Chapter 6

SUMMARY AND DISCUSSION

THE LIPID SECOND MESSENGER PHOSPHATIDYLINOSITOL-5-PHOSPHATE ACTIVATES SIRT1 DEACETYLASE ACTIVITY

Protein lysine acetylation affords a rapid, selective and reversible method for controlling cellular processes such as transcription and gene expression (Scott, 2012). The acetylation signal is terminated primarily by deacetylation realizing a rapid and reversible mechanism for linking protein function to changes in cellular environment (Choudhary *et al.*, 2009; Ellis *et al.*, 2008). However, the differential signaling input that components of lysine (de)acetylation pathways are subjected to and their responses are not completely understood.

In **chapter 2** we identify a novel link between phosphoinositide metabolism and the direct control of acetylation. We show a direct interaction between SIRT1 deacetylase and phosphoinositides, particularly PI(5)P, through a previously unrecognized phosphoinositide binding motif that regulates SIRT1 deacetylation activity *in vitro* and *in vivo*. Increased PI(5)P levels stimulates SIRT1 activity and thereby regulates p53 deacetylation, transcription and the induction of apoptosis. Our results show that phosphoinositides, important signaling inputs in aging and metabolism, are endogenous and direct regulators of SIRT1 deacetylase, thereby linking lipid metabolism with regulation of protein acetylation status in the cell.

Our study shows that SIRT1 controls deacetylation of lys-382 on p53 and inhibits apoptosis in a PI(5)P dependent manner. However, a previous study shows that ING2 stimulates acetylation of p53 on Lys-382 and induces apoptosis, which was also dependent on PI(5)P binding (Gozani *et al.*, 2003). It appears that PI(5)P can both influence acetylation (binds to PHD domain of ING2) and deacetylation (binds to KRKKRK region of SIRT1) of p53. Future efforts should explore the spatial and temporal regulation of PI(5)P through investigation of its generation and conversion in more depth.

How phosphoinositides exactly regulate SIRT1 activity is not yet known. The whole crystal structure SIRT1 has not yet been determined; therefore, its overall structural features remain unknown (Sanders, 2010). However, recent studies have suggested that SIRT1 harbors next to its central conserved catalytic domain, allosteric regulatory domains(Autiero *et al.*, 2009; Pan *et al.*, 2012; Revollo *et al.*, 2013; Zhao *et al.*, 2008). Indeed, of the seven human sirtuin proteins, the SIRT1 protein contains the most extended N- and C-terminal segments that flank a catalytic core domain which have been proposed to play specific regulatory roles. Based on our data one could also speculate that phosphoinositides bind to SIRT1 via the KRKKRK region which resides in the N terminus in close proximity to the catalytic domain, thereby inducing a conformational change that affects SIRT1 deacetylase activity or substrate accessibility. In an effort to examine the intramolecular interaction of N and C termini of SIRT1 Fluorescence-lifetime imaging microscopy (FLIM) measurements were performed (Becker, 2012). We hypothesized that the distances between both termini of SIRT1 would be amenable to detecting FRET and could be used to generate a conformational biosensor to analyze the conformation of SIRT1 in live cells. We fused a donor GFP and an acceptor RFP fluorophore to the amino and carboxyl termini of SIRT1, respectively, and tested this construct under various PI(5)P conditions in live cells using FLIM. One would expect that a change in the intramolecular conformation resulting in a "closed conformation" would yield an increase in FRET efficiency, correlating with a decrease in the

donor lifetime. Alternatively, when SIRT1 in a more "open" conformation, the fluorophores are no longer in close spatial proximity and thus predicted to yield in a reduction in FRET efficiency. Although preliminary results were promising, quantifying a significant difference in donor lifetime between different conditions and experiments proved challenging. To what extent phosphoinositide (PI(5)P) binding may influence SIRT1 protein conformation remains to be examined in further details, perhaps by using an improved mTurquoise–Venus FRET pair sensor which is much more suitable for FLIM determinations. Alternatively, phosphoinostide binding may also affect SIRT1's abililty to bind to its specific protein binding partners like AROS and DBC-1, which in turn could modulate SIRT1 catalytic efficiency (Kim *et al.*, 2007; Kim *et al.*, 2008; Zhao *et al.*, 2008).

How the binding of PI(5)P exactly can allosterically activate SIRT1 remains to be determined. Co-crystallization studies of SIRT1 (or parts if it) bound to phosphoinositides (PI(5)P) will be required to answer this question.

NUCLEAR PHOSPHOLIPIDS AS EPIGENETIC REGULATORS

There is precedent for phospholipids to regulate protein deacetylation and other players in the epigenetic landscape. This provokes the question whether other phospholipids could fulfill a similar role in interacting with chromatin components and regulating gene transcription. **Chapter 3** summarizes the evidence for a role of phospholipids in epigenetic gene regulation. Initially identified for their cytosolic roles, many lipid kinases and phosphatates are also found in the nucleus where they regulate cell cycle progression, gene expression and chromatin remodeling. These enzymes generate distinct nuclear phospholipids such as sphingolipids, inositol polyphosphates and phosphoinositides that associate with several nuclear components (Irvine, 2003). A key component in nuclear lipid signaling involves $PI(4,5)P_2$ and its metabolite IP_3. The latter, through the action of inositol phosphate kinases (IPK's) in the nucleus, is used as a precursor for the generation of higher inositol polyphosphate species, with established roles in gene transcription (Odom, 2000; Zhao, 1998). Additionally, recent evidence has implicated distinct role for enzymes that are involved in histone and protein acetylation (Hait *et al.*, 2009; Watson, 2012). Both members of acetyltransferases and deacetylases can bind and contribute to the activation or inhibition these enzymes, such as IP4 and S1P but also phosphatidylinositol monophosphates like PI(5)P (as decribed in chapter 2). There are also many other lipid kinases and phosphotases that have been found in the nucleus, however their nuclear function have not been defined to the same extent as their IP/PI counterparts, such as kinases that phosphorylate DAG to generate PA (DGKs) (Topham *et al.*, 1999).

An intriguing aspect of nuclear lipid metabolism is the evidence that some of these phospholipids exist not only within the inner nuclear membrane, but in the compartmentalized pools in the nucleus as well. Especially for phosphoinositides these pools have been detected both by biochemical and imaging approaches (Boronenkov, 1998; Divecha, 1993; Mellman, 2008; Osborne, 2001). Current data suggest two compartments for the nuclear phosphoinositide cycle: one associated with the nuclear envelope and another in a subnuclear compartment separate from known membrane structures. PI and PI-generating enzymes that are present at nuclear speckles are separate from known membrane structures (Barlow *et al.*, 2010;

Bunce *et al.*, 2006; Bunce, 2006; Heck *et al.*, 2007). Therefore, in subnuclear compartments, phosphoinositides are hypothesized to be associated with carrier or effector proteins. Such proteins could specifically present phosphoinositides to other effectors. The motifs of PI-binding proteins contain charged residues that are thought to interact with the head group of inositol lipids (Lewis *et al.*, 2011). This would leave the hydrophobic tails free; however, it seems unlikely that the acyl chains would be exposed. How then are these phosphoinositides present in the endonuclear compartment? It is possible that the phosphoinositides and other lipids form a mixed micelle structure, thus protecting the free acyl chains. Another possibility is that the phosphoinositides are associated with carrier proteins in the nucleus that contain phosphoinositide acyl chain-binding pockets. Such proteins would integrate the hydrophobic acyl chain in the binding cleft exposing only the charged inositol head group. However, the exact molecular mechanisms responsible for their endonuclear distrution remain largely elusive.

In conclusion, mounting evidence on intranuclear phospholipid metabolism and signaling has shown that signaling lipid species, particularly the phosphoinositides, can associate with different nuclear proteins and thereby regulate gene transcription (Lewis *et al.*, 2011). This suggests that phospholipids through association with these proteins can translate their nuclear concentration into transcriptional responses. Future efforts should focus on identifying the molecular and cellular consequences of nuclear phospholipid signaling pathways.

PHOSPHATIDYLINOSITOL-5-PHOSPHATE 4 KINASE BETA (PIP4Kβ) BINDS TO AND IS DEACETYLATED BY SIRT1

As described in this thesis, phosphoinositides can bind to SIRT1 and thereby regulate SIRT1 deacetylase activity towards well characterized substrate p53. *In vitro* SIRT1 deacetylase experiments demonstrated that phosphatidylinositol monophosphates, in particular PI(5)P, could stimulate SIRT1 deacetylase activity. This confirmed the binding of SIRT1 to phosphatidylinositol monophosphates in lipid overlay assays. (Jones *et al.*, 2006) previously showed that nuclear PI(5)P is increased after treatment of cells with various stressors such as UV irradiation and etoposide, and that PI(5)P plays a role in regulating the acetylation of p53. Since PI(3)P and PI(4)P are known to be involved in the regulation of intracellular vesicles and Golgi function, respectively, we hypothesized that among the phosphatidylinositol monophosphates PI(5)P (given its nuclear accumulation) was more likely to act as lipid activator of SIRT1 deaceylase (Pendaries, 2005). However, it cannot be completely ruled out that PI(4)P and PI(3)P might also regulate SIRT1 function. Experiments where cellular PI(4)P and PI(3)P levels are manipulated by overexpression of, for example, PI-3-Kinases or PI-4-Kinases should be performed to determine their effect on SIRT1 activity. To establish that PI(5)P indeed can regulate SIRT1 function, the levels of PI(5)P were manipulated by overexpression of two isoforms of the PIP4K family that are able to phosphorylate and remove PI(5)P in the used cellines. However, *in vitro* PIPK4 enzymes can phosphorylate both PI(5)P and PI(3)P to produce PI(4,5)P2 and PI(3,5)P2, respectively (Morris *et al.*, 2000; Rameh *et al.*, 1997). Although many data suggest that PIP4K function trough controlling cellular PI(5)P, it can not be ruled out that PI(3)P can also (in part) stimulate SIRT1 deacetylase activity and therefore p53 acetylation status.

We observed that the manipulation of PIP4K isoforms expression modulated SIRT1 activity, which prompted of the question whether these kinases themselves may have a role in SIRT1 function. **Chapter 4** describes PIP4K isoform β as a novel interacting partner of SIRT1. The interaction of SIRT1 with PIP4Kβ suggested that PIP4Kβ could be modified posttranslationally by (de)acetylation. Mass spectroscopic analysis identified eight distinct lysine residues, one of which lysine 239 appeared to be sensitive to the SIRT1 inhibitor nicotinamide. Further analysis revealed that lysine 239 was acetylated in cells that ectopically expressed wildtype but not K239R mutant as well endogenous PIP4Kβ lysine239 acetylation.

It is noteworthy that lysine 239 acetylated PIP4Kβ was found in untreated cells using the generated acetyl antibody, whereas in the mass spec assays lysine 239 acetylation levels accumulated only after using the SIRT1 inhibitor. It is possible that our K239 acetyl-specific antibody is able to detect the acetylated fraction of PIP4Kβ that was not detectable by mass spectrometry. Alternatively, the difference could be explained by variation in cell lines used for both experiments. Perhaps, differential cues change the acetylation levels of PIP4Kβ via histone acetyl transferases (HAT) in the different cell lines. It will also be important to demonstrate that the manipulation of cellular PI(5)P does not modulate p53 acetylation trough a decrease in HAT activity.

Finally, deacetylation assays demonstrated that PIP4Kβ is deacetylated on lysine 239 by SIRT1 in a nicotinamide-sensitive manner. However, whether SIRT1 can deacetylate PIP4Kβ *in vivo* remains to be determined. Studying PIP4Kβ lysine 239 acetylation in cell lines where SIRT1 is inhibited pharmacologically or trough knockdown should be included in future studies to address this issue.

An important question is how acetylation contributes to PIP4K functions. For instance: does acetylation, particularly lysine 239, effect PIP4K lipidkinase activity and localization or perhaps association with interacting proteins or both? Since preliminary data suggested that 239 acetylation does not regulate PIP4Kβ localization, mostly likely acetylation of PIP4Kβ will modulate the binding to its interacting partners, perhaps PIP4Kβ interaction with PIP4Kα. Based on the recent insight into PIP4Kα/PIP4Kβ heterodimerization, it is tempting to hypothesize that acetylation might regulate this heterodimer formation (Bultsma *et al.*, 2010; Wang *et al.*, 2010). If this is indeed the case, most likely PIP4Kβ lipidkinase activity will be altered since the majority of PIP4K activity associated with PIP4Kβ comes from its interaction with PIP4Kα. Making use of the PIP4Kβ acetylation mutants described in this study, future efforts should focus on their interaction with PIP4Kα.

A second outstanding question is how acetylation of PIP4Kβ will alter cellular PI(5)P levels and what consequence this may have for PI(5)P-dependent SIRT1 activation. An attractive hypothesis proposes a feedback loop where PI(5)P-dependent stimulation of SIRT1 deacetylase activity may be regulated by acetylation of PIP4Kβ as illustrated in chapter 4 figure 6. At this moment it remains unclear if such a regulatory mechanism is indeed in place and whether these would positivity or negatively modulate SIRT1 deacetylase activity. Future studies should preferably focus on determining to what extent (de)acetylation of PIP4Kβ will first changes cellular PI(5)P levels for example by lipid kinase assays and secondly effect SIRT1 deacetylase activity by assessing the p53 acetylation status.

ROLE OF PHOSPHATIDYLINOSITOL 5-PHOSPHATE 4-KINASE ALPHA IN ZEBRAFISH DEVELOPMENT

Vertebrate genomes contain three genes (α, β and γ) that encode PIP4K activity. PIP4kinases exist in all fully sequenced multicellular organisms, including *Caenorhabditis elegans* and the *Drosophila melanogaster*, but are not present in unicellular organisms (Lecompte *et al.*, 2008). Several biochemical studies have highlighted many important cellular functions in different intracellular compartment for the distinct PIP4K isoforms (Clarke *et al.*, 2012; Clarke, 2010). Yet, the physiological significance of which this class of lipid kinase remain largely elusive. The only hint about the *in vivo* function of PIP4K comes from a study in mice. Knockout mice of PIP4Kβ are reported to be growth retarded and hypersensitive to insulin (Lamia *et al.*, 2004). Knockout or knockdown organisms of PIP4Kα or PIP4Kγ have not been reported to date and no developmental functions has been attributed to these genes. In **chapter 5** we identified the *Danio rerio* zebrafish orthologue (zPIP4Kα) of the high-activity PIP4Kα human isoform and analyzed its role in embryonic development (Elouarrat, 2013). Phenotype analysis of zebrafish depleted of zPIP4Kα by specific morpholino's (MOs) reveal anomalies involving primarily the development of the eye, heart and midbody axis. zPIP4Kα catalytic activity was found to be conserved between zebrafish and human and the catalytic role of zPIP4Kα in these phenotypic alterations were confirmed in rescue experiments. Although we did not perform the rescue experiments with a zPIP4Kα resilient to morpholino knockdown we anticipate no difference in rescuing ability between zebrafish and human counterpart as they display high degree of sequence similarity. This is indeed illustrated by the ability of human PIP4Kα to complement reduced zPIP4Kα function in the 'rescued' morphants, supporting the notion that besides the sequence similarities human and zebrafish PIP4Kα also share physiological functions. Importantly, mRNA transcribing PIP4Kα catalytic dead protein was unable to rescue the zPIP4Kα knockdown phenotype, suggesting that the defects in zebrafish development upon knockdown of zPIP4Kα are attributed to the lack of catalysis of PI(5)P to PI(4,5)P2. This observation was further confirmed by the reduced PIP4K kinase activity measured *in vivo* in PIP4Kα ATG morphants. Whereas it is possible that the phenotypes related to loss of zPIP4Kα function may be a consequence of elevated PI(5)P levels, changes in a quantitatively minor pool of PI(4,5)P2 generated by PIP4Kα cannot be excluded. Mass levels measurements of PI(5)P and PI(4,5)P2 should be measured to answer this question and help understand the biochemical consequence of zPIP4Kα. Taken together, these experiments establish the specificity of the phenotype, its dependence on catalytic activity, and conservation of PIP4Kα function between zebrafish and humans.

It is important to note that the ability of other PIP4K isoforms to compensate for the loss of zPIP4Kα could interfere with the severity of the knockdown phenotype in this study. We have searched for PIP4Kβ and γ orthologs and identified for both a predicted protein. However, we were unable to clone and confirm expression of the predicted zPIP4Kβ based on the annotated sequence. zPIP4Kγ expression was confirmed and when targeted by morpholino knockdown the majority of zPIP4Kγ morphants displayed normal development with a low percentage (5-15%) displaying a mild "bent tail" phenotype. Moreover, the combined knockdown of

zPIP4Kγ and zPIP4Kα did not result in more severe phenotype and were in large identical to single zPIP4Kα knockdown (data not shown). This suggests that zPIP4Kγ does not have as a important function as zPIP4Kα in the development of zebrafish midbody. Previous studies have reported that PIP4Kγ lacks catalytic activity, which could explain the lack of detectable gross morphological defects (Clarke *et al.*, 2008). To address the physiological roles of PIP4Kα and other PIP4K isoforms, a more comprehensive approach is required in zebrafish PIP4K models. Such studies should focus on the development of skeletal muscle and other tissues in genetic PIP4K knockout zebrafish strains. These efforts should complement the physiological role of PIP4K in vertebrate embryonic development described in this study.

CONCLUDING REMARKS AND PERSPECTIVES

Overall, the studies presented in this thesis describe a novel role for nuclear phosphoinositide signaling, particular for the PI(5)P/ PIP4K axis, in the regulation of protein deacetylation, through SIRT1 deacetylase. SIRT1 is implicated in numerous age-related diseases and, as such, have become pharmaceutical target for small molecule modulation (Donadini, 2013). Although much attention has focused on the identification of the cellular targets controlled by SIRT1, the mechanisms that regulate SIRT1 activity by biological stimuli have just recently begun to emerge. Our study identified the lipid second messenger PI(5)P as an allosteric activator of the deacetylase SIRT1 and defines an important general role for deacetylases as signal transducing enzymes. Understanding of SIRT1 regulation will help to resolve controversies derived from the opposed physiological effects that were demonstrated for SIRT1, for example, in oncogenic and endocrine responses (Bosch-Presegue *et al.*, 2011; Canto *et al.*, 2012; Revollo *et al.*, 2013; Sebastian *et al.*, 2012). Also various SIRT1-activating compounds (STACs) like resveratrol (a compound found in red wine) have been studied extensively; yet the molecular basis by which such compounds affect SIRT1 have remained somewhat controversial. In part because recent evidence suggested that STACs may not even bind directly to SIRT1 but rather to fluorophores attached for assay purposes (Dai *et al.*, 2010; Pacholec *et al.*, 2010). Our defined small molecule allosteric site within SIRT1 constitutes an advantage in drug discovery for the development of both activators and inhibitors of SIRT1 enzymatic activity. Hence, the allosteric regulation of SIRT1 by PI(5)P is an interesting mechanism to explore in future studies.

PIP4Kβ was identified as target for deacetylation by SIRT1 in our studies, which suggest a regulatory role for PIP4Kβ in the PI(5)P-dependent SIRT1 stimulation. Therefore, the development of pharmacological inhibitors of PIP4K might also be useful as modulators of SIRT1 enzymatic activity. Unfortunately, potent PIP4K inhibitors are still not available. However, Davis *et al.* have developed a new high-throughput screening methodology. This luciferase-coupled bioluminescence assay should enable large chemical library screening to help identify selective inhibitors of PIP4K enzymes (Davis *et al.*, 2013). Potentially these inhibitors of PIP4K also open new avenues for implicating PI(5)P in the many physiological processes and diseases in which SIRT1 is involved. In the end, these compounds would also serve as valuable research tools to investigate the still not fully understood role of PIP4K (activity) in the physiology and development of different organisms.

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