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Regulating the Regulator: Insights into the Cardiac Protein Phosphatase 1 Interactome

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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 unspecific 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 the 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

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CONFLICT OF INTEREST

There are no relevant relationships with industry.

complementary methods has the potential to generate a detailed cardiac PP1 interactome, which is an important step in identifying novel and targeted pharmacological interventions.

Keywords

Atrial fibrillation; heart failure; proteomics; protein phosphatase; regulatory subunit

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

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. On the other hand, PP1c has four isoforms: PP1 α and PP1 β/δ are encoded by *PPP1CA* and *PPP1CB*, respectively; PP1 γ 1 and PP1 γ 2 are splice variants of *PPP1CC*. Because these isoforms all share a high degree of homology (Figure 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.

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]

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 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 (Figure 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.

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).

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, which drastically improved the sensitivity of discovering new PP1 interactors but at the cost of decreasing its 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 both the sensitive Wakula definition and the specific Meiselbach definition in order 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 both 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.

Yeast Two-Hybrid

The yeast two-hybrid (Y2H) system was first described by Fields and Song in 1989 and has become a major contributor to our knowledge of protein-protein 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 PP1 α (*PPP1CA*), PP1 β/δ (*PPP1CB*), and PP1 $\gamma 1/2$ (*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 PP1 α as bait to screen a human heart cDNA library and found 8 potential interactors, although they only reported 2 of them, 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 PP1 α 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 PP1 α 's activity

on its own.[46] Using PP1 co-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 in 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 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.

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 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 monitor dynamic and quantitative 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 PP1 α and PP1 γ 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 PP1 α or PP1 γ in response to ischemia-reperfusion or ischemic tolerance. A similar approach was used in human sperm where PP1 γ 2 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 the lack of a proper negative control.

With these previous 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 PP1 α 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 also 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 (Figure 2).

AP/IP-MS 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 PP1 α IP antibody, it nevertheless has cross-reactivity to the other PP1 isoforms, due to their high degree of homology (Figure 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 require 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 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).

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 perturbation 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.

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REFERENCE

1. Shi Y. Serine/threonine phosphatases: mechanism through structure. *Cell*. 2009; 139:468–484. [PubMed: 19879837]
2. Ceulemans H, Bollen M. Functional diversity of protein phosphatase-1, a cellular economizer and reset button. *Physiol. Rev*. 2004; 84:1–39. [PubMed: 14715909]
3. Heroes E, Lesage B, Gornemann J, Beullens M, Van Meervelt L, Bollen M. The PP1 binding code: a molecular-lego strategy that governs specificity. *FEBS J*. 2013; 280:584–595. [PubMed: 22360570]
4. Christ T, Boknik P, Wohrl S, Wettwer E, Graf EM, Bosch RF, et al. L-type Ca²⁺ current downregulation in chronic human atrial fibrillation is associated with increased activity of protein phosphatases. *Circulation*. 2004; 110:2651–2657. [PubMed: 15492323]
5. El-Armouche A, Boknik P, Eschenhagen T, Carrier L, Knaut M, Ravens U, et al. Molecular determinants of altered Ca²⁺ handling in human chronic atrial fibrillation. *Circulation*. 2006; 114:670–680. [PubMed: 16894034]
6. Heijman J, Dewenter M, El-Armouche A, Dobrev D. Function and regulation of serine/threonine phosphatases in the healthy and diseased heart. *J Mol Cell Cardiol*. 2013; 64:90–98. [PubMed: 24051368]
7. Greiser M, Halaszovich CR, Frechen D, Boknik P, Ravens U, Dobrev D, et al. Pharmacological evidence for altered src kinase regulation of I (Ca,L) in patients with chronic atrial fibrillation. *Naunyn Schmiedebergs Arch Pharmacol*. 2007; 375:383–392. [PubMed: 17593353]
8. Chelu MG, Sarma S, Sood S, Wang S, van Oort RJ, Skapura DG, et al. Calmodulin kinase II-mediated sarcoplasmic reticulum Ca²⁺ leak promotes atrial fibrillation in mice. *J Clin Invest*. 2009; 119:1940–1951. [PubMed: 19603549]
9. Chiang DY, Lebesgue N, Beavers DL, Alsina KM, Damen JM, Voigt N, et al. Alterations in the interactome of serine/threonine protein phosphatase type-1 in atrial fibrillation patients. *J Am Coll Cardiol*. 2015; 65:163–73. [PubMed: 25593058]
10. Voigt N, Heijman J, Wang Q, Chiang DY, Li N, Karck M, et al. Cellular and molecular mechanisms of atrial arrhythmogenesis in patients with paroxysmal atrial fibrillation. *Circulation*. 2013; 129:145–156. [PubMed: 24249718]
11. Greiser M, Neuberger HR, Harks E, El-Armouche A, Boknik P, de Haan S, 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*. 2009; 46:385–394. [PubMed: 19100271]
12. Yeh YH, Wakili R, Qi XY, Chartier D, Boknik P, Kaab S, et al. Calcium-handling abnormalities underlying atrial arrhythmogenesis and contractile dysfunction in dogs with congestive heart failure. *Circ Arrhythm Electrophysiol*. 2008; 1:93–102. [PubMed: 19808399]
13. Wakili R, Yeh YH, Yan Qi X, Greiser M, Chartier D, Nishida K, et al. Multiple potential molecular contributors to atrial hypocontractility caused by atrial tachycardia remodeling in dogs. *Circ Arrhythm Electrophysiol*. 2010; 3:530–541. [PubMed: 20660541]
14. Greiser M, Kerfant BG, Williams GS, Voigt N, Harks E, Dibb KM, et al. Tachycardia-induced silencing of subcellular Ca²⁺ signaling in atrial myocytes. *J Clin Invest*. 2014; 124:4759–4772. [PubMed: 25329692]

15. Li N, Chiang DY, Wang S, Wang Q, Sun L, Voigt N, et al. Ryanodine receptor-mediated calcium leak drives progressive development of an atrial fibrillation substrate in a transgenic mouse model. *Circulation*. 2014; 129:1276–1285. [PubMed: 24398018]
16. Marx SO, Reiken S, Hisamatsu Y, Gaburjakova M, Gaburjakova J, Yang YM, et al. Phosphorylation-dependent regulation of ryanodine receptors: a novel role for leucine/isoleucine zippers. *J Cell Biol*. 2001; 153:699–708. [PubMed: 11352932]
17. Chiang DY, Li N, Wang Q, Alsina KM, Quick AP, Reynolds JO, et al. Impaired local regulation of ryanodine receptor type 2 by protein phosphatase 1 promotes atrial fibrillation. *Cardiovasc Res*. 2014; 103:178–187. [PubMed: 24812280]
18. Neumann J, Eschenhagen T, Jones LR, Linck B, Schmitz W, Scholz H, et al. Increased expression of cardiac phosphatases in patients with end-stage heart failure. *J Mol Cell Cardiol*. 1997; 29:265–272. [PubMed: 9040041]
19. Huang B, Wang S, Qin D, Boutjdir M, El-Sherif N. 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*. 1999; 85:848–855. [PubMed: 10532953]
20. Schwoerer AP, Neuber C, Schmechel A, Melnychenko I, Mearini G, Boknik P, et al. Mechanical unloading of the rat heart involves marked changes in the protein kinase-phosphatase balance. *J Mol Cell Cardiol*. 2008; 45:846–852. [PubMed: 18848565]
21. El-Armouche A, Pamminger T, Ditz D, Zolk O, Eschenhagen T. Decreased protein and phosphorylation level of the protein phosphatase inhibitor-1 in failing human hearts. *Cardiovasc Res*. 2004; 61:87–93. [PubMed: 14732205]
22. Gupta RC, Mishra S, Rastogi S, Imai M, Habib O, Sabbah HN. 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*. 2003; 285:H2373–H2381. [PubMed: 14613911]
23. Respress JL, van Oort RJ, Li N, Rolim N, Dixit SS, deAlmeida A, et al. Role of RyR2 phosphorylation at S2814 during heart failure progression. *Circ Res*. 2012; 110:1474–1483. [PubMed: 22511749]
24. Carr AN, Schmidt AG, Suzuki Y, del Monte F, Sato Y, Lanner C, et al. Type 1 phosphatase, a negative regulator of cardiac function. *Mol Cell Biol*. 2002; 22:4124–4135. [PubMed: 12024026]
25. Pathak A, del Monte F, Zhao W, Schultz JE, Lorenz JN, Bodi I, et al. Enhancement of cardiac function and suppression of heart failure progression by inhibition of protein phosphatase 1. *Circ Res*. 2005; 96:756–766. [PubMed: 15746443]
26. El-Armouche A, Wittkopper K, Degenhardt F, Weinberger F, Didie M, Melnychenko I, et al. Phosphatase inhibitor-1-deficient mice are protected from catecholamine-induced arrhythmias and myocardial hypertrophy. *Cardiovasc Res*. 2008; 80:396–406. [PubMed: 18689792]
27. Wittkopper K, Fabritz L, Neef S, Ort KR, Grefe C, Unsold B, 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*. 2010; 120:617–626. [PubMed: 20071777]
28. Kirchhefer U, Baba HA, Boknik P, Breeden KM, Mavila N, Bruchert N, et al. Enhanced cardiac function in mice overexpressing protein phosphatase Inhibitor-2. *Cardiovasc Res*. 2005; 68:98–108. [PubMed: 15975567]
29. Bruchert N, Mavila N, Boknik P, Baba HA, Fabritz L, Gergs U, et al. Inhibitor-2 prevents protein phosphatase 1-induced cardiac hypertrophy and mortality. *Am J Physiol Heart Circ Physiol*. 2008; 295:H1539–H1546. [PubMed: 18689497]
30. Grote-Wessels S, Baba HA, Boknik P, El-Armouche A, Fabritz L, Gillmann HJ, et al. Inhibition of protein phosphatase 1 by inhibitor-2 exacerbates progression of cardiac failure in a model with pressure overload. *Cardiovasc Res*. 2008; 79:464–471. [PubMed: 18453636]
31. Nicolaou P, Rodriguez P, Ren X, Zhou X, Qian J, Sadayappan S, et al. Inducible expression of active protein phosphatase-1 inhibitor-1 enhances basal cardiac function and protects against ischemia/reperfusion injury. *Circ Res*. 2009; 104:1012–1020. [PubMed: 19299645]
32. Ladilov Y, Maxeiner H, Wolf C, Schafer C, Meuter K, Piper HM. Role of protein phosphatases in hypoxic preconditioning. *Am J Physiol Heart Circ Physiol*. 2002; 283:H1092–H1098. [PubMed: 12181139]

33. Egloff MP, Johnson DF, Moorhead G, Cohen PT, Cohen P, Barford D. Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. *EMBO J*. 1997; 16:1876–1887. [PubMed: 9155014]
34. Wakula P, Beullens M, Ceulemans H, Stalmans W, Bollen M. Degeneracy and function of the ubiquitous RVXF motif that mediates binding to protein phosphatase-1. *J Biol Chem*. 2003; 278:18817–18823. [PubMed: 12657641]
35. Ceulemans H, Bollen M. A tighter RVxF motif makes a finer Sift. *Chem Biol*. 2006; 13:6–8. [PubMed: 16426965]
36. Meiselbach H, Sticht H, Enz R. Structural analysis of the protein phosphatase 1 docking motif: molecular description of binding specificities identifies interacting proteins. *Chem Biol*. 2006; 13:49–59. [PubMed: 16426971]
37. Hendrickx A, Beullens M, Ceulemans H, Den Abt T, Van Eynde A, Nicolaescu E, et al. Docking motif-guided mapping of the interactome of protein phosphatase-1. *Chem Biol*. 2009; 16:365–371. [PubMed: 19389623]
38. Silva JV, Freitas MJ, Felgueiras J, Fardilha M. The power of the yeast two-hybrid system in the identification of novel drug targets: building and modulating PPP1 interactomes. *Expert Rev Proteomics*. 2015; 12:147–158. [PubMed: 25795147]
39. Bennett D, Lyulcheva E, Alphey L, Hawcroft G. Towards a comprehensive analysis of the protein phosphatase 1 interactome in *Drosophila*. *J Mol Biol*. 2006; 364:196–212. [PubMed: 17007873]
40. Esteves SL, Domingues SC, da Cruz e Silva OA, Fardilha M, da Cruz e Silva EF. Protein phosphatase 1alpha interacting proteins in the human brain. *OMICS*. 2012; 16:3–17. [PubMed: 22321011]
41. Esteves SL, Korrodi-Gregorio L, Cotrim CZ, van Kleeff PJ, Domingues SC, da Cruz e Silva OA, et al. Protein phosphatase 1gamma isoforms linked interactions in the brain. *J Mol Neurosci*. 2013; 50:179–197. [PubMed: 23080069]
42. Fardilha M, Esteves SL, Korrodi-Gregorio L, Vintem AP, Domingues SC, Rebelo S, et al. Identification of the human testis protein phosphatase 1 interactome. *Biochem Pharmacol*. 2011; 82:1403–1415. [PubMed: 21382349]
43. Hrabchak C, Varmuza S. 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*. 2004; 279:37079–37086. [PubMed: 15226296]
44. Hrabchak C, Henderson H, Varmuza S. 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*. 2007; 46:4635–4644. [PubMed: 17381077]
45. Kao SC, Chen CY, Wang SL, Yang JJ, Hung WC, Chen YC, et al. Identification of phostensin, a PP1 F-actin cytoskeleton targeting subunit. *Biochem Biophys Res Commun*. 2007; 356:594–598. [PubMed: 17374523]
46. Chen CY, Lai NS, Yang JJ, Huang HL, Hung WC, Li C, et al. FLJ23654 encodes a heart protein phosphatase 1-binding protein (Hepp1). *Biochem Biophys Res Commun*. 2010; 391:698–702. [PubMed: 19945436]
47. Walsh EP, Lamont DJ, Beattie KA, Stark MJ. Novel interactions of *Saccharomyces cerevisiae* type 1 protein phosphatase identified by single-step affinity purification and mass spectrometry. *Biochemistry*. 2002; 41:2409–2420. [PubMed: 11841235]
48. Trinkle-Mulcahy L, Andersen J, Lam YW, Moorhead G, Mann M, Lamond AI. Repo-Man recruits PP1 gamma to chromatin and is essential for cell viability. *J Cell Biol*. 2006; 172:679–692. [PubMed: 16492807]
49. Moorhead GB, Trinkle-Mulcahy L, Nimick M, De Wever V, Campbell DG, Gourlay R, et al. Displacement affinity chromatography of protein phosphatase one (PP1) complexes. *BMC Biochem*. 2008; 9:28. [PubMed: 19000314]
50. Templeton GW, Nimick M, Morrice N, Campbell D, Goudreault M, Gingras AC, et al. Identification and characterization of AtI-2, an Arabidopsis homologue of an ancient protein phosphatase 1 (PP1) regulatory subunit. *Biochem J*. 2011; 435:73–83. [PubMed: 21222654]

51. MacLeod G, Varmuza S. Tandem affinity purification in transgenic mouse embryonic stem cells identifies DDOST as a novel PPP1CC2 interacting protein. *Biochemistry*. 2012; 51:9678–9688. [PubMed: 23140390]
52. Cid C, Garcia-Bonilla L, Camafeita E, Burda J, Salinas M, Alcazar A. Proteomic characterization of protein phosphatase 1 complexes in ischemia-reperfusion and ischemic tolerance. *Proteomics*. 2007; 7:3207–3218. [PubMed: 17683050]
53. Hollin T, De Witte C, Lenne A, Pierrot C, Khalife J. Analysis of the interactome of the Ser/Thr Protein Phosphatase type 1 in *Plasmodium falciparum*. *BMC Genomics*. 2016; 17:246. [PubMed: 26988354]
54. Tsaytler P, Harding HP, Ron D, Bertolotti A. Selective inhibition of a regulatory subunit of protein phosphatase 1 restores proteostasis. *Science*. 2011; 332:91–94. [PubMed: 21385720]
55. Aoyama H, Ikeda Y, Miyazaki Y, Yoshimura K, Nishino S, Yamamoto T, et al. Isoform-specific roles of protein phosphatase 1 catalytic subunits in sarcoplasmic reticulum-mediated Ca(2+) cycling. *Cardiovasc Res*. 2011; 89:79–88. [PubMed: 20675715]
56. Liu R, Correll RN, Davis J, Vagnozzi RJ, York AJ, Sargent MA, et al. Cardiac-specific deletion of protein phosphatase 1 β promotes increased myofilament protein phosphorylation and contractile alterations. *J Mol Cell Cardiol*. 2015; 87:204–213. [PubMed: 26334248]

Highlights

- PP1 plays an important role in cardiac physiology and pathophysiology.
- PP1 is regulated by a large number of regulatory subunits and interactors.
- Most studies to date only focused on catalytic subunits of PP1.
- Detailed mapping of the PP1 interactome may lead to novel therapeutics.
- Affinity-purification MS holds great promise for mapping the PP1 interactome.

Figure 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.

Table 1

Known cardiac PP1c interactors as depicted in Figure 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

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 <i>et al.</i> [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				

Unique (83)	PPP1CA (62)	PPP1CB (15)	PPP1CC (44)	Heroes <i>et al.</i> [3] (52)
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				

Unique (83)	PPP1CA (62)	PPP1CB (15)	PPP1CC (44)	Heroes <i>et al.</i> [3] (52)
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.