“LINKING LIPIDS TO ACETYLATION”

NOVEL ROLES OF PI(5)P AND PIP4K IN SIRT1 REGULATION AND DEVELOPMENT

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Chapter 1

GENERAL INTRODUCTION

1

PREFACE

Every cell is able to communicate by sending, receiving or responding to signals in its environment. In order to trigger a physiological response, these signals must be transmitted across the cell membrane. Cells have developed a complex array of mechanisms to sense changes in the extracellular environment and to transduce these changes into intracellular signals (signal transduction) that can generate a cellular response. Signal transduction can be initiated by a ligand binding to a specific receptor at the plasma membrane. These ligands (primary messengers) bind to a diverse family of receptors, such G protein-coupled receptors, tyrosine kinase receptor or serine-threonine kinase receptors. Upon ligand binding, receptors activate diverse signaling pathways giving rise to change in abundance of intracellular signaling molecules, called second messengers. One important group of second messengers is the phosphoinositide family.

PHOSPHOINOSITIDES

Phosphoinositides are distinct class of phospholipids, consisting of an inositol head group that is linked, via a phosphate diester bond, to the 1, 2-diacylglycerol (DAG) moiety. The hydrophobic DAG moiety remains buried in the membrane (can maintain the phosphoinositide as part of the membrane), whereas the inositol head group is soluble and can interact with components (proteins) in the cytosol. Phosphoinositides can be hydrolysed by phospholipase C family enzymes to generate two important second messengers; DAG, which can stimulates protein kinase C (PKC) activity, and inositol-(1,4,5)trisphosphate (IP3), which triggers calcium release from intracellular stores (Berridge, 1987; Hirasawa, 1985). Phosphatidylinositol (PI) can also be sequentially phosphorylated at the 3', 4' and 5' positions of the inositol ring to generate seven different (biologically active) phosphoinositides, namely PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2 and PI(3,4,5)P_3 (Lietha, 2001). The (de)phosphorylation of phosphoinositides and its derivatives is catalyzed by distinct lipid kinases and phosphatases, each specific for a given hydroxyl or phosphate group at the inositol ring (Sasaki *et al.*, 2009). In this way, phosphoinositide-specific kinases and phosphatases can generate various bioactive phosphoinositides in specific subcellular compartments (Figure 1). This subcellular distribution is regulated by specific protein-protein interactions unique to each kinase or phosphatase (Kutateladze, 2010). This allows the generation of a spatially regulated phosphoinositide signaling system.

Phosphoinositides can regulate numerous cellular processes, including ion channel function, protein localization and enzymatic activity, which in turn can affect many downstream processes such as vesicular transport, cell polarity and migration, gene transcription, cytoskeletal dynamics and cell proliferation and survival (Di Paolo *et al.*, 2006; Irvine, 2005; McCrea *et al.*, 2009). Thus, phosphoinositides function (exert their roles) either as precursors of second messengers (DAG and IP3), or by interacting with target proteins that contain specific phosphoinositide-interacting domains (PIDs). Over the past decades, a number of protein domains have been identified that can bind to phosphoinositides with varying degrees of specificity and affinity (Figure 1). These include the pleckstrin homology domain (PH), Phox domain (PX), epsin N-terminal homology domain (ENTH), FYVE domain and lysine/arginine-rich peptide sequences (Kutateladze, 2010; Lemmon, 2008). The interaction of phosphoinositides with these domains is key to the function of many signaling proteins and networks.

NUCLEAR PHOSPHOINOSITIDES

Most of our knowledge of phosphoinositide signaling is derived from receptor-mediated signaling pathways in the cytosol, yet evidence has emerged on the existence of phosphoinositide pathways in the nucleus. Many studies have shown that nuclear phosphoinositide metabolism and its regulation are independent of their cytoplasmic/plasma membrane counterparts (Irvine, 2003). Nuclear phosphoinositides have been shown to play key roles in a wide range of nuclear events, including cell proliferation and differentiation, DNA repair, mRNA splicing but also chromatin structure and transcription as will be described in **chapter 3** of this thesis.

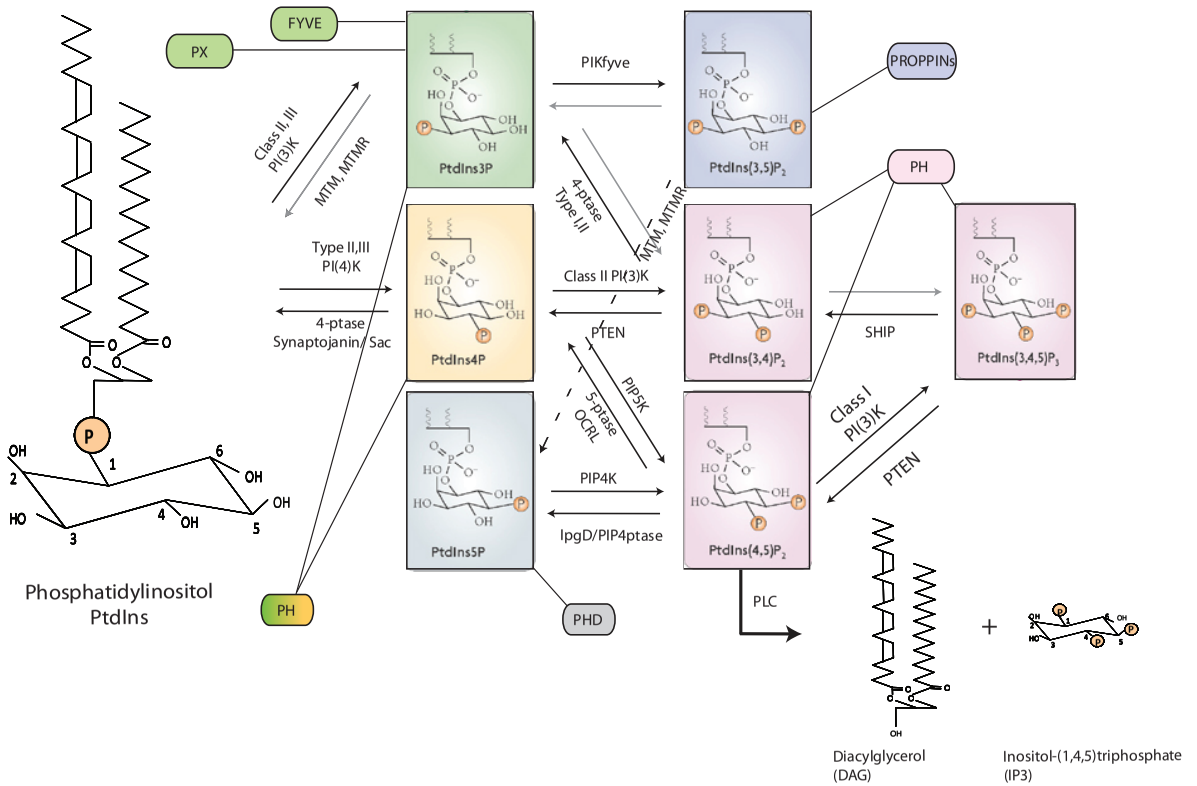

Figure 1. *Pathways of phosphoinositide synthesis and degradation. The phosphatidylinositol (PI) molecule includes inositol 1-phosphate bound via its phosphate group to 1,2-diacylglycerol (DAG) moiety. The structure and interconversion are shown for all seven known phosphoinositides namely PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2 and PI(3,4,5)P_2 in eukaryotes. Grey arrows represent less well-characherized pathways or pathways that have not been established yet. The phospholipid-binding domains that recognize specific phosphoinositides are indicated in different colours. PI(4,5)P_2 and other phosphoinositides can be hydrolysed by phospholipase C (PLC) enzymes to generate inositol 1,4,5-trisphosphate and diacylglycerol.*

PHOSPHATIDYLINOSITOL PHOSPHATE KINASES (PIPKS)

The generation of phosphatidylinositol 4,5-bisphosphate PI(4,5)P_2 by phosphatidylinositol phosphate kinases (PIPK) is central to phosphoinositide signaling not only in the cytosol but also within the nucleus (Bunce, 2006). PI(4,5)P2 can be synthesized by two different classes of PIPKs that utilize different substrates (van den Bout *et al.*, 2009). Phosphatidylinositol-4-phosphate 5 kinase (PIP5K) phosphorylates PI(4)P on the 5 position on the inositol ring, while phosphatidylinositol-5-phosphate 4 kinase (PIP4K) phosphorylates PI(5)P on the 4 position (Loijens *et al.*, 1996; Rameh *et al.*, 1997; Roberts *et al.*, 2005). Although PI(3)P can acts a substrate, it is much less efficiently catalyzed than PI(5)P (Morris *et al.*, 2000). Several isoforms of (consisting of α, β, γ isoforms) PIP5Ks and PIP4Ks have been found to localize in the nucleus. Despite synthesizing the same product, PIP5Ks and PIP4Ks appear functionally nonredundant. For instance, genetic deletion of PIP5K can be rescued by expression of PIP5Ks but not PIP4Ks, suggesting that PIP4Ks do not increase the cellular levels of PI(4,5)P_2 to the same extent as do the PIP5Ks. Because the cellular levels of PI(5)P is present at much lower levels than PI(4)P in the cell, the amount of PI(4,5)P_2 synthesized by PIP4K relative to PIP5K is likely to be much smaller (Clarke *et al.*, 2001; Morris *et al.*, 2000; Rameh *et al.*, 1997; Roberts *et al.*, 2005). Therefore, it is thought that PIP4Ks primarily function to remove the substrate PI(5)P and thereby regulate its potential signaling functions (as will be detailed below).

PHOSPHATIDYLINOSITOL 5-PHOSPHATE 4-KINASE (PIP4K)

The three different PIP4K isoforms, encoded by distinct genes, are found at different cellular locations. The PIP4Kα isoform is predominantly localized in the cytosol, although there is also a significant amount in the nucleus, whereas PIP4Kγ localizes to the Golgi and intracellular vesicles (Boronenkov *et al.*, 1995; Clarke *et al.*, 2008). PIP4Kβ is predominantly nuclear; its 16 amino acid α-helix insertion (not present in the other PIP4K isoforms) is essential for nuclear localization (Ciruela *et al.*, 2000). The relative expression levels of PIP4K isoforms can differ between various tissues. The ratio of PIP4Kβ compaired to PIP4Kα and PIP4Kγ expression is particularly high in skeletal muscle, where there is approximately 10-fold more PIP4Kβ than PIP4Kα and PIP4Kγ (Lamia *et al.*, 2004). Furthermore, in situ hybridation studies and RT-PCR in mice revealed similar expression of PIP4Kα and β in the brain, whereas PIP4Kα is expressed at higher levels than PIP4Kβ and γ in spleen. PIP4Kγ is particularly highly expressed in the kidney compared to PIP4Kα and β (Clarke *et al.*, 2008; Volpicelli-Daley *et al.*, 2010). These studies indicate that the differential expression and subcellular localization of each PIP4K isoform may serve specific functions (depending on cell and tissue context) of each organism (Clarke, 2010). The three isoforms of PIP4Ks also differ greatly in catalytic activity: PIP4Kα has the highest activity, followed by PIP4Kβ which has 2000-fold less activity towards PI(5)P compared to PIP4Kα, whereas PIP4Kγ is generally thought to be inactive (Bultsma *et al.*, 2010; Clarke *et al.*, 2008; Wang *et al.*, 2010). However, recent evidence indicates that distinct isoforms can affect one another's sub-cellular localization (Clarke *et al.*, 2012). For instance, the high activity PIP4Kα isoform can interact with PIP4Kβ possibly by forming a heterodimer. The interaction and targeting of different isoforms suggest a mechanism by which low activity isoforms like

PIP4Kβ can regulate PI(5)P levels in a given cellular compartment (Bultsma *et al.*, 2010). Finally, it should be mentioned that the physiological roles of PIP4Ks remain poorly understood. PIP4Kβ-deleted mice are mildly growth retarded and hypersensitive to insulin (Lamia *et al.*, 2004). However, knockout or knockdown phenotypes of PIP4Kα and PIP4Kγ have not been reported to date. **Chapter 5** examines the role of PIP4Ks in vertebrate embryonic development using zebrafish as a model.

PHOSPHATIDYLINOSITOL 5-PHOSPHATE (PI(5)P) SIGNALING

Function

PI(5)P is the last identified member of the seven known phosphoinositides. Its late discovery is probably due to the fact that PI(5)P is low abundant (1-5%) compared to PI(4)P (Rameh *et al.*, 1997; Sarkes *et al.*, 2010). Therefore, PI(5)P has long been considered to be just an intermediate metabolite in the synthesis of other phosphoinositides. PI(5)P resides in the plasma membrane, intracellular membranes and the nucleus. Interestingly, several physiological and pathological stimuli alter cellular PI(5)P levels. For instance, the levels of PI(5)P are very low in resting cells, but they rise upon thrombin and insulin stimulation in human platelets or by osmotic stress in plant cells (Meijer, 2001; Morris *et al.*, 2000; Ndamukong *et al.*, 2010; Sbrissa *et al.*, 2004). During bacterial infection with Shigella flexneri or Salmonella typhimurium, the levels of PI(5)P are elevated and were shown to facilitate infection (Niebuhr *et al.*, 2002; Terebiznik, 2002). Moreover, a nuclear pool of PI(5)P has been found to increase when cells progress through the cell cycle and in response to oxidative stress and ultraviolet (UV) irradiation (Clarke *et al.*, 2001; Jones *et al.*, 2006; Jones *et al.*, 2012; Keune *et al.*, 2012).

As described above, phosphoinositides exert their function through interaction with specific phosphoinositide-binding domains. Identification of the binding of PI(5)P to ING2 (Inhibitor of Growth Protein 2) through the plant homeobox domain (PHD) motif defined this domain as the first PI(5)P phosphoinositide-binding domain in the nucleus(Gozani *et al.*, 2003). ING2 is a candidate tumor suppressor that induces growth arrest and apoptosis in a p53-dependent manner (Nagashima, 2001; Shi, 2005). Mutations resulting in loss of interaction with PI(5)P affect localization and the activity of ING2, leading to a decrease in ING2-mediated apoptosis and p53 acetylation. Another example of PI(5)P-PHD domain interaction is the relocalization of the histone H3 lysine 4 methylase ATX1 from the nucleus to the cytoplasm in response to dehydration stress (Alvarez-Venegas *et al.*, 2006; Ndamukong *et al.*, 2010b). Furthermore, the activity of the cullin 3 (CUL3) ubiquitination complex appears to be stimulated by nuclear PI(5)P (Bunce, 2008). Together, these studies indicate that nuclear PI(5)P levels play important roles in regulating nuclear protein function, especially in nuclear events such as chromatin remodeling, as discussed in **Chapter 3**.

PI(5)P signaling functions in the cytosol have also been reported (Grainger, 2012). In the cytosol, overexpression of PIP4Kβ resulted in a decrease in Akt activity in response to insulin (Carricaburu, 2003). Conversely, cells expressing IpgD, a bacterial PI(4,5)P_2-4-phosphatase that generates PI(5)P, had higher levels of basal and insulin-stimulated Akt phosphorylation (Pendaries, 2006). Interestingly, deletion of PIP4Kβ in mice also leads to increased insulin-induced Akt activation in muscle (Lamia *et al.*, 2004). Additionally, it has recently been shown

that PI(5)P-dependent Akt activation occurs via translocation of the EGF receptor (Ramel *et al.*, 2011). Next to insulin, H_2O_2-induced PI(5)P levels also regulate the activity of Akt, which in turn is important for cell growth inhibition by H_2O_2(Jones *et al.*, 2012). Furthermore, PI(5)P has been shown to play a role in cell morphology by mediating actin rearrangements downstream of the insulin receptor (Sbrissa *et al.*, 2004). More recently, PI(5)P was shown to promote cell migration through the activity of specific kinases and phosphatases involved in metabolizing PI(3,5)P_2 (Oppelt *et al.*, 2012).

Regulation

How is the abundance of PI(5)P regulated in distinct cellular compartments? Several pathways have the potential to generate PI(5)P, but their relative contributions to PI(5)P regulation *in vivo* are unclear (Figure 2). PI(3)P and PI(4)P phosphoinositides are produced by phosphorylation of PI by their specific kinases; however, no such kinase has been identified for PI(5)P so far (Lecompte *et al.*, 2008). Instead, it appears that PI(5)P production is regulated by specific phosphatases that dephosphorylate PI(4,5)P_2. This pathway was first identified by

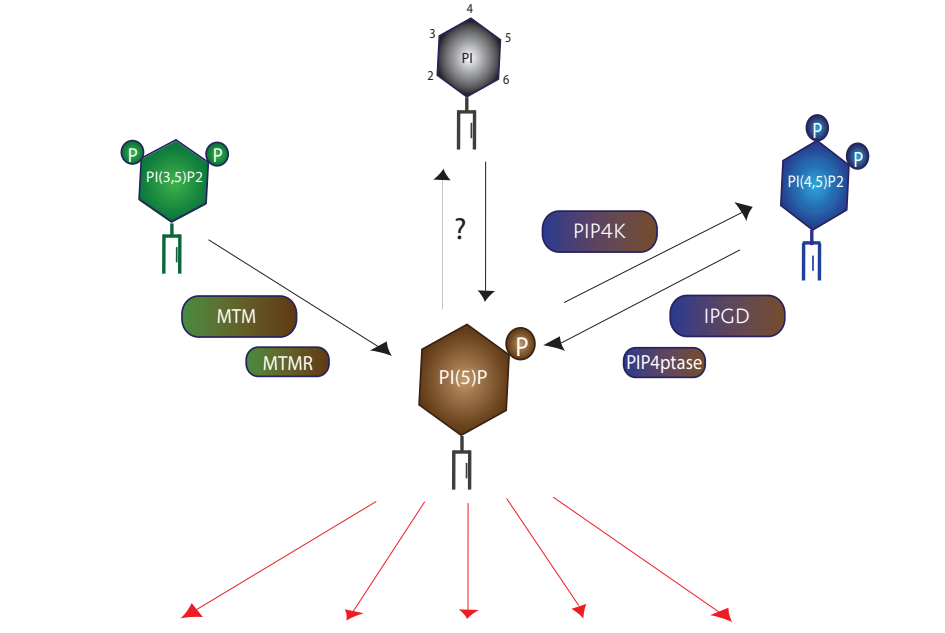

Figure 2. *Regulation of PI(5)P and its distinct roles in nuclear and cytosolic signaling. PI(5)P can be synthesized by MTM/MTMR phosphatases via dephosphorylation of PI(3,5)P_2 and bacterial IpgD and mammalian Type I,II PIP-4-phosphatases that convert PI(4,5)P_2 to PI(4,5)P_2. PIP4K can phosphorylate PI(5)P to PI(4,5)P_2 which is important for the physiological regulation of PI(5)P and downstream signaling. PI(5)P binds to PHD domain present on ING2, ATX1 and other nuclear proteins involved in nuclear signaling. Furthermore PI(5)P levels plays an important role in other cellular functions such as cell migration, insulin responses etc.*

the PI(4,5)P_2 -4-phosphatase IpgD, responsible for the profound increase in PI(5)P observed in S.Flexneri-infected cells (Niebuhr *et al.*, 2002). Subsequently, two human homologous PI(4,5)P_2 -4-phosphatases have been characterized that can translocate to the nucleus upon stress induction (Zou, 2007). The abundance of PI(5)P can also be increased by the stress-activated lipid kinase PIKfyve, whose main function is to produce PI(3,5)P_2 from PI(3)P (Tolias *et al.*, 1998). Overexpression of PIKfyve increases cellular PI(5)P (Sbrissa, 2002). Mice lacking one copy of the gene have reduced PI(5)P levels (Ikonomov, 2011). However, PI(5)P can also be generated by the major product of PIKfyve, PI(3,5)P_2, by myotubularin 3-phosphatases(Schaletzky, 2003). For example, MTM1 overexpression is required for enhanced PI(5)P production in osmotically stressed muscle (Tronchere *et al.*, 2004). However, the ability of myotubularin to dephosphorylate PI(5)P and their operation *in vivo* is not yet established.

Unlike the dephosphorylation to PI(5)P, the PI(5)P removal via PI(5)P phosphorylation is much better established. PIP4Ks plays an important role in the regulation of PI(5)P levels in the nucleus, where stressing cells activates p38 MAP kinase, which phosphorylates PIP4Kβ on Ser^{326} (Jones *et al.*, 2006). This decreases its PIP4K activity leading to an increase in the nuclear abundance of PI(5)P. A possible explanation of how PIP4Kβ, (which has low catalytic activity) can regulate nuclear PI(5)P levels comes from the observation that PIP4Kα and PIP4Kβ can form heterodimers (Bultsma *et al.*, 2010). Thereby PIP4Kβ can target PIP4Kα activity to the nucleus. How phosphorylation by the p38 pathway regulates the PIP4Kα/PIP4Kβ complex and its activity is currently not clear. PIP4Kβ is heavily post-translationally modified: in addition to being phosphorylated, it is also extensively acetylated. Consequently, these modifications might regulate PIP4Kα/PIP4Kβ complex formation. In **Chapter 4**, we describe acetylated residues on PIP4Kβ and we identify it as a target of the SIRT1 deacetylase (see below). In conclusion, the metabolic pathways governing the levels and the signaling roles of PI(5)P are still not well understood, but recent data reveal its function as lipid mediator.

PROTEIN ACETYLATION

Acetylation refers to the addition of an acetyl group on lysine residues present within both histone and non-histone proteins. Acetylation is mediated by histone acetyl transferases (HATs). Since a large number of nonhistone proteins are targeted by HATs, these enzymes are also called K-acetyltransferases (KATs) (Berndsen, 2008). Acetylation of histones directly influences chromatin remodeling, by reducing the positive charge on lysine residues and by decreasing the affinity of histones for negatively charged DNA. This allows a localized "unraveling" of chromatin, making it more accessible for the binding of coactivators and the basal transcription machinery (Jiang, 2009).

The removal of acetyl groups is catalyzed by histone deacetylases (HDACs) and is associated with chromatin condensation and transcriptional repression. HDACs exert their repressive function on transcription either by condensing the chromatin or as components of large multiprotein complexes, by recruiting inhibitory factors to gene promoter regions (Jiang, 2009). Transcriptional regulation exerted by HDACs determines many cellular processes including cell cycle progression, apoptosis, autophagy, response to diverse types of stress, differentiation, and development (Norris *et al.*, 2009). Mammalian HDACs can be classified

into one of four different classes based on their amino acid sequence and structure. Sirtuins, or class III HDACs, have no homology to class I, II, or IV HDACs (TSA-sensitive enzymes and are localized in both the nucleus and the cytoplasm). Their deacetylase activity depends on the cofactor NAD^+, rather than zinc, and they are localized in the nucleus, cytoplasm, or mitochondria and can deacetylate histone and nonhistone proteins (Imai *et al.*, 2000).

SIRTUINS

Sirtuins are highly conserved from bacteria to human and SIR2 (silent mating-type information regulation 2) was originally shown to extend lifespan in budding yeast (Kaeberlein, 1999). In mammals, there are seven sirtuins (SIRT1-7) all containing a conserved NAD-binding and catalytic domain, termed the sirtuin core domain, but they differ in their N and C-terminal domains (Frye, 2000). Sirtuins show significant sequence and functional differences from other classes of HDACs in that they carry out deacetylation via a two-step reaction that consumes NAD^+ and releases, O-acetyl-ADP-ribose (AADPR), the deacetylated substrate and nicotinamide (used as sirtuin inhibitor) (Houtkooper, 2010). Sirtuins, although relatively similar to each other, have divergent biological functions which can be due to distinct cell-type-specific subcellular localisation of each member of the family (Finkel *et al.*, 2009). SIRT1 is located in both the nucleus and the cytoplasm, SIRT2 in the cytoplasm, SIRT3, 4, and 5 are mitochondrial, and SIRT6 and 7 are nuclear (Michan *et al.*, 2007). Apart from intracellular localization, SIRT1, 3, and 5 differ from SIRT2, 4, and 6 in the type of reaction they catalyse. SIRT1, 3, and 5 are NAD^+-dependent deacetylases catalyzing the deacetylation of histones and nonhistone proteins, whereas SIRT6 is a NAD^+-dependent ADP ribosyltransferase (ART) mediating mitochondrial protein ribosylation; SIRT2 and 4 exert both NAD^+-dependent HDAC and ART activities (Westphal, 2007). The enzymatic activity of SIRT7 as well as its specific substrates has not yet been determined.

SIRT1 FUNCTIONS

SIRT1 is the most evolutionarily conserved sirtuin among the seven mammalian homologs, and it has been shown to play crucial roles in complex physiological processes, including metabolism, cancer, and aging (Sebastian *et al.*, 2012). SIRT1 participates in various cellular functions ranging from differentiation and development to metabolism and cell survival by deacetylating diverse substrates, summarized in Figure 3 (Nakagawa *et al.*, 2011). SIRT1 can deacetylate histone H4K16, H3K9 and H1K26 and thereby mediate heterochromatin formation (Liu *et al.*, 2013). Through its enzymatic activity, SIRT1 has also the capacity to regulate the activity of various transcription factors and other regulatory proteins. For example, SIRT1 regulates energy by inducing gluconeogenic while repressing glycolytic gene expressions through deacetylation of PGC-1α and PPARγ (Picard, 2004; Rodgers, 2005). Deacetylation of NFKB, AP1 and Foxp3 by SIRT1 modulates the inflammation and immune pathway (Kong, 2012). And the deacetylation of DNA repair proteins Ku70, NBS1, WRN and XPA by SIRT1 regulates genomic stability (Fan, 2010; Li *et al.*, 2008; Yuan, 2007). SIRT1 regulates cell growth, apoptosis and stress response by deacetylating tumor suppressor protein like forkhead box protein FOXO1,3 and 4, Hif-1α, HSF1, Rb and survivin (Brunet *et al.*, 2004; Lim, 2010; Luo, 2008; Motta *et al.*, 2004; Wang *et al.*, 2008;

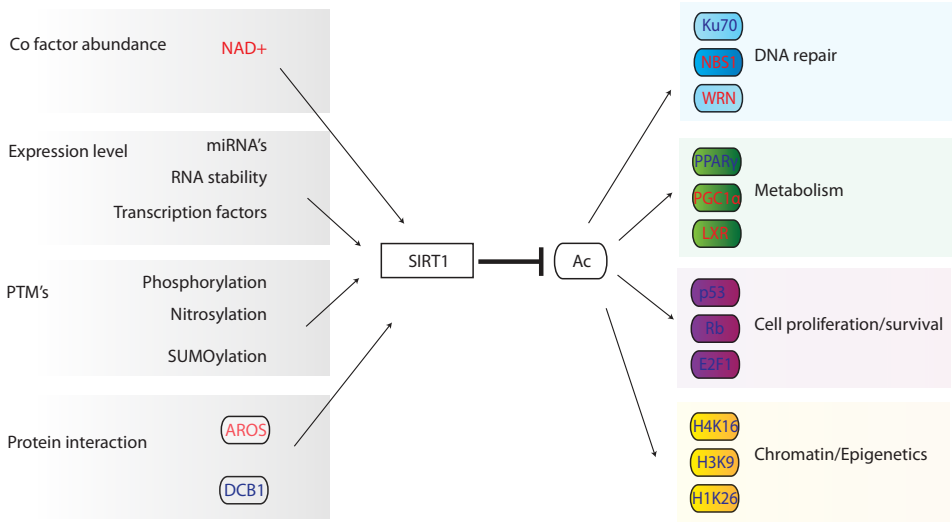

Figure 3. *SIRT1 pathway overview. SIRT1 is an NAD⁺-dependent histone deacetylase that catalyses the removal of acetyl (Ac) groups from a number of non-histone targets. The downstream effects of target deacetylation include changes in chromatin structure, cellular metabolism as well as cell survival and DNA repair. Several factors are involved in the regulation of SIRT1 function. SIRT1 function can be controlled availability of NAD⁺, posttranslational modifications, changes in expression or protein-protein interactions.*

Wong, 2007). The first and most widely known substrate of SIRT1 is tumor suppressor p53, which has critical roles in the cell-cycle regulation and apoptosis(Luo *et al.*, 2001; McCubrey, 2012).

p53 is a short-lived protein that is sustained at low levels under normal physiological conditions. However, p53 is maintained at a relative high level by posttranslational modifications in response to various stresses (Gu, 2012). The acetylation of p53 in response to DNA damage by HAT CBP/p300 was shown to acetylate p53 at K373/K382 and K320. Lysine acetylation at these sites promotes both its DNA binding ability and its transcriptional activity, and include p21, Bax and puma as p53 targets (Smith, 2002). In mice harboring a mutant p53 allele in which all the acetylation sites have been replaced a deficiency in gene transcription in response to DNA damage was shown, which illustrates that acetylation is indispensible for p53 function (Chao *et al.*, 2006; Tang *et al.*, 2008). Conversely, SIRT1 binds to and deacetylates p53, thereby negatively regulating p53-mediated transcriptional activation. SIRT1 functions by deacetylating of p53 which prevents cellular senescence and apoptosis caused by DNA damage and stress (Luo *et al.*, 2001; Vaziri *et al.*, 2001).

SIRT1 REGULATION

Although much attention has been focused on the identification of the cellular targets and functional networks controlled by SIRT1, the mechanisms that regulate SIRT1 activity by biological stimuli have only recently begun to emerge. As an enzyme, the activity of SIRT1 can

be controlled by the availability of its substrates, post-translational modifications, interactions with other proteins and changes in its expression levels (Figure 3) (Revollo *et al.*, 2013).

The basal intracellular NAD^+ levels are maintained relatively constant by the NAD^+ biosynthetic and salvage pathways (Houtkooper, 2010). Proper functioning of these pathways is important for the activation of SIRT1 catalytic activity since the availability of NAD^+ in cells is a limiting step(Revollo *et al.*, 2004). Glucose deprivation and metabolic changes by forexample calorie restriction can cause fluctuations in NAD^+ levels. Since SIRT1 associate with chromatin and its function is NAD^+-dependent, SIRT1 can couple changes in the cellular metabolic state and NAD^+ levels to transcription (Chalkiadaki, 2012).

Furthermore, SIRT1 enzymatic activity can be regulated by several posttranslational modifications. First, prosurvival dual specificity tyrosine phosphorylation-regulated kinases (DYRKs), DYRK1A and DYRK3, have been shown to phosphorylate SIRT1 in response to genotoxic stress (Guo, 2010). This PTM substantially enhances SIRT1 deacetylase activity towards acetylated p53 and protects cells from genotoxic stress-induced apoptosis (Guo, 2012). Another serine residue located at the highly conserved core domain of SIRT1, Ser 434, has been shown to be a phosphorylation target of the cyclic AMP/protein kinase A (cAMP/PKA) signaling pathway (Gerhart-Hines *et al.*, 2011). This PTM rapidly enhances the intrinsic deacetylase activity of SIRT1 independently of cellular NAD+ levels. Finally, C-Jun N-terminal kinase (JNK)1, phosphorylates SIRT1 on several serines, particularly under stressful cellular conditions (Nasrin, 2009). Intriguingly, these phosphorylations appear to increase the deacetylase activity of SIRT1 towards one of its substrates, histone H3, but have no effect towards another substrate, p53. This suggests that some or all of these PTMs can alter the activity of SIRT1 in a substrate-specific manner. SIRT1 is also sumoylated at K734, which in turn increases its activity and nitrosylation at Cys 387 and 390 in the catalytic core reduces SIRT1's ability to deacetylate PGC-1α (Kornberg, 2010; Yang, 2007).

In addition to posttranslational modification, alteration in expression levels can also regulate SIRT1 activity. The "tumor suppressor hypermethylated in cancer" protein (HIC1) has been shown to inhibit SIRT1 expression by forming a repressive complex with SIRT1 on its own promoter (Chen, 2005; Zhang *et al.*, 2007). Conversely, through a DNA damage dependent matter transcription factor E2F1 can induce SIRT1 expression (Wang *et al.*, 2006). Members of the FOXO transcription factors also regulated the expression of SIRT1, like FOXO1, which induces SIRT1 expression by binding to FOXO1 response elements in the SIRT1 promoter (Nemoto, 2004; Xiong, 2011). The SIRT1 promoter also contains several cyclic AMP response element-binding protein (CREB) binding sites and through CREB, a transcription factor whose activation is mediated by PKA in response to low nutrient availability, the expression of SIRT1 is induced (Fusco, 2012; Noriega, 2011). The abundance of SIRT1 is also controlled by translational events and RNA stability. For instance, the Hu antigen (HuR) an mRNA binding protein that binds the 3'UTR of SIRT1 mRNA and plays a major role in stabilizing SIRT1 mRNA transcript (Abdelmohsen, 2007; Yamakuchi, 2012). Furthermore, SIRT1 expression and its activity are also under the influence of several miRNA's as reported by several studies.

Finally, protein–protein interactions also play key roles in the regulation of SIRT1. For instance, interaction with active regulator of SIRT1 (AROS) enhances the activity of SIRT1 towards acetylated p53. AROS appears to bind to the N terminus of SIRT1, but little is known

about how this interaction increases the SIRT1 activity (Kim *et al.*, 2007). In contrast to AROS, deleted in breast cancer 1 (DBC1) is a negative regulator of SIRT1 (Kim *et al.*, 2008; Zhao *et al.*, 2008). The 'essential for SIRT1 activity' (ESA) domain (a 25-amino-acid region in the C terminus of SIRT1), which intramolecularly interacts with the SIRT1 deacetylase core, was also shown to activate its enzymatic activity (Kang *et al.*, 2011). DBC1 also interacts with the SIRT1 deacetylase core, potentially displacing ESA, decreasing the accessibility of the core domain to protein substrates, and thereby inactivating SIRT1. Moreover, cellular stress results in phosphorylation of DBC1 at Thr 454, which appears to create a second binding site for SIRT1 to augment the DBC1–SIRT1 interaction (Yuan, 2012). However, for most of studies where SIRT1 activity was shown to be modulated by protein-protein interactions, it is not clear yet whether allosteric changes or substrate access to catalytic core are responsible for the decrease in SIRT1 activity.

These studies show that SIRT1 is temporally and spatially regulated in response to various environmental cues. In **Chapter 2**, we describe a novel aspect of the regulation of SIRT1 by its interaction with and activation by phosphoinositides.

THESIS OUTLINE

The studies described in this thesis focus on the novel role of the PI(5)P/PIP4K signaling axis in regulating acetylation and understanding their physiological functions. **Chapter 1** is a general introduction giving an overview of the molecules and pathways involved in phosphoinositide signaling and regulation of SIRT1 activity. **Chapter 2** identifies PI(5)P as novel lipid regulator of SIRT1 activity and that SIRT1 is subject to acute regulation in response to lipid signaling. **Chapter 3** reviews the latest insights of phospholipids in the nucleus and their emerging role as important modulators of chromatin and gene transcription. Next, **chapter 4** explores the posttranslational modifications on PIP4Kβ and describes its biochemical interaction and deacetylation by SIRT1 deacetylase**. Chapter 5** provides insight on the physiological roles of PIP4Ks and provides the first description of how lack of PIP4Kα expression and activity affects zebrafish development. In **chapter 6**, the results presented in this thesis are summarized and discussed

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Chapter 2

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

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SUMMARY

Phosphatidyl-5-phosphate (PtdIns5P) is an inositol phospholipid that resides in various subcellular compartments and whose levels are modulated by diverse stress stimuli. However, its putative signaling functions remain largely uncharacterized. Here, we show that PtdIns5P binds to and regulates NAD-dependent deacetylase sirtuin-1 (SIRT1), a key player in metabolic, neurodegenerative and neoplastic processes. PtdIns5P stimulates SIRT1 deacetylase activity thereby relaying signals to p53, an established SIRT1 target. Changes in PtdIns5P levels modulate SIRT1-mediated p53 acetylation, p53-mediated transcription and apoptosis, as do SIRT1 mutants compromised in PtdIns5P binding. Our results define PtdIns5P as an activator of SIRT1 and modulator of SIRT1 physiological outputs.

INTRODUCTION

Phosphoinositides are a family of phosphorylated derivatives of the phospholipid phosphatidylinositol present in various subcellular compartments (Di Paolo and De Camilli, 2006). They can act as signaling molecules through their interaction with specific phosphoinositide binding sites in many proteins, thereby controlling a great diversity of physiological processes (Lemmon, 2008). The phosphoinositide phosphatidylinositol-5-phosphate (PtdIns5P) resides in the plasma membrane,(Tolias *et al.*, 1998) intracellular membranes (Sarkes and Rameh, 2010; Ramel *et al.*, 2011) and the nucleus (Jones *et al.*, 2006; Keune *et al.*, 2011). Its levels are known to be regulated in membranes in response to insulin signaling (Sbrissa *et al.*, 2004) and bacterial invasion (Niebuhr *et al.*, 2002), in the nucleus during cell cycle progression (Clarke *et al.*, 2001), and by cellular stressors such as oxidative stress and DNA damage. Although the regulation of PtdIns5*P* levels has been explored, the function of PtdIns5*P*, as well as the molecular details of how it triggers downstream cascades, are still largely uncharacterized. We reasoned that PtdIns5P might interact with deacetylases, which operate as signal transmitters to regulate key transcriptional events and cellular functional outcomes. In particular, we explored whether, PtdIns5P signal through the NAD+-dependent deacetylase Sirtuin-1 (SIRT1). SIRT1 is involved in the regulation of stress (Motta *et al.*, 2004; Vaziri *et al.*, 2001; Brunet *et al.*, 2004) hormonal and metabolic responses(Canto and Auwerx, 2012) and represents a potential therapeutic target in a wide range of human pathologies (Knight and Milner, 2012; Bosch-Presegue and Vaquero, 2011). SIRT1 influences the acetylation of many downstream targets including p53, thereby controlling its transcriptional activity and the subsequent induction of cell cycle arrest and apoptosis (Luo *et al.*, 2001; Vaziri *et al.*, 2001). SIRT1 deacetylase activity can be regulated by protein-protein interactions and by intramolecular interactions that govern basal SIRT1 activity (Kang *et al.*, 2011; Kim *et al.*, 2007; Zhao *et al.*, 2008). However, there is a paucity of data on the intracellular signaling molecules that may impact on SIRT1 activity (Canto *et al.*, 2009; Yamakuchi *et al.*, 2008). Here, we report that PtdIns5*P* binds to and stimulates SIRT1 activity and downstream p53 acetylation. Thus, the many extracellular cues that modulate intracellular PtdIns5*P* levels may converge onto SIRT1 and thereby regulate SIRT1-mediated physiological responses.

RESULTS

Sirt1 interacts with phosphoinositides

We assessed whether SIRT1 could interact with phosphoinositides in general, and PtdIns5P in particular by performing protein-lipid overlay assays. We found that full-length SIRT1 interacted with PtdIns5P as well as with PtdIns3P, PtdIns4P and PtdIns(3,5)P2 (Figure 1A). Overlay assays using deletion fragments of SIRT1 (Figure 1B), mapped the PtdIns5P binding site to a region within amino acid residues 211-353 (Figure 1D). The 211-353 fragment showed no homology to any of the canonical phosphoinositide interaction domains such as PH, PHOX or PHD fingers (Gozani *et al.*, 2003; Elkin *et al.*, 2005). However, this fragment contained a stretch of positively charged amino acids (KRKKRK), conserved across species (residues 225-230 in mouse SIRT1) (Figure 1C), which may constitute a phosphoinositide interaction site (Lewis *et al.*, 2011). Mutation of Lys227 to Ala [211-253(K3)] as well as double mutation of Lys225 and Arg226 to alanine [(211-253(K1R2)] within

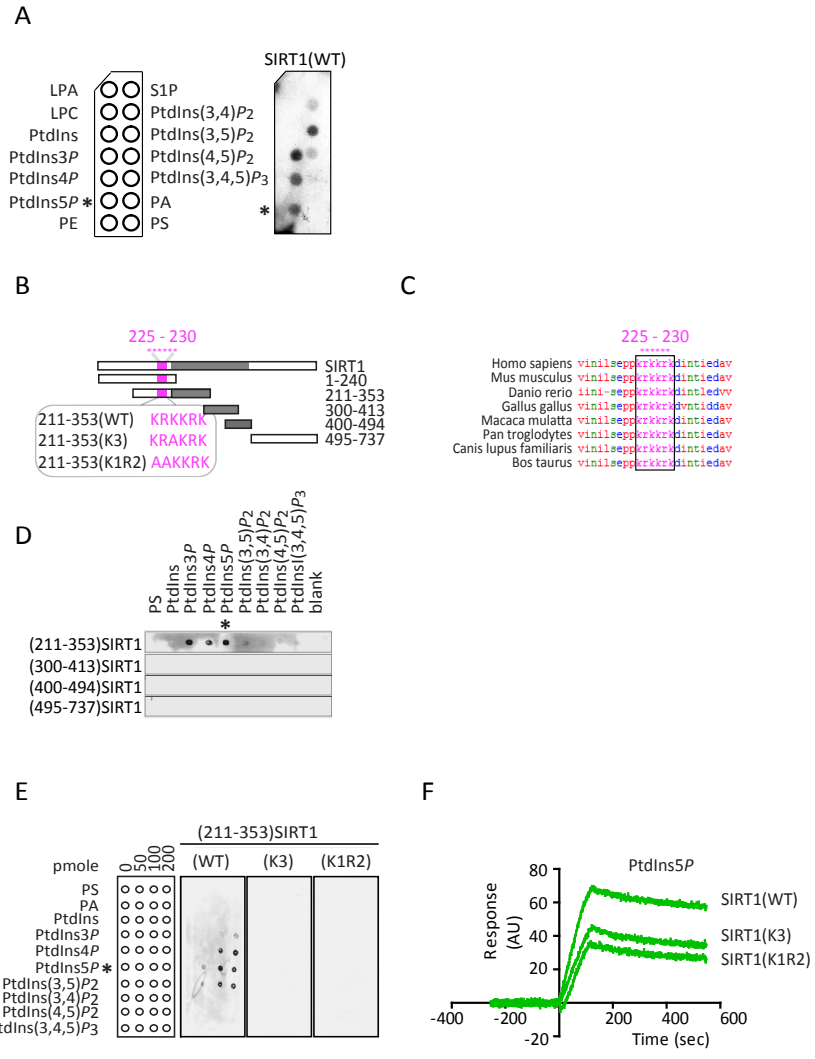


Figure 1. *PtdIns5P interaction with SIRT1; A conserved motif (residues 225-230) outside the catalytic domain is essential for PtdIns5P-SIRT1 interaction. (A) Left: diagram of a PIP Strip (used for protein-lipid overlay assays). Right: protein-lipid overlay assay using full-length SIRT1(WT). Binding of SIRT1(WT) to PtdIns5P is indicated (*). (B) Schematic representation of full-length SIRT1(WT) and SIRT1-deletion mutants (1-240, 211-353, 300-413, 400-494 and 495-737)Residues 225-230 indicated in pink. The 225-230 sequence KRKKRK (WT) and the mutated KRAKRK (K3) and AAKKRK (K1R2) sequences are shown in pink. SIRT1 catalytic domain is shown in grey. (C) The KRKKRK motif (in pink also indicated by ******) is conserved among species. (D) Protein-lipid overlay assay with the indicated SIRT1 deletion domains. (211-353)SIRT1 was the only domain interacting with PtdIns5P (as indicated (*)). (E) Protein-lipid overlay assay using the (211-353)SIRT1 deletion domain carrying the wild type KRKKRK sequence (WT) or the point mutations (K3) and (K1R2) (see 1B). The interaction with PtdIns5P is indicated (*). (F) The interaction of PtdIns5P with SIRT1 [SIRT1(WT)],SIRT1(K3) and SIRT1(K1R2) PtdIns5P was analyzed by Surface Plasmon Resonance (SPR). Data is representative of at least three independent experiments.*

this stretch (Figure 1B) compromised the interaction between SIRT1 and PtdIns5P (Figure 1E), indicating that the KRKKRK motif is essential for SIRT1 and PtdIns5P binding.

To further validate SIRT1-PtdIns5P binding, we analyzed the interactions of full-length SIRT1 wild type [SIRT1(WT)] and SIRT1 mutants [SIRT1(K3) or SIRT1(K1R2)] with PtdIns5P by surface plasmon resonance (SPR). SIRT1(WT) interacted with PtdIns5P while the SIRT1(K3) and SIRT1(K1R2) mutants did not (Figure 1F). Previously defined phosphoinositide-binding domains, notably FYVE domain (interacting with PtdIns3P), PH domain of PLC-delta1 (interacting with PtdIns(4,5)P2), and the PHD finger of ING2 (interacting with PtdIns5P) were used as positive controls (Supplementary Figure S1). The SPR results confirmed that SIRT1 binds PtdIns5P through its KRKKRK motif. We analyzed the subcellular localization of SIRT1 and its mutants and found SIRT1(K3) and SIRT1(K1R2) to have similar nuclear localizations as SIRT1(WT) (Supplementary Figure S2).

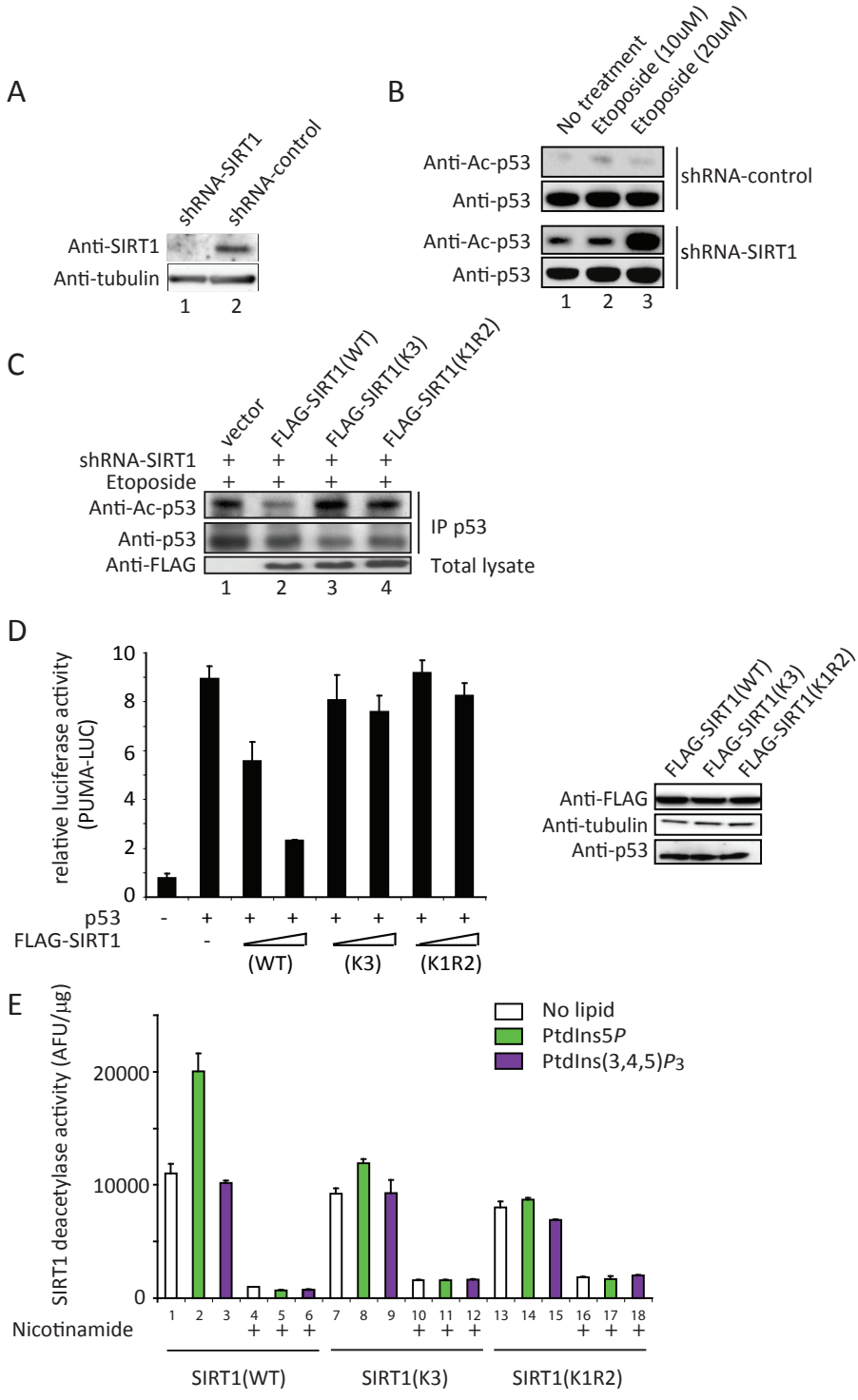
The KRKKRK motif is required for SIRT1 dependent p53 deacetylation

We then analyzed whether PtdIns5P could regulate SIRT1 functions. More specifically, we assessed whether PtdIns5P could modulate SIRT1-mediated regulation of p53 responses, since p53 is a well established SIRT1 substrate (Vaziri *et al.*, 2001; Luo *et al.*, 2001). We assessed whether the abrogation of PtdIns5P-SIRT1 binding could lead to altered p53 protein acetylation. We tested the ability of SIRT1(K3) and SIRT1(K1R2) to deacetylate p53 in response to stress. Sh-RNA targeting SIRT1 in U2OS cells reduced endogenous SIRT1 expression by approximately 90% compared to sh-RNA control (Figure 2A), leading to a robust increase in p53 acetylation at Lys382 in both untreated cells and etoposide-treated cells (Figure 2B). We monitored p53 acetylation after etoposide exposure in SIRT1 knockdown cells reconstituted with either SIRT1(WT) or PtdIns5P binding mutants [SIRT1(K3) or SIRT1(K1R2)]. While SIRT1(WT) blunted etoposide-induced acetylation of p53 at Lys382, SIRT1(K3) and SIRT1(K1R2) mutants failed to deacetylate p53 (Figure 2C). To investigate the functional consequences of a compromised SIRT1PtdIns5P interaction in p53-mediated responses, we analyzed how SIRT1(K3) and SIRT1(K1R2) may affect p53-mediated transcription. p53-deficient H1299 cells were transfected with a luciferase construct driven by the p53-dependent PUMA-promoter together with constructs encoding p53 and SIRT1(WT), SIRT1(K3) or SIRT1(K1R2) (Figure 2D). p53 strongly increased luciferase activity, which was attenuated by the expression of wt SIRT1. SIRT1 mutants unable to bind PtdIns5P failed to attenuate p53-driven transcription. We conclude that PtdIns5P-SIRT1 interaction regulates SIRT1 deacetylase activity and thereby p53 acetylation and p53-mediated transcription.

Next, we assessed SIRT1 catalytic activity *in vitro* in the presence and absence of PtdIns5P. Addition of PtdIns5P increased basal SIRT1 activity about two-fold, whereas PtdIns(3,4,5)P3 did not (and previously shown failing to bind to) SIRT1 (Figure 2E). Crucially, PtdIns5P did not stimulate the activity of SIRT1(K3) and SIRT1(K1R2). This shows that the basal activity of SIRT1(K3) and SIRT1(K1R2) mutants is unperturbed (Figure 2E (lanes 7 and 13 to lane 1). In accordance we showed that of the seven different phosphoinositides tested; only the mono-phosphoinositides were able to stimulate wild type SIRT1 activity but not the mutants (Supplementary Figure S3). Thus, PtdIns5P is an allosteric effector of SIRT1 activity via interaction with the KRKKRK motif.

PtdIns5*P* levels modulate SIRT1 dependent p53 deacetylation

We then examined whether changes in PtdIns5P levels could signal via SIRT1 to regulate p53 acetylation. Three PIP4Kinase isoforms (α, β and γ) are known to phosphorylate PtdIns5P,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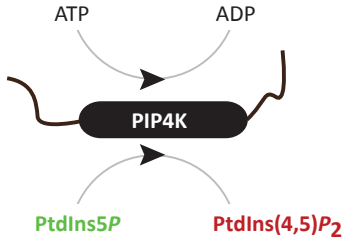
shRNA-control
Anti-SIRT1
Anti-tubulin
1 2
B
No treatment
Etoposide (10uM)
Etoposide (20uM)
Anti-Ac-p53
Anti-p53
shRNA-control
Anti-Ac-p53
Anti-p53
shRNA-SIRT1
1 2 3
C
vector
FLAG-SIRT1(WT)
FLAG-SIRT1(K3)
FLAG-SIRT1(K1R2)
shRNA-SIRT1 + + + +
Etoposide + + + +
Anti-Ac-p53
Anti-p53
IP p53
Anti-FLAG
Total lysate
1 2 3 4
D
relative luciferase activity (PUMA-LUC)
0 2 4 6 8 10
p53 - + + + + + + +
FLAG-SIRT1 - (WT) (K3) (K1R2)
FLAG-SIRT1(WT)
FLAG-SIRT1(K3)
FLAG-SIRT1(K1R2)
Anti-FLAG
Anti-tubulin
Anti-p53
E
SIRT1 deacetylase activity (AFU/μg)
0 10000 20000
No lipid
PtdIns5P
PtdIns(3,4,5)P3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Nicotinamide + + + + + + + + +
SIRT1(WT)
SIRT1(K3)
SIRT1(K1R2)

converting it to PtdIns(4,5)P2 (Figure 3A left). In particular, PIP4Kα is an high specific activity isoform whose over-expression leads to reduced PtdIns5P levels(Bultsma *et al.*, 2010). We generated U2OS cells over-expressing PIP4Kα under the control of a doxycyclin-inducible promoter (U2OS-Myc-PIP4Kα) and confirmed that doxycycline induced PIP4Kα expression, increased PIP4Kα activity and reduced PtdIns5P levels (Supplementary Figure S5). In addition, forced PIP4Kα expression reduced the basal level of nuclear PtdIns5P (Figure 3A right, lane 3 and 1). Etoposide enhanced nuclear PtdIns5P levels (Figure 3A, lane 2 and 1), which was inhibited upon PIP4Kα over-expression (Figure 3A, lane 2 and 4). SIRT1-depleted U2OS-PIP4Kα cells were reconstituted with SIRT1(WT) or SIRT1(K3) (Figure 3B) and used as controls or treated with doxycycline to induce PIP4Kα expression. We analyzed p53 acetylation in response to etoposide. Without PIP4Kα over-expression, SIRT1(WT) deacetylated p53 to a greater extent than did SIRT1(K3) (Figure 3B, lane 2 and 3). PIP4Kα over-expression decreased the ability of SIRT1(WT) to deacetylate p53 (Figure 3B, lane 5 and 2), suggesting that a decrease in PtdIns5P affects SIRT1 activity. Furthermore, PIP4Kα-overexpressing cells showed similar levels of p53 deacetylation mediated by either SIRT1(WT) or SIRT1(K3) (Figure 3B, lane 5 and 6). Thus, a decrease in PtdIns5P reduces SIRT1 activity in accordance with the abrogation of SIRT1-PtdIns5P binding. To confirm that PtdIns5P levels affect SIRT1 activity, we analyzed p53 acetylation in U2OS cells depleted from both PIP4Kα and PIP4Kβ and exposed to etoposide. Knockdown of PIP4Kα-β resulted in reduced p53 acetylation compared to control (Figure 3C, 2 and 1), consistent with increased PtdIns5P levels enhancing SIRT1 activity. To verify that the reduction in p53 acetylation was due to SIRT1, cells were treated with the SIRT1 inhibitor nicotinamide. Nicotinamide caused an increase in p53 acetylation in control cells (Figure 3C, lane 3 and 1) which was not significantly diminished upon knockdown of PIP4Kα and PIP4Kβ (Figure 3C, lane 3 and 4). We conclude that PtdIns5 functions as a SIRT1 activator.

We then investigated whether PIP4K-induced changes in PtdIns5Ps could affect p53-mediated transcription. In a luciferase reporter assay, SIRT1 expression attenuated p53-driven transcription

◄ ***Figure 2.*** *The interaction of PtdIns5P with SIRT1 enhances SIRT1 deacetylase activity. (A) SIRT1 in whole cell lysates was detected by Western Blot in SIRT1 knockdown (shRNA-SIRT1) or control (shRNA-control) U2OS cells. (B) Control (shRNA-control) or SIRT1 knock-down (shRNA-SIRT1) U2OS cells were exposed to etoposide (10-20 μM) for 1 hr and with MG132 (50 μM) for 30 min before harvesting. p53 was immunoprecipitated and p53 acetylation at Lys382 was analyzed by Western Blotting. Total levels of immunoprecipitated p53 are shown. (C) shRNA-SIRT1 U2OS were transfected with the indicated vectors (top). After 48 hrs, cells were exposed to etoposide for 2 hr. p53 was immunoprecipitated and p53 acetylation at Lys 382 was.(D) H1299 cells (p53-deficient) were transfected with PUMA-luciferase renilla-luciferase construct together with p53, SIRT1(WT), SIRT1(K3) and SIRT1(K1R2), as indicated. Relative firefly/Renilla luciferase activity values are presented as means ± s.d. of triplicate samples. The data are representative of three independent experiments. (E) Lysine deacetylase activities of SIRT1 [SIRT1(WT)], SIRT1(K3) and SIRT1(K1R2) were analyzed using a SIRT1 fluorescent activity assay. Time courses, were performed to define optimal assay conditions (Supplementary Figure S4). The effect of PtdIns5P or PtdIns(3,4,5)P3 on SIRT1 activities is shown. Short fatty acid chain (C8) analogues of PtdIns5P and PtdIns(3,4,5)P3 were used to exclude possible sequestration of SIRT1 or 1 acetylated-substrate by liposomes. Nicotinamide (2 mM) was added as indicated. Enzymatic activities, expressed as AFU (arbitrary fluorescence units)/μg of SIRT1, are presented as means ± s.d. of duplicate samples. The data are representative of three independent experiments.*

A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PtdIns5P
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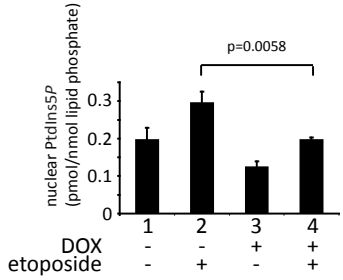
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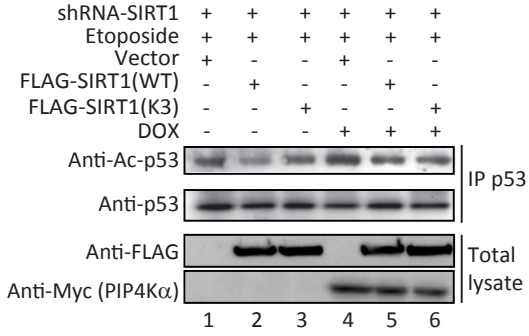
0
0.1
0.2
0.3
1 2 3 4
DOX - - + +
etoposide - + - +

B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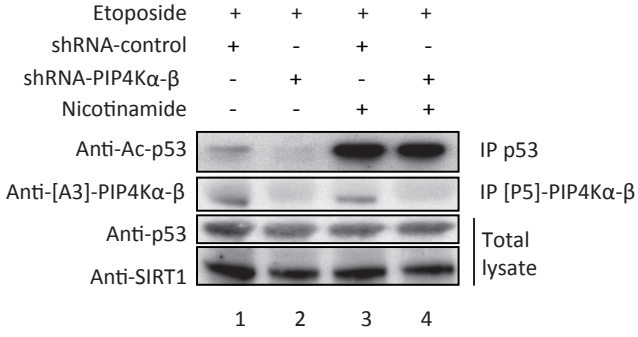
Etoposide + + + + + +
Vector + - - + - -
FLAG-SIRT1(WT) - + - - + -
FLAG-SIRT1(K3) - - + - - +
DOX - - - + + +
Anti-Ac-p53
Anti-p53
IP p53
Anti-FLAG
Anti-Myc (PIP4Kα)
Total lysate
1 2 3 4 5 6


shRNA-control
shRNA-SIRT1

C


Etoposide + + + +
shRNA-control + - + -
shRNA-PIP4Kα-β - + - +
Nicotinamide - - + +
Anti-Ac-p53
IP p53
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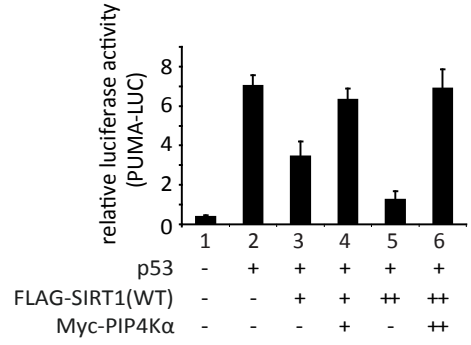
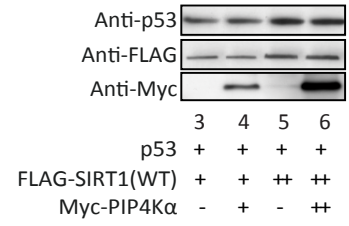
Anti-p53
Anti-SIRT1
Total lysate
1 2 3 4

D


relative luciferase activity (PUMA-LUC)
0
2
4
6
8
1 2 3 4 5 6
p53 - + + + + +
FLAG-SIRT1(WT) - - + + ++ ++
Myc-PIP4Kα - - - + - ++


Anti-p53
Anti-FLAG
Anti-Myc
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p53 + + + +
FLAG-SIRT1(WT) + + ++ ++
Myc-PIP4Kα - + - ++

in a dose-dependent manner (Figure 3D, lanes 3,5 and lane 2). Overexpression of PIP4Kα reduced the ability of SIRT1 to attenuate p53 transcriptional activity, supporting a role for PtdIns5P in SIRT-mediated p53 transcription (Figure 3D, lane 4 and 3 and Supplementary Figure S6). We examined how PtdIns5P-SIRT1 interaction may affect p53-mediated transcription. We analyzed the expression of p21, a p53 target gene regulated by p53 acetylation(Tang *et al.*, 2008), in cells over-expressing SIRT1(K3) compared to cells over-expressing SIRT1(WT). As shown in Figure 4A, the expression of p21 induced by p53 was attenuated after co-expression of SIRT1(WT), but not by SIRT1(K3). Furthermore, p21 expression levels in the presence of SIRT1(WT) or SIRT1(K3) reflected the extent of p53 acetylation.

PtdIns5*P* dependent regulation of SIRT1 in response to stress

We analyzed the effect of PtdIns5P-SIRT1 interaction on p53-mediated apoptosis. SIRT1-depleted U2OS cells were reconstituted with SIRT1(WT) or SIRT1(K3), and etoposide-induced apoptosis was assessed. Etoposide increased apoptosis in SIRT1-depleted cells (Figure 4B left, bar 4 and 2) in a p53-dependent manner, since it was prevented by p53 knockdown (Figure 4B, bar 4 and 10). SIRT1(WT) inhibited etoposide-induced apoptosis (Figure 4B left, bar 6 and 4), whereas SIRT1(K3) was compromised in such inhibition (Figure 4B, bar 8 and 6).

Next, we analyzed how increased PtdIns5P affects SIRT1- and p53-dependent apoptosis. In PIP4Kα-β knockdown U2OS cells exposed to etoposide, apoptosis was significantly reduced (Figure 4C, bars 4 and 3), consistent with increased PtdIns5P levels diminishing p53-induced apoptosis. Since apoptosis was not reduced in PIP4K α-β knockdown cells treated with nicotinamide (Figure 4C, bars 6 and 4), we conclude that PtdIns5P-stimulated SIRT1 activity is responsible for p53 dependent cell survival.

DISCUSSION

The present study identifies the phosphoinositide PtdIns5P as an allosteric activator of the SIRT1 deacetylase, providing new mechanistic insights into how SIRT1 regulates p53 acetylation and

◄ ***Figure 3.*** *PtdIns5P levels regulates SIRT1 activity. (A) Left: Scheme depicting the action of PtdIns5P-4-kinase [PIP4K], catalyzing the phosphorylation of PtdIns5P to form PtdIns(4,5)P2. Right: U2OS cells expressing doxycycline- inducible Myc-PIP4Kα (U2OS-Myc-PIP4Kα). Both untreated and doxycycline-treated cells were exposed to etoposide for 3 hr or maintained as controls e. The nuclear levels of PtdIns5P were measured. The data are presented as means ± s.d. The PIP4Kα-induced decrease of nuclear PtdIns5P was statistically significant (p-value 0.0058). (B) SIRT1-depleted U2OS-Myc-PIP4Kα cells were transfected with empty vector, FLAG-SIRT1(WT) or FLAG- SIRT1(K3), as indicated. Transfected cells were maintained as controls or treated with doxycycline (DOX) for 18 hr and then exposed to etoposide for 2 hr. p53 acetylation at Lys382 was analyzed as above. Cell lysates were assessed with indicated antibodies. Right: SIRT1 expression in the various U2OS cell lines (C) U2OS cells transduced with control shRNA) or with shRNAs targeting PIP4Kα and PIP4Kβ were treated with nicotinamide (6 hr) or left untreated and then exposed to etoposide for 2 hr. p53 acetylation was analyzed as above. Immunoprecipitated PIP4Kα and PIP4Kβ (Anti-[A3]-PIP4K α-β) are shown. (D) Left: Luciferase assays in H1299 cells (p53-deficient) transfected with the indicated constructs and assayed as above. Luciferase activity values are presented as means ± s.d. of triplicate samples. The data are representative of three independent experiments. Right: Expression of p53, FLAG-SIRT1(WT) and Myc-PIP4Kα was assessed by the indicated antibodies.*

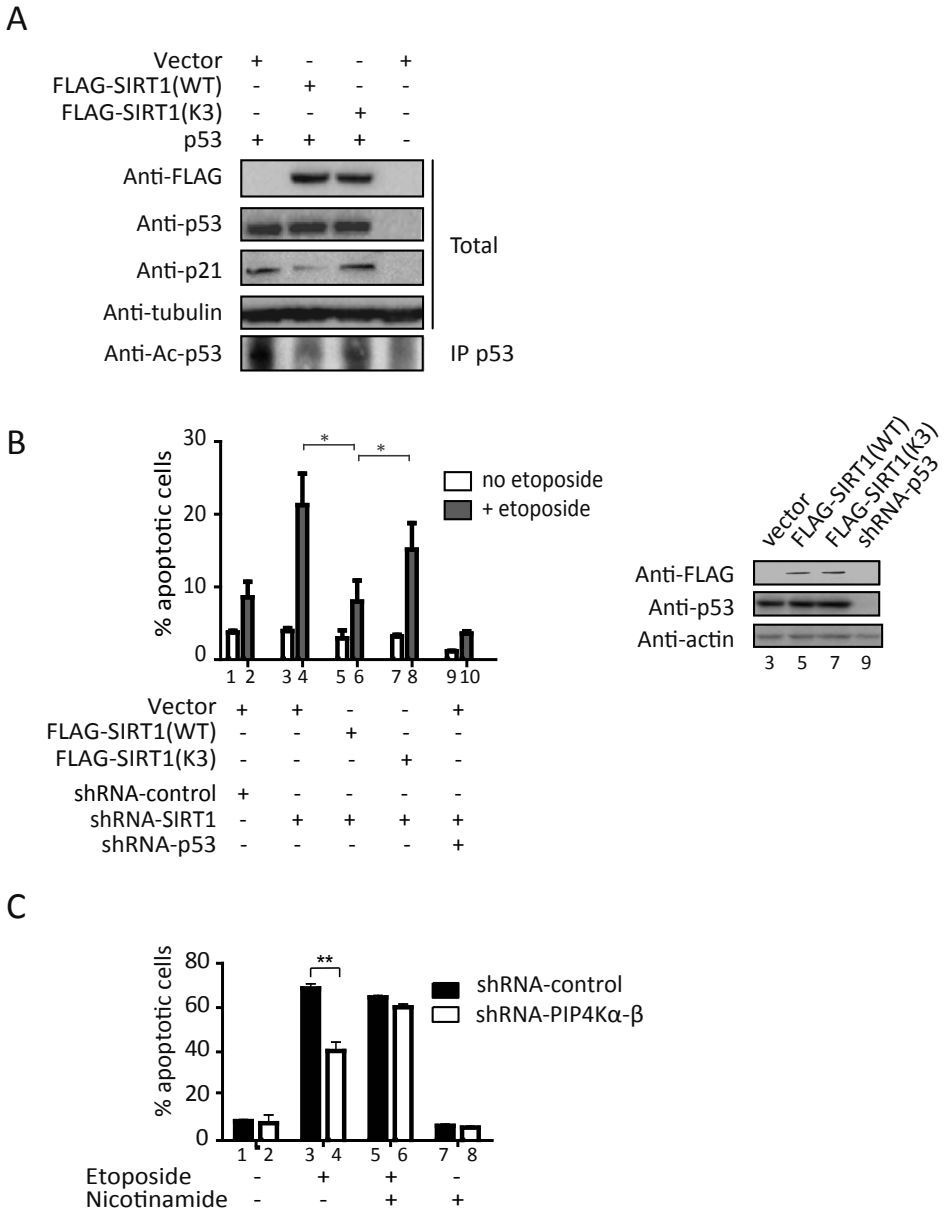

Figure 4. *PtdIns5P dependent SIRT1 regulation is important for cellular stress response. (A) H1299 were transfected with empty vector, FLAG-SIRT1(WT), FLAG- SIRT1(K3) and p53 as indicated (top). Cell lysates were immunoblotted with the indicated antibodies (Total). p53 acetylation at Lys 382 (Anti-Ac-p53) was analyzed as above (B) Left: Indicated knockdown U2OScells were transfected with FLAG-SIRT1(WT) or FLAG-SIRT1(K3) as indicated. After 48 hrs, cells were exposed to 20 μM etoposide for 24 hr or maintained as control. Apoptotic cells were analyzed by Annexin V-FITC and Propidium Iodide staining and flow cytometry. Data are presented as means ± s.d. of three independent experiments. p-values <0.05 determined by Student's t-test were considered to be significant (*).* ►

p53-mediated responses (Figure 4D). Suppression of p53 functions is achieved by coordinating increased PtdIns5*P* levels with increased SIRT1 deacetylation activity. Our findings constitute an important advance in our understanding of nuclear phosphoinositides, particularly PtdIns5P and their transducing mechanisms. Insights into the finely tuned regulation of SIRT1 (Zhao *et al.*, 2008; Kim *et al.*, 2008; Kim *et al.*, 2007; Gerhart-Hines *et al.*, 2011) will help to resolve controversies concerning the opposed physiological effects hitherto demonstrated for SIRT1 as, for example, in oncogenic and endocrine responses (Canto and Auwerx, 2012; Bosch-Presegue and Vaquero, 2011). As nuclear PtdIns5P can influence both acetylation and deacetylation of p53, the temporal and spatial regulation of PtdIns5P likely determines the strength and duration of p53 activation (Gozani *et al.*, 2003). PtdIns5P is the first nuclear lipid associated with SIRT1 suggesting an important general role for deacetylases as signal transducing enzymes.

Interestingy, there is precedent for phosholipids to regulating deacetylase activity in that sphingosine 1-phosphate (S1P) was found to interact with and inhibit the HDAC1,2 deacetylases (Hait *et al.*, 2009). This provoked the question of whether other phospholipids could fulfill a similar role in interacting with chromatin components and regulating gene transcription. Precisely how PtdIns5P signals SIRT1 activation is currently unclear. The N- and C-terminal regions of SIRT1 are important determinants of catalysis (Kang *et al.*, 2011; Pan *et al.*, 2012). Therefore, PtdIns5P might allosterically activate SIRT1 by modulating the interaction with its regulatory termini, or with specific protein binding partners, which in turn could modulate SIRT1 catalytic efficiency. Regardless of the activation mechanism, our findings implicate PtdIns5P as a new player in the many physiological processes and diseases in which SIRT1 is involved (Yeung *et al.*, 2004; Araki *et al.*, 2004; Lee *et al.*, 2008). The definition of an allosteric site within SIRT1 may boost drug discovery efforts to develop pharmacological modulators of SIRT1 activity.

EXPERIMENTAL PROCEDURES

Plasmids and antibodies

FLAG-tagged murine SIRT1 was cloned into pcDNA3 and PGEX-4T-1s and SIRT1 KRKKRK mutant constructs were generated by site-directed mutagenesis. Polyclonal rabbit PIP4Kα and PIP4Kβ antibodies were generated and described as previously, see the Supplemental Experimental Procedures.

Protein-lipid overlay and Surface Plasmon Resonance (SPR)

Protein-lipid overlays were performed using PIP Strips (Echelon Bioscience) and Surface Plasmon Resonance (SPR) was performed using the Bio-Rad Protein Interaction Array System ProteOn XPR36 with lipids from CellSignals Inc, see the Supplemental Experimental Procedures.

► *Right: Expression levels of FLAG-SIRT1(WT), FLAG- SIRT1(K3) and p53 after transfection. Actin was used as loading control. (C) Control U2OS cells and U2OS cells knocked down for PIP4Kα and PIP4Kβ (shRNA-PIP4 α-β) were treated with nicotinamide (10 mM) or left untreated and then further exposed to 20 μM etoposide for 30 hrs. Apoptotic cells were analyzed by Annexin V-FITC and Propidium Iodide staining and flowcytometry. Data are presented as means ± s.d. of three independent experiments p-value <0.01 determined by Student's t-test is indicated (**). (D) The working model of regulation SIRT1 by PI(5)P through binding of KRKKRK motif.*

In vitro SIRT1 deacetylation assay

Lysine deacetylation was measured using a SIRT1 fluorescent activity assay (Biomol International/ Enzo Life Sciences). For complete protocol, see the Supplemental Experimental Procedures.

PIP4Kinase activity assay

PIP4Kα was immunoprecipitated and its activity measured as described (Jones *et al.*, 2006). PtdIns5P measurements were done as described (Ndamukong *et al.*, 2010)

SUPPLEMENTAL INFORMATION

Supplemental Information includes 6 figures, Supplemental Experimental procedures and Supplemental References

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SUPPLEMENTARY INFORMATION

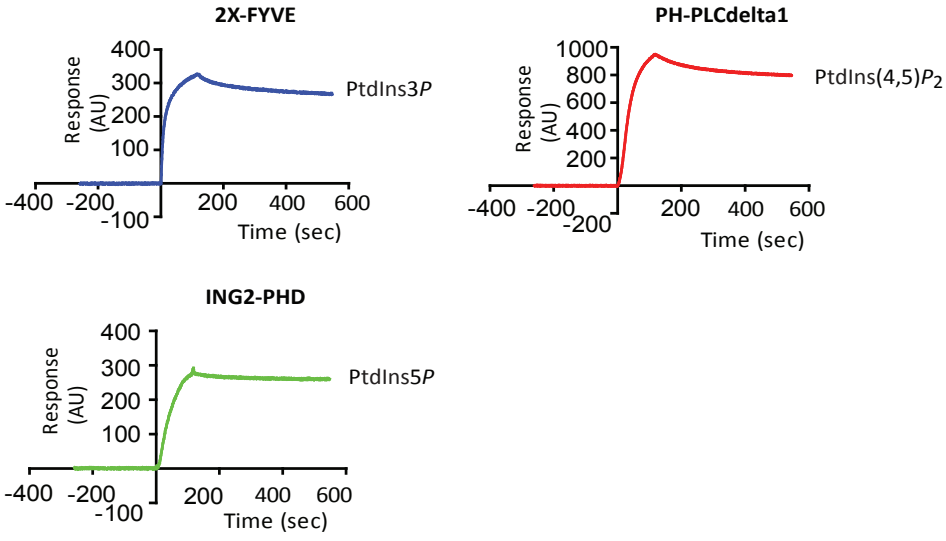

Figure S1. *SPR analysis of canonical phospholinositide binding domains. The respective interactions of the small protein domains 2X-FYVE, PH-PLCdelta1 and ING2-PHD with PtdIns3P, PtdIns(4,5)P2, and PtdIns5P were analyzed by Surface Plasmon Resonance (SPR). Data is representative of at least three independent experiments. Data is representative of at least three independent experiments.*

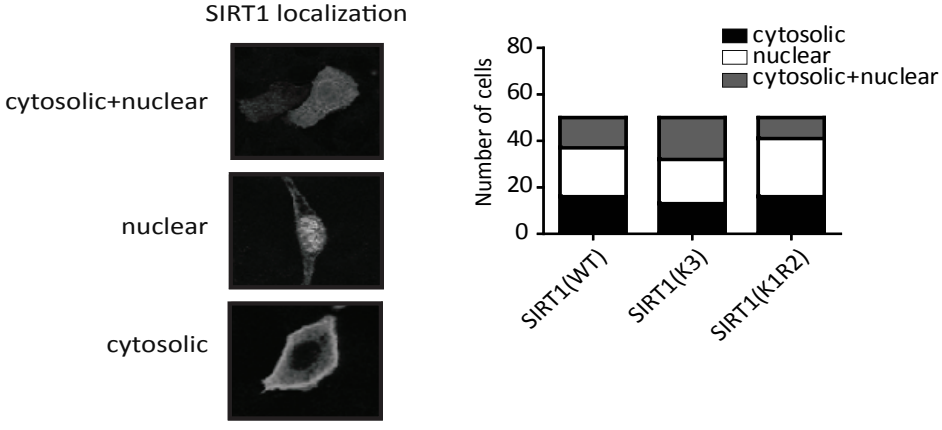

Figure S2. *KKRKK motif in SIRT1 is not a nuclear localization signal. Immunolocalization of full-length SIRT1(WT), SIRT1(K3) and SIRT1(K1R2) was performed in HEK 293T. Left: SIRT1 localizations in the nucleus, cytosol or both are shown. Right: the respective quantifications of SIRT1(WT), SIRT1(K3) and SIRT1(K1R2) localizations are presented.*

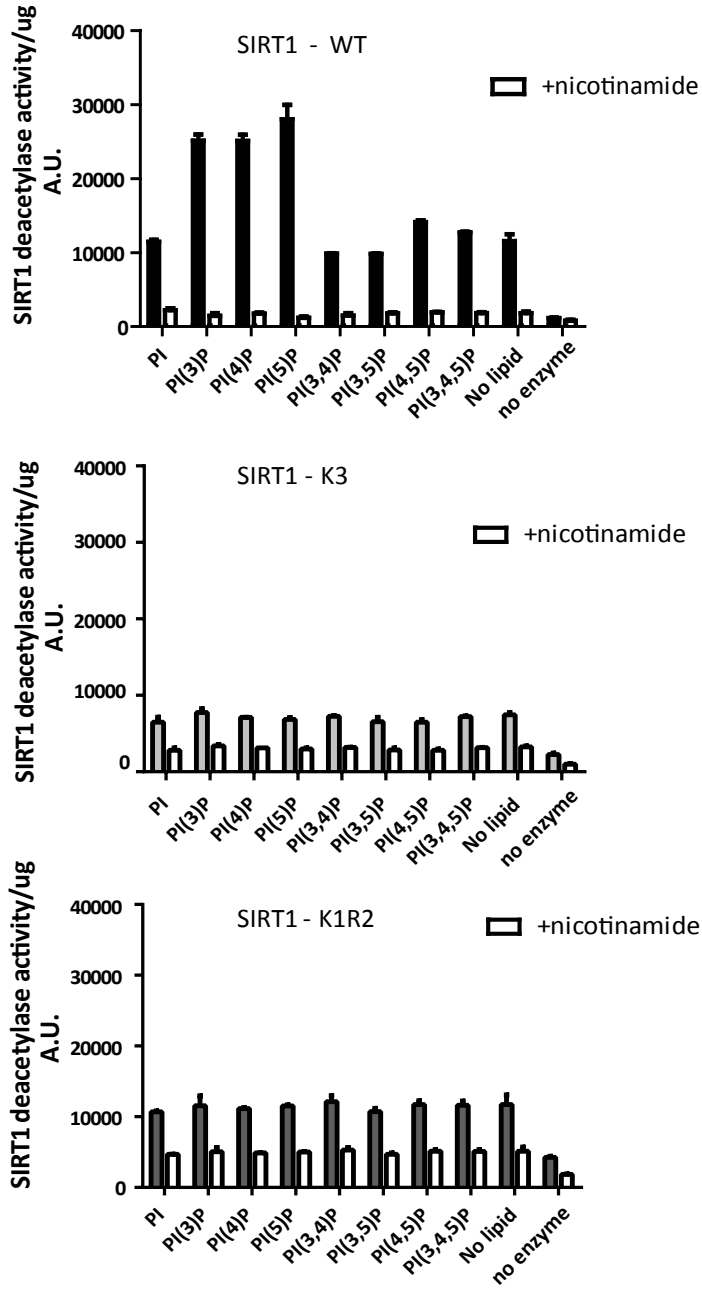

Figure S3. *Phosphoinositides stimulate p53 deaceylation of SIRT1 in vitro. Wild type and mutant SIRT1 were assayed for their deaetylation activity in the absence or presence of di-C8 water soluble phosphoinositides. Only phosphomonoinositides were able to stimulated wild type SIRT1, however importantly, they were not able to stimulate mutant SIRT1 (SIRT1-K3 or SIRT1-K1R) unable to interact with PI. Data is presented as mean ± standard deviation of tree independent experiments each preformed in duplicate.*

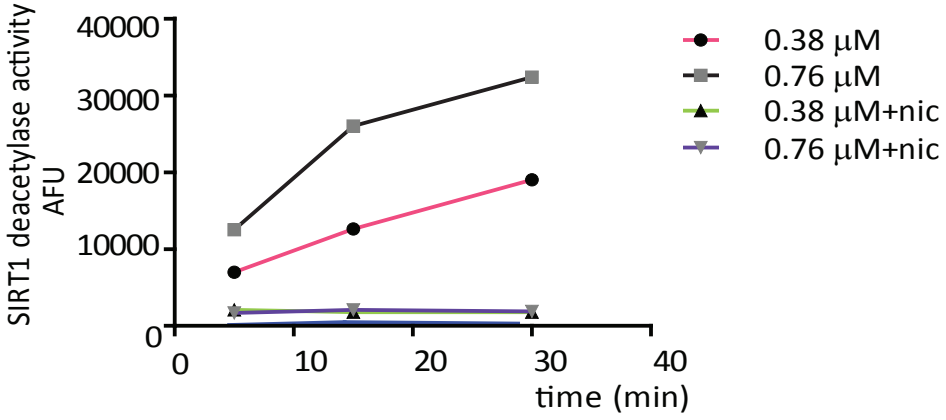

Figure S4. *SIRT1 activity is time- and dose-dependent. Lysine deacetylation activity of purified wild type SIRT1 [SIRT1(WT)] was determined using a fluorescent activity assay. Changes in the amount of deacetylated product formed over time were measured at two different SIRT1(WT) concentrations (0.38 µM and 0.76 µM). The reaction was carried out at 37°C in the presence of 200 µM NAD+ and 100 µM Fluor de Lys- SIRT1 substrate. Nicotinamide (2 mM) was added as indicated. Fluorescent signal values [expressed as AFU (arbitrary fluorescence units)] for deacetylated product were plotted on the y-axis.*

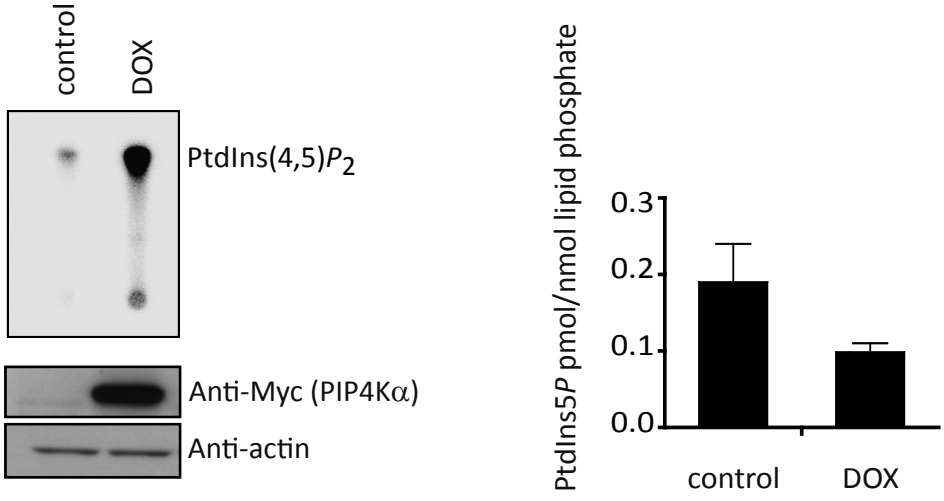

Figure S5. *Doxocycline treatment induces PIPKα expression in U2OS-Myc-PIP4Kα cells. U2OS cells expressing doxycycline-inducible Myc-PIP4Kα (U2OS-Myc-PIP4Kα) were maintained untreated (control) or were treated with doxycycline (DOX) to induce the over- expression of Myc-PIP4Kα. Myc-PIP4Kα was immunoprecipitated from whole cell lysates. Left: Levels of immunoprecipitated Myc-PIP4Kα [Anti-Myc (PIP4Kα)] are shown (bottom panel). PIP4K activity in the immunoprecipitates was assayed by determining the levels of the PIP4K reaction product PtdIns(4,5)P2 (top panel). Right: The cellular level of PtdIns5P was measured. The data are presented as means ± s.d.*

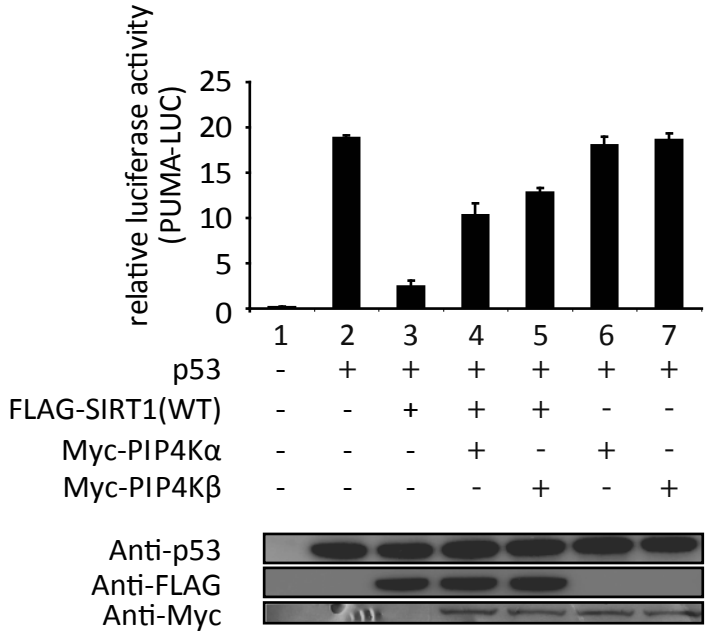

Figure S6. *PIP4Kα,β overexpression and p53-mediated transcription. H1299 cells (p53-deficient) were transfected with the PUMA promoter coupled to firefly luciferase and with the Renilla luciferase vectors together with p53, FLAG-SIRT1(WT), Myc- PIP4Kα or Myc-PIP4Kα as indicated. Top: Relative firefly/Renilla luciferase activity values are presented as means ± s.d. of triplicate samples. The data are representative of three independent experiments. The over-expression of Myc-PIP4Kα or Myc-PIP4Kβ had no effect on transcriptions mediated by p53. Bottom: The expression levels of p53, FLAG-SIRT1(WT), Myc-PIP4Kα and Myc-PIP4Kβ proteins were assessed by Western Blotting (Anti-p53, Anti-FLAG and Anti-Myc).*

Suplemental experimental procedures

Plasmids and antibodies

FLAG-tagged murine SIRT1 was cloned into pcDNA3 and PGEX-4T-1s. SIRT1 deletion mutants (1-240, 211-353, 300-413, 400-494, 495-737) were cloned by PCR into PGEX-4T-1. SIRT1 point mutations [SIRT1(K3), SIRT1(K1R2), (211-353)SIRT1(K3) and (211-353)SIRT1(K1R2)] were generated by site-directed mutagenesis. pcDNA3-Myc-PIP4Kα, pcDNA3-Myc-PIP4Kβ, pCMV-p53, p21-Luc carrying 2.4 kb of the human p21 promoter and PUMA-Luc carrying two p53-responsive regions of the human PUMA promoter, were utilized as specified (El Deiry *et al.*, 1993; Yu *et al.*, 2001). ShRNA targeting SIRT1 was cloned into the pRetroSuper retroviral construct. Polyclonal antibody (P5) recognizing PIP4Kβ and Rat monoclonal antibody A3(recognizing both PIP4Kα and PIP4Kβ) were described (Bultsma *et al.*, 2010; Brooksbank *et al.*, 1993). Antibodies against SIRT1 and Acetyl-p53(Lys 382) were from Upstate Biotechnology and monoclonal p53 antibody (DO-1) from Santa Cruz Biotechnology. Monoclonals against tubulin and Flag-M2 were from Sigma. Anti-Myc antibody conjugated to peroxidase was from Invitrogen.

Cell culture and transfections

U2OS and HEK 293T cells were cultured in DMEM medium supplemented with 10% Foetal Bovine Serum (FBS), penicillin and streptomycin. H1299 cells were cultured in RPMI medium with 10% FBS, penicillin and streptomycin. DNA transfections were performed using polyethylenimine for HT1299 and HEK 293T cells and using FuGENE 6 Transfection Reagent (Roche Applied Science) for U2OS cells. Retroviruses produced in EcoPack 2-293 cells (Clontech) transfected with calcium phosphate, were harvested at 40 and 64 hr after transfection and utilized to infect U2OS cells for 48 hr. Selection with antibiotics after retroviral infection was for seven days. The Tet-On Myc-PIP4Kα-inducible cell line (U2OS- Myc-PIP4Kα) was generated by transducing U2OS cells with both pRetroX-Tet-On Advanced (Clontech) and Myc-PIP4Kα-pRetroX-Tight-Pur retroviral vectors. Myc-PIP4Kα -expression was induced by adding 1 μg/ml doxycycline). Knockdown of PIP4Kα and PIP4Kβ was achieved by retroviral transduction using pRetroSuper carrying the PIP4Kα shRNA targeting sequence 5'-ATAGTGGAATGTCATGGGA-3' and the PIP4Kβ shRNA targeting sequence 5'-AGATCAAGGTGGACAATCA-3'. Cells utilized for analyses of p53 acetylation were treated with MG132 (50 μM) for 30 min before harvesting.

Protein-lipid overlay and Surface Plasmon Resonance (SPR)

Full-length SIRT1 [GST-SIRT1(WT), GST-SIRT1(K3), GST-SIRT1(K1R2)] and SIRT1- deletions [GST-(211-353)SIRT1,GST-(211-353)SIRT1(K3) and GST-(211-353)SIRT1(K1R2)] were produced in E. coli Rosetta 2 induced with 300 μM IPTG and 50 μM ZnSO4. Bacteria were lysed using 30 mM Tris pH 7.5, 300 mM NaCl, 50 μM ZnSO4, 0.5% Triton X-100, 5 mM β-mercaptoethanol, protease inhibitor cocktail (Roche) and GST- tagged proteins were purified using glutathione-sepharose (GE Healthcare). Protein elution was performed using 30 mM Tris pH 7.5, 300 mM NaCl, 50 μM ZnSO4, 5 mM β-mercaptoethanol, 20 mM reduced glutathione. Full-length SIRT1 proteins were further purified by size exclusion chromatography using a S200 column (GE ÄKTA purifier, GE Healthcare) equilibrated in 20 mM Tris pH 7.5, 75 mM NaCl, 10 μM ZnSO4 and 5 mM β-mercaptoethanol. Protein-lipid overlays were performed using PIP Strips (Echelon Bioscience) and using protan nitrocelluse membranes spotted with the indicated serial dilutions of the specified lipids.

Surface Plasmon Resonance (SPR) was performed using the Bio-Rad Protein Interaction Array System ProteOn XPR36. Lipids were from CellSignals Inc. Sonicated lipid solutions were loaded onto a GLC sensor chip derivatized with undecylamine. GST- tagged SIRT1 proteins and small protein domains (2X-FYVE, PH-PLCdelta1, ING2-PHD) were diluted in Phosphate-Buffered Saline (PBS) supplemented with 10 μM ZnSO4. Analyte injections were performed at a flow rate of 50 μl/min. GLC sensor chip temperature was set at 25°C, association time was set as 120 sec, dissociation time as 600 sec. Response units (AU) for SPR signals, were presented using PC as a reference channel.

In vitro SIRT1 deacetylation assay

Lysine deacetylation was measured using a SIRT1 fluorescent activity assay (Biomol International/ Enzo Life Sciences). The reaction conditions were: 50 mM Tris HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1 mg/ml BSA), 200 μM NAD+, 100 μM Fluor de Lys–SIRT1 substrate. Incubation was carried out at 37°C for 30 min. Fluorometric reading on microplates was at wavelength 360 nm for excitation and 460 nm for emission. Water soluble PtdIns5P and PtdIns(3,4,5)P3 were from CellSignals Inc.

Immunoprecipitations and Western Blotting

Cells were lysed in 50 mM Tris HCl pH 8, 10 mM EDTA, 50 mM KCl, 1% NP40, 20 mM Sodium Fluoride, 1 mM Sodium Orthovanadate, 10 mM Nicotinamide, 1 μM TSA and protease inhibitor cocktail (Roche). Lysates were incubated overnight at 4°C with the indicated antibodies. Protein G-agarose was added for 1 hr at 4°C, immune-complexes were collected, washed three times with 50 mM Tris HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.1% Tween 20 and separated by SDS-PAGE followed by Immunoblotting

Luciferase reporter gene assay

A dual-reporter assay (Dual-Luciferase Reporter Assay System; Promega) was utilized. H1299 cells were co-transfected with firefly luciferase reporter (100 ng), Renilla luciferase vector (0.5 ng) and the indicated plasmids (400 ng). Cells were harvested after 24 hr and luciferase activity was measured. Activity was assayed in three separate experiments and shown as the mean of triplicate samples ± standard deviation (s.d.) of one representative experiment.

Apoptosis assay

U2OS cells were treated with etoposide (20 μM for 24 or 30 hr) or maintained as controls. Cells were collected, washed with PBS, incubated with Annexin V-FITC and Propidium Iodide according to the manufacturer's instructions (BD Bioscience Annexin V: FITC Apoptosis Detection Kit) and analyzed by flow cytometry.

Immunofluorescence

HEK 293T cells were fixed with Paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, blocked with 0.5% (BSA in PBS and incubated with primary antibody for 1 hr. Cells were washed and incubated with secondary antibody (Alexa Fluor 594 Goat anti-mouse IgG, Invitrogen) for 30 min Cells were stained with DAPI and were imaged using a Leica TCS SP2 laser scanning confocal microscope.

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Chapter 3

NUCLEAR PHOSPHOLIPIDS AS EPIGENETIC REGULATORS

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PREFACE

Nuclear phospholipids are important for regulating many essential processes such as DNA replication, RNA processing, cell cycle progression and gene expression. Recent advances in the field of epigenetics have indicated involvement of nuclear phospholipids in chromatin remodelling and gene transcription in yeast and animals. However, the molecular function of the nuclear phospholipids has remained elusive. In this review, the potential function of nuclear phospholipids like inositol polyphosphates, sphingolipds and phosphoinositides in remodelling of chromatin and epigenetic regulation of gene expression are discussed within the context of recent literature. Future research will help shed light on the functional significance of nuclear phospholipids as epigenetic therapeutic targets.

INTRODUCTION

Phospholipids are a large family of signaling lipids that play important roles in the control of a very wide range of cellular functions. Most of our knowledge on phospholipid signaling is derived from events occurring at the cytoplasm and plasma membrane; yet phosphoinositide signaling has been established in the nucleus as well. Although the existence of nuclear phospholipids has been known for a while, their functions remain largely unknown. This is partly because this research field has long been hampered by technical difficulties demonstrating nuclear phospholipid synthesis and explaining their endonuclear state. Once their presence was demonstrated in the nucleus and several phospholipids, such as phosphoinositides, phosphatiylcholine, sphingolipids but also inositol polyphosphates, were found associated with several components of the nuclear matrix, the question arose as to what their nuclear function might be. Recent findings have suggested that the interaction of phospholipids with transcription factors or chromatin-modifying enzymes may serve a role in regulation of gene expression. This review will focus on the most significant and recent findings about phospholipid signaling in the nucleus and their emerging role as important modulators of chromatin and gene transcription.

NUCLEAR PHOSPHOLIPIDS

In the cytosol, phospholipids are located in the (plasma) membrane bilayers, whereas in the nucleus a subset of these lipids resides outside membrane structures (Rees, 1963) (Barlow *et al.*, 2010). Several reports (that assessed the nuclear localization of these phospholipids) indicated their presence in nucleoli and were found to colocalize with RNA in interchromatin granules, which are known sites of transcription (La cour Lf *et al.*, 1958; Rose *et al.*, 1965). The cellular phospholipid levels were also found to change in a cell- cycle dependent manner, which suggested a role in transcription (Fakan, 1986; Fraschini, 1992). Thereupon, the idea evolved that phospholipids might be involved in mediating nuclear events like chromatin remodeling and transcription (Fraschini *et al.*, 1999; Manzoli, 1976). This idea was reinforced when evidence accumulated that various phospholipid-metabolizing enzymes (like phospholipases and PIPkinases), largely known for their signaling functions in the cytoplasm and at the plasma membrane, were also present in the nuclear compartment (Capitani, 1989; Payrastre, 1992; Smith, 1983; Vann, 1997).

NUCLEAR PHOSPHOINOSITIDES

Phosphoinositides were among the first families of phospholipids identified in the nucleus. Phosphoinositides represent a small (less than 5%) proportion of the total cell phospholipids, yet they play crucial roles in the regulation of cell metabolism through their involvement in intracellular signaling mechanisms (Hammond, 2004). Phosphoinositides contain two long hydrophobic fatty acyl tails linked to a glycerol group, which is coupled via a phosphodiester linker to an inositol head group. Combination of phosphorylation at the 3, 4 or 5 position of the inositol head group generates different biologically relevant variants that form the basis

of a ubiquitous signaling system. In the "canonical" phosphoinositide cycle, extracellular stimuli trigger the metabolism of phosphoinositides by the action of kinases, phosphatases and phospholipases (Irvine, 2005). Phosphoinositides are sensed by specific phosphoinositide-binding domains present in a diverse array of proteins (Lemmon, 2008). Much of our knowledge about phosphoinositide signaling is derived from cytosolic phosphoinositide pathways. However, early experimental data from Cocco and colleagues demonstrate that isolated intact nuclei from MEL cells were still able to synthesize phosphoinositides *in vitro* (Cocco, 1987). Moreover, they also showed that upon differentiation there was a change in the phospholyration of phosphoinositides present in the isolated intact nuclei. Subsequent biochemical and immunehistochemical studies identified phosphoinositide-metabolizing enzymes in the nucleus, responsible for the change in phosphoinositide levels (Divecha, 1993; Martelli, 1992; Vann, 1997). Collectively, *in vitro* and *in vivo* studies reinforced the existence of an endonuclear phosphoinositide pathway, and that its regulation and metabolizing enzymes are independent of the cytosolic pathway (Gonzales, 2006; Irvine, 1992).

NUCLEAR PI(4,5)P2 METABOLISM AND CHROMATIN STRUCTURE

The identification of an autonomous nuclear phosphoinositide cycle suggested that these phospholipids in the nucleus might function to regulate nuclear processes. In 1965, Rose and colleagues first identified phospholipids that were associated with the chromatin fraction. *In vitro* studies have indicated that nuclear phospholipids could be involved in DNA associated process(Rose *et al.*, 1965). For instance, adding phospholipids to purified nuclei influences DNA replication and transcription (Capitani, 1986; Kuvichkin, 2002). Another study showed that *in vitro* addition of positively charged lipids lead to chromosome condensation whereas negatively charged lipids caused decondensation, however questions regarding the mode of action remained (Kuvichkin, 2002). A clue came from the study of the phosphoinositide species phosphatidylinositol 4,5-bisphosphate PI(4,5)P_2 . This phosphoinositide was shown to bind to the C-terminal tail of histone H1. Depletion of histone H1 unfolds chromatin fibers and increases RNA polymerase II transcriptional activity, and therefore histone H1 is a potent inhibitor of RNA pol II. PI(4,5)P_2 binding to H1 releases H1 from DNA and therefore reverses this inhibition of RNA polymerase II (Yu, 1998). This provided an example regarding how chromatin structure could be regulated by phosphoinositides.

PI(4,5)P2 can also influence chromatin structure by facilitating the interaction between the nuclear matrix and the SWI/SNF-like chromatin remodeling complex called BAF (Zhao, 1998). The mammalian BAF complex is a very large ATP-dependent complex containing BAF53, actin and a central ATPase subunit called BRG-1. Within the BAF complex, BAF53 and actin interact with BRG-1 through two actin-binding domains, one of which contains a lysine-rich patch. Interestingly, this lysine-rich patch of BRG-1 is essential for its function *in vivo* and PI(4,5)P2 inhibits actin binding by this domain *in vitro* (Bourachot, 1999; Rando, 2002). This suggests that PI(4,5)P2 disrupts the interaction of BRG-1 with actin/BAF53 and consequently expose uncapped actin/BAF53 allowing actin to interact with other components of the in

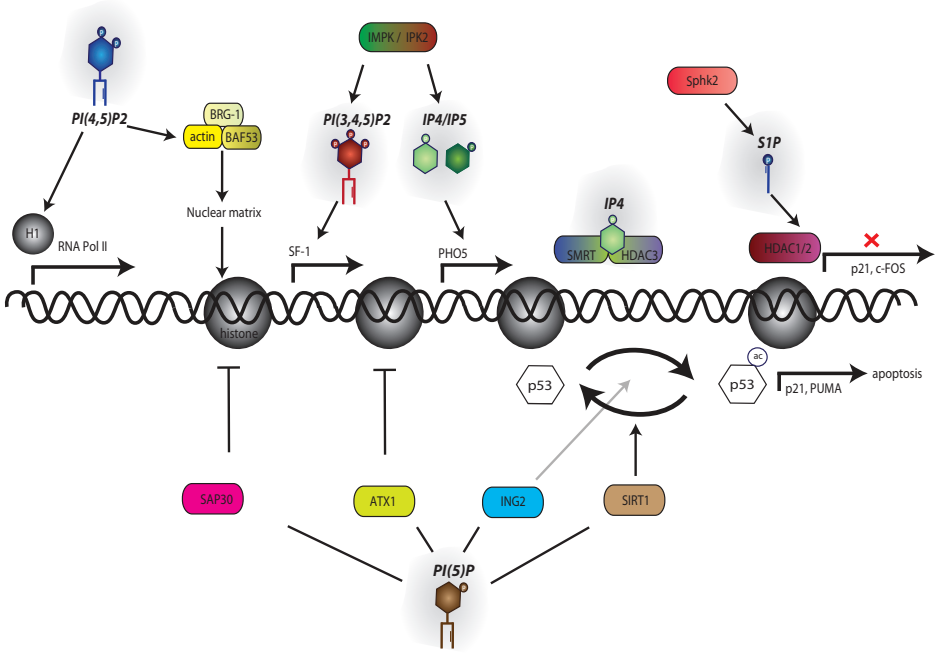

Figure 1. *Examples of regulation of chromatin remodeling and gene expression by phospholipids. Different phospholipids indicated in italic. PI(4,5)P_2 binding to Histone 1 (H1) releases H1 from DNA allowing RNA polymerase II binding. PI(4,5)P_2 binding stabilizes BAF complex by increasing its interaction with nuclear matrix and chromatin. Also PI(4,5)P_2 can mediate SF-1 mediated transcription by interaction with IMPK. Inositol polyphosphates can regulate gene transcription at PHO5 promotor trough the generation of IP_4/IP_5 by Ipk2 in yeast. IP_4 can also act as a modulatory bridging factor between HDAC3 deacetylase and co-repressor component SMRT. S1P formed by SphK2 inhibits histone deacetylases. Complexes containing SphK2 and histone deacetylases are enriched in the promotor regions of p21 and c-fos genes. Binding of PI(5)P to ATX1 inhibits association with chromatin and can also displace SAP30 from chromatin. Furthermore, PI(5)P can influence both acetylation and deacetylation of p53 trough binding of respectively ING2 and SIRT1 deacetylase and induction of p53 target genes and apoptosis.*

nuclear matrix (Janmey *et al.*, 1999; Shen, 2003). PI(4,5)P_2 binding to BRG1 may facilitate recruitment to chromatin and stabilize the chromatin remodeling complex by an increased interaction with nuclear matrix. These findings provide an example of phospholipid-induced chromatin remodeling. A question remains: how is phospholipids synthesis, in this example PI(4,5)P2, regulated at sites where it can affect chromatin (BAF complex) function? An attractive hypothesis suggests that pRB, a tumor suppressor gene that functions trough binding to BAF complex, interacts with and activates PIPKIα (Divecha, 2002). PIPKIα is the enzyme responsible for almost all PI(4,5)P2 synthesis. Therefore, it is plausible that pRB recruits PIPKIα to the BAF complex to control localized PI(4,5)P2 production.

INOSITOL POLYPHOSPHATES REGULATING GENE TRANSCRIPTION

Next to PIPKIα, nuclear PI(4,5)P2 levels can also be regulated via the activity of other metabolizing enzymes such as nuclear phospholipase C isoforms (PLCs). These PLCs catalyze the hydrolysis of PI(4,5)P2, thereby generating the two second messengers DAG and IP_3 (van den Bout *et al.*, 2009). Phosphorylation of IP_3 by inositol polyphosphate kinases (Ipks) produces a set of inositol polyphosphate second messengers. Intriguingly, studies in yeast have implicated these inositol polyphosphates in nuclear transcription (Odom, 2000). In yeast IP3, generated by PI(4,5)P2 hydrolysis, is utilized by Ipk2 to produce IP4 and IP5, which can be further phosphorylated by Ipk1 to IP6 (York, 1999). Strikingly, in yeast this kinase Ipk2 is also known as a transcription factor named ARG82. Steger *et al*. showed that in an *Ipk2/arg82* deficient yeast strain, chromatin remodeling at PHO5, a phosphate responsive promoter was impaired (Steger, 2003). PHO5 transcription depends on either IP4 or IP5, because mutations blocking the pathways leading to IP6 have no effect on transcription. Their data showed that chromatin remodeling at the PHO5 nucleosome required for PHO5 transcription depended on Ipk2/ARG82 inositol kinase activity in yeast. IPMK, the human homologue of ARG82/ Ipk2 has also been reported to phosphorylate IP3 to IP4 and IP5. Blind *et al*. demonstrated a role for IPMK in gene transcription in mammalian cells. IPMK can not only function as an inositol kinase, but also as phosphoinositide 3-kinase (PI3K) that can phosphorylate PI(4,5)P2 to phosphatidylinositol 3,4,5-trisphosphate PI(3,4,5)P3 (Blind, 2012). IPMK was also found to interact with nuclear receptor steroidogenic factor 1 (SF-1) and to phosphorylate its bound ligand PI(4,5)P2 to PI(3,4,5)P3, which subsequently affects SF-1-mediated transcription. A structural study has shown that phosphoinositides, including PI(4,5)P2 and PI(3,4,5)P3, are ligands for nuclear receptor (Krylova *et al*., 2005). The ability of Ipk2 and IPMK to directly remodel and alter the activity of a transcription factor-lipid complex exemplifies how phospholipids can regulate gene expression (Chakraborty, 2011).

LINKING PHOSPHOLIPIDS TO ACETYLATION

The role of the other inositol phosphates and their kinases in mammalian chromatin remodeling complexes, or how they might exert these roles, has not been demonstrated yet. However, a recent study by Watson *et al*., has provided a mechanistic insight into how inositol phosphate IP4 can regulate chromatin structure through the study of histone deacetylases in repressive complexes (Kutateladze, 2012).

Acetylation is a key posttranslational modification on lysine residues in many histone and non-histone proteins. Acetylation of histones by histone acetyltransferases (HAT's) directly influences chromatin remodeling, presumably by reducing the positive charge on lysines hence the affinity of histones for negatively charged DNA. The chromatin is then allowed to become more accessible for binding to the basal transcription machinery (Choudhary *et al*., 2009). Conversely, histone deacetylases can remove acetyl groups from histones and other nuclear proteins, thereby inducing chromatin condensation and transcriptional repression. Watson *et al*. resolved the structure of a complex between HDAC3 deacetylase and the deacetylase activation domain (DAD) of co-repressor SMRT, and revealed IP4 to function as a

modulatory bridging factor between the two proteins, which contributes to the stabilization and activation of HDAC3 (Watson, 2012). IP4 binding was shown to be essential for both the incorporation of the histone deacetylase HDAC3 into a repressive complex and its deacetylase activity. The authors argued that a conformational change of HDAC3, which occurs upon its interaction with IP4 and DAD-SMRT, facilitates the access of substrates to the HDAC3 active site(Kutateladze, 2013). This study provides a possible explanation of why inositol lipids and their kinases are found in the chromatin fraction, and reveals an interesting link between inositol phosphates signaling and epigenetic regulation.

Similarly, another family of phospholipids, nuclear sphingolipids and their kinases can also affect HDAC activity. New evidence revealed that HDAC1 and HDAC2 activity can be inhibited by the sphingolipid metabolite sphingosine-1-phosphate (S1P). Both nuclear S1P and sphingosine kinase 2 (SphK2), the enzyme that synthesizes S1P, were found to be part of a corepressor complex containing HDAC1 and HDAC2 (Hait *et al.*, 2009). SphK2 binding to p21 and c-fos promotors enhanced acetylation of histone H3 and transcription. Furthermore, S1P was shown to inhibit deacetylase activity of HDAC1 and HDAC2. These studies exemplify nuclear lipids that are associated with an epigenetic modification and shows how nuclear phospholipid signaling can regulate chromatin-remodeling enzymes that influence histone acetylation and gene expression (Riccio, 2010). However, it remains to be determined how these nuclear phospholipid signalling pathways can influence HDAC dependent gene repression and how specificity towards acetylated targets is regulated.

CHROMATIN REMODELING AND PI(5)P; DOES IT TAKE A PHD?

Like nuclear S1P and inositol phosphates, nuclear phosphoinositides are implicated in gene transcription and chromatin remodeling by several studies. Many of these studies have been centered on the role of nuclear PI(4,5)P2 in gene expression, as discussed above (Bunce, 2006). There are however, other examples of phosphoinositide species that interact with chromatin. Phosphatidylinositol mono-phosphates (PI3P, PI4P and PI5P) have long been considered to be just intermediate metabolites for the synthesis of PI(4,5)P2 and other phosphoinositides via their (de)phosphorylation by PIP kinases and phosphatases (Doughman *et al.*, 2003). However, it is now clear that phosphatidylinositol monophosphates levels are dynamic and serve important signaling functions by themselves (Pendaries, 2005). For instance, they change abundantly when cells progress through the cell cycle. Nuclear PI(3)P accumulates during G2/M phase in HL-60 cells by activation of the phosphatidylinositol 3 kinase , PI3K-C2β. In MEL cell nuclei, the abundance of PI(4)P and PI(5)P (but not PI(3)P) increases as cells progress through the G1-S phase of the cell cycle, which suggests a potential role for these phosphoinositides in cell cycle progression (Clarke *et al.*, 2001a; Visnjic, 2003). The nuclear levels of phosphoinositide mono-phosphates can be increased by various cellular responses and stresses as well. Notably PI(3)P levels respond to RA treatment and PI (4)P, PI(5)P upon UV etoposide treatment (Jones *et al.*, 2006; Visnjic, 2002).

Recently, it was shown that PI(5)P can act as a redox-regulated second messenger, in that its levels regulate oxidative stress responses (Keune *et al.*, 2012). To date, there is no evidence for a role of nuclear PI3P or PI4P in chromatin remodeling. However, the identification of PI(5)P binding proteins and their nuclear functions are now beginning to be elucidated (Keune *et al.*,

2011). A breakthrough came from the discovery that ING2, which regulates p53 acetylation and function, binds to PI(5)P through its PHD domain (plant homeo domain) (Gozani *et al*., 2003). ING2 promotes the acetylation of p53 and induces p53-mediated apoptosis in response to UV and etoposide. In contrast to wild-type ING2, point mutated ING2 that resulted in loss of PI(5)P binding was compromised in the ability to induce apoptosis and p53 acetylation. In addition, overexpression of PIP4Kβ, which depletes nuclear PI5P by converting it to PI(4,5)P2, significantly inhibited ING2-mediated apoptosis. This study was the first to identify the PHD motif as a PI(5)P-binding module and ING2 as the first PI(5)P binding protein. Expanding on these studies, Jones *et al*. showed that PIP4Kβ becomes phosphorylated by p38 MAPK in response to genotoxic stress (Jones *et al*., 2006). This phosphorylation inhibits PIP4Kβ resulting in accumulations nuclear PI(5)P. This stress induces increase in nuclear PI(5)P causes translocation of ING2 to the chromatin-enriched fraction, where it binds and activates ING2 thereby modulating p53 acetylation and function. The ING2 experimental data exemplifies that phosphoinositides, notably PI(5)P, can modulate the association of remodelling enzymes with chromatin and can be regulated by phosphoinositide metabolizing enzymes (Jones *et al*., 2004). Precisely how PI(5)P promotes binding or detachment of protein-chromatin interactions is currently not known. One could imagine that, similar to PKB activation, interaction of PI(5)P with ING2 could lead to its allosteric activation or recruitment of other binding partners that change ING2 association with chromatin(Franke *et al*., 1997), or that PI(5)P can act as a modulatory bridging factor, similar to the IP4-HDAC association described above (Kutateladze, 2012).

In addition to PI(5)P binding, the PHD motif of ING2 also binds histone H3 trimethylated at lysine 4 (H3K4me3). Upon DNA damage, H3K4me3 binds to ING2, which is essential for the activity of ING2 (Shi, 2006). Together with HDAC1 and Sin3A, ING2 forms a complex that transcriptionally represses active promoters. Interestingly, PHD fingers are mostly found on nuclear proteins involved in regulating gene transcription through the modulation of chromatin structure (Bienz, 2006). An attractive hypothesis is that upon DNA damage, elevated PI(5)P levels recruit ING2 to specific (target) promotors, which would facilitate H3K4me3 binding to ING2 and stabilize ING2 to regulate transcriptional activity at these promotors. However, it is currently not clear how PI(5)P binding can modulate H3K4me3, or vice versa.

There is however some data in plants suggesting that PI(5)P might regulate the levels of H3K4me3 under certain conditions (Alvarez-Venegas *et al*., 2006). ATX1, the Arabidopsis homolog of trithorax, is a trimethyltransferase that trimethylates histone 3 lysine 4 (H3K4me3) at the nucleosome of promotor regions. Others show not only that ATX1 binds PI(5)P through the ATX-PHD domain, but also that PI(5)P binding could negatively affect ATX1 trimethyltransferase-activity and causes ATX1 to detach from promoters and translocate from the nucleus to the cytosol (Ndamukong *et al*., 2010). This is opposite to the effect (described above) where PI(5)P positively affected ING2 activity by translocation into to the nucleus and facilitating recruitment of ING2 to chromatin. This suggests that the effect of the interaction of PI(5)P with PHD domains can have different consequences on the activity of (and therefore association with) PHD-containing proteins with chromatin. In addition, PI(5)P dependent chromatin remodelling can also occur on proteins that do not contain PHD fingers (Viiri, 2009). Components of the Sin3A corepressor complex SAP30 and SAP30L were found to bind

immobilized phosphoinositides, particularly PI(5)P through a zinc motif that overlaps with its DNA binding site. The authors proposed that PI(5)P binding displaces DNA from the Sin3A corepressor complex by modulating SAP30 repressor activity and translocate them from the nucleus to the cytoplasm (Viiri, 2012). Possibly, the displacement of these proteins from the DNA by PI(5)P might be similar to the Histone H1 displacement from RNA by PI(4,5)P2 binding describe above.

Interestingly, PI(5)P can bind to another histone-protein deacetylase that does not contain a PHD domain. We found that nuclear PI(5)P can modulate the activity of another deacetylase namely human sirtuin 1 SIRT1, a NAD+dependent deacetylase that belongs to the class III deacetylases. SIRT1 is recruited to chromatin where it modifies and silenced transcription by histone deacetylation or influences acetylation of many transcriptional regulators including transcription factor p53 (Rajendran *et al.*, 2011; Vaziri *et al.*, 2001). We identified a previously unrecognized polybasic region (PBR) that binds to PI(5)P, and this interaction is essential for the SIRT1-mediated deacetylation of p53 both *in vitro* and *in vivo* (Elouarrat *et al.*, submitted). Subsequently, manipulation of nuclear PI(5)P (by overexpression/depletion of PIP4K) changed SIRT1 deacetylase activity and SIRT1 dependent p53 transcription and apoptosis. As nuclear PI(5)P can influence both acetylation (by binding to PHD domain of ING2) and deacetylation of p53 (by binding to PBR of SIRT1), we expect that the temporal and spatial regulation of PI(5)P likely determines the strength and duration of p53 activation and transcriptional activity. Together, these studies emphasize the role of nuclear phosphoinositide monophosphates in regulating chromatin-modifying enzymes and suggest an important general role for them as phospholipid signal transducing enzymes.

FUTURE PERSPECTIVES AND CONCLUDING REMARKS

Studies over the last decades in plants, yeast and mammalian cells have revealed the existence of phospholipid signaling and metabolism in the nucleus. The multitude of roles they play in nuclear events like chromatin structure and gene expression are only now beginning to be uncovered. The recent advances in identifying the specific nuclear proteins that interact with and are regulated by nuclear phospholipids have been pivotal to reveal these roles. Despite significant advances in this field, many questions still remain regarding the mechanistic regulation of distinct nuclear phospholipids associating with target proteins. One could speculate that (local) changes of phospholipid levels can induce the recruitment of chromatin-remodeling proteins. This recruitment may induce a conformational change of the protein, which changes their interaction partners, localization or activity. It is therefore required to gain a better understanding of how phospholipid levels are regulated by their metabolizing enzymes, as was revealed in the studies on S1P/SphK2 regulating HDAC deacetylase activity or PI(4,5)P2/IMPK regulating SF-1 transcriptional activity (Blind, 2012; Krylova *et al.*, 2005; Watson, 2012). Further studies on these and other phospholipid metabolizing enzymes will provide new insights into how signaling phospholipids are loaded and gain access to their nuclear target protein.

One might wonder why nuclear processes like chromatin remodeling would be regulated by phospholipid signaling. Phospholipids are known to act as acutely regulated signaling

platforms that assemble and coordinately regulate components of cellular pathways (Irvine, 2005). Therefore, they would provide an ideal signal transducing mechanism that can acutely respond to external cues and by transducing to chromatin-associated proteins can rapidly alter gene transcription program. Indications for this come from for example, when chromatin-modifying enzymes associate with active or repressor complexes on promoters. One could speculate that upon the assembly of chromatin complexes on promotors, the production of phospholipid signalling molecules provides a means of rapidly modulating the transcription of their target genes upon differential stimuli and keeping the promotors in an (in) active state.

Recent advances in nuclear phospholipid signaling have opened a field of epigenetics that was previously unknown. Obviously, much work still lies ahead in order to better understand the mechanisms by which phosphoinositides participate in chromatin dynamics and gene transcription. Future efforts should focus on the identification of new (nuclear-specific) phosphoinositide-binding motifs, which will constitute a key step in our comprehension of nuclear phosphoinositides, their transducing mechanism and their functions. A recent unbiased proteomics study for nuclear PI(4,5)P2 interacting proteins has revealed novel nuclear phosphoinositide-binding proteins, many of which have known functional roles in chromatin assembly and no prior history of phosphoinositides interactions (Lewis *et al.*, 2011). Efforts like these and on other nuclear phospholipids will enhance our knowledge on their roles in chromatin remodeling. Additionally, the use of new emerging techniques for sensitive lipidomics profiling of the nucleus will provide a powerful tool for further insight in understanding phospholipid nuclear functions (Hunt, 2006). Elucidating the molecular and cellular consequences of phospholipids as lipid mediator of epigenetic modifications and establishing how widespread this is are going to be crucial. Ultimately, the nuclear roles of phospholipids might offer some new insights in to the development of novel small molecule targets for cancer therapy. Like their counterparts in the cytosol, nuclear phosphoinositides turn out to be essential regulators of diverse cellular processes and functions.

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Chapter 4

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

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ABSTRACT

We have previously shown that phosphoinositides, particularly PI5P, can bind to SIRT1 and regulate its deacetylase activity. Many proteins that are regulated by phosphoinositides also interact with phosphosinositide-metabolizing enzymes. We therefore investigated if SIRT1 interacts with phosphatidylinositol-5-phosphate 4 kinases (PIP4K) which phosphorylate PI(5)P to PI(4,5)P_2. Through immunoprecipitation studies, we found that SIRT1 interacts with PIP4Kβ, but not with the PIP4Kα or PIP4Kγ isoforms, both in transfected cells and endogenously. Mass spectrometric analysis was carried out on purified PIP4Kβ from HEK293T cells that were insulted with UV, cisplatin and nicotinamide. Sites of post translational modification on PIP4Kβ were determined using mass spectrometry. Acetylation of lysine 239 increased in cells treated with nicotinamide suggesting that this acetylation site might be regulated by a NAD dependent deacetylase. Using an antibody against PIP4K239ac, we show that acetylated PIP4Kβ is deacetylated by recombinant SIRT1 in a nicotinamide-sensitive manner. We conclude that upon different insults PIP4Kβ can become acetylated and is deacetylated by SIRT1. The (de) acetylation PIP4Kβ could possibly play a regulatory role in the PI(5)P induced stimulation of SIRT1 deacetylase activity trough a possible feedback loop.

INTRODUCTION

Protein acetylation is a widespread reversible modification, at specific sites lysine residues, which can be subjected to (de)acetylation by numerous enzymes within the cell (Choudhary *et al.*, 2009; Ellis *et al.*, 2008). Following the initial discovery of acetylation as a posttranslational modification on histones in the early 1960, extensive studies over the past four decades have not only identified the enzymes that catalyze reversible acetylation, the protein lysine acetyltransferases and deacetylases (HDACs), but have also identified many nonhistone substrates(Norris *et al.*, 2009). One of these deacetylation enzymes SIRT1, the closest mammalian orthologue to yeast Sir2, is a NAD^+ dependent deacetylase that functions by modulating acetylation of many targets involved in various cellular pathways via deacetylation (Guarente, 2011). By regulating protein lysine acetylation, SIRT1 has numerous functional consequences on its targets including regulation of their catalytic activity, protein stability, subcellular localization and specific protein–protein interactions (Revollo *et al.*, 2013).

Phosphatidylinositol-5-phosphate 4 kinases (PIP4Ks) play a pivotal role in nuclear phosphoinositide metabolism (Clarke *et al.*, 2012). By phosphorylating PI(5)P to produce PI(4,5)P2, the PIP4Ks reduce the abundance of cellular PI(5)P. PIP4Ks are encoded by three distinct genes (α, β and γ), with the α isoform having much higher catalytic activity than the β and γ isoforms (Bultsma *et al.*, 2010; Wang *et al.*, 2010). Also of the three isoforms, which are localized at different subcelllular compartments, PIP4Kβ is predominantly localized in the nucleus. A well understood function of PIP4Kβ comes from the nucleus (Bunce *et al.*, 2006; Ciruela *et al.*, 2000). Upon stress, cells activate p38 MAP kinase, which phosphorylates Ser^{326} on PIP4Kβ causing a decrease in PIP4K activity leading to an increase in the nuclear abundance of PI(5)P (Jones *et al.*, 2006). PI(5)P can interact with PHD-domain-containing proteins like "inhibitor of growth protein 2" (ING2), and a forced increase in PI(5)P levels promotes ING2-mediated apoptosis. ING2 promotes acetylation of p53 and induces p53-mediated apoptosis in response to stress insults (Gozani *et al.*, 2003; Jones *et al.*, 2004).

We have previously shown that PI(5)P can bind to SIRT1 and regulate its deacetylase activity. Many proteins that are regulated by phosphoinositides (PI) also interact with their corresponding PI-metabolizing enzymes. Here we report that PIP4Kβ is posttranslationally modified by acetylation at several lysine residues. We show that Lysine 239 on PIP4Kβ can be deacetylated by SIRT1 deacetylase. Acetylation of PIP4Kβ might modulate its activity and/or localization, and thereby regulate the PI(5)P-SIRT1 signalling pathway.

RESULTS

PIP4Kβ interacts with SIRT1

Since PI(5)P was identified as a novel lipid regulator of SIRT1 activity, we examined the possible regulatory role PI(5)P- metabolizing enzymes. We therefore investigated if SIRT1 can interact with PIP4Ks by performing co-immunoprecipitations using Hek293T cells. Hek293T cells were co-transfected with expression vectors for FLAG-tagged SIRT1 and MYC-PIP4K (α, β or γ isoforms). Cellular extracts from the transfected cells were immunoprecipitated with anti-FLAG antibody and the resulting immune complexes were collected and analysed by immunoblotting

using anti-MYC antibody. Amongst the PIP4Ks, PIP4Kβ was selectively immunoprecipitated with SIRT1, whereas PIP4Kα and PIP4Kγ were not (Figure 1A, B).

To confirm the interaction between endogenous PIP4Kβ and SIRT1, we performed a co-immunoprecipitation assay using U2OS cells. Cell lysates were immunoprecipitated using anti-PIP4Kβ or control preimmune serum, followed by western blot analysis using anti-SIRT1 and anti- PIP4Kβ. Endogenous SIRT1 was detected in the anti-PIP4Kβ immunoprecipitates, but not in those using preimmune serum (Figure 1C); this interaction was lost in cells stably expressing short hairpin RNAi against PIP4Kβ (Figure 1C). These results indicate that PIP4Kβ interacts

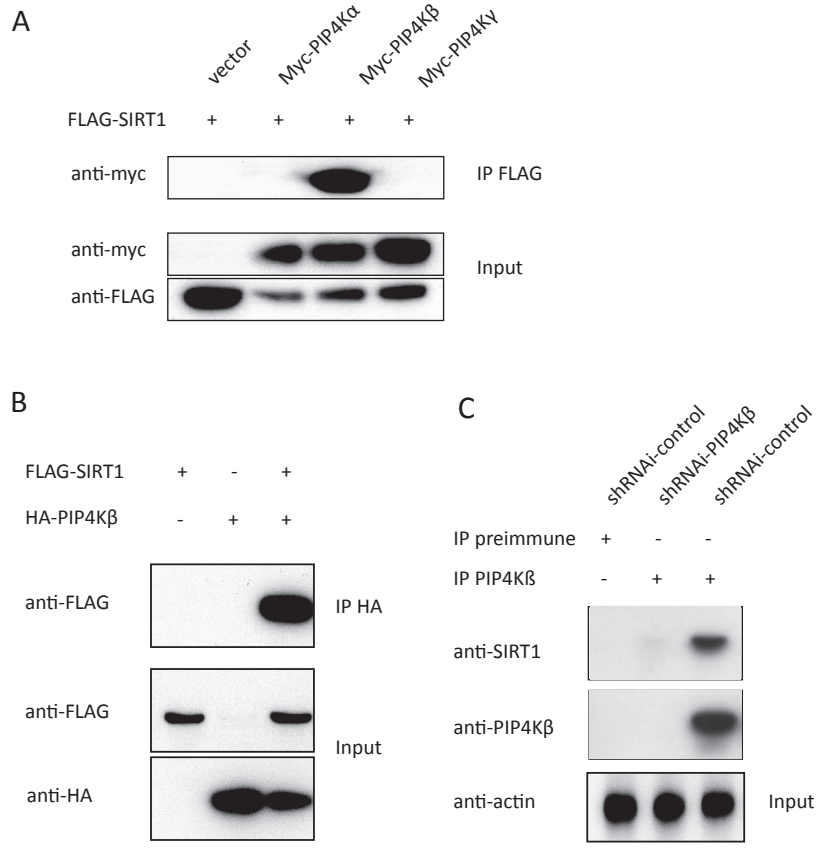

Figure 1. *PIP4Kβ interacts with SIRT1. (A) Co-immunoprecipitation of SIRT1 and PIP4Kβ HEK293T cells transiently transfected with the indicated plasmids. Cell lysates were immunoprecipated with anti-FLAG antibody and immunoblotted with anti-MYC antibody. Input blots as control. (B) Co-immunoprecipitation of FLAG-SIRT1 and HA-PIP4Kβ HEK293T cells transiently transfected with the indicated plasmids. The immunoprecipitates HA-PIP4Kβ were immunoblotted with the reciprocal anti-FLAG antibody. Expression level of HA-PIP4Kβ and FLAG-SIRT1 were assessed in input immunoblot. (C) Endogenous SIRT1 interacts with PIP4Kβ. The lysates of control and PIP4Kβ shRNAi U2OS cells were immunoprecipitated with anti-PIP4Kβ or preimmune serum. The immunoprecipitates were immunoblotted with anti-SIRT1. Anti-actin was used as input control.*

with SIRT1. Further analysis using recombinant purified SIRT1 and PIP4Kβ confirmed that this interaction was direct (data not shown).

Identification of PIP4Kβ acetylation sites

We have previously shown that PIP4Kβ is subject to phosphorylation (Jones *et al.*, 2006; Keune *et al.*, 2012). The binding of SIRT1 to PIP4Kβ suggests that PIP4Kβ could also be modified by acetylation. To map possible acetylated lysine residues on PIP4Kβ, we performed LC-MS/MS. To identify the lysines that might be subject to acetylation, cells were insulted by diverse agents including UV, cisplatin and the sirtuin inhibitor nicotinamide. HA-PIP4Kβ was immunopurified from control HEK293 cells, and from UV-irradiated, cisplatin- and nicotinamide-treated HEK293 cells, excised and digested from SDS gel, and analysed by mass spectrometry. Several acetylated lysine containing peptides were identified. As K239 acetylation levels accumulated only after using nicotinamide, K239 is likely deacetylated by SIRT1 (Figure 2B). We then investigated whether K239 on PIP4Kβ is a target of SIRT1.

A

PIP4Kß acetylated lysines

MSSNCTSTTAVAVAPLSASKTKTKKKHFVCQKVKLFRASEPILSVLMWGVNHTINELSNVPVPVMLMPDD
FKAYSKIKVDNHLFNKENLPSRFKFKEYCPMVFRNLRERFGIDDQDYQNSVTRSAPINSDSQGRCGTRFL
TTYDRRFVIKTVSSEDVAEMHNILKKYHQFIVECHGNTLLPQFLGMYRLTVDGVETYMVVTRNVFSHRLT
VHRKYDLKGSTVAREASDKEKAKDLPTFKDNDFLNEGQKLHVGEESKKNFLEKLKRDVEFLAQLKIMDYS
LLVGIHDVDRAEQEEMEVEERAEDEECENDGVGGNLLCSYGTPPDSPGNLLSFPRFFGPGEFDPSVDVYA
MKSHESSPKKEVYFMAIIDILTPYDTKKKAAHAAKTVKHGAGAEISTVNPEQYSKRFNEFMSNILT

B

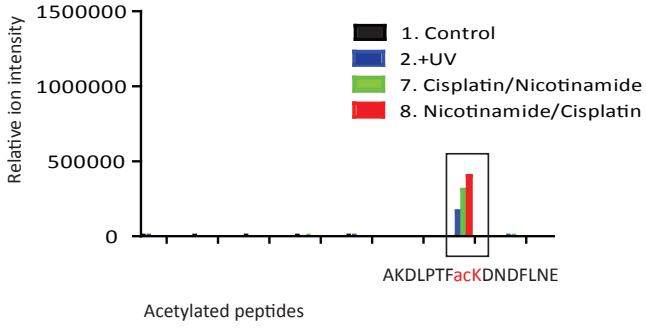

Figure 2. *Identification of acetylation sites in PIP4Kβ by mass spectrometry. (A) Sequence of human PIP4Kβ. The circled red marked lysine residue is acetylated lysine 239 and the other residues marked in red constitute a cluster of basic side chains in the PIP4Kβ-PI(5)P structural model (Rao et al., 1998). Green residues indicate previously identified sites of phosphorylation of threonine 322 and serine 326 (Jones et al., 2006). The letters in dark blue indicate the catalytic domain of PIP4Kβ. (B) Mass spectrometric analysis of HA-PIP4Kβ from control and UV, cisplatin and nicotinamide stimulated cells. Due to a confidationality agreement we can only depict peptide sequence of acetylated lysine 239. Relative ion intensity (y axis) of acetylated peptides (x axis) of PIP4Kβ from control and stimulated cells.*

PIP4Kβ is acetylated *in vitro*

To further characterize K239 acetylation, we developed an acetyl-specific antibody (termed ac-K239- PIP4Kβ) directed against this residue. After negative selection of the rabbit sera using a nonacetylated peptide (corresponding to residues 234-244), ELISA demonstrated that the antibody only recognized the acetylated peptide (Figure 3A).

To demonstrate that ac-K239-PIP4Kβ antibody only recognized acetylated PIP4Kβ, we chemically acetylated recombinant PIP4Kβ purified from bacteria using NHS-acetyl reagent. Benzoylation using NHS-benzyl was used as a control, proteins were separated by SDS-PAGE and immunoblotted using ac-K239-PIP4Kβ antibody. As shown in Figure 3B, ac-K239-PIP4Kβ antibody recognized recombinant PIP4Kβ only after acetylation, but not after benzoylation. To verify the specificity of the ac-K239-PIP4Kβ antibody, we generated mutant GST-PIP4Kβ(K239R). Arginine residues cannot be acetylated while retaining the positive charge. As shown in Figure 3C, recognition is severely comprised by the K239R mutation. As GST-PIP4K2B will still become acetylated at many other lysine residues by NHS-acetyl, this data confirms the specificity of the antibody for Lyisine 239. It should be noted that it appears that the NHS reagent effects the separation of these recombinant proteins by SDS-page, as seen on the total PIP4Kβ.

PIP4Kβ is acetylated *in vivo*

Next, we assessed whether PIP4Kβ was acetylated on lysine 239 in cells. To confirm the acetylation of PIP4Kβ, mutant versions of GFP-tagged PIP4Kβ were mutated on lysine 239 to arginine (acetylation dead) or glutamine (acetylation mimic) and expressed in U2OS cells and immunoprecipitated using GFP-trap beads. The total amount of protein was detected by western blot using anti-GFP antibody, and their K239 acetylation levels were determined using ac-K239-PIP4Kβ antibody. Upon expression of PIP4Kβ acetylation was observed and not then K239 was mutated to arginine (K239R), indicating that in cells K239 is subject to acetylation (Figure 4A). Furthermore, the antibody did also recognized PIP4Kβ (K239Q) as acetylation mimiq, although it was weaker than observed with wild-type PIP4Kβ (Figure 4A).

To confirm endogenous PIP4Kβ acetylation, the protein was immunoprecipated from HT1080 cells and detected using anti-PIP4Kβ antibody (p6). As shown figure 4B, PIP4Kβ is endogenously postranslationally modified by acetylation as detected by the ac-K239-PIP4Kβ antibody. We conclude that PIP4Kβ is acetylated on K239 both *in vitro* and *in vivo*.

SIRT1 targets PIP4Kβ and deacetylates K239

We investigated whether K239 on PIP4Kβ can be deacetylated by SIRT1. We first performed deacetylation assays using bacterially expressed recombinant GST-SIRT1 and GST-PIP4Kβ. PIP4Kβ was incubated in the reaction buffer containing GST-SIRT1 in the presence of NAD^+ or nicotinamide. The samples were resolved by SDS-PAGE, and the acetylation status was monitored by immunoblotting using ac-K239-PIP4Kβ antibody. As shown in Figure 4C, recombinant SIRT1 deacetylated PIP4Kβ only in the presence of NAD^+ but not of nicotinamide. These results indicate that SIRT1 can deacetylate PIP4Kβ in an NAD^+-dependent manner and identify PIP4Kβ as a target of SIRT1 deacetylase.

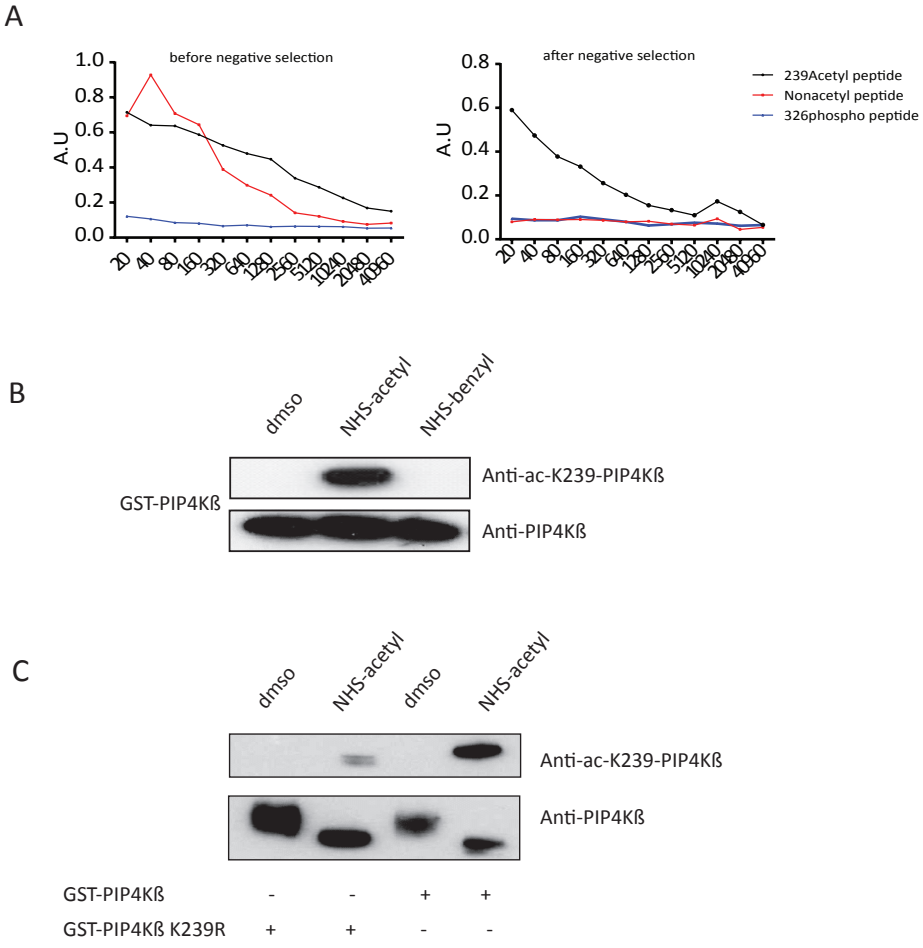

Figure 3. *Generation of an acetylation specific antiserum against 239 acetylated PIP4Kβ. (A) Elisa analysis of serum against 239 acetylated PIP4Kβ; measured O.D of diluted of serum (1:20 till 40960) before and after negative selection on 3 different peptide coated wells (239 non acetyl, 239 acetyl, 326 phospho peptides). This graph shows that after negative selection the serum specifically recognizes the acetylated PIP4Kβ peptide and is a presentative of two replicate experiments. (B) Recombinant GST-PIP4Kβ was chemically acetylated or benzylolated on all lysines, DMSO was used as a control for the reactions. Western blot analysis detected the acetylated fraction using the negatively selected PIP4Kβ serum (anti-ac-239-PIP4Kβ), anti- PIP4Kβ was used for total protein. (C) Western blot analysis of acetylated recombinant wildtype and K239R mutant protein. Chemical acetylation reaction preformed with recombinant wildtype and K239R protein in presence of NHS-acetyl or DMSO. Reactions were stopped with SDS-PAGE lysis buffer and proteins were resolved on SDS-PAGE and immunoblotted with anti-ac-239-PIP4Kβ and for p6-antibody total PIP4Kβ protein.*

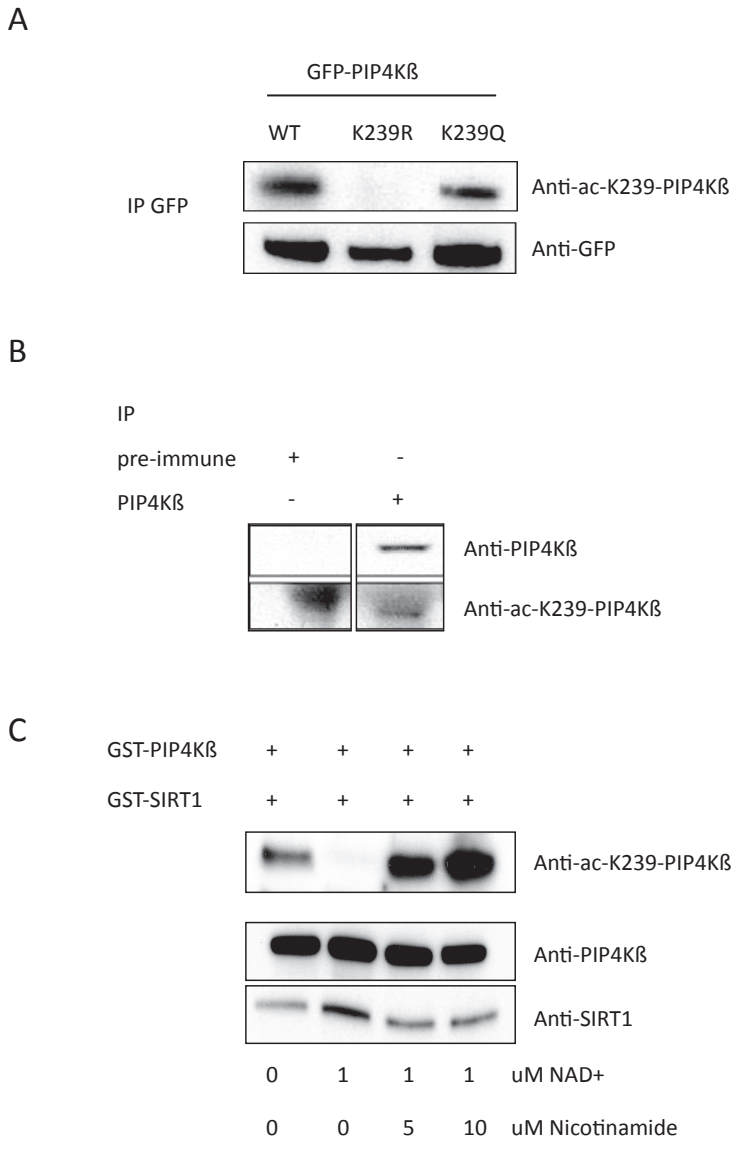

Figure 4. *Acetylated PIP4Kβ is deacetylated by SIRT1 on lysine 239. (A) GFP-PIP4Kβ wildtype K239R and K239Q mutant were transfected in HEK293Tcells that were treated with nicotinamide for 2 hour before harvesting. Lysates were immunoprecipitated using anti-GFP antibody and acetylation level of PIP4Kβ were analyzed by western blot with anti-ac-239-PIP4Kβ. (B) Immunoprecipitation and western blot analysis of endogenous acetylated lysine residue 239 PIP4Kβ in U2OS cells. The acetylated fraction of PIP4Kβ was determined using anti-ac-239-PIP4Kβ and total protein by anti-PIP4Kβ. (C) After the deacetylation reaction (see experimental procedures) the amount of acetylated PIP4Kβ was determined using SDS-page and immunoblotting with anti-ac-239-PIP4Kβ. Immunoblotting also detected the amount of PIP4Kβ and SIRT1 protein used per reaction with the indicated amount of NAD⁺ and nicotinamide.*

Acetylation does not affect the localization of PIP4Kβ in U2OS cells

In an attempt to elucidate what the functional significance of PIP4Kβ acetylation is, we hypothesized that acetylation of PIP4Kβ may affect its subcelullar localization (Ciruela *et al.*, 2000). Like SIRT1, PIP4Kβ is predominantly localized in the nucleus therefore; deacetylation by SIRT1 may target PIP4Kβ to a different subcellular compartment, in addition to altering its kinase activity. We examined the localization of PIP4Kβ and its acetylation mutants control

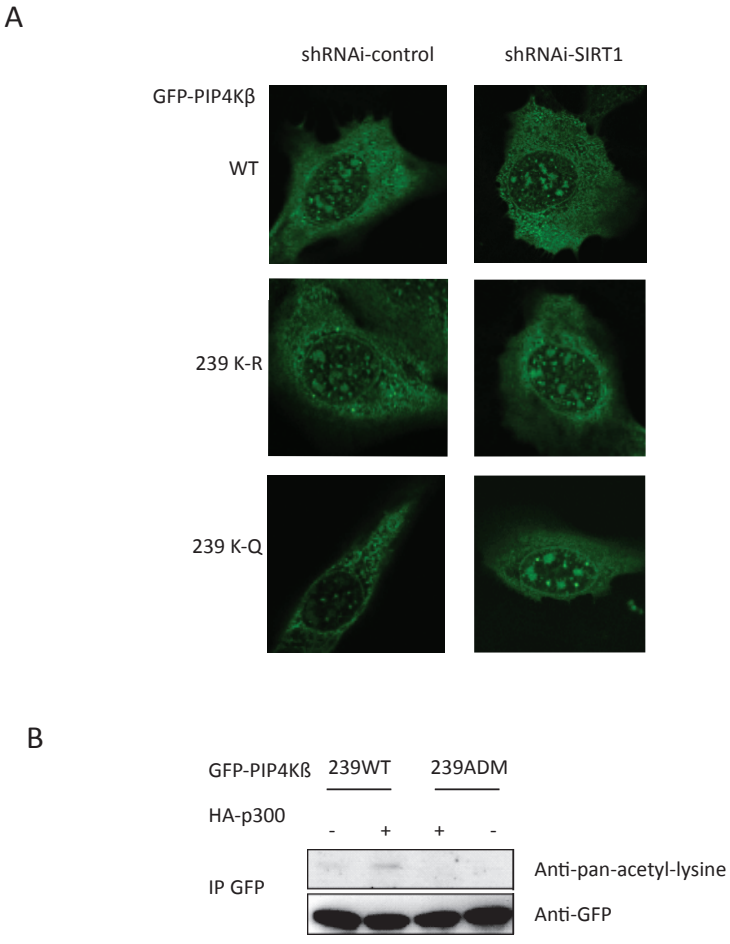

Figure 5. *(A) Acetylation does not affect the localization of PIP4Kβ in U2OS cells. Control and SIRT1 shRNAi U2OS cells transfected with GFP-PIP4Kβ, GFP-K239R-PIP4Kβ and GFP-K239Q-PIP4Kβ on coverslips were fixed with 4% formaldehyde. Confocal analysis shows that all contructs localize to nucleus and cytosol, in presence or absence of SIRT1 knockdown. Images are representative of three independently transcfection experiments. (B) p300 can acetylate lysine 239 of PIP4Kβ. GFP-PIP4Kβ, GFP-K239R-PIP4Kβ and HA-p300 were transiently transfected in HT1299 cells. Immunoprecipitated GFP-PIP4Kβ was analyzed on western blot and acetylated fraction of PIP4Kβ was assessed using anti-ac-239-PIP4Kβ.*

and SIRT1 knockdown U2OS cells. However, we could not detect differences in the localization of either wildtype or mutant PIP4Kβ, regardless of SIRT1 expression (Figure 5A). These results argue against PIP4Kβ (de)acetylation playing a role in regulating PIP4Kβ localization.

DISCUSSION

We previously showed that the phosphoinositide PI(5)P directly binds to SIRT1 via a polybasic region. By manipulating cellular PI(5)P levels, the deacetylase activity of SIRT1 towards on of its targets p53 could be modulated. This present study reveals PIP4Kβ as an interacting partner of SIRT1. PIP4Kβ was found to be acetylated at several distinct lysine residues, one of which (lysine 239) appeared to be regulated by SIRT1. Moreover, we found that ectopically expressed wildtype but not mutant PIP4Kβ(K239R) is acetylated in cells. Using an antibody against ac-K239-PIP4Kβ, endogenous acetylation of PIP4Kβ was verified. Furthermore, PIP4Kβ was shown to be deacetylated by SIRT1 showing that PIP4Kβ not only binds to but also is a target of SIRT1.

PIP4Ks are known, through catalyzing phosphorylation, to control the levels of their substrate PI(5)P (Jones *et al.*, 2006; Rameh *et al.*, 1997). An attractive hypothesis emerges from our identification of PIP4Kβ as an interacting partner of SIRT1 and from our previous finding that PI(5)P can stimulate SIRT1 deacetylase activity. It suggests a regulatory mechanism whereby PIP4Kβ control cellular PI(5)P levels, which in turn regulate PI(5)P stimulation of SIRT1 deacetylase activity. This may be possible by a feedback loop in which the acetylation status of PIP4Kβ is controlled by SIRT1, which in turn could modulate PIP4Kβ activity (Figure 6).

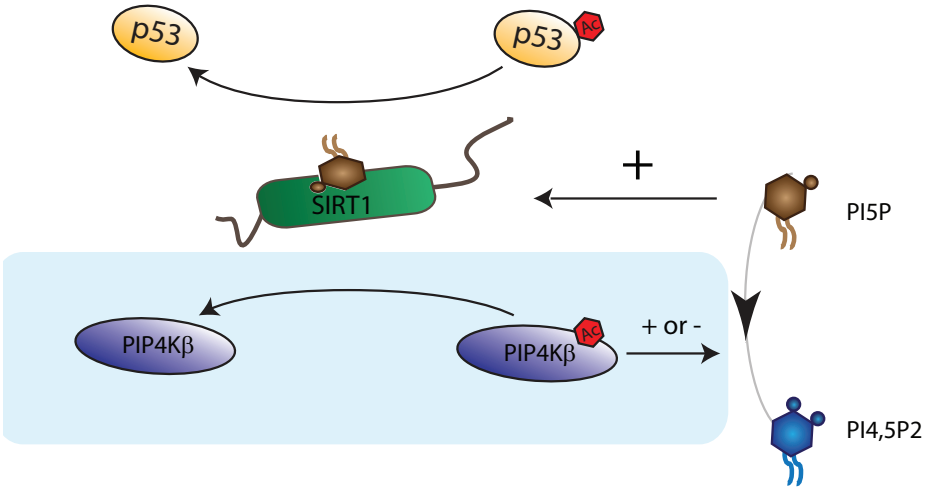

Figure 6. *Working model of PIP4Kβ acetylation. Hypothetical model of a possible feedback loop involving PI(5)P/PIP4K/SIRT1. Acetylation of PIP4Kβ can be regulated by SIRT1 deacetylase. PIP4K lipid activity controls PI(5)P levels by its phosphorylation to PI(4,5)P_2. The accumulation of PI(5)P stimulates SIRT1 deacetylase activity and promotes SIRT1-dependent p53 acetylation. However, how and to what extent (de)acetylated PIP4Kβ might influence PI(5)P levels and its subsequent stimulation of SIRT1 deacetylase activity remain to be determined. See text for further details.*

Protein acetylation can regulate enzyme activity, protein stability or localization (Xiong *et al.*, 2012). It has been shown that PIP4Kβ becomes phosphorylated by p38 MAPK at Ser^{326} and induces a decrease in its PIP4K activity (Jones *et al.*, 2006). Ser326 phosphorylation has been suggested to induce a conformational change resulting in decreased PIP4K activity, but this remains to be established. By analogy to PIP4Kβ phosphorylation, acetylation of PIP4Kβ may also induce changes in its conformation and influence PIP4K catalytic activity. Interestingly, lysine239 of PIP4Kβ resides in a cluster of basic side chains adjacent to the 5-phosphoinositol moiety in the PIP4Kβ-PI(5)P structural model (Rao *et al.*, 1998). Given the negatively charged phosphate groups of phosphoinositides, acetylation (which removes the positive charge) of lysine 239 on PIP4Kβ may reduce the net positive charge of its basic cluster and thereby disrupt the interaction with PI(5)P. This suggests a scenario in which the acetylation status of PIP4Kβ may decrease PIP4Kβ activity(Arif *et al.*, 2007). However, if and how acetylation may affect PIP4Kβ kinase activity needs to be addressed in future studies. Despite several attempts the experiments where comparing kinase activity of wildtype PIP4Kβ to its acetylation mutants still remain to be conducted. This would have helped to elucidate the possible PIP4Kβ/PI(5)P/SIRT1 feedback loop, be it a positive or negative one.

However, we did conduct experiment to confirm an additional or alternative possibility which is that acetylation of PIP4Kβ may affect its subcelullar localization (Ciruela *et al.*, 2000). Despite many efforts, these results shown in figure 5A argue against PIP4Kβ (de)acetylation playing a role in regulating PIP4Kβ localization.

Interestingly, we found SIRT1 to interact only with PIP4Kβ and not with PIP4Kα or PIP4Kγ. The PIP4Kα isoform has a much higher catalytic activity than PIP4Kβ (Bultsma *et al.*, 2010). However, PIP4Kβ can form a heterodimer with PIP4Kα (Bultsma *et al.*, 2010; Wang *et al.*, 2010), which may explain how PIP4Kβ can regulate PI(5)P levels, despite its low intrinsic activity. It is conceivable that SIRT1 interacts indirectly with PIP4Kα in a PIP4Kβ-PIP4Kα complex however experiments to resolve this question remain to be carried out.

Additionally, PIP4Kβ-PIP4Kα heterodimerisation by itself could be regulated by posttranslational modification. Bultsma *et al.* have shown that phosphorylation of PIP4Kβ does not change the interaction between the two PIP4K isoforms. Perhaps acetylated PIP4Kβ regulated by SIRT1 will modulate the amount PIP4Kβ-PIP4Kα complex formation and thereby affect PIP4K activity. The acetylated fraction of PIP4Kβ could either stimulate or reduce PIP4Kα activity in the heterodimer complex, or PIP4Kβ acetylation might modulate PIP4Kα targeting to the nucleus. It is worthy to note that it will be important to identify the lysine acetyl transferase (KAT) that can acetylate PIP4Kβ. Preliminary data show that HAT p300 can acetylate wildtype PIP4Kβ but not a mutant that lacks all possible acetylation sites on PIP4Kβ, but this remains to be studied in more detail (Figure 5B). Further co-IP and confocal microscopy experiments are needed to assess if acetylated PIP4Kβ interacts with PIP4Kα. The possible regulation of PIP4Kβ-PIP4Kα heterodimer formation by acetylation may modulate PI(5)P levels, which in turn could influence SIRT1 deacetylase activity.

In conclusion, we have identified PIP4Kβ as a novel target for SIRT1. PIP4Kβ becomes acetylated on several different lysines in basal conditions and upon insults with UV, cisplatin and nicotinamide. PIP4Kβ is deacetylated on lysine 239 in a SIRT1-dependent manner. Our data suggest a possible mechanism by which the PI(5)P-dependent stimulation of SIRT1 deacetylase activity could be regulated. To what extent PIP4Kβ (de)acetylation may change the levels of PI(5)P, and how acetylation affects its lipid kinase activity (or localization) remains to be examined.

EXPERIMENTAL PROCEDURES

Plasmids and antibodies

FLAG-tagged full length murine SIRT1 was cloned into the pcDNA3 and PGEX-4T-1 vectors. Myc-PIP4Kα, Myc-PIP4Kβ and Myc-PIP4Kγ were derived from rat sequence. HA-PIP4Kβ GFP-PIP4Kβ and GST-PIP4Kβ were derived from human sequences. GFP and GST- PIP4Kβ 239 K-R and 239 K-Q mutants were generated by site-directed mutagenesis.

Antibody used were: p6, 1:10000 is a rabbit polyclonal as described previously (Bultsma *et al.*, 2010j); polyclonal rabbit anti-ac-K239-PIP4Kβ (generated as described below), 1:1000; mouse anti-Myc-HRP, 1:100 (Invitrogen); mouse monoclonal anti-HA, 1:100 (Sigma), rabbit polyclonal anti-SIRT1, 1:1000 (Upstate Biotechnology), mouse monoclonal anti-GFP (Invitrogen), 1:1000 and mouse monoclonal anti-FLAG-M2, 1:5000 (Sigma).

Cell culture and transfections

U2OS, HT1299, HT1080 and HEK 293T cells were cultured in DMEM medium supplemented with 10% Foetal Bovine Serum (FBS), penicillin and streptomycin. DNA transfections were performed using polyethylenimine for HEK 293T cells and using FuGENE 6 Transfection Reagent (Roche Applied Science) for U2OS cells according to manufacturers' instructions. PIP4Kβ shRNAi construct was generated by cloning the sequences below into pRetroSuper, were used to generate viral particles in Phoenix ecotropic cells: RNAi-PIP4Kβ: 5'-AGATCAAGGTGGACAATCA-3' (Bultsma *et al.*, 2010). U2OS cells stably expressing the ecotropic receptor were infected with retroviral particles using Polybrene. Cell populations were selected using puromycin.

Generation and specificity of 239 acetyl antibody for PIP4Kβ

Peptides containing acetylated lysine 239 of PIP4Kβ (CDLPTF**Kac**DNDFL) corresponding to residues 234–244 were coupled to keyhole-limpet haemocyanin and used to immunize New Zealand white rabbits. After negative selection of the rabbit sera using a nonacetylated (CDLPTFKDNDFL) peptide, enzyme-linked immunosorbent assay (ELISA) was used to test the specificity of the negatively selected antibody. 96 well plates were coated with 2ug/50ul PBS antigen o/n. After washing with 1x PBS/Tween, cells were blocked with 1% BSA for 1 hr at 37°C. The negatively selected serum (start dilution 1:20 then by twofold) was incubated for 2 hours at 37°C, after which wells were washed with PBS/Tween six times. After incubation with anti-rabbit (1:1000), wells were washed again six times. The substrate (50 ul) was added (0.615ml Na_2HPO4+ 4.385ml NaH_2PO_4/5ml demi water/9ul H_2O_2/100ul TMB) and reaction was stopped using 50ul of 2M H_2SO_4 and values were determined by elisa reading at 450 nm.

Purification of PIP4Kβ for Mass Spectroscopy

HEK293 cells were transfected with HA-PIP4Kbeta. After 48 hr, cells were either maintained as controls or irradiated with UV (100 J/m^2) or treated with nicotinamide (10mM) or Cisplatin. After 60 min, the cells were lysed in 3 ml of lysis buffer (50 mM Tris [pH 8.0], 10 mM EDTA, 5 mM KCl, 1% NP-40, 20 mM orthovanadate, and 50 mM NaF). Cleared supernatant was subjected to immunoprecipitation with 100 µg of anti-HA antibody. After extensive washing, HA-PIP4Kbeta was eluted using 100 µl of elution buffer (50 mM Tris [pH 8.0] and 0.3 M NaCl containing 1 mg/ml HA peptide) at 30°C with shaking for 20 min.

In-gel digestions were performed as previously described (Shevchenko *et al.*, 2001) with some modifications. Briefly, after colloidal Coomassie brilliant bluestaining, the protein bands were cut and after several washes, the gel pieces were submitted to a reduction step using 10 mM DTT in 100 mM ammonium bicarbonate (NH4HCO3) buffer (56°C, 45 min). Then alkylation was performed using a solution of 55 mM iodoacetamide in 100 mM NH4HCO3 (30 min, room temperature, in the dark). The first digestion was performed using trypsin (7 µg/ml) in 50 mM NH4HCO3 (37°C, over night). Subsequently, V8 was added and the resultant mixture was incubated for 4 hours at room temperature. In order to perform mass spectrometric analysis the mixture was acidified by adding an equal volume of 5% formic acid. Nanoscale liquid chromatography tandem mass spectrometry (nano-HPLC-MS/MS) experiments were performed on an Agilent 1100 HPLC system (Agilent Technologies) connected to a 7-Tesla Finnigan LTQ-FT mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a nanoelectrospray ion source (Jones *et al.*, 2006).

SDS/PAGE and Western blotting

Protein extracts were quantified using Bio-Rad Bradford reagent and adjusted to 1× SDS-loading buffer. After boiling, the extracts containing endogenous proteins were treated with iodoacetamide (50 mM) to modify cysteine residues and then separated by SDS/PAGE. Proteins were transferred on to nitrocellulose, blocked in PBS-T (PBS containing 0.1% Tween 20) containing 5% (w/v) fat-free milk for 1 h, and then incubated with the primary antibody appropriately diluted in MagicMix [PBS-T, 1% Western blocking reagent (Roche) and 3% BSA]. After antibody incubation, the blots were washed with PBS-T and then incubated with the appropriate HRP (horseradish peroxidase)-conjugated secondary antibody diluted in MagicMix [anti-rabbit, anti-mouse used at 1:10000 (GE Healthcare), and anti-rabbit-TrueBlot used at 1:10000 (eBioscience)]. Antibody–protein interactions were visualized using ECL (GE Healthcare) or Supersignal (Pierce). Blots were stripped by incubation at 55°C in 50 ml of strip buffer [50 mM Tris/HCl (pH 7.4), 2% SDS and 50 mM 2-mercaptoethanol] for 20 min followed by extensive washing in PBS-T. The blots were blocked again and then used as above.

Immunoprecipitations

Washed cells were resuspended in lysis buffer [50 mM Tris/HCl (pH 8.0), 50 mM KCl, 10 mM EDTA and 1% Nonidet P40], and after 15 min cell debris were removed by centrifugation (14000 rev./min in a microcentrifuge at 4°C for 10 min). Lysates were incubated with the appropriate antibodies overnight and immune complexes were captured using Protein G–Sepharose (1 h at 4°C). Immunoprecipitates were washed three times with immunoprecipitation wash buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA and 0.1% Tween 20], resuspended in 1× SDS/PAGE loading buffer and subjected to SDS/PAGE and transferred on to nitrocellulose. After incubation with the antibodies indicated, antibody–protein interactions were detected with ECL (enhanced chemiluminescence; GE Healthcare) or Supersignal (Pierce).

Production of recombinant proteins

Fullenght wildtype pGEXT-4T-SIRT1 and pGEXT-4T-PIP4Kβ were transformed in Rosetta 2 bacteria. Cultures were grown at 37°C till OD600 of 0.6 units was reached, induced with 300 µM

IPTG and 50 μM ZnSO4 and after 20 min switched to 16°C overnight for protein expression. All proteins were purified using GST sepharose (GE Healthcare) in buffer (Tris pH 7.5 30mM, 300 mM NaCL, 50uM ZnSO4, 0.5 Triton X-100, 5mM β-mercaptoethanol, protease inhibitor tablet) followed by elution buffer (30mM Tris pH 7.5, 300 mM NaCL, 50uM ZnSO4, 5mM β-mercaptoethanol, 20mM reduced glutathione). All proteins were purified by size exclusion chromatography (GE ÄKTA purifier) using S200 colum and eluted buffer containing 20 mM Tris pH 7.5, 75mM NaCl, 10 uM ZnSO4 and 5mM β-mercaptoethanol. Proteins were further purified by size exclusion chromatography using a S200 column (GE ÄKTA purifier, GE Healthcare) equilibrated in 20 mM Tris pH 7.5, 75 mM NaCl, 10 μM $ZnSO_4$ and 5 mM β-mercaptoethanol.

Chemical modification of PIP4Kβ

Purified recombinant GST-PIP4Kβ and GST-PIP4Kβ 239K-R mutant was diluted in H_2O (100 pmol/ul). The following reagents were added per reaction [5ul 0.1M carbonate buffer (pH 9.2), 1ul recombinant protein or DMSO control, 3ul H_2O and 1ul of NHS-acetyl or NHS-benzyl dissolved in MeCN] and incubated for 30 min at room temperature and stopped by adding 1× SDS/PAGE loading buffer. The reactions were then subjected to SDS/PAGE and western blotting with indicated antibodies.

In vitro deacetylation assay

For the *in vitro* deacetylation reaction, 0.1 μg GST-PIP4Kβ was incubated with 1 μg of GST-SIRT1. The deacteylation reaction was performed in 20 mm Tris, pH 8.0, for 1 hour at 30°C in the presence or absence of the indicated concentration of NAD or nicotinamide. After incubation with GST-SIRT1 the level of acetylated GST-PIP4Kβ was determined as above by Western blot analysis followed by detection using ac-K239-PIP4Kβ antibody.

Confocal microscopy

U2OS shRNAi control and shRNAi SIRT1 knockdown cells were transfected with GFP-PIP4Kβ wildtype and acetylation mutants (K239R and K239Q) on glass coverslips using FuGENE. The cells were fixed using PBS containing 4% formaldehyde (20 min), permeabilized with PBS containing 0.1% Triton X-100 (5 min) and blocked with PBS containing 3% BSA (10 min). Appropriate antibodies were diluted in PBS containing 3% BSA and incubated with the coverslips for 30 min at 37 °C in a humidified incubator. The coverslips were washed with PBS and incubated for 30 min with fluorophore-conjugated secondary antibodies diluted appropriately in PBS containing 3% BSA. The coverslips were mounted using Vectashield, sealed using nail varnish and analysed by confocal microscopy (Zeiss).

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Chapter 5

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

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ABSTRACT

Phosphatidylinositol 5-phosphate 4-kinases (PIP4Ks) phosphorylate phosphatidylinositol 5-phosphate (PI5P) to generate phosphatidylinositol 4,5-bisphosphate; their most likely function is the regulation of the levels of PI5P, a putative signalling intermediate. There are three mammalian PIP4Ks isoforms (α, β and γ), but their physiological roles remain poorly understood. In the present study, we identified the zebrafish orthologue (zPIP4Kα) of the high-activity human PIP4K α isoform and analyzed its role in embryonic development. RT-PCR analysis and whole-mount in situ hybridization experiments showed that zPIP4Kα is maternally expressed. At later embryonic stages, high PIP4Kα expression was detected in the head and the pectoral fins. Knockdown of zPIP4Kα by antisense morpholino oligonucleotides led to severe morphological abnormalities, including midbody winding defects at 48 hpf. The abnormal phenotype could be rescued, at least in large part, by injection of human PIP4Kα mRNA. Our results reveal a key role for PIP4Kα and its activity in vertebrate tissue homeostasis and organ development.

Key words: Zebrafish; Development; Phosphoinositides; Phosphatidylinositol-phosphate kinase; Signalling

INTRODUCTION

Phosphoinositides are a minor but important class of membrane phospholipids that play a key role in a vast array of cellular processes. Through reversible interaction with specific phosphoinositide-binding domains, phosphoinositides can regulate protein localization and enzymatic activities, thereby regulating such diverse cellular processes as cytoskeletal dynamics, migration, vesicle trafficking, gene transcription and proliferation (Di Paolo *et al.*, 2006). Phosphatidylinositol (PI) can be phosphorylated at the 3', 4' and 5' position of the inositol head group by distinct lipid kinases, thereby generating seven different phosphoinositide species (Irvine, 2005). Among these, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) is arguably the most versatile player in diverse signalling pathways, functioning not only as an intermediate in the IP3/diacylglycerol pathway and as substrate of PI 3-kinases, but also as a docking phospholipid and regulator of ion channel activity (Divecha, 2010; Doughman *et al.*, 2003; van den Bout *et al.*, 2009). Two distinct families of lipid kinases, the phosphatidyl inositol 5-phosphate 4 kinases (PIP4Ks) and phosphatidyl inositol 4-phosphate 5-kinases (PIP5Ks), catalyze the formation of PI(4,5)P2 through phosphorylation of PI5P and PI4P on the 4' and 5' position, respectively (Figure 1A). The PIP5Ks are well characterized and likely are the major contributors to the synthesis of PI(4,5)P2 (van den Bout *et al.*, 2009; Volpicelli-Daley *et al.*, 2010). The less well understood PIP4Ks are encoded by three distinct genes (α, β and γ), with the α isoform having much higher catalytic activity than the β and γ isoforms (Bultsma *et al.*, 2010; Clarke *et al.*, 2008; Wang *et al.*, 2010). Since cellular PI4P levels are much more abundant than those of PI5P (Sarkes *et al.*, 2010), the PIP4Ks are probably less important than the PIP5Ks in regulating the bulk of PI(4,5)P2. Thus, PIP4Ks are thought primarily to regulate the levels of their substrate PI5P (Bultsma *et al.*, 2010; Jones *et al.*, 2006; Keune *et al.*, 2012; Sarkes *et al.*, 2010) or to regulate a specific pool of PI(4,5)P2 (Morris *et al.*, 2000). Importantly, PI(5)P has the characteristics of a signaling intermediate. In particular, through interaction with specific protein domains, PI5P has been implicated in modulating chromatin structure and gene transcription (Alvarez-Venegas *et al.*, 2006; Gozani *et al.*, 2003; Ndamukong *et al.*, 2010), membrane trafficking (Lecompte *et al.*, 2008; Ramel *et al.*, 2011), cell migration (Oppelt *et al.*, 2012) and the regulation of oxidative stress responses (Jones *et al.*, 2012; Keune *et al.*, 2012).

The three known isoforms of PIP4K differ not only in catalytic efficiency but also in their subcellular localization and relative expression levels. PIP4Kα localizes to the cytosol, PIP4Kβ is found in the cytoplasm and in the nucleus (Ciruela *et al.*, 2000) and PIP4Kγ is localized to an as yet undefined intracellular membrane compartment (Clarke *et al.*, 2008; Itoh *et al.*, 2000). Recent evidence suggests that distinct isoforms can interact with each other and can affect one another's subcellular localization (Bultsma *et al.*, 2010; Wang *et al.*, 2010). Given their differential expression patterns, catalytic efficiency and subcellular localization, PIP4K isoforms may serve distinct functions depending on cell type and tissue context (Clarke *et al.*, 2012). Mice homozygously deleted for the PIP4Kβ gene are mildly growth retarded and hypersensitive to insulin (Lamia *et al.*, 2004). However, knockout or knockdown phenotypes of PIP4Kα and PIP4Kγ have not been reported to date.

In the present study, we use zebrafish as a model to examine the role of PIP4Ks in vertebrate embryonic development. We identified and cloned the zebrafish orthologue of human PIP4Kα

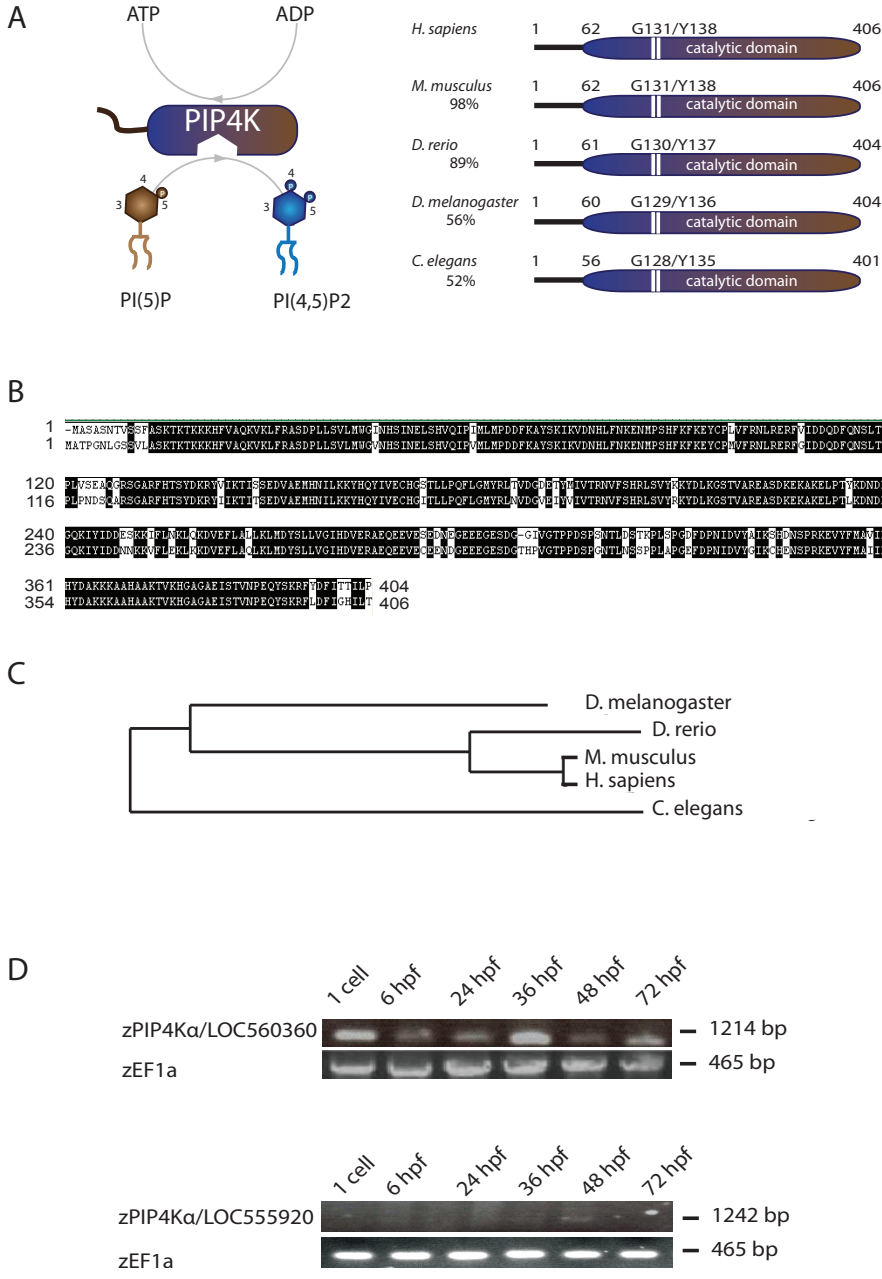

Figure 1. *Identification and developmental expression of zebrafish PIP4Kα. (A) Left panel: Scheme depicting the action of PIP4Kinases, catalyzing the phosphorylation of PI(5)P to PI(4,5)P2. Right panel: the presence of catalytic domain in zPIP4Kα and orthologs from different species. The percentage of sequence identity between human PIP4Kα and amino acids G131 and Y138, critical for catalytic activity, are also indicated. (B) Amino acid sequence comparisons between hPIP4Kα and zPIP4Kα. Sequence alignment using AlignX revealed that the predicted zPIP4Kα (ENSDARG00000003776) is* ▶

(zPIP4Kα) and describe its spatiotemporal expression during embryogenesis. We find that zPIP4Kα is maternally expressed. More importantly, phenotypic analysis of zebrafish depleted of zPIP4Kα by specific morpholino's (MOs) reveals anomalies involving primarily the development of the eye, heart and midbody axis. The importance of PIP4K activity was revealed in rescue experiments using kinase active and inactive hPIP4Kα. Thus, our study uncovers an important role for PIP4Kα in vertebrate development.

MATERIALS AND METHODS

Zebrafish maintenance

Zebrafish (*Danio rerio*) were raised and kept under standard laboratory conditions at 28°C as described previously (Westerfield, 2000). Experiments were performed in accordance with institutional guidelines and as approved by the Animal Experimentation Committee of the Royal Netherlands Academy of Arts and Sciences. Embryos were obtained from natural matings after initiation of the light cycle and staged based on hours post-fertilization (hpf) at 28°C as well as by morphological criteria (Kimmel *et al.*, 1995).

Molecular cloning of zebrafish PIP4Ks

Total RNA was isolated from zebrafish embryos at 36 hpf using Trizol reagent (Invitrogen) according to standard protocols. Total RNA was reverse transcribed using Superscript ™ II RT (Invitrogen). We searched for zebrafish genes with homology to human PIP4Kα, beta and gamma in public databases of the zebrafish genome (the Sanger Institute http://vega.sanger.ac.uk/Danio_rerio/Info/Index) and ESTs using the program BLAST/BLAT (Ensembl). On the basis of the BLAST searches, full-length sequence of predicted zebrafish PIP4Kα http://www.ensembl.org/Danio_rerio/Info/Index/) (ENSDARG00000003776) was amplified by PCR using the following primers F 5'- GGATCCATGGCCTCGGCTAGTAACAC-3' and R 5'-GAATTCCTAGGGCAGGATGGTAGTGA-3' and cloned into pCS2+ All cloning products were confirmed by Big Dye V3 sequencing.

Morpholino antisense oligonucleotide injections

Stock morpholino oligonucleotides (MOs, Gene Tools, LLC) were dissolved in sterile water. The diluted MOs (3.5 ng) were injected into one-cell stage zebrafish embryos. The sequence of ATGMO zPIP4Kα ACAGTGTTACTAGCCGAGGCCATTG was designed against the start codon/5'UTR to block translation and the splice MO was designed against exon 2 – intron 2 (AACAAGTGAGTTCCTCTGAGACGTT) and the standard control MO (Gene Tools) was used as control. The effective concentration for each morpholino was determined through dose-

► *89% identical to hPIP4Kα (identical amino acids shaded in black). (C) Phylogenetic analysis of zPIPK4α and homologues. Zebrafish zPIP4Kα is closely related to mammalian PIP4Kα. Protein sequences used: hPIP4Kα (NP_005019.2), mPIP4Kα (NP_473392.1), zPIP4Kα (NP_001122174.1), D. melanogaster PIP4Kα (NP_001033805.1), C. elegans PIP4Kα (NP_497500.1). (D) RT-PCR analysis monitoring expression of zPIP4Kα/LOC560360 during embryonic development and the indicated stages. Control for input cDNA, EF1α. PIP4Kα expression was detected at the one-cell stage, indicative of maternal contribution. Expression of predicted zPIP4Kα/LOC555920 was not detected.*

response experiments. Specificity of the PIP4Kα morpholino-induced knockdown phenotype was verified by co-injection of 5ng p53 MO (Gene Tools).To generate mRNAs for phenotypic rescue, full-length human PIP4Kα, PIP4KαKD (G131/Y138) and GFP were subcloned into the pCS2+ vector for mRNA synthesis. Capped mRNA was synthesized using the SP6 mMessage mMAchine Kit (Ambion). The mRNAs were then co-injected into MO-treated embryos at the one-two cell stage at 100 ng /µl.

RNA isolation and RT-PCR analysis

Total RNA was extracted from zebrafish embryos at 1 cell, 6 hpf, 24 hpf, 36 hpf, 48 hpf, 72 hpf, using Trizol reagent according to a standard manufacturer's protocol (Invitrogen). For the RT-PCR detection the following forward and reverse primers were used to amplify zPIP4Kα/LOC560360 F 5'- GGATCCATGGCCTCGGCTAGTAACAC-3' and R 5'-GAATTCCTAGGGCAGGATGGTAGTGA-3' ; zPIP4Kα/LOC555920F5'-GGATCCAATGGCCTCTGCAGCCAGCAGandR5'GAATTCTTAGGACAGAA TGGTGGTGA; *zEF1α* F5'-GGCCACGTCGACTCCGGAAAGTCC-3'andR5'-CTCAAAACGAGCCTGGCT GTAAGG-3'. PCR products were analyzed by agarose gel electrophoresis.

In situ hybridization and western blotting

For *in situ* hybridization, digoxigenin-labelled antisense riboprobes were synthesized from PCR-amplified full-length zPIP4Kα cloned into the pCS2+ vector with BamH1/EcoR1 using DIG RNA labelling mix (Roche). As a control, sense riboprobes were synthesized from the opposite strand. Whole-mount in situ hybridizations of zebrafish embryos was performed as described previously (Thisse *et al.*, 1993). Embryos for ISH were fixed in 4% paraformaldehyde in Phosphate-Buffered Solution overnight at 4°C PFA/PBS and stored in 100% methanol. To detect endogenous zPIP4Kα, protein extracts were prepared from 50-dechorionated zebrafish embryos. After lysis in 50 mM Tris pH 8, 10mM EDTA, 50mM KCl and 1% NP40 supplemented with protease inhibitor cocktail (Roche) for 20 min, samples were centrifuged and pellets were resuspended in RIPA lysis buffer containing complete protease inhibitor cocktail tablet (Roche) and 1 mM DTT. After sonication for 5 min, samples were centrifuged and supernatants were separated on 4-12% bis-TRIS precast gels (NuPage). After transfer to PVDF membranes (GE heathcare/Amercham), blots were probed with primary antibodies diluted in PBS with 0.01% triton-X (PBS-T) containing 3% BSA and 2% western blot blocking reagent (Roche). Mouse anti-β-actin antibody (1:5000) was from Abcam (ab6276). Human anti-PIP4Kα polyclonal antibody against peptide CNTLNSSPPLA was raised in rabbits. Horseradish peroxidase-conjugated secondary antibodies were used for detection. Proteins were detected using enhanced chemiluminescence (Pierce Supersignal west-dura ECL) followed by exposure to high-performance autoradiography films.

Immunofluorescence

Wildtype 7 dpf larvae were fixed in 40% ethanol, 5% acetic acid, and 10% formalin (EAF) for 2 h at room temperature, followed by three washes in PBS with Triton-X (PBT) before being embedded in 1.5% low melting agarose. Agarose pellets were dehydrated in ethanol, cleared in xylene, and processed into paraffin. For immunofluorescence analysis, sections were deparaffinized and hydrated, followed by microwave antigen retrieval for 15 min in preheated 1.9 mM citric acid, 8.2 mM sodium citrate, pH 6.0, or in preheated 10 mM Tris, 1 mM EDTA, pH 9.0. Sections

were blocked in PBS containing 5% normal goat serum for 30 min at room temperature and incubated in primary antibody overnight at 4°C. Anti-PIP4Kα polyclonal antibody was used as primary antibody (1:200) and anti-rabbit Alexa Fluor 568 was used as secondary antibody (1:200; Molecular Probes). Nuclei were stained with DAPI and were imaged using a Leica TCS SP2 laser scanning microscope.

PIP4K activity assay

Fifty morphant and control MO-injected zebrafish embryos (48 hpf) were dechorionated and pelleted. For enzyme purification, the extracts applied to a heparin agarose column. After washing, the column was eluted with 25 µl of 1M NaCl. PIP4K activity was measured using liposomes with 1 nmol of PI(5)P and 10 nmol of Pser (phosphatidylserine) as a substrate, 20 µM ATP, 10 µCi [^{32}P]ATP and the heparin sepharose eluate in 100 µl of PIP kinase buffer [50 mM Tris/HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM EGTA and 70 mM KCl] for 10 min as described previously (Jones *et al.*, 2006). [^{32}P]PI(4,5)P_2, the product of PIP4K, was separated by thin layer chromatography (TLC) and quantified using a phosphoimager (Bio-Rad).

RESULTS

Cloning and characterization of zPIP4Kα

To identify the zebrafish orthologs of the different isoforms of human PIP4Ks, we performed database searches (*Danio Rerio* Genebank genomic database) using the sequence of human PIP4K isoform α, β and γ. Analysis of the PIP4Kα isoform identified genomic locus LOC560360 on zebrafish chromosome 24. The predicted gene encodes a transcript that shows very high similarity to human PIP4Kα (77% at the RNA transcript level and 88% at the protein level Figure 1B). PIP4Kα has a highly conserved PIP kinase catalytic domain and has orthologs in many other organisms (Figure 1A; right panel). Phylogenetic analysis of amino acid sequences performed with ClustalW (http://www.ebi.ac.uk/Tools/clustalw/index.html) showed a close relationship of zebrafish PIP4Kα to mammalian PIP4Kα (Figure 1C). The LOC560360 gene is distributed among 10 exons, which is similar to the human equivalent, while the intron-exon boundaries are conserved as well. Due to partial genome duplication, the zebrafish genome often contains different paralogs of genes that are present in a single copy in mammals. In this case, a related gene for PIP4Kα, named "novel protein similar to vertebrate phosphatidylinositol-4-phosphate 5-kinase, type II" or LOC555920 was predicted and mapped to chromosome 2. To validate the predicted transcripts, the coding sequence was amplified and sequenced from RNA purified from zebrafish tissue. ClustalW sequence alignment of LOC560360 revealed 5 silent mutations that are probably due to genetic variation between different zebrafish strains. However, we could not retrieve any transcripts corresponding to LOC555920 from either adult fish or pools of embryos (Figure 1D). This suggested that LOC555920 might be a pseudogene; therefore, we set out to study the developmental expression of LOC560360 (hereafter referred to as zPIP4Kα). To investigate the temporal expression of zPIP4Kα during zebrafish development, we performed RT-PCR analysis on cDNA obtained from different developmental stages. A predicted PCR product of 1214 bp was detected by gel electrophoresis and its identity was confirmed by sequencing. zPIP4Kα was readily detectable at the one-cell stage, indicating maternal

deposition of zPIP4Kα, whereas no mRNA splice variants were detected in the expression analysis. zPIP4Kα expression was detected also at post-gastrulation stages; it peaked at 36 h post-fertilization (hpf) and remained expressed until 72 hpf, the latest developmental time point examined (Figure 1D). Ef-1α gene expression was amplified as a control for cDNA input.

In addition, through database analysis, we identified zebrafish orthologs of PIP4K isoforms β and γ and identified for both a predicted protein. Since mammalian PIP4Kβ shows approx. 2000-fold less catalytic activity than PIP4Kα and PIP4Kγ likely lacks catalytic activity (Bultsma *et al.*, 2010c; Wang *et al.*, 2010c), we focused our study on the zPIP4Kα isoform.

Spatiotemporal expression of zPIP4Kα during zebrafish development

We examined zPIP4Kα mRNA expression by whole-mount in situ hybridization (WISH) in embryos from the 4-cell stage to 48 hpf. A corresponding sense probe was used as negative control (Suppl. Figure S1). Similar to the RT-PCR results, WISH confirmed zPIP4Kα expression during early zebrafish development. zPIP4Kα was ubiquitously expressed from the 1-cell stage to gastrulation (Figure 2A). At 24 hpf, zPIP4Kα was abundant in the brain, CNS, retina and part of the somites. At 48 hpf, robust expression was found in the eye, CNS, brain and pectoral fin buds.

zPIP4Kα knockdown

To analyze the role of zPIP4Kα in zebrafish development, we inhibited the function of zPIP4Kα by injecting antisense morpholinos (MO) at the one-cell stage. We designed two different MOs targeting two different sites of the zPIP4Kα mRNA, so that either splicing or translation events would be disrupted. The splice morpholino (MO1) was aimed at impairing the exon 2- intron 2 splicing in nascent zPIP4Kα mRNA. The ATG MO (MO2) was designed to affect the translation process of both maternal and newly synthesized zPIP4Kα mRNAs by targeting the 5'UTR (Suppl. Figure S2). To test the efficacy of the knockdown approach, we assayed by western blotting the protein levels of zPIP4Kα in MO-injected embryos at 48 hpf. Because zPIP4Kα differed by only four amino acids in the epitope used to generate an antibody to hPIP4Kα we attempted to use this antibody to detect zPIP4Kα by Western blotting. Figure 2B demonstrates that, at 48 hpf, MO1 injection reduced zPIP4Kα expression, whereas zPIPK4α was undetectable in MO2-injected embryos. The region of the genomic DNA corresponding to the MO1 target site was cloned and sequenced to confirm the specificity of the splice MO. On the basis of the sequencing data, we can exclude polymorphisms that might hinder MO1 to bind specifically and efficiently to its target region (data not shown). Therefore, we conclude that the residual zPIP4Kα protein expression in MO1-injected embryos likely arises from translation of residual maternal mRNA.

Developmental effects of zPIP4Kα knockdown

Next, we investigated whether zPIP4Kα expression is critical for normal zebrafish development. We analyzed the effects of zPIP4Kα deficiency by comparing the gross phenotypic alterations produced by the MO1 and MO2 injections into one-cell stage fertilized eggs. MO1-injected embryos were practically indistinguishable from controls, while MO2 injection led to several phenotypic alterations (Figure 2C e-f and i-j). Control injections with an unrelated MO

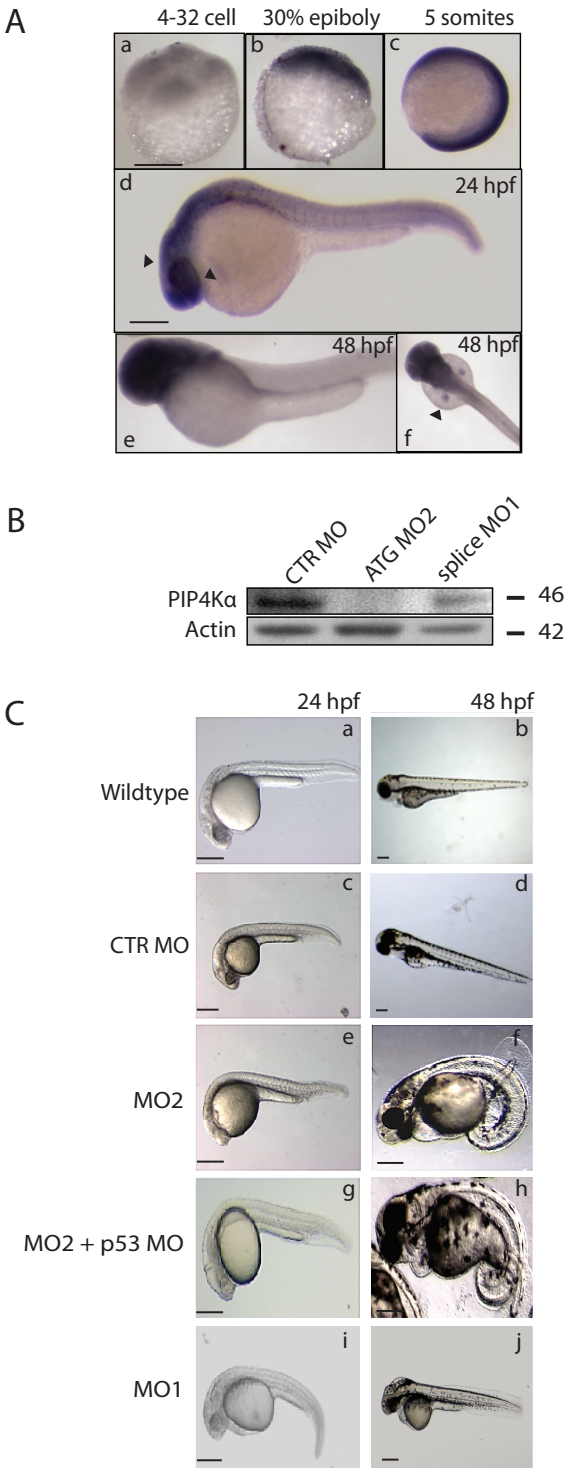

Figure 2. *Knockdown of PIP4Kα in zebrafish. (A) Whole-mount in situ hybridization at the indicated stages. (a) 4-32 cell stage; (b) 30% epiboly; (c) 5 somites; (d) 24 hpf; (e-f) 48 hpf. PIP4Kα mRNA expression was detected in the head region, tail, eyes and pectoral fins (arrowheads) in wild-type embryos. Scale bar, 250 µm. (B) Western blot analysis of two PIP4Kα morpholino-injected embryos. Protein expression PIP4Kα in control MO-injected embryos. Protein levels are strongly reduced in the PIP4Kα ATG MO2 morphants. Splice MO1 injected morphants only showed partial decrease in PIP4Kα expression. MW markers in kDa. (C) Morphological defects in PIP4Kα MO-mediated knockdown at 24 and 48 hpf. (a-b) wild-type non-injected embryos; (c-d) control MO-injected embryos show normal morphology; (e-f) PIP4Kα MO2 morphants display impaired midbody development and severe cardiac oedema; (g-h) co-injection of p53 MO did not affect the PIP4Kα phenotypes; (i-j) PIP4Kα MO1-injected embryos, showing normal morphology. Scale bar, 250 µm.*

(standard control MO, Gene Tools) led to embryos that developed normally and were identical to wild-type at 24 and 48 hpf (Figure 2C a-d). It has previously been shown that some ATG-directed MOs can activate the p53 pathway resulting in apoptosis (Robu *et al.*, 2007), a phenotype that is unrelated to the function of the gene targeted by the MO (off-target effects). To further confirm the specificity of the effects observed by PIP4Kα knockdown, we co-injected the zPIP4Kα MO2 with a p53 MO into zebrafish embryos and found that the embryonic defects of co-injected embryos did not differ from embryos injected with the zPIP4Kα MO2 alone (Figure 2C e-h). At 24 hpf, a variety of developmental defects were observed in the MO2 injected zPIP4Kα morphants. These included heart oedema, eye malformations and strikingly severe midbody axis curvatures (Figure 3). We classified the strong phenotype (severe) as having severe developmental deficiencies with midbody curvatures ranging from over 90 degrees to over 360 degrees and severe cardiac edema. The weak phenotype (mild) was determined as having features with degrees of midbody curvatures ranging till 90 degrees and enlarged pericardium with defects in cardiac looping (Figure 3). Microscopic examination of zPIP4Kα morphants (n=309) revealed that at a dose of 3.5 ng MO2, approximately 75% of injected embryos displayed a severe phenotype: about 20% showed a mild phenotype and <5% had no gross morphological defects (Table 1). However, these phenotypes were not evident in MO1-injected embryos (up to 5 dpf) and therefore were not investigated further (Figure 2C i-j).

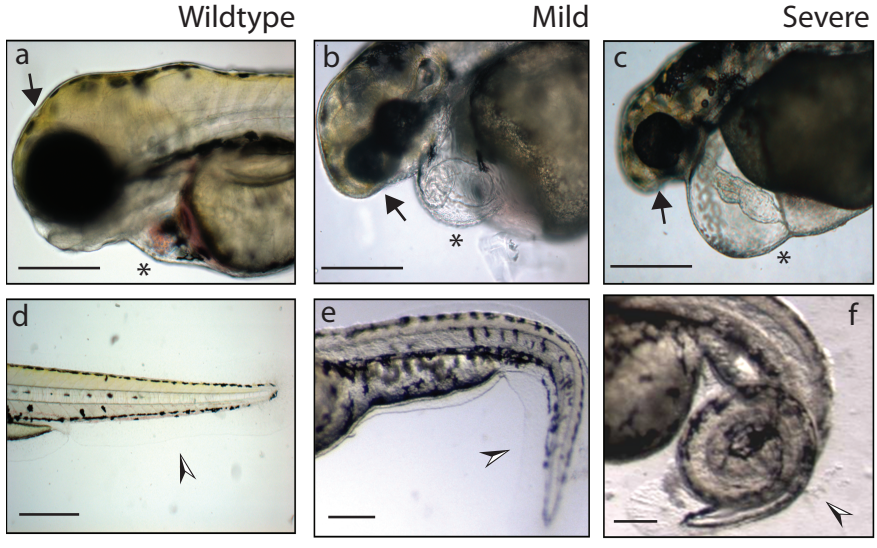

Figure 3. *PIP4Kα knockdown phenotypes. Morphological defects in PIP4Kα morpholino-mediated knockdown at 3 dpf. Left to right displays the range of phenotypic severity observed with PIP4Kα MO. PIP4Kα morphants show overall delay in development and a phenotype affecting several structures. PIP4Kα morphants show curved midbodies with alterations in somatic structure (arrows heads), smaller head with eye malformations (arrows), and pericardial oedema (asterisks). Table 1 summarizes quantification of the indicated developmental defects in PIP4Kα morphants. Scale bar, 300 μm.*

Table 1. *Quantitative analysis of developmental defects in PIP4Kα morphants.*

Phenotype	Control MO (*n* = 126)	MO2 (*n* = 309)		
		Severe	Mild	Wild type
Heart oedema	2	216	65	28
Midbody curvature	0	244	58	7
Eye defects	1	185	92	32

The lack of a phenotype in MO1-injected embryos might be attributed to the presence of maternally supplied mRNA, which may generate enough zPIP4Kα protein to enable normal early embryo development as assessed by western blot (Figure 2B).

The zPIP4Kα morphants eventually died at around 8 dpf, probably due to the severe midbody defects and trunk curvature, which compromises movement of the embryo. Normally, 72 hpf embryos respond to touch stimuli with a rapid and vigorous escape contraction of their trunk and tail, followed by swimming, which propels the embryo forward and results in swimming out of the microscope viewing field. Upon touching, the zPIP4Kα morphants showed rapidly alternating contraction of the trunk and tail, but they failed to propel forward in a straight line and instead swam in circles (data not shown). The altered swimming pattern and delayed hatching from the chorion were most likely due to the midbody curvature. Whether the zPIP4Kα morphants have patterning or structural defects in skeletal somite muscles remains to be determined, but immunohistochemical staining of PIP4Kα in 7 dpf zebrafish larvae (using anti-hPIP4Kα antibody) revealed PIP4Kα localization staining in inter somite structures (Figure Suppl. S3).

Rescue of the zPIP4Kα knockdown phenotype

To examine whether zPIP4Kα and mammalian PIP4Kα are interchangeable, we sought to rescue the knockdown phenotype by exogenous wild-type human PIP4Kα mRNA. Human PIP4K (hPIP4Kα) mRNA was co-injected together with the MOs into one-to two cell stage embryos and the phenotype was scored at 48 hpf (Figure 4A). Exogenously provided hPIP4Kα mRNA partially rescued the zPIP4Kα MO2 phenotype, as the midbody curvatures and reduction in eye size were less severe than in the morphants. About 80% of the MO2-injected embryos exhibited the "severe" zPIP4Kα knockdown phenotype, which was reduced to 38% upon co-injection of hPIP4Ka mRNA with MO2 (n=60 from two independent experiments; Figure 4B; left panel). The defects in cardiac development were less efficiently rescued by hPIP4Kα mRNA (Figure 4A). Injection of hPIP4Kα mRNA into wild-type or control MO-injected embryos did not result in any embryonic abnormalities. Proper expression of hPIP4Kα was confirmed by western blot (Figure 5A).

Kinase activity of zPIP4Kα is indispensable for normal zebrafish development

PIP4Kα is the isoform with the highest catalytic activity (Bultsma *et al.*, 2010; Wang *et al.*, 2010). To confirm that zPIP4Kα can phosphorylate PI(5)P, we measured zPIP4Kα catalytic activity after its partial purification from lysates of 48 hpf zebrafish embryos by heparin sepharose

chromatography. As shown in Figure 5B, morpholino-mediated knockdown of zPIP4Kα led to a significant decrease in the synthesis of PI(4,5)P_2 compared to control MO-injected embryos, indicating that zPIP4Kα is able to phosphorylate PI(5)P. To further confirm the requirement of

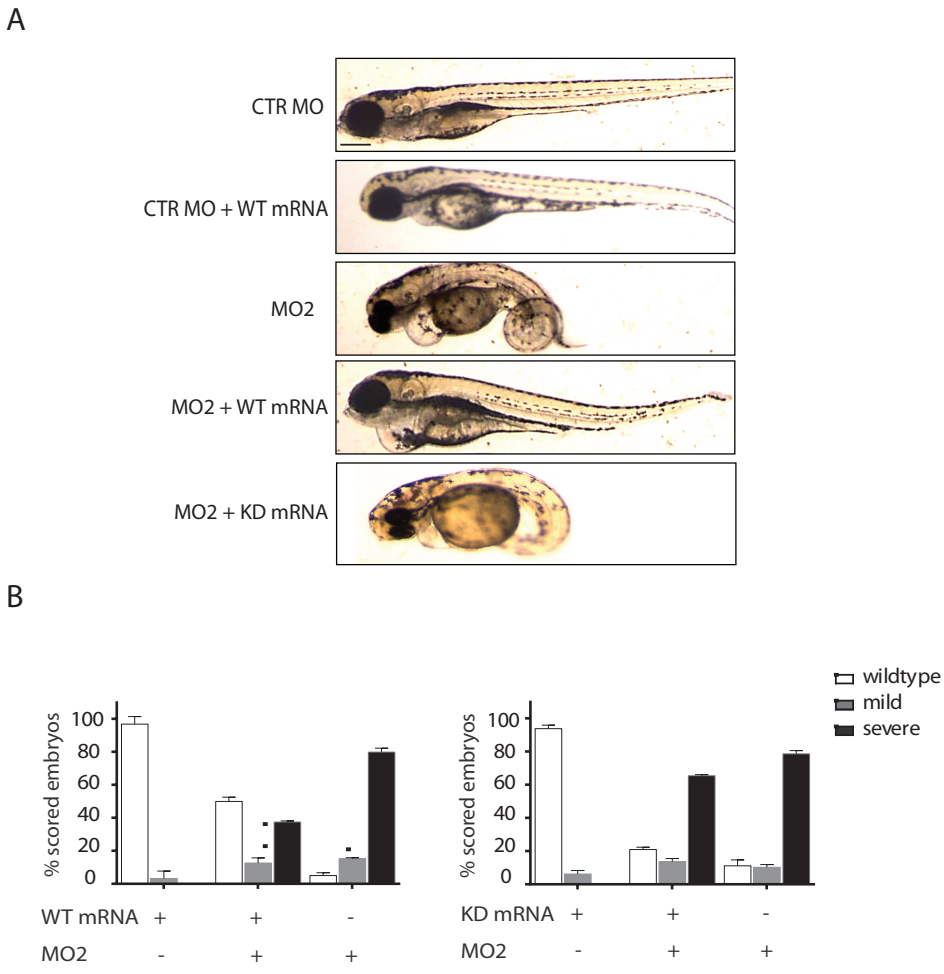

Figure 4. *Rescue of phenotype of zPIP4Kα-deficient embryos by human PIP4Kα RNA variants. (A) Lateral view of embryos at 48 hpf. Expression of hPIP4Kα partial rescues the PIP4Kα morphants from developmental defects. The rescue effect of catalytically dead PIP4Kα mRNA was less than observed with wt PIP4Kα mRNA. Scale bar, 300 µm. (B) Left panel; Quantification of the phenotypes observed upon expression of wt PIP4Kα mRNA in PIP4Kα morphants. Black indicates proportion of PIP4Kα with the classified "severe" morphological defects. Grey indicates the scored embryos displaying a "mild" phenotype and white display a wild type phenotype. Right panel: Quantification of the morphological phenotype obtained upon expression of catalytically dead PIP4Kα mRNA in PIP4Kα morphants with the same classification of phenotypes. Error bars represent standard error (n = 4; total number of embryos analyzed: 415).*

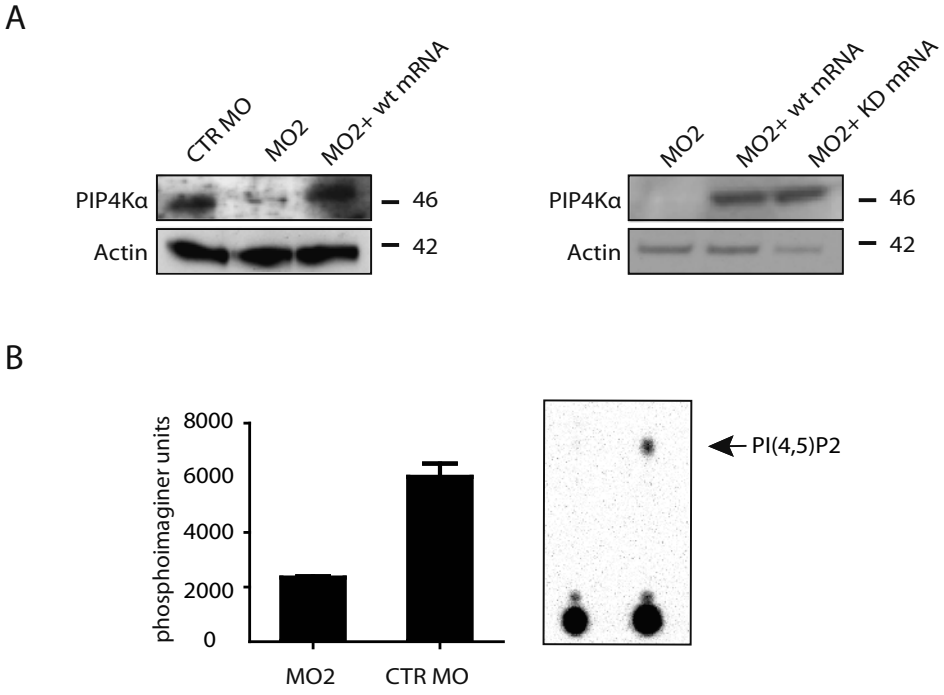

Figure 5. *PIP4K activity in PIP4Kα morphants. (A) Western blot analysis of PIP4Kα expression in "rescued" embryos, as detected by using anti-PIP4Kα polyclonal antibody. Left panel; control MO-injected embryos show PIP4α protein expression, PIP4Kα levels were strongly reduced in the ATG MO2 PIP4Kα morphant embryos. Co-injection of hPIP4Kα mRNA resulted in PIP4Kα expression at 48 hpf. Right panel; reduced PIP4Kα levels in ATG MO2 PIP4Kα morphant embryos at 48 hpf. Co-injection of hPIP4Kα and PIP4Kα-KD mRNA resulted in PIP4Kα expression. MW markers in kDa. (B) PIP4K activity measurements of control and PIP4Kα MO-injected embryos (50 embryos/group). Total lysates were incubated with PtdSer- and PI(5)P-containing liposomes and [^{32}P]ATP. Left panel; quantification of kinase activity of wild-type and PIP4Kα knockdown embryos at 2 dpf. Right panel; autoradiograph showing amount of [^{32}P]PI(4,5)P2, a measure of PIP4K activity, as measured by thin-layer chromatography. Error bars represent standard deviation (n=2). For details see 2.*

zPIP4Kα activity in early development, we examined whether the kinase activity of zPIP4Kα is a prerequisite for normal development. We generated a kinase-dead mutant (G131L/Y138F) (Bultsma *et al.*, 2010) of hPIP4Kα (hPIP4KαKD), and examined whether co-injection of its mRNA with the zPIP4Kα MO2 could rescue the knockdown phenotype. Co-injection of wild-type hPIP4Kα mRNA with zPIP4Kα MO2 significantly reduced the number of embryos that showed gross morphological defects. The hPIP4KαKD mutant also rescued the phenotype, when compared to zPIP4Kα MO injection alone however to a much lesser extent than the kinase active hPIP4K (Figure 4A and B; right panel). Expression of hPIP4KαKD was confirmed by western blot (Figure 5A; right panel). Taken together, these results show that zPIP4Kα catalytic activity is important for normal embryogenesis in zebrafish.

DISCUSSION

Here we report the molecular cloning of a zebrafish orthologue of human PIP4Kα. The high degree of amino acid conservation in the kinase domain, including amino acids G131 and Y138 that are essential for catalysis, strongly suggested that zPIP4Kα has conserved catalytic activity. We found ubiquitous expression of zPIP4Kα during gastrulation and at later stages expression in the eye, brain, somites and pectoral fins. Knockdown of zPIP4Kα caused developmental defects when zPIP4Kα was targeted with a translation-interfering morpholino. Using a morpholino (MO1) that targets the splicing of newly synthesized zPIP4Kα mRNA, zPIP4Kα expression was partly reduced but that did not result in embryonic abnormalities. Using other splice MO's targeting different exon-intron junctions in zPIP4Kα, we also did not detect gross morphological defects (data not shown). The severe phenotypes of the injection of MO2 could be partially rescued by the expression of hPIP4K suggesting that they are unlikely due to off-target mRNA suppression. From these results, we conclude that the translation of maternal zPIP4Kα mRNA provides sufficient levels of zPIP4Kα to ensure normal embryonic development. This may explain why only a morpholino targeting the ATG start codon can induce a knockdown phenotype.

At 48 hpf, zPIP4Kα morphants show developmental defects, namely heart failure, malformation of the eyes and severe midbody winding. These phenotypes can be attributed to zPIP4Kα deficiency, since co-injection of MO2 along with MO2-resistant hPIP4Kα mRNA could partially rescue the phenotype. That hPIP4Kα can complement reduced zPIP4Kα function supports the notion that human and zebrafish PIP4Kα share similar functions. Importantly, catalytically-dead PIP4Kα mRNA rescued the zPIP4Kα knockdown phenotype to a much lesser extent, indicating the importance of PIP4Kα catalytic activity. The partial rescue by the kinase inactive hPIP4K might suggest important scaffolding functions for PIP4K or may be a consequence of residual PIP4K activity in the mutant protein. Taken together, these results establish the specificity of the phenotype, its dependence on catalytic activity, and conservation of PIP4Kα function among vertebrates.

The zPIP4Kα morphants, after 48 hpf, showed an altered swimming pattern and delayed hatching from the chorion. These features were most likely due to the defects in midbody curvature in the zPIP4Kα morphants. It is known that the midbody shape is largely dependent on structurally and functionally intact muscle, especially in somites. Interestingly, several reports have implicated phosphoinositide-metabolizing enzymes in skeletal muscle disorders, especially phosphoinositide phosphatases (McCrea *et al.*, 2009; Nicot *et al.*, 2008). Myotubularins (MTM) are phosphatases that can specifically convert PI(3)P to PI and PI(3,5)P2 to PI(5)P (Robinson *et al.*, 2006) and patients with MTM mutations suffer from severe skeletal myopathy (Jungbluth *et al.*, 2008; Laporte *et al.*, 2000; Tosch *et al.*, 2006). The role of MTMs has been examined in zebrafish muscle development, and knockdown of MTM1 and MTM14 resulted in severe skeletal muscle defects (Dowling *et al.*, 2009; Dowling *et al.*, 2010). The delayed chorion hatching and morphologic changes of the zPIP4Kα morphants are indicative of impaired muscle function. As both PIP4K and MTM's are involved in the regulation of PI(5)P levels, it is tempting to hypothesize a possible link between PI(5)P metabolism and skeletal muscle development. However, whether the zPIP4Kα morphants have patterning or structural defects in skeletal somite muscles remains to be determined. Figure 6 shows a schematic diagram of PIP4K and other 3- or 5- phosphatases in the regulation of PI(5)P and its possible physiological effects relevant to development.

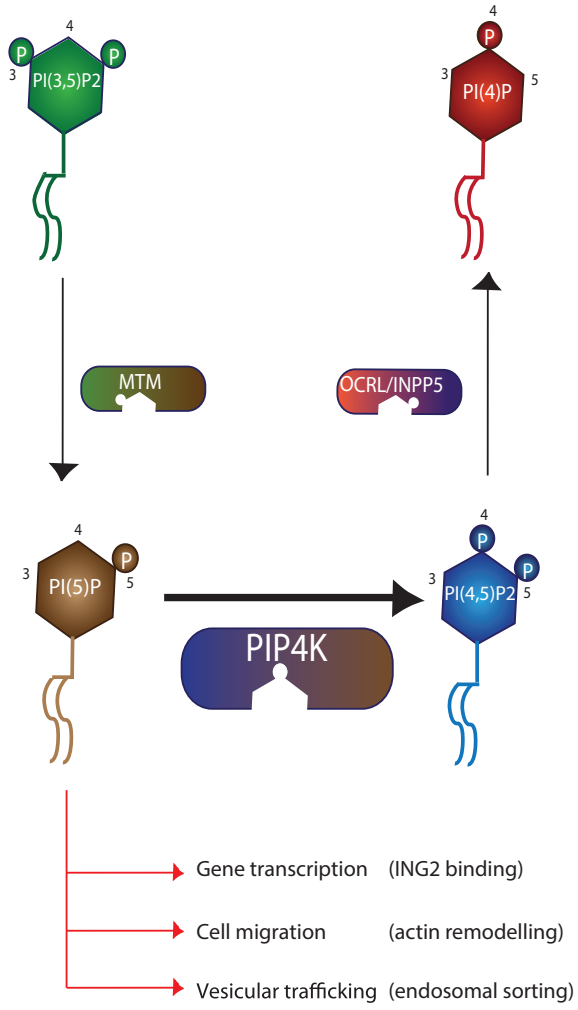

Figure 6. *Scheme of PIP4K and phosphoinositide phosphatases in the regulation of PI(5)P and its possible physiological effects. PI(5)P has been implicated in the regulation of nuclear signalling, gene transcription, membrane dynamics and actin remodelling. To what extent alterations in the pool(s) of PI(5)P and/or PI(4,5)P_2 may underlie the observed phenotypic defects in zPIP4Kα-deficient embryos remains to be established. See text for futher details and references.*

Other phosphoinositide-metabolizing enzymes, notably the inositol polyphosphate 5-phosphatases that can hydrolyze PI(4,5)P_2 (and its water-soluble metabolite Ins(1,4,5)P_3), have also been associated with genetic disorders (Conduit *et al.*, 2012). In particular, *INPP5E* and *OCRL* are associated with Joubert and Lowe's syndrome, respectively, and have been linked to primary cilia defects. Interestingly, OCRL1 knockdown in zebrafish caused developmental defects consistent with disruption of ciliary function, including body axis curvature and pericardial

oedema, similar to what was observed in the zPIP4Kα morphants (Coon *et al.*, 2012; Luo *et al.*, 2012; Ramirez *et al.*, 2012). Collectively, these findings suggest a general role for PI(5)P/PI(4,5)P2-metabolizing enzymes in the formation of cilia, which are implicated in translating signalling cues into the coordinated development of diverse organs.

In conclusion, our study provides the first description of how lack of PIP4Kα expression and activity affects vertabrate development. Our data show that PIP4Kα plays an important role in tissue homeostasis and organ developments. To what extent changes in the levels of PI(5)P and/or those of $PI(4,5)P_2$ in specific tissues may account for the PIP4Kα-deficient phenotype remains to be examined.

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SUPPLEMENTARY INFORMATION

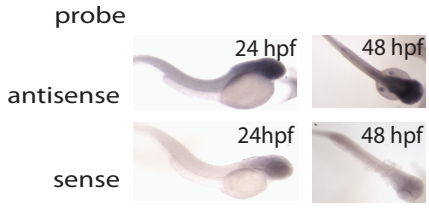

Figure S1. *Whole-mount in situ hybridization; Expression of PIP4Kα at 48 hpf with antisense and sense probe.*

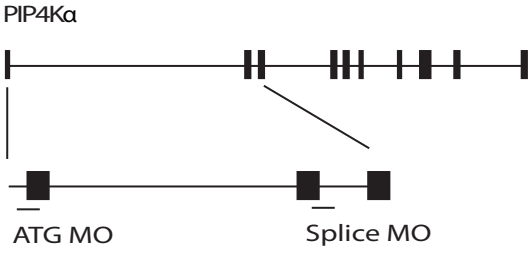

Figure S2. *Schematic representation of splice MO and ATG MO target regions in the PIP4Kα transcript. The sequences of both MOs are shown.*

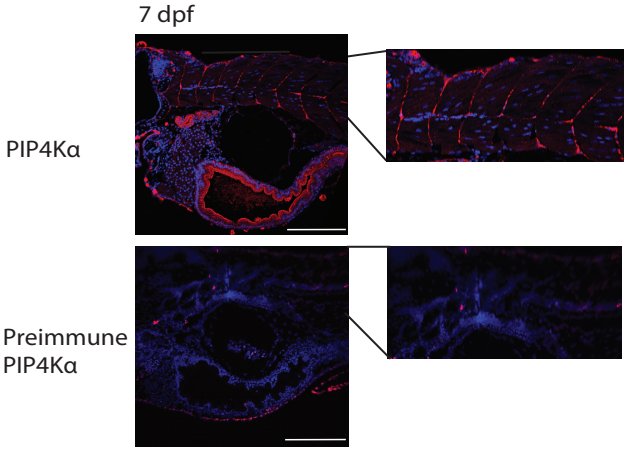

Figure S3. *Analysis of transverse sections of 5 dpf wild-type zebrafish larvae stained by anti-PIP4Kα antibody or preimmune serum (red); nuclei were stained by DAPI (blue). Immunofluorescence reveals expression of PIP4Kα in the lumen, kidneys and intersomite boundaries. Scale bar, 150 μm.*

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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Addendum

NEDERLANDSE SAMENVATTING

Het menselijke lichaam bestaat uit ongeveer 10^{14} (100 biljoen) cellen die alle een specifieke taak hebben. Communicatie tussen deze cellen is van essentieel belang om een goed functionerend organisme te vormen. Onderlinge celcommunicatie of moleculen uit het externe milieu genereren specifieke signalen, die bijvoorbeeld kunnen bepalen welke taak of functie een cel moet uitoefenen. De deling van cellen is een proces waarbij celcommunicatie een grote rol speelt. Als deze communicatie verstoord raakt en cellen niet meer reageren op de juiste signalen kunnen cellen ongecontroleerd gaan groeien wat kan leiden tot kanker. Externe signaalmoleculen, zoals hormonen, groeifactoren en neurotransmitters oefenen hun werking uit via de plasmamembraan. Deze membraan is opgebouwd uit lipiden (vetachtige moleculen) en eiwitten, en vormt een barrière die het celinwendige scheidt van het externe milieu. In de plasmamembraan bevinden zich specifieke receptoren (ontvangers) voor extracellulaire signalen 'first messengers'). Via een opeenvolging van biochemische reacties en 'second messengers', geven deze receptoren signalen door die uitmonden in een respons, bijvoorbeeld het aflezen van bepaalde genen in de celkern of verandering van metabole processen.

Lipiden zijn niet alleen belangrijk voor de opbouw van celmembranen, maar kunnen zelf ook als signaalmoleculen fungeren in de cel. Een belangrijk groep van signaallipiden zijn de zogenaamde fosfoinositiden. Deze bestaan uit een inositol kopgroep die is gekoppeld (via een fosfaatdiester binding) aan diacylglycerol. Een gangbare manier van signaaloverdracht is fosforylering, d.w.z. het modificeren van signaalmoleculen met fosfaatgroepen door specifieke enzymen genaamd kinases. Fosfo-inositiden kunnen worden gefosforyleerd op de 3, 4 en 5 positie van de inositol kopgroep door "PIP kinases".

In dit proefschrift is onderzoek gedaan naar een PIP kinase, genaamd PIP4K, welke een fosfaatgroep koppelt aan fosfatidylinositol 5-fosfaat (PI(5)P) hetgeen leidt tot de vorming van fosfatidylinositol-bisfosfaat (PIP2). Echter, de vermoedelijke functie van PIP4K enzymen (α, β, γ isoformen) is niet de productie van PIP2, maar de verlaging van de hoeveelheid PI(5)P (die toeneemt na cellulaire stress), om zodoende de signaalfunctie van PI5P te remmen (zoals beschreven in hoofdstuk 1). Vroeger werd gedacht dat fosfo-inositiden alleen belangrijk waren voor signaalprocessen aan de plasmamembraan. Tegenwoordig weten we dat fosfo-inositiden ook een belangrijke signaleringsrol spelen in de celkern. PI(5)P is een signaalmolecuul dat diverse eiwitten in het cytosol kan activeren, (zoals de Akt eiwitkinase), maar ook functies in de celkern kan uitoefenen. Het is nu duidelijk dat PI(5)P herkend wordt door eiwitten die een zogenaamd "plant homeobox domain" (PHD) motief bevatten. Dergelijke PHD-bevattende eiwitten worden vooral gevonden in de celkern, zoals bijvoorbeeld het ING2 eiwit ("INhibitor of Growth protein 2") Dit eiwit bindt aan PI(5)P en activeert op deze wijze de tumorsuppressor p53, en remt daardoor de celgroei en stimuleert celdood.

Naast fosforylering, is acetylering een andere veel voorkomende eiwitmodificatie. Acetylering is de koppeling van een acetyl groep aan lysine residuen in eiwitten, zoals histonen in de celkern. Dit is een omkeerbare reactie, waarbij lysine residuen worden geacetyleerd door "histone acetyltransferases" (HATs) en gedeacetyleerd door "histone deacetylases" (HDACs). Histon deacetylering is vaak geassocieerd met chromatine condensatie en repressie (remming) van transcriptie. De HDACs zijn onderverdeeld in vier verschillende klassen, waaronder

klasse III, genaamd sirtuins. Het Sir2 eiwit, de afkorting van Silent Information Regulator 2, is het bekendste voorbeeld van een sirtuin. In bakkersgist cellen is aangetoond dat (verhoogde werking van) Sir2 de levensduur verlengt. Sir2 komt ook voor in vele andere organismen in verschillende varianten. In zoogdieren bestaan zeven sirtuins, SIRT1-SIRT7, die alle een geconserveerd NAD+ (cofactor) – bindings domein en een katalytisch domein bevatten. Ze verschillen vooral in hun N- en C-termini. SIRT1 is de evolutionair meest geconserveerde sirtuin en speelt een cruciale rol in metabolisme, kanker en veroudering.

Door de-acetylering van verschillende substraten (zoals histonen, metabolisme enzymen en transcriptiefactoren), is SIRT1 nauw betrokken bij allerlei fysiologische functies. Het bekendste substraat van SIRT1 is de eerder genoemde tumorsuppressor p53. p53 reguleert genen die betrokken zijn bij remming van celgroei en geprogrammeerde celdood. p53 is vaak onderhevig aan gevaarlijke mutaties: in meer dan 50% van alle menselijke tumoren is een niet-werkend p53 gen aanwezig. Dit defecte p53 eiwit is niet meer in staat om de celdeling te remmen. De mate van acetylering is essentieel voor de activatie van p53. Hoewel veel onderzoek wordt verricht aan SIRT1 substraten en hun functies, blijft het onduidelijk hoe SIRT1 activiteit gereguleerd wordt. In dit proefschrift beschrijven we een nieuwe rol voor PI(5)P en PIP4K in de regulering van SIRT1 activiteit en in de embryonale ontwikkeling van de zebravis.

Hoofdstuk 1 geeft een beknopt overzicht van de huidige status van het onderzoek naar de onderwerpen in dit proefschrift. Hierin staat een overzicht van de moleculen en enzymen die betrokken zijn bij het reguleren van PI(5)P, met nadruk op de rol van de drie PIP4K isoformen en hun biochemische functies. Ook wordt beschreven welke mechanismen betrokken zijn bij het reguleren van SIRT1 activiteit door stimuli zoals de cofactor NAD+, en door interactie met andere eiwitten.

Hoofdstuk 2 definieert PI(5)P als nieuwe regulator van SIRT1 enzymatische activiteit. Hier beschrijven we een directe interactie tussen SIRT1 en fosfo-inositiden, met name PI(5)P, via een fosfoinositiden bindend eiwit motief (KRKKRK) dat niet eerder geïdentificeerd is. Door te binden aan PI(5P), wordt de deacetylerings activiteit van SIRT1 gereguleerd, zowel *in vitro* als *in vivo*. Bovendien hebben we gevonden dat p53 deacetylering door SIRT1 kan worden beïnvloed door de PI(5)P concentraties te manipuleren. Dit resulteert in meer of minder p53-afhankelijke transcriptie en stimulering van celdood. Onze resultaten tonen aan dat PI(5)P als signaalmolecuul kan fungeren, met name wanneer de cel "genotoxische stress" ondervindt. Hoe PI(5) precies de activiteit van SIRT1 reguleert dient nader te worden uitgezocht. Maar de identificatie van een nieuw PI-bindende sequentie in SIRT1 dat de katalytische activiteit van SIRT1 kan beïnvloeden opent nieuwe wegen naar de ontwikkeling van farmacologische middelen gericht tegen SIRT1.

Er zijn literatuuraanwijzingen dat fosfolipiden behalve deacetylatie ook andere epigenetische processen kunnen beïnvloeden. Deze staan beschreven in hoofdstuk 3, met daarin de nieuwste inzichten in de rol van fosfolipiden in processen in de celkern (chromatine en gen transcriptie).

Hoofdstuk 4 is een vervolg op de ontdekkingen beschreven in hoofdstuk 2. Daarin observeerden we o.a. dat PIP4K expressie de activiteit van SIRT1 kon moduleren. Dit leidde tot de vraag of deze kinases zelf (en niet alleen het substraat PI(5)P) een rol in SIRT1 functie kunnen

vervullen. Hoofdstuk 4 verkent niet alleen de posttranslationele modificaties op PIP4Kβ maar beschrijft ook de interactie met SIRT1. Een interactie van SIRT1 met PIP4Kβ suggereert dat PIP4Kβ kan worden gemodificeerd door (de)acetylering. Door middel van massa spectroscopie identificeerden wij acht verschillende lysine residuen, waarvan lysine-239 gevoelig bleek voor de SIRT1 remmer nicotinamide. Ook zagen we dat lysine-239 wordt geacetyleerd door wild-type SIRT1 maar niet door de inactieve SIRT1(K239R) mutant. Gezien de resultaten beschreven in hoofdstuk 2, suggereren we een mechanisme waarin de PI(5)P afhankelijke stimulatie van SIRT1 activiteit kan worden geregeld door PIP4Kβ (de)acetylatie. In hoeverre PIP4Kβ (de)acetylatie de lokale concentraties van PI(5)P kan veranderen, en hoe acetylatie PIP4Kβ activiteit beïnvloedt dient nader onderzocht te worden.

De fysiologische betekenis van de drie PIP4K isovormen is nog onduidelijk. Om dit te onderzoeken in de embryonale ontwikkeling, hebben we gebruik gemaakt van de een modelorganisme, namelijk de zebravis *(Danio Rerio)*. De zebravis wordt veel gebruikt om beter inzicht te krijgen in de complexiteit van de embryonale ontwikkeling. De zebravis biedt vele voordelen : ze leggen meer dan 300 eieren, en de embryonale ontwikkeling verloopt zeer snel. Bovendien vindt de embryonale ontwikkeling plaats buiten de moeder en zijn de embryo's doorzichtig, zodat het ontwikkelingsproces goed kan worden gevolgd.

In Hoofdstuk 5 identificeren we de zebravis ortholoog (zPIP4Kα) van humaan PIP4Kα en analyseren we de rol in de embryonale ontwikkeling. Zebravis embryo's waarin de expressie van zPIP4Kα is onderdrukt door specifieke morfolino's (MO's) vertonen afwijkingen in de ontwikkeling van het oog, het hart en het middenstuk van romp tot staart. De katalytische activiteit van zPIP4Kα is geconserveerd in zebravis en zoogdieren. De morfologische afwijkingen konden grotendeels worden hersteld door re-expressie van actief PIP4Kα. Onze resultaten zijn de eerste beschrijving dat PIP4Kα expressie en activiteit invloed heeft op ontwikkeling van vertebraten.

De resultaten beschreven in dit proefschrift vergroten ons inzicht in het werkingsmechanisme van PIP4Ks en het signaalmolecuul PI(5)P. Dergelijke studies kunnen helpen de (patho-) fysiologische functies te ontrafelen van PIP4K's in het algemeen, en van PI(5)P in het bijzonder. Hopelijk kan de nieuwe kennis in de toekomst vertaald worden in therapeutische toepassingen.

CURRICULUM VITAE

Dalila Elouarrat was born in Amsterdam, the Netherlands, on the 19th of January 1981. She attended Fons Vitae lyceum high school in Amsterdam from 1993 to 1998. In 1998 she studied Biochemistry at the Institute of Life Science & Chemistry, Hogeschool van Utrecht and preformed a research internship on the department of Molecular biology in the group of Prof. Rene Medema at the Netherlands Cancer Institute. She worked under the supervision on dr Suzanne Lens on role of surviving in cytokenesis after which she obtained her bachelor's degree in 2002. In the same year, she started with the Master program in Oncology at the Free University in Amsterdam. From 2003 to 2004 she joined the group of prof. Ronald Plasterk at the Hubrecht institute in Utrecht to work on her MSc internship project studying mismatch repair genes in *C.elegans* under the supervision of dr Marcel Tijsterman. The second Master internship was performed in the Welcome trust/Cancer research UK Gurdon Institute in Cambridge, United Kingdom, in the group of Prof Daniel St Johnston. During her project, where she developed a method to perform targeted RNAi in the germline of *Drosophila melanogaster*, she joined her second Master program Developental Biology at the University of Cambridge which she obtained in 2005. In January 2006 she obtained her Master's degree in Science, Oncology from the Free University. From 2006 to 2007, she worked as a PhD student at the Netherlands Cancer Institute in the group of dr Nullin Divecha, investigating the role of PI(5)P and PIP4K in SIRT1 function. In august 2007 she joined the group of prof. Wouter Moolenaar at the Division of Cell Biology and the results of this research are described in this thesis.

In October 2013, Dalila will continue her scientific career as a postdoctoral fellow in the group of Prof Jerrold Olefsky in the Department of Diabetes, Metabolism & Endocrinology, at the University of California in San Diego, working on the role of lipids in the mechanisms of insulin resistance and type 2 diabetes.

LIST OF PUBLICATIONS

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(DE and MCM contributed equally to this work)

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