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

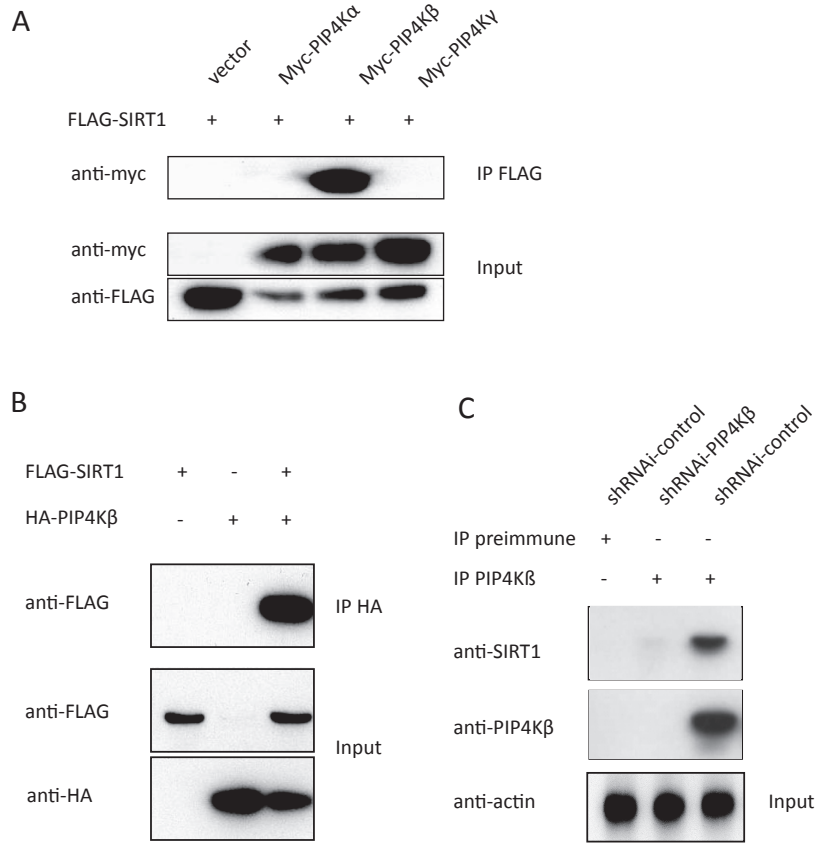

Figure 1. *PIP4Kβ interacts with SIRT1. (A) Co-immunoprecipitation of SIRT1 and PIP4Kβ HEK293T cells transiently transfected with the indicated plasmids. Cell lysates were immunoprecipated with anti-FLAG antibody and immunoblotted with anti-MYC antibody. Input blots as control. (B) Co-immunoprecipitation 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

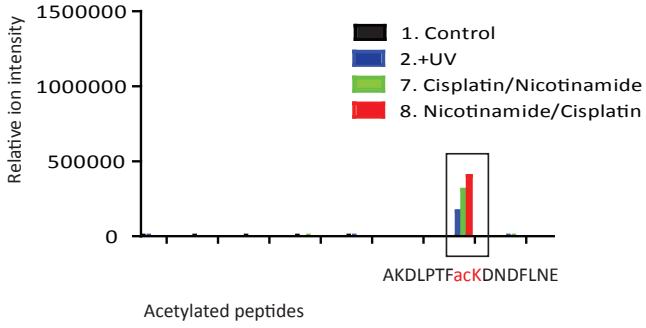

Figure 2. *Identification of acetylation sites in PIP4Kβ by mass spectrometry. (A) Sequence of human PIP4Kβ. The circled red marked lysine residue is acetylated lysine 239 and the other residues marked in red constitute a cluster of basic side chains in the PIP4Kβ-PI(5)P structural model (Rao et al., 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.

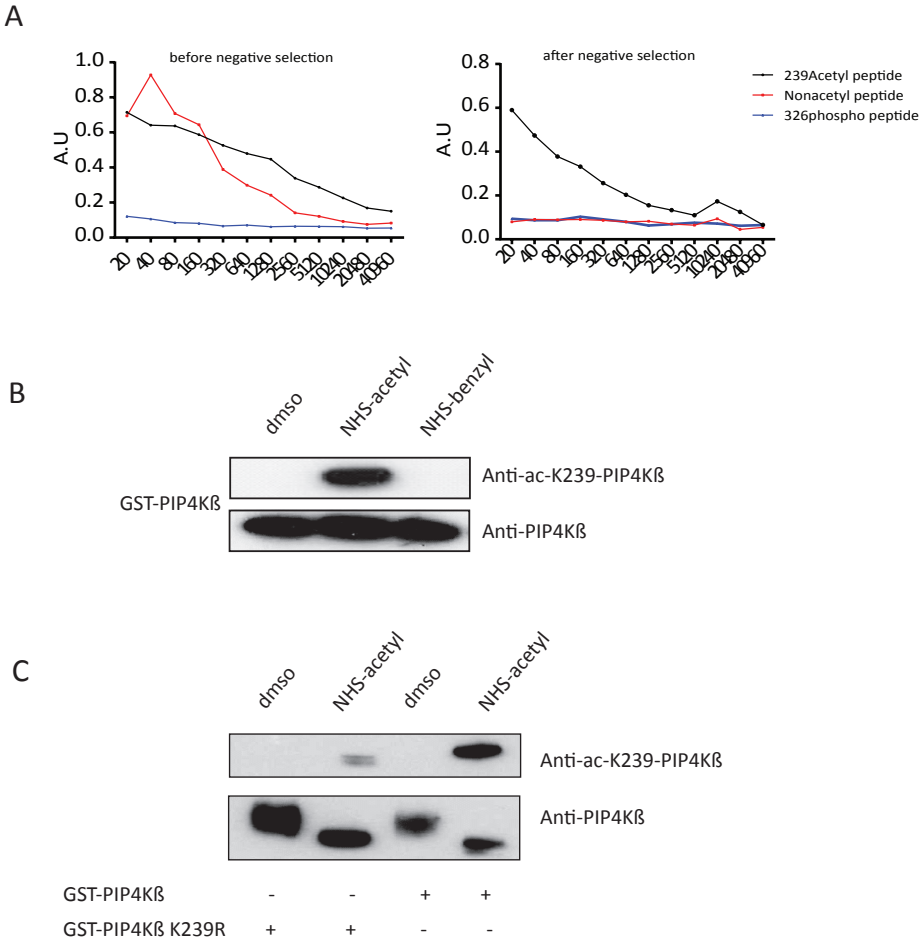

Figure 3. *Generation of an acetylation specific antiserum against 239 acetylated PIP4Kβ. (A) Elisa analysis of serum against 239 acetylated PIP4Kβ; measured O.D of diluted of serum (1:20 till 40960) before and after negative selection on 3 different peptide coated wells (239 non acetyl, 239 acetyl, 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.*

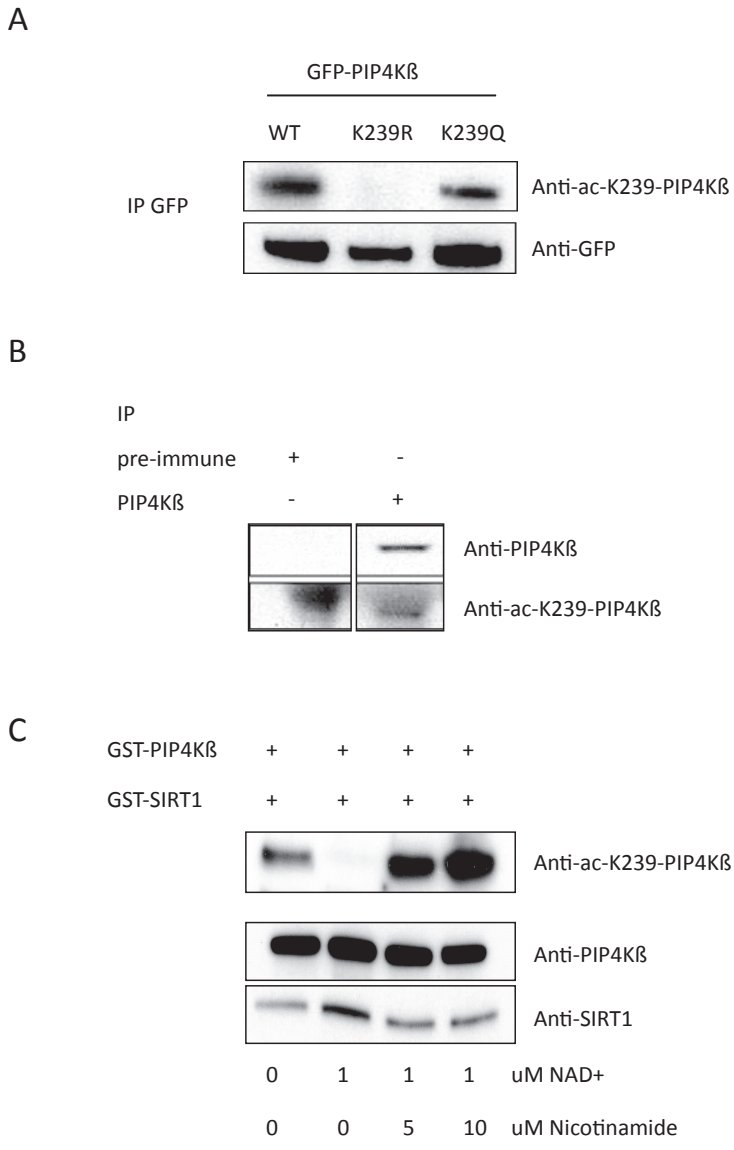

Figure 4. *Acetylated PIP4Kβ is deacetylated by SIRT1 on lysine 239. (A) GFP-PIP4Kβ wildtype K239R and K239Q mutant were transfected in HEK293Tcells that were treated with nicotinamide for 2 hour before harvesting. Lysates were immunoprecipitated using anti-GFP antibody and acetylation level of PIP4Kβ 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

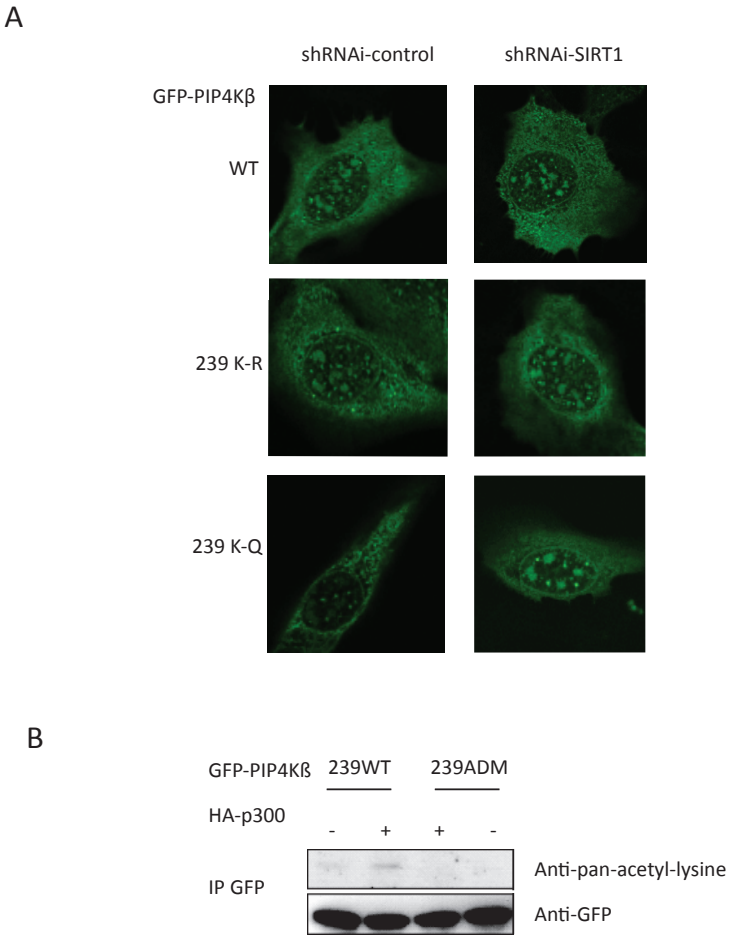

Figure 5. *(A) Acetylation does not affect the localization of PIP4Kβ in U2OS cells. Control and SIRT1 shRNAi U2OS cells transfected with GFP-PIP4Kβ, GFP-K239R-PIP4Kβ and GFP-K239Q-PIP4Kβ on coverslips were fixed with 4% formaldehyde. Confocal analysis shows that all contructs localize to nucleus and 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).

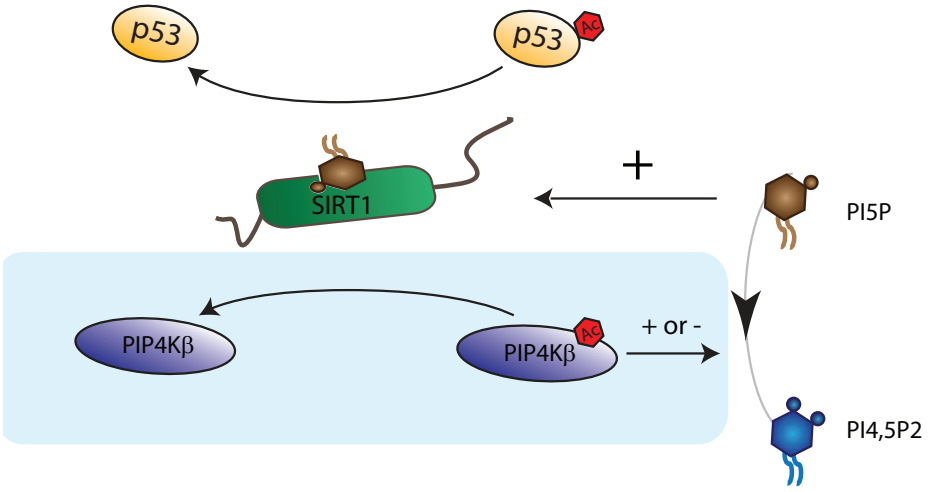

Figure 6. *Working model of PIP4Kβ acetylation. Hypothetical model of a possible feedback loop involving PI(5)P/PIP4K/SIRT1. Acetylation of PIP4Kβ can be regulated by SIRT1 deacetylase. PIP4K lipid activity controls PI(5)P levels by its phosphorylation to PI(4,5)P_2. The accumulation of PI(5)P stimulates 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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