, in various models for first-onset AF, HSPs protect against AF substrate formation and hence AF initiation. In a canine model of (acute) atrial ischemia related AF, geranylgeranylacetone (GGA) pre-treatment induced HSPA1A expression and prevented AF initiation by inhibition of the prolongation of the effective refractory period (ERP) and of atrial conduction abnormalities (Brundel et al., 2006b;Sakabe et al., 2008). Furthermore, a recent study in rat showed that induction of HSPA1A prevents both the angiotensin II mediated atrial fibrosis and increased atrial vulnerability for AF induction (Wakisaka et al., 2007). The findings suggest HSPA1A to play a role in preventing the

development of a non-cardiomyocyte substrate for AF induction. In two clinical studies, HSPA1A has been implicated as cardioprotective in AF, showing a correlation between HSPA1A atrial expression levels and reduced incidence of post-operative AF in patients in sinus rhythm undergoing cardiac surgery (St Rammos et al., 2002;Mandal et al., 2005). In addition to HSPA1A, other HSPs might be involved in primary prevention of AF. In patients with AF, increased mitochondrial HSP expression levels, i.e., HSPD1 (Schafler et al., 2002), HSPE1 and mortalin (HSPA9B), (Kirmanoglou et al., 2004) have been reported. In addition, increased HSPD1 antibody levels in the serum of patients have been associated with the occurrence of post-operative AF (Oc et al., 2007), suggesting HSPD1 as a marker for mitochondrial and cardiac damage and subsequent increased risk for AF. Increased expression of mitochondrial chaperones may protect against oxidative stress. Therefore, these HSP family members may contribute to survival of cardiomyocytes by maintaining mitochondrial integrity and capacity for ATP generation. To date, however, the involvement of mitochondrial HSPs in protection against AF is inconclusive, as studies have shown opposing correlations between their expression and AF (Kirmanoglou et al., 2004;Oc et al., 2007;Yang et al., 2007).

3.2.2. HSPs in the prevention of AF progression and recurrence

Various *in vitro* and *in vivo* models for tachypacing-induced AF progression identified HSPs to protect against the derailment of proteostasis and cardiomyocyte remodeling. In tachypaced HL-1 atrial cardiomyocytes and *Drosophila melanogaster* models for AF, a general HSP induction via a mild heat shock or by a HSP-inducing drug GGA, conserved cardiomyocyte proteostasis during tachypacing and protected against subsequent electrical, contractile, and structural remodeling (Brundel et al., 2006a;Brundel et al., 2006b;Zhang et al., 2011). Furthermore, in canine models for AF progression, GGA pre-treatment induced HSP (HSPA1A and HSPB1) expression and revealed protective effects against shortening of ERP, shortening of APD, reductions in L-type Ca^{2+} current, and AF progression (Brundel et al., 2006a;Sakabe et al., 2008). Also, in clinical studies, a potent Heat Shock Response (HSR) and high HSPB1 levels have been associated with restoration of normal sinus rhythm in patients with permanent AF after mitral valve surgery (Cao et al., 2011). Two other studies comparing paroxysmal vs. persistent AF and sinus rhythm, found higher atrial HSPB1 levels to relate to shorter AF duration and less myolysis (Brundel et al., 2006a;Yang et al., 2007).

These findings suggest that the HSR is temporary activated during a short duration of AF but is exhausted in time. Consequently, cardiomyocytes lose the ability for proteostatic control, which induces or accelerates remodeling, in turn resulting in AF progression and recurrence.
Further studies investigated the role of individual HSPs in protection against tachypacing-induced remodeling. HSPB1, but not HSPA1A, was found to play an important role, as its exclusive overexpression was sufficient to protect against tachypacing-induced remodeling, comparable to GGA pre-treatment (Brundel et al., 2006a). Conversely, the protective effect of a general HSR or GGA pre-treatment on tachypacing-induced changes was annihilated by a selective knockdown of HSPB1.

Taken together, there seems to be a strong case for induction of HSPs to prevent AF initiation, recurrence, and progression, by attenuation of electrical, contractile, and structural cardiomyocyte remodeling. There are strong indications that this effect is via normalization of cellular proteostasis.

3.3 Protective mechanisms of HSPs in AF

It has been recognized that HSPs protect against derailment of proteostasis by preventing cardiomyocyte remodeling at different stages. The exact mechanisms in prevention of AF initiation, maintenance and progression are not known, but are likely due to HSP regulated protection against various AF-promoting factors that induce the substrate for AF initiation and progression. Possible protective modes of action, including direct interaction of HSPs with putative key proteins and known downstream consequences of HSP action, in relation to AF will be discussed below.

3.3.1. Protection against oxidative stress

Interestingly, in AF patients, an increase in oxidative stress markers has been observed. Moreover, anti-inflammatory or anti-oxidant treatment with glucocorticoids and statins (Shiroshita-Takeshita et al., 2004;Shiroshita-Takeshita et al., 2006;Shiroshita-Takeshita et al., 2007;Sanchez-Quinones et al., 2008) suppressed atrial remodeling and prevented post-surgery AF in patients (primary prevention; (Camm et al., 2010)), substantiating a role for oxidative stress in AF-induced remodeling. Glucocorticoids and statins have been reported to induce several HSPs (HSPB1, HSPB5, and HSPA1A; (Nègre-Aminou et al.,

2002;Son et al., 2005)), leaving open the possibility that part of their protective pleiotropic effects is due to expression of HSPs. HSP induction can protect against oxidative stress by several mechanisms. HSPB1 is known to regulate the redox status of cardiomyocytes by maintaining glutathione in its reduced form, thus decreasing the amount of ROS produced in cells exposed to oxidative stress or tumor necrosis factor TNFalpha (Arrigo, 2007). HSPB1 may therefore prevent tachypacing-induced alterations in redox status of cardiomyocytes and thereby preserve cell proteostasis and electrophysiological and contractile function of the cardiomyocyte in AF. In addition to alterations in redox state, oxidative stress can also contribute to actin cytoskeleton instability, resulting in impairment of cardiomyocyte contractile function. Several members of the HSPB family were found to bind the actin filaments and prevent their disruption in response to various stresses, including AF (Sugiyama et al., 2000;Mounier and Arrigo, 2002;Golenhofen et al., 2004;Brundel et al., 2006a;Salinthone et al., 2008;Zhang et al., 2011). In addition, HSPB family members also protected against AF-induced mitochondrial damage (Zhang et al., 2011), thereby limiting the release of ROS.

3.3.2. Protection of ion channel currents

Ion channel function is dependent on the expression level, phosphorylation and redox status of the channel (Hara et al., 2002;Wang, 2003;Dobrev and Voigt, 2011), as well as the stability of the cytoskeleton (Sadoshima et al., 1992) and Rho-GTPase activity (Pochynyuk et al., 2007). The HSP-inducing drug GGA previously showed protective effects against tachypacing-induced reductions in L-type Ca^{2+} current and shortening of APD (Brundel et al., 2006b). Furthermore, several studies have shown protective effects of HSPs on almost all of the aforementioned regulating factors. HSPs are known to directly interact with, and in some cases, inhibit kinases and phosphatases, of which the activity is altered during AF (Ding et al., 1998;Lakshmikuttyamma et al., 2004;Dobrev, 2006;Peng et al., 2010;Fan and Kranias, 2011;Qian et al., 2011), thereby potentially preventing or normalizing the phosphorylation status of ion channels, especially the L-type Ca^{2+} channel (Christ et al., 2004). Furthermore, several HSPs (including HSPB1) were shown to reduce oxidative stress, thereby potentially preventing or restoring the redox status of the ion channels (Kalmar and Greensmith, 2009).

Table 1 Major cardioprotective heat shock proteins, localization, expression, and cardiac disease protective effects.

Stress Protein	Previous family name	Protective member (alternative names)	Cardiac disease	localization	Expression in the heart	References
HSPA	HSP70	HSPA1A	Ischaemic heart disease, hypertrophy	cytosol	+++	(Marber et al., 1995;Plumier et al., 1995;Kim et al., 2006)
DNAJ	HSP40	DNAJA3	Dilated cardiomyopathy	Cytosol/nuclear	+++	(Hayashi et al., 2006)
		DNAJB5	hypertrophy	Cytosol/nuclear	+++	(Ago et al., 2008)
HSPB	Small HSP	HSPB1 (HSP25, HSP27,HSP28)	AF, ischaemic heart disease	cytosol	+++	(Efthymiou et al., 2004;Brundel et al., 2006;Kwon et al., 2007;Ke et al., 2011)
		HSPB5 (αβCrystallin, CRYAB,CRYA1)	(dilated) cardiomyopathy	cytosol	++++	(Inagaki et al., 2006;Rajasekaran et al., 2007)
		HSPB6 (HSP20, p20)	AF, ischaemic heart disease	cytosol	++	(Fan and Kranias, 2011)
		HSPB7 (cvHSP)	AF	cytosol	+++++	(Ke et al., 2011)
		HSPB8 (HSP22,H11)	AF	cytosol	++	(Ke et al., 2011)
HSPD	HSP60	HSPD1 (HSP60)	Heart failure	mitochondria	++++	(Lin et al., 2007)
HSPC	HSP90	HSPCA (HSP90)	Ischaemic heart disease	cytosol	++++	(Kupatt et al., 2004)

Whether HSPs influence the expression level of ion channels is currently not known. Lastly, also the stability of the actin cytoskeleton (through binding of HSPs) and Rho-GTPase activity are regulated by the small HSP family members (Sugiyama et al., 2000;Mounier and Arrigo, 2002;Golenhofen et al., 2004;Brundel et al., 2006a;Salinthone et al., 2008). These findings indicate a protective role of HSPs against AF-induced changes in ion channel current, including the reduction of the L-type Ca^{2+} current.

3.3.3. Prevention of alterations in kinomics activity and target proteins

The activity of various kinases and phosphatases is changed during AF, which contributes to cardiomyocyte remodeling depending on their target proteins (Anderson, 2004;Christ et al., 2004;El-Armouche et al., 2006;Greiser et al., 2007;Dobrev and Nattel, 2008;Ke et al., 2011). In addition to ion channels, known targets are transcription factors, various calcium handling proteins and the actin cytoskeleton. Changes in transcription factor phosphorylation can alter gene expression, possibly contributing to cardiomyocyte hibernation. Interestingly, HSPB1 was shown to interact with certain (downstream) kinases, such as IkappaB kinase and c-Jun N-terminal kinase (JNK), thereby suppressing activation of the transcription factor NF-kappaB (Park et al., 2003;Kammanadiminti and Chadee, 2006). Interestingly, these kinases are also modulated during AF (Li et al., 2001; Cardin et al., 2003). In addition, HSPB1 is known to interact with other kinases and phosphatases and thereby may prevent the activation of downstream transcription factors (Ding et al., 1998;Lakshmikuttyamma et al., 2004;Dobrev, 2006;Peng et al., 2010;Fan and Kranias, 2011;Qian et al., 2011). Changes in phosphorylation status of Ca^{2+} handling proteins will affect the calcium homeostasis in cardiomyocytes. It is generally accepted that AF-induced abnormalities in intracellular Ca^{2+} handling leads to atrial cardiomyocyte stress and induces remodeling that contributes to the progression of AF (Qi et al., 2008;Chelu et al., 2009). A Ca^{2+} overload can be caused by an increase in L-type Ca^{2+} channel activity, or a changed activity of calcium handling proteins such as RyR2, Sarcoplasmic Reticulum Ca^{2+} ATPases (SERCA), or Na^{+}/Ca^{2+} exchanger. These rapid changes in activity of proteins involved in calcium handling are modulated by kinases and/or phosphatases, including CaMKII and PP1, of which the activities are increased during AF (Christ et al., 2004;Vest et al., 2005). Interestingly, studies showed that HSPs interact with CaMKII (Peng et al., 2010), calcineurin (Lakshmikuttyamma et al., 2004), and PP1 (Dobrev, 2006;Fan

and Kranias, 2011). Furthermore, HSPB6 was shown to inhibit PP1 activity (Qian et al., 2011). Also, HSPs increase SR Ca^{2+} ATPase (SERCA) activity and stimulate both the reuptake of Ca^{2+} into the sarcoplasmic reticulum and the extrusion of Ca^{2+} out of the cardiomyocyte via Na^{+}/Ca^{2+} exchanger (Liu et al., 2006;Chen et al., 2010). These findings suggest that HSPs attenuate AF progression by protecting against (tachypacing-induced) changes in calcium handling proteins.

3.3.4. Prevention of protease activation

HSPs also attenuate activation of the cysteine protease calpain and subsequent myolysis and structural remodeling in a *Drosophila* model for AF. In this model, DmHSP23, presumably the ortholog for human HSPB1, prevents induction of calpain activity, mitochondrial damage, and myolysis (Zhang et al., 2011). Other studies have also described the modulating effects of HSPB1 on cysteine proteases such as caspase 3 (Garrido et al., 1999;Concannon et al., 2003).

In summary, AF results in a derailment of cardiomyocyte proteostasis which induces reversible electrical and irreversible structural remodeling. Therapeutic intervention strategies targeted at restoring proteostasis by adjusting the activity of components of the proteostasis network seems a promising approach in AF, as was shown for HSP induction. Pharmacological induction of HSPs help cardiomyocytes to cope with proteotoxic stress (including oxidative stress), protects against alterations in signaling pathways influencing proteostasis (protection against alterations in kinomics) and plays a direct role in protein folding and degradation by preventing the activation of proteases. Therefore, the pleiotropic effects of HSPs influence all layers of the proteostasis network (Figure 2) and can protect against derailment of proteostasis and AF substrate formation. Although studies have been performed on the molecular mechanism(s) of HSP-induced cardioprotection in AF, their exact mode(s) of action remains to be elucidated. Furthermore, modifiers and pathways responsible for AF-induced derailment of proteostasis have not yet been uncovered and may contribute to the identification of novel upstream therapies in addition to HSP induction.

Scope of the thesis

The main goal of this thesis was to gain more insight in the modifiers and signaling pathways contributing to loss of proteostasis in AF and to identify novel targets for upstream therapy. In addition, the efficacy of HSPs to restore AF-induced alterations in the proteostasis network as well as their possible mode(s) of action was further investigated. As Brundel et al. (Brundel et al., 2006a;Brundel et al., 2006b) reported that sole induction of HSPB1 expression is sufficient for protection against tachypacing-induced remodeling in the *in vitro* HL-1 atrial cardiomyocyte model for AF, we investigated in **chapter 2** the therapeutic potential of all individual HSPB family members in preventing tachypacing-induced AF substrate formation and contractile dysfunction. Subsequently we elucidated that the mechanism of action of the protective HSPB family members is via inhibition of pathological RhoA signaling. In **chapter 3**, we continued our investigation on how pathological RhoA activation causes loss of cardiac proteostasis. Therefore, we determined the effects of RhoA activation on the heat shock response (HSR) and the induction of heat shock protein expression. In **chapter 4** we describe our efforts to identify key kinases and substrates involved in AF-induced remodeling employing a kinase array approach in a canine model for AF. Moreover, as GGA treatment prevents tachypacing-induced remodeling (Brundel et al., 2006a;Brundel et al., 2008), we aimed to identifying key kinases, whose change in activity is attenuated by GGA treatment and thus are likely to play an important role in AF-induced remodeling. Another factor of the proteostasis network we investigated consisted of epigenetic regulation via alterations in activity of histone deacetylases (HDAC). As activation of HDAC4 has been implicated in AF (Greiser et al., 2011), we investigated the therapeutic potential of HDAC inhibition in **chapter 5**, applying broad spectrum as well as specific HDAC inhibitors. Furthermore, we elucidated the mode of action of one of the identified protective HDAC inhibitors. In **chapter 6** we investigated the role of autophagy, an important protein and organelle degradation pathway, in cardiac proteostasis and contractile function during tachypacing. As we observed Endoplasmic Reticulum (ER) stress signaling to be involved in tachypacing-induced upregulation of autophagy and contractile dysfunction, we subsequently determined whether pharmacological inhibition of autophagy as well as ER stress inhibition revealed protective effects. Lastly, we examined the potential beneficial effect of HSPA5 (grp78) induction, a HSP localized in the ER, to prevent tachypacing-induced cardiac remodeling. Lastly, in **Chapter 7**,

summarize and discuss the data obtained in our experimental chapters and provide future perspectives for clinical therapy.

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Chapter 2

HSPB1, HSPB6, HSPB7 and HSPB8 protect against RhoA-GTPase-induced remodeling in tachypaced atrial myocytes

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Abstract

Background

We previously demonstrated the small heat shock protein, HSPB1, to prevent tachycardia remodeling in *in vitro* and *in vivo* models for Atrial Fibrillation (AF). To gain insight into its mechanism of action, we examined the protective effect of all 10 members of the HSPB family on tachycardia remodeling. Furthermore, modulating effects of HSPB on RhoA GTPase activity and F-actin stress fiber formation were examined, as this pathway was found of prime importance in tachycardia remodeling events and the initiation of AF.

Methods and Results

Tachypacing (4 Hz) of HL-1 atrial myocytes significantly and progressively reduced the amplitude of Ca^{2+} transients (CaT). In addition to HSPB1, also overexpression of HSPB6, HSPB7 and HSPB8 protected against tachypacing-induced CaT reduction. The protective effect was independent of HSPB1. Moreover, tachypacing induced RhoA GTPase activity and caused F-actin stress fiber formation. The ROCK inhibitor Y27632 significantly prevented tachypacing-induced F-actin formation and CaT reductions, showing that RhoA activation is required for remodeling. Although all protective HSPB members prevented the formation of F-actin stress fibers, their mode of action differs. Whilst HSPB1, HSPB6 and HSPB7 acted via direct prevention of F-actin formation, HSPB8-protection was mediated via inhibition of RhoA GTPase activity.

Conclusion

Overexpression of HSPB1, as well as HSPB6, HSPB7 and HSPB8 independently protect against tachycardia remodeling by attenuation of the RhoA GTPase pathway at different levels. The cardioprotective role for multiple HSPB members, indicate a possible therapeutic benefit of compounds able to boost the expression of single or multiple members of the HSPB family.

Introduction

Atrial Fibrillation (AF) is the most common sustained and progressive clinical tachycardia in the population and it significantly contributes to cardiovascular morbidity and mortality (Dobrev and Nattel, 2010). AF is characterized by specific changes in electrical, structural and contractile function of the atrial myocytes, commonly denoted as 'remodeling'. Tachycardia remodeling underlies contractile dysfunction and the progressive and intractable nature of AF. Therefore, remodeling is believed to have important therapeutic implications, and there is great interest in developing anti-remodeling therapies directed at the targets underlying remodeling (Dobrev and Nattel, 2010).

We recently identified one specific member of the heat shock protein (HSP) family, HSPB1, to protect against AF-induced remodeling (Brundel et al., 2006a;Brundel et al., 2006b). HSPs are molecular chaperones and prevent the accumulation of the misfolded or unfolded proteins in the cells (Morimoto, 2008). HSPB1 is one member of the small heat shock protein (sHSP or HSPB in mammals) family, which comprises a total of at least ten members (Kappe et al., 2003;Kampinga et al., 2009). A characteristic of most HSPBs is their ability to interact with components of the actin cytoskeleton, and this binding protects against cytoskeletal injury during stress, resulting in conservation of the cell function (Landry and Huot, 1995). In addition, HSPBs collectively share important features, including (1) a conserved α-crystallin domain, (2) ability to form large oligomers *in vitro* and (3) increased expression upon exposure to various stresses including heat stress (Vos et al., 2008). Nevertheless, the precise mode of action of HSPB1 to protect from tachycardia remodeling remains elusive and it is unknown whether this is shared between other members of the HSPB family. Therefore, we examined if, in addition to HSPB1, also other HSPB members protect against atrial tachycardia remodeling. Hereto, we utilized tachypaced HL-1 myocytes, an *in vitro* atrial cell line model for tachycardia remodeling (Brundel et al., 2004;Brundel et al., 2006b). In addition to HSPB1, we identified HSPB6, HSPB7 and HSPB8 to protect against tachypacing-induced calcium transient reduction. Because of the known protective actions of HSPBs on actin cytoskeleton, we next examined their effect on tachypacing-induced RhoA GTPase pathway, including RhoA GTPase activity and related F-actin stress fiber formation. Although all protective HSPB members reduced the formation of F-actin stress fibers, their mode of action differs. HSPB1, HSPB6 and HSPB7 were found to directly prevent F-actin stress fiber formation, whereas HSPB8-protection was mediated via inhibition of upstream RhoA GTPase activity.

Materials and Methods

2.1. HL-1 atrial myocyte culture, transfections and constructs

HL-1 atrial myocytes, derived from adult mouse atria, were obtained from Dr. William Claycomb as described before (Brundel et al., 2006b). The myocytes were maintained in Complete Claycomb Medium (JRH, UK) supplemented with 100 µM norepinephrine (Sigma, The Netherlands), 0.3 mM L-ascorbic acid (Sigma), 4 mM L-glutamine (Gibco, The Netherlands) and 10% FBS (Life Technologies, Gaithersburg, MD). They were cultured on coverslips coated with 12.5 µg/ml fibronectin (Sigma) and 0.02% gelatin (Sigma), in a 5% CO_2 atmosphere at 37°C.

To study the influence of HSPBs on Ca^{2+} transient changes, HL-1 myocytes were transiently (co-)transfected, by the use of Lipofectamin (Life Technologies, The Netherlands), with the plasmid CD8 cDNA encoding CD8 antigen and/or pCDNA5/FRT/TO-HSPBX (X indicating 1-10) encoding human HSPB members. Positive myocytes were selected by anti-CD8 Dynabeads (Dynal). To check overexpression of HSPBX proteins in HL-1 myocytes, myocytes were transiently transfected with the fusion proteins V5-HSPBX. For all other experiments HSPBX wildtype constructs were used.

2.2. Tachypacing of HL-1 myocytes

HL-1 myocytes were subjected to tachypacing as described before (Brundel et al., 2004;Brundel et al., 2006a;Brundel et al., 2006b). In short, the spontaneous rate of HL-1 myocytes is ~1 Hz. HL-1 myocytes were subjected to normal electrical field stimulation (1 Hz) for at least 30 min before tachypacing via the C-Pace100™-Culture Pacer (IonOptix Corporation, The Netherlands). Tachypacing was performed at 4 Hz with 20-ms pulses for 8 hours to induce CaT reduction and 1 Hz pacing was used as a control.

2.3. Protein-extraction and Western blot analysis

Western blot analysis was performed as described previously (Brundel et al., 2006a;Brundel et al., 2006b). Equal amount of protein in SDS-PAGE sample buffer was sonicated before separation on 10% PAA-SDS gels. After transfer to nitrocellulose membranes (Stratagene, The Netherlands), membranes were incubated with primary antibodies against HSPB1 (SPA801, StressGen USA), V5 tag (Invitrogen, The Netherlands) or GAPDH (Affinity Reagents, The Netherlands). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit (Santa-Cruz Biotechnology, The Netherlands) was used as secondary antibody. Signals were detected by the ECL-detection method (Amersham, The Netherlands) and quantified by densitometry.

2.4. Live imaging and measurement of CaT

To measure CaT, 2 µM of the Ca^{2+}-sensitive Fluo-4-AM dye (Invitrogen, The Netherlands) was loaded into HL-1 myocytes by 45 min incubation, followed by 3 times washing with DMEM solution. Ca^{2+} loaded myocytes were excited by 488 nm and light emitted at 500–550 nm and visually recorded with a 40x-objective, using a Solamere-Nipkow-Confocal-Live-Cell-Imagingsystem (based on a Leica DM IRE2 Inverted microscope). The live recording of CaT in HL-1 myocytes was performed at 1 Hz of stimulation in a temperature (37°C) controlled system. By use of the software ImageJ (National Institutes of Health, USA), the absolute value of fluorescent signals in live myocytes were recorded and analyzed. To compare the fluorescent signals between experiments, the following calibration was utilized: F_{cal} = F/F0, in which (F) is fluorescent dye at any given time and (F0) is fluorescent signal at rest (Paredes et al., 2008). Mean values from each experimental condition were based on 7 consecutive CaT in at least 50 myocytes.

2.5. Immunofluorescent staining and confocal analysis

Twenty-four hours after transient transfection of HSPB1, HSPB5, HSPB6, HSPB7 and HSPB8, HL-1 myocytes were subjected to (tachy)pacing. Afterwards, the myocytes were fixed with 3.7% formaldehyde for 15 minutes, washed three times with Phosphate-Buffered Saline (PBS), permeabilized with 0.2% Triton-X100 and blocked with 0.1 glycine (10 minutes at room temperature) and 5% BSA (30 minutes at room temperature). Antibodies against HSPB6, HSPB7 and HSPB8 (all Abcam, The Netherlands), and HSPB1 and HSPB5 (StressGen, USA) were used as primary antibody. Fluorescein labeled isothiocyanate (FITC) anti-mouse or anti-rabbit (Jackson ImmunoResearch, The Netherlands) were used as secondary antibodies. To visualize F-actin, rhodamine phalloidin (Invitrogen, The Netherlands) was diluted with PBS at 1:40, followed by incubation for 20 minutes at room temperature and washed three times with PBS. Images of FITC and rhodamine fluorescence were obtained using the Leica confocal laser scanning microscope (Leica SP2 AOBS) with 63X/1.4 oil lens. The captured images were processed using Leica Confocal Software and Adobe Photoshop. For determination of the amount of F-actin stress fibers, the intensity of fluorescence was analyzed by ImageJ in 5 independently taken fields. HL-1 myocytes were treated 4 hours prior and during (tachy)pacing with the ROCK inhibitor Y27632 (10 µM, Sigma, The Netherlands) to prevent F-actin stress fiber formation. For determination of the amount of co-localization of HSPB with stress fibers, ImagePro software was used. The amount of colocalization was determined as the ratio of total red signal (F-actin) divided by yellow signal (colocalization HSPB with F-actin). Between 400 and 500 myocytes were quantified per condition.

2.6. Short interfering RNA of HSPB1 in combination with over-expression of HSPB6, HSPB7 or HSPB8

Downregulation of endogenous HSPB1 was performed as described previously (Brundel et al., 2006a). HL-1 myocytes were transiently transfected with HSPB1 siRNA or mock constructs for 5 days. Furthermore, 24 hours before tachypacing, cells were co-transfected with HSPB6, HSPB7 or HSPB8 construct. After 8-hours (tachy)pacing, CaT were measured and analyzed.

2.7. RhoA GTPase activity measurement with G-LISA

For the quantitative analysis of active RhoA GTP levels, G-LISA RhoA Activation Assay (Cytoskeleton, USA) was performed according to the manufacturer protocol. Briefly, 48 hours after the transfection of HL-1 myocytes, myocytes were subjected to (tachy)pacing for 6 hours or directly lysed in lysis buffer and cells were harvested. After measurement of the protein concentration with the use of Precision Red (supplied), equal amounts of lysates were incubated in RhoA GTP affinity plates. The amount of bound RhoA GTP was detected by using primary anti-RhoA antibody (1:250, supplied) and secondary HRP-labeled antibody (1:62.5, supplied). Colorimetric detection at 490 nm was performed immediately (BioRad, The Netherlands).

2.8. Actin (de-)polymerization-assay

To determine the direct effect of HSPBs on actin polymerization and depolymerization an actin polymerization biochem kit (Cytoskeleton, USA) was used. Twenty-four hours after transient transfection of HL-1 myocytes with HSPB1, HSPB5, HSPB6, HSPB7 or HSPB8, myocytes were lysed in a mild lysis buffer according to the manufacturer protocol. As a control, recombinant human HSPB1 (Stressgen, USA) was dissolved in lysis buffer. Base-line fluorescence of pyrene conjugated actin was measured (Ex. 350 nm; Em. 405 nm) for three minutes, after which cell lysates and recombinant HSPB1 were added to measure the effect on (de-)polymerization. Fluorescence was assayed every 60 s for twenty minutes. Maximum actin polymerization was determined by adding polymerization buffer.

2.9. Statistical analysis

Results are expressed as mean ± SEM. All CaT measurements were performed in at least triple series. Mean values from each experimental condition were based on 7 consecutive CaT in at least 50 myocytes. ANOVA was used for multiple-group comparisons. All p-values were two-sided. $P<0.05$ was considered statistically significant. SPSS version 16.0 was used for statistical evaluation.

Results

3.1. Effect of overexpression of the ten different HSPB members on tachypacing-induced remodeling in HL-1 myocytes

In humans, the HSPB family comprises a group of 10 members with monomeric molecular weight varying between 16 to 28 kDa (Kappe et al., 2003;Vos et al., 2008;Kampinga et al., 2009). Induction of HSPB1 has been shown previously to protect against atrial tachypacing-induced remodeling, including CaT reduction (Brundel et al., 2006b).

To study the effect of individual HSPB members, HL-1 myocytes were transfected with V5 tagged constructs for each member. All members were successfully overexpressed, albeit HSPB8 and HSPB9 at a lower level (Figure 1A). As a control group, HL-1 myocytes were transfected with an empty vector. None of the overexpressed HSPB members changed CaT in control myocytes paced at 1 Hz (data not shown). As observed before (Brundel et al., 2006b), tachypacing at 4 Hz of HL-1 myocytes resulted in a significant and progressive reduction in CaT (Figure S1), which was attenuated by HSPB1 (Figure 1B,C Movie S1, Movie S2, Movie S3). In addition, overexpression of HSPB6, HSPB7 and HSPB8 also protected against tachypacing-induced CaT depression, whereas the other members were ineffective (Figure 1B, C and Movie S1, Movie S2, Movie S3, Movie S4, Movie S5, Movie S6, Movie S7, Movie S8, Movie S9, Movie S10, Movie S11, Movie S12). These results indicate that in addition to HSPB1 also HSPB6, HSPB7 and HSPB8 protect against tachypacing-induced CaT reduction.

3.2. HSPB6, HSPB7 and HSPB8 protection against tachypacing-induced CaT reduction is independent of endogenous HSPB1 expression

HSPB members are known for their ability to form hetero-oligomeric complexes (Sun et al., 2004;Simon et al., 2007) and given the fact that HSPB1 is constitutively expressed in HL-1 myocytes, the possibility existed that the protective effect of HSPB6, HSPB7 or HSPB8 on tachypacing-induced CaT reduction was related to (indirect) effects via (oligomerization with) HSPB1. Also, ectopic HSPB expression may induce a stress response in cells leading to the up-regulation of endogenous HSPB1. To exclude these possibilities, it was first determined whether overexpression of HSPB6, HSPB7 or HSPB8 increased expression of endogenous HSPB1 levels. As shown in Figure 2, endogenous HSPB1 levels were similar after normal pacing (1 Hz) and tachypacing (4 Hz), irrespective of HSPB6, HSPB7 or HSPB8 overexpression. Secondly, the endogenous HSPB1 level was suppressed by short hairpin RNAs (Figure 3A). In HSPB1 depleted myocytes, HSPB6, HSPB7 or HSPB8 overexpression could still protect against tachypacing-induced CaT

reduction (Figure 3B, C, Movie S13, Movie S14, Movie S15, Movie S16, Movie S17, Movie S18). In summary, these results suggest that the protective effects of HSPB6, HSPB7 and HSPB8 against tachypacing-induced CaT reduction are independent of HSPB1.

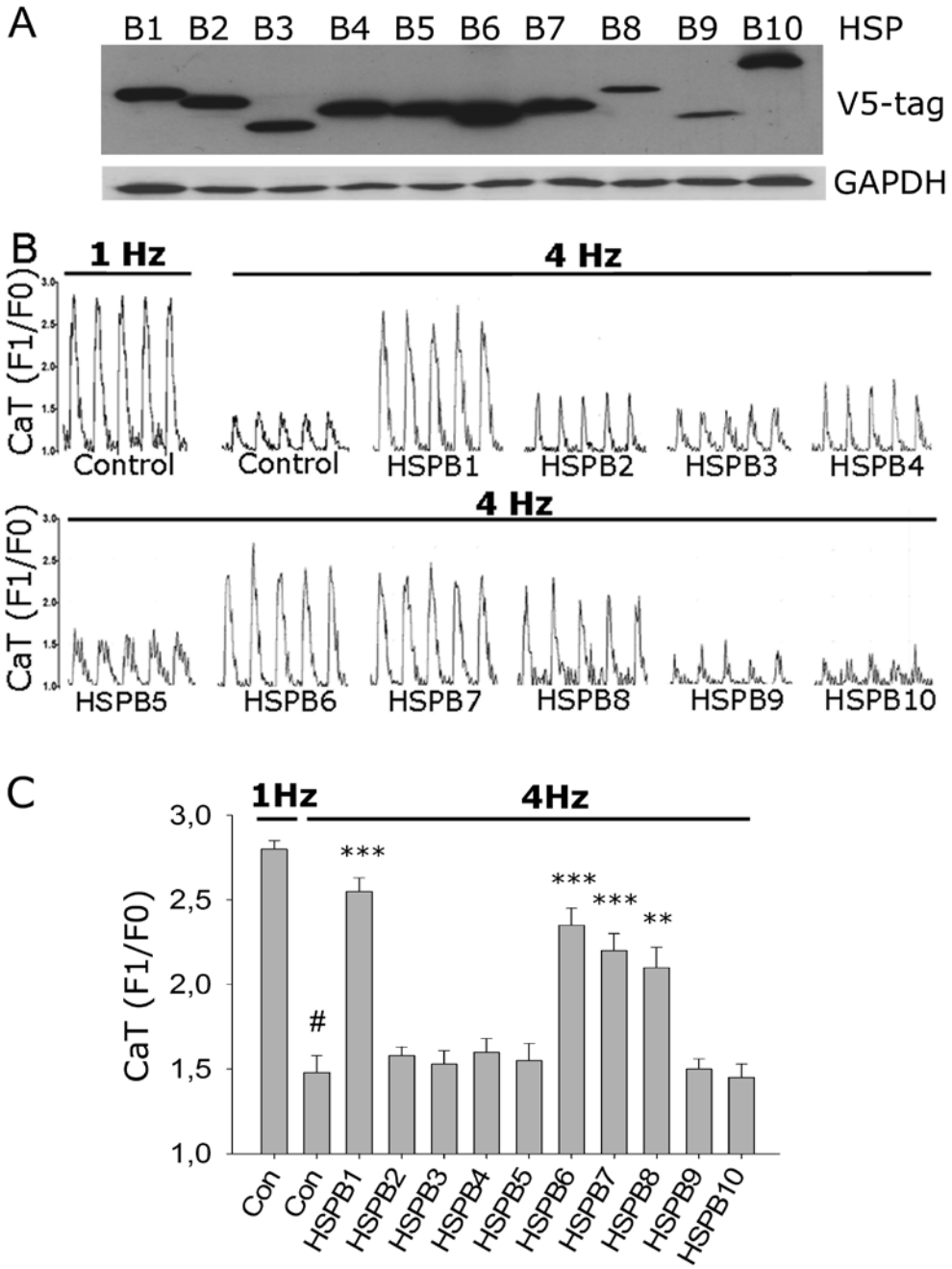

Figure 1 HSPB1, HSPB6, HSPB7 and HSPB8 overexpression prevents against tachypacing-induced CaT reductions in HL-1 myocytes. **A** Representative Western blot showing overexpression of HSPB1-10 in transiently transfected HL-1 myocytes. **B** Original recordings of CaT in 1 myocyte each from groups indicated. **C** Mean CaT data of HSPB1-10 overexpressing myocytes tachypaced (4 Hz) or normal paced cells (1 Hz). **P<0.01, ***P<0.001 vs control tachypaced (4 Hz). # P<0.001 vs control normal paced (1 Hz).

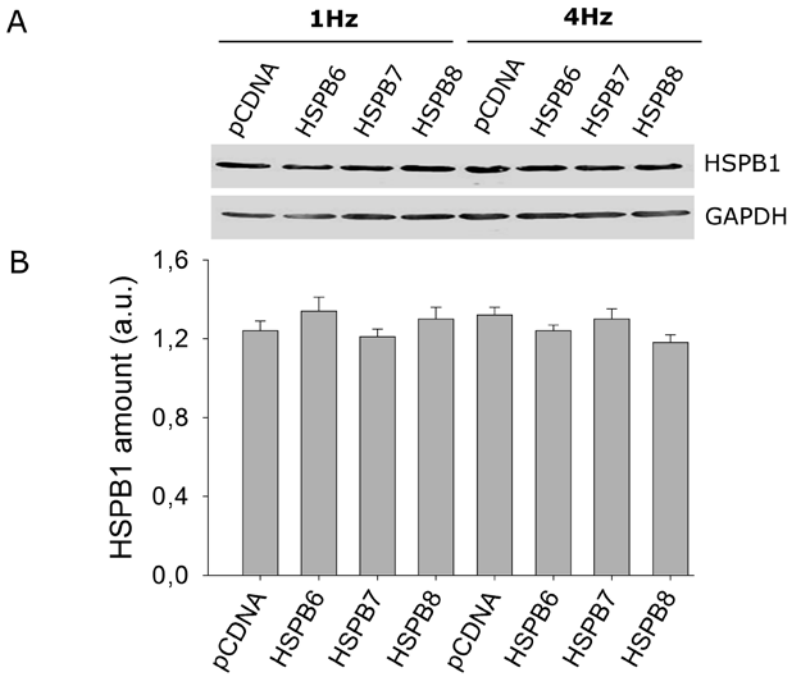

Figure 2 Overexpression of HSPB6, HSPB7 or HSPB8 do not result in changes in endogenous HSPB1 levels.
A Representative Western blot showing that the endogenous HSPB1 levels in transfected HSPB6, HSPB7 and HSPB8 overexpressing HL-1 myocytes are not changed in normal paced myocytes (1 Hz) or tachypaced myocytes (4 Hz).
B Corresponding mean data (n = 3 experiments/group).

3.3. HSPB6, HSPB7 and HSPB8 reduce the amount of F-actin stress fibers after tachypacing in HL-1 myocytes

Calcium signaling is known to be markedly influenced by the stabilization of the cytoskeleton (Kerfant et al., 2001;Johnson et al., 2005;Leach et al., 2005). F-actin is one of the major components of the cytoskeleton and located under the plasma membrane to maintain cell shape, rigidity and integrity (Janmey, 1998;Pollard and Cooper, 2009). Several HSPB members, including HSPB1, HSPB5, HSPB6, HSPB7 and HSPB8, have been reported to be involved in cytoskeletal stability (Hoch et al., 1996;Fan et al., 2004;Golenhofen et al., 2004;Sui et al., 2009). To study if the underlying mechanism for HSPB protection is related to effects on actin, immunofluorescent staining was performed. We observed a 1.7 fold induction in the amount of F-actin stress fibers in tachypaced HL-1 myocytes compared to normal paced control myocytes (Figure 4, 5B), an effect that was significantly reduced by overexpression of HSPB1, HSPB6, HSPB7 or HSPB8 (Figure 5A,B). Overexpression of HSPB5, which did not show protection against tachypacing-induced CaT reductions (Figure 1), also did not lead to a reduction in the amount of tachypacing-induced F-actin stress fibers (Figure 4, 5B). Although in tachypaced HSPB1, HSPB6, HSPB7 or HSPB8 overexpressing myocytes a reduction in the amount of F-actin stress fibers was found, HSPB1, HSPB6, HSPB7 and to a lesser

extent HSPB8 co-localized with the F-actin residues after tachypacing and this was not the case for HSPB5 (Figure 4, 5A,C). Taken together, these results suggest that HSPB1, HSPB6, HSPB7 and HSPB8 prevent the formation of F-actin stress fibers in tachypaced HL-1 myocytes, and thereby stabilize the cytoskeleton and myocyte function.

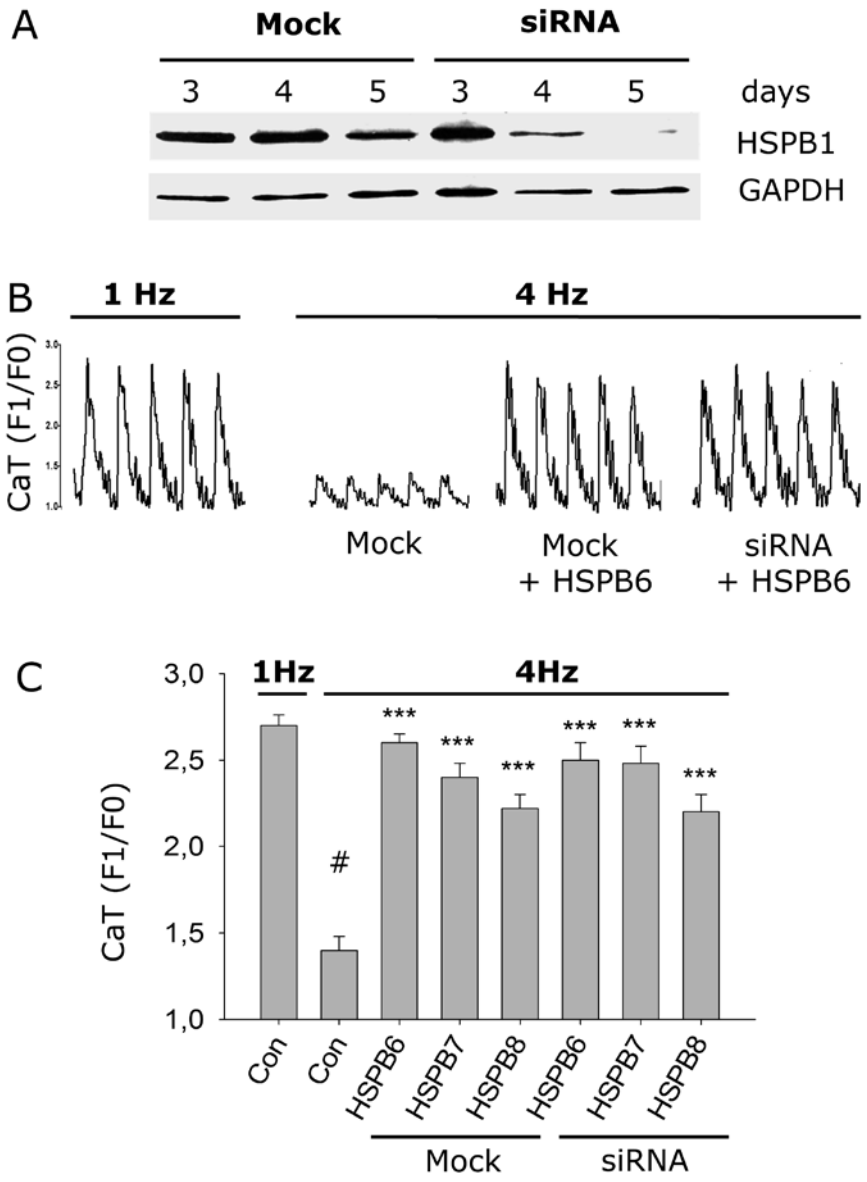

Figure 3 HSPB6, HSPB7, HSPB8 protective effect is independent of HSPB1.

A Western blot showing efficient siRNA-induced HSPB1 knockdown in HL-1 mycoytes. **B** Recordings of CaT for mock and siRNA construct. HL-1 myocytes were transfected with mock or siRNA construct for 5 days before study. One day before tachypacing, the myocytes were transfected with HSPB6, HSPB7 or HSPB8 and subjected to normal pacing (1 Hz) or tachypacing (4 Hz). **C** Mean CaT data. The cardioprotective effect of HSPB6, HSPB7 or HSPB8 was not blocked by HSPB1 suppression. ***P<0.001 vs control tachypaced (4 Hz). # P<0.001 vs control normal paced (1 Hz).

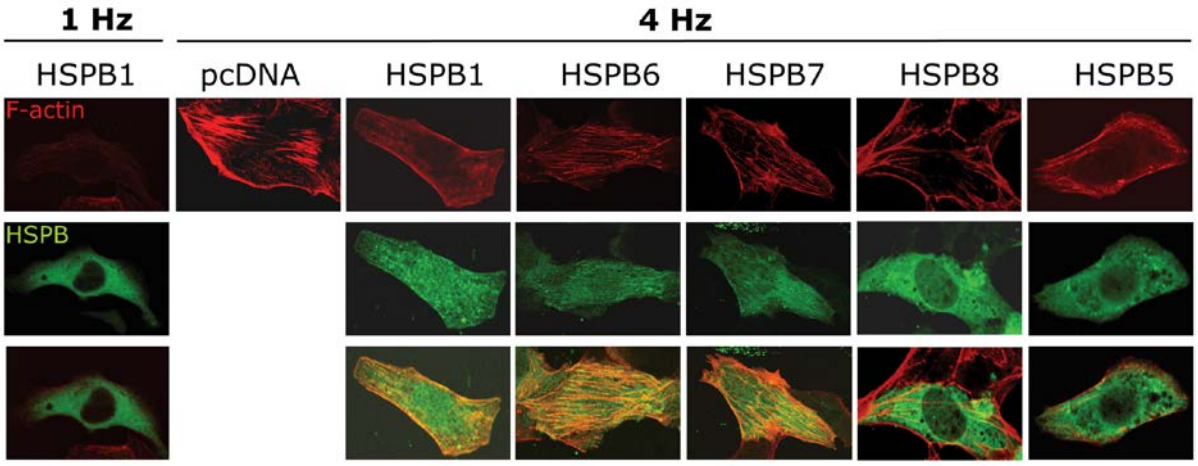

Figure 4 HSPB1, HSPB6 and HSPB7 co-localize with tachypacing-induced F-actin stress fibers in HL-1 myocytes.
Immunofluorescent staining of F-actin stress fibers (red) and HSPB positive myocytes (green), in tachypaced HL-1 myocytes (4 Hz). A normal paced (1 Hz) HSPB1 transfected myocyte was shown as a representative control example.

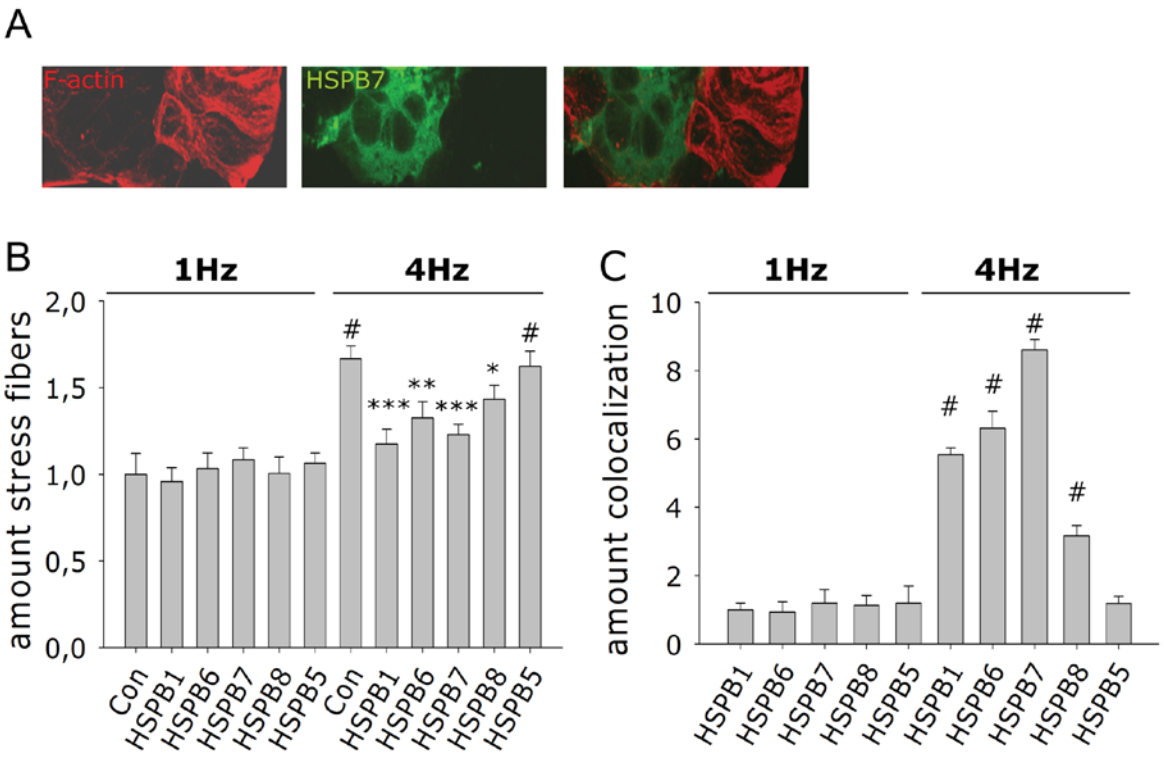

Figure 5 HSPB1, HSPB6, HSPB7 and HSPB8 overexpression is associated with a reduction in the amount of tachypacing-induced F-actin stress fibers in HL-1 myocytes.
A Immunofluorescent staining of F-actin stress fibers (red) and HSPB7 positive myocytes (green), in tachypaced HL-1 myocytes (4 Hz). HSPB7 positive myocytes reveal less stress fibers. **B** Quantification of the amount of F-actin stress fibers in HSPB transfected HL-1 myocytes after normal pacing (1 Hz) or tachypacing (4 Hz). **C** Quantification of the amount of colocalization of transfected HSPB with F-actin stress fibers. **P*<0.05, ***P*<0.01, ****P*<0.001 vs control tachypaced myocytes (4 Hz), #*P*<0.05 vs control normal paced (1 Hz).

3.4. Tachypacing induces RhoA GTPase and ROCK activation, resulting in F-actin formation and reduction in calcium transients

To confirm the role of RhoA GTPase pathway in tachypacing-induced F-actin stress fiber formation and reductions in CaT, HL-1 myocytes were tachypaced for 0-8 hours and RhoA GTPase activity was measured in cell lysates (Figure 6A). A significant induction of the RhoA GTPase activity was observed at 6 hours of tachypacing. In parallel, the amount of F-actin was quantified. A gradual increase in the amount of F-actin was observed during tachypacing, which was prevented by the ROCK inhibitor Y27632 (10 µM) (Figure 6B). In addition, also tachypacing-induced changes in CaT were reduced by Y27632 (Figure 6C), indicating that also this effect is RhoA-mediated.

3.5. HSPB8, but not HSPB1, HSPB6, and HSPB7, reduces activation of RhoA GTPases after tachypacing

To test whether the protective effect of HSPB members is related to a direct modulation of the RhoA GTPase activity, RhoA GTPase activity was measured in normal paced (1 Hz) and tachypaced (4 Hz) HL-1 myocytes transfected with the individual HSPB members. None of the HSPB family members affected RhoA GTPase activity in 1 Hz paced HL-1 myocytes (Figure S2). Only HSPB8 transfected HL-1 myocytes revealed significantly reduced activation of RhoA GTPase upon 6 hours of tachypacing and all other (protective) HSPB members were ineffective (Figure 7), suggesting that their protective effects against tachycardia remodeling are downstream of RhoA GTPase activation.

3.6. HSPB1, HSPB6, and HSPB7 prevent G-to-F actin polymerization

To investigate whether HSPB1, HSPB6, and HSPB7, rather than affecting RhoA GTPase activation, may ameliorate the downstream consequences of activated RhoA GTPase, we measured their effect on the polymerization of G-actin to F-actin and also the depolymerization, using an *in vitro* polymerization kit. Base-line fluorescence of G/F-actin ratios were measured for three minutes, after which cell lysates from HL-1 myocytes transfected with the respective HSPB members or recombinant HSPB1 were added (Figure 8). The non-protective HSPB5 was used as a control. When polymerization buffer was added to the baseline G/F-actin, a rapid increase in the conversion of G-to-F actin ratio was observed, indicative of fast actin polymerization. Addition of lysates from HSPB1 transfected cells as well the addition of 0.5 µg recombinant human HSPB1 induced depolymerization of F-actin. Although less effective, lysates from HSPB6 also induce depolymerization, whereas lysates from HSPB7 transfected myocytes prevent actin polymerization but did not show an effect on depolymerization. In contrast to the findings of HSPB1, HSPB6 and HSPB7

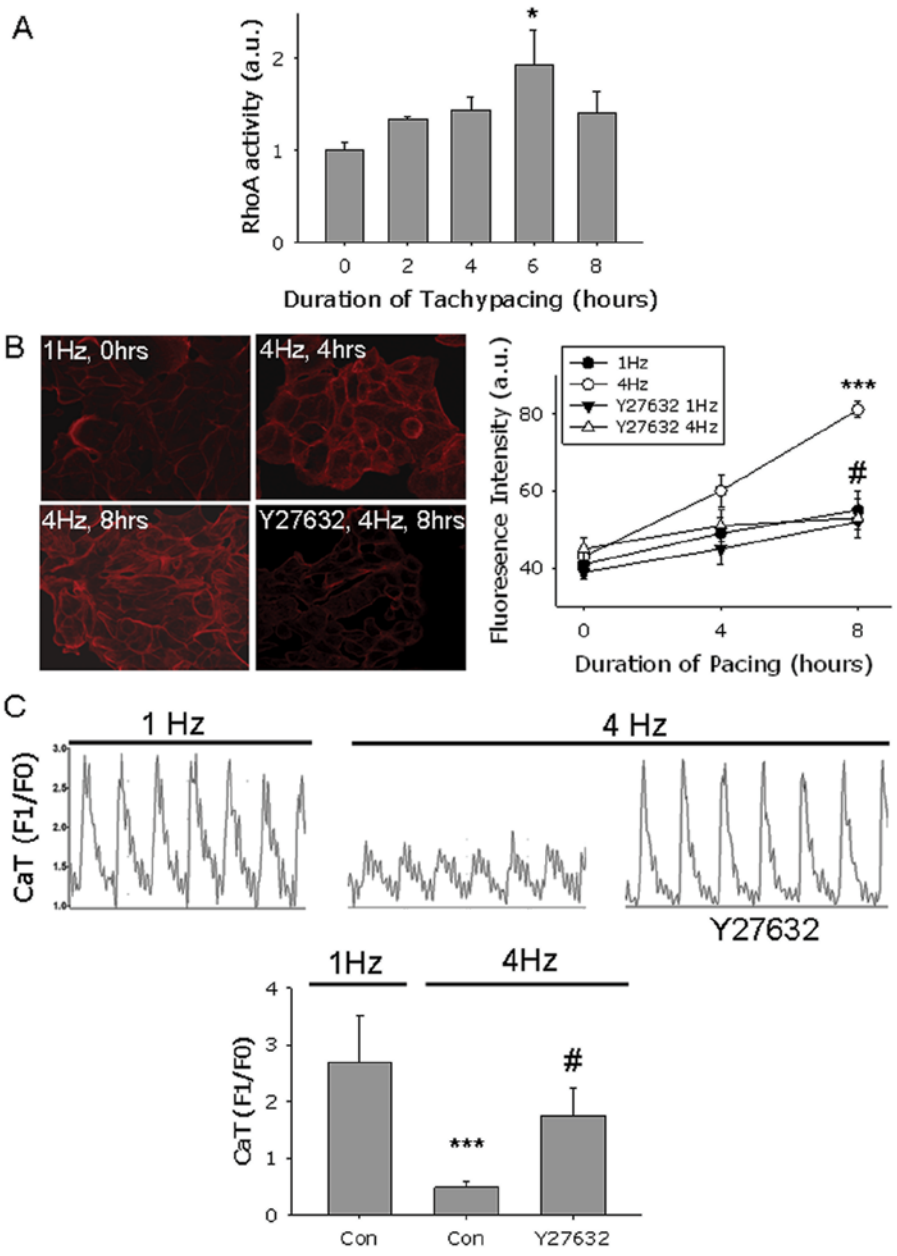

Figure 6 Tachypacing induces gradual activation of RhoA-GTPase and consequently formation of F-actin stress fibers and reduction in CaT in HL-1 myocytes.

A HL-1 myocytes were tachypaced up to 8 hours and activation of RhoA-GTPase was measured. **B** Left: Examples of immunofluorescent staining of F-actin stress fibers (red) in tachypaced HL-1 myocytes (4 Hz, 4 and 8 hours), normal paced (1 Hz) and tachypaced HL-1 myocytes pretreated with the ROCK inhibitor Y27632 (4 Hz, 8 hours). Right: quantification of the fluorescence intensity of F-actin in the conditions as indicated. **C** Top: Original recordings of CaT in 1 myocyte each from groups indicated. Below: Mean CaT data of normal (1 Hz) and tachypaced (4 Hz) HL-1 myocytes and tachypaced myocytes pretreated with Y27632. ***P<0.001 Y27632 tachypaced vs control tachypaced (4 Hz), #P<0.001 vs control normal paced (1 Hz).

in preventing the formation and/or stimulating the depolymerization of F-actin stress fibers, addition of lysates from HSPB8 transfected cells resulted in actin polymerization, although the levels of polymerization were reduced compared to lysates of the non-protective HSPB5 transfected myocytes, which showed near to normal polymerization. These results together suggest that HSPB1, HSPB6 and HSPB7 may prevent tachycardia remodeling by directly preventing the formation and/or stimulating the depolymerization of F-actin stress fibers downstream of active RhoA GTPase, whilst HSPB8 mainly acts at the level of tachypacing-induced RhoA GTPase activation.

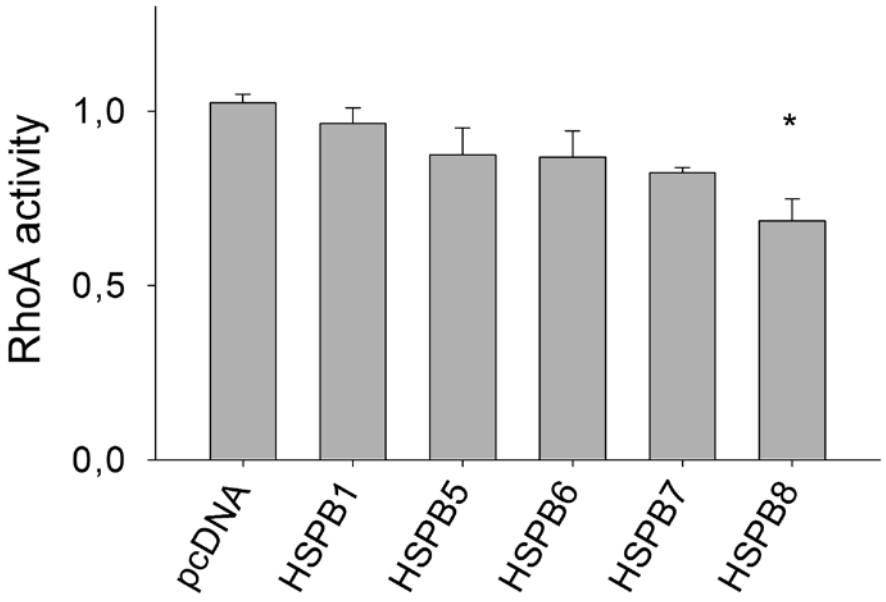

Figure 7 HSPB8 reduces activation of RhoA-GTPase during tachypacing in HL-1 myocytes.
HL-1 myocytes were transfected with HSPB1, HSPB5, HSPB6, HSPB7, HSPB8, or empty plasmid (pcDNA) and subjected to tachypacing (4 Hz, 6 hours). Activation of RhoA-GTPase in tachypaced (4 Hz) HL-1 myocytes, transfected with plasmids as indicated, is shown. * $P<0.05$ vs control tachypaced myocytes (4 Hz).

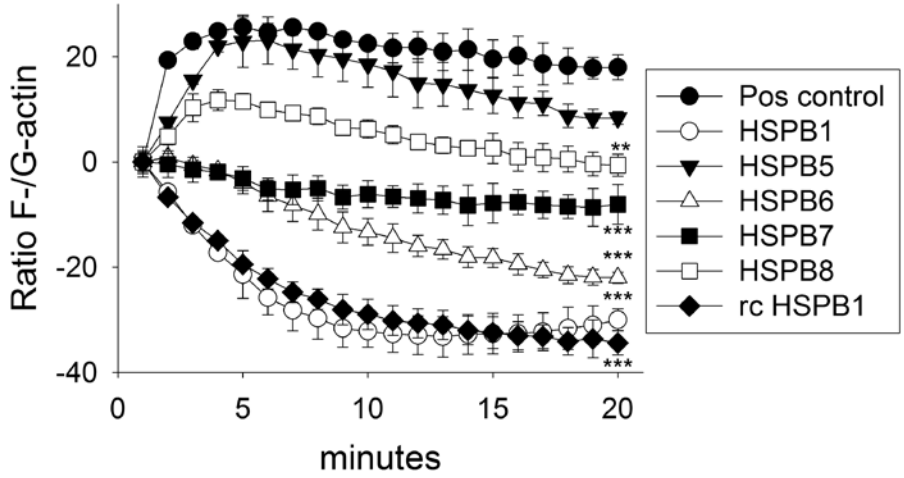

Figure 8 HSPB1, HSPB6, and HSPB7 attenuate F-actin stress fiber formation in vitro.
Cell-lysates from HL-1 myocytes transfected with HSPB1, HSPB5, HSPB6, HSPB7, or HSPB8 and recombinant human HSPB1 were used to measure influence of HSPB members on polymerization of G-actin to F-actin and also the depolymerization. ** $P<0.01$ vs lysates of HSPB5 transfected myocytes at 20 minutes incubation, *** $P<0.001$ vs lysates of HSPB5 transfected myocytes at 20 minutes incubation.

Discussion

Previously, we showed HSPB1 to protect in HL-1 myocytes against tachycardia remodeling and to preserve normal Ca^{2+} transients as well as the actin cytoskeleton upon tachypacing (Brundel et al., 2006a;Brundel et al., 2006b). In the current study we found that, in addition to HSPB1, also some other members of the HSPB family (HSPB6, HSPB7 and HSPB8) display protective effects against tachypacing-induced remodeling. Interestingly, all protective HSPB members reduced the formation of F-actin stress fibers, although their modes of action differ. Whereas HSPB8 interfered with tachypacing-induced RhoA GTPase activity, HSPB1, HSPB6, and HSPB7 did not. HSPB1, HSPB6 and HSPB7 were found to directly inhibit G- to F-actin polymerization and/or stimulate depolymerization, indicating a protective role against tachycardia remodeling downstream of RhoA GTPase activation.

4.1 Role of Rho GTPases in induction of AF

The current study revealed a prime role for tachypacing-induced RhoA GTPase activity and consequently F-actin stress fiber formation in reductions in calcium transients. This finding is in line with studies revealing an important role for Rho GTPases, including RhoA and Rac1, in formation of F-actin stress fibers (Brown et al., 2006) and the initiation of

AF (Sah et al., 1999;Adam et al., 2007). Consistently, experimental studies showed that activation of RhoA GTPases result in conduction disturbances and cardiac dysfunction similar to those described in AF (Ogata et al., 2008;Reil et al., 2010). Rho GTPases represent a family of small GTP-binding proteins involved in cell cytoskeleton organization, migration, transcription, and proliferation. Rho GTPases have gained considerable recognition as powerful regulators of actin cytoskeletal organization in the heart (Brown et al., 2006). It was observed that active Rho GTPases stimulate the conversion of G-actin to F-actin, which results in cytoskeletal injury including changes in calcium signaling, conduction disturbances and contractile dysfunction (Kerfant et al., 2001;Johnson et al., 2005;Leach et al., 2005;Pollard and Cooper, 2009), which are all substrates for the development of AF (Ogata et al., 2008;Reil et al., 2010). Actin stress fiber assembly and contraction are predominantly mediated by Rho-associated serine/threonine kinase (ROCK), a major downstream effector of the Rho pathway. Consistent with all of the above, we now show that tachypacing activates RhoA and that inhibition of ROCK, its effector of actin polymerization, prevents tachypacing-induced reductions in CaT.

4.2 The HSPB family

Whereas all HSPB members are characterized by the presence of a conserved crystallin domain, this domain is flanked by N- and C-termini that shows large sequence divergence between the members (Table 1) (Kappe et al., 2003;Vos et al., 2008). Also, the four members (HSPB1, HSPB6, HSPB7 and HSPB8) that we found to have protective effects against tachycardia remodeling show, besides sequence divergence, a number of structural and functional differences (see below). Interestingly, however, all four members, together with the non-protective HSPB5, show high basal expression in heart tissue (Table 1). In addition, three members (HSPB1, HSPB6, HSPB7) seemed to act similar in AF protection, i.e. preventing actin remodeling downstream of RhoA-activation. Only HSPB8 appears to directly affect RhoA-activation. So, the question is what are the characteristics shared by these members and, in addition, what are the differences between them that can explain their protective effects on tachypacing-induced remodeling?

In cell-free assays, small HSPs have been shown to act as ATP-independent “holdases”, maintaining unfolded or misfolded proteins in a folding competent, non-aggregated state, hereby supporting refolding by ATP-regulated chaperones, in particular the HSP70 machinery (Vos et al., 2008). In cellular assays, however, of the four cardioprotective HSPB members, only HSPB1 seems to support such refolding reaction (Bryantsev et al., 2002;Vos et al., 2010). Moreover, HSPB5 also shares this activity (Vos et al., 2010), but did not reveal protective effects against tachycardia remodeling. This finding indicates that such a

chaperone-like activity is not of prime importance to the HSPB-mediated protective effects as reported in the current study.

Several members of the HSPB family, including HSPB6, HSPB7 and HSPB8 were recently shown to be able to assist in the clearance of stress-induced misfolded proteins, in part through interaction with (HSPB7) or activation of (HSPB8) the macro-autophagy machinery (Carra et al., 2009;Carra et al., 2010;Vos et al., 2010). Yet, this activity is not shared by e.g. HSPB1 whilst HSPB9 that also can enhance clearance of misfolded proteins (Vos et al., 2010), albeit likely via proteasomal degradation (Vos et al., 2011) had no effect on AF. So, the clearance of misfolded proteins seems not to be a common target of all cardioprotective HSP members.

Another feature shared amongst many HSPB members is their dynamic (de)oligomerization (Kappe et al., 2003;Vos et al., 2008). This characteristic has been suggested to be crucial for e.g. the ability of HSPB1 to interact with several cytoskeletal components, including actin, intermediate filaments, and microtubules (Landry and Huot, 1995;Fan and Kranias, 2010). Yet, in cells HSPB7 and HSPB8 do not appear to be present in large oligomeric structures (Carra et al., 2008;Vos et al., 2011) implying also that this does not edify their protective role against tachycardia remodeling. However, all protective HSPB members can be found in cells as non-oligomeric (most likely dimeric) proteins as well. For HSPB1, dimers have been suggested to be the active species in regulating actin (re)polymerization after stress (Lavoie et al., 1995). Also for HSPB6 stress-induced translocation to actin of the myofibrils has been reported, which has been associated with improved heart function (Fan and Kranias, 2010). Also HSPB7 translocates from cytosol to the Z-/I-area of myofibrils, and thereby exerts a protective effect to ischemic stress (Golenhofen et al., 2004). This interaction may be mediated via α-filamin, an actin-binding protein (Krief et al., 1999). All of this is consistent with our current findings that HSPB1, HSPB6 and HSPB7 are associated with F-actin stress fibers upon tachypacing and the fact that they can directly prevent actin polymerization, an effect that occurs in living cells as a down-stream effect of Rho activation. In addition, the findings also suggest that chaperone-like (refolding or clearance) function and actin protection are distinct, uncoupled functions of these HSPB members. For HSPB8, the remaining AF protecting HSPB member, no direct association with actin and/or microtubules has been reported so far. Although HSPB8 is highly expressed in heart and muscle and anti-HSPB8 antibodies decorate sarcomeres (Carra et al., 2010), only a weak association with F-actin stress fibers after tachypacing was observed in the current study. Moreover, we only found weak attenuating effects of HSPB8 on actin polymerization. So, the protective effects of HSPB8 against tachycardia remodeling seem distinct from that of the other HSPB members. Consistently, we indeed observed that HSPB8 was the only member that directly affected tachypacing-induced RhoA activation. How HSPB8 may

modulate this effect remains an enigma, but maybe its unique role within the HSPB family in activating autophagy (Carra et al., 2009;Vos et al., 2010) may be important. Autophagy may prevent protein aggregate formation that served as an early trigger for RhoA activation. Indeed, preventing aggregate formation has been suggested as the mode by which HSPB8 can prevent desmin-related cardiomyopathy (Sanbe et al., 2009).

The present study demonstrates that RhoA activation plays a central role in tachypacing-induced myocyte remodeling. This remodeling can be prevented by some, but not all, members of the HSPB family. This protection is not directly related to canonical chaperone-like function of these HSPB members, but involves prevention of RhoA activation (HSPB8) or its downstream action on actin remodeling (HSPB1, HSPB6, HSPB7). The findings widen the possibilities for the identification of novel therapeutic approaches directed at RhoA activating components or boosting the expression of one or more of the cardioprotective HSPB members.

Table 1 characteristics of small HSPB members (Vos, 2009;Vos et al., 2011)

Gene Name	Protein Name	Alternative Name	Sequence Identity	Molecular Size (kDa)	Heat Inducibility	Expression in Heart	Other tissue expression
HSPB1	HSPB1	Hsp25, HSP27, HSP28	100%	22.783	Yes	+++	Uterus, skin, platelets, brain, kidney, some tumor cells
HSPB2	HSPB2	MKBP	36%	20.233	No	+	Skeletal muscle
HSPB3	HSPB3	HSPL27	23%	16.966		+	Skeletal muscle
HSPB4	HSPB4	αA-crystallin, CRYAA, CRYA1	36%	19.909	No	-	Lens of eye, spleen
HSPB5	HSPB5	αB-crystallin, CRYAB, CRYA2	38%	20.159	Yes	++++	Lens of eye, vascular wall cells, lung, kidney, brain, some tumor cells
HSPB6	HSPB6	Hsp20, p20	34%	17.136	No	++	Skeletal muscle, stomach, liver, lung, kidney, platelet
HSPB7	HSPB7	cvHsp	20%	18.611	?	++++++	Skeletal muscle
HSPB8	HSPB8	Hsp22, H11	34%	21.604	Yes	++	Skeletal muscle, stomach, liver, lung, kidney, brain
HSPB9	HSPB9	FLJ27437	19%	17.486	?	-	Testis
HSPB10	HSPB10	ODF1	17%	28.366	?	-	Testis

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Supplemental information

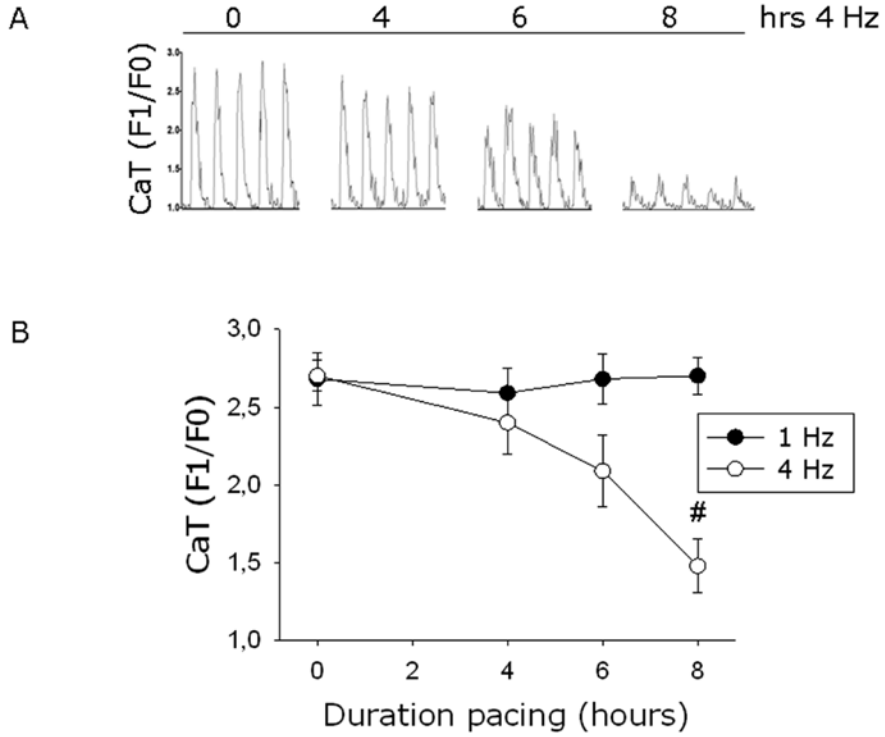

Figure S1 ***Tachypacing induces a progressive reduction in CaT.***
A Original recordings of CaT in 1 myocyte each for a time period as indicated.
B Mean CaT data in myocytes tachypaced at 4 Hz or normal paced at 1Hz. #$P<0.001$

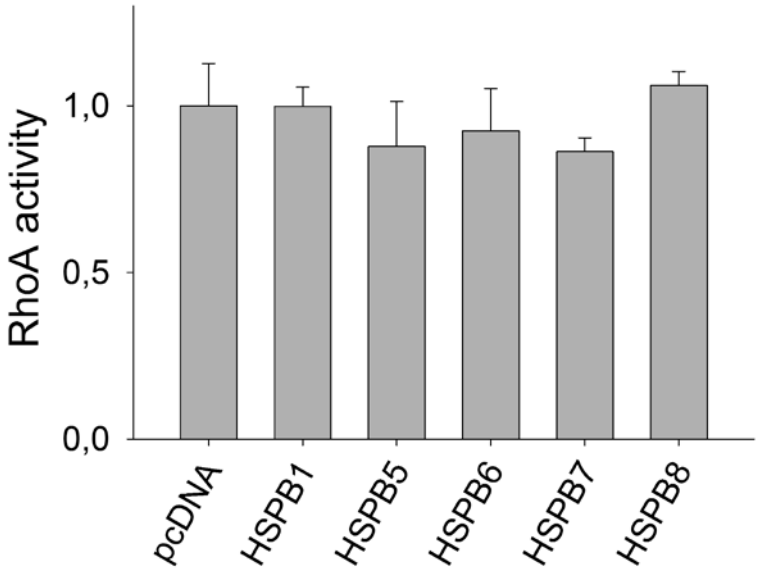

Figure S2 ***No effect of HSPBs on activation of RhoA-GTPase in normal paced HL-1 myocytes.***
HL-1 myocytes were transfected with HSPB1, HSPB5, HSPB6, HSPB7, HSPB8, or empty plasmid (pcDNA) and subjected to normal pacing (1Hz). Activation of RhoA-GTPase was determined by G-LISA.

Chapter 3

RhoA activation abrogates the HSF1-dependent heat shock response

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Abstract

Background

The heat shock response (HSR) is an ancient and highly conserved program of stress-induced gene expression, aimed at reestablishing protein homeostasis to preserve cellular fitness. Cells that fail to activate or maintain this protective response are hypersensitive to proteotoxic stress. The heat shock transcription factor1 (HSF1) is considered as the main regulator of the HSR via binding to conserved heat shock elements (HSE) in the promoter region of heat shock genes. Recently we observed that hyperactivation of RhoA conditions cardiomyocytes for Atrial Fibrillation. In this condition, the HSR was annihilated, yet both pharmacological and genetic induction of the HSR displayed cardioprotective effects. Therefore we hypothesized active RhoA to suppress the HSR.

Methods and Results

Stimulation of RhoA activity, either by calpeptin or RhoA overexpression significantly suppressed the stress-induced HSR in HL-1 cardiomyocytes as determined with a luciferase reporter construct driven by the HSF1 regulated human Hsp70 (HSPA1A) promoter (HSPA1A-luc) and Western blot. Conversely, RhoA inhibition by overexpression of C3T induced the HSR in the absence of exogenous stress and enhanced the HSR in stressed cardiomyocytes. In contrast, pharmacological inhibition with C3T resulted in a HSR comparable to the control. While active RhoA did not preclude HSF1 phosphorylation and translocation to the nucleus upon heat shock, it impaired binding of HSF1 to the *hsp* genes promoter element, the heat shock element (HSE), as determined by Electrophoretic Mobility Shift Assay (EMSA). Ultimately, this results in suppression of HSP expression and enhanced cell death.

Conclusion

RhoA activation suppressed the stress-induced HSR in HL-1 cardiomyocytes by impairing the HSE-binding capability of HSF1. Loss of the protective HSR subsequently sensitizes HL-1 cardiomyocytes to stress-induced cell death. Collectively, these data disclose RhoA activation as a novel pathway suppressing the HSR in stressed cardiomyocytes.

Introduction

The heat shock response (HSR) is one of the main pro-survival stress responses of the cell, restoring cellular homeostasis after exposure to proteotoxic stress stimuli, including heat shock, oxidative stress, heavy metal exposure and inhibition of the proteasome (Akerfelt et al., 2010;Anckar and Sistonen, 2011;Morimoto, 2011). The primary pro-survival mechanism of the HSR is the induction of heat shock proteins (HSPs) expression, which acts as molecular chaperones that assist in the refolding and degradation of damaged proteins (Kampinga and Craig, 2010;Morimoto, 2011). Heat shock transcription factor 1 (HSF1) activity is the main factor governing the HSR through the induction of HSP expression (Kline and Morimoto, 1997;Westerheide et al., 2009;Anckar and Sistonen, 2011). HSF1 activation is a multistep process requiring the release of monomeric HSF1 from its inhibitory chaperones, including HSPCA, HSPA1A and DNAJB1 (Akerfelt et al., 2010), conversion to a DNA-binding competent trimer, nuclear translocation, post-translational modifications and subsequent binding to the heat shock element (HSE) within the promoter region of *hsp* genes (Anckar and Sistonen, 2011). Extensive posttranslational regulation fine-tunes the HSF1 activity and comprises of phosphorylation, acetylation and sumoylation (Soncin et al., 2003;Guettouche et al., 2005;Anckar et al., 2006;Hietakangas et al., 2006;Westerheide et al., 2009;Anckar and Sistonen, 2011). Failure to mount an adequate HSR underlies hypersensitivity to proteotoxic stress and have been associated with disease progression in age-related protein aggregation diseases, such as Huntington's, Alzheimer and Parkinson's disease, and longevity (Anckar and Sistonen, 2011;Morimoto, 2011). Atrial fibrillation (AF) represents another age-related progressive disease in which cardiac cells fail to mount an adequate HSR in response to stress caused by rapid electrical stimulation (Brundel et al., 2006a). While rapid electrical stimulation of cardiomyocytes results in derailment of proteostasis and cell death (Kim et al., 2005;Brundel et al., 2006a;Li et al., 2009;Xu et al., 2013), induction of a proper HSR in these cells fully attenuates the associated changes (Brundel et al., 2006a;Brundel et al., 2006b;Ke et al., 2011). Although altered HSF-1 acetylation and sumoylation have been identified as possible regulators of HSF-1 activity (Hietakangas et al., 2003;Anckar et al., 2006;Hietakangas et al., 2006;Anckar and Sistonen, 2011), the identity of signaling pathways that cause suppression of the HSR (with aging) have remained elusive so far.

In AF, one of the major stress signaling pathways involved in disease progression is RhoA (Brown et al., 2006;Adam et al., 2007;Ke et al., 2011). The role of RhoA signaling on the HSR has never been investigated. The activation of RhoA is controlled by three classes of regulatory proteins, GTPase-activating proteins (GAPs), guanine nucleotide dissociation inhibitors (GDIs), and guanine nucleotide exchange factors (GEFs). GAPs and GDIs inactivate RhoA by promoting the GDP-

bound state and GEFs activate RhoA by stimulating the exchange of GDP for GTP. RhoA signaling, primarily through its downstream effector, RhoA kinase (ROCK), regulates a wide variety of cellular functions, including cytoskeleton reorganization, cell cycle progression, gene expression as well as apoptosis (Coleman et al., 2004;Jaffe and Hall, 2005;Neisch et al., 2010;Tsai and Wei, 2010). As we previously observed cardioprotective effects of small HSPB family members by attenuating the RhoA signaling (Ke et al., 2011), we hypothesized that RhoA signaling negatively regulates stress-induced HSF1 transcriptional activity. Here we show that active RhoA is a dominant suppressor of the HSR by impairing the HSF1 binding to the HSE, ultimately resulting in the inhibition of stress-induced HSP expression and enhanced death after proteotoxic stress.

Materials and methods

2.1 HL-1 atrial cardiomyocyte culture

HL-1 adult mouse-derived atrial cardiomyocytes were obtained from Dr. William Claycomb as described before (Brundel et al., 2006a). The cardiomyocytes were maintained in complete Claycomb medium (JRH, UK) supplemented with 100 mM norepinephrine (Sigma, The Netherlands), 0.3 mM L-ascorbic acid (Sigma, The Netherlands), 4 mM L-glutamine (Gibco, The Netherlands) and 10% FBS (Sigma, The Netherlands). They were cultured on 12.5 mg/ml fibronectin (Sigma, The Netherlands) and 0.02% gelatine (Sigma, The Netherlands) coated surfaces, in a 5 % CO_2 atmosphere at 37°C.

2.2 Constructs

Constructs used in this study are, pcDNA3.1+ (empty plasmid, Invitrogen) and the reporter plasmids, pSRE-luc, to monitor RhoA activity (Dr. Wieland, (Moepps et al., 2008)), or pGL3-HSPA1A-luciferase, to monitor HSPA1A expression (Dr. Kampinga, University Medical Center Groningen). pCDNA5-FRT-TO-RhoA-WT (wild type) and pCDNA5-FRT-TO-C3T (C3-transferase, C3-exoenzyme), were constructed by PCR of the RhoA-WT and C3-exoenzyme coding sequences from pRK5-RhoA and pEF-myc-C3T (Schmidt et al., 1999), (Dr. Schmidt, University Medical Center Groningen). Primers used are C3T fw; GAGGACCTGGGATCCTCTAG, C3T rv; CGGCTCGCCGGCCGCTCATTGCCATATATTGGGTATAAATAGC, RhoA fw; GACCTGGGATCCATGGCTGCCATCCGGAAGAAAC, RhoA rev; AAATATCGCGGCCGCTCACAAGACAAGGCACCCAG. Coding sequences and pCDNA5-FRT-TO plasmids were digested with BamHI and NotI and subsequently ligated to obtain pCDNA5-FRT-TO-RhoWT and pCDNA5-FRT-TO-C3T respectively.

2.3 Antibodies, chemical compounds and transfection reagent

Antibodies used in this study are, HSPA1A (Stressgen, USA), HSF1 (Cell Signaling Technology, USA), eIF2α (Abcam, UK), Acetylated-lysine (Cell Signaling Technology, USA), cleaved caspase-3 (#9661 cell signaling), eIF2α-S51P (Cell Signaling Technology, USA) or GAPDH (Fitzgerald industries international, USA), Horseradish peroxidase-conjugated anti-mouse, anti-rabbit (Santa-Cruz Biotechnology, The Netherlands) and anti-goat (Dako Cytomation, Denmark). Reagents used in this study are C3-exoenzyme (RhoA inhibitor I, Cytoskeleton, USA) and RhoA activator, calpeptin (Cytoskeleton, USA), MG-132 (M7449, Sigma Aldrich), PD15606, calpain inhibitor (Calbiochem). Transient (co)-transfections were performed by the use of Lipofectamin 2000 (Life technologies, The Netherlands).

2.4 Heat shock and compound treatment

Heat shock was induced by submersion of cells in a water bath at 45ºC for 10 minutes. RhoA activity was modulated by treatment of myocytes with a cell permeable C3-exoenzyme (RhoA inhibitor I, Cytoskeleton, USA) and RhoA activator, Calpeptin (Cytoskeleton, USA) according to manufacturer's instructions. Briefly, drugs were dissolved in 50% v/v glycerol/demi for the RhoA inhibitor I (C3T) and DMSO for the RhoA activator calpeptin. Cells were serum deprived on 1% FBS supplemented Claycomb medium for 16h and subsequent serum deprivation on 0% FBS supplemented Claycomb medium for 24h. Cells were treated with RhoA inhibitor I, 1µg/ml for 4h and calpeptin, 1U/ml for 20 min, to induce RhoA activity. ROCK inhibition was achieved by Y27632 treatment; 10 µM for 16h (Sigma, The Netherlands). Proteasome and calpain inhibition was achieved by MG-132; 10uM and 50uM for 20 min. Calpain inhibition was achieved by PD15606 (20uM) pre-treatment for 1h. In case of heat shock treatment, heat shock was applied during the last 10 minutes of compound treatment. After heat shock, cells received fresh serum free medium and were harvested at the below indicated recovery periods. Cardiomyocytes that were used for HSF1 acetylation, translocation and DNA binding experiments were harvested after a 10 min recovery period after heat shock, whereas cells used for cleaved caspase-3 levels were harvested after a 2h recovery period and cells used for HSP expression (protein and mRNA) were harvested after a 4h recovery period.

2.5 Luciferase assay

Luciferase assays were performed 48 hours after transfection and 4h after HS. HL-1 myocytes were lysed and scraped in BLUC (25 mM Tris/H_3PO_4 (pH 7.8), 10 mM $MgCl_2$, 1% (v/v) Triton X-100, 15% glycerol and 1 mM EDTA). Luciferase activity in the samples was measured for 10 s after injecting the substrate buffer (BRLUC, 1.25 mM ATP and 0.087 mg/ml D-luciferin) in a Wallac 1420 Victor3 V plate reader.

2.6 G-LISA RhoA activity measurement

For the quantitative analysis of active RhoA GTP levels, GLISA RhoA Activation Assay (Cytoskeleton, USA) was performed according to the manufacturer's instructions. Briefly, after drug treatment, myocytes were harvested in Rho-GLISA lysis buffer (supplied). After measurement of the protein concentration with the use of Precision Red (supplied), equal amounts of protein were incubated in RhoA-GTP affinity plates. The amount of bound RhoA-GTP was detected by using primary anti-RhoA antibody (supplied) and secondary HRP-labeled antibody (supplied). Subsequently, samples were incubated with HRP detection reagent for 15 min after which a HRP stop buffer was added (supplied). Colorimetric detection at 490 nm was performed immediately in a BioRad Benchmark plus microplate-reader (BioRad, The Netherlands).

2.7 Isolation of cytosolic and nuclear fractions

Cytosolic and nuclear fractions were obtained by harvesting the cardiomyocytes in membrane lysis buffer (10mM Hepes pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1% v/v Igepal-CA630). After centrifugation, the supernatant (cytosolic fraction) was transferred to an eppendorf tube and the pellet is resuspended in nuclear envelope lysis buffer (20 mM Hepes pH 8.0, 1.5 mM $MgCl_2$, 25% v/v glycerol, 0.42 M NaCl, 0.2 mM EDTA, 1mM DTT) to obtain the nuclear fraction.

2.8 Protein-extraction and Western blot analysis

Standard protein-extraction was performed with RIPA lysis buffer. Western-blot analysis was performed as described previously (Brundel et al., 2006a). Briefly, equal amounts of protein in SDS-PAGE sample buffer were homogenized by use of a 26G needle and syringe, before separation on 4-20% PAA-SDS gels (Thermo Scientific, USA). After transfer to nitrocellulose membranes (Stratagene, The Netherlands), membranes were incubated with primary antibodies and subsequently Horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat was used as secondary antibody depending on the origin of the primary antibody. Signals were detected by the SuperSignal-detection method (Thermo Scientific, USA) and quantified by densitometry (GeneGnome/GeneTools from SynGene, USA).

2.9 Immunofluorescent staining and confocal analysis

Myocytes were grown on fibronectin 12.5 mg/ml fibronectin (Sigma) and 0.02% gelatine (Sigma, The Netherlands) coated glass coverslips. After drug treatment as described above, myocytes were fixated with 4% paraformaldehyde for 15 minutes, washed three times with Phosphate-Buffered Saline (PBS), and blocked and permeabilized for 60 min in 5% BSA, 0.3% Triton X-100 in PBS. Samples were subsequently incubated

overnight with HSF1 antibody 1:100 (Cell Signaling Technology, USA) in 1% BSA, 0.3% Triton X-100 in PBS. Fluorescein labeled isothiocyanate (FITC) anti-rabbit (Jackson ImmunoResearch, The Netherlands) was used as secondary antibody 1:200 in combination with TOTO-3 iodide, 2 µM (Life technologies, The Netherlands) as a nuclear counterstain. Cells were mounted in Vectashield without DAPI (Vector Laboratories) and analyzed using an AOBS Leica confocal microscope.

2.10 Quantitative Real Time-PCR analysis

Total RNA from HL1 myocytes was extracted using the RNA extraction kit Nucleospin II (Machery-Nagel, Germany). cDNA synthesis was performed according to standard methods. Briefly, first strand cDNA was synthesized using random primer mix (Promega, USA) and subsequently used (1 µg per reaction) as a template for quantitative real-time reverse-transcriptase PCR (qRT-PCR). All mRNA levels were expressed in relative units on the basis of a standard curve (serial dilutions of a calibrator cDNA mixture). All PCR results were normalized against GAPDH. All reactions were made in triplicates with samples derived from three biological repeats. The sequences for the primers were as follows; sense CATCAAGAAGGTGGTGAAGC antisense ACCACCCTGTTGCTGTAG for HSPA1A, sense GCAAGGAGAAGCAGCAGAGT antisense TTTGTGTTTGGACTCTCCCC for GAPDH, sense ATCTTTGGTTGCTTGTCGCT antisense ATGAAGGAGACTGCTGAGGC for HSPA5, sense TGTATTTCCGGGTGAAGCAC antisense CAGTGAAGACCAAGGAAGGC for HSPB1, sense TGACTTTGCAACAGTGACCC antisense GCTGTAGCTGTTACAATGGGG for HSPD1, sense TCCGTGGAATGTGTAGCTGA antisense GATTTTCGACCGCTATGGAG for DNAJB1, sense ATTGGTTGGTCTTGGGTCTG antisense GCCAGTTGCTTCAGTGTCCT for HSPCA.

2.11 Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed according to manufacturer's instructions of the HSE EMSA kit (Panomics AY1020P). In short, equal amounts of nuclear fractions (4 µg) of heat shocked HL-1 cardiomyocytes with or without calpeptin and C3-exoenzyme treatment were obtained as described above. Transcription factor-DNA-probe complexes were allowed to form by incubating the nuclear extracts with a DNA-probe. The DNA probe consisting of a biotin labelled HSE probe (Heat shock consensus element CTGGAATTTTCCTAGA) or an unlabeled HSE probe (cold probe). As a positive control, nuclear extract prepared from HeLa cell line was used (supplied). Samples were subsequently separated on a 6% non-denaturating polyacrylamide gel and transferred to a positively charged Nylon membrane (Amersham, UK). Proteins were cross-linked by UV crosslinker (Stratagene). After blocking and washing of the membrane in the supplied buffers, the membrane was incubated with a streptavidin-HRP mixture. The HSF1-HSE complexes were detected by applying the

provided detection buffer and subsequent detection with a chemiluminscent imaging system (GeneGnome/GeneTools from SynGene, USA). For control and calpeptin treated cells, a competition assay with unlabeled (cold) probe was performed as control for binding specificity. In both cases addition of the cold probe attenuated the intensity of the observed band, thereby indicating specific binding to the HSE-probe.

2.12 Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was performed by student's t-test or one-way ANOVA with post hoc least significant difference (SPSS 16.0 for Windows), with $P < 0.05$ (two-sided) considered significantly different.

Results

3.1 Active RhoA suppresses HSP expression.

To determine if RhoA signaling affects the HSR, control and heat shocked (HS) HL-1 cardiomyocytes were transfected with a luciferase reporter construct driven by the HSF-1 regulated human Hsp70 (HSPA1A) promoter (HSPA1A-luc). RhoA activity was inhibited by expression of the C3-exoenzyme (pC3T) (Schmidt et al., 1999) or activated by transfection of a RhoA wild type construct (pRhoA) (Schmidt et al., 1999). The effectiveness of these manipulations was validated using co-transfection with a luciferase construct driven by the RhoA-dependent serum response element (SRE) (Figure 1A). pRhoA expression reduced both basal HSPA1A-luc (Figure 1B) and also strongly suppressed the heat-induced increase in HSPA1A-luc expression (Figure 1C). Despite the fact that transient transfection only reached a maximal efficiency of ~ 20%, pRhoA also significantly reduced the level of endogenous HSPA1A protein expression in the pooled population of heat-shocked cells (Figure 1D). In addition, constitutive RhoA activation by RhoA-v14 reduced HSPA1A-luc expression, while it was enhanced by RhoA inhibition in cells transfected with P190RhoGAP or dominant negative RhoA-n19 (supplemental data Figure S1). In line, activation of the RhoA pathway by calpeptin (Calp) treatment (1 U/ml) as determined by increased RhoA-GTP levels using a RhoA-GLISA assay (Figure 1E), strongly reduced basal HSPA1A-luc expression by 80% (Figure 1F) and completely suppressed endogenous HSPA1A expression levels after HS (Figure 1G). Conversely, inhibition of the RhoA pathway by transient transfection of C3T (pC3T, Figure 1A) almost doubled the HSPA1A-luc activity in unstressed cells (Figure 1B) and strongly enhanced activation of the HSPA1A promoter after heat shock (Figure 1C). Pharmacological inhibition by C3T (1µg/ml) did not result in changes in HSPA1A expression as compared to the control or heat shocked control respectively (Figure 1F, G). The findings suggest that the HSR is conserved when RhoA is inhibited and impaired when RhoA is activated. Finally, also in human HEK-293 kidney cells, activation of RhoA reduced HSPA1A-luc expression, while it was strongly activated by inhibition of RhoA with C3T (supplemental data Figure S2), demonstrating that the observed effects are not limited to HL-1 cardiomyocytes. Together, the findings strongly indicate active RhoA to significantly suppress the activity of the HSPA1A promoter both under normal growth conditions and upon heat stress.

Next, we investigated whether the major downstream effector of RhoA, ROCK, is mediating the suppression of the HSR by exploring the effects of its inhibitor, Y27632. Adequate inhibition of ROCK by Y27632 was verified by the SRE-luciferase reporter (Figure 2A). Notably, Y27632 boosted basal HSA1A-luc expression as well as heat shock induced HSPA1A expression comparable to C3T (Figure 2B.C). Induction of HSPA1A expression by C3T

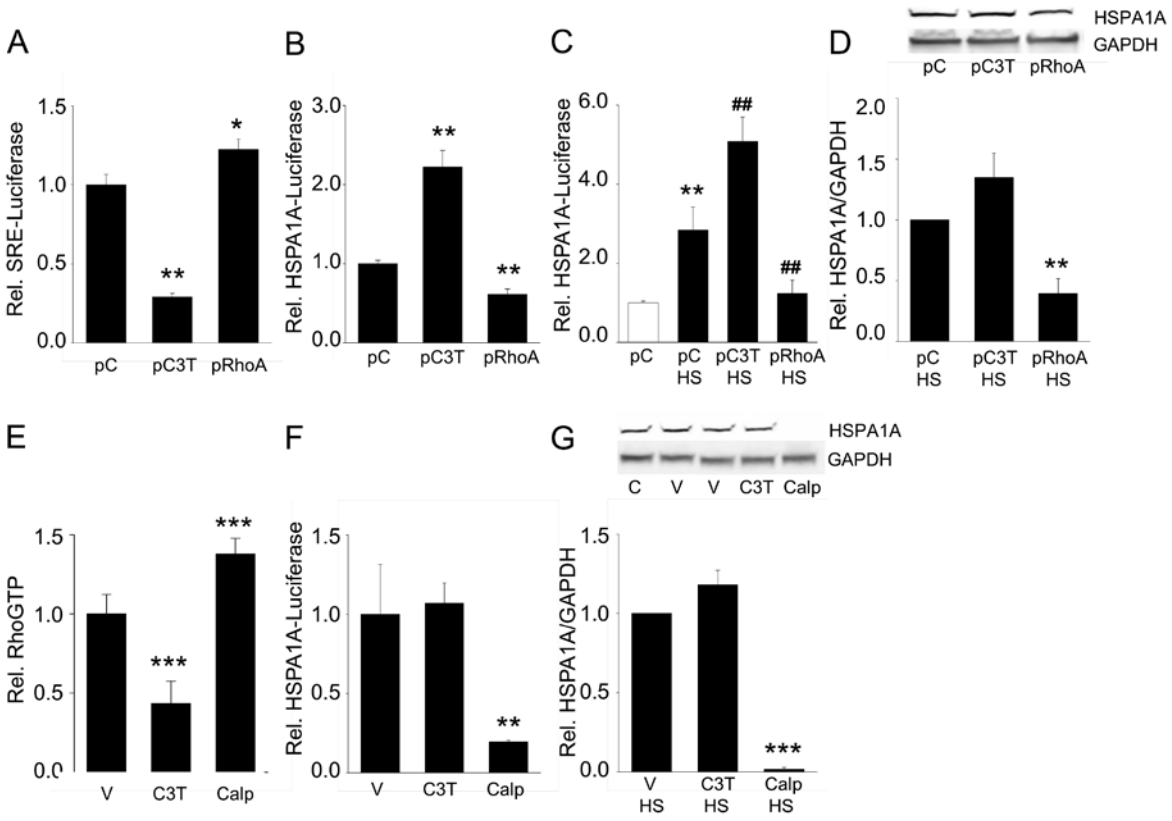

Figure 1 RhoA activation attenuates HSPA1A expression.
A Relative luciferase expression of a reporter construct driven by the SRE promoter (downstream target of RhoA/ROCK signaling) in cells transfected with empty plasmid (pC), C3-Transferase encoding plasmid (pC3T) or RhoA-WT encoding plasmid (pRhoA). **B** Relative luciferase expression of a reporter construct driven by the HSPA1A promoter in cells transfected with pC, pC3T or pRhoA. **C** Relative HSPA1A-luc expression in cells transfected with pC, pC3T or pRhoA and subjected to a HS (45ºC, 10 min). **D** Top panel shows a representative Western Blot with HSPA1A levels of cells transfected with pC, pC3T or pRhoA and subjected to a HS. Below, quantified data of HSPA1A/GAPDH levels for conditions as indicated. **E** Relative Rho-GTP levels as determined by Rho GLISA in cells treated with DMSO (V), a cell permeable C3T or calpeptin (Calp). **F** Relative HSPA1A-luc expression in cells treated with DMSO (V), C3T or Calp. **G** Top panel shows a representative Western Blot with HSPA1A levels of control cells (C), cells treated with DMSO (V), Glycerol (V), C3T, Calp and subjected to a HS. Below, quantified data of HSPA1A/GAPDH levels for conditions as indicated**.** White bars represent control non-HS cells, whereas black bars represent HS cells. * P<0.05, **P<0.01 compared to control pC or V and ## P<0.05 compared to control HS.

was not further enhanced by Y27632 treatment. Importantly, however Y27632 did not affect the calpeptin-induced suppression of HSPA1A expression, indicating that the calpeptin-induced suppression of HSP expression is independent of ROCK activity.

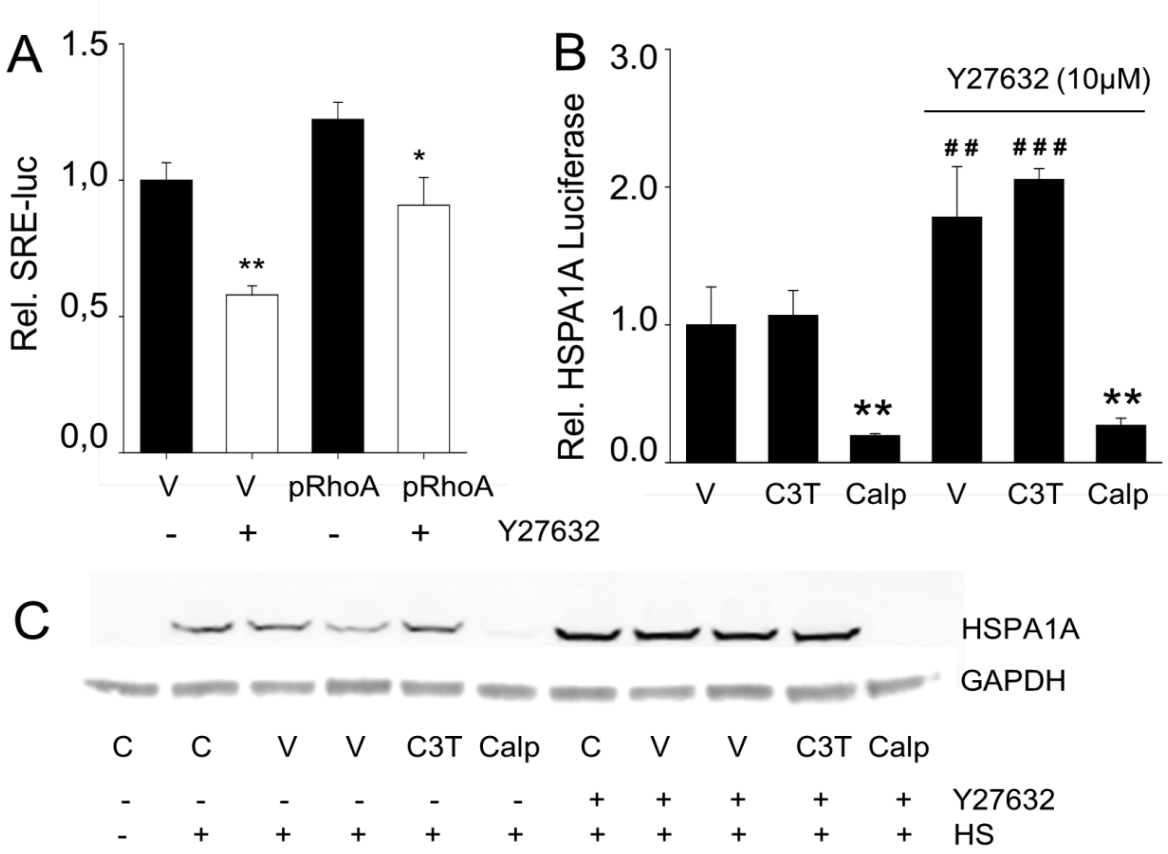

Figure 2 Suppression of the HSR is independent of RhoA's downstream effector ROCK.
A Relative SRE-luciferase expression in cells treated with DMSO (V) or calpeptin (Calp) with or without ROCK inhibitor Y27632. **B** Relative HSPA1A-luc expression in cells for the conditions as indicated. **C** Representative Western Blot of HSPA1A expression for the conditions as indicated. **P<0.01 compared to control and $^{\#\#}$ P<0.01 $^{\#\#\#}$ P<0.001 compared to control HS.

To further examine if active RhoA indeed affects the general HSR, mRNA levels of various endogenous HSP members, including HSPA1A, HSPCA, HSPB1, DNAJB1, HSPD1 and HSPA5, were determined by quantitative PCR (Figure 3). In control cells, modulation of RhoA by C3T or calpeptin revealed no effects on the expression of any of the HSP mRNAs. However active RhoA completely suppressed the induction of HSPA1A, HSPCA, HSPB1, DNAJB1 and HSPD1 mRNA upon heat shock. As a control we show that active RhoA did not affect the expression of the ER-resident Hsp70 family member HSPA5 (Figure 3F), consistent with it being a HSP of which the regulation is independent of HSF1 (Heldens et al., 2011). These results suggest that upon proteotoxic stress, active RhoA suppresses the HSR via inhibition of HSF1 dependent transcription of HSPs.

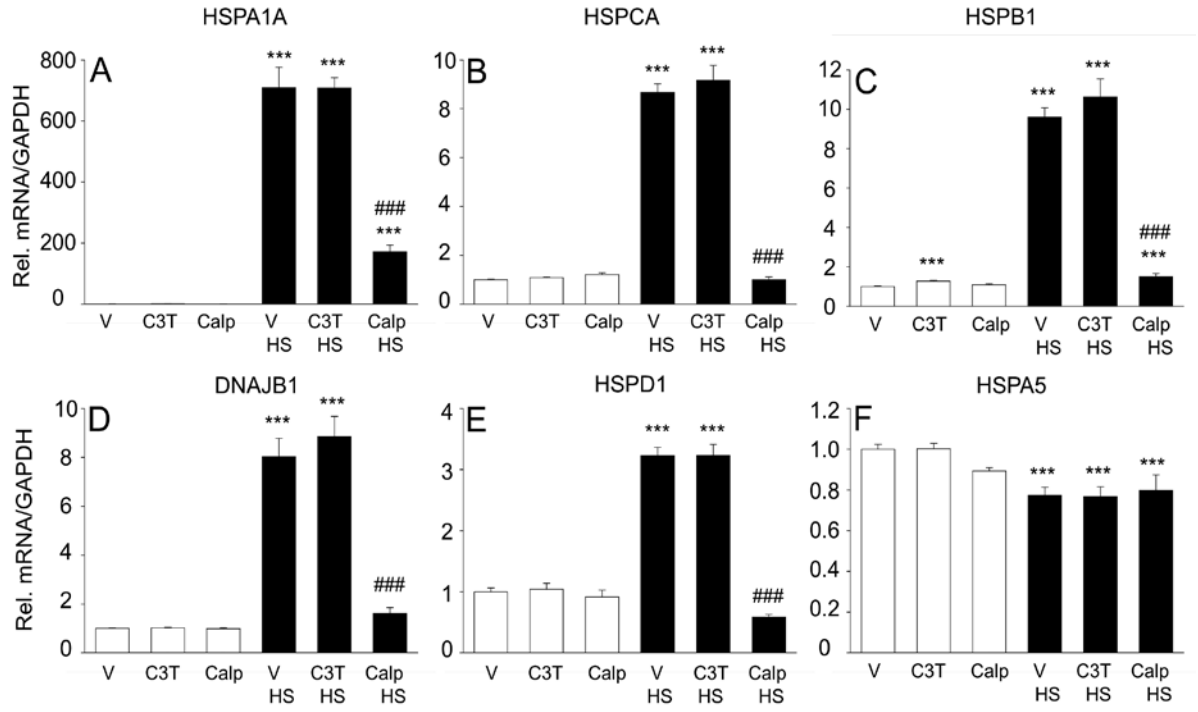

Figure 3 RhoA activation attenuates expression of multiple HSP family members.
Cells were treated with DMSO (V), C3T or RhoA with or without HS (45ºC 10 min) and mRNA levels of **A** HSPA1A **B** HSPCA, **C** HSPB1 **D** DNAJB1, **E** HSPD1 **F** HSPA5 were determined by qPCR. White bars represent non-HS cells, whereas black bars represent HS cells. *** $P<0.001$, compared to control (V) and ### $P<0.001$ compared to control (V) HS.

Although calpeptin activates RhoA (Schoenwaelder and Burridge, 1999), a (weak) effect on proteasome inhibition (Giguere and Schnellmann, 2008) as well as calpain inhibition was previously observed (Figueiredo-Pereira et al., 1994;Pinter et al., 1994). To ensure that the actions of calpeptin were indeed due to activation of RhoA, the effect of MG-132 (10 and 50µM), an inhibitor of the proteasome and PD150606 (20µM), a calpain inhibitor (Figueiredo-Pereira et al., 1994;Lee and Goldberg, 1998) on the HSR was determined. Both, PD150606 and MG-132 (10µM) did not suppress the HS-induced expression of HSPA1A (supplemental Figure S3A, B). Furthermore, MG-132 for prolonged incubation (4h) induced HSPA1A expression under non-heat shocked conditions (supplemental Figure S3B) as previously reported (Bush et al., 1997). Only at the higher MG-132 dose of 50µM a strong decrease in protein levels of HSPA1A was observed (supplemental Figure S3B). However, the opposite effect was observed on mRNA levels of HSPA1A and other HSPs (supplemental Figure S4), demonstrating the effect of the high dose of MG-132 on HSPA1A protein levels to be independent of inhibition of HSF1 transcriptional activity. Together, these results imply a specific effect of calpeptin via activation of RhoA and subsequent suppression of the HSR.

3.2 RhoA suppression results in a stronger damage response and sensitizes HL-1 cardiomyocytes to stress-induced cell death.

To examine the consequence of active RhoA on cellular stress sensitivity, cells were subjected to a HS (45ºC, 10 min) and trypan blue uptake was measured 4h after HS (Figure 4A). In control cells, activation of RhoA by calpeptin also resulted in a small, but significant, increase in cell death (Figure 4A). However, calpeptin treatment grossly enhanced cell death in heat shocked cells (Figure 4A). In parallel, RhoA activation by calpeptin significantly enhanced caspase-3 cleavage in heat shocked cells (Figure 4B). Further, calpeptin treated cells showed a hyperphosphorylation of eIF2alpha (Supplemental Figure 3C), indicative of a stronger damage response with a permanent arrest of protein translation (Aarti et al., 2010;Bevilacqua et al., 2010).

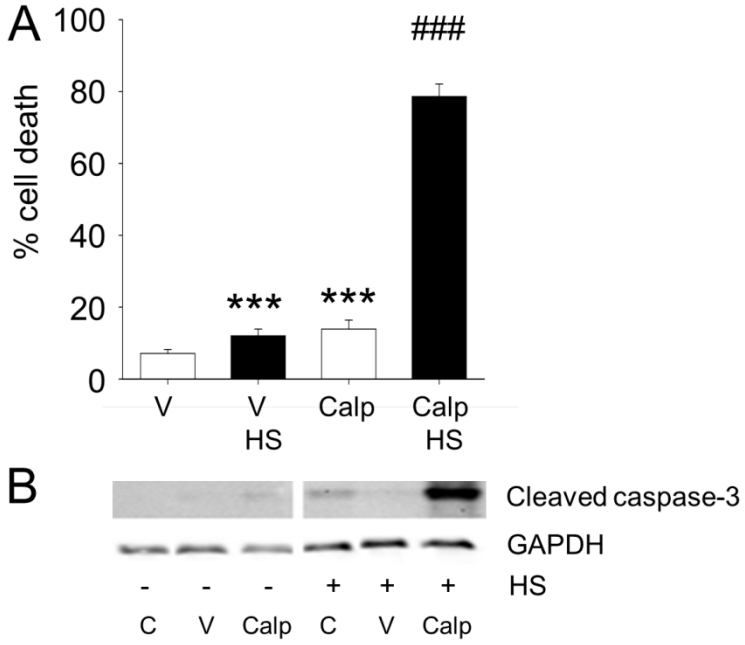

Figure 3 RhoA-induced suppression of the HSR decreases cell stress resistance and induces cell death via apoptosis
A Percentage trypan blue positive cells treated with DMSO (V) or calpeptin (Calp) with or without an HS (45ºC 10 min) with a 4h recovery period. **B** Representative Western Blot of cleaved-caspase 3 in control (C), DMSO (V) or Calp treated cells with or without an HS. **C** Representative Western Blot showing eIF2alpha 51S phosphorylation and eIF2alpha levels for conditions as indicated. White bars represent non-HS cells, whereas black bars represent HS cells. ***P<0.001 compared to control and ### P<0.001 compared to control HS.

3.3 RhoA impairs binding of HSF1 to the HSE.

As active RhoA suppresses the transcription of HSF1 regulated HSPs, we investigated its action on the main steps of HSF1 transcriptional activation, i.e. translocation to the nucleus and binding to HSE. In control cells, HSF1 was mainly located in the cytosol, and heat shock resulted in its translocation to the nucleus and the TX-100 nuclear-containing fraction (Fig. 5A, B). Activation or inhibition of RhoA by calpeptin or C3T respectively did not affect this heat shock-induced HSF1 translocation (Fig. 5A, B). Also, the heat shock-induced hyperphosphorylation, as evidenced by its decreased mobility on SDS-PAGE (Morimoto 1998), was unaffected by RhoA manipulations (Fig. 5B).

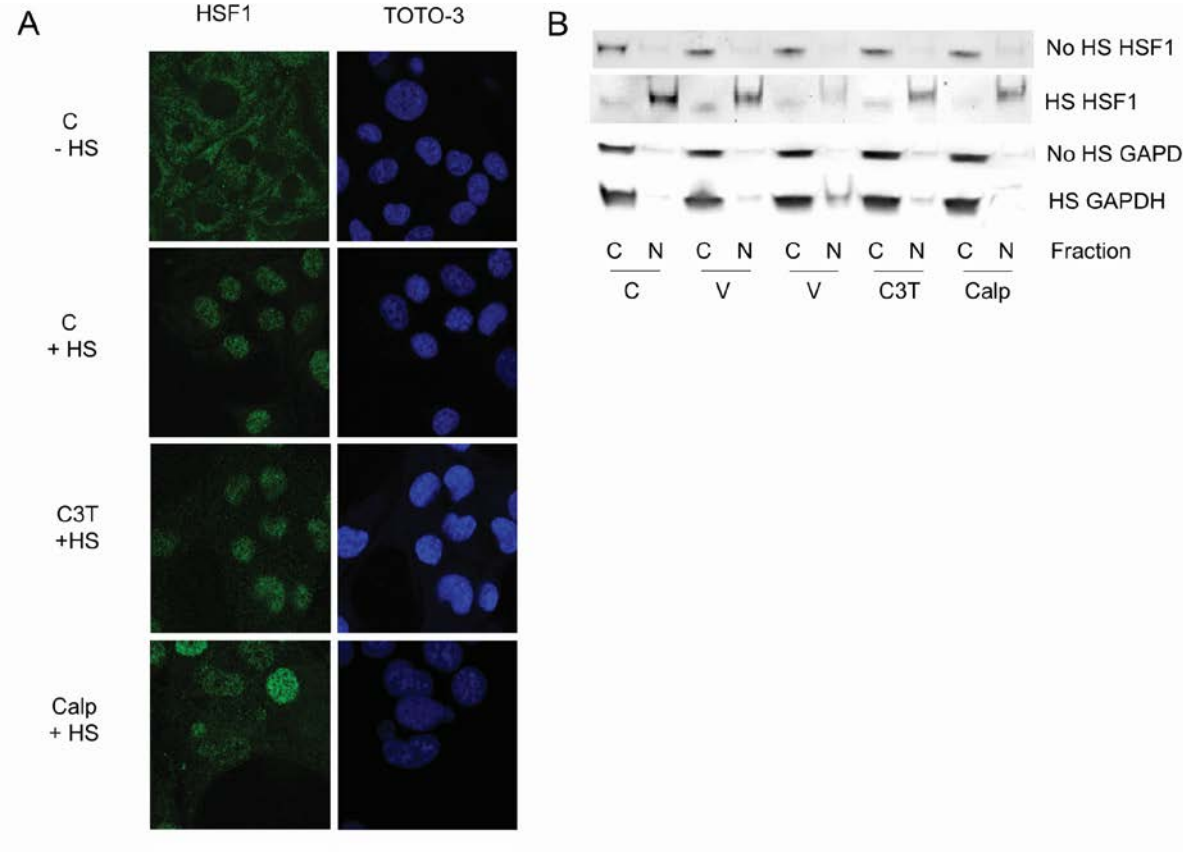

Figure 5 RhoA modulation does not affect nuclear translocation of HSF1 upon heat shock.
A Nuclear translocation of HSF1 in response to HS with and without RhoA modulation by C3T or calpeptin (Calp). **B** HSF1 levels in cytosolic and nuclear fractions of cells with and without HS and RhoA modulation by C3T or Calp.

Next, we asked whether the nuclear translocated HSF1 actually binds the HSE under conditions of Rho activation and heat shock. Hereto, we used a biotin labelled HSE-probe and isolated nuclear fractions from heat shocked cells treated with or without either calpeptin or C3T. HSF1 from heat shocked cells bound the labeled HSE-probe resulting in a HSE-mobility shift (upper band Figure 6, arrow) compared to the unbound HSE-probe (lower band Figure 6, arrow). The specificity of this binding was verified by addition of unlabeled HSE-probe (cold-probe), which reduced the signal-intensity of the upper-band and increased the intensity of the unlabeled probe (lower-band). Interestingly, binding of HSF1 to the labeled HSE-probe was attenuated by calpeptin treatment compared to control and C3T treated cells (Figure 6). Thus, whereas RhoA activation does not affect HSF-1 translocation and phosphorylation, it does affect the DNA binding affinity of HSF-1 after heat shock. Impairment of HSF1 binding to the HSE might explain the inhibitory effect of RhoA activation on HSP expression.

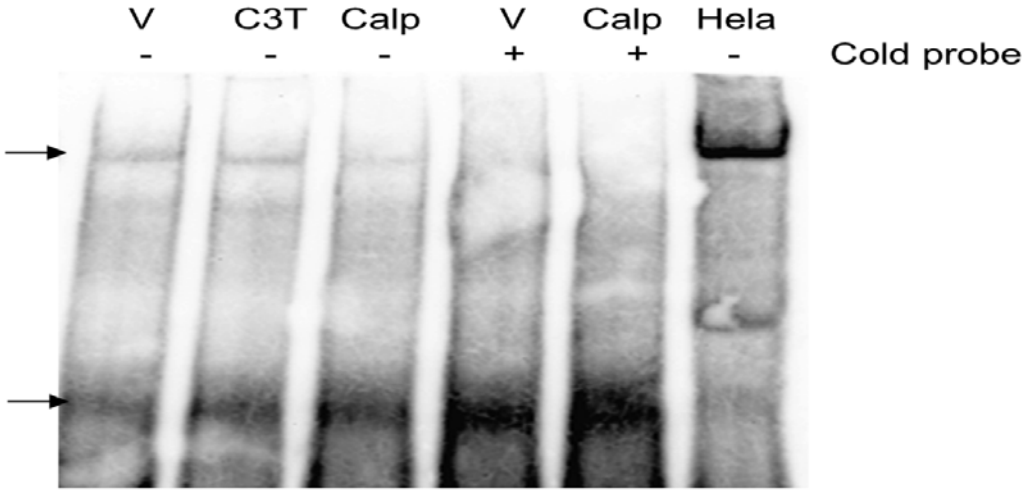

Figure 6 RhoA activation inhibits HSF1 transcriptional activity by inhibiting its ability to bind to HSE.
EMSA for HSF1 binding to the HSE in response to RhoA modulation and HS, with HeLa nuclear cell extract as a positive control and a competition-assay with a non-labeled HSE probe for DMSO (V) and calpeptin (Calp) treated cells, to determine specific binding to the HSE probe. Calpeptin attenuated HSF1-HSE binding compared to C3T and V.

Discussion

The current study shows that active RhoA acts as a dominant suppressor of the HSR by impairing the binding of HSF1 to the HSE in the promoter region of *hsp* genes. Our findings disclose a novel role for active RhoA as a suppressor of the HSR, inducing cellular hypersensitivity to stress.

4.1 Active RhoA suppresses the HSR and sensitizes cells for stress-induced cell death

The regulatory role of active RhoA results from the impaired HSF1 binding to the promoter regions of *hsp* genes. Notably, RhoA activation did not interfere with the translocation of the HSF1 to the nucleus, suggesting that a specific post-translational modification of HSF1 accounts for its loss of DNA binding capacity. Several studies suggest post-translational modifications of HSF1 to play a role in reducing HSF1's DNA binding and transcriptional activity in heat shocked cells (Sarge et al., 1993;Satyal et al., 1998;Anckar and Sistonen, 2011). Future studies should elucidate the exact nature of the post-translational modification of HSF1 elicited by RhoA activation. Furthermore, inhibition of the HSR by RhoA was unaffected by the ROCK inhibitor Y27632, suggesting the HSF1 modification to be independent of the canonical downstream RhoA pathway. Nevertheless, the efficacy of Y27632 still remains to be determined in combination with calpeptin to ensure sufficient inhibition under these conditions. Further, as sole ROCK inhibition by Y27632 results in the boosting of the HSR similar to C3T, we cannot completely exclude a role for ROCK in the regulation of HSF1 activity.

Failure to mount an adequate HSR due to RhoA activation underlies sensitization to heat shock-induced cell death and results in enhanced apoptosis in heat shocked HL-1 cardiomyocytes. RhoA is a known pleiotropic mediator of cardiomyocyte survival and apoptosis. Activation of RhoA can promote cardiomyocyte survival through activation of the FAK-PI3K-Akt signaling pathway, but prolonged activation will induce loss of protective responses and induce apoptosis (Del Re et al., 2007). In addition to this pleiotropic role in cardiomyocytes, published findings on the role of RhoA in apoptosis are conflicting and seem to be cell type specific and dependent on dose or duration of RhoA activation. RhoA activity has been reported to induce apoptosis in HEK293T cells (Navenot et al., 2009), Drosophila (Neisch et al., 2010), erythroblastic cell lines TF1 and D2 (Chang and Lee, 2006) and activation of the Gα12 or Gα13 receptors upstream of RhoA also induce apoptosis in COS-7 and HEK293 cells (Berestetskaya et al., 1998). In contrast, RhoA and/or ROCK inhibition was reported to induce apoptosis in gastric cancer cells (Xu et al., 2012), NIH3T3 cells (Calleros et al., 2006), HUVECs (Li et al., 2002), leukemia NB4 and Jurkat cells (Li et al., 2013). In the current study we observed RhoA activation to impair the HSR both in HL-1 cardiomyocytes

and HEK293 cells. While this suggests that inhibition of the HSR by RhoA represents a generalized mechanism, it remains to be determined in other cell types because of the pleiotropic role of RhoA.

RhoA mediated induction of apoptosis is thought to be mediated by activation of the JNK pathway (Calleros et al., 2006;Neisch et al., 2010;Tsai and Wei, 2010), inhibition of the ERK pathway (Li et al., 2013) and/or upregulation of the pro-apoptotic protein Bax (Del Re et al., 2007). Inhibition of HSF1 function could also contribute to its pro-apoptotic role. Interestingly, JNK is known to phosphorylate HSF1 at its transcriptional activation domain under conditions of severe stress and promote HSPA1A expression (Park and Liu, 2001). Furthermore, ERK1 was reported to phosphorylate HSF1 on serine 307 leading to secondary phosphorylation on serine 303 (Wang et al., 2003) which is a priming signal for sumoylation of HSF1 inhibiting HSF1 activity (Hietakangas et al., 2003;Anckar and Sistonen, 2011). Based on these findings, RhoA activation would be expected to rather enhance HSF1 function than impair its function as was observed in this study. However, as we did not determine the activation state of these pathways and as these may be dependent on the duration of RhoA activation, we cannot exclude an influence of these pathways on HSF1 function. Nevertheless, the currently most likely explanation is that HSF1 function is regulated by additional pathways downstream of RhoA. Together our data and those of previous studies illustrate the complex regulation of HSF1 and future studies should elucidate the molecular mechanism by which RhoA activation attenuates HSF1 DNA binding.

In summary, the current study shows a previously undisclosed role for active RhoA in the sensitization towards cell death by a HSF1 dependent inhibition of the HSR. While active RhoA did not preclude HSF1 translocation to the nucleus upon HS, it impaired HSF1 binding to the *hsp* promoter, resulting in suppression of HSP expression and subsequent sensitization to stress-induced apoptosis. These findings identify a novel role for active RhoA as suppressor of the HSR in stressed cells and disclose its prominent role in the decision between cell survival or cell death. Finally, the inhibition of RhoA in cardiomyocytes may have a therapeutical application in AF by enhancing the expression of cardioprotective HSPs.

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Supplemental Information

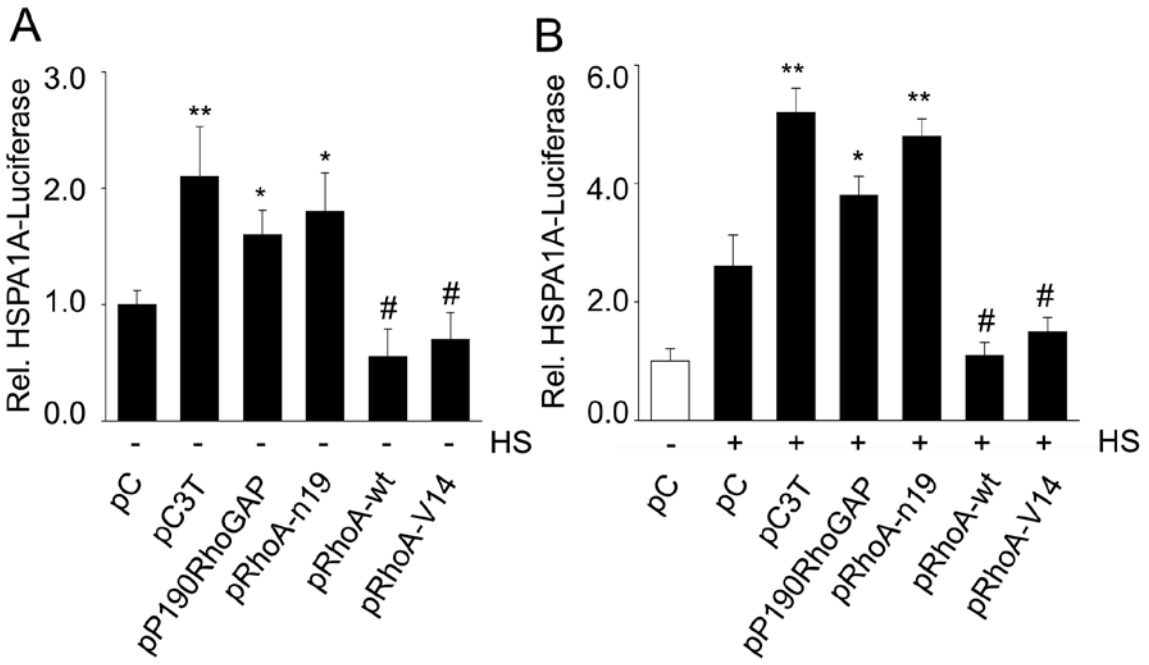

Figure S1 RhoA activation attenuates HSPA1A expression.
Relative luciferase expression of a reporter construct driven by the HSPA1A promoter in cells transfected with empty plasmid pC, pC3T, pP190RhoGAP, pRhoAn19, pRhoA or pRhoAv14 without **A** and with an HS **B**. White bar in panel **B** represents control non-HS cells, whereas black bars represent HS cells. * P<0.05, **P<0.01 compared to control pC and # P<0.05 compared to pC or pC HS.

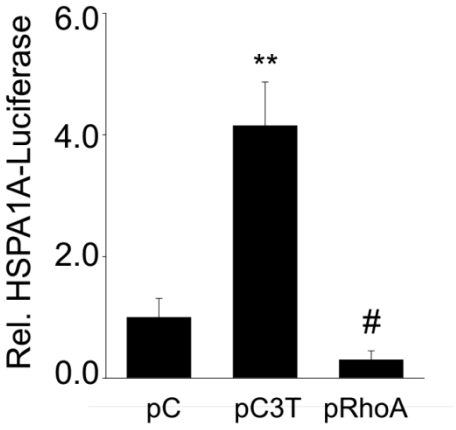

Figure S2 RhoA activation attenuates HSPA1A expression in human HEK293 cells.
Relative luciferase expression of a reporter construct driven by the HSPA1A promoter in cells transfected with empty plasmid pC, pC3T, or **P<0.01 compared to control pC and # P<0.05 compared to pC.

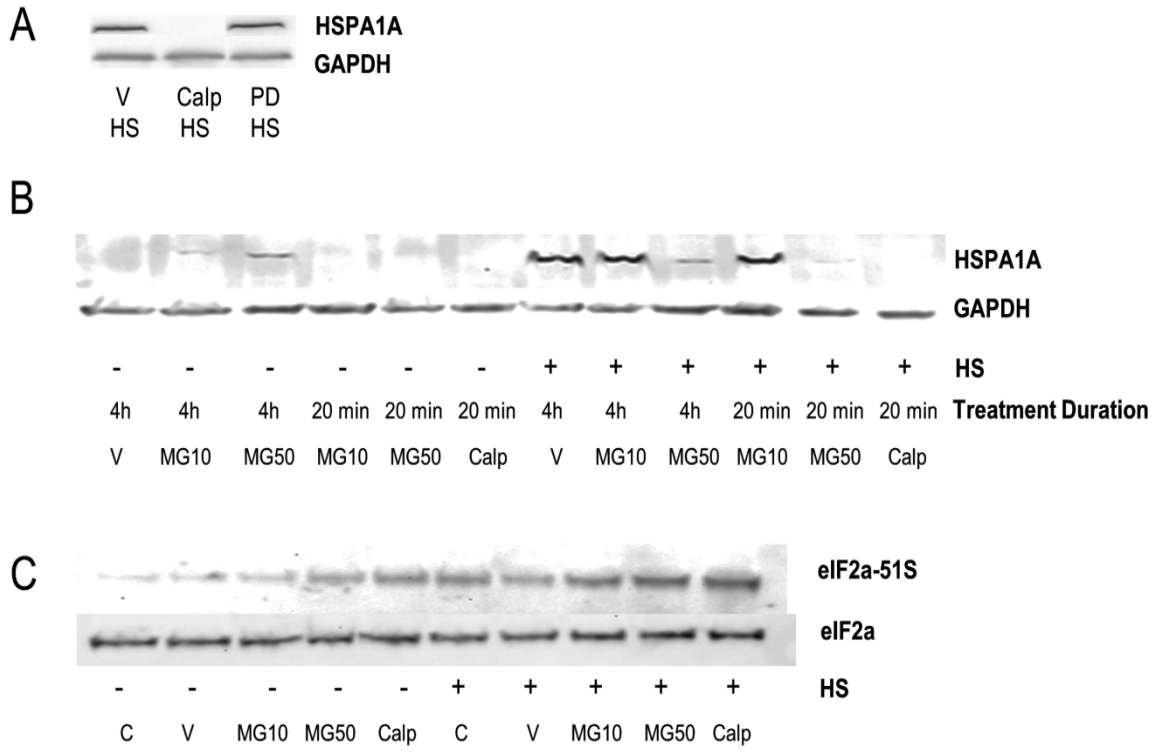

Figure S3 Effective suppression of the HSR by calpeptin and not MG132 or PD150606 treatment.
A Representative Western Blot of HSPA1A and GAPDH for cells treated with DMSO (V), calpeptin (Calp) or calpain inhibitor PD150606 (PD) after HS (10 min 45ºC). **B** Representative Western Blot of HSPA1A and GAPDH for cells treated with DMSO (V), Calp or proteasome/calpain inhibitor MG132 (10 and 50 µM) with and without HS.

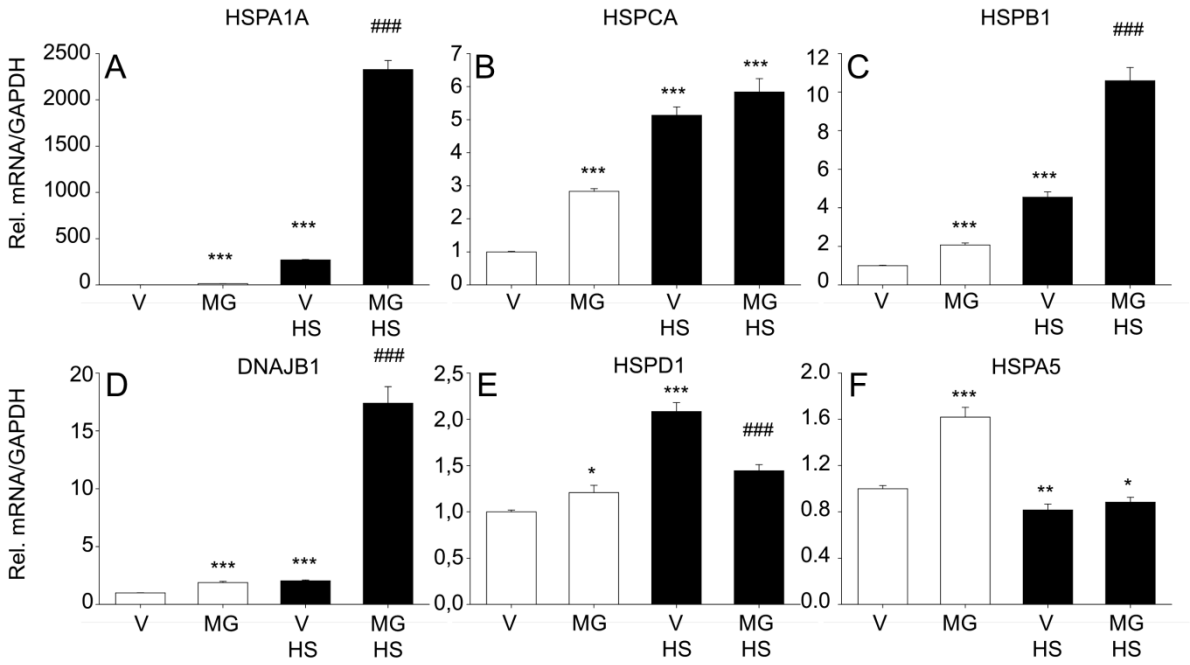

Figure S4 Proteasome inhibition does not inhibit HSP expression on the transcriptional level.
Quantified qPCR data of **A** HSPA1A **B** HSPCA, **C** HSPB1 **D** DNAJB1, **E** HSPD1 **F** HSPA5 mRNA levels for cells treated with DMSO (V) or MG (50µM) with or without an HS. White bars represent control non-HS cells, whereas black bars represent HS cells. ***P<0.001 compared to control and ### P<0.001 compared to control HS.

Chapter 4

Boosting the heat shock response attenuates tachypacing-induced kinomic changes in an *in vivo* canine model for Atrial Fibrillation

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Abstract

Background

Dysregulation of protein kinase mediated signaling plays an important role in a variety of diseases, including atrial fibrillation (AF). As phosphorylation is the predominant mechanism regulating protein function with central roles in virtually every cellular process, dysregulation of kinase and/or phosphatase activity in the heart may result in disturbances in cardiomyocyte proteostasis and cardiac remodeling.

Methods and Results

In this study we applied a kinase array approach to determine whether the heat shock protein (HSP) inducer GGA protects the heart against AF by attenuation of tachypacing-induced alterations in kinase activity and phosphorylation status of their downstream substrates in a canine model for AF. Three groups of mongrel dogs were assigned a different pacing schedule; the control group (C) received no tachypacing whereas the TP-group and TP+GGA group were atrial tachypaced at 400 bpm for the duration of 7 days. GGA treatment of the TP+GGA group consisted of administering GGA orally (120 mg/kg per day), starting 3 days before and continuing throughout the duration of atrial tachypacing. After 7 days left atrial appendages were snap frozen and subsequently lysed for application on the kinase array (Pepchip kinomics array) containing 1024 different kinase pseudo-substrates, each spotted in triplicate. In total the phosphorylation of 232 substrates and activity of 52 kinases was significantly changed during TP. GGA treatment attenuated these changes in case of 131 substrates (56%) and 34 kinases (65%), including AKT kinase, the Src-family of kinases (Src and Fyn) and kinases involved in the cell cycle and apoptosis (ASK1, CHK1, CHK2, CDC2, CCNB2, CCNB1, CDK2, CCND1, CDK4) as well as members of the MAPK pathway (ASK1, MEK5, MAPK4, MAP3K7, ERK5).

Conclusion

Our findings imply that tachypacing significantly alters kinome homeostasis. Importantly, GGA treatment attenuates tachypacing-induced changes in kinase activity, which may underlie the loss of structure and contractile function of the cardiomyocyte.

Introduction

Atrial fibrillation (AF) is the most prevalent and persistent clinical tachyarrhythmia (Nattel et al., 2008;Nattel and Dobrev, 2012). Its maintenance and progression is driven by AF-induced structural and electrophysiological changes of cardiomyocytes, commonly denoted as remodeling (Ausma et al., 1997a;Nattel et al., 2008;Allessie et al., 2010) Cardiac remodeling creates a substrate for AF, which limits the efficacy of current drug and cardioversion therapies (Van Gelder et al., 1996;Nattel et al., 2008). Therefore, identifying the molecular mechanisms underlying AF-induced atrial remodeling may provide insight into AF maintenance and progression and identify novel therapeutic targets.
At the molecular level, dysregulation of kinase and phosphatase activity has been reported to contribute to AF-induced remodeling and contractile dysfunction. In experimental models for AF as well as in patients, aberrant activity of several kinases (Goette et al., 2002;Anderson, 2004;Hove-Madsen et al., 2004;Vest et al., 2005;Greiser et al., 2007;Qi et al., 2008a;Nattel, 2009;Ke et al., 2011;Zhang et al., 2013), and phosphatases (Christ et al., 2004) has been reported. Their altered activity is thought to contribute to AF-induced remodeling and contractile dysfunction through (de)phosphorylation of diverse downstream effector proteins. Currently known downstream proteins involved in AF remodeling include diverse ion channels (Tessier et al., 1999;Christ et al., 2004;Vest et al., 2005;Greiser et al., 2007;Dobrev and Nattel, 2008;Voigt et al., 2012), calcium handling proteins (Ohkusa et al., 1999;El-Armouche et al., 2006;Carter et al., 2011;Nattel and Dobrev, 2012), gap-junction proteins (van der Velden et al., 2000;Burstein et al., 2009;Kato et al., 2012), contractile proteins (Greiser et al., 2007;Wakili et al., 2010), cytoskeletal proteins (Ke et al., 2011) and transcription factors (Lin et al., 2004;Gao and Dudley, 2009). Together, changes in phosphorylation status of these proteins during AF result in the modulation of electrical properties, calcium homeostasis, contractility and gene transcription (Dobrev and Nattel, 2008;Liang et al., 2008;Nattel et al., 2008;Qi et al., 2008a;Chelu et al., 2009;Neef et al., 2010;Xu et al., 2013b). These findings imply a key role for alterations in kinase and phosphatase activity in AF-induced cardiac remodeling and hence AF maintenance and progression.

Although several kinases and phosphatases have been implicated in AF-induced remodeling, a complete overview of AF-induced kinases and phosphatases and their targets is still lacking. A broad inventory of dysregulated kinases and their downstream targets may thus help to identify key kinases and substrates, possibly representing novel targets for pharmacological interventions to stop, slow down or even reverse cardiac remodeling in AF. Therefore, in the current study, we applied a kinomics array (1024 substrates) approach to determine the kinase activity profile for an *in vivo* canine model for AF (Brundel et al., 2006b). Hereto, we determined and contrasted the kinase profiles of left atrial

tissue of atrially tachypaced versus nonpaced control dogs. In addition, we further contrasted these profiles to a third group of tachypaced dogs orally treated with geranyl-geranyl acetone (GGA), a potent Heat Shock Protein (HSP) inducer, known to protect against tachypacing-induced cardiac remodeling (Brundel et al., 2006a;Brundel et al., 2006b;Hoogstra-Berends et al., 2012).

Materials and Methods

2.1 Canine *in vivo* model for AF

The material obtained for the kinase profiling was obtained from experiments performed at the Montreal Hearth Institute as described before (Brundel et al., 2006b). The animal experiments were according to the guidelines for animal-handling of the National Institutes of Health and approved by the Animal Research Ethics Committee of the Montreal Heart Institute. All three groups consisted of five mongrel dogs (28 to 38 kg), which were anesthetized with ketamine (5.3 mg/kg IV), diazepam (0.25 mg/kg IV), and halothane (1.5%) before undergoing the insertion of unipolar pacing leads into the right ventricular apex and right atrial (RA) appendage. Subsequently the pacing leads were connected to pacemakers (Vitatron), which were implanted in subcutaneous pockets in the neck. Radiofrequency catheter ablation was performed to create an atrioventricular block to prevent excessively rapid ventricular responses due to atrial tachypacing. The groups were assigned different pacing schedules; the control group (C) was nonpaced, for the TP-group and the TP+GGA-group, pacemaker frequency was set to 80 bpm (right ventricle) and after a recovery period of 24 hours, atrial tachypacing was set at 400 bpm for the duration of 7 days. GGA treatment of the TP+GGA group consisted of administering GGA orally (120 mg/kg per day), starting 3 days before and continuing throughout the duration of atrial tachypacing. After 7 days of atrial tachypacing, dogs were anesthetized with morphine (2 mg/kg SC) and α-chloralose (120 mg/kg IV, followed by 29.25 mg/kg per hour) and ventilated mechanically. AF vulnerability and effective refractory period (ERP), as well as cardioprotective effects of GGA on tachypacing-induced myocyte cell death, I_{cal}, APD alterations and HSP protein expression were determined and reported previously (Brundel et al., 2006b). For kinomics analysis left atrial appendages were snap-frozen and stored at -80ºC.

2.2 Kinome array

Kinome arrays were performed according to the manufacturer's instructions. The exact protocol of the kinome array is described in detail by Pepscan Presto (http://www.pepscan.com/presto/products-services/kinase-profiling/). The peptide arrays (Pepscan, Lelystad, the Netherlands, containing 1024 different kinase pseudo-substrates, each

spotted in triplicate to confirm reproducibility of the results), were incubated with 150 µg of pooled (per group) left atrial appendage lysates and ^{33}P-γ-ATP for 90 minutes in a humidified stove at 37ºC. Subsequently the array was washed twice in 2M NaCl, twice in 1x PBS containing 1% SDS, once under tap water and a final wash in distilled water. Slides were air dried and exposed to a phosphor-imaging screen for 72 hours and scanned on a cyclone plus storage phosphor system (Perkin Elmer, Waltham USA). Per group a technical replicate of 3 array slides was applied for enhanced reliability of the results.

2.3 Peptide array imaging and data analysis

After imaging, the scanned phosphor images were analyzed using Mathlab. In short, a grid tool was applied to the arrays and substrate (spot) intensities as well as background and slide gradient levels were determined. Background was subtracted for all individual spots. Inconsistent data identified by a flagging procedure was excluded from further analysis. Flags assigned included; Kolmogorov-Smirnoff statistic at a cut-off $p<0.01$ to exclude spots with a value distribution that cannot be distinguished from the background, flags indicating artefacts, overshine effects by neighboring bright spots, no contrast at the spot position, incorrect position in the grid, the spot shape was larger than the defined cutoff of 1.6, or the presence of too many saturated pixels with a cutoff of 3 or more pixels. Further, outliers were determined and excluded, as defined by <> average ± 2*SD. After exclusion of flagged data and outliers, the remaining data was median normalized to the control. For further analyses, the relative difference between spot intensities of TP and TP+GGA groups were compared to the control group. Relevant substrates likely involved in tachypacing-induced remodeling were further defined as substrates whose change in phosphorylation was attenuated by GGA treatment with at least 50%. For determination of effects of TP and GGA treatment on kinase activity, substrates sharing the same upstream kinases were grouped per kinase. Subsequently, the average of overall intensity was determined per group. All the substrate phosphorylation intensities as well as overall kinase activities displayed in the figures and tables are displayed as log2 transformed data. The software BRB array tools was applied to perform hierarchical clustering and to generate heat maps of the obtained data set. Substrate intensity change was considered significant at a p-value <0.05 as determined by Student t-test.

Results

3.1 Kinase activity profiles

To identify key kinases and downstream targets involved in tachypacing-induced remodeling, the kinase profile was determined in three groups of dogs, i.e. control nonpaced (C), atrially tachypaced (TP) and atrially tachypaced with GGA pre-treatment (TP+GGA). Kinase profiles were generated by incubating PepChip^{TM} kinomic arrays (1024 peptide substrates), with canine left atrial lysates and ^{33}P-γ-ATP (see Methods). Active kinases in the lysates induce the covalent transfer of the radioactive labeled ^{33}P to the peptide substrates, resulting in incorporation of radioactivity on the arrays. As phosphatase activity was inhibited in the assay, differences in substrate phosphorylation are specific of altered kinase activity. All arrays showed substantial radioactivity and representative phosphor images are shown in figure 1A. To assess the technical quality of the profiles, Pearson correlation coefficients were determined. The within-slide correlation was 91.1 ± 2.5, 91.8 ± 4.9 and 82.8 ± 3.8 %, while between-slide correlation of groups was 82.4 ± 6.6, 81.3 ± 7.3 and 84.7 ± 3.6 % for C, TP and TP+GGA, respectively. Based on the correlations between the technical replicates and according to the manufacturer's guidelines the slide quality and data quality is good (correlations >80%). The data was further processed by a flagging and outlier removal procedure (see Methods), ensuring the data quality of each individual spot. Of the 1024 analyzed substrates, 911, 920 and 909 substrates passed the quality procedure for C, TP and TP+GGA, respectively.

3.2 Tachypacing-induced changes in kinase activity

To obtain an overview of tachypacing-induced changes in the kinase profile and the effect of GGA treatment on these changes, a heat-map was generated for all three groups excluding substrates that had missing values (Figure 1B). The heatmap of the remaining 824 substrates shows tachypacing-induced changes in the phosphorylation intensity patterns compared to the control, which appear to be partially attenuated by GGA treatment. However, the pattern of TP+GGA treatment is not completely similar to the C group, possibly indicating only partial protection for some substrates or an effect of GGA treatment itself on phosphorylation of a subset of substrates. To substantiate overall differences between groups, hierarchical clustering using Euclidean distance and average linkage was determined (Figure 1C). This analysis shows that the TP+GGA group clustered with the C group, indicating that it is more similar to the C than the TP group.

As GGA treatment has been shown to protect against tachypacing-induced cardiac remodeling, it is likely that the substrates and kinases, that are

changed due to tachypacing but remain similar to control due to GGA treatment, play an important role in tachypacing-induced cardiac remodeling. To identify these targets, we first determined the number of substrates that significantly changed in phosphorylation due to tachypacing and subsequently contrasted these substrates to the TP+GGA group. In comparison to the C, tachypacing caused a significant change in spot intensity of 232 substrates, of which 95 decreased (see supplemental Table 1) and 137 increased (see supplemental Table 2). Subsequently, we compared these 232 substrates to the TP+GGA treated group by generation of a heat-map and hierarchical clustering (see Figure 2A and B). Again, profiles of the TP affected substrates were partially attenuated by GGA treatment. Substrates whose change in phosphorylation was attenuated by GGA treatment were identified on the basis of 2 criteria. First, GGA treatment needed to reduce the TP-induced change by 50% or more. Secondly, their intensity of phosphorylation with GGA treatment did not differ significantly from control. According to these criteria, GGA treatment attenuated the tachypacing-induced change in phosphorylation of 131 substrates (56%). Thus, both hierarchical clustering and the above analysis in phosphorylation change show that GGA treatment prevents tachypacing-induced changes in phosphorylation status of these substrates (see Figure 3 and 4).

Of the 131 substrates whose phosphorylation was changed due to tachypacing and attenuated by GGA, 43 were protected against a reduction in phosphorylation (see Table 1) and 88 were protected against an increase in phosphorylation (see Table 2). The substrates that showed reduced phosphorylation while being attenuated by GGA treatment included many substrates being involved in cell signaling, representing themselves kinases or protein phosphatases, receptors and guanine nucleotide exchange factors. In addition, adapter, adhesion molecules and transcription factors were differently phosphorylated (see Table 1). Diverse substrates were less phosphorylated on multiple sites, such as the adhesion molecule catenin beta 1 (CTNNB1), which is also known to mediate the activation of transcription of WNT-signaling target genes (MacDonald et al., 2009). Further, the receptor tyrosine kinase KIT, a known upstream regulator of diverse signaling pathways, including the AKT pathway (Blume-Jensen et al., 2000), was significantly less phosphorylated in TP compared to C. In addition, multiple substrates shared a common upstream kinase. The kinases that were represented multiple times included, AKT (3 times, AKT signaling), CK2 (6 times, WNT signaling), insulin receptor (2 times, INSR), EGF receptor (2 times, EGFR) and PKA (2 times). Also, multiple kinases of the Src family were identified by multiple substrates, i.e. Src, Fyn and Lck (total 4 times).

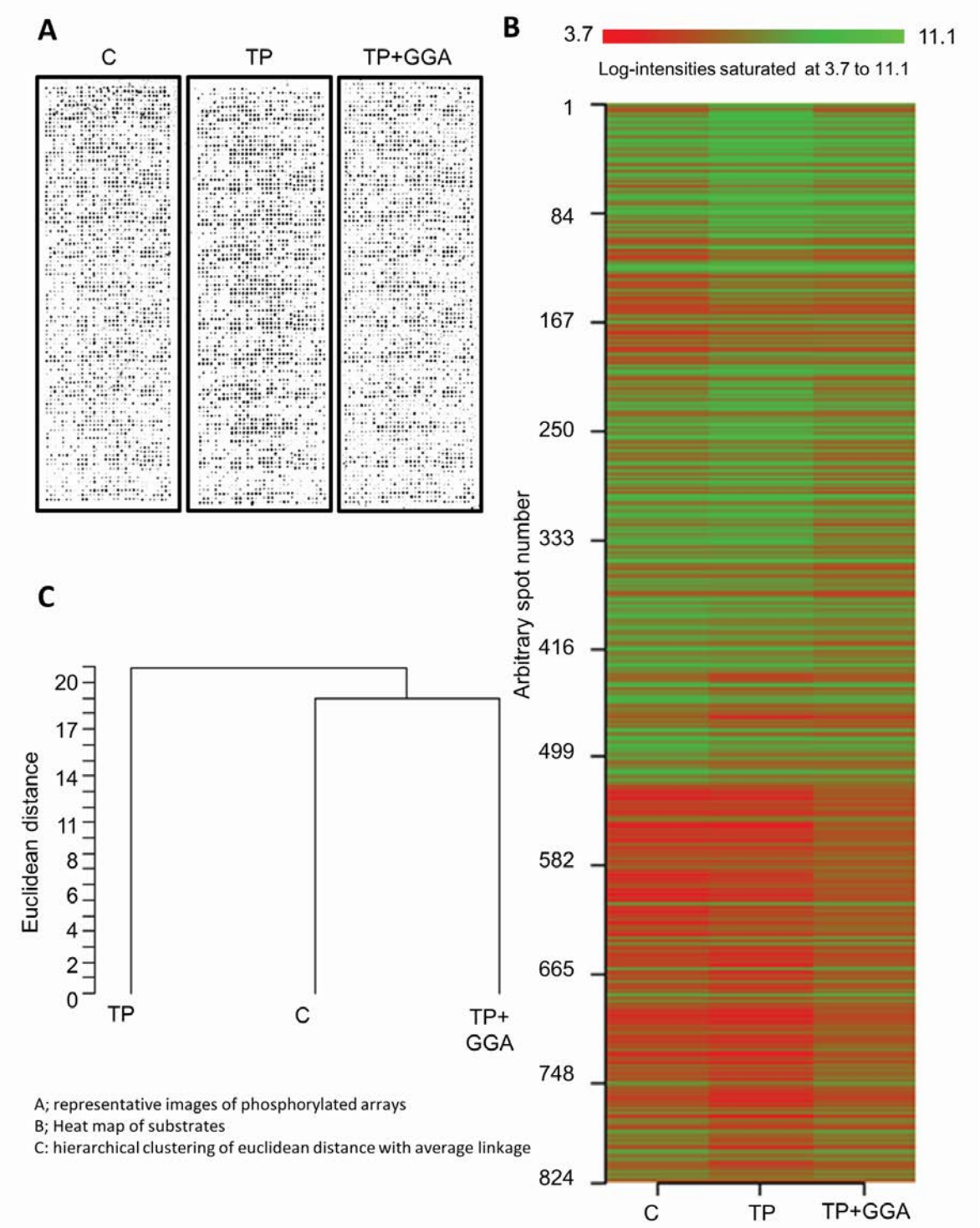

Figure 1 GGA treatment partially protects against overall tachypacing-induced changes in substrate phosphorylation.
A A representative phosphor image of the kinase array per group, (C) nonpaced controls, (TP) tachypaced, (TP+GGA) tachypaced and GGA treated. **B** heat-map of overall substrate phosphorylation changes in response to tachypacing (TP) and tachypacing and GGA treatment (TP+GGA) in comparison to nonpaced control group (C). **C** Dendogram showing the hierarchical clustering of Euclidean distance with average linkage for all three groups.

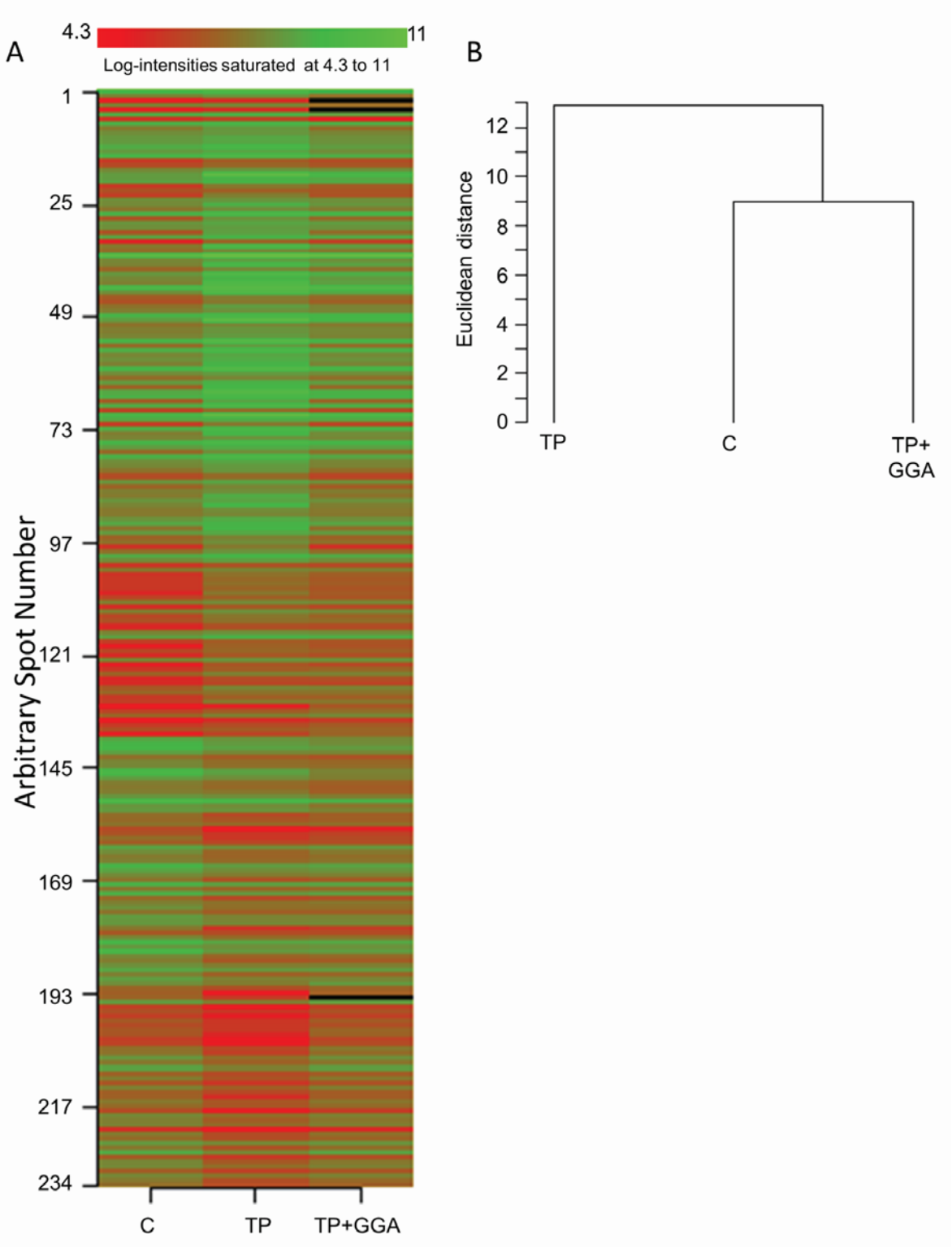

Figure 2 GGA treatment partially protects against tachypacing-induced changes in substrate phosphorylation.
A heat-map of overall substrate phosphorylation changes in response to tachypacing (TP) and tachypacing and GGA treatment (TP+GGA) in comparison to nonpaced control group (C). **B** Dendogram showing the hierarchical clustering of Euclidean distance with average linkage for all three groups.

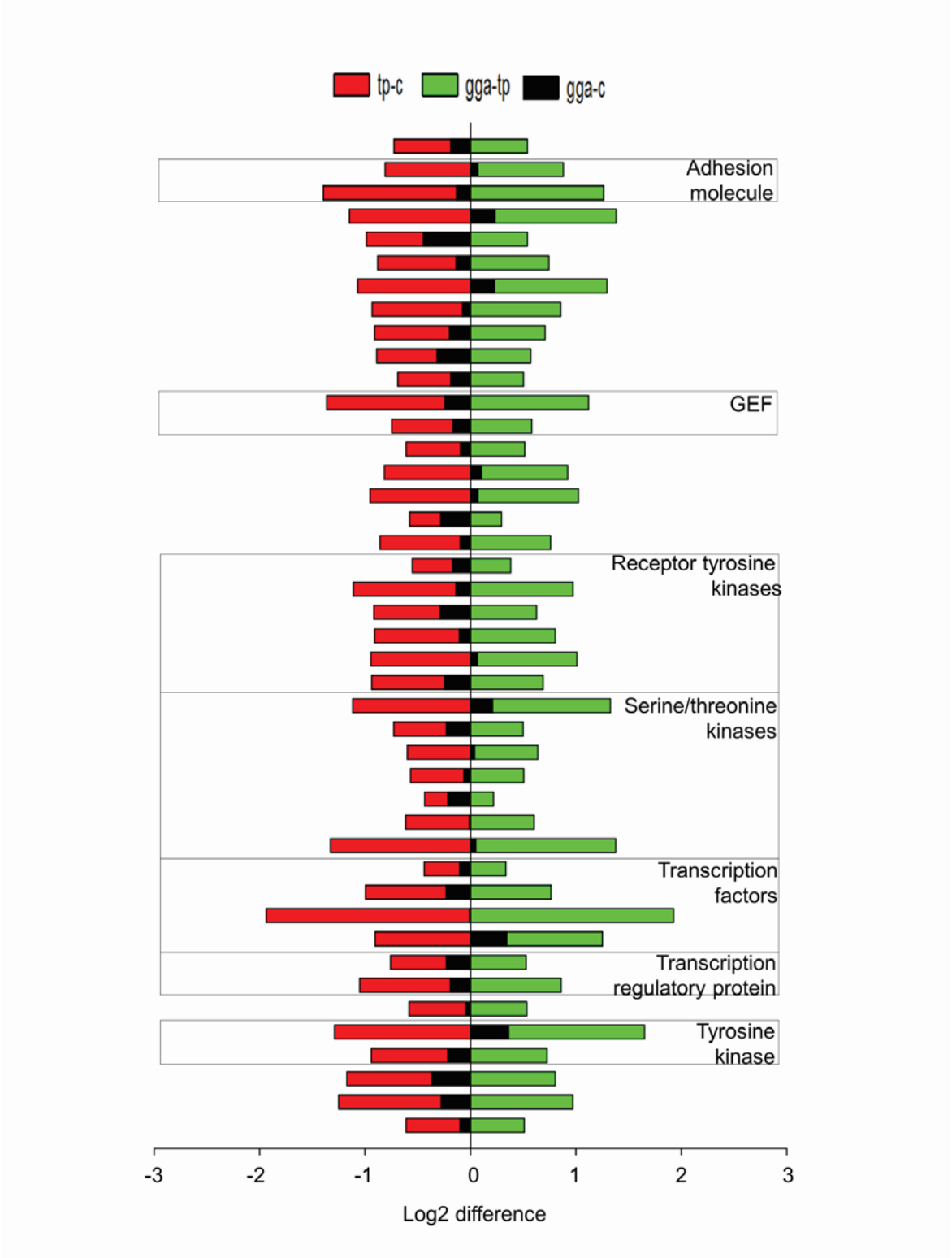

Figure 3 GGA treatment partially protects against tachypacing-induced decrease in substrate phosphorylation.
Differences between the indicated groups of 2 log normalized substrate phosphorylation intensities are displayed and clustered for biological function to indicate substrates with decreased phosphorylation upon tachypacing (TP) and conserved by GGA treatment (TP+GGA) towards control levels (C). Substrates according to the same order as in Table 1.

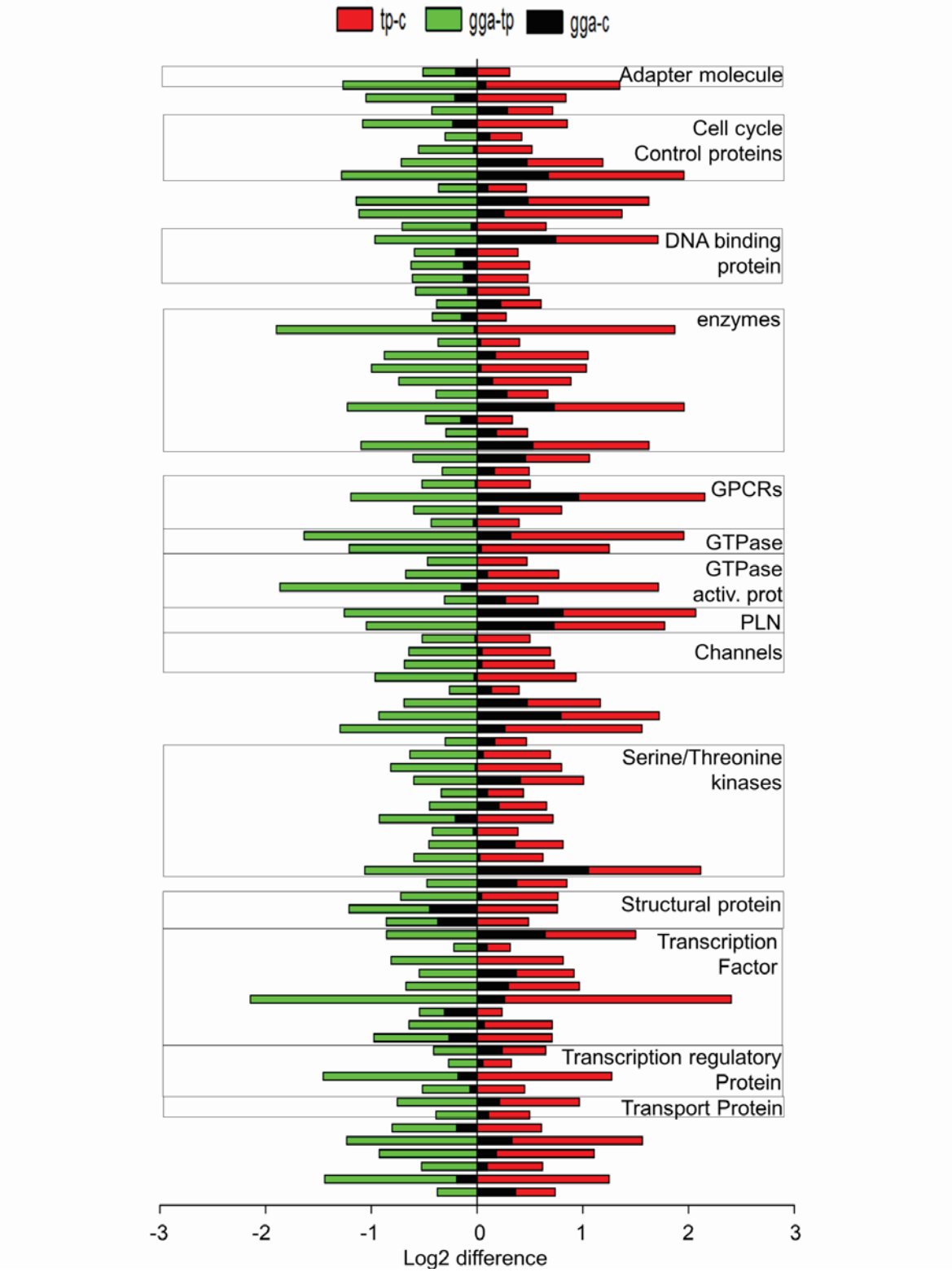

Figure 4 GGA treatment partially protects against tachypacing-induced increase in substrate phosphorylation.
Differences between the indicated groups of 2 log normalized substrate phosphorylation intensities are displayed and clustered for biological function to indicate substrates with increased phosphorylation upon tachypacing (TP) and where the change in phosphorylation was attenuated by GGA treatment (TP+GGA) towards control levels (C). Substrates according to the same order as in Table 2.

The substrates that displayed increased phosphorylation by TP while being attenuated by GGA treatment represented a different set of cell functions (see Table 2). They included more substrates involved in transcription, structural proteins, ion-channels, GTPase activating proteins, diverse enzymes, DNA binding proteins and cell cycle control proteins. Substrates that showed increased phosphorylation on multiple sites included the beta adrenergic receptor kinase 1 (ADRBK1), the serine/threonine kinase B-Raf (a signaling and anti-apoptotic protein (Muslin 2005)), the transcription factors MEF2A and TFII-I, phospholamban (PLN), RAP1GAP, a GTPase activating protein, and the cell cycle control protein RBL2. Common kinases that were represented by multiple substrates included AKT (9 times), CDC2 (8 times), CDK2 (4 times), CDK4 (2 times), PKA (19 times), PKC (19 times) and ZAP70 (2 times).

It should be noted that on the array used, multiple substrates represent a single upstream kinase. Thus, we examined the overall activity for each kinase based on all its substrates in response to tachypacing, excluding those substrates that had missing values. When contrasting the TP to the control group using clustered data from changes in substrates, a total of 52 kinases differed significantly in activity, of which 41 kinases showed normalization of their activity upon GGA treatment (see Table 3). Further, of these 41 kinases, 7 kinases were still significantly different from the control, as the activity of PKA, MLCK, SRC, TXL, CCNB1 and EphB2 was only partially conserved to control level by GGA treatment. Conversely, PTPP showed enhanced activity in the TP+GGA group compared to TP. Compared to the upstream kinases identified by the analysis of separate substrate phosphorylation, we again identified the AKT, the Src-family of kinases (Src and Fyn), KIT and kinases involved in the cell cycle and apoptosis (ASK1, CHK1, CHK2, CDC2, CCNB2, CCNB1, CDK2, CCND1, CDK4) as well as members of the MAPK pathway (ASK1, MEK5, MAPK4, MAP3K7, ERK5) to be significantly altered. Thus, by identifying those kinases with an activity change in TP in multiple substrates which is conserved by GGA treatment, we further strengthen the notion that these kinases play an important role in tachypacing-induced cardiac remodeling.

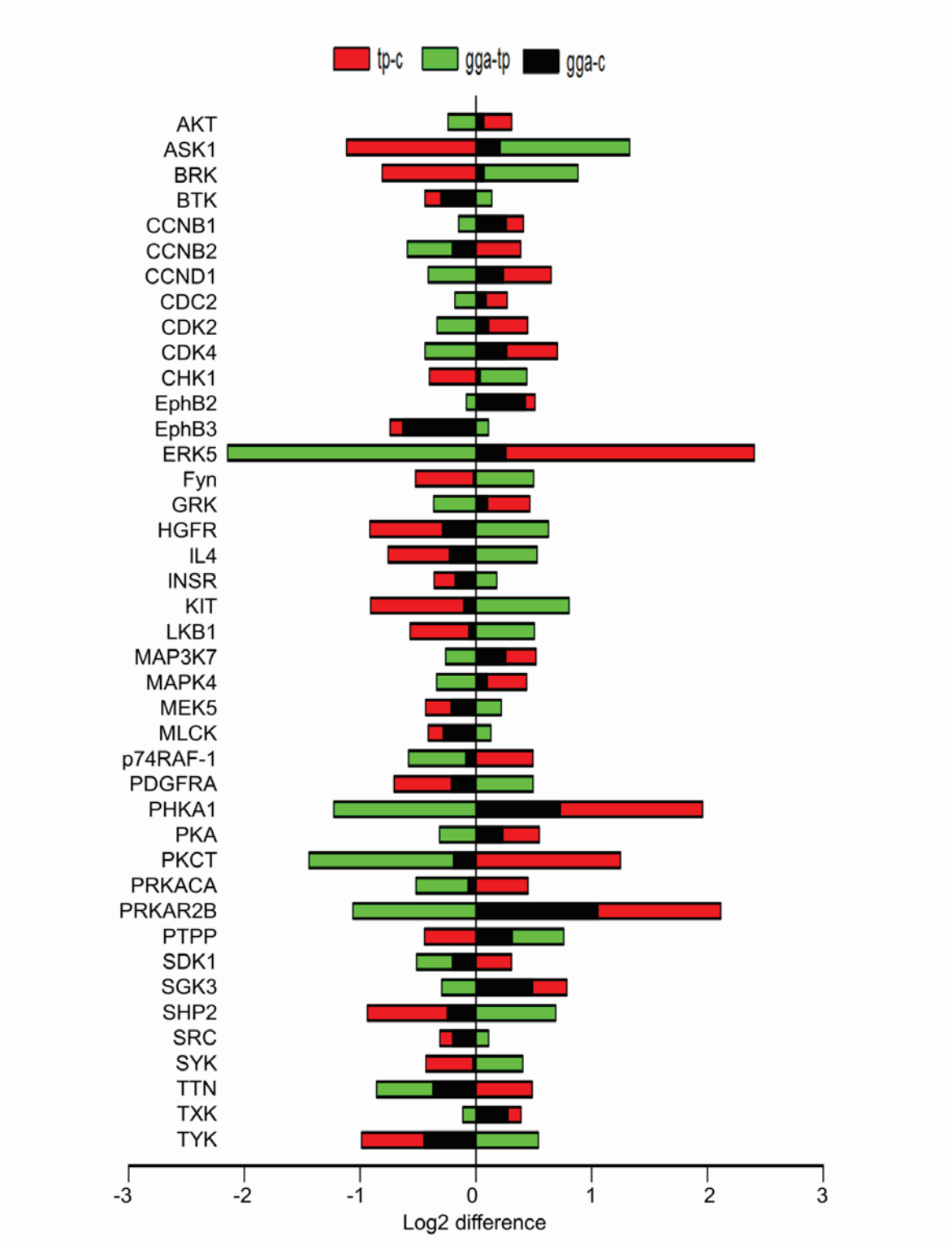

Figure 5 GGA treatment partially protects against tachypacing-induced changes in overall kinase activity.
Kinases with changed activity were derived from changes in substrate phosphorylation. Differences between the indicated groups of 2 log normalized overall kinase activity are displayed. Only kinases are displayed whose activity is (partially) attenuated by GGA treatment. C, nonpaced controls, TP, Tachypacing, GGA, tachypacing and GGA treatment.

Discussion

In the current study we applied a kinase array approach to identify substrates and upstream kinases which are involved in the protective effect of GGA against AF-induced cardiac remodeling. By contrasting the kinase profiles of atrial tissue from nonpaced, tachypaced and tachypaced/GGA treated dogs, we identified tachypacing-induced alterations in activity or phosphorylation status of 52 kinases and 232 substrates of which 34 kinases (65%) and 131 substrates (56%) were conserved to control level by GGA treatment. GGA treatment was previously shown to protect against tachypacing-induced structural remodeling (Brundel et al., 2006a;Ke et al., 2011) and contractile dysfunction (Brundel et al., 2006b) via upregulation of HSP expression (Brundel et al., 2006b). Induction of HSP expression has been shown to protect against various AF-promoting factors that can result in a substrate for AF initiation and progression, as reviewed in Meijering et al. (Meijering et al., 2012). Findings in this study suggest GGA to protect against cardiac remodeling by maintaining kinome homeostasis. The reported ability of HSPs to interact and, in some cases, alter kinase and phosphatase activity is likely playing a major role in GGA mediated protection of the kinome (Ding et al., 1998;Park et al., 2003;Lakshmikuttyamma et al., 2004;Dobrev, 2006;Kammanadiminti and Chadee, 2006;Peng et al., 2010;Fan and Kranias, 2011;Ke et al., 2011;Qian et al., 2011). Further, HSPs may indirectly affect the activity of specific kinases and phosphatases by providing protection against diverse proteotoxic stresses. In addition, HSPs may protect diverse substrates through direct binding e.g. the cytoskeleton and ion channels to shield phosphorylation sites (Brundel et al., 2008). GGA mediated protection of kinome homeostasis may therefore represent a prominent factor to attenuate AF progression in patients.

How the identified candidate substrates and kinases might be involved in AF remodeling is discussed below with respect to the modulation of ion channel properties, calcium handling, contractility (contractile and cytoskeletal proteins), gene transcription, oxidative stress and cardiomyocyte apoptosis, all of which are important factors contributing to AF substrate formation and hence its maintenance and progression (Ausma et al., 1995b;Ausma et al., 1997a;Ausma et al., 2001;Goette et al., 2002;Ausma et al., 2003;Schotten et al., 2003;Thijssen et al., 2004;Nattel et al., 2008;Van Wagoner, 2008;Li et al., 2009;Ke et al., 2011;Dobrev et al., 2012;Meijering et al., 2012;Nattel and Dobrev, 2012;Xu et al., 2013a).

4.1 Modulation of ion channel properties and calcium handling

Among the tachypacing-induced altered substrates, increased phosphorylation of connexin 43 (Ram, 2008), phospholamban (El-

Armouche et al., 2006), the ryanodine receptor (Li et al., 2012) and phosphodiesterase 4 (Hua et al., 2012;Molina et al., 2012;Van Wagoner and Lindsay, 2012) have been reported previously in human and experimental models for AF. Phosphorylation of connexin 43, part of the cardiac gap junction complex responsible for cell-to-cell coupling, was shown to impair gap junction function and is thought critical for the development of cardiac re-entry arrhythmias, like AF (Polontchouk et al., 2001;van der Velden et al., 2002;Lampe and Lau, 2004;Kjolbye et al., 2007;Ram, 2008;Burstein et al., 2009). Phospholamban (Currie and Smith, 1999;Mattiazzi et al., 2005) and the ryanodine receptor (Bers, 2006) both play important roles in cardiac calcium homeostasis and their phosphorylation was suggested to contribute to sarcoplasmic calcium leakage and increased delayed after-depolarizations (Nattel and Dobrev, 2012) as well as increased arrhythmogenicity during catecholaminergic stimulation (Bers, 2006). Phosphodiesterases, especially PDE4 play a critical role in controlling cAMP levels and thereby Calcium influx and release in human atrial cardiomyocytes (Molina et al., 2012) and are therefore critical regulators of atrial contractility and arrhythmogenesis (Van Wagoner and Lindsay, 2012). Other calcium handling proteins or binding proteins present on the kinase array consisted of Calmodulin 1 (CALM1) and their upstream calcium/calmodulin dependent kinases CAMK1, CAMK2 and CAMK4. In contrast to diverse other studies (Nattel and Dobrev, 2012), we did not find an altered CAMK2 activity, which might be due to limitations of the kinase array technique as is discussed below. Although modulation of other ion-channels has been reported in AF (Dobrev and Voigt, 2011) we were not able to assess the phosphorylation of these channels as their representative consensus peptide sequences were not present on the currently applied kinase array. However, the overall activity of known ion-channel modulating kinases was assessed. Among the significantly altered kinases, three kinases, namely PKA (Allessie et al., 2001;Mattiazzi et al., 2005;Vest et al., 2005;Dobrev and Voigt, 2011), PKC (Allessie et al., 2001;Dobrev and Voigt, 2011) and the Src-family (Greiser et al., 2007), were previously reported to regulate ion-channel function and may represent therapeutic targets in AF. Although no novel substrates or kinases involved in ion channel function and calcium handling were identified with this kinase array approach, we identified many key substrates known to be important in AF maintenance and progression indicating the kinase array a valuable and reliable technique in identifying kinases involved in AF pathology.

4.2 Contractility

Contractility is determined by calcium handling as well as the proteostasis of contractile and cytoskeletal proteins (de Tombe, 1998;Ke et al., 2008;Ke et al., 2011). During AF, the loss of proteostasis and subsequent altered calcium handling results in contractile dysfunction and cardiac hibernation and involves an altered expression and organization of

contractile and cytoskeletal proteins, including re-expression of alpha smooth muscle actin, induction of F-actin stress fibers, altered localization and degradation of sarcomeric proteins such as troponins, myosin and titin, and degradation of cytoskeletal proteins being part of microtubules (tubulin) and intermediate filaments (desmin, α-actinin and spectrin) (Ausma et al., 1995a;Ausma et al., 1995b;Ausma et al., 1997a;Ausma et al., 1997b;Ausma et al., 2000;Ausma et al., 2001;Allessie et al., 2002;Brundel et al., 2002;Ke et al., 2011). In the current kinase array, various contractile and cytoskeletal proteins were represented, including cortactin, desmin, actin, tubulin (beta-4A chain), caldesmon, myosin light chain and titin. Of these substrates, only titin, caldesmon and myosin light chain were significantly differently phosphorylated in response to tachypacing. Of these substrates only the change in phosphorylation of titin was prevented by GGA treatment. The effect of enhanced phosphorylation of titin on Serine 157 is currently unknown, but may contribute to its altered localization or degradation in response to tachypacing (Ausma et al., 2003). For overall kinase activity, not much is known of upstream kinases involved in modulation of cytoskeletal and contractile proteins in AF. However, we previously showed Rho-GTP and its downstream RhoKinase (ROCK) involved in tachypacing-induced cardiac remodeling and loss of contractile function (Ke et al., 2011). In the current array, however, we did not observe a significant change in substrate intensities reflecting overall ROCK activity. Differences between the diverse models and duration of AF might explain these discrepancies. Another upstream kinase, myosin light chain kinase (MLCK) and its substrate myosin light chain 2 (MLC2) showed significantly reduced phosphorylation in TP, which was partially attenuated by GGA treatment, which was also previously found in the canine model for atrial tachycardia (Wakili et al., 2010). The tachypacing-induced change in phosphorylation of myosin light chain 2 may modulate cardiac muscle contractility by increasing the sensitivity of the contractile element to activation by calcium (Moore et al., 1991;van der Velden et al., 2003). To our current knowledge therapeutic modulation of MLCK activity in models for AF have not yet been attempted and may prove beneficial in preserving cardiac contractility.

4.3 Gene transcription

An important molecular mechanism of cardiac remodeling involves the (re-)expression of pathological cardiac genes via activation of diverse signaling pathways and their downstream transcription factors, as well as transcription regulators and histone acetylation modulators such as HDACs (Ausma et al., 1995b;Ausma et al., 1997a;Ausma et al., 1997b;Allessie et al., 2002;Thijssen et al., 2004;Backs et al., 2006;Qi et al., 2008a;Cao et al., 2011;Zhang et al., 2011;McKinsey, 2012). Diverse transcription factors, transcription regulators as well as HDACs were represented on the array. Of the HDAC family HDAC1, HDAC2 and HDAC4

were represented on the array, but no alterations in their phosphorylation status in response to tachypacing was observed. Of the transcription factors represented on the array that were previously reported involved in cardiac remodeling in AF, i.e. NFAT and NFκB (Lin et al., 2004;Gao and Dudley, 2009), a significant increase in phosphorylation status in response to tachypacing was observed. However these targets were not conserved by GGA treatment and therefore were not identified as primary targets in the current analysis. Of the significantly different transcription regulators and transcription factors we will discuss MEF2A, MAX, STAT6, FOXO3A, FOXO4 (ATFX1) and beta-catenin (CTNNB1), which were previously reported to play a role in cardiac remodeling. However, to our current knowledge they represent novel targets in tachypacing-induced remodeling. Of these transcription factors STAT6 and CTNNB1 showed a tachypacing-induced decrease in phosphorylation whereas the other transcription factors showed an increase in phosphorylation, which likely represents their activation. As STAT6 was reported to protect the heart from hemodynamic stress (Hikoso et al., 2004), the decreased phosphorylation of STAT6 may render cardiomyocytes more vulnerable to tachypacing-induced damage. CTNNB1 has a dual role as transcription factor and as part of the catenin/cadherin complex important for cell adhesion and cell structure. In the current kinase array, we observed a decrease in phosphorylation on two sites, Y142 and T102. Phosphorylation of these two sites regulates its interaction with critical components of the catenin/cadherin complex and may act as a switch between its membrane bound (catenin-cadherin complex) and transcriptional role (nuclear) (Piedra et al., 2001;Krejci et al., 2012). The tachypacing-induced decrease in phosphorylation of beta-catenin may therefore result in increased membrane association of beta-catenin and a reduction in Wnt/beta-catenin signaling. Similarly, increased membrane localization and reduced nuclear beta-catenin levels were found in cardiac tissue from patients with inherited cardiac hypertrophy and in samples from experimental cardiac hypertrophy (Masuelli et al., 2003). As there is increasing evidence linking the Wnt signaling to regulation of cardiac function (Dawson et al., 2013), our results indicate a novel role in tachypacing-induced remodeling. MEF2A, has been reported to play an important role in the induction of the myocardial hypertrophic genetic program in response to stretch (Nadruz et al., 2005) as well as angiotensin II mediated cardiac remodeling (McCalmon et al., 2010). Further, its activation is required for maladaptive cardiac remodeling downstream of calcineurin signaling and is involved in the response to pressure overload and subsequent cardiac remodeling (Akazawa and Komuro, 2003;van Oort et al., 2006). Furthermore, knockout of MEF2A has been shown essential for energy metabolism and sarcomeric organization in adult hearts (van Eldik and Passier, 2013). Activation of MEF2A in response to tachypacing indicates its involvement in tachypacing-induced remodeling. Further, also the increased activity of its upstream kinase ERK5 (Lee et al., 2011) substantiates our findings and

indicates also ERK5 as a possible novel therapeutic target in AF. The transcription factor MAX was reported as one of the factors important in regulating alpha-myosin heavy chain expression (Gupta et al., 1997). The observed alterations in its phosphorylation, and therefore possible activation, may affect the expression of this contractile protein in AF. The last transcription factors discussed are the FOXO family members, FOXO3 (FOXO3A) and FOXO4. FOXO3 is critical in maintaining cardiac function (Ronnebaum and Patterson, 2010) and is known to have pleiotropic effects in the heart, as it is involved together with ARC (an anti-apoptotic protein) to constitute an anti-apoptotic pathway which regulates calcium homeostasis and apoptosis in cardiomyocytes (Lu et al., 2013). Further, FOXO3 is involved in cardiac hypertrophy signaling downstream of the calcium-calcineurin pathway as it can reduce the activity of calcineurin (Schips et al., 2011) and has been reported to promote autophagy in cardiomyocytes (Sengupta et al., 2009). Furthermore, it is involved in transcription and can interact with the 14-3-3 protein. Increased phosphorylation of FOXO3A by AKT, as found in TP, has been reported to mask its nuclear localization sequence. Consequently, increased phosphorylation of FOXO3 promotes its interaction with nuclear 14-3-3 proteins causing translocation of FOXO3-14-3-3 complex to the cytoplasm where it is rendered inactive (Clavel et al., 2010;Tzivion et al., 2011). Since FOXO3 nuclear localization is critical in counteracting hypertrophic stress (Ronnebaum and Patterson, 2010), maintaining FOXO3 activity via AKT inhibition might prove beneficial in preventing tachypacing-induced cardiac remodeling. In addition to FOXO3 also FOXO4 (ATFX1) showed a tachypacing-induced increase in its phosphorylation. Although less is known about FOXO4 function in the heart, also FOXO4, as FOXO3, is reported critical in maintaining cardiac function (Ronnebaum and Patterson, 2010). As also FOXO4 is a downstream target of AKT, inhibition of AKT could also restore FOXO4 activity. Whether AKT inhibition has therapeutic potential remains to be determined as short-term activation of AKT is known to be cardioprotective whereas long-term activation causes pathological hypertrophy and heart failure (Chaanine and Hajjar, 2011).

4.4 cardiomyocyte apoptosis

Increased apoptosis has been reported in experimental models for AF and is thought to occur when adaptive mechanisms of cardiac survival are no longer able to sustain cardiac homeostasis (Kim et al., 2005;Li et al., 2009;Xu et al., 2013a). Increased apoptosis leads to cardiomyocyte loss, contractile dysfunction, electrical remodeling and disease progression (Aime-Sempe et al., 1999;Kang and Izumo, 2000;Cardin et al., 2003;Shih et al., 2011). Recently, attenuation of apoptosis was suggested to represent an innovative approach for the treatment of AF (Trappe, 2012). However, a better understanding of underlying pathways is necessary to identify targets for specific therapeutic strategies. On the kinase array diverse targets involved in apoptosis regulation are

represented, including BCL2, B-Raf, p53, cell cycle control proteins and the transcription factor FOXO3A. The phosphorylation status of these targets, including FOXO3A (discussed previously), B-Raf, and a substantial amount of cell cycle control proteins (including downstream targets such as p53 and Rb), were significantly changed in response to tachypacing. While these targets have not been reported in relation to AF previously, they function in apoptosis. Firstly, B-RAF/MEK/ERK signaling modulates cell cycle progression and cell survival (Chang et al., 2003), but has also been reported to mediate cardiac hypertrophy (Harris et al., 2004). Tachypacing-induced alterations in its phosphorylation may hence result in shifting the balance between cardiomyocyte survival and apoptosis or induction of hypertrophic signaling contributing to cardiac remodeling. Further, the most interesting and largest group of novel targets, consists of cell cycle control proteins, including CHK1, CHK2, CDC2, CCNB2, CCNB1, CDK2, CCND1 and CDK4. These proteins however do not only control the cell cycle of mitotic cells but also regulate apoptosis and hypertrophic growth in post-mitotic cardiomyocytes (Hauck et al., 2002;Ahuja et al., 2007). Activation of the cell cycle control signaling pathways may thus enhance tachypacing-induced cardiomyocyte apoptosis and hypertrophic signaling. Although their exact role in AF should be elucidated, prevention of apoptosis by targeting cell cycle proteins and b-Raf signaling might represent an innovative therapeutic approach in AF.

4.5 Oxidative stress

Oxidative stress and inflammation contribute to AF substrate formation (Van Wagoner, 2008;Bonilla et al., 2012;Meijering et al., 2012). On the current array only two substrates involved in oxidative stress were found significantly altered in their phosphorylation status in response to tachypacing and conserved by GGA treatment, namely HMG-Co-Reductase (HMGCR) and NOS3 (endothelial NO-synthase 3). To our knowledge, a tachypacing-induced increase in HMG-CoA-Reductase phosphorylation has not been previously described in AF. Importantly, this mechanism may explain part of the therapeutic effects of statins in AF, as statins are HMG-CoA-Reductase inhibitors (Kumagai et al., 2004;Pinho-Gomes et al., 2013). Future research should elucidate the role of HMG-CoA-Reductase phosphorylation in AF. The second target is NOS3, a nitric oxide synthase which showed increased phosphorylation at S1177. In AF a downregulation of endocardial NO synthase expression and NO production has been reported (Cai et al., 2002). Further, NOS can under certain conditions become uncoupled, shifting from NO production to superoxide anion production, hence generation of a potent free radical and thereby likely inducing oxidative stress (Bonilla et al., 2012). Phosphorylation of NOS3 at S1177 represents a key switch in the regulation of superoxide anion generation, altering both the enzyme's Calcium sensitivity and maximal rate of superoxide anion generation (Chen et al., 2008). Enhanced tachypacing-induced phosphorylation of

NOS3 may therefore contribute to oxidative stress in AF and play a role in AF maintenance and progression. If so, inhibition of AKT or PKA, which have been reported to phosphorylate NOS3 at S1177, may prove beneficial as a novel therapeutic approach.

4.6 Miscellaneous targets

In addition to the above described targets, our results also indicated five other targets with already established roles in general cardiac remodeling, namely lamin A and B1, the receptor KIT and the beta-adrenergic receptor kinase 1 (ADRBK1) and beta-2 adrenergic receptor (ADRB2). The receptor KIT is a known upstream regulator of diverse signaling pathways, including the AKT pathway (Blume-Jensen et al., 2000) and has been shown to mediate myocardial repair in response to HDAC inhibition (Zhang et al., 2012). Its reduction in phosphorylation upon tachypacing might hamper its contribution to myocardial repair, although the significance of its phosphorylation status in relation to its function is currently not known. The other targets, Lamin A and B1 (LMNA, LMNB1) play a key role in maintaining nuclear architecture as well as regulating transcription and LMNA is the most prevalent gene in dilated cardiomyopathy associated with a high risk of arrhythmias, sudden death and heart failure ((Malhotra and Mason, 2009). Further, mutations in lamins have been associated with numerous diseases, including familial atrial fibrillation (Pan et al., 2009;Saj et al., 2012). However, Ausma et al. found unchanged nuclear levels and location of lamin in models for chronic atrial fibrillation (Ausma et al., 1997a). However, as phosphorylation of lamin A was not evaluated in this study, the exact role of increased phosphorylation of lamin A and B1 in tachypacing-induced remodeling remains to be elucidated. The final miscellaneous targets are beta-adrenergic receptor kinase 1 (ADRBK1) and beta-2-adrenergic receptor, which play pivotal roles in beta-adrenergic signaling (Takahashi et al., 2004) and have been implicated in atrial fibrillation induced cardiac remodeling (Goette et al., 2002). ADRBK1 can be induced by protein kinase C (PKC) and is activated in the failing myocardium, resulting in uncoupling of the beta-adrenergic receptor from its G proteins through phosphorylation (Pitcher et al., 1998;Luttrell et al., 1999). Indeed, an impaired beta adrenergic modulation in fibrillating atrial tissue was observed as the inotropic response to isoproterenol was reduced compared to sinus rhythm (Schotten et al., 2001). However, this was suggested to be independent of alterations in beta adrenergic signal transduction, but rather caused by downregulation of the L-type calcium channel (Schotten et al., 2001). Thus, if and how the increased activity of ADRBK1 and phosphorylation of ADRB2 contributes to modulation of beta adrenergic signaling in AF needs further clarification.

To conclude, the current study reveals that GGA protects against AF-induced remodeling by conserving kinome homeostasis. Furthermore, a broad overview of tachypacing-induced alterations in phosphoproteome

and kinase activity is provided, which broadly supports existing knowledge of the pathophysiology of AF and extends this by identifying several novel kinases and their downstream substrates, which may offer future novel therapeutic options. Future studies will be aimed at the verification of the candidate substrates and kinases by other molecular biological techniques and the elucidation of their exact roles in AF maintenance and progression.

4.7 Current limitations of the kinase array technique

The kinase array technique is currently the most powerful high-throughput technique to identify alterations in kinase activity without *a priori* assumption. Kinase arrays have been successfully applied in diverse other studies (Diks et al., 2003;Diks et al., 2007;Parikh et al., 2009;Hoogendijk et al., 2011a;Hoogendijk et al., 2011b;Ter Elst et al., 2011), and also the current study broadly supports existing knowledge of the pathophysiology of AF. However, some major kinases and targets represented on the array and known to be involved in AF pathophysiology were not altered in their phosphorylation status or activity in the current study. Apart from differences in models, limitations of the array technique and analysis may possibly explain these discrepancies (Sikkema et al., 2009;Arsenault et al., 2011). First, there is loss of cellular compartmentalization caused by cell lysis, which results in the loss of necessary co-activators or co-factors. Secondly the consensus peptide may not represent the preferred substrate of a kinase because of interspecies differences between dog (used in this study) and human optimized consensus peptides on the array. Although this technique has been applied in different species (Diks et al., 2007;Arsenault et al., 2011;Hoogendijk et al., 2011a;Hoogendijk et al., 2011b), interspecies differences have been reported (Arsenault et al., 2011). Therefore we cannot exclude artifacts due to interspecies differences. A third limitation is the application of phosphatase inhibitors in the current array technique. As the phosphorylation status is a balance between kinase activity and phosphatase activity, verification of the phosphoproteome status by western blot is necessary to verify our current results. Further false positive results likely include consensus peptide sequences on the array representing proteins which are not expressed in cardiac tissue. Lastly the current analysis of determining the overall kinase activity (per kinase) may have a limited predictive value, as not all potential substrates of a protein kinase are phosphorylated to the same extend on the array and even opposite directions were observed, as has been previously reported in other studies (Diks et al., 2004;El-Armouche et al., 2006;de Borst et al., 2007;Parikh et al., 2009;Sikkema et al., 2009;Hennig et al., 2012). This may be partly due to kinases which are themselves regulated and therefore alter their substrate specificity (Yung et al., 2011), resulting in phosphorylation of only a specific subset of substrates. Thus, while this study provides an overview of altered kinase activity in tachypaced dogs,

its limitations warrant the verification of the phosphorylation status and biological effect of the candidate targets in AF by other molecular biological techniques.

Table 1. Overview of substrates protected by GGA from tachypacing induced decrease in phosphorylation

Spot	C	TP	TP+GGA	P-Site	Up Kin	Prot	Function
841	7.81	7.09	7.63	Y627	INSR	GAB1	Adapter molecule
321	6.41	5.60	6.48	Y142	BRK	CTNNB1	Adhesion molecule
786	6.84	5.45	6.71	T102	CK2B	CTNNB1	Adhesion molecule
467	6.05	4.90	6.28	T436	nd	TNFR2	Cell surface receptor
975	7.22	6.23	6.77	Y20	TYK	FABP4	Chaperone
923	8.52	7.64	8.38	S22	nd	FGA	Coagulation factor
811	7.21	6.14	7.43	S56	CDC2	VIM	Cytoskeletal protein
916	7.43	6.50	7.36	Y28	ABL	RAD9	DNA repair protein
173	5.54	4.63	5.34	S19	CK2	PIN4	Enzyme: Isomerase
842	7.19	6.30	6.87	Y1252	Fyn	NR2B	Extracellular ligand gated channel
273	7.70	7.01	7.51	T312	PKA	MC4R	G protein coupled receptor
203	7.35	5.98	7.10	Y172	EGFR	VAV2	Guanine nucleotide exchange factor
129	7.11	6.36	6.95	Y174	Lck	VAV1	Guanine nucleotide exchange factor
257	6.89	6.28	6.80	T140	nd	CRYAA	Heat shock protein
998	8.27	7.45	8.37	S335	CK2	PSEN2	Integral membrane protein
985	5.88	4.93	5.95	S380	AKT	PTEN	Lipid phosphatase
509	7.08	6.50	6.79	S491	PKA	GLUT2	Membrane transport protein
281	7.60	6.75	7.51	S22	nd	STATH	nd
779	7.82	7.27	7.65	Y721	CHK1	KIT	Receptor tyrosine kinase
961	7.55	6.44	7.41	Y1248	HER2	HER2	Receptor tyrosine kinase
962	6.77	5.85	6.48	Y1349	HGFR	HGFR	Receptor tyrosine kinase
194	6.60	5.69	6.49	Y568	KIT	KIT	Receptor tyrosine kinase
515	6.57	5.63	6.64	Y754	PDGFRA	PDGFRA	Receptor tyrosine kinase
271	7.68	6.74	7.43	Y751	SHP2	PDGFRB	Receptor tyrosine kinase
87	5.93	4.81	6.14	Y718/T838	ASK1	ASK1	Serine/threonine kinase
221	7.89	7.16	7.66	T68	CHK2	CHK2	Serine/threonine kinase
259	6.32	5.72	6.36	Y90	Lck	PKCT	Serine/threonine kinase
343	6.76	6.20	6.70	T189	LKB1	LKB1	Serine/threonine kinase
288	7.26	6.83	7.05	T218	MEK5	ERK5	Serine/threonine kinase
153	6.92	6.31	6.91	S207	nd	MAP2K6	Serine/threonine kinase
138	6.01	4.68	6.06	Y658	SYK	PKCA	Serine/threonine kinase
592	8.19	7.75	8.09	Y29	nd	CRYBB3	Structural protein
387	7.44	6.45	7.21	Y398	BTK	TFII-I	Transcription factor
169	6.78	4.84	6.77	S11	CK2	MAX	Transcription factor
316	6.61	5.70	6.96	S263	CK2	N-myc	Transcription factor
780	6.78	6.02	6.55	Y641	IL4	STAT6	Transcription factor
282	8.19	7.14	8.00	S784	ATM	p53BP1	Transcription regulatory protein
522	7.08	6.50	7.03	Y537	Src	ERA	Transcription regulatory protein
932	6.52	5.23	6.88	S116	AKT; CAMK2	PEA15	Transport/cargo protein
809	8.67	7.73	8.46	S465	AKT	ABL	Tyrosine kinase
963	7.79	6.61	7.42	Y397	FAK	FAK	Tyrosine kinase
769	7.49	6.24	7.21	Y152	INSR	PTPN1	Tyrosine phosphatase
925	7.49	6.88	7.40	T16	CK2A1	HCLS1	Unclassified

* All substrate intensities are log2 normalized data

Table 2. Overview of substrates protected by GGA treatment from tachypacing induced increase in phosphorylation

Spot	C	TP	TP+GGA	P-Site	Up Kin	Prot	Function
500	8.35	8.66	8.15	S59;S60	SDK1	14-3-3B	Adapter molecule
325	7.77	9.12	7.85	Y273	ZAP70	LNK	Adapter molecule
666	7.35	8.19	7.14	S1152	p90RSK	L1CAM	Adhesion molecule
128	9.52	10.23	9.81	S451	GSK3	ACLY	ATPase
432	6.94	7.79	6.71	S1068	CDC2	RBL2	Cell cycle control protein
184	6.36	6.78	6.48	S20	CDC2	RRM2	Cell cycle control protein
680	7.48	8.00	7.45	S151	CDK2	hCDH1	Cell cycle control protein
374	7.86	9.04	8.33	S964	CDK4	RBL1	Cell cycle control protein
118	6.51	8.46	7.18	S1035	CDK4	RBL2	Cell cycle control protein
943	9.02	9.48	9.12	S336	GRK	CCR5	Cell surface receptor
441	6.00	7.63	6.48	S285	PKC	F3	Coagulation factor
667	8.72	10.09	8.97	T80	nd	CAPN1	Cysteine protease
174	8.13	8.78	8.07	S72	ROCK1	VIM	Cytoskeletal protein
879	7.76	9.47	8.50	S417	AKT	HTT	DNA binding protein
670	8.28	8.67	8.08	T463	CCNB2	KIF22	DNA binding protein
918	7.88	8.38	7.75	T53	CDC2	HMGA1	DNA binding protein
730	8.11	8.59	7.98	S273	CDK2	ORC1	DNA binding protein
952	7.61	8.10	7.52	S222	p74RAF-1	MEK1	Dual specificity kinase
990	7.32	7.93	7.55	S198	PLK1	CDC25C	Dual specificity phosphatase
685	7.99	8.26	7.84	S80	AMPK	ACC	Enzyme: Carboxylase
154	5.68	7.55	5.65	S293	PDK1	PDHA1	Enzyme: Dehydrogenase
899	7.99	8.40	8.03	Y989	TK	OGT	Enzyme: Glucosaminyltransferase
428	6.32	7.37	6.50	S99	CDC2	dUTPase	Enzyme: Hydrolase
877	8.97	10.00	9.00	S294	AKT	PDE3A	Enzyme: Phosphodiesterase
637	8.66	9.55	8.81	S133	PKA	PDE4B	Enzyme: Phosphodiesterase
170	7.92	8.59	8.21	S561	PKCA	PLD1	Enzyme: Phosphodiesterase
410	8.42	10.37	9.15	S1018	PHKA1	PHKA1	Enzyme: Phosphorylase
695	8.86	9.19	8.70	S483	AKT	PFKFB2	Enzyme: Phosphotransferase
107	7.68	8.16	7.87	S872	PKA	HMG1	Enzyme: Reductase
45	6.59	8.21	7.12	S1177	AKT	NOS3	Enzyme: Synthetase
299	6.97	8.03	7.43	S715	PKA	GRIK2	Extracellular ligand gated channel
377	8.16	8.66	8.33	S397	PKA	VTN	Extracellular matrix protein
248	7.94	8.44	7.92	S314	nd	C5aR	G protein coupled receptor
100	7.38	9.54	8.34	S222	PKA	PTGER4	G protein coupled receptor
766	8.67	9.47	8.87	S262	PKC	ADRB2	G protein coupled receptor
510	8.61	9.00	8.57	S373	PKC	BKR2	G protein coupled receptor
55	6.76	8.71	7.08	S123	CDC2	Rab5B	GTPase
638	7.21	8.46	7.25	S179	PKA	RAP1B	GTPase
373	8.22	8.69	8.22	S939	AKT	TSC2	GTPase activating protein
940	8.20	8.97	8.30	S484	CDC2	RAP1GAP1	GTPase activating protein
230	7.39	9.10	7.24	S499	PKA	RAP1GAP1	GTPase activating protein
690	8.38	8.96	8.65	S176	PKA	RGS10	GTPase activating protein
210	7.72	9.79	8.53	T17	CAMK2	PLN	Integral membrane protein
108	8.07	9.84	8.79	S16	PKA	PLN	Integral membrane protein
691	8.69	9.19	8.67	S368	nd	CXN43	Intercellular channel
689	8.79	9.48	8.84	S233	PKC	CNX32	Intercellular channel
382	8.11	8.85	8.16	S2808	PKA;CAMK2	RYR2	Intracellular ligand gated channel
946	7.19	8.13	7.16	S686	PKC	CFTR	Membrane transport protein
234	8.00	8.39	8.13	S687	PKC	MEP1B	Metallo protease
878	7.27	8.43	7.74	S21	ERK1/2	PPARA	Nuclear receptor

Spot	C	TP	TP+GGA	P-Site	Up Kin	Prot	Function
411	5.38	7.11	6.18	S117	nd	GAL1	Peptide hormone
938	6.02	7.58	6.29	S1064	PKC	INSR	Receptor tyrosine kinase
369	8.97	9.43	9.13	S199	PKA	NIPP1	RNA binding protein
747	7.21	7.90	7.27	S473	AKT	AKT	Serine/threonine kinase
885	7.18	7.98	7.17	S364	AKT;SGK1	B-Raf	Serine/threonine kinase
438	6.43	7.44	6.84	S159	CK1	CDK5	Serine/threonine kinase
696	7.82	8.25	7.91	S196	MAPK4	MAPK4	Serine/threonine kinase
505	7.43	8.08	7.63	S685	PKA	ADRBK1	Serine/threonine kinase
629	6.39	7.11	6.19	S428	PKA	B-Raf	Serine/threonine kinase
697	7.52	7.91	7.49	S29	PKC	ADRBK1	Serine/threonine kinase
445	7.28	8.09	7.63	S645	PKCD	PKCD	Serine/threonine kinase
624	7.80	8.42	7.82	S738	PKCmu	PKD1	Serine/threonine kinase
324	8.38	10.49	9.43	S114	PRKAR2B	PRKAR2B	Serine/threonine kinase
632	10.20	11.05	10.57	S48	RSK1	PPP1R3A	Serine/threonine phosphatase
427	7.90	8.66	7.94	S22	CDC2	LMNA	Structural protein
132	7.55	8.31	7.10	Y24917	nd	TTN	Structural protein
163	9.11	9.59	8.73	S157	TTN	TCAP	Structural protein
117	8.22	9.72	8.87	S197	AKT	AFX1	Transcription factor
213	7.92	8.24	8.02	T32	AKT	FOXO3A	Transcription factor
599	6.93	7.74	6.93	T494	CDK2	B-Myb	Transcription factor
942	6.75	7.67	7.12	S408	CDK5	MEF2A	Transcription factor
380	7.92	8.88	8.21	S146	CK2	SPIB	Transcription factor
474	6.90	9.30	7.16	S355	ERK5	MEF2A	Transcription factor
881	7.38	7.62	7.07	S623	PKA	NOLC1	Transcription factor
433	9.13	9.84	9.19	S132	PKC	VPS72	Transcription factor
99	8.90	9.61	8.63	S412	PRKG1	TFII-I	Transcription factor
944	7.58	8.23	7.82	S780	CCND1	RB1	Transcription regulatory protein
746	8.60	8.92	8.65	S305	PAK1	ERA	Transcription regulatory protein
587	7.54	8.81	7.36	S365	PKA	WT1	Transcription regulatory protein
492	8.16	8.61	8.09	S1050	PRKACA	CIITA	Transcription regulatory protein
954	7.19	8.16	7.40	S1042	PKA	ABCA1	Transport/cargo protein
750	8.36	8.86	8.47	S313	PKC	STXBP1	Transport/cargo protein
331	8.06	8.67	7.87	Y319	ZAP70	ZAP70	Tyrosine kinase
378	7.69	9.25	8.02	S83	PKA	PTPN7	Tyrosine phosphatase
381	9.38	10.49	9.57	S10	PKA	SYN2	Unclassified
426	8.85	9.47	8.94	S36	PKCA	NRGN	Unclassified
883	7.84	9.09	7.65	S488	PKCT	WIP	Unclassified
255	6.25	6.99	6.62	S331	PKA	AQP0	Water channel

* All substrate intensities are log2 normalized data

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Supplemental information

Supplementary Table 1

Overview of substrates reduced in phosphorylation upon tachypacing

Spot	C	TP	TP+GGA	P-Site	Up Kin	Prot	Function
777	7.45	6.40	7.05	Y192	EGFR	STAM2	Adapter molecule
841	7.81	7.09	7.63	Y627	INSR	GAB1	Adapter molecule
513	8.50	7.88	7.82	Y629	INSR	IRS2	Adapter molecule
5	8.31	8.11	7.61	Y95	nd	SH2D3A	Adapter molecule
321	6.41	5.60	6.48	Y142	BRK	CTNNB1	Adhesion molecule
786	6.84	5.45	6.71	T102	CK2B	CTNNB1	Adhesion molecule
876	8.00	7.56	8.32	S147	PTPP	SPP1	Adhesion molecule
845	7.10	6.25	6.52	Y196	Lyn	CD79B	B cell antigen receptor
828	9.16	8.58	8.32	Y453	Lck	CD5	CD antigen
53	8.29	8.06	7.64	S54	CDK2	CDC6	Cell cycle control protein
50	7.56	6.94	7.22	S90	PKA	CCND1	Cell cycle control protein
773	7.85	7.12	7.24	Y478	nd	PKP4	Cell junction protein
148	6.90	5.84		Y531	Lyn	CD19	Cell surface receptor
467	6.05	4.90	6.28	T436	nd	TNFR2	Cell surface receptor
181	6.48	5.61	5.94	S564	CK2	CANX	Chaperone
975	7.22	6.23	6.77	Y20	TYK	FABP4	Chaperone
923	8.52	7.64	8.38	S22	nd	FGA	Coagulation factor
790	8.62	8.02	8.19	T1031	CK1	C3	Complement protein
552	8.58	7.95	8.22	S206	CK2	C1R	Complement protein
811	7.21	6.14	7.43	S56	CDC2	VIM	Cytoskeletal protein
285	7.45	7.01	7.16	T555	ROCK1	DPYSL2	Cytoskeletal protein
916	7.43	6.50	7.36	Y28	ABL	RAD9	DNA repair protein
554	9.06	8.36	8.54	S116	CDC2	CDC25A	Dual specificity phosphatase
173	5.54	4.63	5.34	S19	CK2	PIN4	Enzyme: Isomerase
569	8.37	8.07	8.12	S320	PKCA	p47-phox	Enzyme: Oxidase
333	8.48	8.06	7.89	Y771	Lyn	PLCG1	Enzyme: Phospholipase
833	7.38	6.81	6.82	Y753	Lyn	PLCG2	Enzyme: Phospholipase
729	6.77	6.15	6.39	S61	nd	NME3	Enzyme: Phosphotransferase
785	7.72	7.15	7.42	T475	PKC	PFKFB2	Enzyme: Phosphotransferase
588	7.98	7.54	7.66	Y391	nd	ST6GAL1	Enzyme: Sialyltransferase
842	7.19	6.30	6.87	Y1252	Fyn	NR2B	Extracellular ligand gated channel
827	9.39	8.27	8.41	S1416	PKC	GRIN2A	Extracellular ligand gated channel
801	7.44	7.04	6.70	S236	nd	FPRL1	G protein coupled receptor
273	7.70	7.01	7.51	T312	PKA	MC4R	G protein coupled receptor
926	8.65	8.19	8.26	S374	PKC	PTGER4	G protein coupled receptor
702	6.66	6.22	6.31	S53	PKA	GMFB	Growth factor
216	7.41	6.48	6.74	Y142	EGFR	VAV2	Guanine nucleotide exchange factor
203	7.35	5.98	7.10	Y172	EGFR	VAV2	Guanine nucleotide exchange factor
129	7.11	6.36	6.95	Y174	Lck	VAV1	Guanine nucleotide exchange factor
257	6.89	6.28	6.80	T140	nd	CRYAA	Heat shock protein
998	8.27	7.45	8.37	S335	CK2	PSEN2	Integral membrane protein
29	9.36	8.51	7.73	T723	PKC	APLP2	Integral membrane protein
985	5.88	4.93	5.95	S380	AKT	PTEN	Lipid phosphatase
60	7.13	5.69	6.24	S14	CK2	SYN1A	Membrane transport protein
645	9.22	8.53	8.65	Y37	EGFR	SCAMP1	Membrane transport protein
389	8.80	8.17	8.36	Y41	EGFR	SCAMP3	Membrane transport protein
509	7.08	6.50	6.79	S491	PKA	GLUT2	Membrane transport protein
94	8.31	7.99	7.38	T702	PKCA	TRPV6	Membrane transport protein

Spot	C	TP	TP+GGA	P-Site	Up Kin	Prot	Function
281	7.60	6.75	7.51	S22	nd	STATH	nd
88	7.94	7.27	7.11	S1604	ALK	ALK	Receptor tyrosine kinase
779	7.82	7.27	7.65	Y721	CHK1	KIT	Receptor tyrosine kinase
12	7.84	7.11	6.97	Y596	EphA4	EphA4	Receptor tyrosine kinase
464	8.41	7.67	7.78	Y614	EphB3	EphB3	Receptor tyrosine kinase
961	7.55	6.44	7.41	Y1248	HER2	HER2	Receptor tyrosine kinase
14	7.41	5.62	5.96	Y280	FGFR1	FGFR1	Receptor tyrosine kinase
258	7.27	6.70	6.52	Y591	FLT3LG	FLT3	Receptor tyrosine kinase
962	6.77	5.85	6.48	Y1349	HGFR	HGFR	Receptor tyrosine kinase
194	6.60	5.69	6.49	Y568	KIT	KIT	Receptor tyrosine kinase
515	6.57	5.63	6.64	Y754	PDGFRA	PDGFRA	Receptor tyrosine kinase
271	7.68	6.74	7.43	Y751	SHP2	PDGFRB	Receptor tyrosine kinase
504	8.39	8.01	7.63	S366	nd	SSB	RNA binding protein
972	6.24	4.30	5.01	Y154	nd	PRSS1	Serine protease
87	5.93	4.81	6.14	Y718/T838	ASK1	ASK1	Serine/threonine kinase
65	8.07	7.19	7.21	S370	CDC2	CK2A1	Serine/threonine kinase
221	7.89	7.16	7.66	T68	CHK2	CHK2	Serine/threonine kinase
664	8.00	7.02	7.46	Y246	FES	BCR	Serine/threonine kinase
259	6.32	5.72	6.36	Y90	Lck	PKCT	Serine/threonine kinase
343	6.76	6.20	6.70	T189	LKB1	LKB1	Serine/threonine kinase
288	7.26	6.83	7.05	T219	MEK5	ERK5	Serine/threonine kinase
153	6.92	6.31	6.91	S207	nd	MAP2K6	Serine/threonine kinase
42	8.91	8.37	8.52	S19	PKA	CHK2	Serine/threonine kinase
850	7.62	7.39	6.76	T508	ROCK1	LIMK1	Serine/threonine kinase
578	7.63	6.55	6.70	Y130	Src	PAK2	Serine/threonine kinase
322	7.63	7.21	6.88	Y432	Src	PKCM	Serine/threonine kinase
138	6.01	4.68	6.06	Y658	SYK	PKCA	Serine/threonine kinase
56	7.68	7.27	7.40	S19	MLCK	MLC2	Structural protein
592	8.19	7.75	8.09	Y29	nd	CRYBB3	Structural protein
387	7.44	6.45	7.21	Y398	BTK	TFII-I	Transcription factor
85	8.35	7.73	7.77	T142	CK2	HSF1	Transcription factor
169	6.78	4.84	6.77	S11	CK2	MAX	Transcription factor
316	6.61	5.70	6.96	S263	CK2	N-myc	Transcription factor
780	6.78	6.02	6.55	Y641	IL4	STAT6	Transcription factor
834	8.28	7.83	7.87	Y679	Src	STAT5B	Transcription factor
38	8.11	7.52	7.60	S664	ATM	CTIP	Transcription regulatory protein
282	8.19	7.14	8.00	S784	ATM	p53BP1	Transcription regulatory protein
522	7.08	6.50	7.03	Y537	Src	ERA	Transcription regulatory protein
932	6.52	5.23	6.88	S116	AKT;CAMK2	PEA15	Transport/cargo protein
809	8.67	7.73	8.46	S465	AKT	ABL	Tyrosine kinase
416	7.76	6.96	7.08	Y223	BTK;ABL	BTK	Tyrosine kinase
963	7.79	6.61	7.42	Y397	FAK	FAK	Tyrosine kinase
74	7.96	7.03	7.14	Y420	Fyn	TXK	Tyrosine kinase
340	9.06	8.36	8.03	Y577	Src	FAK	Tyrosine kinase
769	7.49	6.24	7.21	Y152	INSR	PTPN1	Tyrosine phosphatase
925	7.49	6.88	7.40	T16	CK2A1	HCLS1	Unclassified
63	9.66	9.37	8.91	S929	PKC	PAM	Unclassified

* All substrate intensities are log2 normalized data

Supplementary Table 2

Overview of substrates enhanced in phosphorylation upon tachypacing

Spot	C	TP	TP+GGA	P-Site	Up Kin	Prot	Function
500	8.35	8.66	8.15	S59;S60	SDK1	14-3-3B	Adapter molecule
268	6.14	6.96	6.79	Y174	SYK	3BP2	Adapter molecule
325	7.77	9.12	7.85	Y273	ZAP70	LNK	Adapter molecule
188	5.91	7.46	6.82	S1076	CAMK2	ADCY3	Adenylate cyclase
666	7.35	8.19	7.14	S1152	p90RSK	L1CAM	Adhesion molecule
700	7.50	8.01	7.83	S2523	CAMK2	FLNA	Anchor protein
128	9.52	10.23	9.81	S451	GSK3	ACLY	ATPase
435	7.64	8.02	7.93	S26	PKC	ANXA2	Calcium binding protein
432	6.94	7.79	6.71	S1068	CDC2	RBL2	Cell cycle control protein
184	6.36	6.78	6.48	S20	CDC2	RRM2	Cell cycle control protein
680	7.48	8.00	7.45	S151	CDK2	hCDH1	Cell cycle control protein
374	7.86	9.04	8.33	S964	CDK4	RBL1	Cell cycle control protein
118	6.51	8.46	7.18	S1035	CDK4	RBL2	Cell cycle control protein
943	9.02	9.48	9.12	S336	GRK	CCR5	Cell surface receptor
570	5.90	6.82	6.37	S303	PKA	NGFR	Cell surface receptor
441	6.00	7.63	6.48	S285	PKC	F3	Coagulation factor
667	8.72	10.09	8.97	T80	nd	CAPN1	Cysteine protease
437	7.70	8.08	8.01	S724	CDC2	CALD1	Cytoskeletal protein
1018	5.81	7.31	7.33	S100	PKA	PPP1R9B	Cytoskeletal protein
127	8.87	9.48	9.06	S693	PKCA	ADD3	Cytoskeletal protein
174	8.13	8.78	8.07	S72	ROCK1	VIM	Cytoskeletal protein
879	7.76	9.47	8.50	S417	AKT	HTT	DNA binding protein
670	8.28	8.67	8.08	T463	CCNB2	KIF22	DNA binding protein
918	7.88	8.38	7.75	T53	CDC2	HMGA1	DNA binding protein
730	8.11	8.59	7.98	S273	CDK2	ORC1	DNA binding protein
540	4.53	5.44		S11	nd	H3F3A	DNA binding protein
1024	4.81	5.50		S66	CK2A1	LIG1	DNA ligase
481	5.54	6.70	6.28	S336	nd	RAD9A	DNA repair protein
952	7.61	8.10	7.52	S222	p74RAF-1	MEK1	Dual specificity kinase
507	5.72	7.20	6.73	T48	CDC2	CDC25C	Dual specificity phosphatase
990	7.32	7.93	7.55	S198	PLK1	CDC25C	Dual specificity phosphatase
685	7.99	8.26	7.84	S80	AMPK	ACC	Enzyme: Carboxylase
154	5.68	7.55	5.65	S293	PDK1	PDHA1	Enzyme: Dehydrogenase
899	7.99	8.40	8.03	Y989	TK	OGT	Enzyme: Glucosaminyltransferase
428	6.32	7.37	6.50	S99	CDC2	dUTPase	Enzyme: Hydrolase
497	6.81	7.46	7.77	S272	MAPK2	ALOX5	Enzyme: Lipase
753	6.80	7.59	7.67	S554	nd	HSL	Enzyme: Lipase
605	8.64	8.89	8.26	T154	PKC	NCF4	Enzyme: Oxidase
650	6.28	6.67	6.94	Y33	JNK1	WWOX	Enzyme: Oxidoreductase
877	8.97	10.00	9.00	S294	AKT	PDE3A	Enzyme: Phosphodiesterase
637	8.66	9.55	8.81	S133	PKA	PDE4B	Enzyme: Phosphodiesterase
798	7.79	8.32	8.05	S14	PKA	PDE4D	Enzyme: Phosphodiesterase
254	8.41	8.79	8.09	S9	PKC	CNPase	Enzyme: Phosphodiesterase
170	7.92	8.59	8.21	S561	PKCA	PLD1	Enzyme: Phosphodiesterase
410	8.42	10.37	9.15	S1018	PHKA1	PHKA1	Enzyme: Phosphorylase
695	8.86	9.19	8.70	S483	AKT	PFKFB2	Enzyme: Phosphotransferase
107	7.68	8.16	7.87	S872	PKA	HMG1	Enzyme: Reductase
45	6.59	8.21	7.12	S1177	AKT	NOS3	Enzyme: Synthetase
816	6.08	6.87	6.57	S21	SGK3	GSK3B	Enzyme: Synthetase
959	8.33	8.89	8.76	S645	GSK3	GYS1	Enzyme: Synthetase

Spot	C	TP	TP+GGA	P-Site	Up Kin	Prot	Function
299	6.97	8.03	7.43	S715	PKA	GRIK2	Extracellular ligand gated channel
377	8.16	8.66	8.33	S397	PKA	VTN	Extracellular matrix protein
248	7.94	8.44	7.92	S314	nd	C5aR	G protein coupled receptor
100	7.38	9.54	8.34	S222	PKA	PTGER4	G protein coupled receptor
766	8.67	9.47	8.87	S262	PKC	ADRB2	G protein coupled receptor
510	8.61	9.00	8.57	S373	PKC	BKR2	G protein coupled receptor
55	6.76	8.71	7.08	S123	CDC2	Rab5B	GTPase
267	5.79	6.51	6.30	Y66	EphB2	RRAS	GTPase
638	7.21	8.46	7.25	S179	PKA	RAP1B	GTPase
373	8.22	8.69	8.22	S939	AKT	TSC2	GTPase activating protein
940	8.20	8.97	8.30	S484	CDC2	RAP1GAP1	GTPase activating protein
230	7.39	9.10	7.24	S499	PKA	RAP1GAP1	GTPase activating protein
690	8.38	8.96	8.65	S176	PKA	RGS10	GTPase activating protein
210	7.72	9.79	8.53	T17	CAMK2	PLN	Integral membrane protein
108	8.07	9.84	8.79	S16	PKA	PLN	Integral membrane protein
765	5.78	6.78	6.80	S10	PKA	STOM	Integral membrane protein
1017	8.40	9.06	8.77	S340	PKC	OCLN	Integral membrane protein
691	8.69	9.19	8.67	S368	nd	CXN43	Intercellular channel
689	8.79	9.48	8.84	S233	PKC	CNX32	Intercellular channel
382	8.11	8.85	8.16	S2808	PKA;CAMK2	RYR2	Intracellular ligand gated channel
946	7.19	8.13	7.16	S686	PKC	CFTR	Membrane transport protein
349	9.52	9.86	9.17	T695	PKC	GRM1	Membrane transport protein
379	5.70	6.91	6.50	S63	PKC	KEL	Membrane transport protein
234	8.00	8.39	8.13	S687	PKC	MEP1B	Metallo protease
878	7.27	8.43	7.74	S21	ERK1/2	PPARA	Nuclear receptor
411	5.38	7.11	6.18	S117	nd	GAL1	Peptide hormone
636	4.64	5.40	5.86	Y812	FGF2	FGFR2	Receptor tyrosine kinase
938	6.02	7.58	6.29	S1064	PKC	INSR	Receptor tyrosine kinase
369	8.97	9.43	9.13	S199	PKA	NIPP1	RNA binding protein
747	7.21	7.90	7.27	S473	AKT	AKT	Serine/threonine kinase
885	7.18	7.98	7.17	S364	AKT;SGK1	B-Raf	Serine/threonine kinase
438	6.43	7.44	6.84	S159	CK1	CDK5	Serine/threonine kinase
696	7.82	8.25	7.91	S196	MAPK4	MAPK4	Serine/threonine kinase
276	4.35	5.01	6.62	T180	MST2	TPK4	Serine/threonine kinase
360	5.97	6.62	7.22	T320	PDPK1	SGK3	Serine/threonine kinase
505	7.43	8.08	7.63	S685	PKA	ADRBK1	Serine/threonine kinase
629	6.39	7.11	6.19	S428	PKA	B-Raf	Serine/threonine kinase
697	7.52	7.91	7.49	S29	PKC	ADRBK1	Serine/threonine kinase
445	7.28	8.09	7.63	S645	PKCD	PKCD	Serine/threonine kinase
624	7.80	8.42	7.82	S738	PKCmu	PKD1	Serine/threonine kinase
324	8.38	10.49	9.43	S114	PRKAR2B	PRKAR2B	Serine/threonine kinase
632	10.20	11.05	10.57	S48	RSK1	PPP1R3A	Serine/threonine phosphatase
427	7.90	8.66	7.94	S22	CDC2	LMNA	Structural protein
171	8.81	9.35	8.43	S23	CDC2	LMNB1	Structural protein
874	6.61	7.71	7.70	S11	CK2	CLTB	Structural protein
132	7.55	8.31	7.10	Y24917	nd	TTN	Structural protein
163	9.11	9.59	8.73	S157	TTN	TCAP	Structural protein
117	8.22	9.72	8.87	S197	AKT	AFX1	Transcription factor
213	7.92	8.24	8.02	T32	AKT	FOXO3A	Transcription factor
180	5.39	6.47	6.37	S387	BMK1	MEF2C	Transcription factor
599	6.93	7.74	6.93	T494	CDK2	B-Myb	Transcription factor
595	5.73	7.03	6.63	T518	CDK2	B-Myb	Transcription factor
858	5.83	6.32	5.06	S315	CDK2	p53	Transcription factor

Spot	C	TP	TP+GGA	P-Site	Up Kin	Prot	Function
942	6.75	7.67	7.12	S408	CDK5	MEF2A	Transcription factor
425	6.35	7.26	7.15	S142	CK2	MAX	Transcription factor
380	7.92	8.88	8.21	S146	CK2	SPIB	Transcription factor
823	5.71	6.28	6.65	S329	DYRK1A	FOXO1A	Transcription factor
166	5.72	6.74	6.99	S274	EphB2	NEUROD1	Transcription factor
422	7.70	8.14	7.28	S668	EphB2	TFII-I	Transcription factor
474	6.90	9.30	7.16	S355	ERK5	MEF2A	Transcription factor
978	5.31	6.30	6.43	T38	HGF	ETS1	Transcription factor
300	4.95	6.56	6.27	S536	IKKB	NFKB3	Transcription factor
559	6.90	7.30	7.31	S103	KCNQ1	SRF	Transcription factor
799	5.34	6.17	6.13	T293	MAPK14	MEF2C	Transcription factor
580	5.28	7.32	6.63	Y343	nd	HOXA10	Transcription factor
101	6.20	7.38	6.96	S451	nd	RUNX2	Transcription factor
442	4.88	6.89	6.48	S294	PKA	NFATC1	Transcription factor
881	7.38	7.62	7.07	S623	PKA	NOLC1	Transcription factor
433	9.13	9.84	9.19	S132	PKC	VPS72	Transcription factor
99	8.90	9.61	8.63	S412	PRKG1	TFII-I	Transcription factor
944	7.58	8.23	7.82	S780	CCND1	RB1	Transcription regulatory protein
866	4.92	6.12	6.96	S165	CDC2	PTTG1	Transcription regulatory protein
103	5.94	6.77	6.58	S422	MAP3K7	SMAD3	Transcription regulatory protein
622	5.66	7.28	6.81	S266	MAPK14	PGC1A	Transcription regulatory protein
746	8.60	8.92	8.65	S305	PAK1	ERA	Transcription regulatory protein
587	7.54	8.81	7.36	S365	PKA	WT1	Transcription regulatory protein
492	8.16	8.61	8.09	S1050	PRKACA	CIITA	Transcription regulatory protein
489	7.45	7.86	7.72	S1881	CCNB1	NUP210	Transport/cargo protein
954	7.19	8.16	7.40	S1042	PKA	ABCA1	Transport/cargo protein
750	8.36	8.86	8.47	S313	PKC	STXBP1	Transport/cargo protein
897	7.34	7.73	7.62	Y91	TXK	TXK	Tyrosine kinase
331	8.06	8.67	7.87	Y319	ZAP70	ZAP70	Tyrosine kinase
378	7.69	9.25	8.02	S83	PKA	PTPN7	Tyrosine phosphatase
381	9.38	10.49	9.57	S10	PKA	SYN2	Unclassified
426	8.85	9.47	8.94	S36	PKCA	NRGN	Unclassified
883	7.84	9.09	7.65	S488	PKCT	WIP	Unclassified
255	6.25	6.99	6.62	S331	PKA	AQP0	Water channel

* All substrate intensities are log2 normalized data

Chapter 5

Activation of Histone Deacetylase-6 (HDAC6) induces contractile dysfunction through derailment of α-tubulin proteostasis in experimental and human Atrial Fibrillation

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Abstract

Background

Atrial Fibrillation (AF) is the most common persistent clinical tachyarrhythmia, characterized by structural remodeling resulting in electric dissociation, contractile dysfunction and AF progression. Recent research showed histone deacetylases (HDACs) to cause derailment of cardiomyocyte proteostasis and subsequent structural and contractile remodeling. The role of HDACs in AF is unknown. Therefore, we investigated the involvement of different HDACs in tachypacing-induced contractile dysfunction in experimental and clinical AF.

Methods and Results

Tachypacing of HL-1 atrial cardiomyocytes and the *Drosophila* pupae heart significantly impaired contractile function (amplitude of Ca^{2+} transients (CaT) and heart wall contractions, respectively), which was prevented by inhibition of HDAC6 (Tubacin) and Sirtuins (Nicotinamide). Tachypacing induced a specific activation of HDAC6, resulting in α-tubulin deacetylation, depolymerization and degradation by calpain and subsequent disruption of the microtubule structure. Tachypacing-induced contractile dysfunction was completely rescued by dominant negative HDAC6 mutants with loss of deacetylase activity in the second catalytic domain, which bears α-tubulin deacetylase activity (TDAC). Atrial tissue from patients with AF also showed a significant increase in HDAC6 activity and a reduction in both acetylated and total α-tubulin, which correlated inversely with calpain activity.

Conclusion

AF induces loss of contractile function through HDAC6 activation and subsequent derailment of α-tubulin proteostasis and disruption of the cardiomyocyte microtubule structure. HDAC6 action is conveyed via its TDAC domain, and therefore offers excellent opportunities to design specific TDAC-inhibitors to conserve proteostasis and attenuate cardiomyocyte remodeling in AF.

Introduction

Atrial Fibrillation (AF) is the most common persistent clinical tachyarrhythmia and a significant contributor to cardiovascular morbidity and mortality (Dobrev et al., 2012). It is widely acknowledged that the progressive nature of AF hampers the effective functional conversion to sinus rhythm in patients and explains the limited efficacy of current drug therapies (Dobrev et al., 2012). Therefore, research is directed at preventing new-onset and the recurrence of AF by limiting the development of substrates underlying AF promotion, i.e. the molecular changes that cause structural remodeling and drive the development and progression of the arrhythmia (Allessie et al., 2010). Once known, druggable targets in these pathways will be identified to modify the atrial substrate ("upstream therapy") (Meijering et al., 2012).
Several observations suggest a prime role for epigenetic regulation in AF promotion. Epigenetic regulation refers primarily to processes that influence the packaging of the chromatin of the nuclear DNA, thus controlling the on/off states of multiple genes with a single switch thereby modifying the homeostasis of protein production, degradation and function known as cellular proteostasis (Balch et al., 2008;Haberland et al., 2009). Epigenetic dysregulation causes a derailment of proteostasis, which was found to result in many systemic diseases, including cardiovascular disorders (Balch et al., 2008). Importantly, the packaging of chromatin is largely dependent on the acetylation status of histones (Jenuwein and Allis, 2001), which is controlled by histone acetyl transferases and deacetylases (HATs and HDACs, respectively). Evidence for epigenetic regulation in AF originates from observations that the (re)activation of the fetal gene program in cardiomyocytes promotes AF (Ausma et al., 1997;Thijssen et al., 2004). Furthermore, genetically modified mice with increased HDAC activity reveal substrates for AF, such as reductions in connexin 40 expression, cardiac hypertrophy and fibrosis, and consequently were prone to develop atrial arrhythmia (Liu et al., 2008). Thus, HDACs substantially affects cardiomyocyte proteostasis and may represent a substrate for AF.

Notably, protein acetylation modification is not only confined to histones, but also affects many cytoplasmic proteins, including structural proteins such as α-tubulin (Hubbert et al., 2002;Matsuyama et al., 2002;Xu et al., 2013). Alpha-tubulin is a key component of the microtubule network and its acetylation influences microtubular composition and organization, thereby modulating Ca^{2+} signaling and contractility (Gomez et al., 2000;Gupta et al., 2008). Thus, α-tubulin acetylation regulates cardiomyocyte contractile function and structure, and may represent a second mechanism by which HDACs influence cardiomyocyte proteostasis and AF progression (Balch et al., 2008).

Recently, substantial progress is made in understanding the crucial role of HDACs in cardiac development and pathogenesis (Bush and McKinsey, 2009;Haberland et al., 2009). Eighteen mammalian HDACs are classified into four classes based on their structure, complex formation, and expression pattern: Class I (HDAC1, 2, 3 and 8), class IIa (HDAC4, 5, 7 and 9), class IIb (HDAC6 and 10), class III (Sirt1 to 7) and class IV (HDAC11) (Haberland et al., 2009). Various HDAC inhibitors are developed which reveal promising effects on attenuation of cardiac hypertrophy, fibrosis and contractile dysfunction (Liu et al., 2008;Haberland et al., 2009). Their role in prevention of the AF substrate is unknown. The aim of the present study is to identify HDACs that are involved in the induction of structural and functional remodeling which underlie AF progression in experimental and human AF. Hereto, both the established HL-1 cardiomyocyte model (Brundel et al., 2006;Ke et al., 2008) and *Drosophila melanogaster* model (Bier and Bodmer, 2004;Wessells et al., 2004;Zhang et al., 2011) were used and findings were verified in human AF. Comparable to human AF, both models develop cardiac contractile dysfunction and structural remodeling, and therefore have been utilized successfully to identify novel therapeutic targets (Neely et al., 2010;Zhang et al., 2011). In the models and in human AF, HDAC activity and acetylation of proteins was determined, revealing a specific induction of HDAC6 activity and α-tubulin deacetylation. Furthermore, we demonstrated HDAC6 to result in a TDAC domain dependent deacetylation, depolymerization and calpain-mediated degradation of α-tubulin and subsequent disruption of the microtubular network, thereby causing contractile dysfunction. Therefore, our results indicate that inhibition of the TDAC domain of HDAC6 is a promising upstream therapeutic target to conserve α-tubulin proteostasis and attenuate cardiomyocyte remodeling in AF.

Materials and Methods

2.1 HL-1 cardiomyocyte tachypacing, transfection, drugs and calcium transient measurements

HL-1 cardiomyocytes derived from adult mouse atria were obtained from Dr. William Claycomb (Louisiana State University, New Orleans, LA, USA) and cultured as previously described (Brundel et al., 2006;Ke et al., 2008). The cardiomyocytes were normal paced (1Hz) or tachypaced (5Hz) with a C-Pace100^{TM}-culture pacer (IonOptix Corporations). Calcium transient measurements were performed as previously described (Brundel et al., 2006;Ke et al., 2008). Before pacing, HL-1 cardiomyocytes were treated with HDAC inhibitors described in Table 1. All inhibitors were obtained from Sigma. The non-active analogue Niltubacin (1µM) was added as a control for Tubacin. To study the effect of HDAC6 on CaT, HL-1 cardiomyocytes were transfected with pcDNA3.1+ (empty plasmid), HDAC6 wild type (wt), HDAC6 mutant in the first catalytic domain (HDAC6 m1), HDAC6 mutant in the second catalytic domain (HDAC6 m2), or HDAC6 mutant in both domains (HDAC6 m1-2), by the use of Lipofectamin 2000 (Invitrogen). The HDAC6 plasmids were a kind gift from Dr. Alexander Bershadsky.

2.2 Drosophila stocks, tachypacing and heart wall contraction assays

Wild type W1118 strains were obtained from Genetic Services Inc., Massachusetts. All flies were maintained at 25^{o}C on standard medium. After fertilization, adult flies were removed and HDAC inhibitors (Table 1) were added into the medium containing fly embryos (Figure 2 A I). After 2 days, pre-pupae were selected for tachypacing as previously described (Zhang et al., 2011). Briefly, transparent pupae were placed on 1% agarose gel in PBS (Figure 2 A II). Groups of 5 pupae were subjected to tachypacing (4Hz, 20min) by the use of a C-Pace100™-Culture Pacer (IonOptix Corporation). Before and after tachypacing, movies of whole pupae visualized through a microscope at 10x magnification were obtained in triplicate periods of 10 seconds. Heart wall contractions were analyzed using *image J* software.

2.3 Patients

Prior to surgery, one investigator assessed patient characteristics (Table 2) as described before (Ke et al., 2008). Right and left atrial appendages (RAAs and LAAs, respectively) were obtained from patients with paroxysmal (PAF) and permanent AF (PeAF) and control patients in sinus rhythm (SR). The PeAF and PAF group contained patients with lone AF or AF with underlying mitral valve disease (MVD). After excision, atrial appendages were immediately snap-frozen in liquid nitrogen and stored at

−80 °C. The study conforms to the principles of the Declaration of Helsinki. The Institutional Review Board approved the study, and patients gave written informed consent. Tissue was used to perform various assays. Unfortunately, the amount of tissue per patient was insufficient to be utilized for all the assays. However, at least n=3 samples per group were used for the individual tests, as indicated in the legend section.

2.4 Calpain activity measurement

The calpain activity measurement in human tissue was performed as described previously (Ke et al., 2008). Suc-Leu-Leu-Val-Tyr-7-amino-4-methyl-coumarin (AMC, Sigma), was used as calpain substrate. Protein extracts (25µg) were added to 20µM AMC in 300µl Tris-buffered saline. AMC release was measured by fluorometry (360nm excitation; 430nm emission, Spectrometer LS50B, PerkinElmer) after 30 minutes incubation at room temperature.

2.5 HDAC activity measurements

The overall HDAC activity was measured by utilizing a fluorimetric HDAC activity assay kit (Sigma) according to the manufacturer's instructions. Briefly, normal or tachypaced HL-1 cardiomyocytes or atrial tissue samples were lysed in non-denaturing buffer (CelLytic™ MT Cell Lysis Reagent, Sigma). Protein concentrations were determined by Bio-Rad Protein Assay Kit (Bio-Rad). Fluorescence was measured with a fluorimeter (Bio-tek instruments FLx800) at 360nm excitation and 460nm emission wavelength. To test efficiency of HDAC inhibitors in HL-1 cardiomyocytes, lysates were incubated with the HDAC inhibitors as described in Table 1. Blank samples and samples with HeLa cell nuclear extracts were used as negative and positive controls respectively.

2.6 HDAC6 activity assay

The HDAC6 activity assay was developed as previously described with minor changes (Lemon et al., 2011). Atrial tissue samples were lysed in non-denaturing buffer (CelLytic™, Sigma) supplemented with protein inhibitor cocktail (Roche). HL-1 cardiomyocytes were lysed in PBS (pH7.4) containing Triton X-100 and protein inhibitor cocktail, followed by sonification and centrifugation. Protein concentrations were determined by Bio-Rad Protein Assay Kit (Biorad) and 30µg of HL-1 cardiomyocytes or 100µg of tissue extracts were diluted in PBS buffer (total volume 100µl) in a 96-well plate. HDAC6 inhibitor Tubacin (1µM) or vehicle was added (30 min at 37 °C). Then, 5µl of 1mM stock solution of synthetic HDAC class I/IIb substrate I-1875 (Bachem) was added (2.5hr at 37°C). The reaction was stopped with 50µl of stop solution (PBS with 1.5% Triton X-100, 3µM TSA, and 0.75mg/ml trypsin). AMC (Alfa Aesar) fluorescence was measured by a BioTek Synergy 4 plate reader (360nm excitation and

460nm emission). Background signals from buffer blanks were subtracted. To correct for quenching of the AMC signal by colored tissue extracts, an additional reference set of samples and buffer blanks with identical aliquots of AMC instead of substrate was included. The reference AMC signals were used to calculate quenching ratios relative to the AMC buffer blank signal. Each raw fluorescence value from the substrate-containing samples was divided by the corresponding quenching ratio, followed by subtraction of the appropriate substrate background. HDAC6 activity was calculated as the amount of HDAC activity blocked by HDAC6 inhibitor Tubacin.

2.7 Protein extraction and Western blot analysis

HL-1 cardiomyocytes or tissue samples were lysed in RIPA buffer as described before (Ke et al., 2008). In short, equal amounts of protein were separated on SDS-PAGE gels, transferred onto nitrocellulose membranes and probed with anti-acetylated α-tubulin (Sigma), anti-α-tubulin (Sigma), anti-human HDAC6 (Cell Signaling), anti-mouse HDAC6 (Cell Signaling), anti-HA tag (Sigma) or anti-GAPDH (Fitzgerald). Blots were subsequently incubated with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (Dako). Signals were detected by the ECL-detection method (Amersham) and quantified by densitometry (Syngene, Genetools).

2.8 Polymerized and depolymerized α-tubulin fractions and drug Treatment

Polymerized and depolymerized (soluble monomers) α-tubulin were fractionized as previously described with minor changes (Li et al., 2009). In brief, after one rinse in PBS, HL-1 cardiomyocytes were washed in microtubule-stabilizing buffer (MSB) (0,1M PIPES, pH 6.93, 1mM EGTA, 1mM $MgCl_2$, 2M glycerol) and then incubated for 3min with 0.5ml MSB containing 0.5% Triton X-100 and protein inhibitor cocktail (Roche). After incubation, the supernatant contained the soluble depolymerized α-tubulin fraction and was transferred to a new tube. The remaining HL-1 cardiomyocytes were washed once with MSB buffer and then lysed with RIPA buffer. The collected lysate contains the polymerized tubulin. For testing the effect of drugs on (de-)polymerization status of α-tubulin, 20μM calpain inhibitor, PD150606 (Calbiochem), was added to HL-1 cardiomyocytes 2h prior to tachypacing as described before (Ke et al., 2008). HDAC6 inhibitor, Tubacin (1μM), was added to cells 12h prior to tachypacing. HL-1 cardiomyocytes were tachypaced for 8h, followed by isolation of polymerized and depolymerized α-tubulin fractions as described above.

2.9 Statistical analysis

Results are expressed as mean ± SEM. All experimental procedures were performed in at least duplicate series. Student *t* tests were used for comparisons involving two groups. One-way ANOVA was used for multiple group comparisons. All *P* values were two-sided. $P<0.05$ was considered statistically significant.

Table 1 Overview protective effects of HDAC inhibitors in tachypaced HL-1 cardiomyocytes and *Drosophila melanogaster.*

	Class	*HDACs*	*Concentration in HL-1*	*Concentration in Drosophila*	*Protection in HL-1*	*Protection in Drosophila*
TSA	I IIa IIb IV	HDAC 1,2,3,8 HDAC 4,5,7,9 HDAC 6,10 HDAC 11	10nM	1mM	-	-
Sodium Butyrate	I IIa	HDAC 1,2,3,8 HDAC 4,5,7,9	2mM	20mM	-	-
Nicotinamide	III	Sirt1-7	10mM	100mM	+++	++
Tubacin	IIb	HDAC6	1µM	10µM	++	++

-, no significant protective effect; ++ $P<0.01$ vs control TP; +++ $P<0.001$ vs control TP.

Table 2 Baseline demographic and clinical characteristics of patients with paroxysmal AF (PAF), permanent AF (PeAF) and control patients in sinus rhythm.

	SR	PAF	PeAF
N	17	13	28
RAA (n)	17	13	25
LAA (n)	14	11	27
Age	58 ± 5	50 ± 3	61 ± 3
Duration of AF (median, range (months)	-	-	8 (0.1-56)
Duration of SR before surgery (median, range (days))	-	10 (0.5-210)	-
Duration of last episode AF (median, range h))	-	12 (0.2-24)	-
AF/day (median, range(%))	-	2 (0.2-70)	-
Underlying heart disease (n) and/or surgical procedure			
Lone AF/Maze	0	7	7
MVD/MV replacement/repair	17	6	21
Medication (n)			
ACE-ARB	10	5	15
Digoxin	1	1	11
Ca channel blocker	5	3	8
B-blocker	13	3	8

Values are presented as mean value ± SEM or number of patients Maze: atrial arrhythmia surgery; MVD: mitral valve disease.

Results

3.1 HDAC6 and Sirtuins mediate tachypacing-induced contractile dysfunction in cardiomyocytes and Drosophila.

We initially determined which HDAC classes were involved in cardiomyocyte remodeling by utilizing various HDAC inhibitors (Table 1) in tachypaced HL-1 cardiomyocytes and *Drosophila*. Hereto, HL-1 cardiomyocytes were pre-treated with an inhibitor, followed by tachypacing and assessment of calcium transients (CaT). Inhibition of HDAC6 by Tubacin and of Sirtuins by Nicotinamide protected against the tachypacing-induced reduction of CaT (Figure 1A, B; Supplementary movies S1-S7). In contrast, Niltubacin (the inactive analogue of Tubacin), and the pan-HDAC inhibitors, TSA and Sodium Butyrate, did not protect from CaT loss, despite being used at concentrations reducing overall HDAC activity (Figure 1C, D). These findings indicate that HDAC6 and Sirtuins are involved in tachypacing-induced cardiomyocyte remodeling in the *in vitro* HL-1 cardiomyocyte model for AF. To extend these findings to a second experimental model, the tachypaced *Drosophila* model for contractile dysfunction was used (Zhang et al., 2011). Non-treated *Drosophila* showed tachypacing-induced contractile dysfunction (Figure 2A, B). Similar to the findings in tachypaced HL-1 cardiomyocytes, inhibition of HDAC6 and Sirtuins attenuated tachypacing-induced contractile dysfunction in *Drosophila*, while TSA and Sodium Butyrate were ineffective (Figure 2A, B, Supplementary Figure S1, movies S8-S18). Moreover, both in HL-1 cardiomyocytes and *Drosophila*, tachypacing did not affect the overall HDAC activity significantly (Supplementary Figure S2A, B), supporting the activation of specific HDACs during tachypacing.

3.2 Tubulin catalytic domain (TDAC) of HDAC6 modulates the loss of CaT and microtubule network through deacetylation of α-tubulin.

As Tubacin is a specific HDAC6 inhibitor and protects against tachypacing-induced contractile dysfunction in both tachypaced HL-1 cardiomyocytes and *Drosophila*, we investigated whether HDAC6 becomes activated by tachypacing. Hereto, we measured HDAC6 activity and expression in tachypaced HL-1 cardiomyocytes. A significant increase in HDAC6 activity and expression (Figure 3A, B) was observed after 6 and 8 hours tachypacing. To conclusively prove HDAC6 to modulate tachypacing-induced CaT reductions, genetically modified HDAC6 was used. As HDAC6 contains two catalytic domains, we used dominant negative HDAC6 mutants to investigate which domain is involved in CaT modulation.

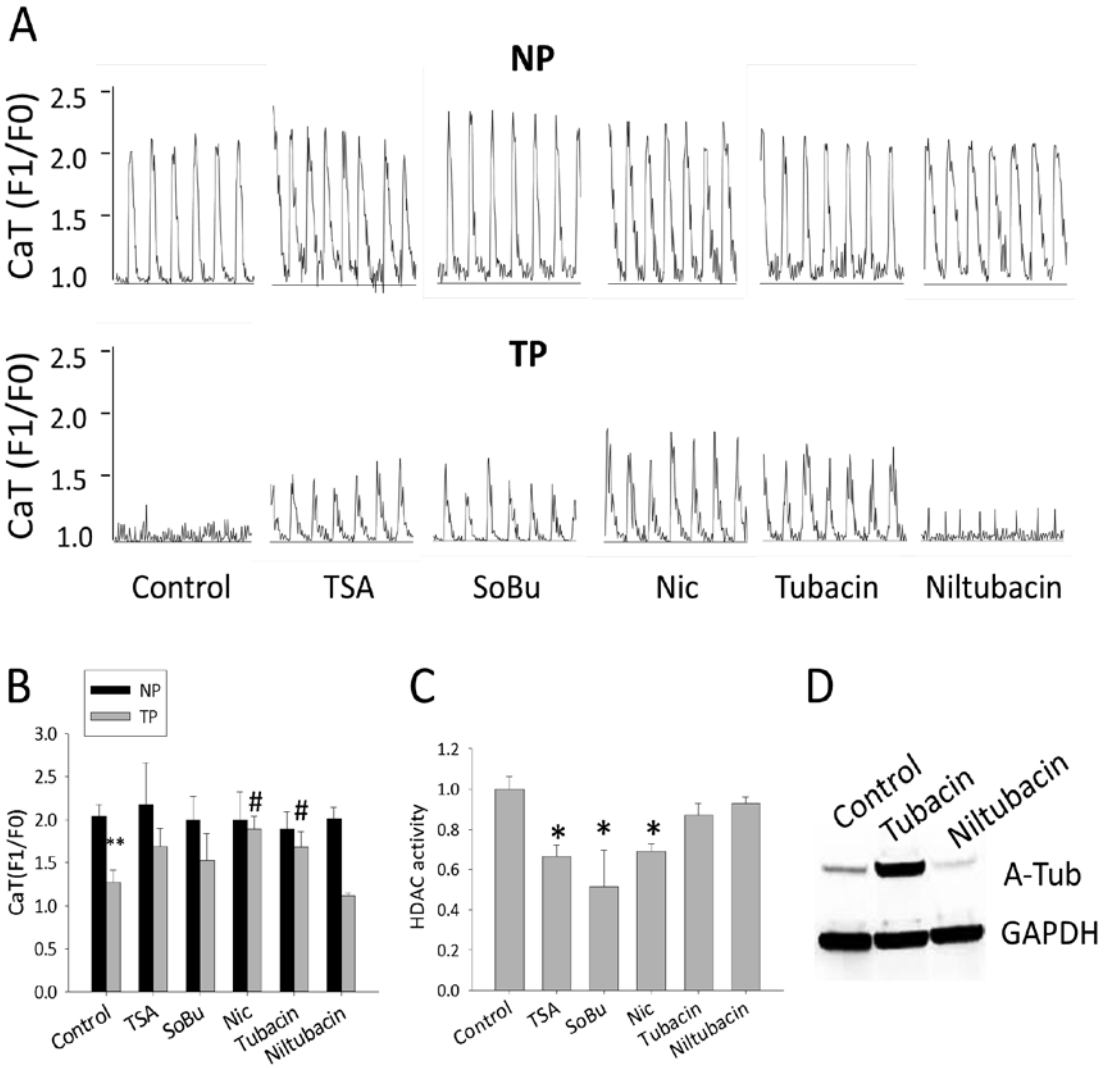

Figure 1 HDAC6 and Sirtuin inhibitors protect against CaT reductions in tachypaced HL-1 cardiomyocytes.
A Representative CaT of HL-1 cardiomyocytes after normal pacing (NP, 1 Hz) or tachypacing (TP, 5Hz). HL-1 cardiomyocytes were pre-treated with TSA, Sodium butyrate (SoBu), Nicotinamide (Nic), Tubacin or Niltubacin, followed by normal or tachypacing and measurement of CaT. **B** Quantified CaT amplitude of NP and TP HL-1 cardiomyocytes, each from groups indicated. ***P*<0.01 vs control NP; #*P*<0.05 vs control TP. n>=15 cardiomyocytes for each group. **C** Efficiency of pan-HDAC inhibitors was tested by using an HDAC activity assay and **D** the specific HDAC6 inhibitor Tubacin was tested by Western blot analysis of acetylated α-tubulin (A-Tub). Concentration of drugs used as mentioned in Table 1. **P*<0.05 vs control.

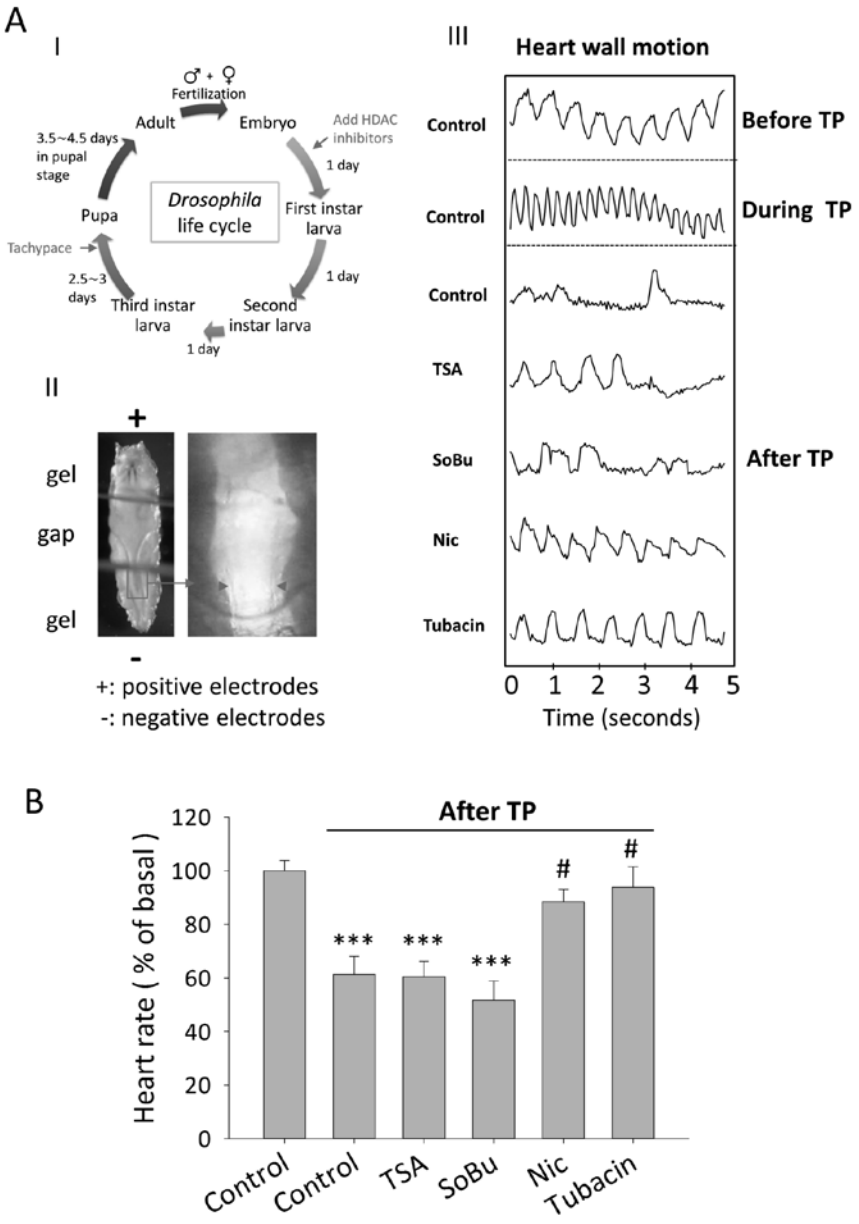

Figure 2 HDAC6 and Sirtuin inhibitors protect against tachypacing-induced contractile dysfunction in the heart wall of Drosophila melanogaster.

A) (I) HDAC inhibitors were added to the food and early pupae were selected for normal (NP) or tachypacing (TP) (arrows). (II) Early pupae were subjected to tachypacing by placing them on an agarose gel connected to electrodes, followed by monitoring of the heart wall contraction rate. (III) Representative heart wall contractions monitored before TP, during TP and after TP with or without pre-treatment with TSA, Sodium butyrate (SoBu), Nicotinamide (Nic), or Tubacin at concentrations as indicated in Table 1. **B** Quantified data showing heart wall contraction rates from each group as indicated. ***P<0.001 vs control before TP; #P<0.01 vs control TP, n=9-18 pupae for each group.

Hereto, HL-1 cardiomyocytes were transfected with HDAC6 constructs, including wild type (wt), and dominant negative mutants of HDAC6 with loss of deacetylase activity in the first catalytic domain (HDAC6 m1), second catalytic domain (HDAC6 m2), or both domains (HDAC6 m1-2). Successful transfection of the various constructs was assessed by Western blot (Figure 3C). HL-1 cardiomyocytes transfected with the control plasmid pCDNA3.1+, HDAC6 wt, and HDAC6 m1 persisted in significant reductions in CaT after tachypacing (Figure 3D, E). In contrast, the dominant negative HDAC6 m2 and HDAC6 m1-2 mutants rescued tachypaced HL-1 cardiomyocytes from CaT loss (Figure 3D, E). Our finding demonstrates that tachypacing-induced CaT reduction is mediated by the TDAC activity of HDAC6.

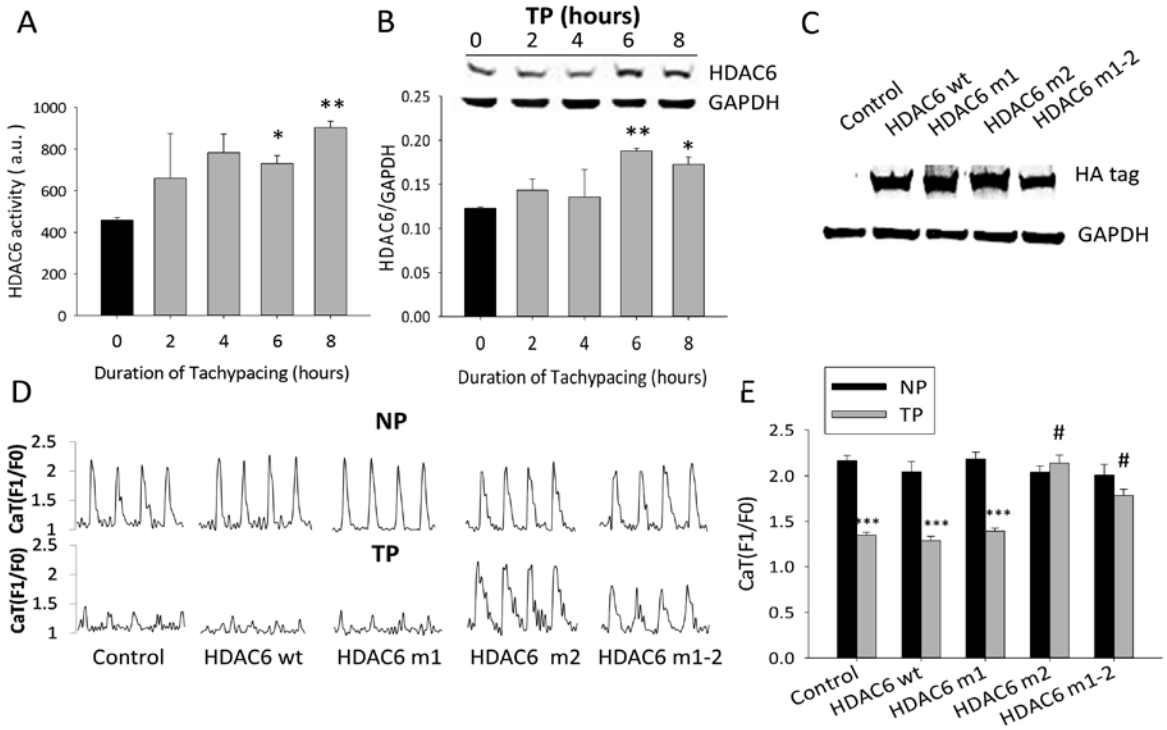

Figure 3 Tachypacing activates HDAC6 and inhibition of TDAC catalytic domain of HDAC6 rescues tachypacing-induced CaT loss in HL-1 cardiomyocytes.

A Time-course of tachypacing (TP)-induced HDAC6 activity. HDAC6 activity is significantly increased after 6h and 8h TP. **P*<0.05, ***P*<0.01 vs control 0h TP. **B** Western blot showing TP to increase HDAC6 expression level after 6h and 8h TP. **P* <0.05, ***P* <0.01 vs control 0h **C** Representative Western blot showing transfected HL-1 cardiomyocytes overexpressing the wild-type HDAC6 (HDAC6 wt), and the three mutants with impaired deacetylating activity i.e. HDAC6 m1, HDAC6 m2 or HDAC m1-2. Control cells are transfected with empty plasmid, pCDNA3.1. **D** Representative CaT of normal paced (NP) or TP HL-1 cardiomyocytes transfected with HDAC6 wt, HDAC6 m1, HDAC6 m2 or HDAC6 m1-2. **E** Quantitative data showing HL-1 cardiomyocytes transfected with HDAC6 m2 or HDAC6 m1-2 reveal protective effects against tachypacing-induced CaT reductions. ****P*<0.001 vs control NP, #*P*<0.001 vs control TP, n=32-80 cardiomyocytes for each group.

Since the TDAC domain of HDAC6 conveys the deacetylation of α-tubulin (Xu et al., 2013), it further suggests α-tubulin acetylation to be crucial for CaT regulation. To study the effect of tachypacing on α-tubulin acetylation, the level of acetyl α-tubulin during a time-course of tachypacing was determined. It was observed that tachypacing induces a significant reduction in α-tubulin acetylation after 6 hours (Figure 4A, B), which was followed by a significant reduction in total α-tubulin levels after 8 hours (Figure 4A, C). Tubacin attenuated both the tachypacing-induced deacetylation and degradation of α-tubulin (Figure 4D, E, F). The reduction in acetyl α-tubulin is specific, since no significant changes in the overall acetylation level of proteins or histones were observed between control and tachypaced HL-1 cardiomyocytes (supplemental data Figure S3).

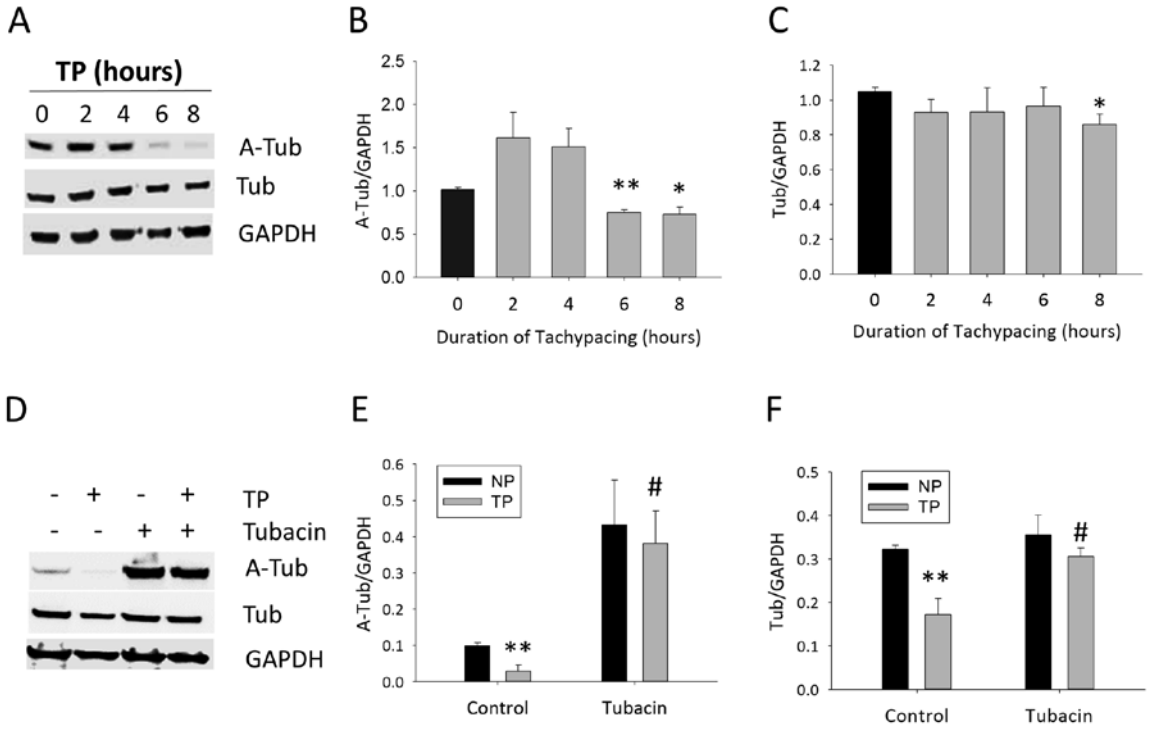

Figure 4: Tachypacing induces deacetylation and degradation of α-tubulin, which is prevented by HDAC6 inhibition. **A** Representative Western blot showing tachypacing (TP)-induced reductions in acetyl α-tubulin (A-Tub) and degradation of α-tubulin (Tub). **B** and **C**, Quantified data of 3 independent western blots showing a significant reduction in acetyl α-tubulin after 6h and α-tubulin after 8h tachypacing. **P*<0.05, ***P* <0.01 vs control 0h TP **D** Representative Western blot showing HDAC6 inhibition by Tubacin protects against tachypacing-induced deacetylation and degradation of α-tubulin. **E** and **F** Quantified data of 3 independent Western blots showing the amount of acetyl α-tubulin (**E**) and α-tubulin Tub (**F**) for the treatments as indicated. Control HL-1 cardiomyocytes were treated with DMSO (solvent of Tubacin). ***P*<0.01 vs Control NP. #*P*<0.01 vs Control TP.

Alpha-tubulin together with β-tubulin form the microtubule network, which are structural polymers supporting the architecture, contractile function of cardiomyocytes and also the active transport of cytoplasmic constituents, including mitochondria and endoplasmic reticulum, thereby controlling cell proteostasis and function (Verdel et al., 2000;Balch et al., 2008). Moreover, HDAC6-induced deacetylation of α-tubulin results in the depolymerization of microtubules (Matsuyama et al., 2002;Zilberman et al., 2009;Nam et al., 2010), and thereby regulates CaT (Gomez et al., 2000). Therefore, HDAC6 may cause tachypacing-induced CaT loss via modulation of the microtubule polymerization status. To this end, HL-1 cardiomyocytes were stained for α-tubulin and acetyl α-tubulin and the structure of microtubules was determined after normal pacing and tachypacing with or without HDAC6 inhibition. As expected, confocal microscopy showed tachypacing to significantly reduce the amount of total α-tubulin and acetyl α-tubulin, which was prevented by Tubacin (Figure 5A, B). In addition, tachypacing induced the disruption of the microtubule structure, since the amount of polymerized microtubules was significantly reduced (Figure 5A, B). Tubacin protected against tachypacing-induced depolymerization of microtubules (Figure 5A, B). To further confirm the role of HDAC6 in microtubule modulation, we separated polymerized from depolymerized microtubules in normal paced and tachypaced HL-1 cardiomyocytes. Tachypacing reduced the amount of polymerized and depolymerized α-tubulin (Figure 5C), which were both attenuated by Tubacin (Figure 5C). The observations indicate that tachypacing induces deacetylation and depolymerization of α-tubulin, and subsequent disruption of microtubules. Although acetyl α-tubulin is a substrate for both HDAC6 and Sirt2, the Sirtuin inhibitor Nicotinamide did not prevent tachypacing-induced deacetylation, depolymerization and degradation of α-tubulin (Supplementary Figure S4). Overall, the findings indicate a prime role for HDAC6 in tachypacing-induced cardiomyocyte remodeling through TDAC-induced deacetylation and depolymerization of α-tubulin and subsequent disruption of the microtubule network and loss of CaT.

3.3 Tachypacing induces calpain-mediated degradation of depolymerized α-tubulin

Our findings described above reveal tachypacing-induced HDAC6 activation to cause deacetylation of α-tubulin and subsequent depolymerization and disruption of microtubules, which underlie loss in CaT. Previous studies showed calpain to degrade depolymerized but not polymerized α-tubulin (Kawaguchi et al., 2003) and we previously demonstrated a key role for activated calpain in the degradation of contractile proteins in experimental and human AF (Iwata et al., 2005;Ke et al., 2008). Therefore, we tested if calpain caused the tachypacing-induced degradation of depolymerized α-tubulin as observed in Figure 4 and 5. Hereto, HL-1 cardiomyocytes were pre-treated with the calpain

inhibitor PD150606 and its effect on degradation of polymerized and depolymerized α-tubulin was determined. PD150606 mitigated tachypacing-induced degradation of total α-tubulin (Figure 6A). Further, PD150606 only attenuated the degradation of depolymerized α-tubulin without affecting the depolymerization status of microtubules or deacetylation of α-tubulin (Figure 6B, C and Supplementary Figure S5), suggesting that deacetylated and depolymerized microtubules are susceptible to accelerated degradation by calpain.

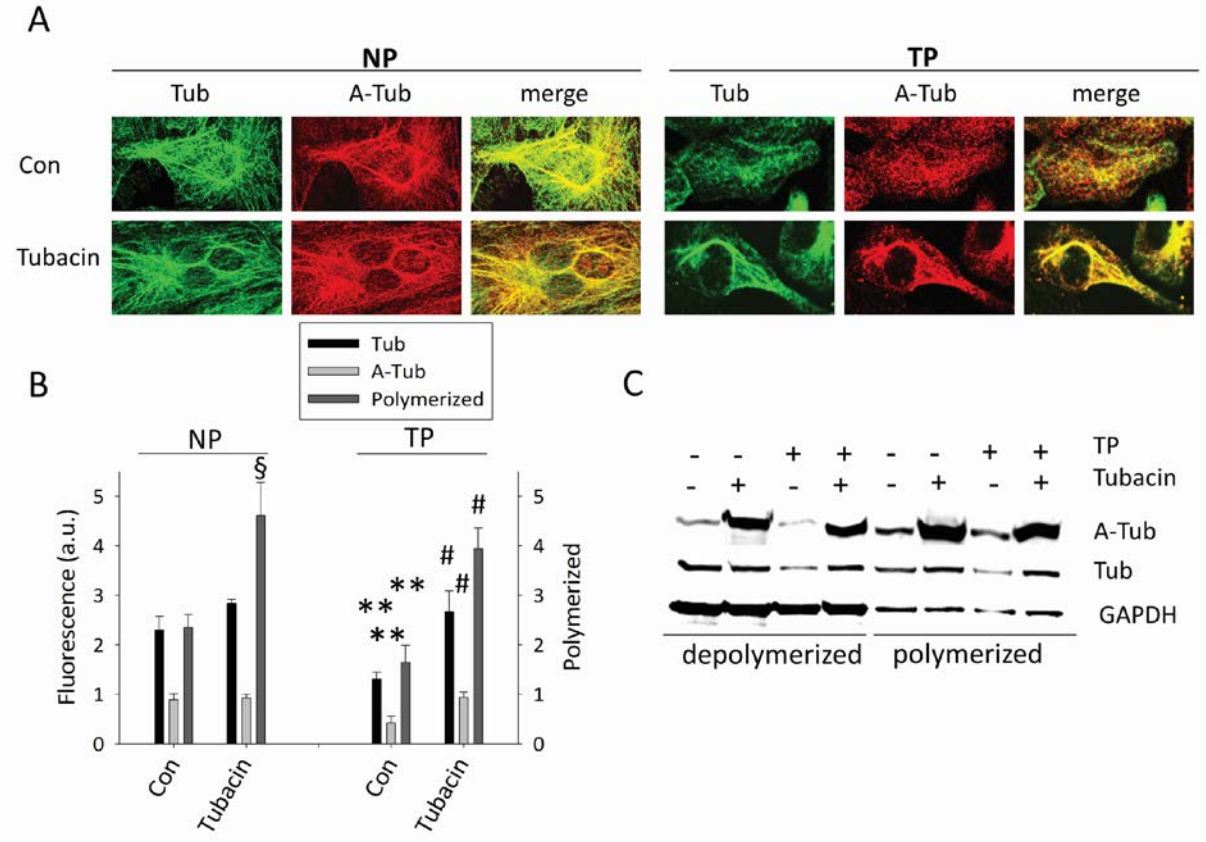

Figure 5 Inhibition of HDAC6 protects against tachypacing-induced degradation of α-tubulin and depolymerization of microtubules. **A** Typical example of an immunofluorescent staining of α-tubulin (Tub, green), acetyl α-tubulin (A-Tub, red) in normal paced (NP) and tachypaced (TP) HL-1 cardiomyocytes. Tachypacing induces depolymerization of microtubules (yellow) which is preserved by Tubacin. **B** Quantification of the amount of acetyl- and α-tubulin and polymerized microtubules in NP and TP HL-1 cardiomyocytes. TP significantly decreased the amount of acetyl- and α-tubulin and polymerized α-tubulin, which was prevented by Tubacin. Number of cardiomyocytes per condition: 100-250. **C** Representative Western blots showing tachypacing-induced reductions in polymerized α-tubulin and degradation of depolymerized α-tubulin (Tub), in line with decreased acetylation of polymerized and depolymerized α-tubulin (A-Tub). Tubacin protected against the depolymerization and degradation of α-tubulin, and revealed increased levels of acetyl α-tubulin. GAPDH was used to show successful separation of polymerized and depolymerized fractions. Control HL-1 cardiomyocytes were treated with DMSO (solvent of Tubacin). ***P*< 0.01 vs Control NP. #*P*< 0.01 vs Control TP. §*P*<0.01 vs control NP.

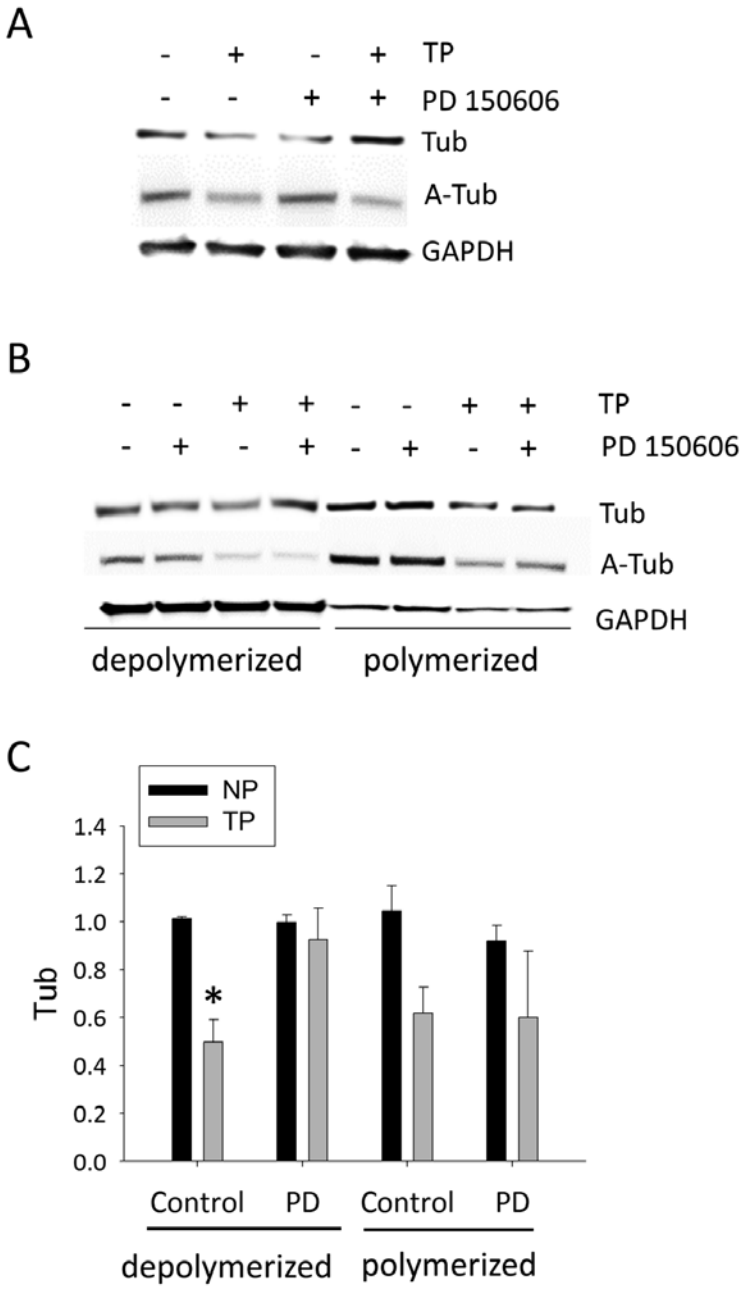

Figure 6 Calpain inhibitor PD150606 attenuates tachypacing-induced degradation of depolymerized α-tubulin. A Representative Western blot showing pre-treatment with calpain inhibitor PD150606 to attenuate tachypacing (TP)-induced α-tubulin (Tub) degradation. **B** Representative Western blot showing the effect of PD150606 on tachypacing-induced reductions in polymerized α-tubulin and degradation of depolymerized α-tubulin. Tachypacing-induced degradation of depolymerized α-tubulin is attenuated by PD150606. No effect of PD150606 on the amount of TP-induced depolymerization (Tub) or deacetylation of α-tubulin (A-Tub) was observed. GAPDH was used to indicate the successful separation of polymerized and soluble α-tubulin. **C** Quantification of three independent experiments showing TP-induced degradation of depolymerized α-tubulin, which was prevented by PD150606. *P<0.05 vs Control NP

Thus, our findings show that tachypacing induces HDAC6 activity, which mediates deacetylation of α-tubulin, and subsequent depolymerization of the microtubule network. As depolymerized α-tubulin is susceptible for accelerated degradation by calpain, HDAC6 finally results in structural disruption of the microtubule network and functional remodeling. In accord, inhibition of HDAC6 attenuates deacetylation, depolymerization and degradation of α-tubulin, resulting in the conservation of the microtubule network and maintenance of contractile function.

3.4 Increased HDAC6 activity modulates calpain-induced degradation of α-tubulin in patients with AF.

To investigate whether a similar modulating role of HDAC6 is also found in patients with AF, the amount of acetylated α-tubulin and total tubulin was determined in left and/or right atrial appendages (LAA and/or RAA) of patients with paroxysmal (PAF) or permanent AF (PeAF) and in controls with normal sinus rhythm (SR). A significant reduction in the amount of acetylated α-tubulin was observed in PAF and PeAF patients compared to patients with SR (Figure 7A, B). Furthermore, reduction of acetylated α-tubulin was more pronounced in LAA compared to RAA of patients with PeAF (Figure 7A, D). Further, similar to the findings in experimental models, both the total HDAC activity and overall acetylation level of proteins did not differ between patients with PeAF, PAF and SR (Supplementary Figure S2C, S3C). Also in human AF, total α-tubulin levels were significantly reduced in PAF and PeAF (Figure 7A, C, E), and α-tubulin correlated inversely with calpain activity in LAA (Figure 7F, G). Finally, HDAC6 activity and expression was determined in RAA and LAA of controls and PeAF patients. HDAC6 activity and expression were both significantly increased in patients with PeAF compared to SR (Figure 8A, B). Thus, the results in human AF are in line with the experimental findings in tachypaced HL-1 cardiomyocytes, and indicate a prime role for HDAC6 as a major α-tubulin deacetylating enzyme and inducer of derailed proteostasis by modulating α-tubulin degradation by calpain, which underlies the progression of AF.

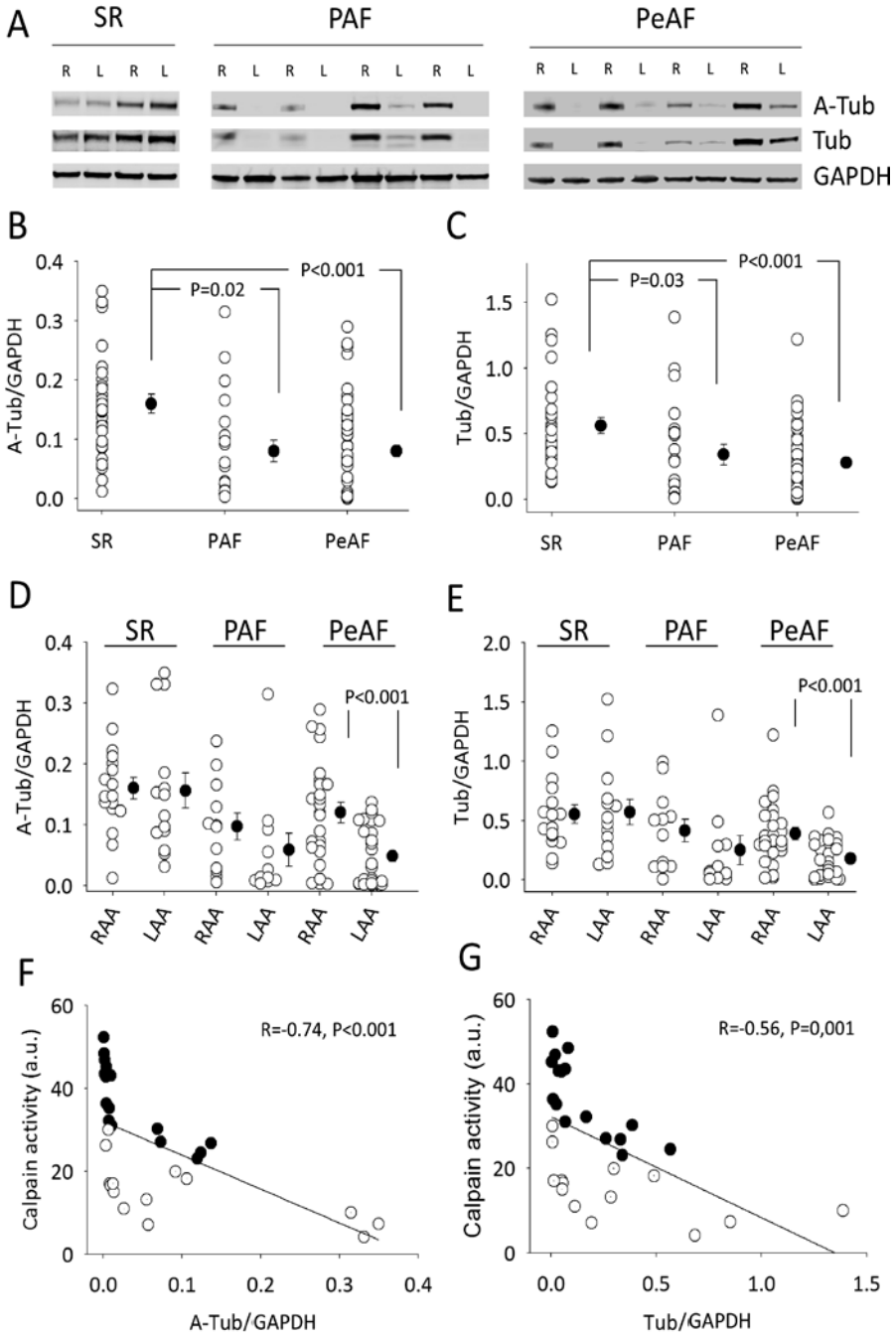

Figure 7 Patients with AF reveal reduced levels of acetylated and total α-tubulin, especially in the left atria. **A** Representative Western blot of α-tubulin (Tub) and acetyl α-tubulin (A-Tub) and GADPH in patients with PAF, PeAF and control SR. **B** Quantification of acetyl α-tubulin and **C** α-tubulin in patients with SR, PAF, and PeAF, showing a significant reduction in α-tubulin and acetyl α-tubulin in PAF and PeAF compared to SR. **D** Quantification of acetyl α-tubulin and **E** α-tubulin in RAA and LAA. In patients with PeAF, LAAs show significant reductions in acetyl and total α-tubulin compared to RAAs. (RAA, n=16 for SR, n=12 for PAF, n=25 for PeAF; LAA, n=14 for SR, n=11 for PAF, n=27 for PeAF) **F** Significant inverse correlation between tissue calpain activity and the amount of acetyl α-tubulin and **G** α-tubulin in LAA. (●) represents PAF (n=11), (ʘ) represents PeAF (n=16), (○) represents SR (n=3).

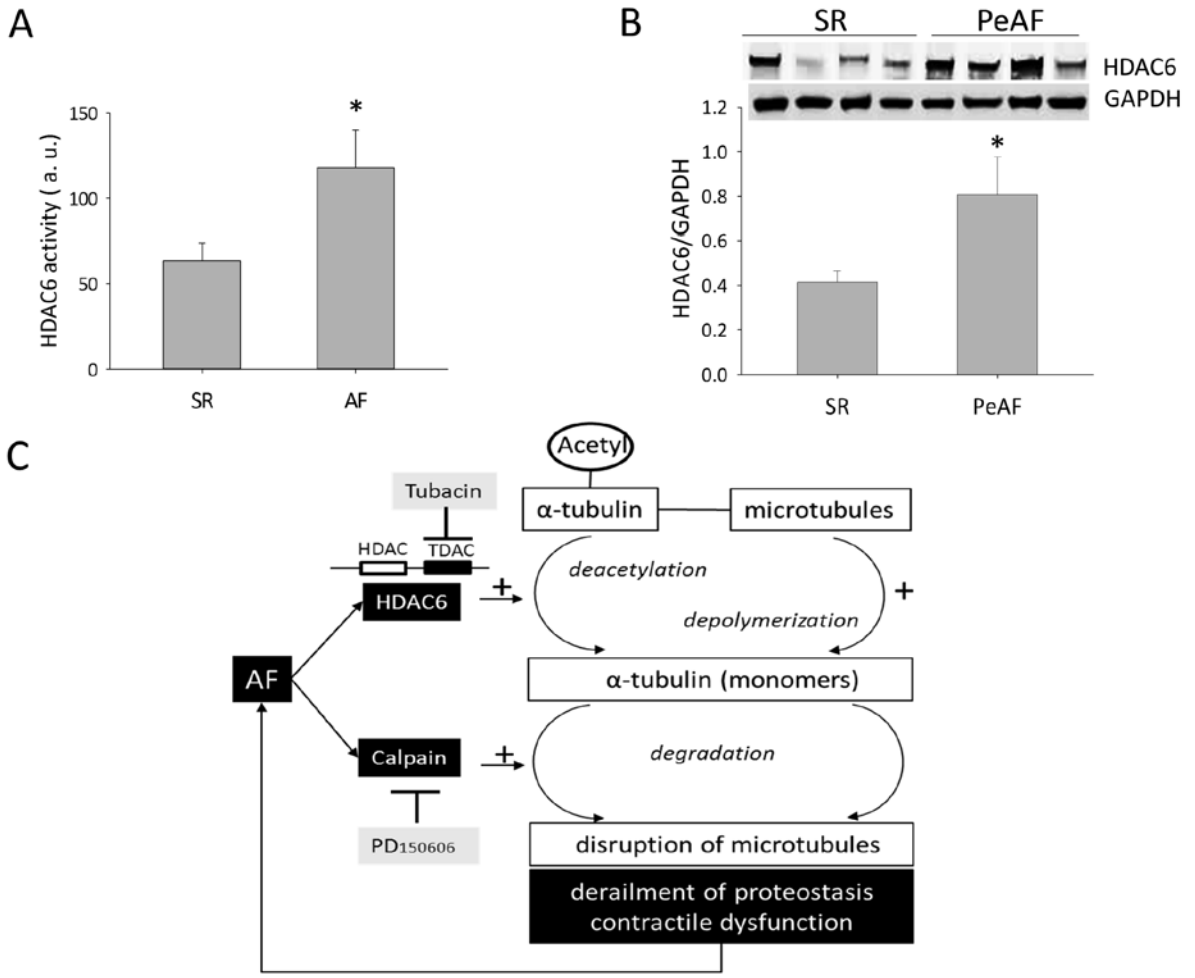

Figure 8 Patients with PeAF reveal induced HDAC6 activity compared to control patients in SR.
A HDAC6 activity is induced in PeAF (n=15) compared to SR (n=18). **P*<0.05 vs SR. **B** Top panel: representative Western blot of HDAC6 and GADPH expression levels in patients with PeAF and controls in SR. Bottom, quantified data showing a significant increase in HDAC6 expression in PeAF compared to SR (n=23 for SR, n=21 for PeAF). **C** Proposed model for AF-induced HDAC6 activity as a key enzyme in the derailment of α-tubulin proteostasis, microtubule disruption and contractile dysfunction, which underlies AF progression. HDAC6 causes α-tubulin deacetylation and subsequent depolymerization of microtubules into monomeric α-tubulin, which are degraded by calpain. Tubacin blocks TDAC activity of HDAC6 and thereby prevents the initial α-tubulin deacetylation and further downstream effects of derailment. PD150606 blocks activation of calpain, thereby preventing degradation of α-tubulin, but not AF-induced deacetylation and depolymerization of microtubules.

Discussion

Here, we identify HDAC6 as a key enzyme in the development of a substrate underlying AF development and progression. Tachypacing of HL-1 cardiomyocytes increases HDAC6 activity and expression, resulting in a TDAC domain dependent deacetylation, depolymerization and calpain-mediated degradation of α-tubulin and subsequent disruption of the microtubule network, thereby causing contractile dysfunction (proposed model Figure 8C). Consistent with our experimental data, patients with permanent AF reveal induced HDAC6 activity and expression, and also induced deacetylation and degradation of α-tubulin, which inversely correlates with calpain activity. HDAC6 inhibition by Tubacin conserved the microtubule structure and prevented depolymerized α-tubulin from degradation by calpain. Together, our results identify inhibition of the TDAC domain of HDAC6 as a promising upstream therapeutic target to conserve α-tubulin proteostasis and attenuate cardiomyocyte remodeling in AF.

4.1 Prime role for HDAC6 in AF-induced loss of proteostatic control

The current study identifies HDAC6 as the most prominent deacetylase and key enzyme of proteostasis derailment, which underlies structural and functional remodeling and AF progression (Meijering et al., 2012). HDAC6 is a member of the class IIb HDACs and is essentially cytoplasmic (Verdel et al., 2000). HDAC6 deacetylates α-tubulin (Hubbert et al., 2002), which impairs control of cell proteostasis by depolymerization of the microtubule network (Matsuyama et al., 2002;Zilberman et al., 2009;Nam et al., 2010). This function of HDAC6 was found to be involved not only in protein misfolding diseases, such as Parkinson's (Kawaguchi et al., 2003) and Huntington's disease (Iwata et al., 2005), but also in cancer (Witt et al., 2009). Furthermore, HDAC6 is the only cytosolic HDAC with two catalytic domains (Boyault et al., 2007), one of those is mainly responsible for its TDAC activity (Kolodney and Elson, 1995;Haberland et al., 2009). Several lines of evidence suggest that HDAC6 promotes AF by deacetylation of α-tubulin. First, the current study clearly shows tachypacing-induced remodeling to be fully prevented by treatment with the specific HDAC6 inhibitor Tubacin, a drug previously found to be mainly directed against the TDAC domain of HDAC6, thereby promoting acetylation of α-tubulin with no effect on other proteins including histones (Xu et al., 2013). Indeed, also others showed that inhibition of HDAC6 by Tubacin, significantly increases the acetylation of α-tubulin (Zilberman et al., 2009). In accord, we observed inhibition of HDAC6 by Tubacin to exclusively change acetylation status of α-tubulin, without an effect on overall protein or histone acetylation. Secondly, dominant negative mutation of the TDAC domain of HDAC6, the domain conveying deacetylation of α-tubulin, prevented tachypacing-induced reduction of

CaT, while dominant negative mutation of the histone acetylating domain was without protective effect. In accord with these findings in experimental AF, we found increased HDAC6 activity in patients with permanent AF to coincide with deacetylation of α-tubulin. Together, the findings implicate that HDAC6 mediated deacetylation of α-tubulin plays a prime role in cardiomyocyte remodeling and AF progression.

Further to this, we identified depolymerization of the microtubule network as the next downstream step conveying the action of tachypacing-induced HDAC6 activation underlying AF progression. Our results demonstrate HDAC6-induced deacetylation of α-tubulin to promote a shift from the polymerized microtubule structure into its depolymerized form. Also, we show deacetylated and depolymerized α-tubulin susceptible to accelerated degradation by the protease calpain. Accordingly, in patients with permanent AF, we found activation of HDAC6, next to deacetylation and degradation of α-tubulin, which correlated inversely with calpain activity. These results are consistent with previous reports, demonstrating that microtubule network disruption resulted in changed calcium signaling of cardiomyocytes, including reductions in contractions (Kolodney and Elson, 1995), L-type calcium current (Galli and DeFelice, 1994) and CaT amplitude (Leach et al., 2005), which is known to underlie AF progression (Meijering et al., 2012). Besides, the involvement of calpain is in accord with our previous observations that calpain is strongly activated during experimental and clinical AF (Brundel et al., 2002;Ke et al., 2008;Zhang et al., 2011) and with data showing degradation of brain α-tubulin by calpain (Van Wagoner, 2008).

Since Tubacin and Nicotinamide inhibit HDACs involved in deacetylation of α-tubulin, HDAC6 and Sirt2 respectively, and both HDACs were reported to form a cytoplasmic complex that functions in concert (North et al., 2003), it would be conceivable that this molecular mechanism also underlies the cardioprotective effect of Nicotinamide. However, in contrast to previous studies (North et al., 2003), Nicotinamide did not prevent deacetylation and depolymerization of α-tubulin after tachypacing, indicating other mechanism(s) to convey its cardioprotective effect, such as increasing NAD+ bioavailability (Yang and Sauve, 2006), which was found to be depleted in AF (Kalifa et al., 2008). Moreover, it is unclear what member(s) of the Sirtuin family mediate the protective effects of Nicotinamide (Yang et al., 2007). Further research is warranted to elucidate the molecular mechanism(s) involved in Nicotinamide mediated cardioprotection.

Taken together, our data imply that inhibition of HDAC6 represents a novel upstream target for the conservation of the microtubule network and contractile function in AF.

4.2 Therapeutic implications

Pharmacological approaches preventing or limiting the substrate for the promotion of AF are being studied ("upstream therapy"), with the aim to identify useful therapeutic agents in treating AF (Dobrev et al., 2012). So far, the efficacy of commonly used drugs on remodeling is limited (Dobrev et al., 2012). There are strong indications that loss of proteostatic control in cardiomyocytes represents an important substrate for the development and progression of AF (Meijering et al., 2012). The current study shows HDAC6-induced α-tubulin deacetylation and microtubule disruption to play a prime role in AF. Therefore, pharmacological inhibition of the major α-tubulin deacetylating enzyme, HDAC6, seems to represent a promising target for upstream therapy. The present study shows the beneficial effect of the specific HDAC6 inhibitor Tubacin. Since Tubacin has low druglikeness and is not suitable for in vivo studies (Butler et al., 2010), other promising HDAC6 inhibitors, such as Tubastatin A and ACY-1215 have been recently developed, which revealed beneficial effects in mice models for neurodegenerative diseases and cancer (Butler et al., 2010;d'Ydewalle et al., 2011;Santo et al., 2012). Pan-HDAC inhibitors, such as sodium butyrate and TSA, both inhibiting class I and II HDAC activity, have been shown to block agonist and pressure-overload induced cardiac hypertrophy (Liu et al., 2008). In addition, in homeobox gene overexpressing mice, TSA was shown to attenuate fibrosis and susceptibility to atrial arrhythmia (Liu et al., 2008). In contrast to these studies, we did not observe a protective effect of TSA and sodium butyrate. The discrepancies might be due to different signaling pathways underlying the hypertrophic versus tachypacing-induced response in cardiomyocytes. Moreover, pan-HDAC inhibitors have toxic off-target effects, which were found to abolish cardioprotection (Rivieccio et al., 2009). Unlike pan-HDAC inhibitors, specific inhibition of HADC6 has not been associated with any serious toxicity, making it an excellent drug target (Witt et al., 2009).

In summary, the current study demonstrates that AF activates HDAC6, leading to deacetylation, depolymerization and subsequent degradation of α-tubulin by calpain. As a consequence, the microtubule structure becomes disrupted resulting in contractile dysfunction which comprises a further substrate for the progression of AF. Therefore, the major α-tubulin deacetylating enzyme, HDAC6, represents a promising upstream therapeutic target to conserve cardiomyocyte structure and function in AF.

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Supplemental information

Supplemental Methods

5.1 Slot Blotting and Western Blotting

Slot blot analysis was performed as described previously (Brundel et al., 2001). In short, frozen RAA and LAA of patients, pre-pupae of *Drosophila* or HL-1 cardiomyocytes were lysed in RIPA buffer. Protein concentration was measured according to the Bradford method (Bio-Rad, The Netherlands). Equal amounts (10µg) of heat-denatured protein were used for Western blotting or spotted on nitrocellulose membranes (Stratagene) by the use of a slot blot apparatus (Bio-Rad) and checked by staining with Ponceau S solution (Sigma). After blocking with skim milk, membranes were incubated with primary antibody against acetyl lysine (Cell Signaling), acetyl Histone 3 (Cell Signaling), or GAPDH (Fitzgerald), followed by incubation with secondary HRP-conjugated anti-rabbit antibody (Amersham). Signals were detected by the ECL-detection method (Amersham) and quantified by densitometry (Syngene Genetools).

5.2 In vitro calpain mediated α-tubulin degradation assay

To measure α-tubulin degradation by calpain *in vitro,* α-tubulin was isolated from HL-1 cardiomyocytes by immunoprecipitation with non-denaturing lysis buffer (Tris HCl pH 8, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40 (NP-40), and 2 mM EDTA). A/G beads (Santa Cruz) were coated with α-tubulin antibody (Sigma) to pull down α-tubulin from HL-1 cardiomyocyte lysate. To detach α-tubulin from the beads, the beads were incubated with elution buffer (0.2M glycine pH2.5) at room temperature for 10min. After centrifugation, beads were removed and the eluate containing α-tubulin was transferred to a new tube, followed by adjustment to physiological pH by adding neutralization buffer (1M Tris PH9.5, 1/10 v/v of elution buffer). To detect degradation of α-tubulin by calpain, calpain I (Merck/Calbiochem) was incubated with the same amount of α-tubulin eluate in reaction buffer (PBS with 2mM $CaCl_2$) for 1 hour at room temperature. After incubation, loading buffer (10% SDS, 50% glycerol, 0.33M Tris HCl pH=6.8, 10% beta-mercaptoethanol, 0.05% bromophenol blue) was added to the tube followed by 5 min boiling. Western blot analysis was used to detect α-tubulin.

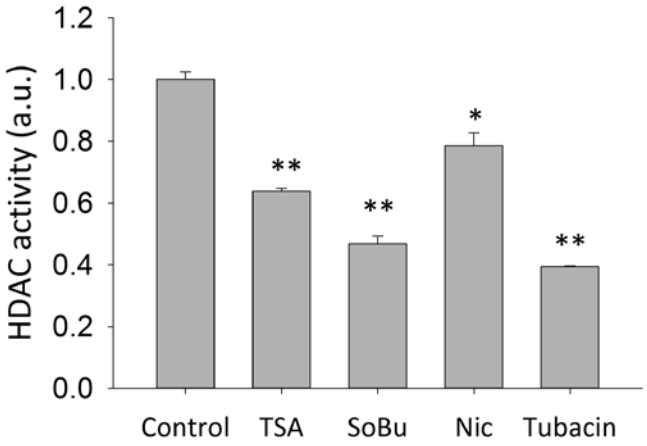

Figure S1 Efficacy of HDAC inhibitors to reduce overall HDAC activity in Drosophila.
All the HDAC inhibitors significantly reduced HDAC activity at the concentrations used as described in Table 1. *P<0.05 vs control, **P<0.01 vs control (without HDAC inhibitor).

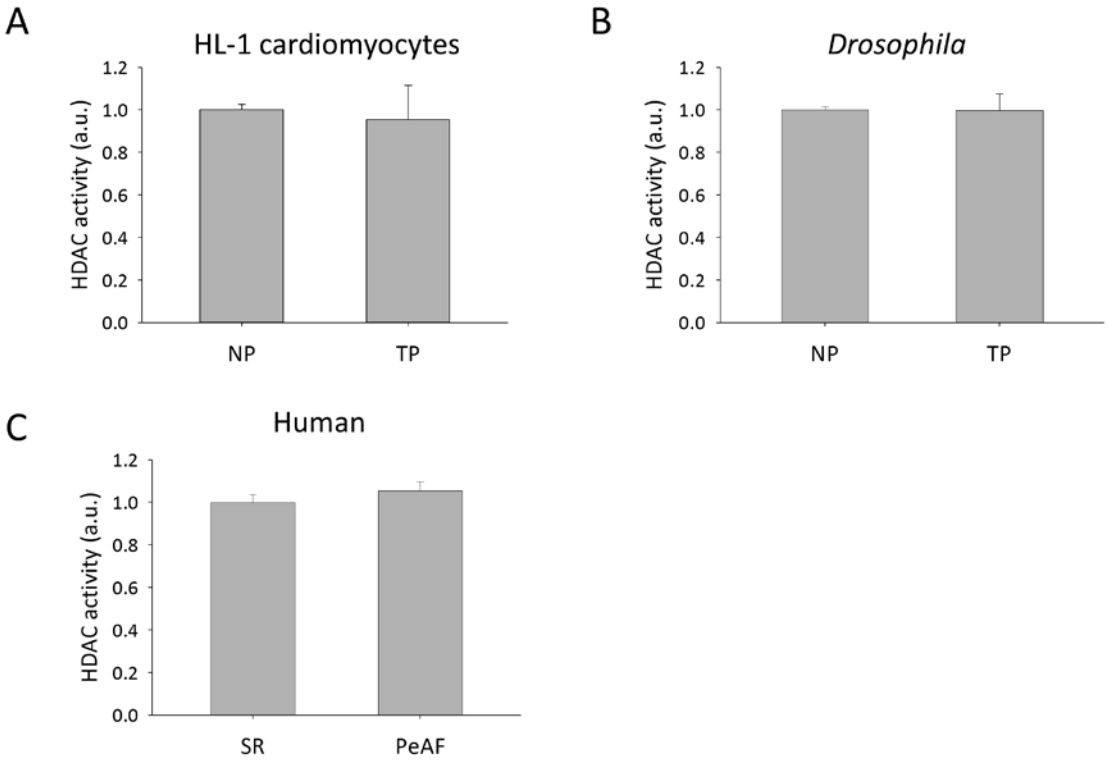

Figure S2 No significant changes in overall HDAC activity after tachypacing of HL-1 cardiomyocytes, Drosophila and in clinical AF.
Overall HDAC activity was tested using the HDAC activity assay kit. No changes in HDAC activity were observed between **A** normal (NP) and tachypaced (TP) HL-1 cardiomyocytes and **B** *Drosophila* and **C** between patients in normal sinus rhythm (SR, n=6) and permanent AF (PeAF, n=6). Experiments were performed in at least in duplicate series.

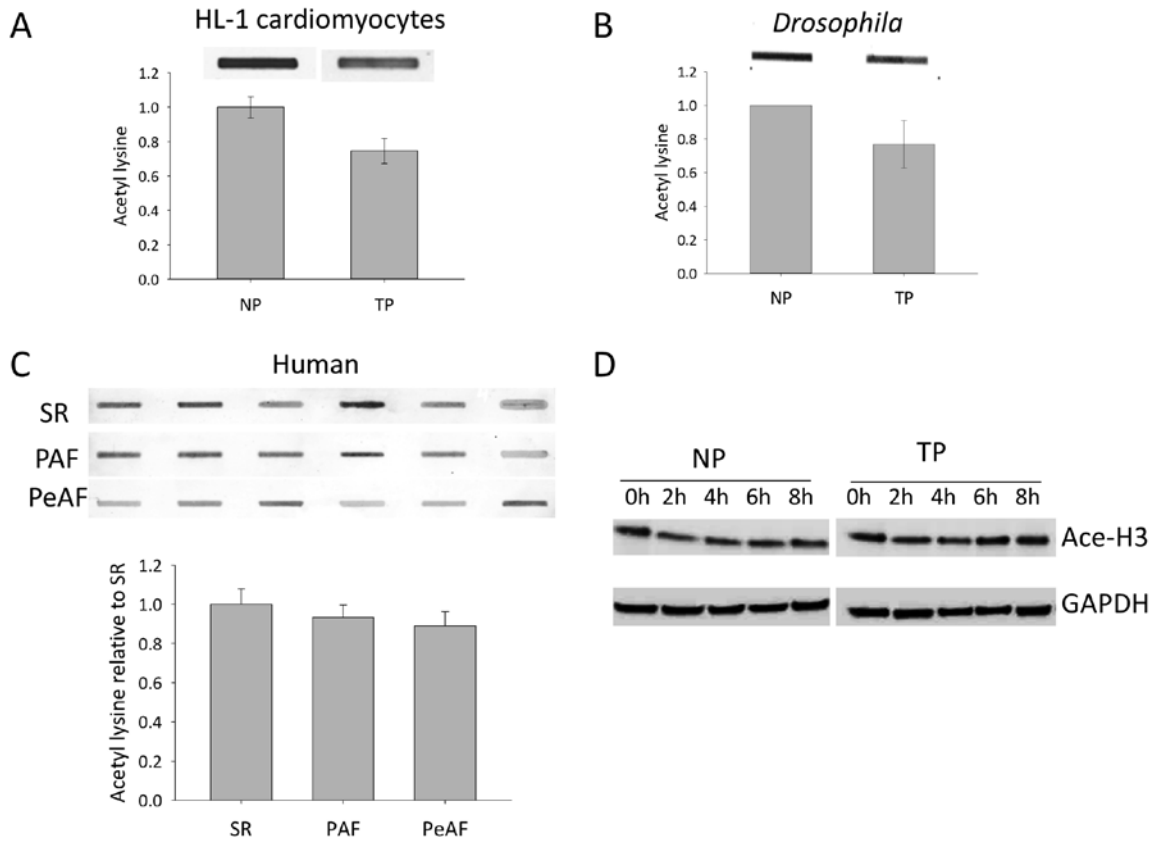

Figure S3 No significant changes in overall acetyl lysine and acetyl histone H3 levels after tachypacing of HL-1 cardiomyocytes, Drosophila or during clinical AF. On top: a representative slot blot showing overall lysine levels in normal (NP) or tachypaced (TP) **A** HL-1 cardiomyocytes, **B** *Drosophila* and **C** human paroxysmal (PAF), permanent AF (PeAF) and controls in sinus rhythm (SR). Below: quantified data showing no significant changes between the groups as indicated. **D** Western blot showing no significant differences in acetyl histone H3K9 (Ace-H3) levels between NP and TP HL-1 cardiomyocytes. All experiments were performed in at least duplicate series.

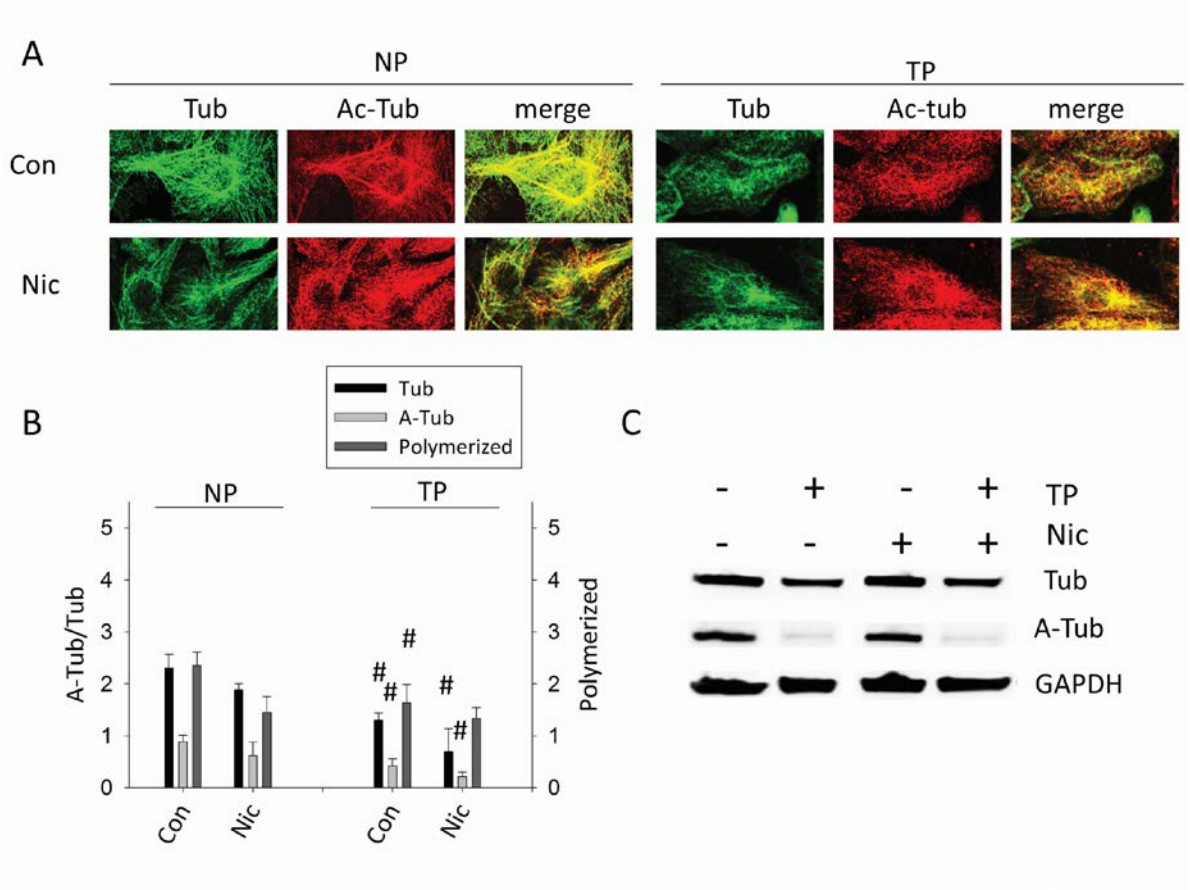

Figure S4 Nicotinamide does not prevent tachypacing-induced deacetylation of α-tubulin and depolymerization of microtubules. **A** Typical example of an immunofluorescent staining of α-tubulin (green), acetyl α-tubulin (red) in normal paced (NP) and tachypaced (TP) HL-1 cardiomyocytes. Tachypacing with or without Nicotinamide treatment, induces depolymerization of microtubules (yellow) which is not prevented by pre-treatment with Nicotinamide. **B** Quantification of α-tubulin (Tub), acetylated α-tubulin (A-Tub) and amount of polymerized tubulin (microtubules) after NP and TP. TP significantly reduced the amount of Tub, A-Tub and polymerized α-tubulin, which was not prevented by Nicotinamide (100-250 cells per condition). **C** Representative Western blot showing tachypacing-induced reductions in total α-tubulin and acetyl tubulin which was not prevented by Nicotinamide pretreatment. # $P < 0.01$ vs Control NP.

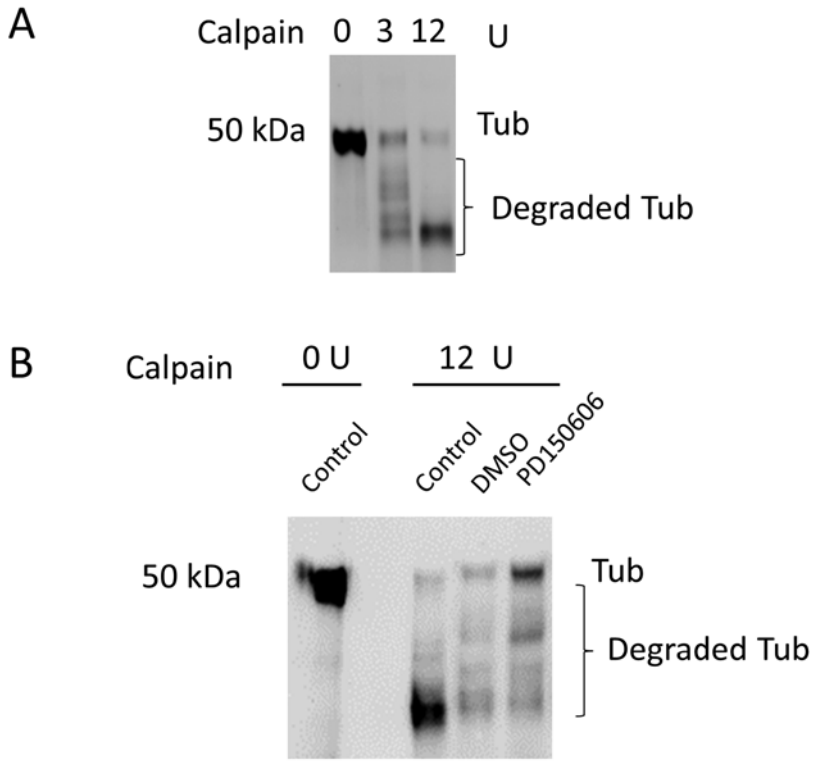

Figure S5 Depolymerized α-tubulin is degraded by calpain in vitro.
A Depolymerized α-tubulin was incubated with increasing amounts of calpain, which resulted in accelerated degradation of α-tubulin. **B** Calpain-induced α-tubulin degradation was attenuated by the calpain inhibitor PD150606.

Chapter 6

Inhibition of ER stress-induced autophagy preserves proteostasis and protects against cardiomyocyte dysfunction in experimental and human Atrial Fibrillation

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Manuscript in preparation

Abstract

Background

Atrial fibrillation (AF) is characterized by its self-perpetuating nature, which is rooted in structural remodeling of cardiomyocytes, resulting in electric dissociation, contractile dysfunction and limited success of cardioversion. Recent views on its pathogenesis suggest an important role for the derailment of protein homeostasis (proteostasis). Stress-induced excessive autophagy, by degradation of proteins and organelles, plays an important role in derailed proteostasis. We investigated the role of stress-induced autophagy in AF progression.

Methods and results

Tachypacing of HL-1 atrial cardiomyocytes resulted in a gradual and significant activation of autophagy, evidenced by increased autophagic flux, autolysosomes formation, p62 degradation and processing of LC3B-I to LC3B-II. The autophagy inhibitors bafilomycin A1 and pepstatin A prevented tachypacing-induced calcium transient loss. Autophagy was initiated by upstream ER stress in response to tachypacing, as evidenced by significant eIF2α phosphorylation and activation of ER stress markers ATF6, HSPA5, ATF4, ATG12, and CHOP. Also, inhibition of endoplasmic reticulum (ER) stress by 4-phenyl-butyric-acid (4-PBA) and overexpression of the ER stress protein HSPA5 attenuated tachypacing induced loss of calcium transients. The findings were extended to human AF. Autophagosomes and autolysosomes were observed in atrial tissue of patients with permanent AF, together with a significant reduction in p62 and HSPA5 levels. Similar changes were found in atrial tissue from tachypaced dogs. Finally, in patients, p62 levels correlated with cTnI, cTnT, α-tubulin and correlated inversely with the amount of myolysis.

Conclusions

AF causes ER stress, which subsequently activates autophagy resulting in structural remodeling and functional loss of cardiomyocytes. The findings suggest a beneficial role for inhibitors of ER stress and autophagy to conserve cell proteostasis in clinical AF.

Nonstandard Abbreviations and Acronyms

AF; Atrial Fibrillation, ATF4; Activating Transcription Factor 4, ATF6; Activating Transcription Factor 6, ATG12; Autophagy related protein 12, ATP; Atrial Tachypacing, BAF; Bafilomycin A1, CHOP; C/EBP-homologous protein, eIf2α; eukaryotic initiation factor 2α, ER; Endoplasmic Reticulum, HSPA5; Heat Shock Protein A5, LC3B; microtubule-associated proteins 1 light chains 3B, mTOR; mammalian target of rapamycin, 4-PBA; 4-phenyl butyric acid, PERK; protein kinase RNA-like endoplasmic reticulum kinase. RAA/LAA; Right or Left Atrial Appendages, RA/LA; Right or Left Atrial, SR; Sinus Rhythm.

Introduction

Atrial fibrillation (AF) is the most common persistent clinical tachyarrhythmia contributing to increased cardiovascular morbidity and mortality (Dobrev et al., 2012). The self-perpetuating nature of AF is driven by structural remodeling, electric dissociation and contractile dysfunction of cardiomyocytes (Ausma et al., 2003;Schotten et al., 2004;Allessie et al., 2010). As these changes ultimately limit the effectiveness of current drug and cardioversion therapies, research is increasingly directed at uncovering the underlying mechanisms of AF progression (Dobrev et al., 2012).

Derailment of proteostasis, i.e. the homeostasis of protein production, breakdown and function, has been implicated in AF-substrate formation in various experimental models for AF as well as in patients with AF (Brundel et al., 2002;Ausma et al., 2003;Qi et al., 2008;Meijering et al., 2012;Zhang et al., 2013). An element of proteostasis derailment consists of activation of proteases, including calpain. Studies in experimental and human AF revealed activation of calpain to induce breakdown of contractile and structural cytoskeletal proteins (myolysis) (Brundel et al., 2002;Ke et al., 2008). In addition to protease activation, activation of auxiliary cellular protein degradation pathways, such as macro-autophagy (hereafter autophagy) (Kroemer et al., 2010;Yang and Klionsky, 2010), may play an important role in AF progression. Stress-induced excessive activation of autophagy has already been associated with myolysis (Chen et al., 2011). Nevertheless, the contribution of autophagy to the initiation and progression of AF has not yet been investigated.

Autophagy is an evolutionary conserved cellular degradation pathway to maintain cell proteostasis by removing damaged or long-lived proteins and organelles (Kroemer et al., 2010;Yang and Klionsky, 2010). Following sequestration of proteins in cytoplasmic isolation vesicles, called autophagosomes, they are subsequently degraded in a lysosomal dependent manner (Kroemer et al., 2010;Yang and Klionsky, 2010). Controlled autophagy during (mild) cardiac stress conditions, such as nutrient deprivation, hypoxia, and oxidative stress, supports cardiomyocyte survival. In contrast, excessive activation of autophagy causes derailment of cell proteostasis by degradation of essential proteins and organelles and thereby triggers autophagic cell death, as found in mitral valve regurgitation (Gurusamy and Das, 2009b;Chen et al., 2011) and cardiac hypertrophy (Zhu et al., 2007). As permanent AF is related to the degradation of contractile proteins and structural remodeling of atrial cardiomyocytes (Brundel et al., 2002;Ke et al., 2008), we investigated the role of autophagy in functional and structural remodeling. Hereto, we utilized *in vitro* tachypaced HL-1 atrial cardiomyocytes, atrial tissue of patients with AF, and dogs subjected to atrial tachypacing (ATP) and compared the findings to controls in sinus rhythm (SR).

Here we show that tachypacing results in ER stress, which induces substantial autophagy in cultured atrial cardiomyocytes leading to

contractile dysfunction. In addition, we found autophagy being activated in atrial tissue of AF patients and dogs subjected to ATP. In patients, autophagy markers correlated significantly with loss of contractile proteins and myolysis.

Materials and Methods

2.1 HL-1 atrial cardiomyocyte cell culture, transfections, constructs

HL-1 atrial cardiomyocytes, derived from adult mouse atria, were obtained from Dr. William Claycomb (Louisiana State University, New Orleans, USA) (Claycomb et al., 1998). The cardiomyocytes were maintained in complete Claycomb Medium (JHR, UK) supplemented with 10% FBS (PAA Laboratories GmbH, Austria), 100U/ml penicillin (PAA Laboratories GmbH), 100 µg/ml streptomycin (PAA Laboratories GmbH), 4 mM L-glutamine (Gibco, The Netherlands), 0.3 mM L-ascorbic acid (Sigma, The Netherlands) and 100 µM norepinephrine (Sigma, The Netherlands). HL-1 cardiomyocytes were cultured on cell culture plastics or on glass coverslips coated with 12.5 µg/ml fibronectin (Sigma, The Netherlands) and 0.02% gelatin (Sigma, The Netherlands) in a humidified atmosphere of 5% CO_2 at 37°C. Where indicated, HL-1 cardiomyocytes were transiently transfected with the LC3B-GFP plasmid (Pankiv et al., 2007) by the use of Lipofectamin 2000 (Life Technologies, The Netherlands).

2.2 Tachypacing of HL-1 cardiomyocytes, live imaging and measurement of CaT

HL-1 cardiomyocytes were subjected to tachypacing as described before (Brundel et al., 2006;Ke et al., 2008). In short, HL-1 cardiomyocytes were subjected to normal pacing at 1 Hz (control) or 4.5 Hz (tachypacing), 40V and 20ms pulses, for a duration of maximal 8 hours via the C-Pace100TM-culture pacer (IonOptix Corporation, The Netherlands). To measure calcium transients (CaT), HL-1 cardiomyocytes were incubated for 45 min with 2µM of the Ca^{2+}-sensitive Fluo-4-AM dye (Invitrogen, The Netherlands), followed by 3 times washing with DMEM (Gibco, The Netherlands). Ca^{2+} loaded cardiomyocytes were excited by a 488nm laser and emission at 500-550nm was visually recorded with a 40x-objective, using a Solamere-Nipkow-Confocal-Live-Cell-Imaging system (based on a Leica DM IRE2 inverted microscope). The live recording of CaT in HL-1 cardiomyocytes was performed at 1Hz stimulation at 37^0C. Live recordings were further processed by use of the software ImageJ (National Institutes of Health, USA); the absolute value of fluorescent signals between experiments was determined utilizing the following calibration; Fcal=F/F0, where F is the fluorescent dye signal at any given time and F0 is the fluorescent signal at rest. Mean values and

SEM from each experimental condition were based on 7 consecutive CaT in at least 50 cardiomyocytes.

2.3 Antibodies and reagents

The antibodies used in the study were purchased from the following vendors. Rabbit polyclonal anti-GFP (A6544, Molecular Probes, The Netherlands) rabbit monoclonal anti-Akt (#4691), rabbit monoclonal anti-phospho-Akt (Ser473) (#4060), rabbit polyclonal anti-LC3B (#2775), rabbit polyclonal anti-phospho-eIF2α (Ser51) (#9721), mouse monoclonal anti-eIF2α (ab5369) (Abcam, Cambridge, UK), rabbit monoclonal anti-phospho-S6 Ribosomal Protein (Ser235/236) (#4858), mouse monoclonal anti-S6 Ribosomal Protein (#2317), rabbit polyclonal anti-SQSTM1/p62 (#5114), rabbit monoclonal anti-mTOR (#2983) (all Cell Signaling Technology, The Netherlands), rabbit polyclonal anti-phospho-mTOR (Ser2481, #2974), rabbit polyclonal anti-phospho-mTOR (Ser2448, #2971), and mouse monoclonal anti-GAPDH (#10R-G109a) (Fitzgerald, MA, USA). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit (Santa Cruz Biotechnology, The Netherlands) were used as secondary antibodies. Wortmannin, Pepstatin A, Tunicamycin, Rapamycin, Bafilomycin A1 and 4-phenylbutyric acid (4-PBA) were purchased from Sigma (The Netherlands) and dissolved according to manufacturer's instructions. HL-1 cardiomyocytes were treated with tunicamycin (5 µg/ml), 4-PBA (10 mM) and rapamycin (50nM) 8 hours prior to pacing. Pepstatin A (10 µM) and bafilomycin A1 (10nM) were added 30 minutes prior to pacing.

2.4 Protein-extraction and Western blot analysis

Western blot analysis was performed as previously described (Ke et al., 2008). Equal amounts of total protein in SDS-PAGE sample buffer, were separated on SDS-PAGE 4-20% Precise^{TM} Protein gels (Thermo Scientific, The Netherlands). After transfer to nitrocellulose membranes (Stratagene, The Netherlands), membranes were incubated with primary antibodies and corresponding horseradish peroxidase-conjugated secondary antibodies. Signals were detected by the Western Lightning Ultra (PerkinElmer, Waltham, MA, USA) method and quantified by densitometry via the software Gene Gnome, Gene tools (Syngene, Cambridge, UK).

2.5 Quantitative RT-PCR

Total RNA was isolated from cardiomyocytes utilizing the nucleospin RNA isolation kit (Machery-nagel, The Netherlands). First strand cDNA was generated by M-MLV reverse transcriptase (Invitrogen, The Netherlands) and random hexamer primers (Invitrogen, The Netherlands). Relative changes in transcription level were determined using the CFX384 Real-time system C1000 Thermocycler (BioRad, The Netherlands) in

combination with SYBR green supermix (Bio-rad, The Netherlands). Calculations were performed using the comparative CT method according to User Bulletin 2 (Applied Biosystems). Fold inductions were adjusted for GAPDH levels. Primer pairs used included; ATF4 F: GTCCGTTACAGCAACACTGC and R: CCACCATGGCGTATTAGAGG; ATF6 F: AAGAGAAGCCTGTCACTG and R: GGCTGGTAGTGTCTGAAT; ATG12 F: CTCCACAGCCCATTTCTTTG and R: AACTCCCGGAGACACCAAG; CHOP F: GACCAGGTTCTGCTTTCAGG and R: CAGCGACAGAGCCAGAATAA; HSPA5 (BiP) F: ATCTTTGGTTGCTTGTCGCT and R: ATGAAGGAGACTGCTGAGGC; GAPDH F: CATCAAGAAGGTGGTGAAGC and R: ACCACCCTGTTGCTGTAG. The PCR efficiencies for all primer pairs were between 90-110%.

2.6 Immunofluorescent staining and confocal analysis

Forty eight hours after transient transfection of GFP-LC3B, or without transfection in case of endogenous LC3B, cardiomyocytes were subjected to normal- (NP) or tachypacing (TP) followed by fixation with 4% formaldehyde (Klinipath, The Netherlands) for 15 minutes RT, washing three times with phosphate buffered saline (PBS) and permeabilization with 0.2% Triton-X100 and blocking in 5% BSA (30 min RT). Nuclei were visualized by TOTO-3 (Molecular Probes, The Netherlands) according to manufacturer's instructions. Endogenous LC3B was visualized by an anti-LC3B antibody as described above and subsequent alexa488 labeled anti-rabbit antibody. Confocal images were obtained by confocal laser microscopy (Leica SP2 AOBS), captured at 125x magnification to demonstrate the formation of GFP-LC3 punctae (dots), indicative of autophagosomes. The number of GFP dots, were counted manually from at least two independent experiments using imagePro. Mean values and SEM from each experimental condition were based on at least 20 cardiomyocytes.

2.7 Canine in vivo model for AF

Experiments with the canine in vivo model for AF were performed at the Montreal Hearth Institute as described before (Brundel et al., 2006) and were according to the guidelines for animal-handling of the National Institutes of Health and approved by the Animal Research Ethics Committee of the Montreal Heart Institute. Ten mongrel dogs (28 to 38 kg) were anesthetized with ketamine (5.3 mg/kg IV), diazepam (0.25 mg/kg IV), and halothane (1.5%). Unipolar pacing leads were inserted into the right ventricular apex and right atrial (RA) appendage under fluoroscopic guidance and were connected to pacemakers (Vitatron) in subcutaneous pockets in the neck. Atrioventricular block was created by radiofrequency catheter ablation to avoid excessively rapid ventricular responses during atrial tachypacing (ATP). The right ventricular demand pacemaker was programmed to 80 bpm. After 24-hour recovery, 7-day ATP at 400 bpm was instituted. At the end of the ATP period, dogs were sacrificed and right atrial and left atrial tissue was snap frozen in liquid

nitrogen and stored at -85ºC. Results in 5 ATP dogs were compared with 5 NP control dogs.

2.8 Patient material

Prior to surgery, one investigator assessed patient characteristics (Table 1) as described before (Brundel et al., 2001). The permanent and paroxysmal AF group contained patients with lone AF undergoing MAZE surgery. All patients were euthyroid and had normal left ventricular function. Coumarin therapy was interrupted 3 days before surgery and class I and II anti-arrhythmic drugs were discontinued for at least 5 half lifes. Right and or left atrial appendages (RAA and LAAs respectively) were obtained from all patients, except for the control patients (SR). From SR patients undergoing Coronary Bypass surgery (CABG) only the RAA was obtained prior to CABG (Table 1). After excision, the atrial appendages were immediately snap frozen in liquid nitrogen and stored at -85ºC. The study conforms to the principles of the Declaration of Helsinki. The institutional review board approved the study and patient gave written informed consent.

2.9 Statistical analysis

Results are presented as mean ± SEM of at least two independent experiments and statistically evaluated for difference by a one way ANOVA (Bonferroni) with SPSS version 18. Correlations were determined using the Spearman correlation test, which is a non-parametric measure of correlation for small group numbers. A value of $P<0.05$ was considered statistically significant.

Table 1 Baseline characteristics of patients with paroxysmal AF (PAF), permanent AF (PeAF) and control patients in sinus rhythm (SR).

	SR	**PAF**	**PeAF**
n	6	7	7
RAA (n)	6	6	6
LAA (n)		6	5
Age	56±8	51±7	61±10
Duration of AF (median, range (months))	-	-	14.6 (8-56)
Duration SR before surgery (median, range (days))	-	1.5 (0-30)	-
Duration of last episode AF (median, range (h))	-	12 (0.2-24)	-
Underlying heart disease (n) and/surgical procedure			
Coronary artery disease/CABG	6	0*	0*
Lone AF/Maze	0	7*	7*
New York Heart Association for exercise tolerance			
Class I	3	5	3
Class II	3	2	4
Echocardiography			
Left atrial diameter (parasternal, mm)	42±3	42±4	48±4
Left ventricular end-diastolic diameter (mm)	50±4	52±3	52±3
Left ventricular end-systolic diameter (mm)	34±4	38±3	34±5
Medication (n)			
ACE-inhibitors	1	0	3
Digoxin	2	0	3
Verapamil	4	0	3
Beta-Blocker	5	1	2

Values are represented as mean ± SEM or number of patients. CABG; Coronary Artery Bypass Grafting, MAZE; atrial arrhythmia surgery, MVD; mitral valve disease. *$p<0.05$ vs SR

Results

3.1 Tachypacing induces autophagy

Autophagy was examined by determining the levels of the autophagy markers p62, LC3B-I and LC3B-II and via visualization of autophagosomes by confocal microscopy (Gottlieb and Mentzer, 2010). P62 is a poly-ubiquitin binding protein, which is sequestrated to autophagosomes during autophagy and is subsequently degraded upon fusion with the lysosome. Hence its levels are inversely proportional to activation of autophagy (Wang et al., 2006;Maejima et al., 2013). LC3B-II is a protein produced from LC3B-I upon autophagy initiation and is also incorporated into autophagosomes. LC3B-II levels are proportional to the number of autophagosomes (Wang et al., 2006;Maejima et al., 2013). HL-1 atrial cardiomyocytes were subjected to tachypacing and the levels of autophagy markers were determined by Western Blot (Figure 1A-C). Tachypacing induced a time-dependent decrease in p62 levels and increase in LC3B-II levels (Figure 1A-C), indicating the activation of autophagy. Consistent with western blot results, confocal analysis of tachypaced HL-1 cardiomyocytes revealed a time-dependent increase in LC3B levels in both LC3B-GFP transfected cardiomyocytes (Figure 1D) as well as in untransfected cardiomyocytes stained for endogenous LC3B (Figure 1E). Also, a clear redistribution of LC3B into discrete perinuclear punctae were observed (Figure 1F), indicative of autophagosome formation.
Next, we determined the autophagic flux to discern between induction of autophagy and diminished degradation of autophagosomes. Autophagic flux was measured as the difference in LC3B-II protein levels in the absence and presence of the lysosomal inhibitor bafilomycin A1 (BAF), which prevents the fusion to lysosomes and subsequent clearance of autophagosomes (Hansen and Johansen, 2011). BAF pretreatment further increased the levels of LC3B-II compared to those induced by tachypacing alone (Figure 1G-H). Together, these observations demonstrate that tachypacing induces the activation of autophagy in HL-1 atrial cardiomyocytes.

3.2 Induction of autophagy is via ER stress

Activation of autophagy is regulated by various cellular signal transduction pathways. A key regulator is mammalian target of rapamycin (mTOR). mTOR assembles into two complexes, mTOR complex 1 (mTORC1) and complex 2 (mTORC2). Both complexes become activated by mTOR phosphorylation, although at different sites, after which they attenuate autophagy (Gurusamy and Das, 2009a;Jung et al., 2010). To determine if tachypacing-induced autophagy results from the inhibition of mTOR signaling, we determined total mTOR, phosphorylation of mTOR at

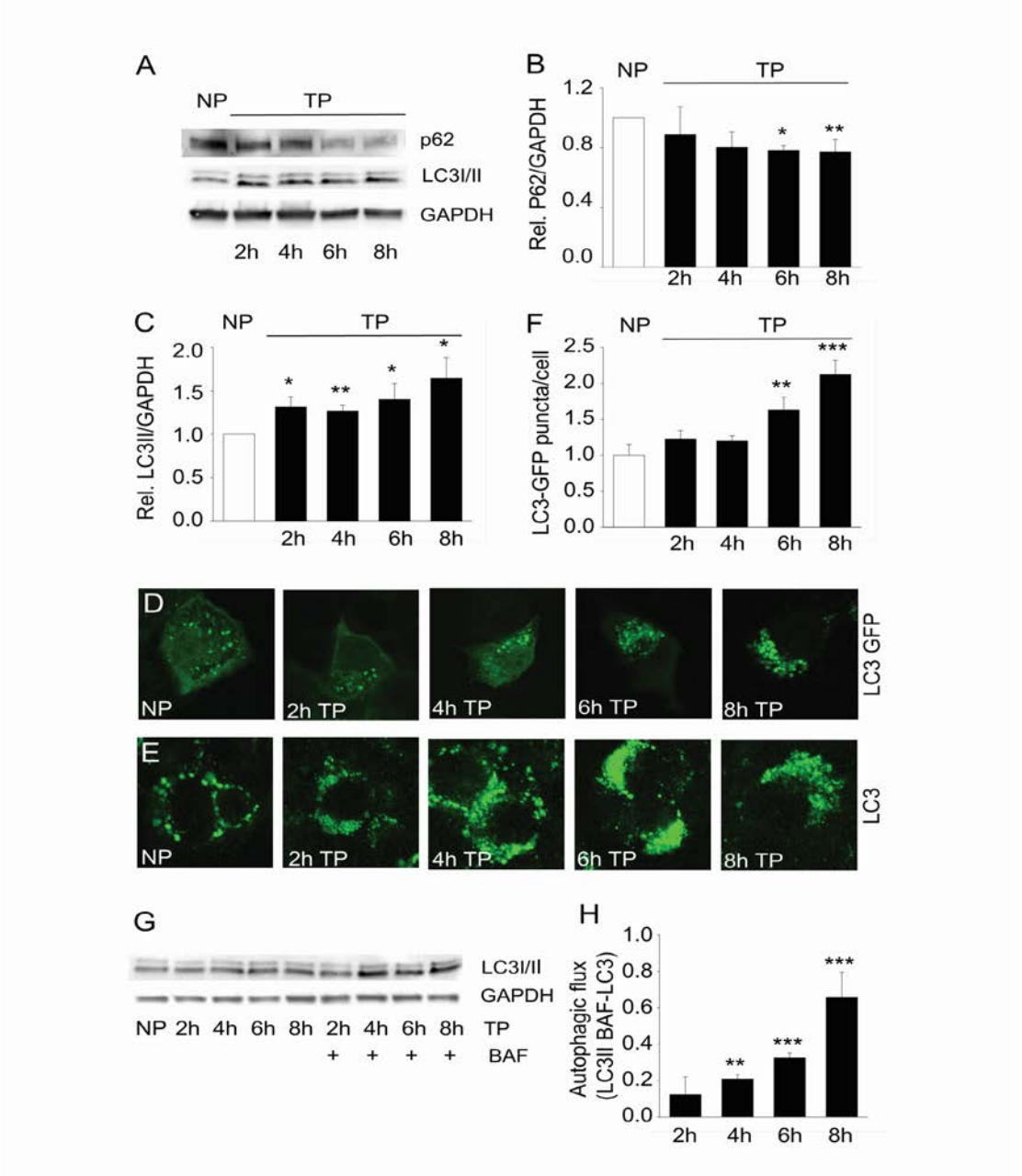

Figure 1 Tachypacing induces autophagosome formation and activation of autophagy.

A Representative western blot of tachypacing induced autophagy markers p62, LC3B I, LC3B II and GAPDH. HL-1 cardiomyocytes were NP (normal paced) or TP (tachypaced) for the duration as indicated. **B** Quantification of p62 levels relative to GAPDH compared to control (NP). **C** Quantification of LC3B II level relative to GAPDH compared to control (NP). **D** Confocal images of tachypaced HL-1 cardiomyocytes, transfected with LC3B-GFP plasmid. **E** Confocal images of tachypaced HL-1 cardiomyocytes for the period as indicated. Endogenous LC3B was visualized by immunostaining. Green punctae indicate autolysosomes. **F** Quantified data showing accumulation of LC3B-GFP punctae/cell during TP. **G** Representative western blot of HL-1 cardiomyocytes NP versus TP for the duration as indicated, in the presence or absence of bafilomyocin A1 (BAF). **G** Quantification of the autophagic flux by determining the difference in LC3BII levels in the presence versus absence of bafilomycin A1 (BAF). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus NP.

S2448 for mTORC1, S2481 for mTORC2 and their respective downstream targets ribosomal protein S6 (S6RP) and Akt (Figure 2). Tachypacing did not significantly affect mTOR phosphorylation at S2448, S2481 and S6RP at S235-236. However, tachypacing resulted in a significant increase in phosphorylation of Akt at S473. Since an increase phosphorylation of Akt at S473 was previously observed during ER stress (Yung et al., 2011), and ER stress is an important regulator of autophagy (Kouroku et al., 2007;Kroemer et al., 2010), the involvement of ER stress signaling in tachypacing-induced autophagy was examined. To this end, levels of the downstream ER stress marker phosphorylated eIF2α (Kim et al., 2008), were quantified. Tachypacing induced a strong increase in eIF2α phosphorylation without affecting total eIF2α levels indicating the presence of ER stress (Figure 3A). To substantiate the role of ER stress signaling in tachypacing-induced autophagy, levels of additional ER stress markers and the induction of the downstream autophagy gene ATG12, which is involved in autophagosome formation (Kouroku et al., 2007), were determined by qPCR (Figure 3B). Tachypacing induced the transcription of different markers of ER stress, i.e. ATF6, HSPA5, ATF4, CHOP, and ATG12. Furthermore, the chemical chaperone and ER stress inhibitor 4-PBA prevented tachypacing-induced phosphorylation of eIF2alpha and activation of autophagy, as evidenced by attenuation of p62 breakdown and LC3B processing (Figure 3C). In addition, inhibition of autophagy by pepstatin A (lysosomal cathepsin D/E inhibitor) and BAF (lysosomal fusion inhibitor) also attenuated p62 degradation upon tachypacing, without affecting LC3B processing. In contrast, tunicamycin (activator of ER stress) and rapamycin (stimulator of autophagy) did not protect against tachypacing-induced p62 degradation and LC3B processing. Thus, the results indicate induction of the ER stress signaling to be upstream of tachypacing-induced autophagy in HL-1 atrial cardiomyocytes.

3.3 Inhibition of autophagy protects against tachypacing-induced CaT loss

To determine if autophagy is involved in tachypacing-induced contractile dysfunction, the effect of autophagy modulating drugs on calcium transients (CaT) were determined. Autophagy was pharmacologically inhibited by 4-PBA (ER-stress inhibitor), pepstatin A and BAF. All these inhibitors of autophagy significantly protected tachypaced cardiomyocytes from loss of calcium transients (CaT) (Figure 4A, B). In contrast, stimulators of autophagy (rapamycin and tunicamycin), applied at a low dose to prevent cell toxicity and CaT loss under normal pacing conditions, did not protect against tachypacing-induced CaT loss (Figure 4A, B). Since 4-PBA is a chemical chaperone and an inhibitor of ER stress, the role of the ER chaperone HSPA5 was tested. Overexpression of HSPA5 protected against tachypacing-induced loss of CaTs (Figure 4C). The results indicate that both inhibition of ER stress, via 4-PBA or overexpression of HSPA5,

and inhibition of autophagy protects against tachypacing-induced loss of contractile function.

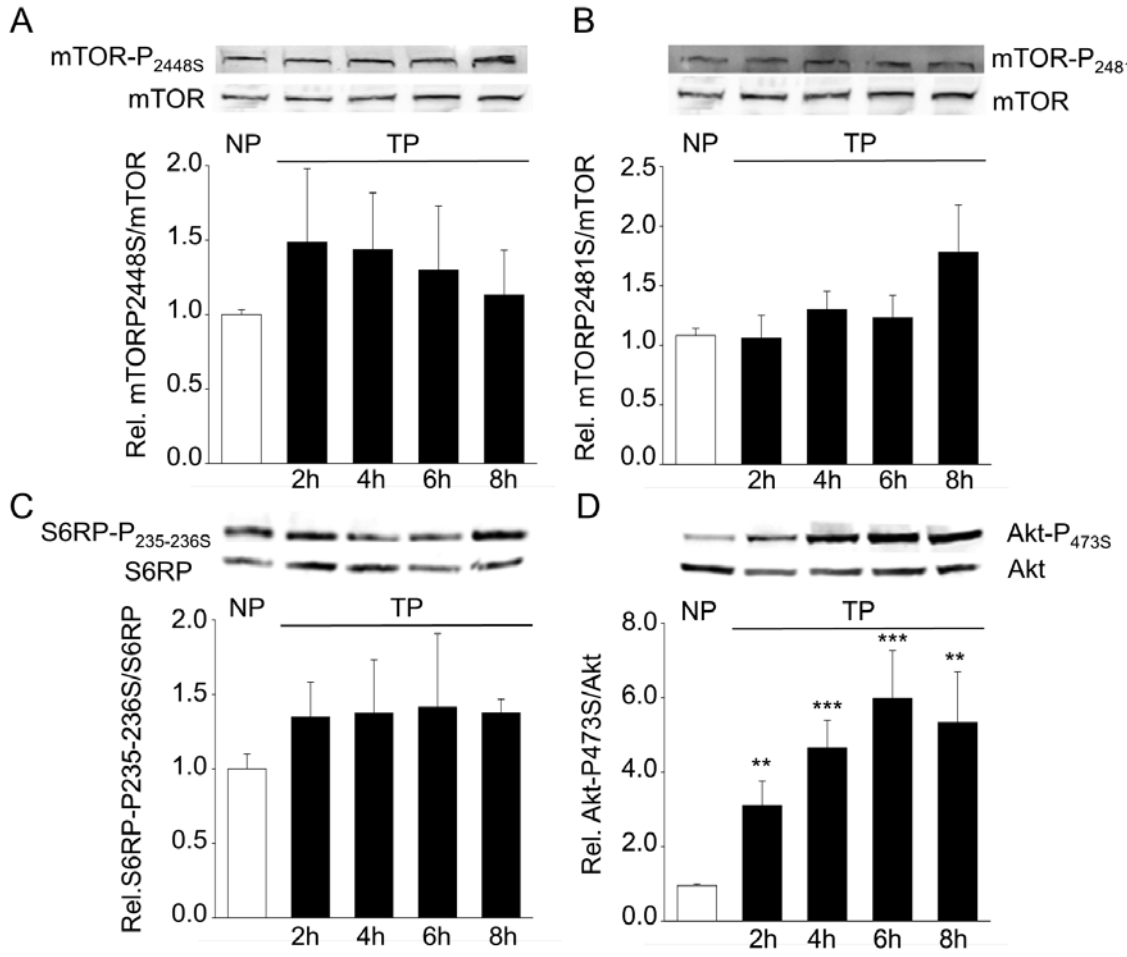

Figure 2 Tachypacing-induced autophagy does not involve mTORC signaling.
Top panels represent western blots of proteins within the mTORC signaling and lower panels reveal quantified data of the ratio phosphorylated proteins normalized for basal protein levels. **A** Phospho-mTOR 2448S (mTORC1). **B** Phospho-mTOR 2481S (mTORC2), **C** Phospho-S6RP 235-236S (downstream of mTORC1) and **D** phospho-Akt 473S (downstream of mTORC2) in response to tachypacing for the duration as indicated compared to normal paced (NP).
** $p < 0.01$, *** $p < 0.001$ versus NP.

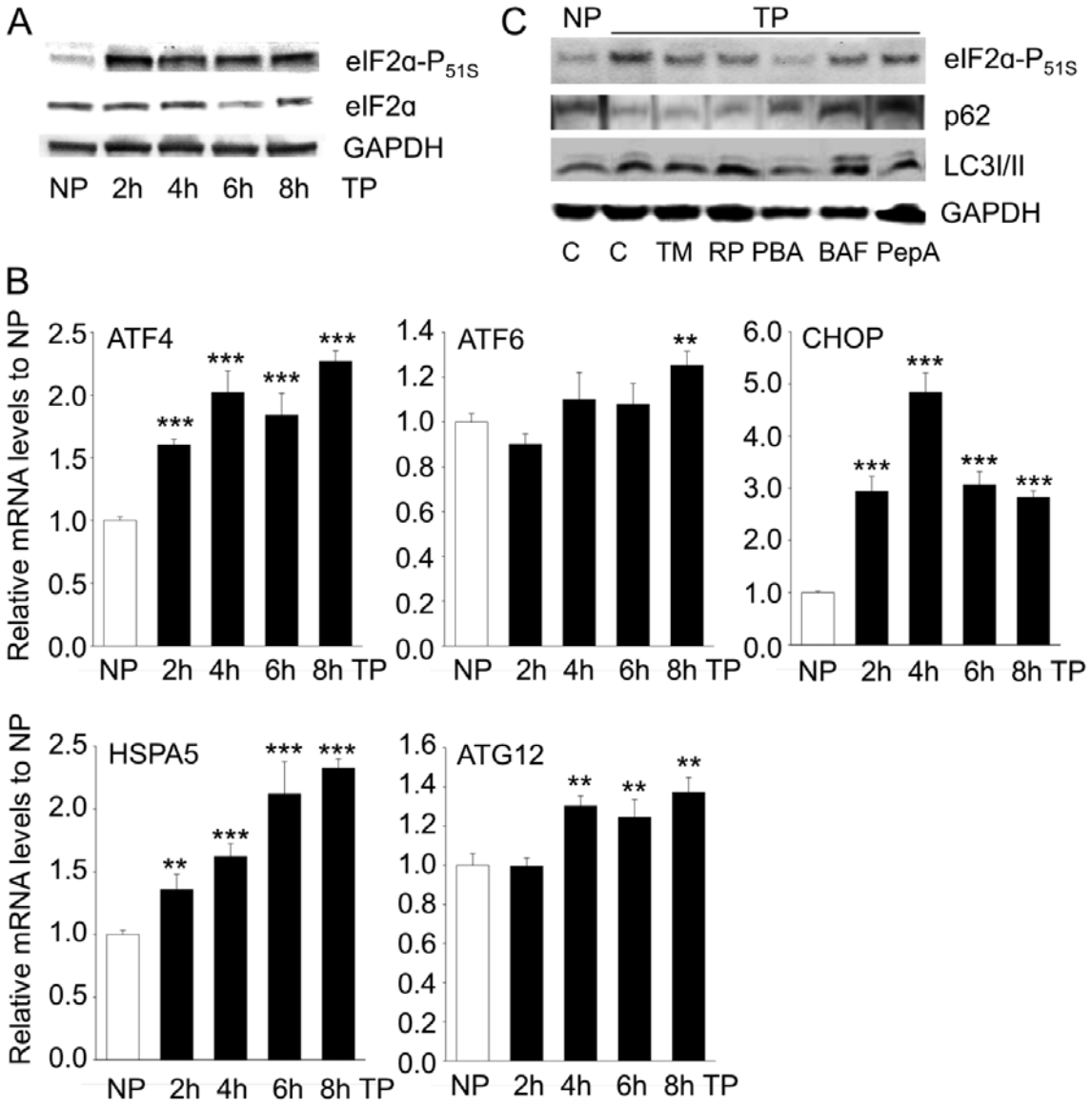

Figure 3 Tachypacing induces ER stress and mRNA levels of autophagy gene ATG12.

A Representative western blot of phospho-eIF2alpha 51S, an ER stress marker, and basal eIF2alpha and GAPDH levels in response to tachypacing for the indicated duration or normal pacing (NP). **B** Quantitative real time PCR of ER stress markers (ATF4, ATF6, CHOP, HSPA5) and autophagy related genes (ATG12) in response to tachypacing for the indicated duration relative to normal pacing (NP). **C** Representative western blot of ER stress markers (eIF2alpha P51S) and autophagy markers (LC3B and p62) in response to autophagy modulators. ** p <0.01, *** p <0.001 versus NP. Abbreviations, TM;Tunicamycin, RP;Rapamycin, PBA; 4-Phenyl Butyric Acid, BAF; Bafilomycin A1, PepA; Pepstatin A.

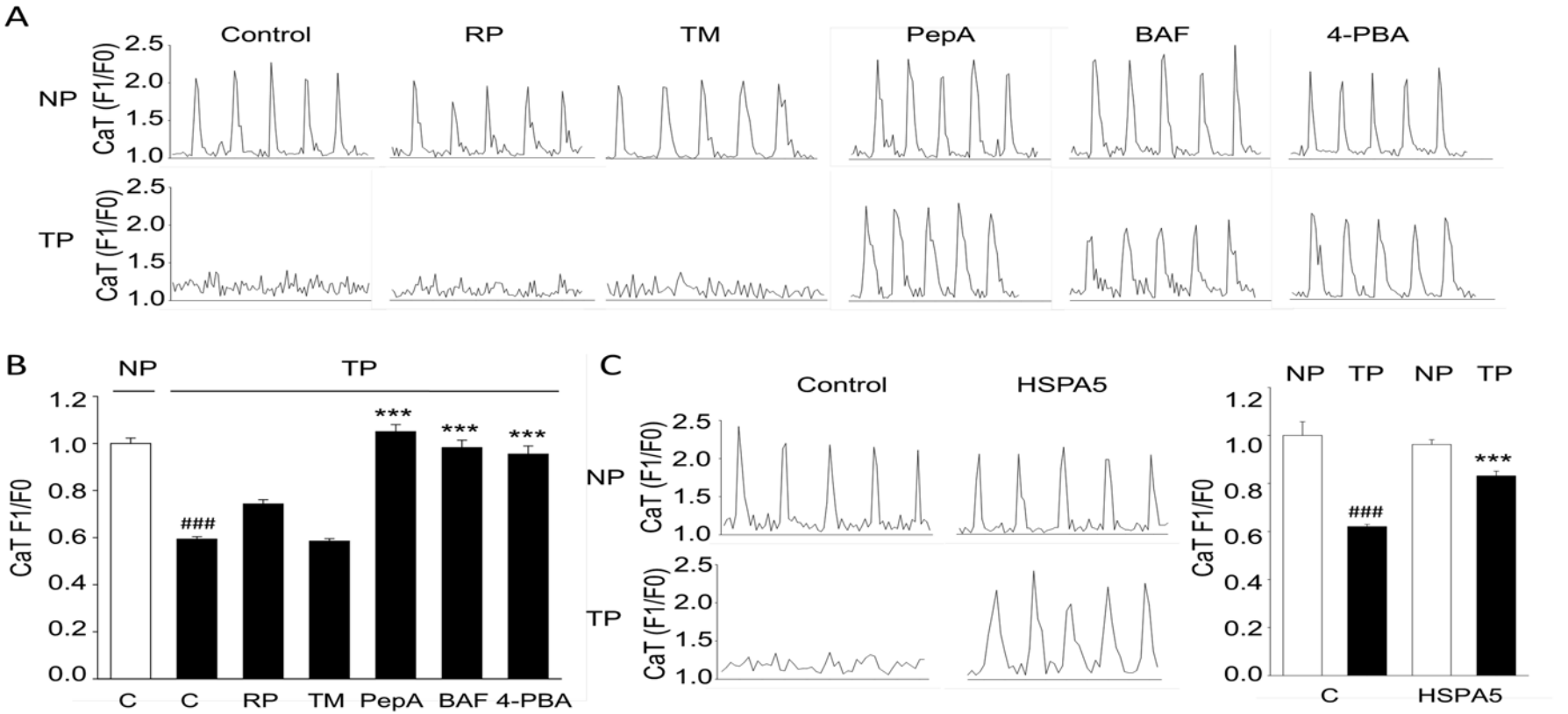

Figure 4 Autophagy inhibition and overexpression of ER chaperone HSPA5 protect against tachypacing-induced loss of CaT.

A Representative CaT of HL-1 cardiomyocytes pretreated with autophagy modulators as indicated after 8h of normal versus tachypacing. **B** Quantified CaT of HL-1 cardiomyocytes pretreated with autophagy modulators and subjected to normal versus tachypacing. **C** Left panel, representative CaT of HL-1 cardiomyocytes transfected with empty plasmid (Control) or ER chaperone HSPA5 as indicated after 8h of normal versus tachypacing. Right panel, Quantified CaT of HL-1 cardiomyocytes transiently transfected with empty plasmid or the ER chaperone HSPA5 and subjected to normal versus tachypacing. *** p <0.001 versus TP, ### p<0.001 versus NP. Abbreviations, RP; Rapamycin, TM; Tunicamycin, PepA; Pepstatin A, BAF; Bafilomycin A1, 4-PBA; 4-phenyl butyric acid.

3.4 Active autophagy is observed in patients in permanent AF and in a dog model for AF

To extend our findings to human AF, markers of autophagy were determined in RAA and LAA of patients with paroxysmal (PAF) or permanent AF (PeAF) and of control patients in sinus rhythm (SR). Electron microscopic analysis in atrial tissue of PeAF and SR, demonstrated that patients with PeAF accumulate autophagosomes (Chen et al., 2011;Garcia et al., 2012;Maejima et al., 2013) and show myolysis (degradation of sarcomeres) in both RAA and LAA, which was absent in SR patients (Figure 5A-D). Furthermore, PeAF patients displayed a significant decrease in p62 levels in LAAs compared to RAAs (Figure 6E). Also, the levels of the ER chaperone HSPA5 were significantly reduced in LAA compared to RAA of both patients with PeAF and of controls in SR (Figure 5F), suggesting the ER stress response to underlie AF progression. To further substantiate the involvement of autophagy in structural remodeling and AF progression, the p62 levels were correlated with the levels of contractile proteins and amount of myolysis. A significant correlation was found between p62 expression and cTnI, cTnT and alpha-tubulin expression (Figure 6A, B, C). Also, the p62 levels correlated inversely with the amount of myolysis (Figure 6D). In addition, the p62 levels correlated with HSPA5 levels (Figure 6E), suggesting ER stress response to underlie autophagy and AF progression. No changes in LC3BI and LC3BII levels were observed (supplemental data Figure 2).
To investigate if autophagy is caused by tachypacing, autophagy markers were also tested in left atrial (LA) and right atrial (RA) tissue of dogs subjected to ATP for 1 week and non-paced control dogs. Comparable to the findings in patients, a significant reduction in p62 and HSPA5 levels and an increased phosphorylation of eIF2alpha was observed in LA of ATP dogs (Figure 7A, B, C). Furthermore, as found in patients no changes in LC3B levels were found between ATP and control groups (Figure 7D).

Together, these results demonstrate activation of autophagy in patients with permanent AF. Most likely, autophagy is initiated by ER stress in response to the increased activation rate of the atrial cardiomyocytes. The degree of autophagy correlates with the degradation of contractile proteins and the amount of structural remodeling.

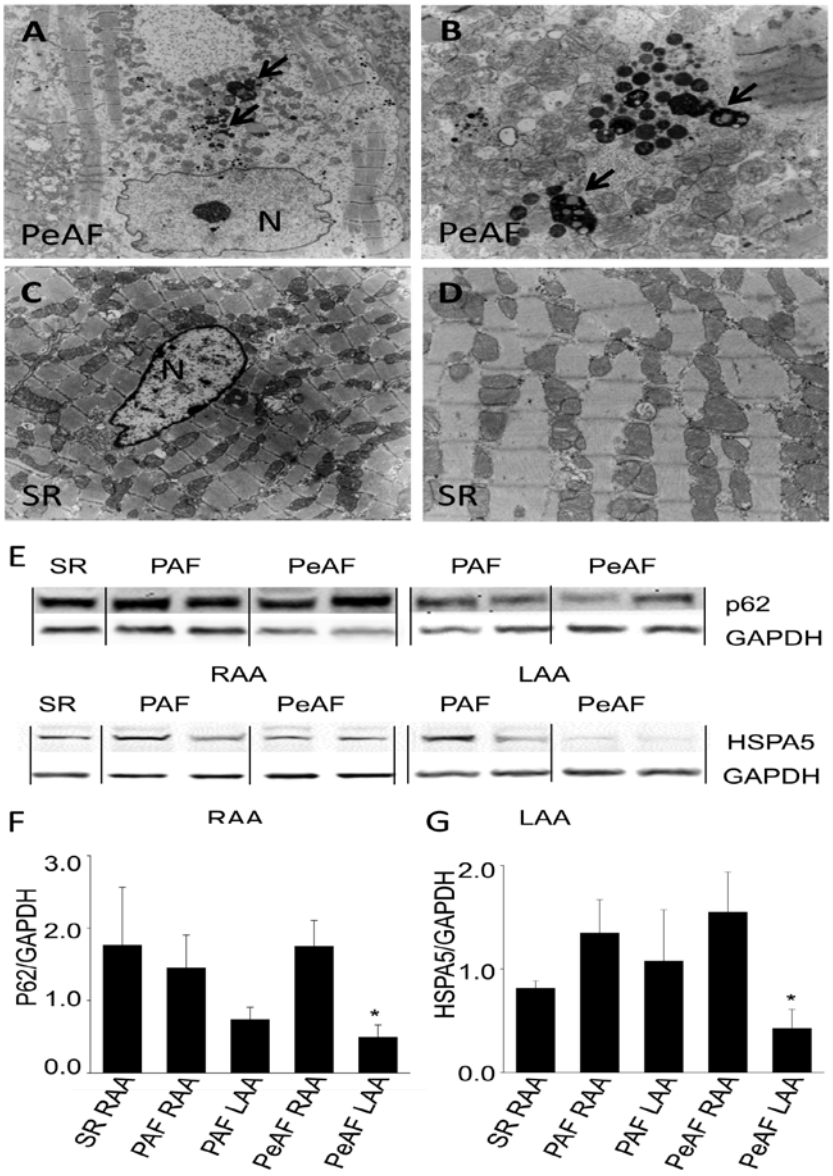

Figure 5 Markers for ER stress and autophagy are present in patients with permanent AF.

A SEM image of left atrial appendage of a patient with permanent atrial fibrillation (PeAF), arrows indicate the presence of autophagosomes and autolysosomes with a perinuclear (N) localization. **B** SEM image of left atrial appendage of a patient with permanent atrial fibrillation at a higher magnification, showing the presence of autophagosomes and autolysosomes. **C** SEM image of left atrial appendage of a patient in SR, showing normal sarcomere structures and absence of perinuclear autophagosomses. **D** SEM image of left atrial appendage of patient in SR, showing normal sarcomere structures and absence of perinuclear autophagosomses at a greater magnification. **E** On top: Representative western blot of the autophagy marker p62 in right (RAA) and left atrial appendages (LAA) of patients in paroxysmal (PAF) and permanent AF (PeAF) versus SR. Below: Representative western blot of ER stress marker HSPA5 of right and left atrial appendages of patients in PAF and PeAF versus SR. **F and G** Quantified data of autophagy marker p62 and ER stress marker HSPA5, in right (RAA) and left atrial appendages (LAA) of patients with paroxysmal AF (PAF), permanent (PeAF) and control patients in sinus rhythm (SR). * $p < 0.05$ versus SR.

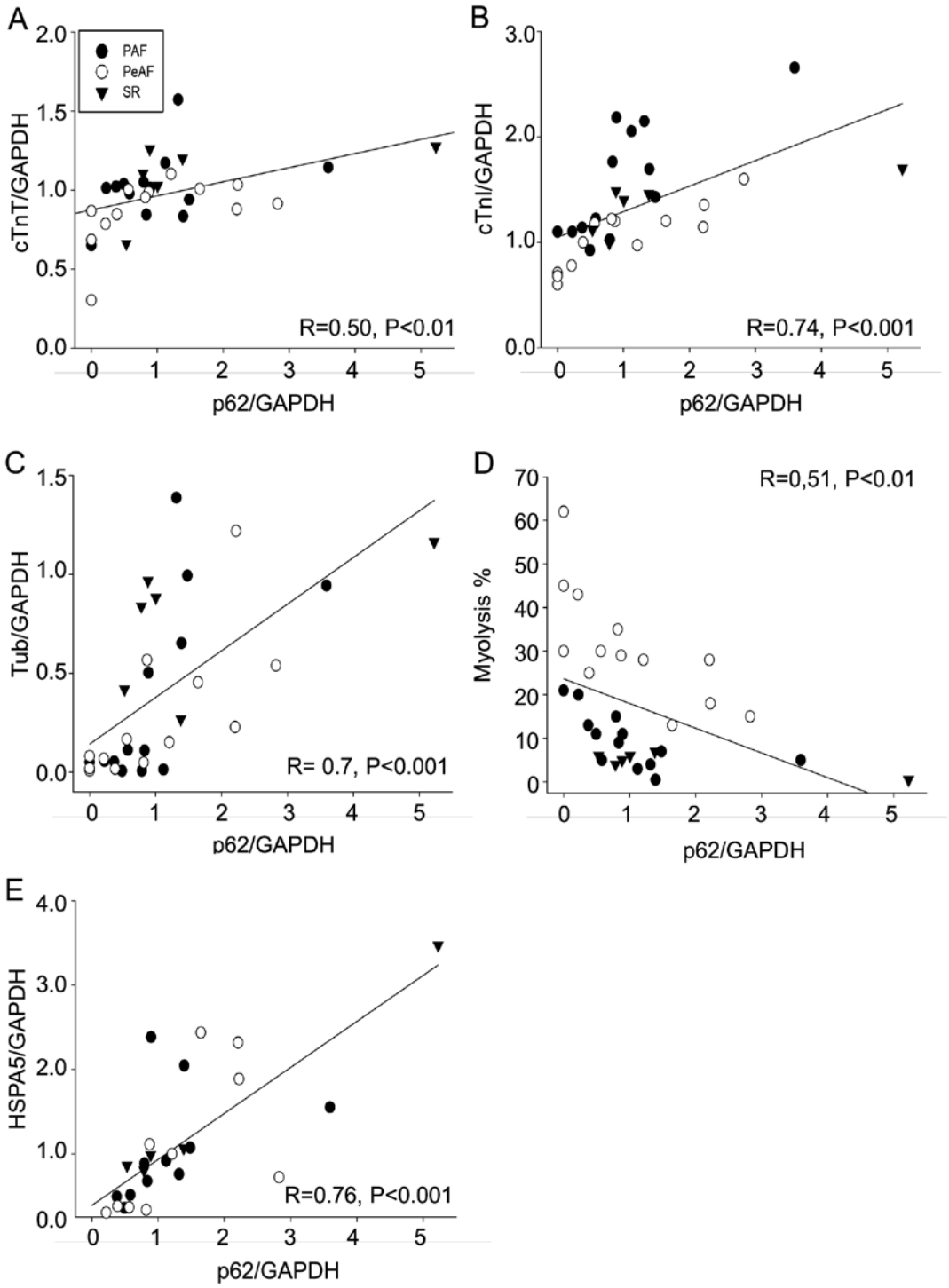

Figure 6 Significant correlations between levels of the autophagy marker p62 and markers of cardiomyocyte structural remodeling in patients with paroxysmal (PAF) and permanent (PeAF) and SR. A Cardiac troponin T (cTnT), **B** Cardiac troponin I (cTnI), **C** α-tubulin (tub), **D** Myolysis and **E** HSPA5.

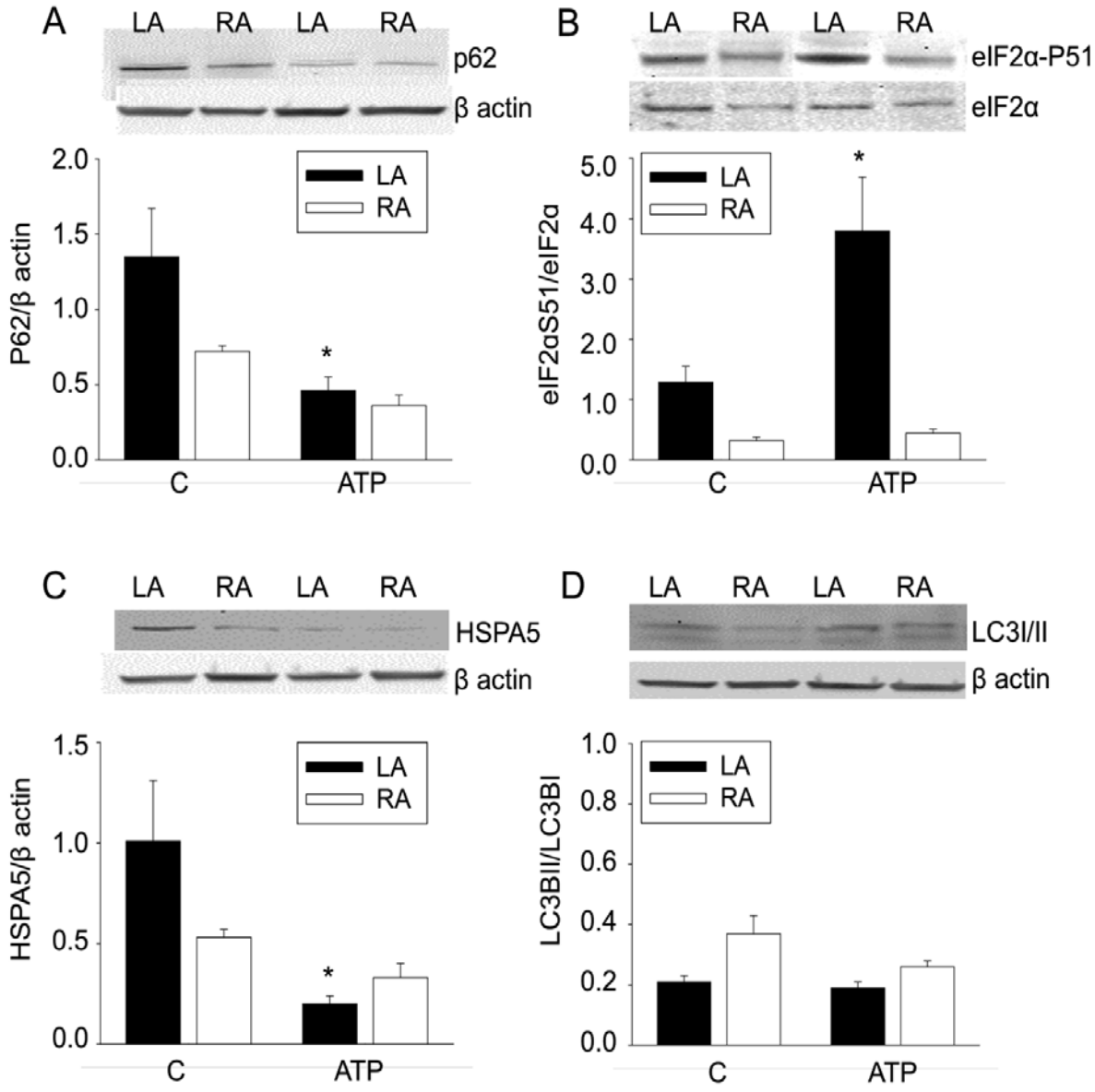

Figure 7 Dogs subjected to atrial tachypacing reveal induction of ER stress and downstream autophagy in right and left atrial tissue. A Representative western blot and quantified data of the autophagy marker p62 in right (RA) and left atrial (LA) tissue of dogs with and without atrial tachypacing (ATP). **B** Representative western blot and quantified data of ER stress marker eIF2α-P51S levels in RA and LA of dogs with and without ATP. **C** Representative western blot and quantified data of ER stress marker HSPA5 in RA and LA of dogs with and without ATP. **D** Representative western blot and quantified data of autophagy marker LC31/II in RA and LA of dogs with and without ATP. * $p < 0.05$ versus C.

Discussion

In the present study, we reveal tachypacing of cardiomyocytes to activate autophagy. Apparently, activation of autophagy plays a major role in cardiomyocyte remodeling, as inhibition of autophagy by pepstatin A and Bafilomycin A1 fully protected against tachypacing-induced loss of CaT. Furthermore, we show that activation of autophagy is caused by upstream induction of the ER stress response, as autophagy was attenuated by the inhibition of ER stress, by 4-PBA, and by overexpression of HSPA5. In accord, tachypacing induced phosphorylation of the ER stress marker eIF2α and thereby stimulated ATF6, HSPA5, ATF4, CHOP, and ATG12 expression. Importantly, 4-PBA blocked the phosphorylation of eIF2α, and the subsequent activation of autophagy and loss of CaT. In addition, the activation of autophagy was observed in patients with permanent AF, as demonstrated by perinuclear localized autophagosomes and reduced levels of the autophagy marker p62. In these patients, a reduction in the ER chaperone HSPA5 was found, suggesting a role for the ER stress response as activator of autophagy in human AF. Moreover, the correlation of p62 levels with levels of contractile proteins and myolysis suggests that activation of autophagy may also contribute to cardiomyocyte structural damage and dysfunction and thereby disease progression in human AF. Data from atrial tachypaced dogs corresponded with those found in patients. Together, our data indicate that activation of autophagy is involved in the derailment of cardiomyocyte proteostasis, contributing to functional loss and AF progression.

4.1 Autophagy via ER stress

The first finding of our study is that tachypacing of cardiomyocytes activates autophagy, a known key modulator of cell proteostasis (Wong and Cuervo, 2010;Ryter et al., 2013). Activation of autophagy is evidenced by the accumulation of autolysosomes, p62 degradation, and LC3B processing. Moreover, two inhibitors of autophagy, Bafilomyocin A1 and pepstatin A, prevented tachypacing-induced loss of contractile function. In addition, patients with permanent AF revealed active autophagy, which correlates with structural remodeling. The second finding was that tachypacing-induced autophagy is activated via upstream ER stress signaling, and this also seems to apply for patients with permanent AF and atrial tachypaced dogs. Furthermore, inhibition of ER stress, via 4-PBA or overexpression of HSPA5, protects HL-1 cardiomyocytes against tachypacing-induced contractile dysfunction. Since inhibition of basal autophagy, by Bafilomycin A1 and lysosomal enzyme inhibitors (such as pepstatin A), have recently been shown to induce ER stress and the expression of HSPA5 (Mathew et al., 2009;Xu et al., 2012;Guo et al., 2013), the observed protective effects of autophagy inhibitors in the current study may be due to the induction of HSPA5

expression and remains to be investigated. Thus, the current study identifies a prominent role for ER stress in the induction of autophagy and derailment of cell proteostasis which underlies AF progression.

On the mechanistic level, the current study reveals tachypacing to induce ER stress markers via phosphorylation of eIF2alpha. It is well known that the ER is involved in protein synthesis, folding and maturation. Also the ER constitutes a source of the autophagic isolation membrane, also called phagophore, which is necessary for the inclusion of autophagy-prone proteins (Figure 8) (Hayashi-Nishino et al., 2009). Upon ER stress, eIF2α gets phosphorylated at S51, as observed in our study, which then initiates a cascade of events aiming to decrease the ER stress. These events include a general inhibition of protein translation as well as the selective translation of stress-responsive transcripts including ATF4 and ATF6 (Figure 8) (Kroemer et al., 2010). As a consequence, ATF4 and ATF6 signaling induces expression of CHOP, ATG12, LC3 and HSPA5 (also named BiP, Grp78 or ER chaperone). During prolonged ER stress, the phosphorylated eIF2alpha replenishes cellular supplies of ATG12 and LC3, allowing for sustained and excessive autophagy flux (Kouroku et al., 2007;Rzymski et al., 2010;Verfaillie et al., 2010;Fleming et al., 2011). Consistent with the upstream role of ER stress, we found the chemical chaperone 4-PBA to attenuate eIF2alpha phosphorylation at S51 and subsequent activation of autophagy, resulting in conservation of contractile function after tachypacing. Thus, our data identifies ER stress and downstream activation of autophagy as the prevailing pathway that is activated in tachypaced cardiomyocytes.

4.2 The role of autophagy in AF

Our results obtained in atrial tissue samples from patients substantiate a role for the activation of autophagy in AF. Patients with permanent AF displayed perinuclear autophagosomes and reduced levels of p62. In addition, correlations between p62 and the contractile proteins cTnT, cTnI and alpha-tubulin were observed as was an inverse correlation between p62 and the amount of myolysis. Also in the dog model for AF, reduced levels of p62 were observed. Since reduced levels of HSPA5 were observed in both patients with permanent AF and in the dog model for AF, ER stress seems a prominent pathway to induce autophagy in AF (Figure 8). Reduction in p62 and HSPA5 levels were mainly observed in left atrial tissue of patients with permanent AF and in the dog model for AF, suggesting that ER stress-induced autophagy is more pronounced in the left atrium. This finding is in line with previous reports describing also marked changes in ion channel expression in the left atrium compared to the right atrium in the dog model for AF and in patients with AF (Li et al., 2001;Voigt et al., 2010;Zhang et al., 2013). Thus, our data from clinical AF patients indicate that activation of autophagy, probably via ER stress, may play a prominent role in the derailment of proteostasis, structural

remodeling and progression of AF. Comparable to our findings, also an association between autophagy and the presence of myolysis was found in patients with mitral valve regurgitation (Chen et al., 2011). Furthermore, an accumulation of autophagosomes was observed in patients who developed post-surgery AF (Garcia et al., 2012). These studies substantiate a role for autophagy in structural remodeling and AF progression.

Our results in tachypaced HL-1 cardiomyocytes demonstrate that autophagy promotes the progression of cardiomyocyte remodeling. Whether autophagy conveys a beneficial or detrimental action seems to relate to the time frame and level of activation of autophagy. While ER-stress-induced autophagy initially attenuates ER stress by activation of ER chaperones (Kroemer et al., 2010), excessive activation of autophagy, reflected by eIF2α phosphorylation and increased expression of ATF4, autophagy genes and LC3B, is associated with various chronic diseases, including heart diseases (Kim et al., 2008;Chen et al., 2011). Likewise, excessive autophagy is present mainly in permanent AF, contributing to myolysis and structural remodeling of the cardiomyocytes, finally, resulting in AF progression.

4.3 Autophagy and ER stress modulation as a therapeutic intervention strategy

Pharmacological approaches preventing or limiting AF progression and substrate formation are extensively being studied, with the aim of identifying novel effective therapeutic agents for AF treatment (Dobrev et al., 2012). Given our results, pharmacological intervention to inhibit autophagy may constitute a promising therapeutic strategy in clinical AF. At present, autophagy can be modulated by a number of small molecules (Balgi et al., 2009;Cao et al., 2011;Fleming et al., 2011;Baek et al., 2012;Sciarretta et al., 2012). Testing whether these drugs are effective in humans warrants their clinical development. Since basal autophagy is crucial for normal cell physiology, chronic treatment with autophagy inhibitors, such as in permanent AF, may be detrimental to the cardiomyocyte (Hara et al., 2006;Nakai et al., 2007;Maruyama et al., 2008;Gurusamy and Das, 2009b;Kaminskyy et al., 2012). For example, the high toxicity of bafilomycins precludes its use in the clinical setting (Drose and Altendorf, 1997). Currently, the clinical options for autophagy inhibition are limited. Alternatively, as we demonstrated ER stress to cause tachypacing-induced autophagy, (novel) compounds directed against ER stress may prove effective in limiting AF progression. The most promising approach seems the chemical chaperone 4-PBA, as this compound inhibits ER-stress induced upregulation of autophagy and has been approved for clinical use. More importantly, 4-PBA was reported to have few side effects and is considered safe in patients (Carducci et al., 2001).

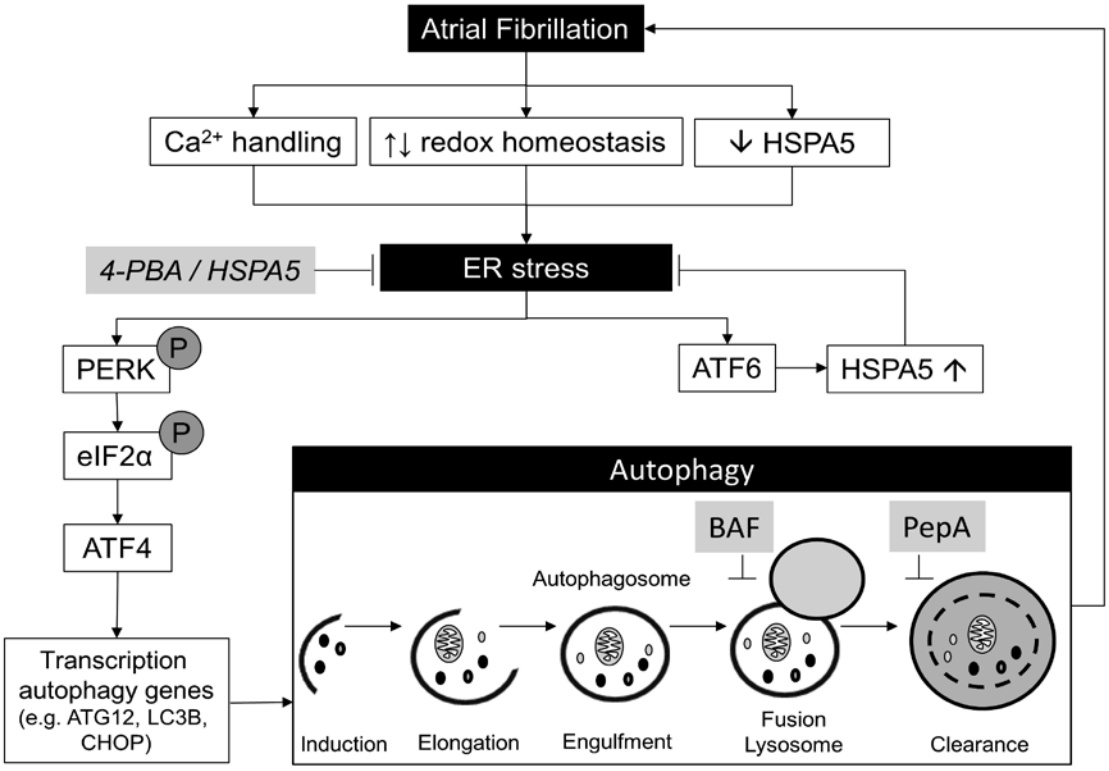

Figure 8 Proposed model for the role of AF-induced autophagy and disease progression.
AF causes changes in the cardiomyocytes, which trigger ER stress. These changes include altered Ca^{2+} handling, derailment in redox homeostasis and reduction in ER chaperone HSPA5 levels (Demaurex et al., 2000;Schonthal, 2012). Subsequently, ER stress induces the activation of the ER stress response, which includes the activation and phosphorylation of PERK and downstream phosphorylation of eIF2α. This ultimately results in the activation of the transcription factor ATF4, which regulates the expression of autophagy genes (e.g. ATGs) and LC3B involved in autophagy induction and elongation. Another part of the ER stress response results in activation of ATF6, which upregulates the transcription of HSPA5 in an attempt to restore ER homeostasis. Initially AF-induced activation of autophagy may attempt to restore cardiomyocyte proteostasis, however excessive stress-induced autophagy contributes to loss of contractile function and cardiac remodeling. Stress-induced autophagy appears maladaptive, as inhibition of autophagy via 4-PBA, HSPA5 overexpression, BAF and PepA prevented AF-induced loss of calcium transients.

Abbreviations, ATF4; Activating Transcription Factor 4, ATF6; Activating Transcription Factor 6, ATG12; Autophagy related protein 12,BAF; Bafilomycin A1, eIf2α; eukaryotic initiation factor 2α , ER; Endoplasmic Reticulum, HSPA5; Heat Shock Protein A5, LC3B; microtubule-associated proteins 1 light chains 3B, 4-PBA; 4-phenyl butyric acid, PePA; Pepstatin A, PERK; protein kinase RNA-like endoplasmic reticulum kinase.

Therefore, our findings suggest 4-PBA as a therapeutic agent with great potential in AF.

In summary, the present study demonstrates AF to activate autophagy, which is involved in cardiomyocyte remodeling, as inhibition of autophagy protected against loss of CaT. Furthermore, activation of autophagy is caused by upstream stimulation of the ER stress signaling. These findings imply that prevention of autophagy, possibly by compounds stimulating the expression of ER chaperones, might form a novel therapeutic target for intervention.

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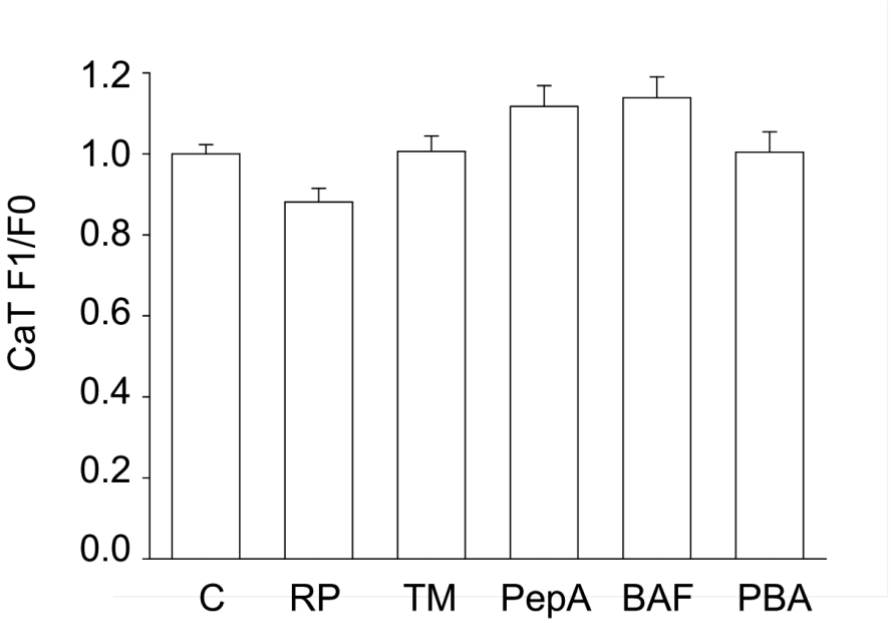
Supplemental Information



Supplemental Figure 1 Pharmacological modulation of autophagy in normal-paced HL-1 cardiomyocytes does not affect calcium transients.
Quantified CaT of HL-1 cardiomyocytes pretreated with autophagy modulators and subjected to normal-pacing.

Summary, discussion, the past and present

Partially adapted from:

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Loss of proteostatic control as a substrate for atrial fibrillation: a novel target for upstream therapy by heat shock proteins.

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Summary

1.1 Loss of proteostasis in AF

Atrial fibrillation (AF) is the most common sustained clinical tachyarrhythmia associated with increased mortality and morbidity (Nattel et al., 2008;Dobrev et al., 2012). Conventional treatment options against AF still lack efficacy and are ineffective in preventing the recurrence of AF (Van Gelder et al., 1996;Nattel et al., 2008;Dobrev et al., 2012). As AF induction, maintenance and progression requires the presence of a suitable substrate (Nattel et al., 2008;Allessie et al., 2010;Dobrev et al., 2012), prevention of substrate formation may improve the therapeutic outcome in AF. We and others have recently obtained evidence that derailment of proteostasis is a key factor involved in AF substrate formation (Brundel et al., 2001;Allessie et al., 2002;Ausma et al., 2003;Cha et al., 2004;Todd et al., 2004;Brundel et al., 2006;Allessie et al., 2010;Dobrev et al., 2012). Derailment of proteostasis is defined as the loss of protein homeostasis, which is necessary for the maintenance of normal cardiac function. The derailment affects processes controlling the concentration, conformation, binding-interaction, kinetics, and location of individual proteins. In addition to contributing to AF induction, derailment of proteostasis is aggravated by AF itself, resulting in electrical and structural remodeling and subsequently AF progression (Brundel et al., 2006;Nattel et al., 2008;Ke et al., 2011;Dobrev et al., 2012;Meijering et al., 2012).

In this thesis we investigated AF-induced derailment of proteostasis and identified several druggable targets to conserve cardiomyocyte proteostasis and prevent AF substrate formation. Firstly, in experimental models for AF, we have identified altered activation of diverse kinases to contribute to AF substrate formation and observed protective effects by pharmacological and genetic boosting of HSP expression. In **chapter 2**, we show tachypacing-induced RhoA and ROCK activation to result in F-actin stress fiber formation and subsequently loss of calcium transients (CaT). Conservation of actin proteostasis by ROCK inhibition or expression of HSPB family members (HSPB1, HSPB6, HSPB7 and HSPB8) was sufficient to protect against F-actin stress fiber formation and loss of CaT. HSPB family members, HSPB1, HSPB6 and HSPB7, directly prevented F-actin formation, whereas HSPB8 inhibited upstream RhoA activity. In **chapter 3**, we further elucidated the role of pathological RhoA activation on cardiac proteostasis. We show RhoA activation to suppress the protective heat shock response (HSR) in HL-1 atrial cardiomyocytes. In **chapter 4**, we applied a kinomics array approach to identify key kinases involved in atrial tachypacing-induced cardiac remodeling in dogs with and without pre-treatment of the cardioprotective HSP booster GGA. Tachypacing-induced alterations in kinome profile were to a large extent restored by GGA, indicating conservation of kinase activity as an

important cardioprotective mechanism of GGA. In addition to altered kinase activation we show in **chapter 5** that tachypacing-induced activation of histone deacetylase HDAC6 results in loss of α-tubulin proteostasis, through deacetylation of α-tubulin and subsequent depolymerization and degradation of the microtubule network. Tachypacing-induced contractile dysfunction was rescued by HDAC6 inhibition and by expression of HDAC6 with a mutation in the α-tubulin deacetylation domain. This study, and our findings on the role of F-actin stress fibers (chapter 2), suggest cytoskeletal proteostasis to play an important role in AF substrate formation, as α-tubulin and F-actin are major components of the cytoskeleton. Lastly, in **chapter 6** we show the role of tachypacing-induced autophagy, through activation of ER stress signaling, in cardiomyocyte remodeling in experimental models for AF as well as in clinical AF. Conservation of cardiac proteostasis was achieved by pharmacological inhibition of autophagy and relief from upstream ER-stress, which protected against tachypacing-induced loss of CaT. Interestingly, also overexpression of the ER-chaperone, HSPA5, revealed protective effects. These findings imply that prevention of autophagy, possibly by compounds stimulating the expression of ER chaperones, might prove a novel therapeutic intervention strategy in AF.

In summary, our data indicate loss of proteostasis as an important determinant of AF substrate formation. Furthermore, we identified several novel druggable targets directed at conservation of cardiac proteostasis, and prevention of AF substrate formation. An overview of AF-induced derailment of proteostasis and druggable targets is depicted in figure 1.

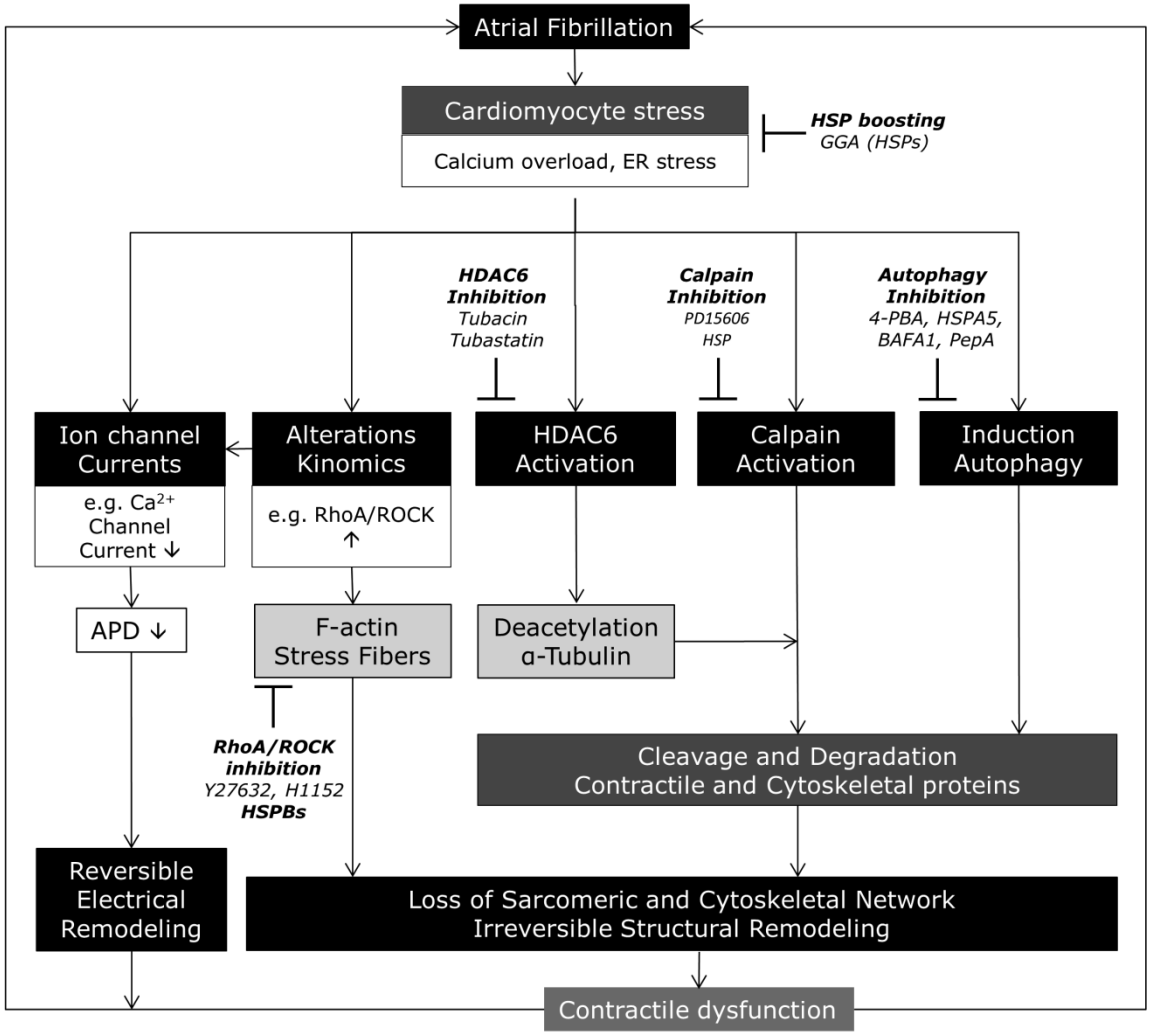

Figure 1 Overview of AF-induced derailment of cardiomyocyte proteostasis.
AF causes cardiomyocyte stress due to cellular Ca^{2+} overload, oxidative stress and ER stress, which results in altered activation of kinases and phosphatases. These kinases and phosphatases regulate early electrical remodeling resulting in the reduction of L-type Ca^{2+} channel current, shortening of action potential duration (APD), and contractile dysfunction. These changes occur within minutes and are reversible. These early responses protect the cardiomyocyte against Ca^{2+} overload but at the expense of creating a substrate for AF maintenance. When AF persists, derailment of proteostasis continues, including RhoA and ROCK activation induced F-actin stress fiber formation, HDAC6 activation and activation of protein degradative pathways, including autophagy and calpain. Ultimately, derailment of proteostasis results in irreversible cleavage and breakdown of sarcomeric and cytoskeletal proteins leading to AF persistence and progression. Druggable targets are indicated and include, pharmacological HSP boosting by GGA, treatment with the chemical chaperone 4-PBA, ROCK inhibition by Y27632 and H1152, HDAC6 inhibition by tubacin or tubastatin A, autophagy inhibition by 4-PBA, HSPA5 expression, bafilomycin A1 and pepstatin A and lastly calpain inhibition by PD15606.

Abbreviations, ER; Endoplasmic reticulum, BAFA1; Bafilomycin A1, PepA; Pepstatin A, HSP; Heat Shock Protein, APD; Action Potential Duration.

Discussion

2.1 Targeting loss of proteostasis in AF

Accumulating evidence suggests loss of cardiac proteostasis to play an important role in AF substrate formation contributing to AF induction as well as maintenance and progression (Brundel et al., 2001;Allessie et al., 2002;Ausma et al., 2003;Cha et al., 2004;Todd et al., 2004;Brundel et al., 2006;Nattel et al., 2008;Ke et al., 2011;Dobrev et al., 2012;Meijering et al., 2012). In this thesis we have elucidated novel mechanisms involved in AF-induced derailment of cardiomycoyte proteostasis, including changes in kinome homeostasis (chapter 2, 3 and 4), cytoskeleton proteostasis (chapter 2 and 5) as well as activation of autophagy (chapter 6), an important protein degradation pathway. Interestingly, conservation of cardiac proteostasis via pharmacological and genetic modulation of various key targets provided equal protection against loss of CaT and contractile dysfunction. It is still an open question how to explain the apparent effectiveness of interventions targeting only a single factor in these pathways. Accumulating evidence from our studies suggest that their effectiveness is due to an interplay between these factors in the cardiac proteostasis network, which may include the protection of shared downstream targets (as discussed below). However, it should be noted that in all our studies, drugs were administrated before the induction of AF. Therefore, the efficacy of the drug on prolonged conservation of the cardiac proteostasis in patients with persistent, permanent AF remains to be determined. Nevertheless, if drug administration results in a short term conservation of proteostasis, such intervention may prove promising in patients developing postoperative AF. Especially HDAC6 inhibition may be promising, as colchicine (another microtubule modulator) has already been successfully applied in preventing postoperative AF and early AF recurrence after ablation (Imazio et al., 2011;Deftereos et al., 2012). In addition to targeting specific downstream processes in the proteostasis network, application of HSP inducers such as GGA may demonstrate superior therapeutic efficacy, as they have pleiotropic effects on cardiac proteostasis. HSP induction not only provided protection against loss of actin proteostasis (chapter 2), but also protects against alterations in kinome homeostasis (chapter 2 and 6), activation of proteases (Garrido et al., 1999;Concannon et al., 2001;Zhang et al., 2011) and oxidative stress (Kalmar and Greensmith, 2009). Furthermore, HSP induction was effective against AF initiation, maintenance and progression in diverse models for AF (Brundel et al., 2006;Sakabe et al., 2008;Zhang et al., 2011). In clinical AF, a protective role for HSPs is implicated by diverse studies reporting the association of high HSP levels and reduced incidence of post-operative AF (St Rammos et al., 2002;Mandal et al., 2005) and spontaneous restoration of normal sinus rhythm (Cao et al., 2011). In addition, an inverse correlation between HSPB1 atrial expression and AF duration and extend of myolysis

was observed (Brundel et al., 2006;Yang et al., 2007). Because of its pleiotropic cardioprotective effects on AF substrate formation, HSP inducers currently represent a class of drugs with the most promising therapeutic potential in clinical AF. Whether HSP induction also protects against loss of α-tubulin proteostasis or counteracts the induction of autophagy remains to be determined. Nevertheless, our results warrant the testing of HSP inducers, particularly GGA, in human studies.

As mentioned above, the therapeutic efficacy of the diverse key modulators of cardiac proteostasis may be due to central downstream targets. A highly interesting target identified in this thesis comprises the loss of cardiac cytoskeletal proteostasis (chapter 2 and 5) as an important determinant for AF substrate formation and loss of contractile function. As the cardiac cytoskeleton is involved in diverse cellular functions, we discuss the possible molecular mechanisms that contribute to AF substrate formation.

2.2 The cardiac cytoskeleton

The cytoskeleton of cardiomyocytes consists of cortical (non-sarcomeric) actin filaments, desmin (intermediate) filaments and microtubules consisting of polymerized α- and β-tubulin. These cytoskeletal proteins interact with membrane associated proteins in the juxtamembrane costamere (e.g. dystrophin, spectrin, paxillin, talin, vinculin, ankyrin, metavinculin), sarcomeric and sarcomeric Z-disc proteins (e.g. sarcomeric actin, titin, C-protein, α-actinin) as well as proteins of the intercalated disk, including proteins comprising desmosomes (e.g. desmoplakin, desmocollin, desmoglein), adherens junctions (catenins and vinculin) and gap junctions (connexins) (Hein et al., 2000;Knoll et al., 2011;Knoll and Buyandelger, 2013). This complex network, of which a conceptual model is depicted in Figure 2, plays an important role in the transmission of mechanical and chemical signals within and between cells. (Hein et al., 2000;Knoll et al., 2011). The complex interactions between the sarcomere and the cytoskeleton are important for the fixation of the sarcomere to the sarcolemma and subsequently to the extracellular matrix via integrins and dystroglycans. The resulting network provides structural support but is also important for force transmission from the sarcomere to the sarcolemma as well as mechanosensation and mechanotransduction, which enables cells to sense and respond to mechanical stimuli by activation of cytoskeletal signaling involving integrins and Z-disc protein complexes (Hellberg et al., 1999;Hein et al., 2000;Knoll et al., 2011). In addition to these functions, the cytoskeletal filaments are also involved in transport of diverse proteins and organelles, as well as (in)direct anchoring of subcellular structures and organelles, including the t-tubule, mitochondria, nuclei and the sarcoplasmic reticulum (Hein et al., 2000;Kamal and Goldstein, 2000;Rogers and Gelfand, 2000;Leach et al., 2005;Knoll et al., 2011;Smyth et al., 2012).

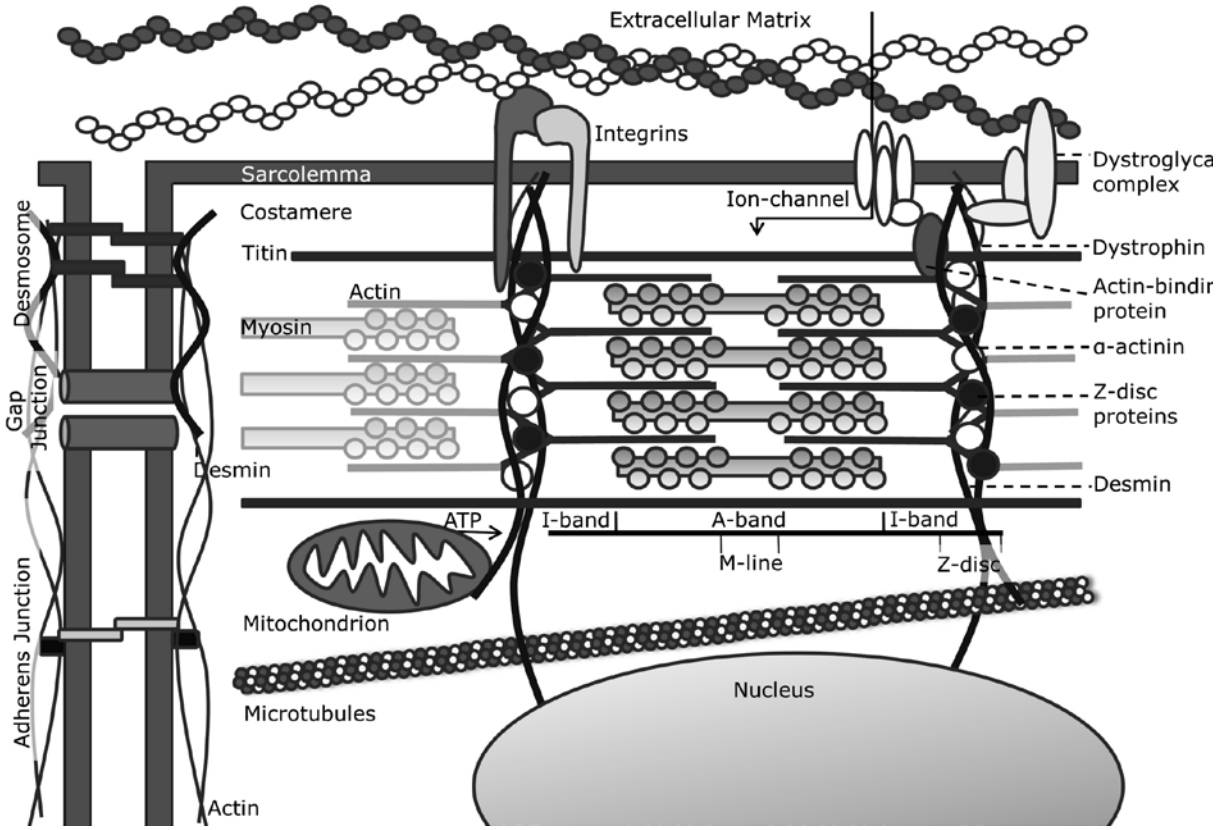

Figure 2 Conceptual model of cytoskeletal interactions
Sarcomeric proteins are organized in an I-band (isotropic) which consists of mainly actin filaments, an A-band (anisotropic) consisting of both myosin and actin filaments. The M-line which forms the center of the sarcomere and consists of only myosin filaments. Finally, actin filaments are crosslinked at the Z-disc where it interacts with desmin (intermediate filaments), dystrophin, α-actinin and other actin-binding proteins. Actin filaments are also known to interact with microtubules, which in turn regulate mitochondrial transport, amongst other functions. The desmin filaments not only provide an interaction between the sarcolemma and the sarcomere but also provide an interaction between the nucleus and the sarcomere, as well as can bind to mitochondria and is present at gap junctions and desmosomes. Contractions are the result of ATP-dependent sliding of actin and myosin filaments. Interactions between the sarcomeric and cytoskeletal proteins are pivotal for transmission of the force generated by the sarcomere to the sarcolemma and subsequently to the extracellular matrix via integrins and dystroglycans. In addition to force transmission the interactions between the sarcomere and the cytoskeleton also play a role in mechanotransduction, which allows cardiomyocytes to sense and respond to mechanical stimuli via activation of cytoskeletal signaling involving integrins and Z-disc proteins. Ion channel function is modulated by actin-binding proteins, which link the channel to the cytoskeleton.

2.3 Cardiac cytoskeletal proteostasis and AF substrate formation

2.3.1. Cytoskeleton and ion channel function and expression

AF is associated with reduced ion-channel currents and expression, with a prominent role for I_{Cal} (Nattel et al., 2008). Cardiac ion channel

expression, trafficking, location, as well as function are known to be influenced by cytoskeleton dynamics. (Calaghan et al., 2004;Vatta and Faulkner, 2006;Steele and Fedida, 2013). The effect of cytoskeletal dynamics on ion-channel function are likely due to the interaction of the ion-channels with the actin network through diverse actin-binding proteins, including AHNAK and AnkyrinB (Hohaus et al., 2002;Alvarez et al., 2004;Haase et al., 2005;Smith et al., 2012). Indeed diverse ion channel currents including, I_{CaL}, I_{Na}, I_{KATP} and I_{SAC} are modulated by actin-binding proteins and changes in the actin cytoskeleton (Lader et al., 1999;Rueckschloss and Isenberg, 2001;Hohaus et al., 2002;Alvarez et al., 2004;Calaghan et al., 2004;Haase et al., 2005;Leach et al., 2005). In addition to the role of actin filaments, microtubules were also suggested to modulate ion channel current, as modulation of microtubule dynamics by taxol (microtubule stabilizer) and colchicine (microtubule disruptor) were reported to affect ion channel current, including I_{CaL} (Pascarel et al., 1999;Kerfant et al., 2001;Malan et al., 2003;Gomez et al., 2004). In addition, also nocodazole (microtubule disruptor), regulates I_{CaL}, although via prevention of phosphorylation of the channel by CaMKII (Dzhura et al., 2002). As microtubules interact with actin (Cunningham et al., 1997) and desmin filaments (Gurland and Gundersen, 1995), indirect effects on I_{CaL} via these filaments cannot be excluded. However, it is unclear whether desmin filaments can modify ion-channel currents.

In relation to AF, disruption of cytoskeletal proteostasis or mutations in cytoskeletal or actin binding proteins may create a substrate for AF initiation and maintenance. Recently, Ankyrin-B dysfunction has been linked to AF, as a loss of function mutation in ANK2 presented with a high incidence of early-onset AF which progressed to permanent AF (Cunha et al., 2011). Also Ankyrin deficient mice (+/-) revealed atrial arrhythmias and showed increased susceptibility to AF induction in response to burst pacing. Further, isolated primary atrial myocytes from these mice displayed shortened action potential duration and decreased I_{CaL} due to loss of Cav1.3 expression (Le Scouarnec et al., 2008).

2.3.2. Cytoskeleton and intercalated disc function

Individual cardiomyocytes are connected at the intercalated disc by desmosomes and adherens junctions to provide mechanical continuity, while gap junctions provide a passage for ions and small molecules between cells, allowing propagation of the action potential (van der Velden and Jongsma, 2002;Bar et al., 2004). Desmosomes, adherens junctions as well as gap junctions interact with the cytoskeleton, which provides stabilization of these structures at the intercalated disc (Hein et al., 2000;Calaghan et al., 2004). Further, actin filaments and microtubules, transport membrane components from the ER to the cell membrane, including connexins (Smyth et al., 2012). Changes in the cytoskeleton may affect the stability of these structures at the

intercalated disc as well as affect the presence of connexins. Indeed, the actin-binding protein Ankyrin-G has been implicated in the formation and maintenance of the intercalated disk (Cunha and Mohler, 2006;Sato et al., 2011). Loss of ankyrin-G in cardiac myocytes was found to result in a reduction in desmosome and gap junction (connexin 43) densities at the intercalated disc along with a decrease in intercellular adhesion strength and electrical coupling (Sato et al., 2011). A role for alterations in adherens junctions (N-cadherin) and gap-junction/connexin physiology in creating a substrate for AF has already been established. Loss of N-cadherin is associated with a reduced connexin expression resulting in conduction slowing and arrhythmogenesis (Li et al., 2005). Further, AF is associated with changes in connexin expression and distribution, which are expected to have profound effects on cardiac conduction and refractoriness heterogeneity, both important reentry mechanisms (Van der Velden et al., 1998;van der Velden et al., 2000;van der Velden and Jongsma, 2002;van der Velden et al., 2002;Lampe and Lau, 2004;Kjolbye et al., 2007;Ram, 2008;Burstein et al., 2009;Igarashi et al., 2012;Kato et al., 2012). Desmin mutations, which can affect desmosome formation have not been directly linked to AF, although some desmin mutations are linked to arrhythmogenic ventricular cardiomyopathies (Lorenzon et al., 2013).

2.3.3. Cytoskeleton and oxidative stress

Accumulating evidence implicates a role for oxidative stress in the pathogenesis of AF, which likely contributes to structural and electrical remodeling in the heart (Ausma et al., 1997;Tsuboi et al., 2001;Lai et al., 2003;Dudley et al., 2005;Bukowska et al., 2008;Van Wagoner, 2008). Oxidative stress is known to affect cytoskeletal proteostasis as it induces depolymerization of microtubules (Lee et al., 2005) and reorganization of actin (Huot et al., 1997). Hence, oxidative stress induced disruption of the cytoskeleton may contribute to cardiac remodeling and AF maintenance. Indeed, we observed in chapter 2 and 5 that protection of loss of cytoskeletal proteostasis prevented cardiac remodeling.

Interestingly, a link between cytoskeleton dynamics and activation of enzymes related to reactive oxygen species (ROS) production, including NADPH oxidase, has been reported (Roberge et al., 1996;Kiley and Parker, 1997;Kustermans et al., 2005;Devillard et al., 2006;Xiao et al., 2010). Further, microtubules and microtubule-associated proteins are involved in mitophagy, which may reduce oxidative stress by removal of damaged mitochondria (Xie et al., 2011;Hanna et al., 2012). Treatment with microtubule modulators, e.g. taxol, nocodazole and colchicine were observed to indeed decrease ROS levels (Devillard et al., 2006;Xiao et al., 2010). Further, in clinical and *in vitro* models for AF, colchicine was reported to reduce post-operative AF and AF-recurrence after pulmonary vein isolation in patients with paroxysmal AF (Imazio et al., 2011;Deftereos et al., 2012), while taxol was reported to prevent AF in an

in vitro model (Xiao et al., 2010). Since taxol induces microtubule polymerization, while nocodazole and colchicine induce microtubule depolymerization, it remains unclear how all three compounds reduce ROS levels. Moreover, whether their effect is conveyed through modulation of microtubule dynamics remains to be elucidated.

2.3.4. Cytoskeleton and mitochondrial regulation

In AF, alterations in mitochondrial shape and size have been reported (Ausma et al., 1997) and mitochondrial dysfunction has recently been reported to represent an arrhtyhmogenic substrate associated with the occurrence of post-operative AF (Montaigne et al., 2013). The cytoskeleton plays an important role in mitochondrial regulation as it is involved in mitochondrial transport, localization and function, e.g. mitochondrial ADP sensitivity (Thornell et al., 1997;Milner et al., 2000;Capetanaki, 2002;Bar et al., 2004;Nekrasova et al., 2011;Kuznetsov et al., 2013;Varikmaa et al., 2013). Whether alterations in the cytoskeleton indeed influence mitochondrial morphology and function in AF is currently unknown.

2.3.5. Cytoskeleton and signaling

In addition to providing mechanical stability and force transmission, the interactions between the sarcomere and the cytoskeleton play a pivotal role in mechanosensation and mechanotransduction (Hellberg et al., 1999;Knoll et al., 2011;Knoll and Buyandelger, 2013). Furthermore, the Z-disc where sarcomeric proteins and cytoskeletal proteins (amongst others) interact with each other, is also a major signaling site. Several key molecules of transmembrane signaling are (temporarily) located at the Z-disk as well as the I-band of the sarcomere, suggesting a role for the Z-disc in compartmentalization and regulation of signaling (Knoll et al., 2011;Knoll and Buyandelger, 2013). As we observed, activation of calpain and autophagy (chapter 5 and 6) in AF are involved in the degradation of cytoskeletal (tubulin) and contractile proteins (troponins) (Ke et al., 2008). The subsequent loss of cytoskeletal and sarcomeric proteostasis likely contributes to the loss of mechanosensing and subsequent mechanotransduction, as well as to the loss of signaling compartmentalization provided by the Z-disc, resulting in further derailment of cardiac proteostasis and AF progression. Reduced mechanosensing and mechanotransduction may reduce integrin signaling, responsible for RhoA activation and subsequent ROCK and Serum response (transcription) factor (SRF) activation. As SRF regulates the expression of several sarcomeric genes including cardiac actin, α-MHC and β-MHC, myosin light chain (MLC)2a and 2v, myomesin, troponin C, and titin (Balza and Misra, 2006), loss of SRF activation may further contribute to loss of sarcomeric integrity and possibly to cardiac hibernation. Further, loss of sarcomeric and/or cytoskeletal proteostasis

may also affect Z-disc signaling which can have a broad range of effects as diverse proteins involved in a wide range of signaling pathways and cellular processes are located at or temporarily translocate to or near the Z-disc were they regulate signaling involved in apoptosis and cell survival (telethonin-Siva-p53-MDM2), gene expression (telethonin-calsarcin-1, myostatin, myopodin (Faul 2007), BMP10, NFAT-calcineurin, MURC-RhoA/ROCK), metabolism (CLOCK, MEF2A), ion channel activity (telethonin-minK), myofibrillar calcium sensitivity (MLP-HDAC4), Unfolded Protein Response/Protein degradation (E3 ubiquitin ligases, mURF1 and mURF3, HSPB family members, atrogin1, calpain1) and autophagy (BAG3) (Frey and Olson, 2002;Hoshijima et al., 2002;Faul et al., 2007;Gupta et al., 2008;Ogata et al., 2008;Mihatsch et al., 2009;Frank and Frey, 2011;Knoll et al., 2011;Knoll and Buyandelger, 2013). Furthermore, diverse kinases and phosphatases translocate to the Z-disc (PKC isoforms, PKD, Erk2, p38, calcineurin). Multiple of these factors are involved in AF, e.g. activation of calcineurin-NFAT signaling (Lin et al., 2004), calpain, ERK1/2 and PKC isoforms (Makary et al., 2011) contribute to pathological gene expression and action potential duration shortening. Further, p38 is a key regulator of the atrial stretch response (Kerkela et al., 2011). These findings suggest the sarcomeric Z-disc to be a key structure in AF induced remodeling, linking myofilament activity, mechanosensation, mechanotransduction, transmembrane signaling and transcriptional activity (Frank and Frey, 2011;Knoll et al., 2011;Knoll and Buyandelger, 2013). Disruption of cytoskeletal and/or sarcomeric proteostasis, as reported in AF, could therefore affect several downstream processes. In addition, loss of sarcomeric structure (including titin and desmin), as has been reported in AF (Ausma et al., 1997;Thijssen et al., 2000), can induce loss of compartmentalization of key signaling components, leading to deregulated signaling. Hence, in addition to altered signaling in response to altered calcium handling, alterations in signaling may be due to deregulation of integrin and Z-disc transcriptional coupling, resulting in subsequent alterations in cardiac gene expression and proteostasis. Loss of sarcomeric proteins and cytoskeletal proteins can induce loss of Z-disc compartmentalization and hence result in a variety of downstream effects further contributing to loss of proteostasis.

2.3.6. Cytoskeleton and contractility

In addition to the effects of microtubule modulation on ion-channel current, intercalated disk formation and transport of proteins and organelles, microtubules also affect the beating rate and amplitude of contraction in cultured neonatal cardiomyocytes (Klein 1983, Lampidis 1992, Webster 2000). These studies showed a negative regulatory effect of tubulin polymerization on beating rate, suggested to be due to mechanical interference with sarcomere motion. Subsequent disruption of all microtubule populations increased beating rate and contractile function. In contrast, this effect was not observed in adult

cardioymyocytes (Tagawa et al., 1998). However, microtubule stabilization by taxol-treatment in adult cardiomyocytes resulted in reductions in contraction amplitude (Tagawa et al., 1998), demonstrating that the maintenance of the normal tubulin and microtubule proteostasis is important for maintaining sarcomere function.

In conclusion, the cytoskeleton has multiple roles in regulating normal electrical and mechanical activity of cardiomyocytes, including organization of organelles, transport of proteins, autophagy, cell motility, contractility, mechanotransduction, mechanosensing, modulation of receptor and enzyme activities and ion channel function. Pathophysiological mechanisms involving loss of cytoskeletal and/or sarcomeric proteostasis that might be implicated in AF include a defective force generation, due to degradation of sarcomeric and cytoskeletal proteins, a defective force transmission, due to alterations in actin and tubulin dynamics or actin/tubulin binding proteins (loss of cytoskeletal proteostasis), alterations in mitochondrial regulation, an abnormal Ca^{2+} homeostasis, due to modulation of cytoskeleton on L-type Ca^{2+} current and abnormal ion currents due to modulation of other ion channels. Furthermore, loss of mechanotransduction (integrin and Z-disc mechanotranscriptional coupling) may drive cardiac dedifferentiation by altering signaling and gene-expression. The results from this thesis strongly suggest that maintenance of cytoskeletal proteostasis will preserve cardiomyocyte structure and may prove an important therapeutic strategy in AF. Deepening our understanding of the exact interplay and functions of the cytoskeleton comprises a first step towards that goal.

3.1 Novel therapeutic options in AF

In this thesis we identified several novel pharmacological approaches to prevent AF substrate formation by conserving cardiac proteostasis. These therapeutic strategies included, ROCK inhibition by Y27632 (chapter 2), HDAC6 inhibition by tubacin (chapter 4), inhibition of autophagy by pepstatin A and bafilomycin A1, as well as inhibition of upstream ER stress by the chemical chaperone 4-PBA (chapter 5). In addition, the induction of HSP expression by the HSP inducer GGA protected against cardiac remodeling by conserving actin proteostasis (chapter 2) and kinome homeostasis (chapter 6). Below we discuss their possible application in more detail and provide an overview of clinical trials involving these and comparable compounds (table 1).

3.1.1. ROCK/RhoA inhibition

RhoA and its downstream effector ROCK regulate the polymerization of G-actin to filamentous F-actin. On the downside, this impairs calcium homeostasis and induces cardiac remodeling (chapter 2). On the upside,

their activation maintains sarcomeric protein expression (Balza and Misra, 2006) through induction of serum response factor (SRF) transcriptional activity (Sotiropoulos et al., 1999;Geneste et al., 2002;Liu et al., 2003). Hence, the therapeutic efficacy of RhoA/ROCK inhibition may be limited due to the dual role of this pathway in cardiomyocyte function. Indeed, this is confirmed in studies reporting overexpression of RhoA to result in sinus and atrioventricular (AV) nodal dysfunction, AF, and ventricular contractile heart failure (Sah et al., 1999;Yang et al., 2013). Furthermore, the inhibition of Rho family members is also detrimental for cardiac function as it resulted in an AV block with atrial enlargement and ventricular hypertrophy (Wei et al., 2004;Brown et al., 2006). These studies suggest that fine-tuning of RhoA/ROCK signaling is required to maintain proper cardiac function and warrants further investigation of RhoA/ROCK activation dynamics in AF to determine the appropriate therapeutic approach. In case of pathological (excessive) activation of this signaling pathway, partial inhibition may be a valid therapeutic approach. Further, excessive activation of the RhoA GTPase likely in combination with another stress may contribute to HSP depletion and cardiomyocyte apoptosis, as described in chapter 3.

3.1.2. HDAC6 inhibition

HDAC6 inhibition, by tubacin, conserves α-tubulin proteostasis, prevents its degradation by calpain 1 and protects against loss of calcium transient and cardiac remodeling in experimental model systems for AF (chapter 5). However, tubacin is not suitable for *in vivo* studies as it has low druglikeness (Butler et al., 2010). Other promising HDAC6 inhibitors, such as tubastatin A and ACY-1215 have been recently developed and reveal beneficial effects in mice models for neurodegenerative diseases and cancer (Butler et al., 2010;d'Ydewalle et al., 2011;Santo et al., 2012). We very recently demonstrated tubastatin A to protect from tachypacing-induced cardiac remodeling in a canine model for AF (Zhang et al., 2013), strengthening the notion that HDAC6 inhibitors represent a novel therapeutic approach in AF.

3.1.3. Inhibition of autophagy

In chapter 6 we identified tachypacing induced upregulation of autophagy via ER stress in experimental models for AF. We extended these findings to patients with permanent AF, as autophagosomes and autolysosomes were increased in atrial tissue of chronic AF patients. Further HSPA5 and p62 levels were decreased, which indicates the presence of ER stress and the activation of autophagy, respectively. In addition, p62 levels correlated significantly with cardiac troponin cTnI, cTnT, α-tubulin and inversely correlated with the amount of myolysis in patients with paroxysmal and persistent AF, suggesting autophagy to be involved in the degradation of sarcomeric contractile proteins and cytoskeletal proteins.

In chapter 6 we show that autophagy inhibition by bafilomycin A1 and pepstatin A prevented tachypacing-induced reductions in calcium transients. Further, also inhibition of upstream endoplasmic reticulum (ER) stress by 4-phenyl butyric acid (4-PBA) protected against tachypacing-induced loss of CaT as well as overexpression of HSPA5 (an ER located chaperone). Although we observed protective effects in our models for AF, long term or excessive inhibition of autophagy may be harmful, as (basal) autophagy is critical for protein and organelle quality control. Therefore, rather than a general blockade of autophagy, a sound approach avoiding side-effects would consist of targeting specific stress-induced mechanisms responsible for the excessive activation of autophagy. As we uncovered a prominent role for ER stress to induce downstream autophagy, ER stress inhibitors might represent a promising therapeutic target in AF. Currently, the clinical options for autophagy inhibition are limited. Bafilomycins are prohibited for clinical applications due to their high toxicity (Drose and Altendorf, 1997). Hydroxychloroquine has entered early phase clinical trials in cancer, see table 1. Furthermore, pepstatin A has been investigated in the past in clinical trials on gastric ulcers (Bonnevie et al., 1979;Svendsen et al., 1979). In those studies no beneficial effect of pepstatin was shown and clinical trials were discontinued. However, our results indicate a possible novel application for pepstatin A in AF. The most promising compound seems 4-phenyl butyric acid, as this compound inhibits ER-stress induced upregulation of autophagy and has been approved for clinical use, see table 1. More importantly, 4-PBA was reported to have few side effects and is considered safe for patients (Carducci et al., 2001). Therefore our findings suggesting 4-PBA as a therapeutic agent with great potential in AF.

3.1.4. HSP inducers

HSPs display pleiotropic effects on cardiomyocyte proteostasis and protect against AF-induced early and late cardiac remodeling. HSP induction provided protection against loss of actin proteostasis (as further elucidated in chapter 2), protection against alterations in kinome homeostasis (as further elucidated in chapter 2 and 4) and finally protection against activation of proteases (Brundel et al., 2006;Zhang et al., 2011). In this thesis we also determined HDAC activation and downstream tubulin proteostasis as an important factor in AF as well as autophagy to contribute to myolysis and hence irreversible remodeling. Whether HSP induction by GGA protects from AF substrate development via a direct inhibition of these factors is currently unknown. However, we found GGA to block the vast majority of changes in kinomic profile in tachypaced dogs (chapter 6), suggesting that the drug interferes with a plethora of cellular signaling pathways involved in AF-induced remodeling. As GGA treatment induces HSP proteins via the activation of HSF1, it is likely that the induction of the cardioprotective HSPA5 (chapter 6), which

is not under the control of HSF1 is limited. Indeed, GGA treatment was reported to only induce HSPA5 (grp78) expression at high concentrations which triggered both pro-apoptotic and anti-apoptotic unfolded protein response (UPR) (Endo et al., 2007). As we identified ER stress an important target in AF remodeling, co-treatment of HSP-inducer GGA with 4-PBA, which relieves ER stress, may represent a valid therapeutic strategy. Furthermore, as we show in chapter 3 that RhoA signaling is involved in inhibition of the HSR, co-treatment of GGA with a RhoA inhibitor would further boost the HSP expression and provide superior therapeutic efficacy. Currently, GGA represents the most efficacious compound for the pharmacological induction of HSPs and it has already been applied clinically in Japan since 1984 as an antiulcer drug with no reported serious adverse reactions (Murakami et al., 1981;Unoshima et al., 2003;Katsuno et al., 2005;Yanaka et al., 2007;Fujimura et al., 2012). Hence, induction of HSP expression seems at date the most promising therapeutic approach with pleiotropic protective effects and its efficacy as single or co-therapies in AF should be assessed in future clinical trials.

Table 1: Clinical application of drugs with potential clinical benefit in preventing AF substrate formation.

drug	target	phase	indication	ref (clinical trials.gov identifier)
GGA (teprenone)	HSP induction	Phase IV	Gastric ulcers Gastritis Gastric lesion	NCT01190657 NCT01547559 NCT01284647 NCT01397448
NYK9354	HSP induction	Pre-clinical	Atrial Fibrillation	(Hoogstra-Berends et al., 2012)
4-PBA (Buphenyl)	ER stress inhibitor, Chemical chaperone	Phase II/III Phase II/III Phase II Phase I/II Phase I/II Phase I/II Phase II	Maple Syrup Urine Disease Urea cycle disorder Huntington Cystic Fibrosis ALS Spinal Muscular Atrophy Thalassemia	NCT01529060 NCT00345605 NCT00992459 NCT00212316 NCT00590538 NCT00107770 NCT00528268 NCT00005934
Tubastatin	HDAC6	Pre-clinical	Arthritis/ anti-inflammatory	(Vishwakarma et al., 2013)
ACY-1215	HDAC6	Phase I/II	Myeloma	NCT01323751 NCT01583283
Nicotinamide	Sirtuins	Phase III Phase II/III Phase I Phase II	Phosphatemia control Psoriasis Alzheimer Friedreichs ataxia	NCT01011699 NCT01763424 NCT00580931 NCT01589809
Colchicine	Microtubules ROS	Phase II Phase III	AF AF	NCT01755949 NCT01552187
Fasudil **Ezetimibe** **AR-12286**	ROCK	Phase III Phase II Phase II Phase IV Phase II	Raynaud's Phenomenon Vascular function study Atherosclerosis Atherosclerosis Glaucoma	NCT00498615 NCT00120718 NCT00670202 NCT00560170 NCT01936389
CCG-1423 **Rhosin**	RhoA	Pre-clinical		(Evelyn et al., 2007) (Shang et al., 2012)
Hydrochloro-quine	Autophagy inhibitor	Phase II	Cancer	NCT01292408
PepA	Aspartic protease inhibitor	Phase I/II	Duodenal ulcer	(Bonnevie et al., 1979; Svendsen et al., 1979)

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Chapter 7 Part II The Future

HSP-inducing compounds as therapeutics to restore proteostasis in Atrial Fibrillation

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Abstract

Atrial Fibrillation (AF) is the most common clinical tachyarrhythmia associated with significant morbidity and mortality and is expected to affect about 30 million North Americans and Europeans by 2050. AF is a persistent disease, caused by progressive, often age-related, derailment of proteostasis resulting in structural remodeling of the atrial cardiomyocytes. It has been widely acknowledged that the progressive nature of the disease hampers the effective functional conversion to sinus rhythm in patients and explains the limited effect of current drug therapies. Therefore, research is directed at preventing new-onset AF by limiting the development of substrates underlying AF promotion. Upstream therapy refers to the use of drugs that modify the atrial substrate- or target-specific mechanisms of AF, with the ultimate aim to prevent the occurrence (primary prevention), recurrence of the arrhythmia following (spontaneous) conversion or prevent the progression of AF (secondary prevention).

Recently, we observed Heat Shock Proteins (HSPs)-inducing drugs, such as geranylgeranylacetone (GGA), to prevent derailment of proteostasis and remodeling of cardiomyocytes, and thereby attenuate the AF substrate in cellular, *Drosophila melanogaster* and animal experimental models. Also, correlative data from human studies were consistent with a protective role of HSPs in preventing the progression from paroxysmal AF to permanent AF and in the recurrence of AF.

In this review, we discuss novel HSP-inducing compounds as emerging therapeutics for the primary and secondary prevention of AF.

1.1 Factors underlying Atrial Fibrillation initiation and progression

Atrial Fibrillation (AF) represents the arrhythmia with the highest prevalence, accounting for one third of hospitalizations related to cardiac rhythm disturbances (Dobrev et al., 2012). As age is a main risk factor to develop AF, it is expected to put increasing (financial) burden on societies, which will rise even further with the ageing of the population (Dobrev et al., 2012). In addition to age, other conditions that limit cardiac or vascular performance are associated with AF, including cardiac surgery, valvular heart disease, congestive heart disease, ischemic cardiomyopathy, obesity, hypertension and diabetes mellitus, causing atrial stretch and dilation (Dobrev et al., 2012). While these adaptations in the atria provide a substrate for the arrhythmia, AF itself also promotes AF by inducing specific changes to cardiomyocytes. Together, these changes obstruct the effective conversion to sinus rhythm in AF patients (Dobrev et al., 2012). During the past decade, evidence increases that the expansion of irreversible structural remodeling of cardiomyocytes comprises a main component of the progressive nature and the impaired functional recovery of AF (Figure 1) (Dobrev et al., 2012). However, current drug therapy, which mainly alleviates reversible electrical changes, has limited effect on patient outcome (Dobrev et al., 2012). Thus, therapeutic approaches that block the mechanisms conveying the AF-induced structural remodeling, referred to as 'upstream therapy', may offer superior therapeutic perspectives. We have identified the induction of small heat shock proteins (HSPs) to adequately suppress the substrate for AF (Brundel et al., 2006b). Recent research has identified several compounds that boost the HSP response, which may represent novel classes of drugs capable of attenuation of new onset AF (primary prevention) and/or progression of AF or recurrence of the arrhythmia after cardioversion (secondary prevention) (Brundel et al., 2006b;Sakabe et al., 2008).

1.2 Main concepts of cardiomyocyte remodeling and AF progression

1.2.1 Reversible electrical changes

Initiation of AF has been recognized to substantially alter electrophysiological properties of cardiomyocytes, which in turn strongly promote recurrence and maintenance of the arrhythmia (Dobrev et al., 2012). A conceptual model for the mechanisms underlying AF progression is depicted in Figure 2, in which the Ca^{2+} overload due to rapid activation (400-600 times per min) takes a central stage. In turn, Ca^{2+} overload causes a limitation of Ca^{2+} influx through inhibition of the L-type Ca^{2+} channel conductance and its down-regulation. Consequently, the action potential duration (APD) is shortened. Moreover, decreased Ca^{2+} influx initiates contractile dysfunction (hypocontractility). Together, these changes provide an expansion of the substrate for AF (Figure 2) (Dobrev

et al., 2012). While these changes have an immediate onset, they are reversible, and consequently are addressed by drug therapy providing rhythm control (Schotten et al., 2003).

1.2.2 Irreversible structural remodeling: role for derailment of proteostasis

In contrast to electrical changes, remodeling of the structural elements of cardiomyocytes seems far less reversible. Persistent AF induces structural changes comprising dissociation of atrial muscle bundles and hibernation of cardiomyocytes (Allessie et al., 2010), during which cardiomyocytes turn into a non-functional phenotype (Ausma et al., 2003). One of the key features of irreversible remodeling is the degradation of the myofibril structure (myolysis), which gives rise to contractile dysfunction (Figure 2) (Ke et al., 2008;Allessie et al., 2010). Other changes characteristic of structural remodeling are the redistribution of nuclear chromatin (Brundel et al., 2008), NFAT regulated suppression of L-type Ca channel expression (Qi et al., 2008) and the re-expression of a specific panel of genes, including ssTnI and α-smooth muscle actin (Brundel et al., 2008). Importantly, the structural remodeling of the atria causing impaired reversibility and persistence of AF (Allessie et al., 2010) is not addressed by the current symptomatic drug therapy.

Thus, structural remodeling represents currently the main target to provide upstream therapy. The various molecular mechanisms that underlie the progression and recurrence of AF (Brundel et al., 2006b;Ke et al., 2008;Ke et al., 2011), hold the promise of being highly instrumental in drug development. Thinking along these lines, a main target includes calpain, whose activation in AF results in degradation of contractile and structural proteins (Ke et al., 2008). Also, cardiomyocytes are unable to re-express (contractile) proteins, even after conversion of AF to normal sinus rhythm (Brundel et al., 2008). Most likely, this blockade is controlled by epigenetic factors (Ausma et al., 2003), and may be addressed by therapeutic strategies that are currently developed, mainly in de field of oncology (Fullgrabe et al., 2011). In addition, a target to counteract structural remodeling, that seems within reach, is post-translational modifications in structural proteins (Ke et al., 2011;Zhang et al., 2011b). To our current knowledge, key targets comprise of the formation of F-actin stress fibers due to early activation of RhoA GTPases (Figure 2) (Ke et al., 2011) and the deacetylation of α-tubulin by HDAC6 (Zhang et al., 2011b). Together, the above findings strongly suggest that AF is characterized by a derailment of the cardiomyocytes' proteostasis (i.e. the homeostasis of protein production, breakdown and function), which may serve as a key substrate for the induction and progression of AF. Indeed, loss of proteostasis is associated with many age-related diseases, including cardiovascular disorders (Balch et al., 2008).

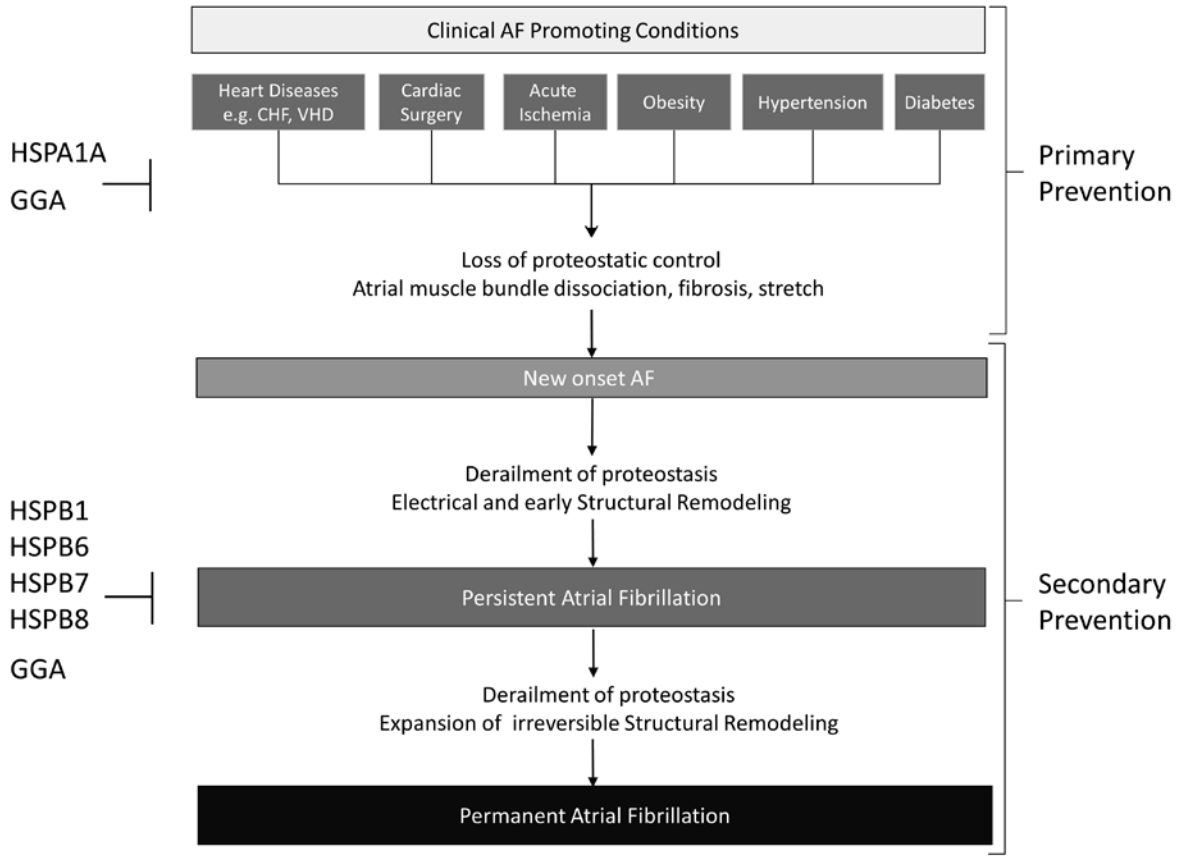

Figure 1 Overview of AF promotion conditions.
AF can be caused by many cardiac and non-cardiac conditions, including valvular heart disease, congestive heart disease, ischemic cardiomyopathy, obesity, hypertension and diabetes mellitus, which result in cell damage including atrial muscle bundle dissociation, stretch, and fibrosis. In addition to the underlying heart disease, AF itself can also cause further damage to the cardiomyocytes, by derailment of proteostasis and consequently electrical and structural remodeling. When AF is not converted to normal sinus rhythm, the arrhythmia will further progress from persistent into permanent AF. This progressive nature of AF hampers the effective functional conversion to sinus rhythm in patients and is rooted in AF-induced derailment of proteostasis causing irreversible structural cardiomyocyte remodeling. Current research showed HSP-inducing compounds such as GGA or overexpression of individual HSPs (HSPB1, HSPB6, HSPB7 and HSPB8) to prevent new onset AF (primary prevention), recurrences after cardioversion or even the progression of the arrhythmia (secondary prevention).

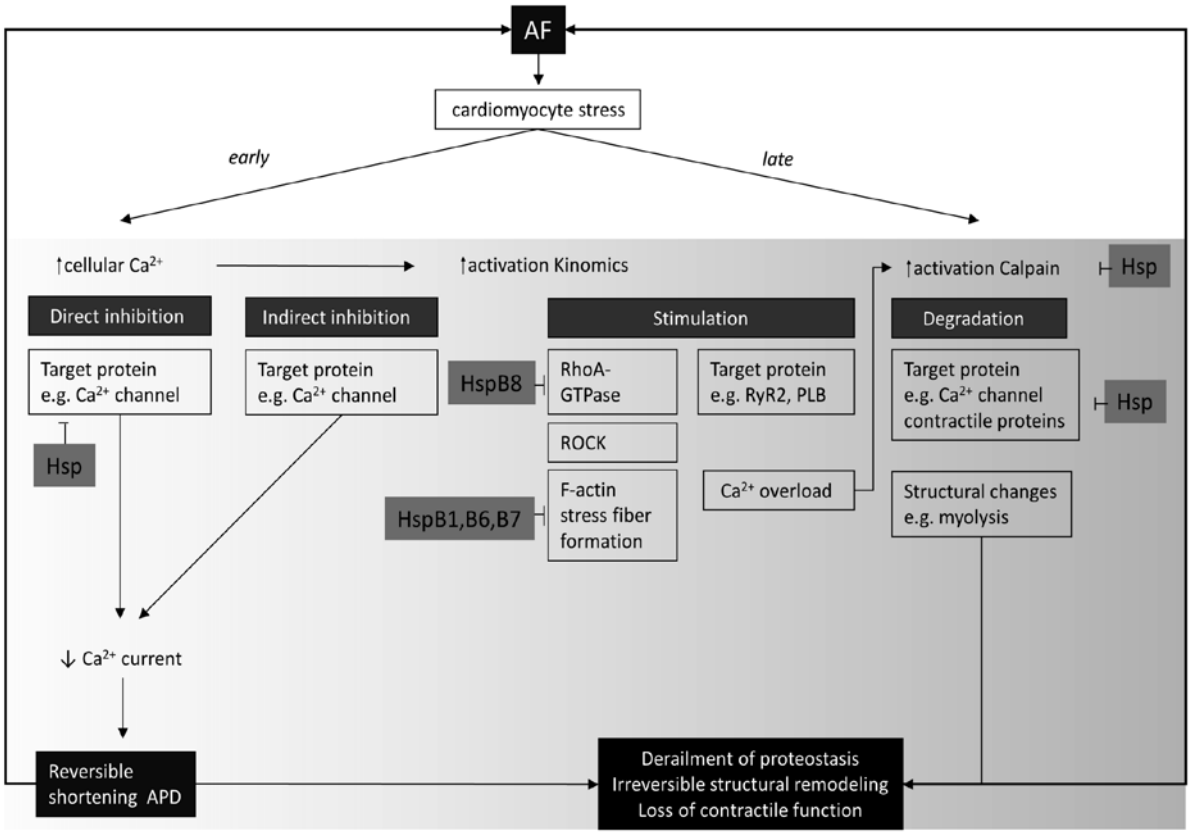

Figure 2 Overview of AF-induced derailment of cardiomyocyte proteostasis.
AF induces time-related atrial cardiomyocyte remodeling. First, AF causes a stressful cellular Ca^{2+} overload, which leads to a direct inhibition of the L-type Ca^{2+} channel. In addition AF induces changes in the kinomic profile causing an indirect inhibition or stimulation of target proteins. Both direct and indirect effects on target proteins result in shortening of action potential duration (APD) and loss of contractile function. Recently, we found AF to activate RhoA-GTPase and downstream ROCK resulting in formation of F-actin stress fibers and contractile dysfunction. When the tachycardia persists calcium overload induces calpain activation. Activated calpain can degrade the L-type Ca^{2+} channel and contractile proteins resulting in structural remodeling (myolysis). During myolysis tissue integrity is maintained, however, at the expense of contractile function.
Putative target sites for HSP protection against atrial cardiomyocyte remodeling are indicated. HSPB1, HSPB6 and HSPB7 prevent formation of stress fibers and subsequent contractile dysfunction. HspB8 prevents activation of RhoA-GTPase. Also HSPB1 was found to prevent AF-induced calpain activation. HSPs can bind to myofibrils and ion-channels to conserve their function.

One of the key defense mechanisms of cells against derailment of proteostasis is the heat shock response. Sensing of cellular stress activates the transcription factor heat shock factor 1 (HSF-1) and induces the expression of HSPs. HSPs, through their chaperone function, assist in balancing proteostasis by facilitating protein (re-)folding or degradation (Calamini et al., 2012;Xu et al., 2013). It is known that the activation of HSF-1 and downstream expression of HSPs is declining with aging, which is related to the loss of key longevity factors like SIRT-1 (Westerheide et al., 2009). Consequently, drugs that are able to boost HSP expression constitute an emerging therapeutic class for upstream therapy of AF.

In this review, we examine the evidence that HSP induction is able to restore proteostasis in experimental and human AF. In addition, we discuss the properties and potential of HSP-inducing agents, such as GGA and GGA derivatives, as therapeutic drugs to alleviate the occurrence and recurrence of AF.

1.3 Upstream therapy by heat shock protein induction

Previous research has unequivocally demonstrated that the induction of the heat shock response provides protection against derailment of proteostasis (Akerfelt et al., 2010;Calamini et al., 2012;Morimoto, 2012) and is beneficial in various cardiac diseases (Balch et al., 2008;Powers et al., 2009). Several recent studies thus addressed the potential of HSPs to limit remodeling in experimental models of AF and examined HSP in AF patients. HSPs consist of five HSP families, i.e. HSPA, HSPB, HSPC, HSPD, and DnaJB of which various members have been suggested to convey cardioprotection (Brundel et al., 2008). While the expression of HSPs is not exclusively dependent on HSF-1 activation, the majority of HSPs that seem involved in control of proteostasis do, including HSPB1 (HSP27), HSPA1A (HSP70) and HSPC1 (HSP90) (Balch et al., 2008;Powers et al., 2009;Calamini et al., 2012).

1.3.1 Prevention of first onset AF (Primary prevention)

There is evidence, both from experimental and human studies, that HSPs provide protection against the first onset of AF. In dog, pre-treatment with the HSP inducer, GGA, attenuates changes in action potential and prevents initiation of AF in (acute) ischemia, while augmenting cardiac expression of HSPA1A and HSPB1 (Sakabe et al., 2008). A further indication for the protective effect of HSPA1A is obtained from 2 studies in patients undergoing cardiac surgery, in which higher levels of HSPA1A expression in atrial tissue were related to a lower incidence of post-operative AF (St Rammos et al., 2002;Mandal et al., 2005). In addition, induction of HSPA1A in an experimental study in mice infused with angiotensin II prevents vulnerability to develop AF and limits atrial fibrosis (Wakisaka et al., 2007). Together, these results suggest that induction of

HSPs, especially HSPA1A, mitigates the development of the atrial substrate for the induction of AF (Figure 1).

1.3.2 Prevention of AF recurrence and progression (Secondary prevention)

If upstream therapy limits the substrate for AF by preventing derailment of proteostasis and cardiomyocyte remodeling, it is expected that induction of HSPs also protects against the recurrence of AF. Several studies support such action, including studies in tachypaced HL-1 atrial cardiomyoyctes, in which a general HSP induction by a heat shock or GGA treatment, normalizes proteostasis, as well as contractile function and structural integrity (Figure 1 and 2) (Brundel et al., 2006b;Brundel et al., 2008). Similar protection by general HSP induction was obtained in a dog model, in which GGA pretreatment attenuated tachypacing-induced AF promotion and limited recurrence of AF after cardioversion, most likely by limiting the shortening of APD and maintaining L-type Ca^{2+} current (Brundel et al., 2006b). Further studies into the members of the HSP family that mediate these protective effects have set the limelight to members of the HSPB family of small HSPs. Induction of HSPB1 was found sufficient to prevent tachypacing-induced structural remodeling, while overexpressing HSPA1A was not protective (Brundel et al., 2006b). Other specific HSPB family members also show protection in cellular models, including HSPB6, HSPB7 and HSPB8, but not the other HSPBs (Ke et al., 2011). Importantly, selective knockdown of HSPB1 fully attenuates the protective effect of a general heat shock response, demonstrating a main role for HSPB1. Thus, multiple HSPB family members attenuate remodeling following tachypacing and preserve cell proteostasis and thus are supposed to prevent the progression of the AF substrate once the arrhythmia starts (secondary prevention).
A similar protective effect of HSPB as observed in HL-1 cardiomyocytes has been found in *Drosophila melanogaster* (fruit fly) model for tachypacing-induced contractile dysfunction. In the transparent fruit fly pupae, the heart wall can be visualized. In this model, tachypacing induces both contractile dysfunction and structural remodeling (Figure 3) (Zhang et al., 2011a). This model also allows for induction of HSPs, either by heat shock, or by HSP-inducing compounds, such as GGA. Indeed, induction of HSPs by these strategies prevents tachypacing-induced contractile dysfunction and structural remodeling in *Drosophila* pupae. The *Drosophila* is an organism that can be easily genetically manipulated to overexpress proteins, i.e. small HSPBs. Of the six members of the *Drosophila* family of small HSPB that were overexpressed (DmHSP22, DmHSP23, DmHSP26, DmHSP27, CG14207, and Hsp67Bc), only overexpression of DmHSP23 was found to protect against tachypacing-induced contractile dysfunction and structural remodeling (Zhang et al., 2011a). Most likely, the attenuation of remodeling was by limiting the activation of calpain (Zhang et al., 2011a). Of note is that DmHSP23 probably represents a functional ortholog of the human HSPB1, as both HSPs share many features including chaperone activity, phosphorylation

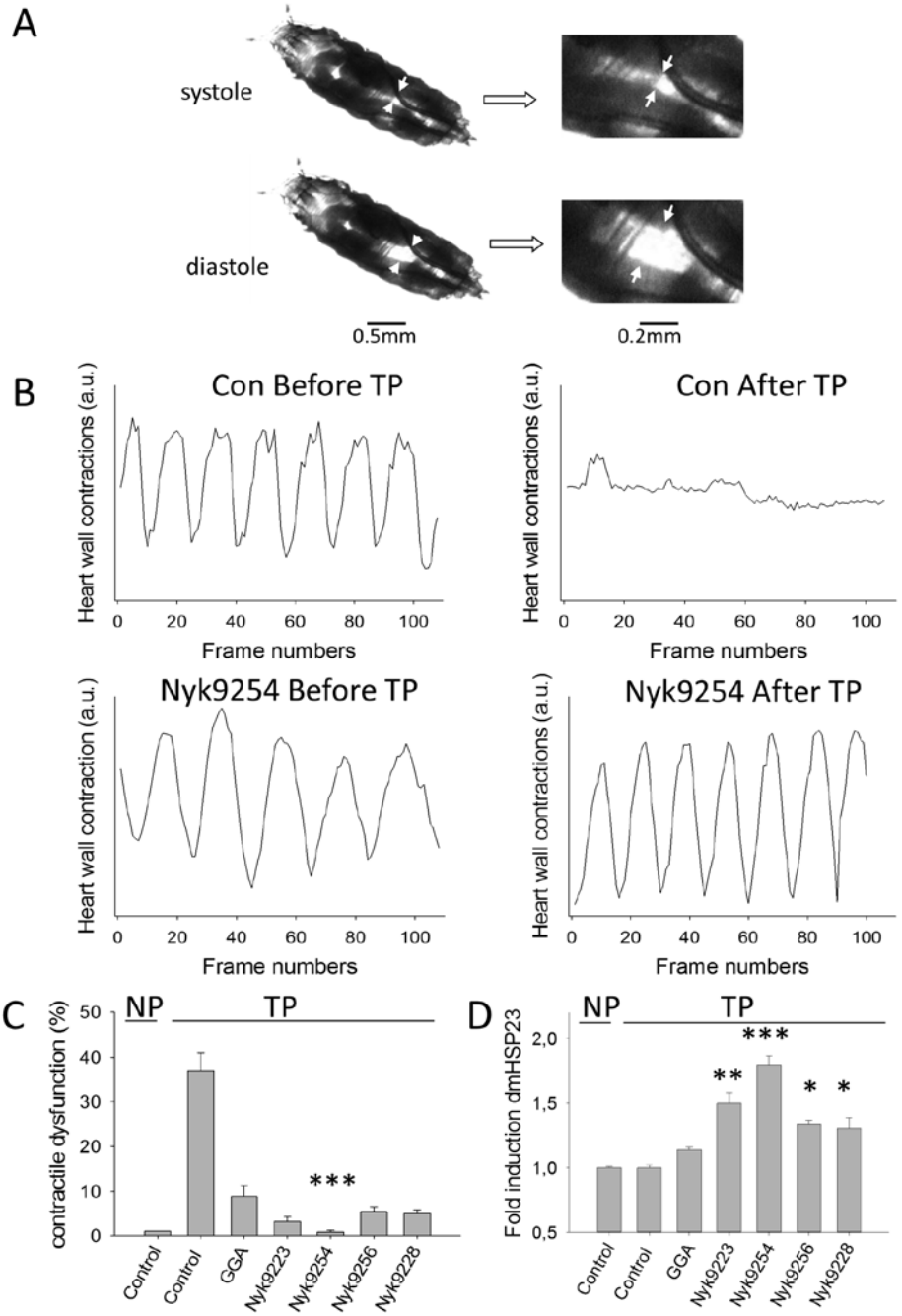

Figure 3 GGA derivates show improved protective effect against tachypacing-induced contractile dysfunction in Drosophila.
Pre-treatment with GGA-derivates Nyk9223, Nyk9254, Nyk9256 and Nyk9228 in *Drosophilae* reveal an improved protective effect against tachypacing-induced loss of contractile function compared to GGA. (A) Images of early *Drosophila* pupae in systole and diastole. The heart wall is marked with an arrow. (B) Illustrations of heart wall contractions are depicted after 20 minutes normal pacing (2,2Hz) and tachypacing (5Hz) of early pupae of a W1118 genetic background with and without pretreatment with Nyk9254 (100µM). Controls were treated with DMSO (100µM). (C) Quantified heart wall contraction rate for normal paced (NP) and tachypaced (TP) with or without GGA (1mM), GGA derivate Nyk9223, Nyk9254, Nyk9256, Nyk9228 (100µM) pretreatment. (D) QPCR data showing effect of GGA and GGA-derivatives on dmHSP23 expression. *P<0.05, **P<0.01, ***P<0.001 vs GGA tachypaced.

dependent dynamic oligomerization and protection of the cytoskeletal integrity (Vos et al., 2008).
Also in human AF there are hints that HSPB1 may protect from cardiac remodeling and attenuate progression of AF. Two independent studies report higher atrial expression levels of HSPB1 to relate to a shorter duration of AF and less extensive atrial myolysis (Brundel et al., 2006a;Yang et al., 2007). This suggests that in short duration AF the HSP response gets activated, while it exhausts in time with longer duration of AF. Thus lower levels of HSPB1 may signify loss of proteostatic control, in turn resulting in progression of structural remodeling paving the way to persistence of AF. Thus, securing HSP(B) levels at an adequate level, e.g. by treatment with HSP inducers, may thus limit the expansion of the AF substrate during paroxysmal and short term AF and attenuate progression from paroxysmal to persistent and permanent AF (Figure 1). In agreement with this hypothesis, restoration of sinus rhythm in patients with permanent AF after mitral valve surgery is related to a heat shock response and induced HSPB1 levels (Cao et al., 2011).
Several mechanisms have been coined to explain how small HSPB members protect against AF-induced derailment of proteostasis and structural remodeling (Figure 2). As stated above, we identified a range of members of the HSPB family to protect from tachypacing in cardiomyocytes, i.e. HSPB1, HSPB6, HSPB7 and HSPB8. Moreover, we demonstrated these HSPBs to reduce the formation of F-actin stress fibers during tachypacing, indicating that actin stabilization may represent a key mechanism employed by HSPBs to limit the substrate of AF. Nevertheless, different HSPBs seem to employ different modes to stabilize actin. HSPB1, HSPB6 and HSPB7 inhibit G- to F-actin polymerization and/or stimulate depolymerization of F-actin, while HSPB8 interferes with the upstream RhoA GTPase activation (Figure 2) (Ke et al., 2011). While the mechanism of action of various HSPBs seems to differ, it is yet unclear whether induction of multiple HSPBs offers therapeutic advantage over induction of a single HSPB member in limiting expansion of the AF substrate and/or increasing the success of cardioversion.

Taken together, induction of HSPs attenuates electrical, contractile and structural remodeling of cardiomyocytes in AF through normalization of cell proteostasis and thus seems to represent an effective upstream therapy to prevent initiation and progression of AF.

1.4 Novel HSP-inducing agents as a therapeutic approach in AF

Pharmacological approaches to prevent mechanisms underlying atrial cardiomyocyte remodeling are being studied, with the hope that they might be useful therapeutic agents in treating AF (Dobrev et al., 2012). So far, the efficacy of commonly used drugs on remodeling is limited (Dobrev et al., 2012). Interestingly, some of these drugs, such as glucocorticoids and statins induce HSPB1 expression and its phosphorylation (Dobrev et al., 2012), leaving open the possibility that the protective effect of these

drugs is due to expression of HSPB1. As the protective action of HSPs depends on their timely induction, drugs that boost the endogenous heat shock responses are of particular interest in prevention of first onset (primary prevention), recurrence and progression (secondary prevention) of AF (Figure 1 and 2) (Brundel et al., 2006a;Brundel et al., 2006b). A drug often used to boost HSP expression is GGA, a drug originally used as an anti-ulcer agent (Brundel et al., 2008). GGA is a nontoxic acyclic isoprenoid compound with a retinoid skeleton that induces HSP synthesis in various tissues, including gastric mucosa, intestine, liver, myocardium, retina, and central nervous system. GGA induces HSP expression through the activation of heat shock transcription factor HSF-1 (Brundel et al., 2008). The protective effect of GGA-induced HSP expression on tachycardia-induced structural remodeling has been observed in experimental models of promotion of atrial tachycardia-induced AF, suggesting that the induction of HSPs by GGA might have a potential value for clinical AF (Brundel et al., 2006a;Brundel et al., 2006b;Brundel et al., 2008) In *in vivo* tachypaced *Drosophila*, GGA treatment protected against contractile dysfunction of the heart wall and structural remodeling (Zhang et al., 2011a). Also in a canine model for (acute) atrial ischemia and tachypacing-induced AF promotion, a HSP-inducing GGA treatment revealed protective effects against cardiomyocyte remodeling, and consequently occurrence and recurrence of AF after cardioversion (Brundel et al., 2006b). Although protective effects with GGA were observed, an important disadvantage of GGA is its high LogP value (around 9) and therefore generally high dosages are required, as observed in the canine model for AF (Brundel et al., 2006b). Therefore we synthesized various derivatives of GGA, with improved pharmaco-chemical properties, and tested their ability to induce heat shock response in HL-1 cardiomyocytes (Figure 4). Some of these novel GGA derivatives were more potent to boost the heat shock response then GGA (Figure 4) and subsequently showed improved protection against tachypacing-induced contractile dysfunction in *Drosophila* (Figure 3). In comparison, GGA was found to protect against tachypacing-induced contractile dysfunction in *Drosophila* at a concentration of 1mM (Figure 3) (Zhang et al., 2011a), several of the GGA derivatives showed significant enhancement of protective effects compared to GGA at a concentration of 100µM, indicating a more potent profile for these GGA derivatives in the protection against tachypacing-induced heart wall remodeling (Figure 3). Interestingly, the GGA derivatives only revealed enhancement of the heat shock response in cells pretreated with a mild heat shock, yet did not induce a heat shock response under non-stressed conditions. This characteristic indicates that enhancement of the heat shock response by GGA and its derivatives are confined to stressed cells. As a consequence, such compounds may display lower incidence of side effects. Currently, one of the selected GGA derivatives is further tested in *in vivo* animal models for AF and prepared to be developed until a clinical phase II protocol to test the prevention of post-surgery AF. GGA is currently awaiting proof of clinical effectiveness in a similar setting in cardiosurgical patients. These initiatives underscore

that GGA derivatives are promising candidates for upstream treatment of AF initiation (primary prevention), recurrences and progression (secondary prevention) (Figure 1).

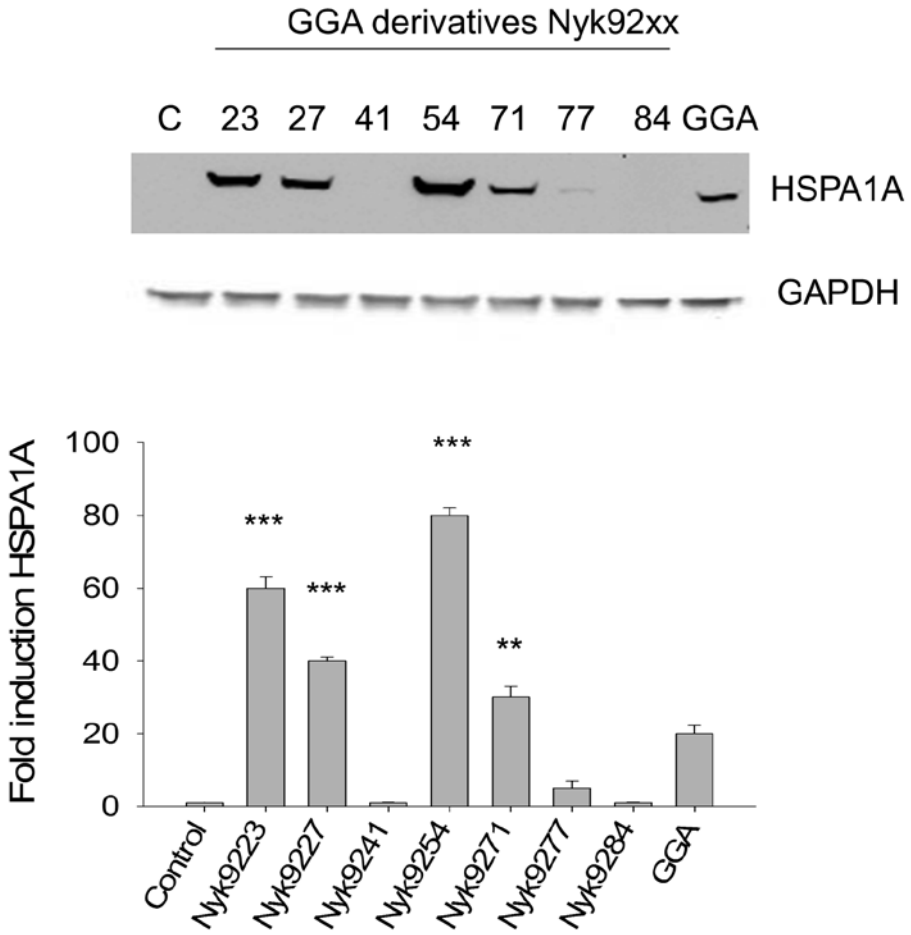

Figure 4 GGA derivatives show an improved HSP-inducing response in mild heat shocked HL-1 cardiomyocytes compared to GGA.
HL-1 cardiomyocytes were subjected to a mild heat shock (43^0C, 10 minutes) followed by incubation for 6 hours with a GGA derivative (Nyk92xx, 10 µM), or GGA (10µM) or with DMSO (10µM) as a control. After incubation proteins were harvested and loaded on a SDS-PAGE followed by Western blotting. (A) Representative Western blot showing HSPA1A levels. GAPDH expression was used as loading control. (B) Quantified data showing significant enhancement of HSPA1A expression for Nyk9223, Nyk9227, Nyk9254, Nyk9271 vs GGA. ** P<0.01, ***P<0.001 compared to GGA.

In summary, AF causes a derailment of cardiomyocyte proteostasis by inducing irreversible structural remodeling. There is strong evidence that induction of HSPs, in particular HSPA1A and HSPB family members, preserve proteostasis and thereby attenuate the structural remodeling that underlies the promotion of AF. Known upstream targets for HSP protection include L-type Ca^{2+} channel, calcium handling proteins, calpain, RhoA pathway and actin stress fibers. Ultimately, the induction of HSPs, by proteostasis regulators such as GGA and GGA derivatives, may prevent AF occurrence (primary prevention) and expansion of the structural remodeling during AF, resulting in improved outcome of cardioversion and/or delay in progression towards permanent AF (secondary prevention).

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Nederlandse Samenvatting

Het ontsporen van de eiwit homeostase zorgt voor de ontwikkeling en progressie van boezemfibrilleren

Boezemfibrilleren is de meest voorkomende en hardnekkige ritmestoornis die gepaard gaat met een verhoogde kans op sterfte en morbiditeit (Nattel et al., 2008;Dobrev et al., 2012). Conventionele behandelingen tegen boezemfibrilleren zijn nog niet effectief genoeg en kunnen het terugkeren van boezemfibrilleren niet voorkomen (Van Gelder et al., 2002;Nattel et al., 2008;Dobrev et al., 2012). Het onstaan, maar ook het handhaven en de progressie van boezemfibrilleren, is afhankelijk van de aanwezigheid van een geschikt aritmogeen substraat, welke een gevoeligheid voor boezemfibrilleren creëert (Nattel et al., 2008;Allessie et al., 2010;Dobrev et al., 2012). Dus het voorkomen van een aritmogeen substraat kan bijdragen aan het verbeteren van de therapeutische uitkomsten. Wij en anderen hebben recent bewijs verkregen dat verlies van eiwit homeostase een belangrijke factor is voor het ontwikkelen van een aritmogeen substraat voor boezemfibrilleren (Brundel et al., 2001;Allessie et al., 2002;Ausma et al., 2003;Cha et al., 2004;Todd et al., 2004;Brundel et al., 2006;Allessie et al., 2010;Dobrev et al., 2012). Verlies van eiwit homeostase kan leiden tot veranderingen in de lokalisatie, de activiteit en/of de afbraak van een eiwit. Verlies van de normale functie van (specifieke) eiwitten kan een aritmogeen substraat creëeren en de hartfunctie negatief beïnvloeden. Het verlies van eiwit homeostase draagt hierdoor bij aan het onstaan van boezemfibrilleren. Ook leidt boezemfibrilleren zelf tot verder verlies van eiwit homeostase, wat uiteindelijk resulteert in electrische en structurele veranderingen met progressie van boezemfibrilleren tot gevolg (Brundel et al., 2006;Nattel et al., 2008;Ke et al., 2011;Dobrev et al., 2012;Meijering et al., 2012).

In dit proefschrift hebben we het verlies van eiwit homeostase door boezemfibrilleren onderzocht. Daarnaast hebben we verschillende nieuwe therapeutische aangrijpingspunten kunnen identificeren die zorgdragen voor het behoudt van de eiwit homeostase in de hartspiercel, waardoorhet ontstaan van boezemfibrilleren kon worden voorkomen. Ten eerste, in experimentele modellen voor boezemfibrilleren hebben we aangetoond dat veranderingen in kinase activiteit bijdragen aan het onstaan van een aritmogeen substraat voor boezemfibrilleren. Farmacologische en genetische verhoging van heat shock eiwit (HSP) expressie beschermen de hartspiercellen. In hoofdstuk 2 laten we zien dat versnelde electrische stimulatie van hartspiercellen RhoA en ROCK (RhoKinase) activeert, wat vervolgens resulteert in de vorming van F-actine stress vezels en verlies van contractiele functie (calcium transienten (CaT)). Bescherming van actine homeostase door ROCK inhibitie of expressie van HSPB familieleden (HSPB1, HSPB6, HSPB7 en HSPB8) was voldoende om bescherming te bieden tegen de vorming van

F-actine stress vezels en het verlies in CaT. HSPB familieleden, HSPB1, HSPB6 en HSPB7 voorkwamen het ontstaan van F-actine mogelijk door binding met het cytoskelet in tegenstelling tot HSPB8 die de bovenliggende RhoA activatie verminderde. In hoofdstuk 3 hebben we de rol van pathologische RhoA activatie op de hartcel proteostase nader bestudeerd. Hier laten we zien dat RhoA activatie de inductie van de beschermende heat shock respons (HSR) kan verminderen in het HL-1 celmodel voor boezemfibrilleren. In hoofdstuk 4, hebben we een kinomics array uitgevoerd om kinases en substraten te kunnen identificieren die een belangrijke rol spelen bij de veranderingen in de hartspiercel in een hondenmodel voor boezemfibrilleren. De arrays hebben we uitgevoerd op hartweefsel van honden zonder en met voorbehandeling met de HSP inducerende stof geranylgeranylaceton (GGA). Tachypacing leidt tot veranderingen in het kinoom profiel, welke grotendeels worden voorkomen door GGA.. Deze bevinding wijst erop dat behoud van de normale kinase activiteit een belangrijke rol speelt in het mechanisme dat bijdraagt aan het beschermende effect van GGA. Naast een verandering in kinase activiteit laten we in hoofdstuk 5 zien dat versnelde electrische stimulatie van hartspiercellen leidt tot activatie van de histone deacetylase HDAC6. HDAC6 activatie resulteert in het verlies van tubulin homeostase door deacetylatie van tubuline en vervolgens depolimerisatie en afbraak door calpain. Dit heeft uiteindelijk de verstoring van microtubuli en contractiele dysfunctie tot gevolg. HDAC6 inhibitie of expressie van een HDAC6 mutant, met een mutatie in het tubuline deacetylatie domein, voorkwam tachypacing geïnduceerd verlies van contractiele functie. Deze studie en onze bevindingen met betrekking tot de rol van F-actine stress fibers (hoofdstuk 2) suggereren een belangrijke rol voor het behoud van eiwit homeostase van het cytoskelet, met tubuline en F-actine als belangrijke componenten, om het ontstaan van een substraat voor boezemfibrilleren tegen te gaan.
Tenslotte, in hoofdstuk 6 laten we de rol zien van tachypacing-geïnduceerde autofagie, via de activatie van de Endoplasmatisch Reticulum (ER) stress signaaltransductie cascade, op de veranderingen van hartspiercellen in experimentele modellen voor boezemfibrilleren als ook in patientenmateriaal. Behoud van hartspiercel eiwit homeostase werd bereikt door farmacologische remming van autofagie en bovenliggende ER-stress, wat verlies van contractiele functie (calcium transienten) voorkwam. Interessant was ook dat overexpressie van de ER-chaperone, HSPA5 beschermende effecten had. Deze bevindingen impliceren dat voorkomen of remming van autofagie, waarschijnlijk door stoffen die de expressie van ER-chaperones verhogen, een nieuwe therapeutische interventie voor boezemfibrilleren kan zijn.

Samenvattend, onze studies geven aan dat verlies van eiwit homeostase een belangrijke factor speeltin het ontstaan van een aritmogeen substraat voor boezemfibrilleren. Verder hebben we diverse nieuwe therapeutische aangrijpingspunten geïdentificeerd die proteostase van hartspiercellen

behoudt en het onstaan van een substraat voor boezemfibrilleren voorkomt. Ons onderzoek laat zien dat remmers van HDAC6, RhoGTPase, ER stress en HSP inducerende middelen interessante kandidaten zijn voor toekomstige behandeling van patienten met boezemfibrilleren.

Een overzicht van boezemfibrilleren geïnduceerde ontsporing van eiwit homeostase en therapeutische mogelijkheden is weergegeven in figuur 1.

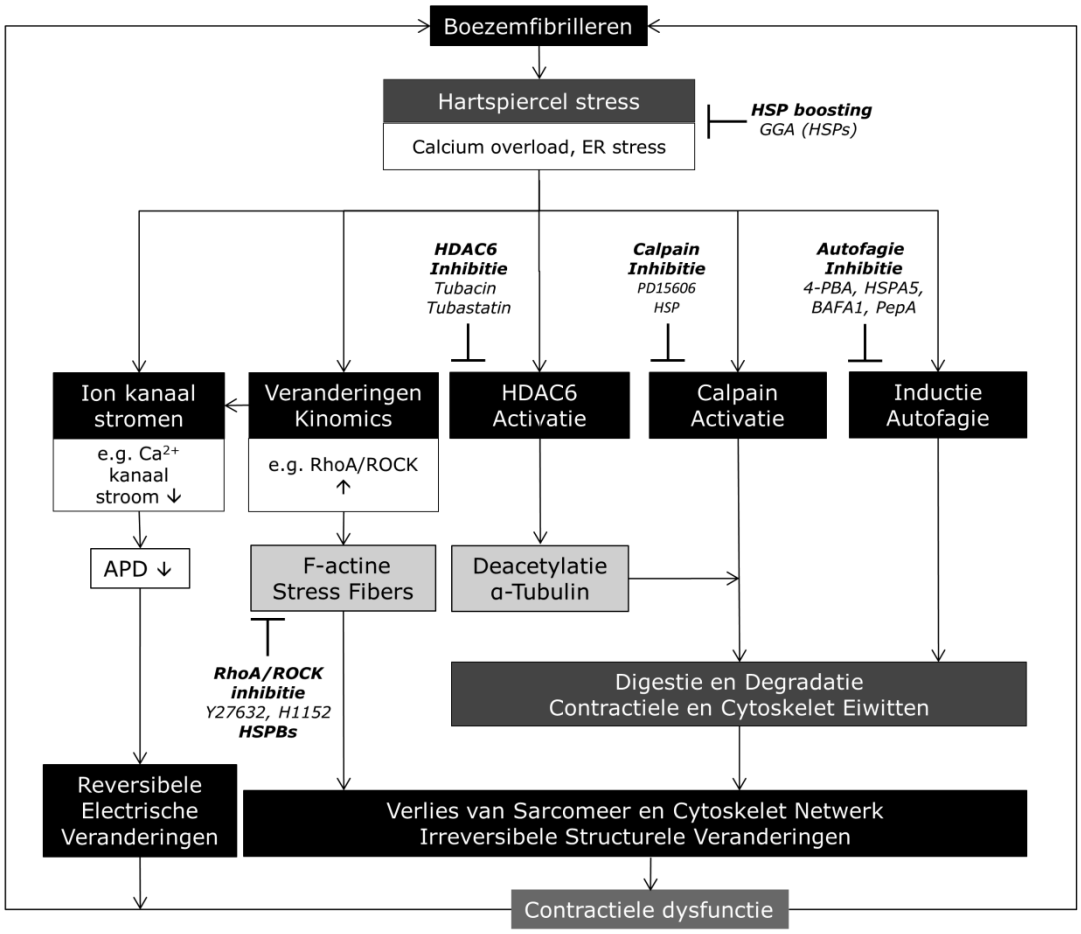

Figure 1 Overzicht van boezemfibrilleren geïnduceerd verlies van hartspiercel eiwit homeostase.
Boezemfibrilleren induceert hartpsiercel stress ten gevolge van een te hoge Ca^{2+} concentratie (overload) en endoplasmatisch reticulum (ER) stress. Deze stress reguleert vroege electrische veranderingen door middel van een verminderde L-type Ca^{2+} kanaal stroom met als gevolg een verkorting van de actie potentiaal duratie (APD) en uiteindelijk het optreden van contractiele dysfunctie. Deze vroege veranderingen vinden plaats binnen enkele minuten en beschermen de hartspiercel tegen de te hoge Ca^{2+} concentratie, echter met als gevolg dat er een aritmogeen substraat voor het in stand houden van boezemfibrilleren ontstaat. In het geval dat boezemfibrilleren aanhoudt, vindt verder verlies van proteostase plaats, inclusief veranderingen in kinomics, formatie van RhoA en ROCK gereguleerde F-actine stress vezels, HDAC6 activatie en activatie van eiwit afbraak processen zoals inductie van autofagie en proteases (calpain). Uiteindelijk leidt het verlies van eiwit homeostase tot irreversibele veranderingen inclusief de degradatie van sarcomeer (contractiele) en cytoskelet eiwitten. De afbraak van deze eiwitten draagt bij aan het verslechteren van de contractiele functie en het in stand houden van boezemfibrilleren alsmede de progressie ervan. Therapeutische

mogelijkheden zijn aangegeven en omvatten, farmacologische boosting van de expressie van Heat Shock Eiwitten (HSP) door GGA, overexpressie van HSPA5, ROCK inhibitie door behandeling met Y27632 of H1152, HDAC6 inhibitie door tubacin of tubastatin, autofagie inhibitie met de chemische chaperone 4-PBA, bafilomycin A1 (BAFA1) en pepstatin A (PepA) en ten slotte inhibitie van calpain door behandeling met PD15606.

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Curriculum Vitae

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2006-2008 Supervision of the practical course "Identification of micro-organisms" for dentistry students.

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