Chapter 2

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

Dalila Elouarrat[1]*, Maria Carla Motta[1,2]*, Satwinder Kaur Singh[1], Yvette Bultsma[2], David R Jones[2], Wouter H Moolenaar[1] and Nullin Divecha[1,2 $]

[1] Division of Cell Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[2] The CRUK Inositide Laboratory, The Paterson Institute for Cancer Research, Wilmslow Road, Manchester M20 4BX, UK
* Joint first authors
$ To whom correspondence should be addressed: ndivecha@picr.man.ac.uk

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

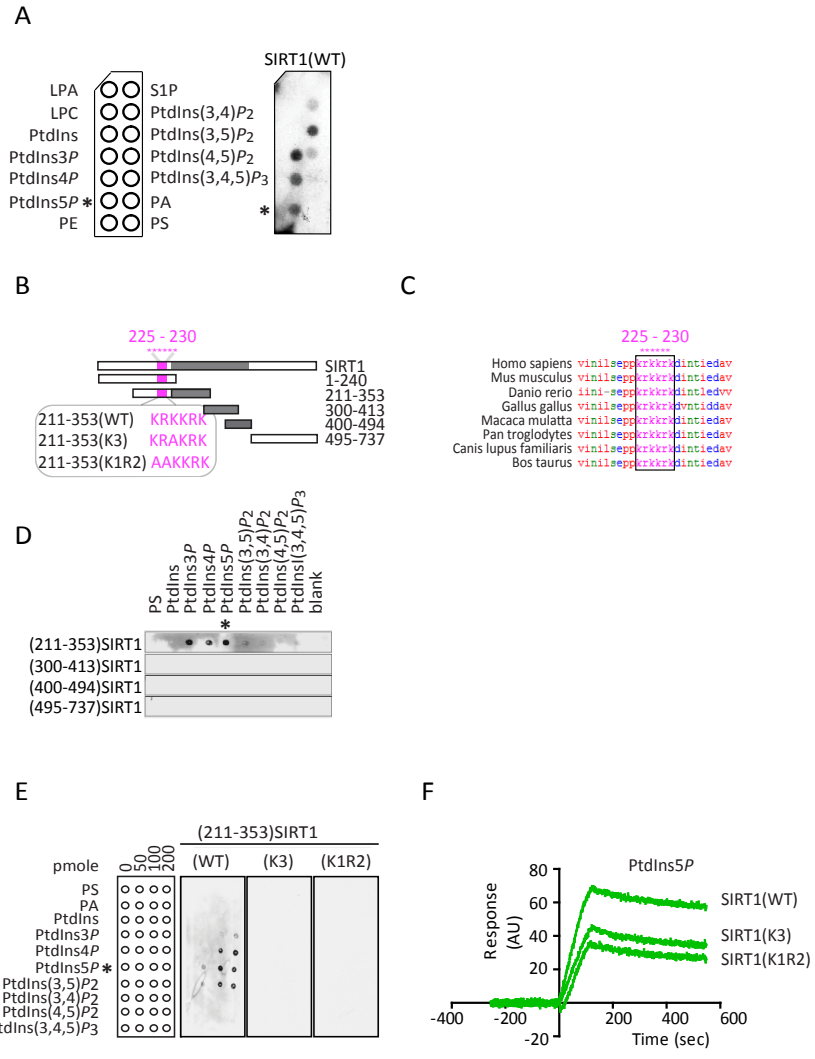


Figure 1. *PtdIns5P interaction with SIRT1; A conserved motif (residues 225-230) outside the catalytic domain is essential for PtdIns5P-SIRT1 interaction. (A) Left: diagram of a PIP Strip (used for protein-lipid overlay assays). Right: protein-lipid overlay assay using full-length SIRT1(WT). Binding 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).

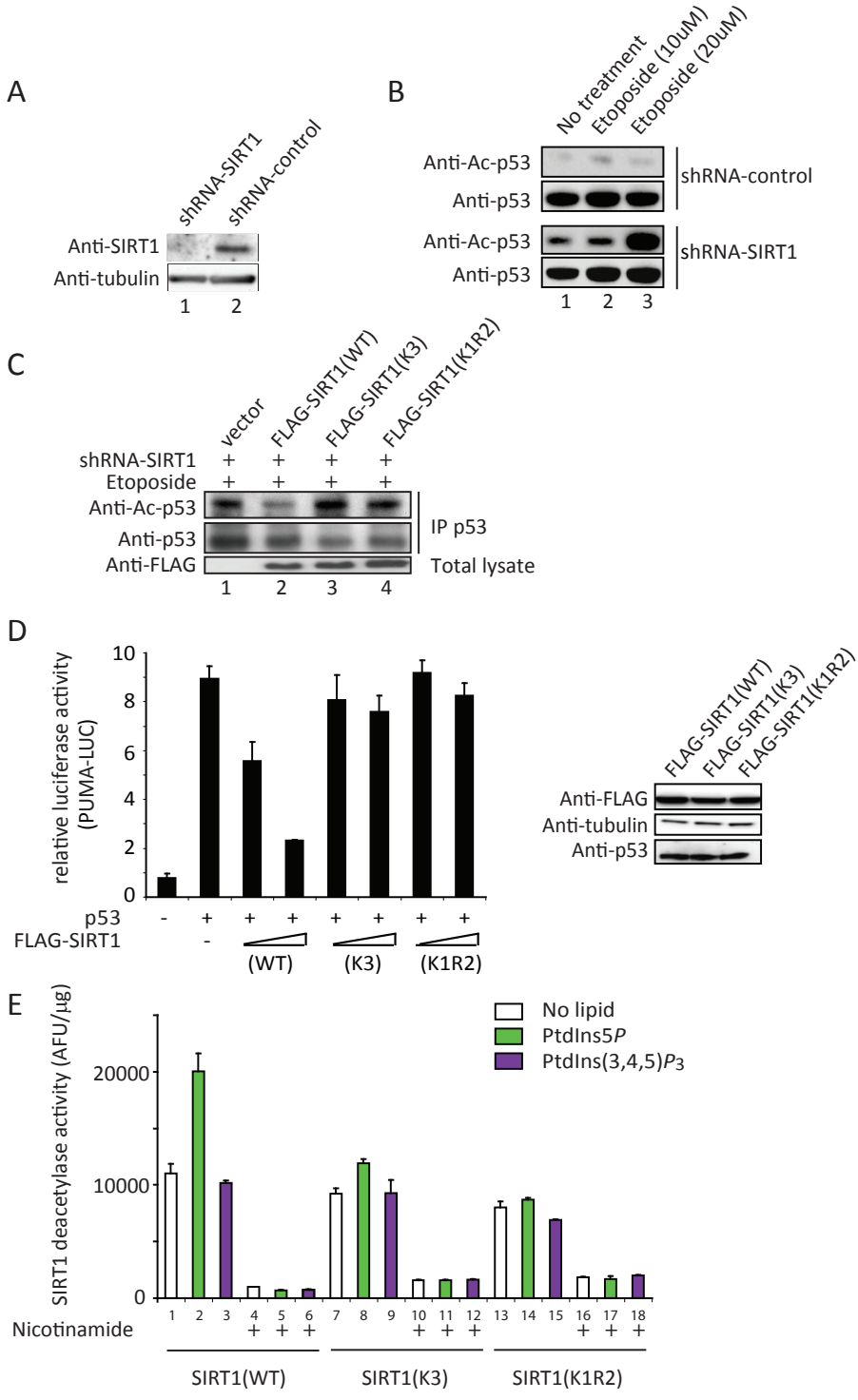
The KRKKRK motif is required for SIRT1 dependent p53 deacetylation

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,

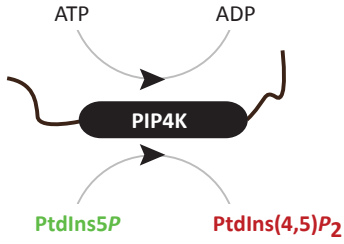

A
shRNA-SIRT1
shRNA-control
Anti-SIRT1
Anti-tubulin
1 2
B
No treatment
Etoposide (10uM)
Etoposide (20uM)
Anti-Ac-p53
Anti-p53
shRNA-control
Anti-Ac-p53
Anti-p53
shRNA-SIRT1
1 2 3
C
vector
FLAG-SIRT1(WT)
FLAG-SIRT1(K3)
FLAG-SIRT1(K1R2)
shRNA-SIRT1 + + + +
Etoposide + + + +
Anti-Ac-p53
Anti-p53
IP p53
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)

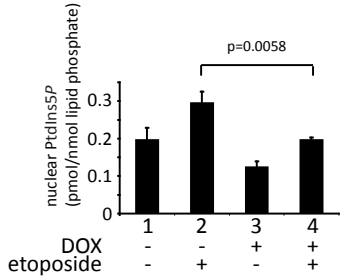
converting it to PtdIns(4,5)P2 (Figure 3A left). In particular, PIP4Kα is an high specific activity isoform whose over-expression leads to reduced PtdIns5P levels(Bultsma *et al.*, 2010). We generated U2OS cells over-expressing PIP4Kα under the control of a doxycyclin-inducible promoter (U2OS-Myc-PIP4Kα) 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

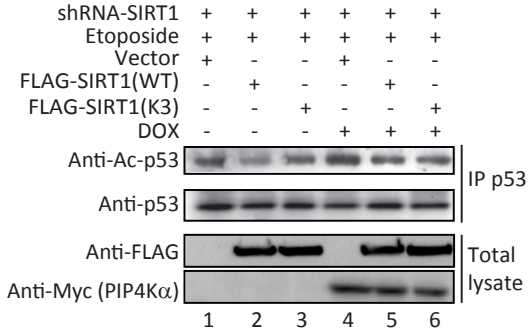
◄ ***Figure 2.*** *The interaction of PtdIns5P with SIRT1 enhances SIRT1 deacetylase activity. (A) SIRT1 in whole cell lysates was detected by Western Blot in SIRT1 knockdown (shRNA-SIRT1) or control (shRNA-control) U2OS cells. (B) Control (shRNA-control) or SIRT1 knock-down (shRNA-SIRT1) U2OS cells 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


ATP
ADP
PIP4K
PtdIns5P
PtdIns(4,5)P2

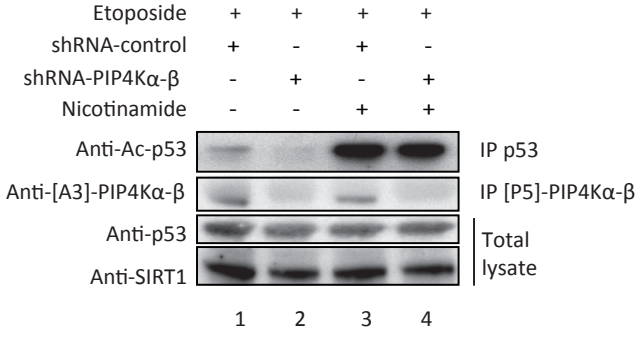

nuclear PtdIns5P (pmol/nmol lipid phosphate)
p=0.0058
0
0.1
0.2
0.3
1 2 3 4
DOX - - + +
etoposide - + - +

B

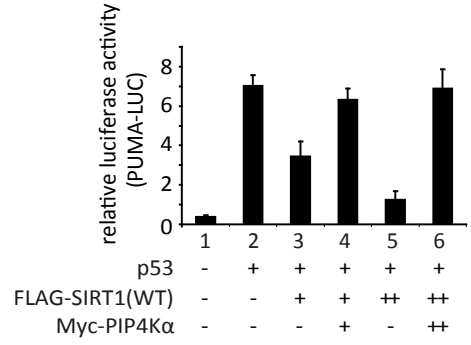

shRNA-SIRT1 + + + + + +
Etoposide + + + + + +
Vector + - - + - -
FLAG-SIRT1(WT) - + - - + -
FLAG-SIRT1(K3) - - + - - +
DOX - - - + + +
Anti-Ac-p53
Anti-p53
IP p53
Anti-FLAG
Anti-Myc (PIP4Kα)
Total lysate
1 2 3 4 5 6

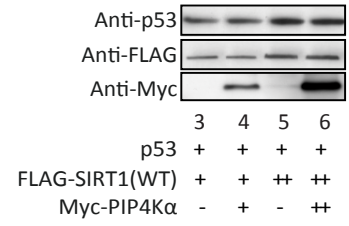

shRNA-control
shRNA-SIRT1

C


Etoposide + + + +
shRNA-control + - + -
shRNA-PIP4Kα-β - + - +
Nicotinamide - - + +
Anti-Ac-p53
IP p53
Anti-[A3]-PIP4Kα-β
IP [P5]-PIP4Kα-β
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
3 4 5 6
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.*

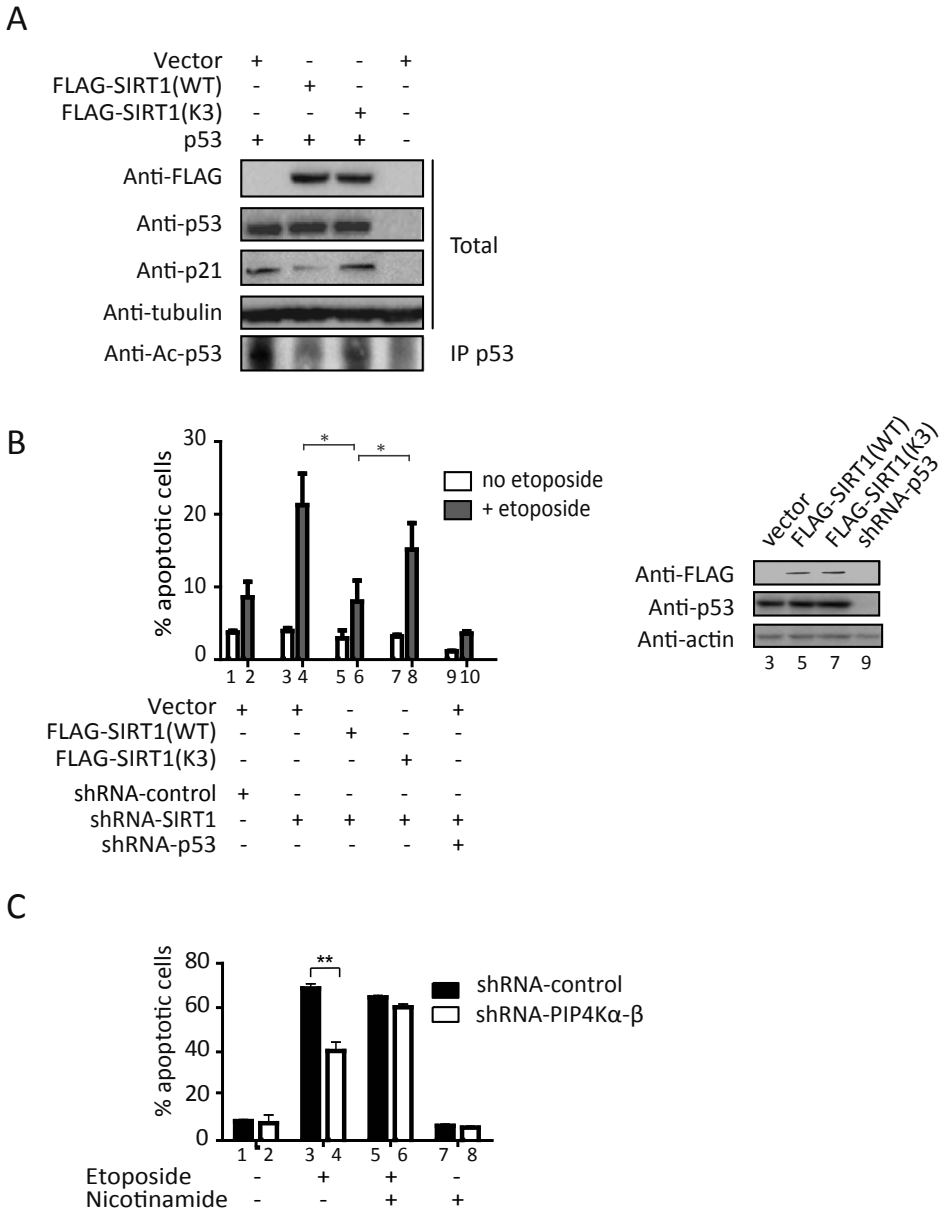


Figure 4. *PtdIns5P dependent SIRT1 regulation is important for cellular stress response. (A) H1299 were transfected with empty vector, FLAG-SIRT1(WT), FLAG- SIRT1(K3) and p53 as indicated (top). Cell lysates were immunoblotted with the indicated antibodies (Total). p53 acetylation at Lys 382 (Anti-Ac-p53) 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

ACKNOWLEDGEMENTS

We thank Patrick Celie, Bernat Blasco and Magda Stadnik at the Netherlands Cancer Institute for protein production and Jarno Drost for generating luciferase constructs and to members of the Inositide laboratory for helpful discussions. D.E was supported by a grant from the Dutch Cancer Society and work in the Inositide laboratory was funded by CRUK.

REFERENCES

1. Araki,T., Sasaki,Y., and Milbrandt,J. (2004). Increased Nuclear NAD Biosynthesis and SIRT1 Activation Prevent Axonal Degeneration. Science 305, 1010-1013.
2. Bosch-Presegue,L. and Vaquero,A. (2011). The dual role of sirtuins in cancer. Genes Cancer 2, 648-662.
3. Brunet,A., Sweeney,L., Sturgill,J.F., Chua,K., Greer,P., Lin,Y., Tran,H., Ross,S., Mostoslavsky,R., Cohen,H., Hu,L., Cheng,H., Jedrychowski,M., Gygi,S., Sinclair,D., Alt,F., and Greenberg,M. (2004). Stress-Dependent Regulation of FOXO Transcription Factors by the SIRT1 Deacetylase. Science 303, 2011-2015.
4. Bultsma,Y., Keune,W., and Divecha,N. (2010). PIP4Kalpha interacts with and modulates nuclear localization of the high-activity PtdIns5P-4-kinase isoform PIP4Kalpha. Biochemical Journal 430, 223-235.
5. Canto,C. and Auwerx,J. (2012). Targeting Sirtuin 1 to Improve Metabolism: All You Need Is NAD+? Pharmacological Reviews 64, 166-187.
6. Canto,C., Gerhart-Hines,Z., Feige,J.N., Lagouge,M., Noriega,L., Milne,J.C., Elliott,P.J., Puigserver,P., and Auwerx,J. (2009). AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature 458, 1056-1060.
7. Clarke,J.H., Letcher,A.J., D'santos,C.S., Halstead,J.R., Irvine,R.F., and Divecha,N. (2001). Inositol lipids are regulated during cell cycle progression in the nuclei of murine erythroleukaemia cells. Biochem. J. 357, 905-910.
8. Di Paolo,G. and De Camilli,P. (2006). Phosphoinositides in cell regulation and membrane dynamics. Nature 443, 651-657.
9. Elkin,S., Ivanov,D., Ewalt,M., Ferguson,C., Hyberts,S., Sun,Z., Prestwich,G., Yuan,J., Wagner,G., Oettinger,M., and Gozani,O. (2005). A PHD Finger Motif in the C Terminus of RAG2 Modulates Recombination Activity. Journal of Biological Chemistry 280, 28701-28710.
10. Gerhart-Hines,Z., Dominy,J., Bluttler,S., Jedrychowski,M., Banks,A., Lim,J., Chim,H., Gygi,S., and Puigserver,P. (2011). The cAMP/ PKA Pathway Rapidly Activates SIRT1 to Promote Fatty Acid Oxidation Independently of Changes in NAD+. Molecular Cell 44, 851-863.
11. Gozani,O., Karuman,P., Jones,D.R., Ivanov,D., Cha,J., Lugovskoy,A.A., Baird,C.L., Zhu,H., Field,S.J., Lessnick,S.L., Villasenor,J., Mehrotra,B.,

Chen,J., Rao,V.R., Brugge,J.S., Ferguson,C.G., Payrastre,B., Myszka,D.G., Cantley,L.C., Wagner,G., Divecha,N., Prestwich,G.D., and Yuan,J. (2003). The PHD finger of the chromatin-associated protein ING2 functions as a nuclear phosphoinositide receptor. Cell 114, 99-111.

12. Hait,N., Allegood,J., Maceyka,M., Strub,G., Harikumar,K., Singh,S., Luo,C., Marmorstein,R., Kordula,T., Milstien,S., and Spiegel,S. (2009). Regulation of Histone Acetylation in the Nucleus by Sphingosine-1-Phosphate. Science 325, 1254-1257.
13. Jones,D., Bultsma,Y., Keune,W., Halstead,J., Elouarrat,D., Mohammed,S., Heck,A., D'Santos,C., and Divecha,N. (2006). Nuclear PtdIns5P as a Transducer of Stress Signaling: An In Vivo Role for PIP4Kbeta. Molecular Cell 23, 685-695.
14. Kang,H., Suh,J., Jung,Y., Jung,J., Kim,M., and Chung,J. (2011). Peptide Switch Is Essential for Sirt1 Deacetylase Activity. Molecular Cell 44, 203-213.
15. Keune,W., Bultsma,Y., Sommer,L., Jones,D., and Divecha,N. (2011). Phosphoinositide signalling in the nucleus. Adv. Enzyme Regul. 51, 91-99.
16. Kim,E., Kho,J., Kang,M., and Um,S. (2007). Active Regulator of SIRT1 Cooperates with SIRT1 and Facilitates Suppression of p53 Activity. Molecular Cell 28, 277-290.
17. Kim,J., Chen,J., and Lou,Z. (2008). DBC1 is a negative regulator of SIRT1. Nature 451, 583-586.
18. Knight,J.R. and Milner,J. (2012). SIRT1, metabolism and cancer. Curr. Opin. Oncol. 24, 68-75.
19. Lee,I., Cao,L., Mostoslavsky,R., Lombard,D., Liu,J., Bruns,N., Tsokos,M., Alt,F., and Finkel,T. (2008). A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy. Proceedings of the National Academy of Sciences 105, 3374-3379.
20. Lemmon,M.A. (2008). Membrane recognition by phospholipid-binding domains. Nat. Rev. Mol. Cell Biol. 9, 99-111.
21. Lewis,A., Sommer,L., Arntzen,M., Strahm,Y., Morrice,N., Divecha,N., and D'Santos,C. (2011). Identification of Nuclear Phosphatidylinositol 4,5-Bisphosphate-Interacting Proteins by Neomycin Extraction. Molecular & Cellular Proteomics 10.
22. Luo,J., Nikolaev,A., Imai,S., Chen,D., Su,F., Shiloh,A., Guarente,L., and Gu,W. (2001). Negative Control of p53 by Sir2alpha Promotes Cell Survival under Stress. Cell 107, 137-148.
23. Motta,M., Divecha,N., Lemieux,M., Kamel,C., Chen,D., Gu,W., Bultsma,Y., McBurney,M., and Guarente,L. (2004). Mammalian SIRT1 Represses Forkhead Transcription Factors. Cell 116, 551-563.
24. Ndamukong,I., Jones,D.R., Lapko,H., Divecha,N., and Avramova,Z. (2010). Phosphatidylinositol 5-phosphate links dehydration stress to the activity of ARABIDOPSIS TRITHORAX-LIKE factor ATX1. PLoS One 5, e13396.
25. Niebuhr,K., Giuriato,S., Pedron,T., Philpott,D., Gaits,F., Sable,J., Sheetz,M., Parsot,C., Sansonetti,P., and Payrastre,B. (2002). Conversion of PtdIns(4,5)P2 into PtdIns(5)P by the S.flexneri effector IpgD reorganizes host cell morphology. EMBO J 21, 5069-5078.
26. Pan,M., Yuan,H., Brent,M., Ding,E.C., and Marmorstein,R. (2012). SIRT1 contains N- and C-terminal regions that potentiate deacetylase activity. J. Biol. Chem. 287, 2468-2476.
27. Ramel,D., Lagarrigue,F., Pons,V., Mounier,J., Dupuis-Coronas,S., Chicanne,G., Sansonetti,P., Gaits-Iacovoni,F., Tronchere,H., and Payrastre,B. (2011). Shigella flexneri Infection Generates the Lipid PI5P to Alter Endocytosis and Prevent Termination of EGFR Signaling. Sci. Signal 4, ra61.
28. Sarkes,D. and Rameh,L. (2010). A novel HPLC-based approach makes possible the spatial characterization of cellular PtdIns5P and other phosphoinositides. Biochemical Journal 428, 375-384.
29. Sbrissa,D., Ikonomov,O., Strakova,J., and Shisheva,A. (2004). Role for a Novel Signaling Intermediate, Phosphatidylinositol5-Phosphate, in Insulin-Regulated F-Actin Stress Fiber Breakdown and GLUT4 Translocation. Endocrinology 145, 4853-4865.
30. Tang,Y., Zhao,W., Chen,Y., Zhao,Y., and Gu,W. (2008). Acetylation Is Indispensable for p53 Activation. Cell 133, 612-626.
31. Tolias,K., Rameh,L., Ishihara,H., Shibasaki,Y., Chen,J., Prestwich,G., Cantley,L., and Carpenter,C. (1998). Type I Phosphatidylinositol-4-phosphate 5-Kinases Synthesize the Novel Lipids Phosphatidylinositol 3,5-Bisphosphate and Phosphatidylinositol 5-Phosphate. Journal of Biological Chemistry 273, 18040-18046.
32. Vaziri,H., Dessain,S.K., Ng Eaton,E., Imai,S.I., Frye,R.A., Pandita,T.K., Guarente,L., and Weinberg,R.A. (2001). hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell 107, 149-159.

33. Yamakuchi,M., Ferlito,M., and Lowenstein,C.J. (2008). miR-34a repression of SIRT1 regulates apoptosis. Proc. Natl. Acad. Sci. U S A 105, 13421-13426.

34. Yeung,F., Hoberg,J., Ramsey,C., Keller,M., Jones,D., Frye,R., and Mayo,M. (2004). Modulation of NF-[kappa]B-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J 23, 2369-2380.

35. Zhao,W., Kruse,J.P., Tang,Y., Jung,S.Y., Qin,J., and Gu,W. (2008). Negative regulation of the deacetylase SIRT1 by DBC1. Nature 451, 587-590.

SUPPLEMENTARY INFORMATION

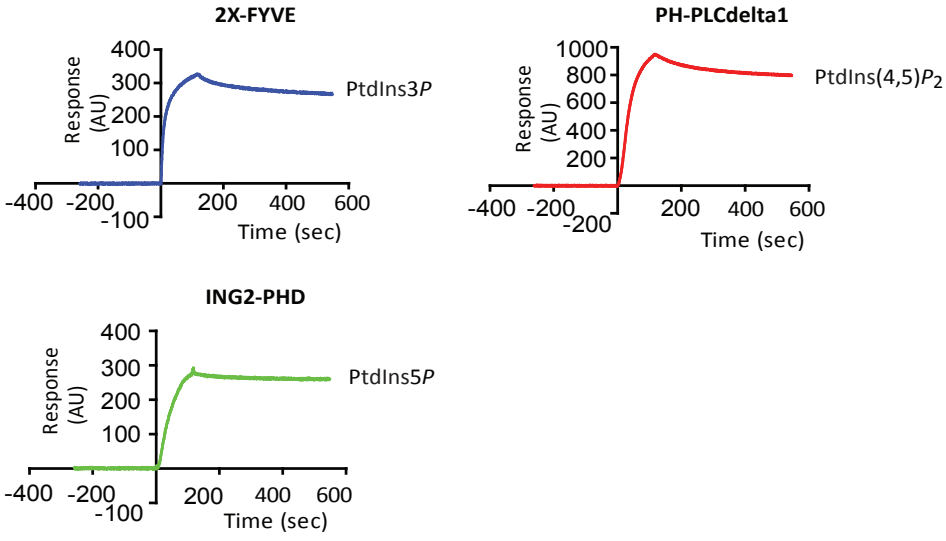


Figure S1. *SPR analysis of canonical phospholinositide binding domains. The respective interactions of the small protein domains 2X-FYVE, PH-PLCdelta1 and ING2-PHD with PtdIns3P, PtdIns(4,5)P2, and PtdIns5P were analyzed by Surface Plasmon Resonance (SPR). Data is representative of at least three independent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

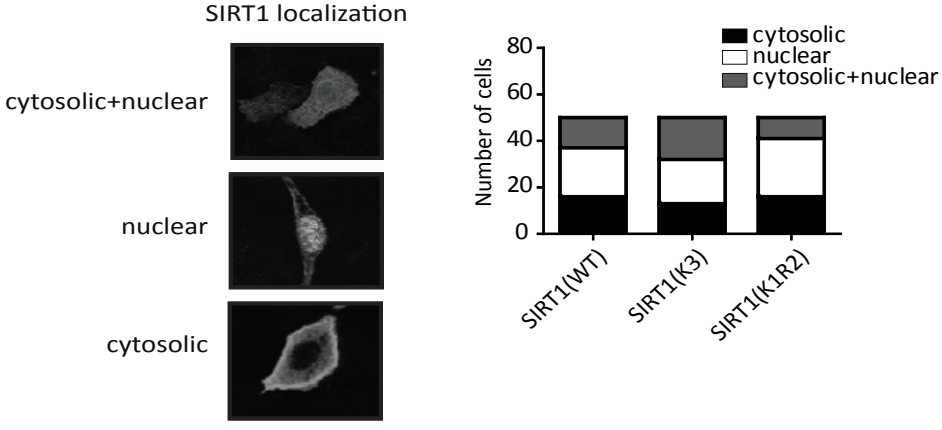


Figure S2. *KKRKK motif in SIRT1 is not a nuclear localization signal. Immunolocalization of full-length SIRT1(WT), SIRT1(K3) and SIRT1(K1R2) was performed in HEK 293T. Left: SIRT1 localizations in the nucleus, cytosol or both are shown. Right: the respective quantifications of SIRT1(WT), SIRT1(K3) and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

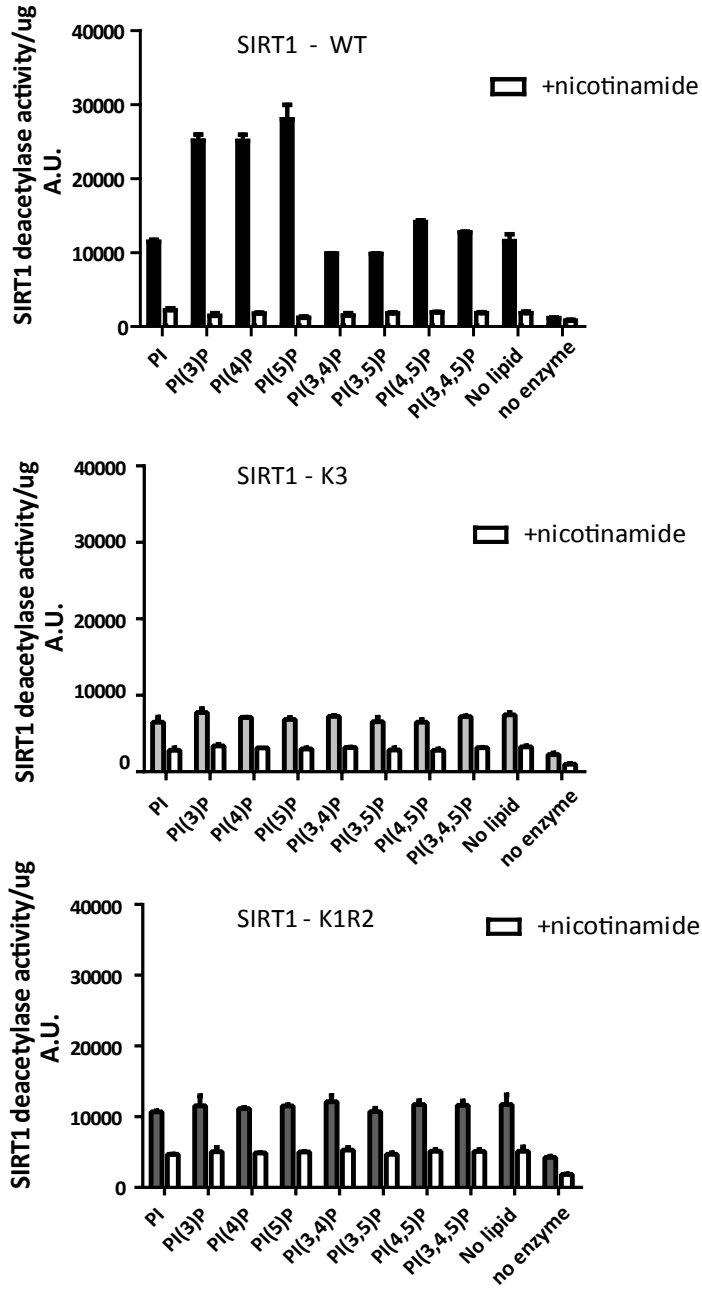


Figure S3. *Phosphoinositides stimulate p53 deaceylation of SIRT1 in vitro. Wild type and mutant SIRT1 were assayed for their deaetylation activity in the absence or presence of di-C8 water soluble phosphoinositides. Only phosphomonoinositides were able to stimulated wild type SIRT1, however importantly, 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.*

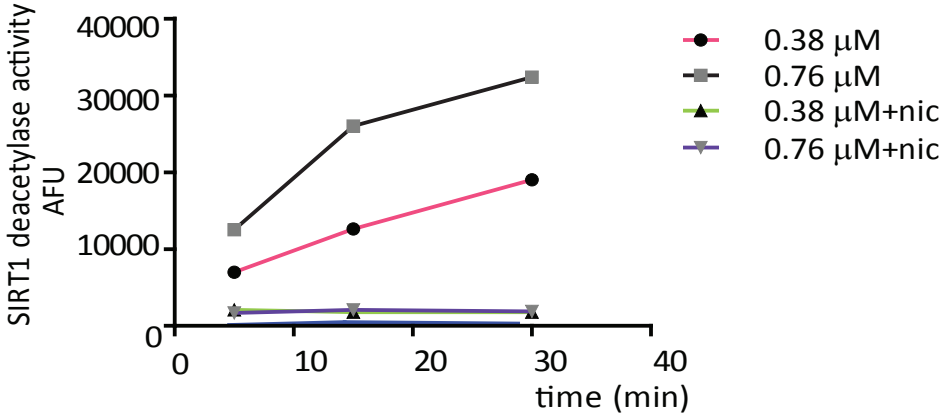


Figure S4. *SIRT1 activity is time- and dose-dependent. Lysine deacetylation activity of purified wild type SIRT1 [SIRT1(WT)] was determined using a fluorescent activity assay. Changes in the amount of deacetylated product formed over time were measured at two different SIRT1(WT) concentrations (0.38 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.*

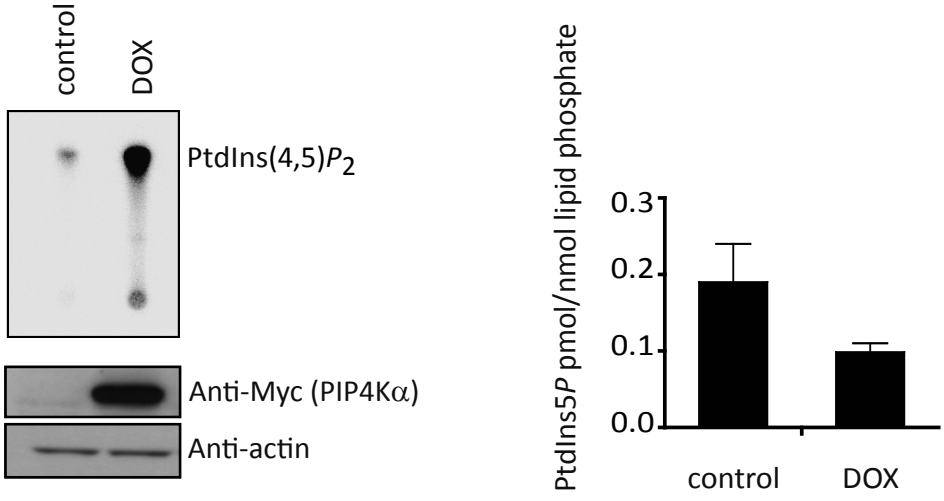


Figure S5. *Doxocycline treatment induces PIPKα expression in U2OS-Myc-PIP4Kα cells. U2OS cells expressing doxycycline-inducible Myc-PIP4Kα (U2OS-Myc-PIP4Kα) were maintained untreated (control) or were treated with doxycycline (DOX) to induce the over- expression of Myc-PIP4Kα. Myc-PIP4Kα was immunoprecipitated 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.*

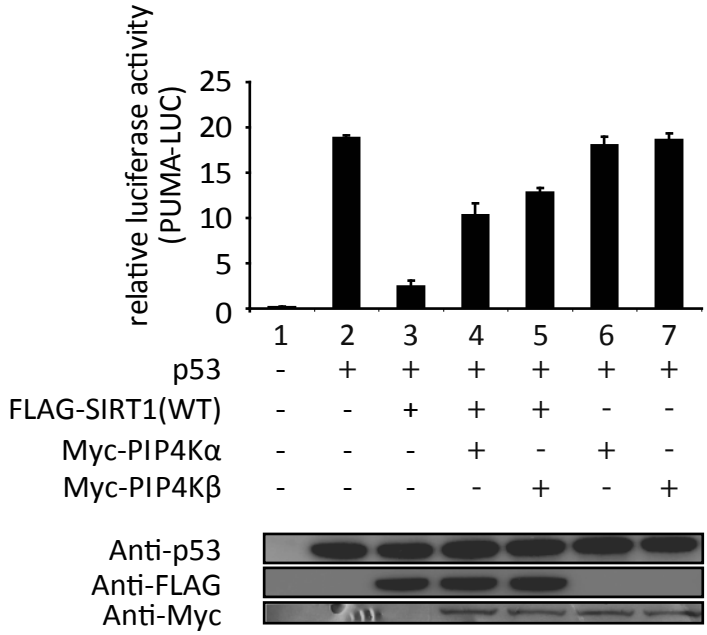


Figure S6. *PIP4Kα,β overexpression and p53-mediated transcription. H1299 cells (p53-deficient) were transfected with the PUMA promoter coupled to firefly luciferase and with the Renilla luciferase vectors together with p53, FLAG-SIRT1(WT), Myc- PIP4Kα or Myc-PIP4Kα as indicated. Top: Relative firefly/Renilla 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.

Suplemental references

1. Brooksbank,C.E., Hutchings,A., Butcher,G.W., Irvine,R.F., and Divecha,N. (1993). Monoclonal antibodies to phosphatidylinositol 4-phosphate 5-kinase: distribution and intracellular localization of the C isoform. Biochem. J. *291 (Pt 1)*, 77-82.
2. Bultsma,Y., Keune,W., and Divecha,N. (2010). PIP4Kalpha interacts with and modulates nuclear localization of the high-activity PtdIns5P-4-kinase isoform PIP4Kalpha. Biochemical Journal *430*, 223-235.
3. El Deiry,W., Tokino,T., Velculescu,V., Levy,D., Parsons,R., Trent,J., Lin,D., Mercer,W.E., Kinzler,K., and Vogelstein,B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817-825.
4. Yu,J., Zhang,L., Hwang,P., Kinzler,K., and Vogelstein,B. (2001). PUMA Induces the Rapid Apoptosis of Colorectal Cancer Cells. Molecular Cell *7*, 673-682.