Journal of Molecular and Cellular Cardiology 101 (2016) 165–172

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Regulating the regulator: Insights into the cardiac protein phosphatase 1 interactome

David Y. ChiangMD, PhD [a,b,c], Albert J.R. HeckPhD [b], Dobromir DobrevMD, PhD [d], Xander H.T. WehrensMD, PhD [a,e,f,g,*]

[a] Cardiovascular Research Institute, Baylor College of Medicine, Houston, TX, USA
[b] Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands
[c] Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA
[d] Institute of Pharmacology, University Duisburg/Essen, Essen, Germany
[e] Department of Molecular Physiology & Biophysics, Baylor College of Medicine, Houston, TX, USA
[f] Department of Medicine (Cardiology), Baylor College of Medicine, Houston, TX, USA
[g] Department of Pediatrics (Cardiology), Baylor College of Medicine, Houston, TX, USA

ARTICLE INFO

Article history:
Received 22 August 2016
Received in revised form 15 September 2016
Accepted 18 September 2016
Available online 20 September 2016

Keywords:
Atrial fibrillation
Heart failure
Proteomics
Protein phosphatase
Regulatory subunit

ABSTRACT

Reversible phosphorylation of proteins is a delicate yet dynamic balancing act between kinases and phosphatases, the disturbance of which underlies numerous disease processes. While our understanding of protein kinases has grown tremendously over the past decades, relatively little is known regarding protein phosphatases. This may be because protein kinases are great in number and relatively specific in function, and thereby amenable to be studied in isolation, whereas protein phosphatases are much less abundant and more nonspecific in their function. To achieve subcellular localization and substrate specificity, phosphatases depend on partnering with a large number of regulatory subunits, protein scaffolds and/or other interactors. This added layer of complexity presents a significant barrier to their study, but holds the key to unexplored opportunities for novel pharmacologic intervention. In this review we focus on serine/threonine protein phosphatase type-1 (PP1), which plays an important role in cardiac physiology and pathophysiology. Although much work has been done to investigate the role of PP1 in cardiac diseases including atrial fibrillation and heart failure, most of these studies were limited to examining and manipulating the catalytic subunit(s) of PP1 without adequately considering the PP1 interactors, which give specificity to PP1's functions. To complement these studies, three unbiased methods have been developed and applied to the mapping of the PP1 interactome: bioinformatics approaches, yeast two-hybrid screens, and affinity-purification mass spectrometry. The application of these complementary methods has the potential to generate a detailed cardiac PP1 interactome, which is an important step in identifying novel and targeted pharmacological interventions.

1. Introduction

Reversible phosphorylation of proteins is a process fundamental to life, the dysregulation of which underlies numerous disease processes. Whereas much attention has been paid to protein kinases in terms of our understanding and development of pharmacologic interventions, relatively little investment has been made to the investigation of protein phosphatases, which play an equally important role in maintaining cellular homeostasis. This may be partly due to the fact that protein kinases are widely diverse and thereby specific in their subcellular localization and substrate selection, which are of paramount importance to drug design considerations. For example, the human genome is thought to contain at least 428 kinases that phosphorylate serine/threonine residues, but only about 30 phosphatases that dephosphorylate the same residues [1]. In order to counteract the action of these kinases, protein phosphatases depend on combinatorial formation of holoenzymes where the same catalytic subunit can partner with a large number of regulatory (R)-subunits or interactors [1]. This added layer of complexity presents a significant barrier to their study but at the same time holds the key to unexplored opportunities for novel pharmacologic intervention. In this review we will focus on one particular serine/threonine protein phosphatase, protein phosphatase type-1 (PP1), which is expressed ubiquitously and has a wide range of cellular targets. In particular, we will review the challenges encountered by previous studies of PP1 in

* Corresponding author at: Baylor College of Medicine, One Baylor Plaza BCM335, Houston, TX 77030, USA.
E-mail address: wehrens@bcm.edu (X.H.T. Wehrens).

http://dx.doi.org/10.1016/j.yjmcc.2016.09.009
0022-2828/

cardiovascular diseases and the three major methods that have been used in mapping the PP1 interactome.

2. Fundamental challenge in studying PP1

The PP1 holoenzyme consists of a catalytic subunit (PP1c) and one or more R-subunits or interactors. Whereas all R-subunits are considered interactors, not all interactors have been formally defined or renamed as R-subunits although they are functionally the same. PP1c has four isoforms: PP1α and PP1β/δ are encoded by *PPP1CA* and *PPP1CB*, respectively; PP1γ1 and PP1γ2 are splice variants of *PPP1CC*. Because these isoforms all share a high degree of homology (Fig. 1), the spatial and temporal specificity of the holoenzyme is largely dependent on their R-subunits/interactors, although the isoforms themselves may also drive these interactions and thereby influence substrate selection [2]. To date, close to 200 PP1 interactors have been identified with estimated hundreds more to be discovered [3]. However, most prior studies focused primarily on quantifying and then manipulating the global PP1c level and/or activity. Although such generalization is useful as an initial approach, these studies are limited in scope and sometimes lead to conflicting results. In this section we discuss this concept by highlighting PP1 studies in atrial fibrillation (AF) and heart failure (HF), which are two areas of intense research focus in the field.

2.1. Atrial fibrillation

Atrial fibrillation is the most commonly encountered sustained arrhythmia in the clinical setting, yet to date only a handful of studies have investigated the role of PP1 in AF pathogenesis. In patients with chronic AF, a number of studies have found an increase in the global level and/or activity of PP1c [4–7]. However, instead of finding decreased phosphorylation across known PP1 targets, inhomogeneous changes of target phosphorylation levels have been reported across different subcellular compartments [5]. For example, the phosphorylation levels of myosin binding protein-C and of the L-type Ca^{2+} channel were decreased whereas the phosphorylation levels of phospholamban (PLN) and ryanodine receptor 2 (RyR2) were increased [4,5,8]. On the other hand, in patients with paroxysmal AF, there is no significant change in the expression levels of PP1c [9], yet the phosphorylation of at least one target protein, PLN, is increased [9,10]. Finally, in experimental AF models, both unchanged PP1c levels with or without increased PP1c activity have been reported, and again with inconsistent changes in the phosphorylation of its known targets [6,8,11–14].

These apparent inconsistencies may be due to the fact that PP1 is regulated at the level of its R-subunits/interactors, which underlie the heterogeneity in protein phosphorylation patterns within cardiomyocytes. With this in mind, our group sought to investigate PP1's local regulation of RyR2, which is often dysregulated in cardiac arrhythmias including AF [15]. Since PP1 is believed to be primarily targeted to RyR2 via the R-subunit spinophilin (*Ppp1r9b*) [16], we studied the spinophilin knockout mouse and found a reduction in the association of PP1 with RyR2 by 70% while the global PP1 level was unchanged [17]. At the whole animal level, this disruption of PP1's regulation of RyR2 also resulted in increased atrial ectopy and susceptibility to pacing-induced AF [17]. This study demonstrated for the first time that targeted disruption of PP1's local regulation could have profound phenotypical consequences. Motivated by this study, we undertook a large-scale unbiased study to map changes in PP1's interactome in the setting of paroxysmal AF, discussed in a later section [9].

2.2. Heart failure

Several studies examined the role of PP1 in the pathogenesis of HF, a major cause of mortality and morbidity worldwide. In 1997, Neumann and colleagues found an increase in both the activity and mRNA levels of PP1c in patients with HF secondary to idiopathic cardiomyopathy [18]. Later, two other studies also found an increase in PP1 activity in rat models of HF, albeit from different causes [19,20]. In order to explain these findings, El-Armouche and colleagues reported that the protein level and activity of inhibitor 1 (I-1, *PPP1R1A*), a potent inhibitor of PP1c, are downregulated in HF [21]. However, this study found no difference in the protein level of PP1c among HF patients both from idiopathic cardiomyopathy and dilated cardiomyopathy [21]. Nevertheless, this study was corroborated by another study using a dog model of HF in which I-1 level was decreased in the sarcoplasmic reticulum, where key PP1 targets RyR2 and PLN reside [22]. Although

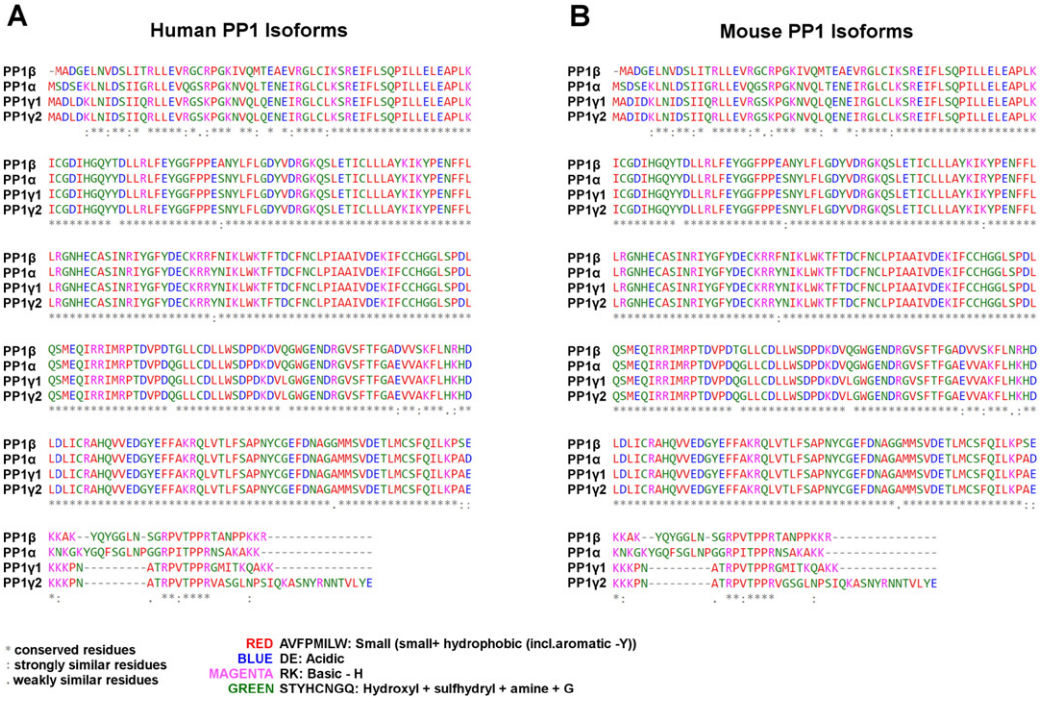

Fig. 1. Sequence alignment of human and mouse PP1 isoforms using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

this may explain the increase in PP1 activity and subsequent decrease in PLN phosphorylation at Ser16, it does not explain the increase in RyR2 phosphorylation at Ser2814 during HF progression as reported by our group [23].

In spite of these inconsistencies, the hypothesis of increased PP1 activity driving HF development remained highly attractive, motivating several groups to generate mouse models to either increase the overall PP1 activity in order to induce HF or decrease its activity to protect against HF. This was nicely demonstrated by Kranias and her group who found that cardiac-specific overexpression of PP1c caused HF in mice, whereas I-1 ablation caused impaired cardiac function without overt HF at baseline [24]. Furthermore, they showed that expression of a constitutively active I-1 (Thr35Asp mutant) rescued the phenotype in myocytes isolated from HF patients [24]. In a follow-up study, cardiac-specific expression of this active I-1 was associated with decreased PP1 activity, increased PLN phosphorylation at both Ser16 and Thr17 (but no change in RyR2 phosphorylation at Ser2808 or troponin I phosphorylation), and augmented cardiac contractility with enhanced β-adrenergic response [25]. Importantly, when this mouse model was subjected to transaortic constriction (TAC), hypertrophy was attenuated with no decompensation to HF. Furthermore, acute adenoviral gene delivery of this active I-1 restored function and partially reversed remodeling in a rat model of HF [25]. In contrast, studies by El-Armouche and others showed that cardiac-specific I-1 transgenic mice developed cardiac hypertrophy and mild dysfunction, as well as a paradoxical or compensatory increase in PP1 abundance and activity [26]. In addition, conditional expression of a truncated constitutively-active I-1 or I-1 Ser67Ala mutant increased contractile function in young mice but was associated with arrhythmias and cardiomyopathy after adrenergic stress and with aging [27]. Although these opposite outcomes may be due to differences between the structures and functions of the different forms of I-1, they call into question whether increased PP1 activity is always detrimental.

Using a different approach to counteract the increased PP1 activity in HF, Neumann and his group overexpressed a functional truncated form of the PP1 inhibitor-2 (I-2, *Ppp1r2*) in mouse cardiomyocytes [28]. Total PP1 activity was reduced by 80%, associated with an increase in PLN phosphorylation at Ser16 but not Thr17, although PP1c protein level was increased nearly 7-fold, presumably due to protection from degradation via association with the excess (truncated) I-2. Phenotypically, the mice showed enhanced cardiac contractility with no associated morbidity and mortality. Compared to the PP1c transgenic mice which developed HF, the PP1c and I-2 double mutant mice had normalized PP1 activity, contractile function, heart size and histology, and mortality rate [29]. In contrast, another study showed that transgenic mice with a constitutively active form of I-2 developed worsening HF following TAC associated with decreased PP1 activity [30]. The authors therefore concluded that long-term inhibition of PP1 using I-2 is not a viable therapeutic option in the treatment of HF.

In summary, whereas a number of studies supported the notion that increased PP1 activity contributes to HF development and that global inhibition by I-1 or I-2 may be therapeutic [24,25,28,29,31], a few studies showed conflicting results [26,27,30,32]. These inconsistencies may be partially explained by differences in experimental setup and the exact molecular manipulation. However, the fundamental challenge faced by these studies lies in the fact that PP1 is not a single enzyme but an integral part of an extensive interactome with a large number of interactors and targets, which are part of numerous pathways (Fig. 2). Therefore, a more detailed understanding of the PP1 interactome and its key interactors is essential to dissect PP1's precise role in the healthy and diseased heart.

3. Mapping the PP1 interactome

To date close to 200 PP1 interactors have been identified. These interactors are diverse in their sizes, structures, and functions, but share a number of PP1-docking motifs, the most common of which is the short sequence of Arg-Val-x-Phe (RVxF; where x denotes any residue) [3]. The RVxF motif is present in the vast majority of known PP1 interactors and binds to a hydrophobic channel on PP1 that is remote from its catalytic site (Table 1) [3,33,34]. Besides RVxF there are at least 8 other known motifs, which are uncommon among the known PP1 interactors, but may act in combination with one another [3]. The discovery of these interactors has drastically accelerated over the past decade through a concerted effort of studies using large-scale, unbiased, and complementary methods. In this section we will critically review the three main methodologies that have contributed significantly to the mapping of the PP1 interactome. These are bioinformatics approaches, yeast-two-hybrid (Y2H), and affinity-purification mass spectrometry (AP-MS).

3.1. Bioinformatics

The RVxF motif was defined for the first time in 1997 by Egloff and colleagues who reported the crystal structure of PP1c in complex with a peptide from a known PP1 R-subunit, G_M [33]. Later, this motif was refined by Wakula and colleagues with drastically improved sensitivity in discovering new PP1 interactors but at the cost of decreased specificity [34,35]. An alternative definition was developed by Meiselbach and colleagues, which had a 10-fold higher specificity than the Wakula definition but with a low sensitivity [35,36]. In 2009, Bollen and his group combined the sensitive Wakula definition and the specific Meiselbach definition to screen for putative PP1 interactors *in silico* [37]. Moreover, since the RVxF motif is known to be present in a flexible loop [33] and the fact that PP1 is an intracellular protein, they excluded proteins where the RVxF motif is in a globular domain or an extracellular or transmembrane domain [37]. These stringent criteria yielded 115 novel candidate interactors although it filtered out I-2, which has an RVxF motif that does not conform to the definitions, and possibly other unknown interactors. They validated 78 of these interactors by pull-down assays using PP1c and His-tagged fragments of the putative interactors expressed in bacteria. Using phosphorylase assay they also showed that 49 out of the 78 exhibited inhibitory effects on PP1c [37]. Altogether, this study doubled the then known PP1 interactome from 65 to 143 interactors. In 2013 this number reached 189 as reviewed by Bollen and his group [3]. Based on these interactors, the authors described the PP1 binding code as a "molecular-lego strategy" consisting of 9 different PP1 docking motifs working in combination to give rise to a vast number of specific interactions. These 9 docking motifs are RVxF (the most common motif), SILK, MyPhoNE, SpiDoC, IDoHA, RNYF, BiSTriP, pseudosubstrate, and AnkCap [3].

By leveraging the power of bioinformatics, these studies have drastically increased our knowledge of the PP1 interactome. However, there are two limitations inherent to this general approach. First, the biochemical validations are carried out in artificial systems and sometimes with protein fragments, which may lead to false positive results. And even if two proteins can interact, it does not mean that they do interact in their native cellular environment. Second, the prediction of novel interactors depends on knowledge of previous interactors. In other words, it is based on the characteristics of the previously validated interactors that the searches for new interactors are carried out. It is conceivable, however, that there are entire new classes of interactors that do not harbor any of the known motifs but instead interact via entirely different mechanisms. If so the current bioinformatics approach will be unable to predict their existence without at least some prior knowledge, which must be derived from complementary approaches such as the ones discussed below.

3.2. Yeast two-hybrid

The Y2H system was first described by Fields and Song in 1989 and has become a major contributor to our knowledge of protein-protein

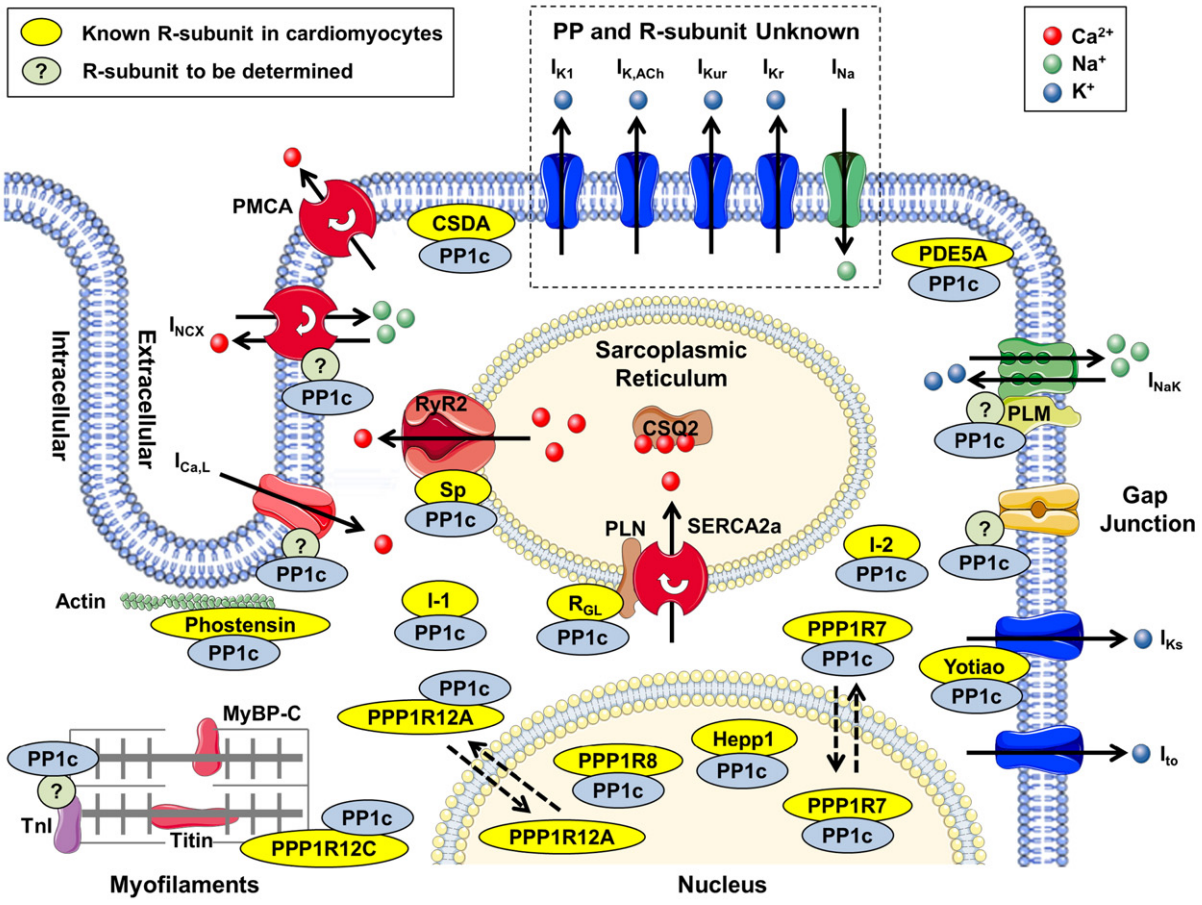

Fig. 2. The cardiac PP1 interactome.
Modified from Chiang et al. [9] and incorporated data from various studies as well as from the Human Protein Atlas (http://www.proteinatlas.org/). All known PP1 regulatory (R)-subunits or interactors (yellow) in the heart are listed in Table 1.

interactions. Y2H screens are scalable, relatively inexpensive, and easy to implement. Importantly, they interrogate binary interactions with high fidelity, which is important in teasing apart primary from secondary and tertiary interactions common in protein complexes [38]. One particular database, the CCSB Human Interactome Database (http://interactome.dfci.harvard.edu/H_sapiens/index.php), includes data from several large-scale Y2H screens and curation of 7 other databases, and thereby serves as an invaluable tool in studying any protein interactome including the PP1 interactome. Searches done for PP1α (*PPP1CA*), PP1β/δ (*PPP1CB*), and PP1γ1/2 (*PPP1CC*) found 83 unique interactors that bind to at least 1 of the PP1c isoforms (Table 2). This is a much shorter list compared to the list of 189 reviewed by Bollen and his group in 2013 [3], and only 52 of these 83 interactors were included on that list, suggesting a significant discrepancy between the ways these lists were compiled. In addition, only 9 of these 83 interactors were directly identified from the large-scale Y2H screens whereas the vast majority was curated from the other databases, suggesting that the large-scale screens have very low sensitivity.

Besides these large-scale screens, a number of studies have employed more targeted screens using one or more of the PP1c isoforms as bait. These include screens done in *Drosophila* [39], human brain [40,41], human testis [42], and mouse testis [43,44], among others [38]. Only one group has published studies utilizing Y2H in investigating the PP1 interactome in the heart [45,46]. Huang and his group used a rabbit PP1α as bait to screen a human heart cDNA library and found 8 potential interactors although they only reported 2, both of which were novel proteins, and validated their interaction with PP1 using co-IP, GST pull-down assays, and site-directed mutagenesis [45,46]. The authors named the first interactor PP1 F-actin cytoskeleton targeting subunit or phostensin (*PPP1R18*), and found that it co-localized with F-actin and could therefore play a role in targeting PP1c to the F-actin cytoskeleton [45]. The second interactor was named Hepp1 for heart PP1-binding protein and its mRNA was found exclusively in heart and testis by Northern blot [46]. When the catalytic activity of PP1α was probed in the presence of I-1 and I-2, Hepp1 antagonized the inhibition of both phospho-I-1 and I-2 and non-significantly increased PP1α's activity on its own [46]. Using PP1 co-immunoprecipitation (IP) followed by MS, we also found an interaction between PP1 and phostensin in both mouse ventricles and human atria but did not detect Hepp1 [9]. In any case, the precise functional roles of these proteins in the heart will need to be explored and defined by future studies.

Although Y2H is a powerful tool in studying protein-protein interactions, the contributions of both large-scale and targeted screens to our understanding of the PP1 interactome have been somewhat limited. This is likely due to the low sensitivity of these screens, secondary to a number of intrinsic limitations. These include potential alterations in the tertiary structure of proteins from fusion with the yeast transcription factor, non-native environment where the interaction takes place (yeast nucleus), and toxicity of some proteins to the yeast cell [38]. Although some of these may be circumvented with newer iterations of

Table 1
Known cardiac PP1c interactors as depicted in Fig. 2 and their RVxF motifs.

Name	Alternate name(s)	RVxF motif	Start position
PPP1R1A	I-1	RKIQF	8
PPP1R2	I-2	KSQKW	43
PPP1R3A	RGL, GM	RRVSF	62
PPP1R7	SDS22	–	–
PPP1R8	NIPP1	SRVTF	199
PPP1R9B	Spinophilin, Neurabin-II	RKIHF	445
PPP1R12A	MBS, MYPT1	TKVKF	34
PPP1R12C	MBS85	RTVRF	45
PPP1R18	Phostensin	LKISF	538
PPP1R39	HEPP1, SH3RF2	KTVRF	98
CSDA	YBX3	–	–
PDE5A		MQVGF	816
Yotiao	AKAP-9	DKVSF	1063

the Y2H system, the fact that these systems are artificial will always be an inherent limitation to our understanding of protein interactions in their native environment. Nevertheless, Y2H remains a powerful tool especially when used in conjunction with complementary methods such as AP-MS.

3.3. Affinity-purification mass spectrometry

In recent years, AP-MS has increasingly been employed for the mapping of protein interactomes. This rapid growth in application is partly due to advances in both affinity-purification strategies and tandem MS. The basic work-flow involves 1) the purification of target protein complexes using some kind of affinity-based biochemical methods such as antibody-capture with or without a resin column, 2) separation of the protein complexes on 1D or 2D gel, and 3) digestion of the separated proteins and analysis by MS. One major advantage of AP-MS is scalability, since it can be performed on as many proteins as can be tagged and expressed or immunoprecipitated directly, although the dynamic range of protein identification by the MS becomes a limiting factor. Another important advantage is that AP-MS identifies not only binary interactions but also entire protein complexes, in which most proteins normally exist and function. When applied to the mapping of PP1 interactome, AP-MS has the potential to quantitatively monitor dynamic changes under different physiological or pathological conditions. In addition, AP-MS has the ability to identify and quantify post-translational modifications such as phosphorylation, which may be of particular interest in mapping the PP1 interactome.

To date a good number of studies have applied the principles of AP-MS in studying the PP1 interactome [42,47–52]. Due to the lack of a good IP antibody for PP1c, several groups first tagged PP1c, such as with protein-A [47] or EGFP [48], in exogenous expression systems before purifying it and using it to pull-down interactors. To better mimic endogenous PP1c expression and localization, one group created knock-in alleles of doubly-tagged PP1c in embryonic cell lines before purifying the PP1 complexes with specific resins. However, they only identified 11 putative PP1 interactors, 9 of which were already known [51]. Instead of tagging PP1c, another group captured PP1c directly using different Sepharose columns before either competitively displacing the interactors off using a RVxF-based peptide [49] or incubating with lysates from another source before eluting everything off of the column for MS analysis [50]. Unfortunately, all these studies were limited by exogenous expression, non-native interaction environment and/or a modified (tagged) PP1c. To circumvent these limitations, Alcazar and his group directly immunoprecipitated PP1α and PP1γ from rat brain lysates under different physiologic stresses and analyzed their interactors with 2D gel and MS. [52] The authors identified 14 PP1 interactors that exhibited significant changes in their association with PP1α or PP1γ in response to ischemia-reperfusion or ischemic tolerance. A similar approach was used in human sperm where PP1γ2 was immunoprecipitated followed by gel separation and MS although only 7 proteins were identified [42]. As a variation of AP-MS, these two IP-MS studies directly captured endogenous PP1c from tissue lysates, and thus were able to preserve native interactions to a greater extent. The success of such approach depends on the existence of high-affinity and high-specificity IP antibodies in order to adequately and specifically pull-down PP1 complexes. However, both studies were limited by lack of a proper negative control.

With these studies and their limitations in mind, our group conducted a similar but larger-scale study based on IP-MS, with the aim to 1) capture as many endogenous PP1 complexes as possible directly from primary tissues, 2) quantify these interactions with PP1c using high resolution MS, and 3) measure changes in these interactions in diseased state [9]. As mentioned earlier, one limiting factor faced by IP-MS is the inadequate capturing of the bait since there is not always a good antibody, thus forcing many studies to using a tagged PP1c *in vitro* instead. After testing a number of commercial antibodies and several homogenization methods, we identified a strong IP antibody for PP1α and established a robust co-IP protocol which was then coupled to state-of-the-art nanoscale liquid chromatography followed by tandem MS for increased sensitivity and dynamic range. This allowed us to quantify 135 and 78 putative PP1c interactors from mouse and human cardiac lysates, respectively, which were many times more than all previous studies. Also, our methodology was validated by the fact that we captured a large number of known R-subunits (the most identified in a single AP/IP-MS experiment, to the best of our knowledge). Moreover, a large percentage of the putative interactors contained 3 common PP1c docking motifs (RVxF, SILK, and MyPhoNE). Finally, we applied our method to studying atrial samples from patients with paroxysmal AF or in sinus rhythm and found alterations in the interaction between PP1c and PPP1R7, cold-shock domain protein A (CSDA), and phosphodiesterase type-5A (PDE5A). Together this study offered a straight-forward protocol for high resolution mapping of the PP1 interactome directly from primary tissues, as well as doubled the known cardiac PP1 interactome (Fig. 2).

The AP/IP-MS approach promises great contribution to our understanding of the PP1 interactome, although it has its limitations. First, it will always be limited by available reagents such as antibodies. Although we were fortunate to find a strong PP1α IP antibody, it nevertheless has cross-reactivity to the other PP1 isoforms, due to their high degree of homology (Fig. 1). Second, because MS is highly sensitive and therefore able to identify hundreds of proteins in complexes, a robust AP/IP-MS experiment requires good and proper controls in order to distinguish true positive from false positive hits. Finally, MS is in general more expensive to implement and requires expertise in both instrumentation and data analyses, which may pose a significant barrier to research groups.

Ultimately, it is the complementation of bioinformatics, Y2H, and AP-MS (among others) that hold the greatest potential in mapping the PP1 interactome with high resolution and fidelity. One recent study implemented all three methods in an effort to map the PP1 interactome in

Table 2
PP1c interactors as found in the CCSB Human Interactome Database (searched on July 31, 2016). Brackets indicate the numbers of proteins that had a positive hit with the respective PP1c isoforms from the database. The last column cross-references the lists from the database with the list of 189 vertebrate PP1c interactors reviewed by Heroes and colleague [3].

Unique (83)	PPP1CA (62)	PPP1CB (15)	PPP1CC (44)	Heroes et al. [3] (52)
APAF1	■			
ATM	■		■	
AURKB	■			■
BAD	■			
BCL2	■	■	■	■
BCL2L1	■		■	■
BIRC5			■	
BRCA1		■		■
C15orf59			■	
C1QA			■	
CASC5	■			■
CCND1		■	■	
CCND3		■	■	
CD2BP2	■		■	■
CDC34		■	■	
CDH1	■			
CDK1	■		■	
CDK2	■		■	
CDK4	■			
CDKN2A		■	■	
CEP192	■			■
CHCHD3	■			■
CNST	■		■	■
COPS5			■	
CSRNP1*	■		■	
CSRNP2*	■	■	■	■
DLG3	■			■
ELL	■			■
ESR1			■	
HDAC6			■	■
KANK1	■			
LMTK2	■		■	■
MAX	■	■	■	
MPHOSPH10	■			■
NEK2			■	■
PCNA			■	
PHACTR1	■			
PHACTR3	■			■
PPP1R10	■			■
PPP1R12A		■		■
PPP1R13B	■		■	■
PPP1R13L	■			■
PPP1R15A	■			■
PPP1R15B	■		■	■
PPP1R16A*		■		■
PPP1R18	■		■	■
PPP1R2	■		■	■
PPP1R26	■			■
PPP1R27	■			■
PPP1R2P3*	■	■	■	
PPP1R32	■		■	■
PPP1R37	■			■
PPP1R3C*		■		■
PPP1R7		■		■
PPP1R8	■		■	■
PPP1R9A	■			■
PPP1R9B	■		■	■
PTEN	■			
RANBP9	■		■	
RB1	■			■
RIF1	■		■	
SFI1	■			■
SFRP1			■	
SH2D4A*	■	■		■
SH3RF2	■		■	■
SKP1	■		■	
SPRED1	■			■
STAU1	■			■
SYTL2	■			■
TCTEX1D4			■	
TMEM132D	■			■
TOR1AIP1	■			
TP53			■	
TP53BP2*	■	■	■	■
TPRN	■		■	
TRPC4AP	■			■
TSC2	■			■
VDR			■	■
WBP11	■		■	■
WNK1	■			■
YLPM1	■		■	■
ZFYVE1	■			■
ZFYVE9*	■		■	■

* Interactors that were identified directly from large-scaled Y2H screens and not curated from other databases.

the malaria parasite *Plasmodium falciparum* [53]. Although this study used all three methods, the overlap between the hits from the different methods was quite poor, mainly due to the fact that their AP-MS protocol yielded too few putative interactors (only 6). Ideally, a work-flow will begin with a large-scale AP/IP-MS screen for endogenous PP1 complexes followed by targeted Y2H experiments to map the binary

interactions among the proteins, and finally analyzed using bioinformatics both retrospectively (e.g. confirming presence of known motifs), and prospectively (e.g. building new motifs to search for novel interactors).

4. Summary and future directions

Although much effort has already been made to understand the PP1 interactome, much work remains to be done especially in the context of the cardiac system. Once a robust cardiac PP1 interactome is benchmarked, investigation to understand its pertubation in disease and identification of novel therapeutic strategies can quickly advance. One promising example of this concept comes from a study that used guanabenz, a small molecule, to specifically disrupt the binding between PP1c and PPP1R15A (aka. GADD34) [54]. This disruption decreased PP1's dephosphorylation of the α-subunit of translation initiation factor 2 during stress, leading to modulation of protein production rates and cellular rescue from protein misfolding stress [54]. As this is a common mechanism underlying a number of diseases, this strategy of targeting a specific PP1 interaction holds great therapeutic potential.

To achieve this ultimate goal, several milestones need to be reached (but not necessarily sequentially). First, although there are some recognition of the differences between the PP1c isoforms in terms of their interactors and functions [2], sufficiently detailed and comprehensive information is missing to confidently delineate and compare their multifarious roles. This may be difficult to accomplish due to the limitations with reagents and the inherent similarity among the isoforms, although recent studies have made significant advances in this regard [55,56]. Second, efforts must be made to validate interactors in the literature that do not have strong evidence behind their interaction with PP1c (e.g. two or more stringent orthogonal biochemical methods). This will help filter out false positive results which may hinder or slow down future efforts. Third, besides seeking to understand the primary interactors of PP1, it is equally important to identify its downstream targets and secondary/tertiary interactors in order to place PP1 into distinct regulatory pathways relevant to cellular physiology. For this the combination of AP/IP-MS and Y2H methods may be very useful. Fourth, once such networks are constructed, methods of modulating local interactions between PP1c and its interactors must be developed while monitoring their effects on the rest of the interactome. Even though these are ambitious undertakings which require much investment of time and resources, they are attainable through our concerted efforts and will greatly advance both the cardiac field and science in general.

Funding sources

This work was supported by the Basic Research Fellowship from the European Society of Cardiology (D.Y.C.); Proteins At Work (project 184.032.201), a program of The Netherlands Proteomics Centre financed by The Netherlands Organisation for Scientific Research (NWO) as part of the National Roadmap Large-scale Research Facilities of The Netherlands (A.J.R.H.); the DZHK (German Center for Cardiovascular Research) (D.D.); the National Institutes of Health grants R01-HL089598, R01-HL091947, R01-HL117641, R41-HL129570 (X.H.T.W.), and R01-HL131517 (D.D.); and the American Heart Association grant 13EIA14560061 (X.H.T.W.).

Conflict of interest

There are no relevant relationships with industry.

References

[1] Y. Shi, Serine/threonine phosphatases: mechanism through structure, Cell 139 (2009) 468–484.
[2] H. Ceulemans, M. Bollen, Functional diversity of protein phosphatase-1, a cellular economizer and reset button, Physiol. Rev. 84 (2004) 1–39.
[3] E. Heroes, B. Lesage, J. Gornemann, M. Beullens, L. Van Meervelt, M. Bollen, The PP1 binding code: a molecular-lego strategy that governs specificity, FEBS J. 280 (2013) 584–595.
[4] T. Christ, P. Boknik, S. Wohrl, E. Wettwer, E.M. Graf, R.F. Bosch, et al., L-type Ca^{2+} current downregulation in chronic human atrial fibrillation is associated with increased activity of protein phosphatases, Circulation 110 (2004) 2651–2657.
[5] A. El-Armouche, P. Boknik, T. Eschenhagen, L. Carrier, M. Knaut, U. Ravens, et al., Molecular determinants of altered Ca^{2+} handling in human chronic atrial fibrillation, Circulation 114 (2006) 670–680.
[6] J. Heijman, M. Dewenter, A. El-Armouche, D. Dobrev, Function and regulation of serine/threonine phosphatases in the healthy and diseased heart, J. Mol. Cell. Cardiol. 64 (2013) 90–98.
[7] M. Greiser, C.R. Halaszovich, D. Frechen, P. Boknik, U. Ravens, D. Dobrev, et al., Pharmacological evidence for altered src kinase regulation of I (Ca, L) in patients with chronic atrial fibrillation, Naunyn Schmiedeberg's Arch. Pharmacol. 375 (2007) 383–392.
[8] M.G. Chelu, S. Sarma, S. Sood, S. Wang, R.J. van Oort, D.G. Skapura, et al., Calmodulin kinase II-mediated sarcoplasmic reticulum Ca^{2+} leak promotes atrial fibrillation in mice, J. Clin. Invest. 119 (2009) 1940–1951.
[9] D.Y. Chiang, N. Lebesgue, D.L. Beavers, K.M. Alsina, J.M. Damen, N. Voigt, et al., Alterations in the interactome of serine/threonine protein phosphatase type-1 in atrial fibrillation patients, J. Am. Coll. Cardiol. 65 (2015) 163–173.
[10] N. Voigt, J. Heijman, Q. Wang, D.Y. Chiang, N. Li, M. Karck, et al., Cellular and molecular mechanisms of atrial arrhythmogenesis in patients with paroxysmal atrial fibrillation, Circulation 129 (2013) 145–156.
[11] M. Greiser, H.R. Neuberger, E. Harks, A. El-Armouche, P. Boknik, S. de Haan, et al., Distinct contractile and molecular differences between two goat models of atrial dysfunction: AV block-induced atrial dilatation and atrial fibrillation, J. Mol. Cell. Cardiol. 46 (2009) 385–394.
[12] Y.H. Yeh, R. Wakili, X.Y. Qi, D. Chartier, P. Boknik, S. Kaab, et al., Calcium-handling abnormalities underlying atrial arrhythmogenesis and contractile dysfunction in dogs with congestive heart failure, Circ. Arrhythm Electrophysiol. 1 (2008) 93–102.
[13] R. Wakili, Y.H. Yeh, X. Yan Qi, M. Greiser, D. Chartier, K. Nishida, et al., Multiple potential molecular contributors to atrial hypocontractility caused by atrial tachycardia remodeling in dogs, Circ. Arrhythm Electrophysiol. 3 (2010) 530–541.
[14] M. Greiser, B.G. Kerfant, G.S. Williams, N. Voigt, E. Harks, K.M. Dibb, et al., Tachycardia-induced silencing of subcellular Ca^{2+} signaling in atrial myocytes, J. Clin. Invest. 124 (2014) 4759–4772.
[15] N. Li, D.Y. Chiang, S. Wang, Q. Wang, L. Sun, N. Voigt, et al., Ryanodine receptor-mediated calcium leak drives progressive development of an atrial fibrillation substrate in a transgenic mouse model, Circulation 129 (2014) 1276–1285.
[16] S.O. Marx, S. Reiken, Y. Hisamatsu, M. Gaburjakova, J. Gaburjakova, Y.M. Yang, et al., Phosphorylation-dependent regulation of ryanodine receptors: a novel role for leucine/isoleucine zippers, J. Cell Biol. 153 (2001) 699–708.
[17] D.Y. Chiang, N. Li, Q. Wang, K.M. Alsina, A.P. Quick, J.O. Reynolds, et al., Impaired local regulation of ryanodine receptor type 2 by protein phosphatase 1 promotes atrial fibrillation, Cardiovasc. Res. 103 (2014) 178–187.
[18] J. Neumann, T. Eschenhagen, L.R. Jones, B. Linck, W. Schmitz, H. Scholz, et al., Increased expression of cardiac phosphatases in patients with end-stage heart failure, J. Mol. Cell. Cardiol. 29 (1997) 265–272.
[19] B. Huang, S. Wang, D. Qin, M. Boutjdir, N. El-Sherif, Diminished basal phosphorylation level of phospholamban in the postinfarction remodeled rat ventricle: role of beta-adrenergic pathway, G(i) protein, phosphodiesterase, and phosphatases, Circ. Res. 85 (1999) 848–855.
[20] A.P. Schwoerer, C. Neuber, A. Schmechel, I. Melnychenko, G. Mearini, P. Boknik, et al., Mechanical unloading of the rat heart involves marked changes in the protein kinase-phosphatase balance, J. Mol. Cell. Cardiol. 45 (2008) 846–852.
[21] A. El-Armouche, T. Pamminger, D. Ditz, O. Zolk, T. Eschenhagen, Decreased protein and phosphorylation level of the protein phosphatase inhibitor-1 in failing human hearts, Cardiovasc. Res. 61 (2004) 87–93.
[22] R.C. Gupta, S. Mishra, S. Rastogi, M. Imai, O. Habib, H.N. Sabbah, Cardiac SR-coupled PP1 activity and expression are increased and inhibitor 1 protein expression is decreased in failing hearts, Am. J. Physiol. Heart Circ. Physiol. 285 (2003) H2373–H2381.
[23] J.L. Respress, R.J. van Oort, N. Li, N. Rolim, S.S. Dixit, A. deAlmeida, et al., Role of RyR2 phosphorylation at S2814 during heart failure progression, Circ. Res. 110 (2012) 1474–1483.
[24] A.N. Carr, A.G. Schmidt, Y. Suzuki, F. del Monte, Y. Sato, C. Lanner, et al., Type 1 phosphatase, a negative regulator of cardiac function, Mol. Cell. Biol. 22 (2002) 4124–4135.
[25] A. Pathak, F. del Monte, W. Zhao, J.E. Schultz, J.N. Lorenz, I. Bodi, et al., Enhancement of cardiac function and suppression of heart failure progression by inhibition of protein phosphatase 1, Circ. Res. 96 (2005) 756–766.
[26] A. El-Armouche, K. Wittkopper, F. Degenhardt, F. Weinberger, M. Didie, I. Melnychenko, et al., Phosphatase inhibitor-1-deficient mice are protected from catecholamine-induced arrhythmias and myocardial hypertrophy, Cardiovasc. Res. 80 (2008) 396–406.
[27] K. Wittkopper, L. Fabritz, S. Neef, K.R. Ort, C. Grefe, B. Unsold, et al., Constitutively active phosphatase inhibitor-1 improves cardiac contractility in young mice but is deleterious after catecholaminergic stress and with aging, J. Clin. Invest. 120 (2010) 617–626.
[28] U. Kirchhefer, H.A. Baba, P. Boknik, K.M. Breeden, N. Mavila, N. Bruchert, et al., Enhanced cardiac function in mice overexpressing protein phosphatase inhibitor-2, Cardiovasc. Res. 68 (2005) 98–108.

[29] N. Bruchert, N. Mavila, P. Boknik, H.A. Baba, L. Fabritz, U. Gergs, et al., Inhibitor-2 prevents protein phosphatase 1-induced cardiac hypertrophy and mortality, Am. J. Physiol. Heart Circ. Physiol. 295 (2008) H1539–H1546.
[30] S. Grote-Wessels, H.A. Baba, P. Boknik, A. El-Armouche, L. Fabritz, H.J. Gillmann, et al., Inhibition of protein phosphatase 1 by inhibitor-2 exacerbates progression of cardiac failure in a model with pressure overload, Cardiovasc. Res. 79 (2008) 464–471.
[31] P. Nicolaou, P. Rodriguez, X. Ren, X. Zhou, J. Qian, S. Sadayappan, et al., Inducible expression of active protein phosphatase-1 inhibitor-1 enhances basal cardiac function and protects against ischemia/reperfusion injury, Circ. Res. 104 (2009) 1012–1020.
[32] Y. Ladilov, H. Maxeiner, C. Wolf, C. Schafer, K. Meuter, H.M. Piper, Role of protein phosphatases in hypoxic preconditioning, Am. J. Physiol. Heart Circ. Physiol. 283 (2002) H1092–H1098.
[33] M.P. Egloff, D.F. Johnson, G. Moorhead, P.T. Cohen, P. Cohen, D. Barford, Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1, EMBO J. 16 (1997) 1876–1887.
[34] P. Wakula, M. Beullens, H. Ceulemans, W. Stalmans, M. Bollen, Degeneracy and function of the ubiquitous RVXF motif that mediates binding to protein phosphatase-1, J. Biol. Chem. 278 (2003) 18817–18823.
[35] H. Ceulemans, M. Bollen, A tighter RVxF motif makes a finer sift, Chem. Biol. 13 (2006) 6–8.
[36] H. Meiselbach, H. Sticht, R. Enz, Structural analysis of the protein phosphatase 1 docking motif: molecular description of binding specificities identifies interacting proteins, Chem. Biol. 13 (2006) 49–59.
[37] A. Hendrickx, M. Beullens, H. Ceulemans, T. Den Abt, A. Van Eynde, E. Nicolaescu, et al., Docking motif-guided mapping of the interactome of protein phosphatase-1, Chem. Biol. 16 (2009) 365–371.
[38] J.V. Silva, M.J. Freitas, J. Felgueiras, M. Fardilha, The power of the yeast two-hybrid system in the identification of novel drug targets: building and modulating PPP1 interactomes, Expert Rev. Proteomics 12 (2015) 147–158.
[39] D. Bennett, E. Lyulcheva, L. Alphey, G. Hawcroft, Towards a comprehensive analysis of the protein phosphatase 1 interactome in *Drosophila*, J. Mol. Biol. 364 (2006) 196–212.
[40] S.L. Esteves, S.C. Domingues, O.A. da Cruz e Silva, M. Fardilha, E.F. da Cruz e Silva, Protein phosphatase 1alpha interacting proteins in the human brain, OMICS 16 (2012) 3–17.
[41] S.L. Esteves, L. Korrodi-Gregorio, C.Z. Cotrim, P.J. van Kleeff, S.C. Domingues, O.A. da Cruz e Silva, et al., Protein phosphatase 1gamma isoforms linked interactions in the brain, J. Mol. Neurosci. 50 (2013) 179–197.
[42] M. Fardilha, S.L. Esteves, L. Korrodi-Gregorio, A.P. Vintem, S.C. Domingues, S. Rebelo, et al., Identification of the human testis protein phosphatase 1 interactome, Biochem. Pharmacol. 82 (2011) 1403–1415.
[43] C. Hrabchak, S. Varmuza, Identification of the spermatogenic zip protein Spz1 as a putative protein phosphatase-1 (PP1) regulatory protein that specifically binds the PP1cgamma2 splice variant in mouse testis, J. Biol. Chem. 279 (2004) 37079–37086.
[44] C. Hrabchak, H. Henderson, S. Varmuza, A testis specific isoform of endophilin B1, endophilin B1t, interacts specifically with protein phosphatase-1c gamma2 in mouse testis and is abnormally expressed in PP1c gamma null mice, Biochemistry 46 (2007) 4635–4644.
[45] S.C. Kao, C.Y. Chen, S.L. Wang, J.J. Yang, W.C. Hung, Y.C. Chen, et al., Identification of phostensin, a PP1 F-actin cytoskeleton targeting subunit, Biochem. Biophys. Res. Commun. 356 (2007) 594–598.
[46] C.Y. Chen, N.S. Lai, J.J. Yang, H.L. Huang, W.C. Hung, C. Li, et al., FLJ23654 encodes a heart protein phosphatase 1-binding protein (Hepp1), Biochem. Biophys. Res. Commun. 391 (2010) 698–702.
[47] E.P. Walsh, D.J. Lamont, K.A. Beattie, M.J. Stark, Novel interactions of *Saccharomyces cerevisiae* type 1 protein phosphatase identified by single-step affinity purification and mass spectrometry, Biochemistry 41 (2002) 2409–2420.
[48] L. Trinkle-Mulcahy, J. Andersen, Y.W. Lam, G. Moorhead, M. Mann, A.I. Lamond, Repo-man recruits PP1 gamma to chromatin and is essential for cell viability, J. Cell Biol. 172 (2006) 679–692.
[49] G.B. Moorhead, L. Trinkle-Mulcahy, M. Nimick, V. De Wever, D.G. Campbell, R. Gourlay, et al., Displacement affinity chromatography of protein phosphatase one (PP1) complexes, BMC Biochem. 9 (2008) 28.
[50] G.W. Templeton, M. Nimick, N. Morrice, D. Campbell, M. Goudreault, A.C. Gingras, et al., Identification and characterization of Atl-2, an *Arabidopsis* homologue of an ancient protein phosphatase 1 (PP1) regulatory subunit, Biochem. J. 435 (2011) 73–83.
[51] G. MacLeod, S. Varmuza, Tandem affinity purification in transgenic mouse embryonic stem cells identifies DDOST as a novel PPP1CC2 interacting protein, Biochemistry 51 (2012) 9678–9688.
[52] C. Cid, L. Garcia-Bonilla, E. Camafeita, J. Burda, M. Salinas, A. Alcazar, Proteomic characterization of protein phosphatase 1 complexes in ischemia-reperfusion and ischemic tolerance, Proteomics 7 (2007) 3207–3218.
[53] T. Hollin, C. De Witte, A. Lenne, C. Pierrot, J. Khalife, Analysis of the interactome of the Ser/Thr protein phosphatase type 1 in plasmodium falciparum, BMC Genomics 17 (2016) 246.
[54] P. Tsaytler, H.P. Harding, D. Ron, A. Bertolotti, Selective inhibition of a regulatory subunit of protein phosphatase 1 restores proteostasis, Science 332 (2011) 91–94.
[55] H. Aoyama, Y. Ikeda, Y. Miyazaki, K. Yoshimura, S. Nishino, T. Yamamoto, et al., Isoform-specific roles of protein phosphatase 1 catalytic subunits in sarcoplasmic reticulum-mediated Ca($^{2+}$) cycling, Cardiovasc. Res. 89 (2011) 79–88.
[56] R. Liu, R.N. Correll, J. Davis, R.J. Vagnozzi, A.J. York, M.A. Sargent, et al., Cardiac-specific deletion of protein phosphatase 1beta promotes increased myofilament protein phosphorylation and contractile alterations, J. Mol. Cell. Cardiol. 87 (2015) 204–213.