Targeting proteostasis in atrial fibrillation

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Chapter 8

Summary, general discussion and future perspective

1. SUMMARY

Atrial fibrillation (AF) is the most common clinical tachyarrhythmia and is expected to affect about 30 million North Americans and Europeans by 2050.[1] It is widely acknowledged that one of the main features of AF is its progressive nature, which hampers the effective functional conversion to sinus rhythm in patients with persistent forms of AF.[2-4] This progressive nature and impaired functional recovery are rooted in AF-induced irreversible cardiomyocyte remodeling, especially structural remodeling, which promotes the persistence of AF.[4-7] Current AF drug therapies target the reversible electrical changes and therefore have limited effect on patients' outcome.[8] A balanced eukaryotic protein homeostasis, i.e. a proper proteostasis, enables healthy cell and organismal development and protects against diseases.[9] Recently, our lab revealed evidence that derailment of proteostasis is a main contributor to the development of structural remodeling in AF and underlies AF progression. In addition, HSP-inducing compounds are promising therapeutics to prevent AF progression by preserving proteostasis, which is reviewed in chapter 2.[7,10-12] Besides HSPs, many other modifiers, including HDACs and autophagy, influence proteostasis.[9] However, the detailed molecular pathways contributing to derailment of proteostasis in AF remain unidentified. Therefore, the main goal of this thesis is to elucidate if key modulators of proteostasis get derailed in AF and whether these key modulators represent druggable targets to attenuate AF initiation and progression.

1.1. Identify key modulators involved in derailment of proteostasis in AF

To identify key modulators involved in the derailment of proteostasis in AF, we first developed a *Drosophila* model for AF to screen for compounds that prevent AF progression, as described in chapter 3**.** Tachypaced *Drosophila* revealed characteristics of AF-induced alterations in experimental animal and cardiomyocyte models and in clinical AF, including increased arrhythmicity, hypocontractility of the heart wall, activation of calpain, myolysis and damaged mitochondria.[13] We previously found cardioprotective effects by HSP overexpression, especially the small HSPB1, and the HSP-inducing compound GGA, against important features of tachycardia remodeling, such as electrical and structural remodeling and contractile dysfunction.[14-17] In this chapter, we demonstrated for the first time, that such protective effects are also observed in the *Drosophila* model for tachypacing-induced contractile dysfunction of the heart wall. We observed that HSP-inducing agents (GGA and BGP-15), a heat shock pretreatment and overexpression of one small HSP, dmHSP23, protect against tachypacing-induced contractile dysfunction. Our results show that findings in *Drosophila* match those of *in vitro* tachypaced HL-1 and dog atrial cardiomyocytes, and therefore the *Drosophila* model can be used to study tachycardia remodeling. Since *Drosophila's* gene expression can be easily manipulated in a highly

precise spatial and temporal fashion, this model seems to represent an excellent tool to study molecular mechanism underlying tachycardia remodeling and AF progression. Combined with short life-cycle of *Drosophila*, cost efficiency and the powerful techniques for genetic and molecular manipulations, the *Drosophila* model is also highly suitable for (high-throughput) compound screening.[18]

The newly developed *Drosophila* model and previously developed HL-1 cardiomyocyte model for AF, were both utilized to screen broad HDAC inhibitors, including Trichostatin A (TSA), sodium butyrate (SoBu), nicotinamide, and the specific HDAC6 inhibitor tubacin, as described in chapter 4. In this chapter we observed nicotinamide and tubacin to protect against contractile dysfunction in both experimental models. As tubacin is a specific inhibitor of HDAC6 and nicotinamide can inhibit Class III HDACs, sirtuins, we further focused on the role of HDAC6 and sirtuins in AF progression. We identified HDAC6 as a key enzyme in the development of a substrate for AF progression. Tachypacing of HL-1 cardiomyocytes increased HDAC6 activity and expression, resulting in TDAC-domain dependent deacetylation/depolymerization and calpain-mediated degradation of α-tubulin with subsequent disruption of the microtubule network. HDAC6 inhibition by tubacin conserved the microtubule structure and prevented depolymerized α-tubulin from degradation by calpain. Ultimately, this derailment of α-tubulin proteostasis causes contractile dysfunction. Consistent with our experimental data, patients with permanent AF show increased HDAC6 TDAC domain activity and expression, and increased deacetylation and degradation of α-tubulin. In these patients the amount of α-tubulin degradation correlates with calpain activity. Finally, we obtained proof of concept for HDAC6 as a therapeutic target by testing the HDAC6 inhibitor tubastatin A in a dog model of AF. Tubastatin A protected against tachypacing-induced electrical and structural remodeling and AF progression in dogs. Together, our results identify inhibition of HDAC6 as a promising therapeutic target to conserve α-tubulin proteostasis and attenuate cardiomyocyte remodeling in AF.

In addition to HDAC6 inhibitors, we also found that the class III HDAC (sirtuins) inhibitor, nicotinamide, protects cardiomyocytes and *Drosophila* from tachypacing-induced remodeling, as described in chapter 4. It is conceivable that inhibition of deacetylation of α-tubulin also underlies the protective effect of nicotinamide, since HDAC6 and sirtuin both deacetylate α-tubulin. However, in contrast to previous reports[19], nicotinamide did not prevent deacetylation and depolymerization of α-tubulin.[20] This observation suggests that other mechanism(s) convey the protective effect of nicotinamide in AF, such as increased availability of NAD^+ by the inhibition of poly(ADP-ribose) polymerases (PARPs).[21,22] In chapter 5, we describe that nicotinamide inhibits PARP but not sirtuins. In addition, we found that AF induces DNA damage and subsequent PARP1 activation. Active PARP1, in turn, consumes NAD^+, resulting in metabolic remodeling and functional loss in tachypaced

cardiomyocytes and *Drosophila*. Accordingly, replenishment of NAD^+ protects against tachypacing-induced contractile dysfunction in cardiomyocytes and *Drosophila*. Moreover, inhibition of PARP, by another broad PARP inhibitor **3-AB** or the specific PARP1/2 inhibitor **ABT-888**, protects against tachypacing-induced contractile dysfunction in HL-1 cardiomyocytes and *Drosophila*. Consistent with these findings, PARP is also activated in atrial tachypaced dogs and permanent AF patients, and PARP activation correlates with the level of DNA damage. Taken together, these findings suggest a dominant role of PARP1 in AF-induced metabolic and functional remodeling and consequently disease progression.

Having identified the role of class IIb member HDAC6 in AF in chapter 4, we continued exploring the role of class I and class IIa HDACs in tachypacing-induced cardiomyocyte remodeling in chapter 6. We found that class I HDAC1 and HDAC3 overexpression have detrimental effects on contractile function in tachypaced HL-1 cardiomyocytes. In contrast, overexpression of class IIa HDAC5 and HDAC7 revealed protective effects. Their protective effects were suppressed in cardiomyocytes overexpressing HDAC5 or HDAC7 with a mutation in the binding domain for MEF2, revealing MEF2 as a downstream effector of HDAC5 and HDAC7. Moreover, tachypacing induced HDAC5 phosphorylation, nuclear export and downstream fetal gene activation (BNP, β-MHC) in HL-1 cardiomyocytes. Similar results were observed in permanent AF patients, suggesting a role for HDAC5 in the progression of clinical AF.

Recently, HDAC inhibitors have been found to attenuate cardiac hypertrophy by suppressing autophagy.[23] In addition, HDAC6 and microtubules are required for proper degradation of misfolded proteins in cells via selective autophagy.[24-27] Since the microtubule network is disrupted due to HDAC6 activation in AF [20], one could speculate that autophagy is also dysregulated in AF. Therefore, in chapter 7, we studied the role of autophagy in AF. Indeed, we found tachypacing of cardiomyocytes to induce excessive autophagy through activation of ER stress signaling in experimental model systems for AF as well as in clinical AF. Conservation of cardiac proteostasis was achieved by inhibition of ER stress-induced autophagy with the chemical chaperone, 4-PBA, as well as with overexpression of ER chaperone, HSPA5, thus limiting AF progression. These findings suggest that rather than inhibition of autophagy, the inhibition of ER stress by compounds stimulating the expression of ER chaperones, may represent the preferred novel therapeutic intervention strategy for targeting excess autophagy in AF.

In summary, we identified several key modulators in the derailment of proteostasis to contribute to structural remodeling and hence AF progression. HDAC6, class I HDACs (HDAC1 and HDAC3), class IIa HDACs (HDAC5 and HDAC7) and ER stress-induced autophagy contribute directly to derailment of proteostasis by inducing disruption of microtubules, pathological fetal gene expression and myolysis, respectively. Moreover, maintaining proteostasis is an ATP dependent process in cells.[28] PARP1 activation resulted

in depletion of NAD^+ levels, which is a coenzyme in ATP production. Therefore, PARP1 activation contributes to derailment of proteostasis via impairment of ATP production. An overview of AF-induced derailment of proteostasis and druggable targets is depicted in Figure 1.

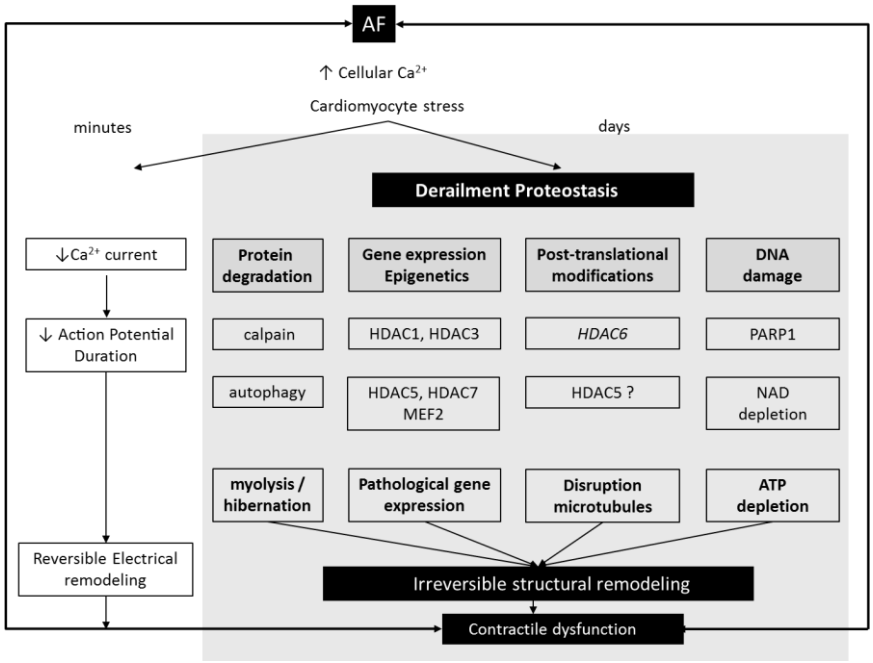

Figure 1: An overview of AF-induced derailment of proteostasis and druggable targets. AF induces time-related progressive cardiomyocyte remodeling. First, AF causes cellular Ca^{2+} overload and oxidative stress, which results in a direct inhibition of the L-type Ca^{2+} channel, shortening of action potential duration 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 continues, including myolysis by calpain and autophagy, pathological gene expression by HDACs (HDAC1, HDAC 3, HDAC5 and HDAC 7), disruption of microtubules by HDAC6, and depletion of NAD^+ by PARP1 activation and consequent exhaustion of ATP. Derailment of proteostasis results in irreversible alterations in structural proteins, thereby creating substrates for impaired contractile function and AF persistence. Novel druggable targets include inhibitors of ER stress-induced autophagy, HDAC5-MEF stabilizers, HDAC6 inhibitors, PARP1 inhibitors, and NAD supplements, which are listed in Table 1.

1.2. novel therapeutic options in AF

AF-induced remodeling contributes to AF progression and hampers the effective treatment of patients. As a result, there is an urgent need for new therapies that slow, arrest or even reverse the pathologic changes that occur with AF. The efficacy of drugs presently used in AF is limited.[1,8] Thus, pharmacological approaches preventing or limiting the substrate for the promotion of AF ("upstream therapy") are warranted.[1,8] There are strong indications that loss of proteostatic control in cardiomyocytes represents an important substrate for the development and progression of AF.[10,29] We previously discovered HSP inducers to attenuate AF-induced remodeling and can therefore serve as novel therapeutic

options in AF[29], which is further confirmed in a newly-developed *Drosophila* model system (chapter 3).[13] Beside HSP inducers, we identified several novel therapeutic options in AF, which are discussed below (Table 1).

Table 1: Drugs with potential benefit in preventing AF substrate formation currently developed for human application.

Drug	target	phase	indication	Ref/identifier (clinicaltrials.gov)
GGA (teprenone)	HSP induction	Phase IV	Gastric ulcers Gastritis Gastric lesionAB	NCT01190657 NCT015475559 NCT01397448
BGP-15	HSP activator, PARP inhibitor	Phase II	Diabetes Mellitus	NCT01069965
NYK9354	HSP induction	Pre-clinical	Atrial Fibrillation	[29]
Tubastatin A	HDAC6	Pre-clinical	Arthritis	[47]
ABT-888	PARP1 inhibitor (K_i = 5.2 nM) PARP2 inhibitor (K_i = 2.9 nM)	Phase II Phase II Phase I Phase II Phase II	metastatic breast cancer Hepatocellular Carcinoma Adult Solid Neoplasm Ovarian Cancer Colorectal Cancer	NCT01009788 NCT01205828 NCT01154426 NCT01113957 NCT01051596
Nicotinamide	PARP inhibitor Sirtuin inhibitor NAD precursor	Phase II/III Phase III Phase II Phase I/II	Lung Carcinoma Chronic Kidney Disease Neurodegenerative Disorders Alzheimer's Disease	NCT02416739 NCT02258074 NCT01589809 NCT00580931
AG014699	K_i = 1.4 nM (PARP1)	Phase II	BRCA1- or BRCA2-mutant tumours	NCT00664781
AZD2281 (olaparib)	PARP1 inhibitor (IC_{50} = 5 nM) PARP2 inhibitor (IC_{50} = 1 nM) Tankyrase 1 inhibitor (IC_{50} = 1.5 μM)	Phase I Phase I Phase II Phase II	Neoplasm Metastasis Advanced Solid Malignancies Platin-sensitive ovarian cancer Ovarian Carcinoma and Breast Cancer	NCT00710268 NCT00572364 NCT00753545 NCT00679783
BSI-201	PARPs	Phase III Phase II	Breast Cancer Ovarian Cancer Advanced Solid Tumors	NCT00938652 NCT01033123
MK-4827	PARP1 inhibitor (IC_{50} = 3.2 nM) PARP2 inhibitor (IC_{50} = 4 nM)	Phase I	Solid tumours and ovarian cancer	NCT01294735
Niacin	dietary NAD precursor	Phase IV Phase III Phase IV	Coronary Disease Hypolipoproteinemia Coronary Heart Disease	NCT00298909 NCT00461630 NCT01126073
Nicotinamide riboside	NAD precursor	Phase 0 Phase 1	Dietary Supplement in healthy Participants Pharmacokinetics	NCT02300740 NCT02191462
4-PBA (Buphenyl)	ER stress inhibitor, chemical chaperone	Phase I/II Phase II Phase II/III Phase IV Phase II/III Phase I	Cystic Fibrosis Pulmonary Tuberculosis Maple Syrup Urine Disease Diabetes Urea Cycle Disorders Lymphoma	NCT00590538 NCT01580007 NCT01529060 NCT00533559 NCT00947544

HDAC6 inhibitors

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 4). However, tubacin is not suitable for *in vivo* studies as it has low druglikeness.[30] Other HDAC6 inhibitors, such as tubastatin A and ACY-1215 have been developed, and show beneficial effects in mice models for neurodegenerative diseases and cancer.[30-32] We very recently provided the first evidence for the efficacy of HDAC6 inhibitors in the dog model for AF.[20] Dogs treated with tubastatin A were protected against atrial tachypacing-induced electrical remodeling, cellular Ca^{2+} handling/contractile dysfunction and AF progression. These *in vivo* findings strengthen the notion that HDAC6 inhibitors represent a novel therapeutic approach in AF.

PARP inhibitors and NAD^+ supplementation

The inhibition of PARP proteins has become a promising therapeutic approach in several human diseases, including cardiovascular diseases.[22] In chapter 5, we identified PARP1 activation in experimental models and in human permanent AF, and demonstrate the protective effect of ABT-888 in HL-1 cardiomyocytes and *Drosophila*. Recently, novel PARP inhibitors have entered clinical development for various cardiovascular indications.[33,34] Early PARP inhibitors, such as 3-AB and nicotinamide, are designed to compete with NAD^+ at the active site of the enzyme. They have little specificity for individual PARP proteins, with half-maximal inhibitory concentration (IC50) values in the micromolar range, and they elicit significant off-target effects and toxicity.[35,36] The newer PARP inhibitors exhibit increased potency and specificity, with IC50 values reaching the low nanomolar range, and even PARP family member selectivity for some inhibitors. For example, ABT888 inhibits only PARP1 and PARP2 with high potency[37], and is now in phase I and II for clinical cancer studies.[34,36] Consequently, our findings call for the exploration of the action of ABT-888 in large animal models and in human AF. In addition, NAD^+ supplementation protects against tachypacing-induced remodeling in HL-1 cardiomyocytes and *Drosophila*. Currently, NAD^+ precursors, such as niacin and nicotinamide riboside, which are currently tested in clinical trials in patients with heart diseases and in healthy patients (Table 1), might also represent therapeutic options in AF.

HDAC5-MEF2 complexes stabilizer

In **chapter 6**, we observed a key role for class IIa HDAC5 in AF remodeling via suppression of MEF2 activity. So compounds inhibiting the release of HDAC5 from MEF2 might have a therapeutic potential for treatment of AF and it is of interest to test these compounds in experimental and clinical AF. One interesting compound is MC1568, since it inhibits the activity of HDAC4 and HDAC5, thereby leaving MEF2-HDAC complexes in a repressed state.[38] In addition, inhibitors of upstream kinases which phosphorylate HDAC5

and thus its subsequent nuclear export, would also be of interest. A more upstream approach is to inhibit CaMK and PKC, since these represent two main kinases involved in HDAC5 phosphorylation and nuclear export in cardiomyocytes.[39] In accord, CaMK inhibitors have been reported to prevent AF[40], while the PKC inhibitor Go6983 blocked HDAC5 nuclear export and also α-tubulin deacetylation after nerve injury.[41] Given that deacetylation of α-tubulin is involved in AF structural remodeling[20], PKC inhibitor Go6983 is also an interesting candidate to test in our experimental model systems. Thus, our study suggests that AF induces HDAC5 phosphorylation, leading to derepression of MEF2 responsive genes, in turn contributing to cardiomyocyte remodeling and AF progression. Therefore, a stabilizer of HDAC5-MEF2 complex, which prevents AF-induced HDAC5 release from MEF2, such as MC1568 and the PKC inhibitor Go6983, might represent novel therapeutic approaches to attenuate AF progression.

ER stress inhibitors

In chapter 7, we revealed that inhibition of ER-stress induced autophagy preserves proteostasis and protects against cardiomyocyte dysfunction in experimental model systems for AF. Therefore, pharmacological intervention to inhibit autophagy may constitute a promising therapeutic strategy in clinical AF. Currently, autophagy can be modulated by a number of small molecules.[42] 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.[43,44] Such view is corroborated by the high toxicity of bafilomycins, which precludes its use in the clinical setting.[45] As the clinical options for autophagy inhibition are currently limited, inhibitors of ER stress may represent a suitable alternative, as identified in chapter 7. From the available compounds, the chemical chaperone 4-PBA seems the most promising, as this compound not only inhibits ER-stress induced autophagy, but also has been approved for clinical use (Table 1). More importantly, 4-PBA was reported to have minor side effects and is considered safe in patients.[46] Currently, we conduct a 4-PBA study in the dog model for AF, in collaboration with Dr. S. Nattel, which so far shows encouraging results. Therefore, our findings suggest 4-PBA as a therapeutic agent with great potential in clinical AF.

2. DISCUSSION: key role of HDACs in cardiac proteostasis

As reviewed in chapter **2**, loss of proteostasis creates a substrate for AF initiation and progression.[10] We previously found HSPs to protect against AF by preserving proteostasis in experimental models for AF as well as in clinical AF.[14,15,48,49] Here, we identified a group of enzymes, HDACs, to be an important modulator of proteostasis in AF (chapter 4 and chapter 6).[20] HDACs have been recently implicated in various heart diseases, especially heart failure. Liu et al. were the first to show that HDAC inhibition reverses atrial

arrhythmia inducibility and fibrosis in cardiac hypertrophy.[50] We discovered that class I HDACs (HDAC1 and HDAC3), class IIa (HDAC5 and HDAC7), and class IIb (HDAC6) to be involved in derailment of proteostasis and subsequent tachypacing-induced contractile dysfunction and structural remodeling in AF. The potential mechanisms of HDACs to regulate proteostasis in heart diseases include by epigenetic regulation, by deacetylation of contractile and structural proteins or by modulation of autophagy and/or HSP production (Figure 2). The detailed mechanisms are discussed below.

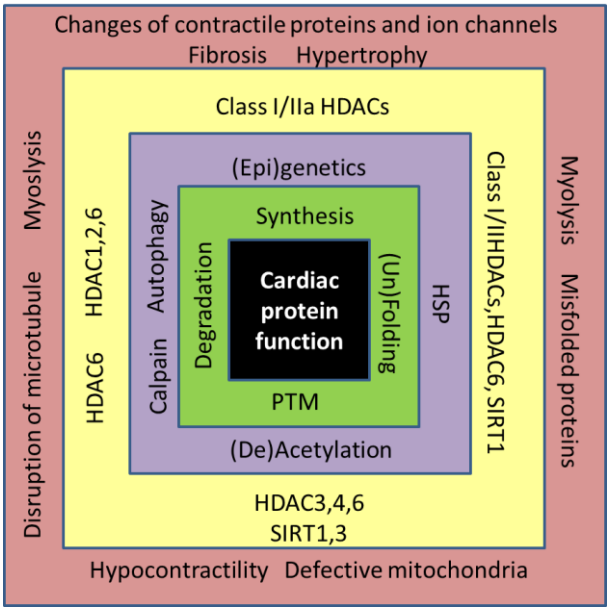

Figure 2: The role of HDACs in cardiac proteostasis network, regulating cardiac protein function and structural remodeling. Shown are the interactions that comprise the cardiac proteostasis network responsible for cardiac protein function. Central components of the cardiac proteostasis network are outlined in the inner layer (green) including synthesis, folding, post translational modification (PTM) and degradation. The second layer (purple) consists of major modifiers of each central component. The specific HDACs regulating each modifier in the second layer are indicated in the third layer (yellow). Stress activates these HDACs, thereby contributing to derailment of proteostasis, details of which are indicated in the fourth layer (red).

2.1. Aspects of epigenetic regulation by HDACs: transcriptional reprogramming

Transcriptional reprogramming, especially by activation of the fetal gene program, is associated with AF susceptibility and progression.[51,52] For example, the thick filament of the mammalian sarcomere consists of two myosin isoforms, fast-contracting alpha-myosin heavy chain (α-MHC) and slow-contracting beta-myosin heavy chain (β-MHC). Stress signals enhance the expression of fetal β-MHC and reduce the expression of adult α-MHC. The consequences include diminished myofibrillar ATPase activity and impaired contractility.[53] In AF patients, myolytic cardiomyocytes are in a dedifferentiated state resembling that of immature muscle cells. Here, β-myosin heavy chain (MHC) and smooth

muscle α-actin (α-SMA), two proteins of the fetal program, were re-expressed.[54] In both, tachypaced HL-1 cardiomyocyte model and canine model for AF, transcriptional expression of α-MHC was decreased.[55,56] However, the mechanisms underlying transcriptional reprogramming and fetal gene expression in AF is not yet elucidated.

HDACs can deacetylate histones and numerous transcriptional factors[57], thereby affecting multiple processes by altering chromatin structure and gene expression. Studies on heart failure and development strongly indicate, that class I and IIa HDACs are involved in transcriptional (i.e., chromatin-related) reprogramming.[58-60] Class IIa HDACs (HDAC4, HDAC5, HDAC7, and HDAC9), and especially HDAC4 and HDAC5, are highly expressed in the heart. Class IIa HDACs normally repress pathological cardiac fetal gene expression. In response to stress signals, class IIa HDACs are phosphorylated and undergo nuclear export, resulting in derepression of downstream target pathological fetal genes.[60] Generally, class IIa HDACs regulate gene expression through recruitment of class I HDACs and interaction with various transcription factors. Numerous transcription factors have been implicated in stress-dependent gene expression in the heart. Most of these transcription factors do not change abundantly in the stressed myocardium, suggesting that their enhanced activity depends on posttranslational mechanisms. HATs and HDACs play a central role in modulating the activities of these transcription factors during pathological cardiac growth and development[61], and our findings strongly indicate that HDACs play also a key role in AF.

Table 2: Overview of cardiac transcription factors (TGs) regulated by HDACs

TG	HDACs	Pathological pathways	References
MEF2	HDAC4 HDAC5 HDAC7 HDAC9	Heart failure Cardiac hypertrophy	38,61-68
KLF4	HDAC2	Cardiac hypertrophy	71
YY1	HDAC2 HDAC4 HDAC 5	Cardiac hypertrophy	75,76
NKX 2.5	HDAC5	Heart development, NCX1 expression	92
Myocardin/SRF	HDAC5	Differentiation of smooth muscle cells	93
FOXO	HDAC4 HDAC5 HDAC7	Glucose Homeostasis	94,95
PGC-1α	HDAC5 SIRT1	Mitochondrial biogenesis, fatty acid oxidation	65,96
NF-κB	HDAC1 HDAC5	Cardiac infarction	97

HDACs in cardiac hypertrophy and heart failure

One transcriptional mechanism by which HDACs regulate the phenotype of the heart, involves the hypertrophic transcription factor MEF2.[38,62-68] In pathological cardiac remodeling, MEF2 may serve as a platform on which HATs and HDACs converge as positive and negative regulators of pathologic cardiac gene expression. Despite high levels of MEF2 expression in heart, MEF2 induced proteins display only basal levels of transcriptional activity in adult myocardium.[69] Hypertrophic agonists or biomechanical stress result in a dissociation of class IIa HDACs from MEF2, the export of HDACs from the nucleus, exchange of HDAC for HAT binding and consequent activation of MEF2 target genes leading to pathological cardiac growth.[70] Another transcription factor regulated by HDACs is krüppel-like factor 4 (KLF4). KLF4 overexpression blocks cardiac hypertrophy in cultured cells and *KLF4* knockout mice develop exaggerated cardiac hypertrophy and fibrosis in response to pressure overload.[71-73] Pan-HDAC inhibitors increase the expression of KLF4 in cultured cardiomyocytes, and the resulting increase in KLF4 expression appears to be sufficient to block agonist-dependent hypertrophy of the cells.[71,72]

In patients with heart failure, reactivation of a fetal gene program, including atrial natriuretic peptide (*ANP*) and brain natriuretic peptide (*BNP*), is a hallmark for maladaptive remodeling of the left ventricle.[74] Expression of BNP is enhanced in ventricular cardiomyocytes during pathological cardiac hypertrophy, and circulating BNP levels are used clinically as a surrogate measure for heart failure. Employing cultured neonatal rat cardiac myocytes, Gardner and colleagues demonstrated that upregulation of BNP expression in response to endothelin signaling is dependent on association of HDAC2 with the Yin Yang 1 (YY1) transcription factor on the *BNP* gene promoter.[75] YY1 is acetylated in cardiac myocytes, and deacetylation of this transcription factor by HDAC2 enhances its ability to stimulate BNP gene transcription. TSA treatment disrupts YY1-HDAC2 complexes and suppresses endothelin-induced BNP expression. In addition, the interaction of HDAC4 and HDAC5 with YY1 was also found necessary for the repressor activity of YY1 in cardiac specific promoters.[76] Recently, it was shown that in isolated working murine hearts, an acute increase of cardiac preload induced HDAC4 nuclear export, H3K9 demethylation, HP1 dissociation from the promoter region, and activation of the ANP gene. Increased cardiac preload and/or activated CaMKIIδB induces nucleo-cytoplasmic shuttling of HDAC4 and dissociation of its corepressor complex with SUV39H1 and HP1. This relieves H3K9me3, resulting in chromatin condensation, and repression of ANP and BNP gene transcription in response to MEF2.[74] Given the apparent role of HDACs in AF, these results in heart failure warrant the exploration of the involvement of above mentioned transcription factors in AF-induced cardiomyocyte structural and functional remodeling.

HDACs in cardiac fibrosis

An important substrate for AF induction is fibrosis. Interestingly, much of the beneficial effects of HDAC inhibitors in models of heart failure are likely due to inhibition of pathological fibrosis, although surprisingly little is known about the anti-fibrotic mechanisms of HDAC inhibitors in the heart.[77] It seems likely that HDAC inhibitors block cardiac fibrosis by multiple mechanisms, including inhibition of cardiac fibroblast proliferation or migration, induction of genes that suppress extracellular matrix production from fibroblasts, suppression of proinflammatory cues for fibrosis, and blockade of the endothelial-to-mesenchymal transition (Endo-MT).

Endo-MT is a form of epithelial-to-mesenchymal transition (EMT) that occurs during the embryonic development of the heart: the mesenchymal cells that form the atrioventricular cushion, the primordia of the valves and septa of the adult heart, are derived from the endocardium by Endo-MT.[78] Endo-MT initiates a process of pathological dedifferentiation of vascular endothelial cells into matrix-producing mesenchymal cells. During this process, excessive numbers of cardiac fibroblasts are produced in adult hearts in response to pressure overload [79] and myocardial infarction.[80] Cardiac Endo-MT is stimulated by Transforming Growth Factor-Beta (TGF-β) and suppressed by Bone Morphogenic Protein-7 (BMP-7)[79], which blocks fibrosis.[81] Endothelin-1, a potent vasoconstrictor with promitogenic properties, stimulates cardiac fibrosis by promoting Endo-MT.[82] TSA blocks EMT, by inhibition of HDAC1 and HDAC2.[83] Shan et al. investigated the role of HDAC6 in TGF-β1-induced EMT and showed that TGF-β1 induces HDAC6-dependent deacetylation of α-tubulin in human lung epithelial cells, which was concurrent with the expression of EMT markers. Inhibition of HDAC6 attenuated the TGF-β1-induced expression of EMT markers as well as activation of SMAD3. In addition, inhibition of SMAD3 activation abrogated HDAC6-dependent deacetylation of α-tubulin and the expression of EMT markers induced by TGF-β1.[84,85] Yu et al. found that high concentrations of glucose induced EMT, suggested by a decreased expression of E-cadherin and increased expression of α-SMA, fibronectin, and type I collagen and by increased cell migration.[86] As such, future studies should address whether HDAC inhibition alters Endo-MT in the heart. Interestingly, these initiators of Endo-MT, such as TGF-β1[87], fibrosis[88], HDAC6 activation[20]and high glucose[89] are all related to AF induction and progression. Whether Endo-MT occurs in AF and contributes to AF progression remains also to be studied.

HDACs in regulation of ion channels

NKX2.5 is a transcription factor that regulates cardiac development in humans. NKX2.5 works along with MEF2, HAND1, and HAND2 transcription factors to direct heart looping during early development. NKX2.5 in vertebrates is equivalent to the

'tinman' gene in *Drosophila* and directly activates the MEF2 gene to control cardiomyocyte differentiation.[90] NKX2.5 operates in a positive feedback loop with GATA transcription factors to regulate cardiomyocyte formation.[91] NKX2.5 influences HAND1 and HAND2 transcription factors that control essential asymmetrical development of the heart's ventricles. NKX2.5 recruits HDAC5 to the sodium-calcium exchanger gene (NCX1) promoter, where HDAC5 complexes with HDAC1. Chandrasekaran et al. demonstrated that acetylation of NKX2.5 induces its association with HDAC5, whereas deacetylated NKX2.5 is in a complex with p300. Notably, TSA treatment prevents p300 from being recruited to the endogenous NCX1 promoter, resulting in the repression of the NCX1 gene.[92]

2.2. Direct modulation of contractile function by deacetylating structural and contractile protein

HDACs can deacetylate numerous non-histone and structural proteins[57], thereby affecting multiple processes beyond altering chromatin structure and gene expression. Work by Gupta and colleagues revealed that class I HDAC3 localizes to cardiac sarcomeres, thereby regulating the cardiac contractility.[98] Deacetylation of both α- and β-MHC by HDAC3 reduces their affinity for actin, resulting in decreased actin sliding velocity of the myosin heads.[98]

Class IIa HDAC4 is also localized to cardiac sarcomeres, where it appears to decrease myofilament calcium sensitivity by promoting deacetylation of muscle LIM protein (MLP). It remains unclear whether MLP is a direct substrate of HDAC4.[99] Indeed, for many years it was believed that class IIa HDACs lacked intrinsic catalytic activity, because recombinant forms fail to deacetylate canonical HDAC substrates. Instead, the catalytic activity of class IIa HDACs was attributed to their association with class I HDACs.[100] However, a synthetic substrate that is efficiently deacetylated by class IIa HDACs has been identified recently.[101] Nevertheless, the endogenous substrates of class IIa HDACs in the heart have not been identified. Further investigation is needed to address the role of HDAC4 in the control of cardiac contractility, as well as the general role of class IIa HDAC catalytic activity in the heart.

Class IIb HDAC6 activation leads to microtubule structure disruption and contractile dysfunction.[20] Microtubules together with cortical (non-sarcomeric) actin filaments, desmin (intermediate) filaments forms the cytoskeleton of cardiomyocytes. Microtubule dysregulation has been reported to be involved in various heart diseases.[102-105] Microtubules are necessary for enhanced gap junction growth and likely facilitate connexin trafficking under basal conditions.[106] HDAC6 is a key enzyme to deacetylate α-tubulin causing microtubule depolymerization and influencing microtubule-dependent cell mobility.[102,103]

In addition to its role in microtubule-dependent cell motility, HDAC6 influences actin-dependent cell motility by altering the acetylation of cortactin, which in turn changes the F-actin binding activity of cortactin.[107] In accord with a main function of HDAC6 in preservation of the cytoskeleton, its deletion in mice dramatically improves myofibril force generation without blocking cardiac hypertrophy or fibrosis.[108] HDAC6 co-purifies with cardiac myofibrils, suggesting a possible role for HDAC6 in the control of sarcomere protein acetylation and function.[108] Furthermore, various extracellular stress stimuli consistently increase HDAC6 activity in myocardium, cultured cardiomyocytes and fibroblasts.[109] Finally, HDAC6 contributes to pathological responses of heart and skeletal muscle to chronic angiotensin II signaling[108], substantiating the important role of HDAC6 in cardiac diseases.

In summary, a combination of chromatin and non-chromatin substrates for HDACs will play key roles in derailment of cardiac proteostasis under stress (Figure 3).

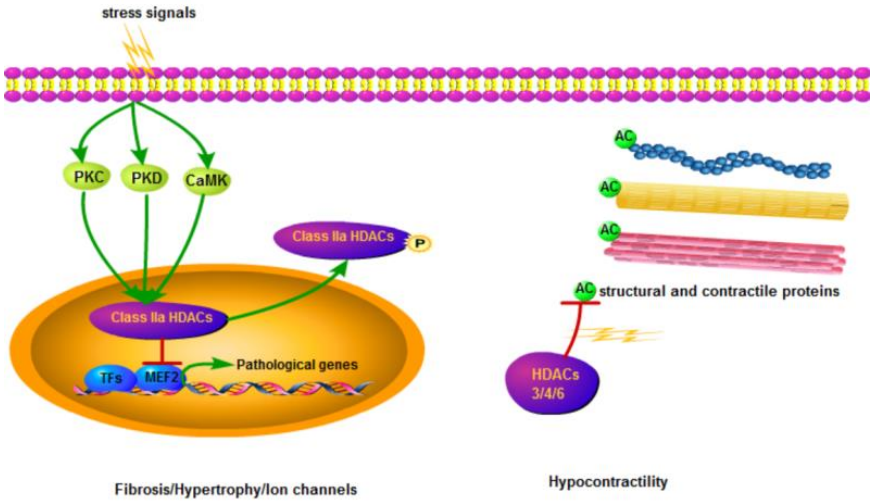

Figure 3: Effects of cardiac stress signals on HDACs in proteostasis regulation. Class IIa HDACs normally repress pathological cardiac gene expression. In response to stress signals, kinases, including protein kinase C (PKC), protein kinase D (PKD) and calcium/calmodulin-dependent kinase (CaMK), directly phosphorylate class IIa HDACs to trigger their nuclear export. Class IIa HDACs undergo nuclear export, resulting in derepression of downstream target pathological genes contributing to structural remodeling such as fibrosis and hypertrophy and changes of ion channel expression. In the cytoplasm, cardiac stress activates HDAC6, HDAC3 and HDAC4. HDAC6 deacetylates substrates such as tubulin and cortactin. During stress, HDAC3[98], HDAC4[99] and HDAC6[108] colocalize with sarcomeric proteins and contribute to hypocontractility by deacetylation of sarcomeric and cytoskeletal proteins.

2.3. Involvement of HDACs in major proteostasis pathways

Role of HDACs in autophagy

Autophagy is primarily considered a non-selective degradation process induced by starvation. Nutrient-independent basal autophagy, in contrast, imposes intracellular quality control by selective disposal of aberrant protein aggregates and damaged organelles. Controlled autophagy during (mild) cardiac stress conditions, such as nutrient deprivation, brief 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[43,110] and cardiac hypertrophy.[111] Recently, autophagy has been identified as an obligatory element in pathological cardiac remodeling and point to HDAC1 and HDAC2 as required effectors.[23] HDAC6 is the first HDAC to be found involved in autophagy and is a component of the aggresome.[27] HDAC6 has the capacity to bind polyubiquitinated misfolded protein cargo to dynein motors for transport to aggresomes. Indeed, cells deficient in HDAC6 fail to clear misfolded protein aggregates from the cytoplasm, cannot form aggresomes properly, and are hypersensitive to accumulation of misfolded proteins. These findings identify HDAC6 as a crucial player in the cellular management of misfolded proteins.[27] HDAC6 recruits and deacetylates cortactin, thereby promoting F-actin remodeling important for autophagosome-lysome fusion and protein aggregate clearance and defective mitochondria clearance.[24,112] HDAC6 overexpression activates c-Jun NH2-terminal kinase (JNK) and activates autophagic cell death through the c-Jun NH2-terminal kinase (JNK)/Beclin 1 pathway in liver cancer.[113] In summary, HDAC1, HDAC2 and HDAC6 are involved in the regulation of autophagy, which we demonstrate in chapter 7 to be activated by ER-stress in AF. However, to which extend HDACs are involved in the regulation of autophagy in AF remains unclear and needs future investigation.

Role of HDACs in HSP production

With respect to maintenance of proteostasis in case of protein misfolding, HDACs may also have an indirect effect via HSPs, which are known to (re)fold misfolded proteins and thereby prevent proteotoxic effects in the cell. Reports about the role of HDACs in the regulation HSP production are limited and reveal seemingly conflicting conclusions among different HDAC classes.

Inhibitors of class I/II HDACs have been reported to boost HSP production in various cells, suggesting that protective effects of these inhibitors may be related to HSP expression. In mouse and human embryonic stem (ES) cells, class I/II HDAC inhibitors, TSA, SoBu, suberoylanilide hydroxamic acid (SAHA) and valproic acid trigger early differentiation of mouse ES cells and induction of HSP70. In contrast, a class III HDAC inhibitor, nicotinamide, fails to induce HSP70 expression or differentiation in these ES cells.[114] TSA but not nicotinamide induces an association of HSF1 with the HSP70 promoter, indicating that HSF1 is activated and bind to the HSP70 promoter in response to

TSA.[114] In *Drosophila*, class I/II HDAC inhibitors, TSA and SoBu, also affect the chromatin structure at the site where HSP70 gene is located and significantly promote the HSP70 gene transcription and hence play important roles in HSP gene regulation.[115] Furthermore, these two inhibitors, TSA and SoBu, promote HSP22 and HSP70 expression and extend the lifespan in *Drosophila*.[116] Interestingly, we found TSA to enhance the reversibility of tachypacing-induced remodeling in HL-1 cardiomyotes and boost HSP70 expression (unpublished data). Taken together, these data suggest class I/II HDACs negatively regulate HSP production and inhibitors of class I/II HDACs can boost HSP production. Notably, the use of broad spectrum inhibitors precludes further identification of specific class I/II HDACs that are responsible for HSP induction. However, as the Class II HDACs have low activity *in vivo*, it is conceivable that mainly class I HDACs convey the inhibition of HSPs.

In contrast, a class IIb HDAC, HDAC6, and a class III HDACs, SIRT1, have both been implicated in boosting/facilitating the heat shock response (HSR) via regulation of HSF1 in cells under proteotoxic stress. Hsp90-HSF1 complex is present in the unstressed cell in cytoplasm and dissociates during stress.[117] In stressed cells, HDAC6 senses ubiquitinated cellular aggregates via its ubiquitin binding domain and consequently mediates the dissociation of the repressive HSP90-HSF1 complex leading to subsequent release and activation of HSF1, in turn inducing the expression of major cellular chaperones including HSP70 and HSP25.[118] Notably, the ubiquitin binding domain and not the catalytic domain of HDAC6 is required for stress-induced HSF1 activation.[118] Thus, tubacin, which inhibits the HDAC6 activity and conserves microtubule structure in AF[20], will not interfere with the role of HDAC6 in boosting HSP expression under stress. Furthermore, a class III HDAC, SIRT1, boosts HSP production by deacetylation of HSF1 K80, thereby prolonging HSF1 binding to the promoter of HSP70 by maintaining HSF1 in a deacetylated, DNA-binding competent state. Conversely, down-regulation of SIRT1 accelerates the attenuation of the HSR and release of HSF1 from its cognate promoter elements.[119] These results provide a mechanistic basis for the requirement of HSF1 in the regulation of life span and establish a role for SIRT1 in protein homeostasis and the HSR.[119]

In summary, HDACs role in the regulation of HSP production is class dependent. The deacetylase activity of Class I/IIa HDACs negative regulate HSP expression. Class IIb HDAC6 induces HSP expression in the cytoplasm, which is dependent of the ubiquitin binding domain. SIRT1 positively regulates HSP expression through its deacetylase domain. One possible explanation of the different actions of class I/II HDACs and SIRT1 on HSR may constitute of the different substrates they address. Class I/II HDACs, especially class I HDACs, deacetylate histones, resulting in chromatin condensation, thus rendering the heat shock element (HSE) to become unaccessible for HSF1 to bind the HSE

in the promoter sequence of *hsp* genes. In contrast, the SIRT1 deacetylates HSF1 itself[119], which increases the affinity of HSF1 to HSE and thus positively regulate HSR.

2.4. Proteostasis and redox homeostasis: crosstalk between sirtuins and PARPs via NAD^+

A proper proteostasis requires a well-maintained energy balance, i.e. a proper redox homeostasis. Like other cell types, cardiomyocytes encompass the basic features of proteostasis, but they are postmitotic, highly specialized, force-generating, beating cells.[120] In terms of proteostasis, there is a permanent turnover of contractile proteins in an environment under pressure to maintain the redox homeostasis due to the high number of mitochondria, which produce the required ATP energy by redox reactions while generating potentially deleterious ROS.[121] Nicotinamide adenine dinucleotide (NAD^+) is a coenzyme and essential in redox reactions producing ATP. Enzymes that consume NAD^+ thus interfere with ATP production and redox state.

Interestingly, NAD^+ is a rate-limiting co-substrate for the class III HDACs or sirtuins (SIRTs 1-7), implying that NAD^+ modulation may regulate sirtuin function and, consequently, oxidative metabolism. Sirtuins target a wide range of cellular proteins in the nucleus, cytoplasm, and mitochondria for post-translational modification by acetylation (SIRT1, SIRT2, SIRT3 and SIRT5) or ADP ribosylation (SIRT4 and SIRT 6).[122] The orthologs of sirtuins in lower organisms play a critical role in regulating lifespan.[122] SIRT1 functions in glucose homeostasis as a modulator of PGC1-α in a NAD^+ dependent manner.[96] It is important to note that although SIRT1 is not itself physically associated with mitochondria, it also impacts mitochondrial function.[123] The mitochondrial localization of SIRT3–5 is especially intriguing, because mitochondrial dysfunction is associated with mammalian aging and many diseases, including cardiac diseases, neurodegenerative diseases and cancer.[124] There is growing evidence linking mitochondrial sirtuins with regulating energy equilibrium and mammalian lifespan.[125] Very recently, Gupta et al. found that activation of mitochondrial SIRT3 by a novel SIRT3 activator, honokiol (HKL), blocks and reverses cardiac hypertrophy in mice.[126] HKL is present in mitochondria, enhances SIRT3 expression nearly twofold and further increase its activity. Increased SIRT3 activity is associated with reduced acetylation of mitochondrial SIRT3 substrates, MnSOD and oligomycin-sensitivity conferring protein (OSCP). HKL-treatment increases mitochondrial rate of oxygen consumption and reduces ROS synthesis in wild type, but not in SIRT3-KO cells. Moreover, HKL-treatment blocks cardiac fibroblast proliferation and differentiation to myofibroblasts in a SIRT3-dependent manner.[126] Thus, NAD^+-dependent sirtuin activity seems essential in maintaining redox homeostasis and consequently essential for proper preoteostasis.

Activation of PARP enzymes, a family of major NAD^+ consumers, deplete NAD^+ levels thereby inhibiting sirtuins activity.[123] In line with this premise, decreased activity of PARP1 increases NAD^+ bioavailability, resulting in SIRT1 activation and protection against metabolic disease.[123] Canto et al. evaluated whether similar effects could be achieved by increasing the supply of nicotinamide riboside (NR), a recently described natural NAD^+ precursor with the ability to increase NAD^+ levels. They show that NR supplementation in mammalian cells and mouse tissues increases NAD^+ levels and activates SIRT1 and SIRT3, culminating in enhanced oxidative metabolism and protection against high fat diet-induced metabolic abnormalities. Consequently, their results indicate that the natural vitamin, NR, could be used as a nutritional supplement to ameliorate metabolic and age-related disorders characterized by defective mitochondrial function.[127] Both defective mitochondrial and derailed redox homeostasis is involved in AF.[128,129] In chapter 5, we show that inhibition of NAD^+ depletion caused by tachypacing-induced PARP1 activation, resulted in remodeling in experimental models systems for AF. How PARP1 activation induces NAD^+ depletion and thereby influences sirtuins function, downstream energy metabolism and proteostasis in AF remains to be elucidated.

3. CONCLUSIONS AND FUTHER PERSPECTIVES

In summary, we discovered several novel key modulators of proteostasis, including HDAC6, HDAC5, PARP1 and ER-stress induced autophagy, to be involved in AF initiation and progression. Targeting of these modulators with compounds revealed protective effects against AF progression in experimental model systems for AF. However, it is still an open question how to explain the apparent effectiveness of interventions that target only one single factor in these pathways controlling proteostasis. 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. Studies from cardiac conditions other than AF, such as heart failure and hypertrophy, shed additional light on how HDACs regulate proteostasis by themselves or by the interplay with other modulators including HSPs, autophagy and energy metabolism. The pathways constituting this interplay still remain to be elucidated in AF. Furthermore, although we obtained proof of concept that some pharmacological interventions directed at these key modulators of proteostasis prevent AF initiation and progression, it is unknown whether such interventions can reverse AF structural remodeling. Since most AF patients are subjected to cardiomyocyte remodeling at the moment they enter a clinic, therapies directed at enhancing the reversibility of cardiomyocyte remodeling and thereby improving functional recovery after AF conversion are clinically of major importance.

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