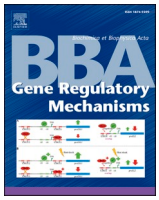
BBA - Gene Regulatory Mechanisms 1865 (2022) 194887

Contents lists available at ScienceDirect

BBA - Gene Regulatory Mechanisms

journal homepage: www.elsevier.com/locate/bbagrm



The VRK1 chromatin kinase regulates the acetyltransferase activity of Tip60/KAT5 by sequential phosphorylations in response to DNA damage

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ARTICLE INFO

Keywords:
Tip60
VRK1
Phosphorylation
Acetylation
DNA damage
Histone

ABSTRACT

The regulation of histone epigenetic modifications mediates the adaptation of chromatin to different biological processes. DNA damage causes a local relaxation of chromatin associated to histone H4 acetylation in K16, mediated by Tip60/KAT5. In this work, we have studied the role that the VRK1 chromatin kinase plays on the activation of Tip60 during this process. In the DNA damage response induced by doxorubicin, VRK1 directly phosphorylates Tip60. However, the phosphorylated Tip60 residues and their functional roles are unknown. In DDR, we have identified these two Tip60 phosphorylated residues and the cooperation of the participating kinases. The T158 phosphorylation, mediated by VRK1, is early and transient, preceding that of S199, which is more sustained in time, and mediated by DNA-PK. The role of each phosphorylated residues was determined by using phosphomimetic and phosphonull mutants and their combination. T158 phosphorylation protects Tip60 from ubiquitin-mediated degradation, promotes its recruitment to chromatin from the nucleoplasm, and is necessary for its full *trans*-acetylase activity. The phosphorylation in S199 by DNA-PK directly facilitates Tip60 autoacetylation, but it is not enough for *trans*-acetylation of two of its targets, histone H4 and ATM, which requires a double phosphorylation of Tip60 in T158 and S199. DNA-PK inhibitors block the phosphorylation of S199. We propose a model in which the cooperation between VRK1 and DNA-PK mediates the sequential phosphorylation of Tip60/KAT5, and contributes to the recruitment of this protein to initiate the sequential remodeling of chromatin in DDR. Both proteins are candidates for novel synthetic lethality strategies in cancer treatment.

1. Introduction

Chromatin remodeling requires the sequential coordination of different epigenetic modifications of histones in order to adapt its local organization to specific cellular functions, ranging from transcription, replication, recombination and DNA damage responses [1–4]. Moreover, mutations in genes coding for chromatin epigenetic enzymes have been associated to cancer [3,5–7], neurodegenerative diseases [8] and developmental alterations [9,10].

DNA damage is a problem to which each individual cell has to detect and respond independently of its particular situation regarding cell cycle phase, differentiation stage, cellular interactions or local microenvironment. DNA damage caused by either DNA base modifications, or strand breaks, and their impact on nucleosomal histones, triggers the initiation of the DNA damage repair (DDR) [11]. At this early stage, all types of DNA damage share a locally altered chromatin, and once identified the type of damage the specific DNA repair pathway is engaged in the response. Therefore, DNA damage triggers a dynamic sequential remodeling of chromatin as DDR progresses [12]. Chromatin remodeling is associated to different epigenetic modifications of histones, mainly acetylation, methylation, ubiquitination and phosphorylation, which have different functional roles in time and space [13]. These epigenetic modifications often occur on the same specific lysine residue in histones [3,14,15]. The addition of these modifications to histones is mediated by different types of enzymes, such as lysine acetyl transferases (KAT), histone deacetylases (HDAC), lysine methyl

Abbreviations: VRK1: Vaccinia Related Kinase-1, DDR: DNA damage response.

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https://doi.org/10.1016/j.bbagrm.2022.194887
Received 13 September 2022; Received in revised form 13 October 2022; Accepted 15 October 2022
Available online 21 October 2022

transferases (KMT) and lysine demethylases (KDM). Therefore, these alternative histone epigenetic modifications need to be coordinated in the context of specific biological roles. Likely candidates for playing a coordinating role among these different epigenetic enzymes are kinases, which by phosphorylation can regulate their activity, stability and localization, as well as their interactions with other proteins in different chromatin functional processes.

An essential epigenetic modification involved in chromatin remodeling is the acetylation of H4 in K16, a modification that is associated with chromatin relaxation and implicated in several processes such as DNA damage [16,17], transcription [18] and replication [1,19,20].

In DNA damage, the initial and local alteration of chromatin is associated with the accumulation of histone H4K16ac [17,20,21]. In this context, the H4K16 acetylation is mediated by histone acetylases such as Tip60/KAT5 in G0/G1 [22], and MOF in G2/M [23] in different phases of the cell cycle, and both regulate genome stability, for which this specific histone acetylation is necessary [24–26]. This histone acetylation represents an early process that occurs prior to the selection of a specific DDR pathway, once the specific type of DNA damage is identified. However, the activation mechanisms of the histone acetylases implicated in the regulation of genome stability are unknown. In this context, the activation of histone acetyl transferases is likely to be the consequence of a phosphorylation mediated by kinases associated with chromatin and DNA damage responses. Among the kinase family, the human chromatin kinase VRK1 (vaccinia-related kinase 1), also known as NHK1 (Nucleosomal histone kinase-1) in *Drosophila melanogaster* [27], is a candidate to play a regulatory role of Tip60. This kinase is the most abundant chromatin kinase [28], regulates gene transcription [29–31], replication [32] and DNA damage responses [33,34]. All these processes require a dynamic chromatin remodeling. VRK1 is activated in response to DNA damage independently of its type [34,35], such as pyrimidine dimers induced by ultraviolet light [36], double strand breaks induced by ionizing radiation or doxorubicin [35], or strand breaks caused by transcriptional block and oxidative stress [37]. The interaction of histones with the VRK1 C-terminus regulates its kinase activity [37–39]. On chromatin, VRK1 specifically phosphorylates histones H2A in Thr120 [40,41], H3 in Thr3 [37,38,42,43], both associated with chromatin remodeling, and sequential downstream proteins associated with specific DDR pathways, such as H2AX phosphorylation in Ser139 [16,37,44], phosphorylation of NBS1 in Ser343 [45], and 53BP1 in Ser25/29 [35] required for foci formation, as well as the phosphorylation of p53 in Thr18 [36,46], which is a regulator of DDR pathways. Moreover, VRK1 is an upstream kinase necessary for the activation of several kinases involved in DDR, such as ATM, ATR and DNA-PK [35]. DNA damage, before triggering a specific DDR, causes a local chromatin alteration associated with the acetylation of H4K16. This specific H4 acetylation is rescued by kinase active VRK1 [22], which phosphorylates Tip60/KAT5 in an unidentified residue [22], and is not rescued by kinase-dead VRK1(K179E) mutant [22].Therefore, VRK1 is a candidate to play a significant role in the regulation of histone modifications associated to early DNA damage responses, including H4K16ac. This initial role of VRK1 and Tip60 connects the DNA lesion with the specific response to repair DNA, in which both have additional roles. Furthermore, the acetylase activity of Tip60/KAT5 is regulated in other processes by its specific phosphorylation in Ser86 and Ser90 mediated by cyclin B/Cdc2 complex in G2/M cell cycle progression [47], GSK3 in apoptosis [48] and autophagy [49], CDK9 in transcription [47,50], and phosphorylation in Thr158 by p38α in p53-dependent apoptosis [51], in which p53 is stabilized by its phosphorylation by VRK1 [36,52].

In this report, we have identified the sequential phosphorylation of Tip60 mediated by VRK1, in cooperation with DNA-PK, in the very early steps, and prior to the response to DNA damage. Furthermore, we have characterized the specific role of each phosphorylation of Tip60 on this protein stability, its nuclear recruitment to altered chromatin, and the regulation of Tip60 acetyltransferase activity, processes that lead to the acetylation of H4K16 in order to facilitate the subsequent steps of specific DDR pathways.

2. Material and methods

2.1. Reagents

All chemical reagents were from Sigma-Aldrich-Merck (Darmstadt, Germany). The inhibitors used in this work are listed in Table 1.

2.2. Plasmids and mutagenesis

Human VRK1 was expressed from plasmid pCEFL-HA-VRK1 [22,42,45,53]. Human Tip60 was expressed from plasmid pcDNA3.1-TIP60-V5-His from D. Maurer [48]. Murine VRK1 (mVRK1) was expressed from plasmid pCMV6-mVRK1-myc-DKK (OriGene, Rockville, MD). A kinase-dead construct of the murine VRK1(K179E) was generated with the Quick-Mutagenesis system (Stratagene, San Diego, CA) [53]. Other plasmids were used to express RNF8 (pCDNA3.1-RNF8-HA) [54] mdm2 (pECFL-mdm2-HA) [55], and ubiquitin (pCDNA3-ubiquitin-His) [53].

All Tip60/KAT5 residues that are candidates as phosphorylation substrates, and identified in Phosphosite (https://www.phosphosite.org) [56], were mutated to alanine (phospho-null) or aspartic acid (phosphomimetic) in the pcDNA3.1-TIP60-V5-His plasmid as the target for site directed mutagenesis using the QuickChange Site-Directed Mutagenesis kit (Stratagene, San Diego, CA). The primers used to generate the different Tip60 phospho mutants are listed in Table 2. The reaction mixture in a final reaction volume of 30 μL contained: 50 ng of plasmid, 10 μM of each primer (Table 2), 0.2 mM of deoxynucleotide mix (Hoffman-La Roche), 1 unit of PFU DNA polymerase (Biotools, B&M Labs, Madrid, Spain), and the PCR buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl pH 8,8, 20 mM $MgSO_4$, 1 % Triton X-100 y BSA 1 mg/mL). The PCR was performed in an iCycler thermocycler (BioRad, Hercules, CA). After the PCR, the plasmid used as template was digested with *Dpn1* (Thermo Fisher Scientific, Waltham, MA). The sequences of the mutant plasmids generated were confirmed by DNA Sanger sequencing.

2.3. Cell lines, culture and transfections

The following validated cell lines A549 (CCL-185) and HT-144 (HTB-63) were from the *American Type Culture Collection* (ATCC, Manassas, VA), and are mycoplasma free. Cells were grown in DMEM (Gibco-Life Technologies, Carlsbad, CA) supplemented with 10 % fetal calf serum, 2

Table 1
Inhibitors used in this work.

Inhibitor	Targeted kinase	Supplier	Catalog reference
Kinase inhibitors			
Caffeine	PIKK	SIGMA	C8960
KU55933	ATM	Tocris Bioscience	3544
VE-281	ATR	Selleckchem	S8007
NU7026	DNA-PK	Selleckchem	S2893
JNK-IN-8	JNK (1, 2, 3)	Selleckchem	S4901
Roscovitine	Cdk1	Selleckchem	S1153
SB 203580	p38	Selleckchem	S1076
TZD8	GSK3	Selleckchem	S2926
PD98059	MEK1	Merck/ Calbiochem	513,000
Other inhibitors			
MG132	Proteasome	Calbiochem	474,790
C646	P300 acetyl transferase	Selleckchem	S7152
MG149	KAT5/Tip60	Axon Medchem	1785
Cycloheximide	protein synthesis inhibitor	Sigma-Aldrich	C7698

Table 2
Primers for introduction of phospho mutations in human Tip60.

Mutation	Primers
Tip60 (S155A)	*Forward:* ACGGAAGGTGGAGGTGGTT**GCA**CCAGCAACTCCAGTGCCCAGC *Reverse:* GCTGGGCACTGGAGTTGCTGG**TGC**AACCACCTCCACCTTCCGT
Tip60 (T158A)	*Forward* AGGTGGTTTCACCAGCA**GCT**CCAGTGCCCAGCGAGACAGCCC *Reverse:* GGGCTGTCTCGCTGGGCACTGG**AGC**TGCTGGTGAAACCACCT
Tip60 (T158D)	*Forward:* AGGTGGTTTCACCAGCA**GAT**CCAGTGCCCAGCGAGACAGCCC *Reverse:* GGGCTGTCTCGCTGGGCACTGG**ATC**TGCTGGTGAAACCACCT
Tip60 (S190A)	*Forward:* GCCAGGACGGAAGCGAAAA**GCG**AATTGTTTGGGCACTGATGAG *Reverse:* CTCATCAGTGCCCAAACAATT**CGC**TTTTCGCTTCCGTCCTGGC
Tip60 (S199A)	*Forward:* GGGCACTGATGAGGAC**GCC**CAGGACAGCTCTGATGGAATACCG *Reverse:* CGGTATTCCATCAGAGCTGTCCTG**GGC**GTCCTCATCAGTGCCC
Tip60 (S199D)	*Forward:* GGGCACTGATGAGGAC**GAC**CAGGACAGCTCTGATGGAATACCG *Reverse:* CGGTATTCCATCAGAGCTGTCCTG**GTC**GTCCTCATCAGTGCCC
Tip60 (T158/S199A)	*Forward:* GGCACTGATGAGGAC**GCC**CAGGACAGCTCTGATGGAATACCG *Reverse:* CGGTATTCCATCAGAGCTGTCCTG**GGC**GTCCTCATCAGTGCCC
Tip60 (T158/S199D)	*Forward:* GGCACTGATGAGGAC**GAC**CAGGACAGCTCTGATGGAATACCG *Reverse:* CGGTATTCCATCAGAGCTGTCCTG**GTC**GTCCTCATCAGTGCCC
Tip60 (T158A/S199D)	*Forward:* GGCACTGATGAGGAC**GAC**CAGGACAGCTCTGATGGAATACCG *Reverse:* CGGTATTCCATCAGAGCTGTCCTG**GTC**GTCCTCATCAGTGCCC
Tip60 (T158D/S199A)	*Forward:* GGCACTGATGAGGAC**GCC**CAGGACAGCTCTGATGGAATACCG *Reverse:* CGGTATTCCATCAGAGCTGTCCTG**GGC**GTCCTCATCAGTGCCC

mM de L-glutamine (Gibco-Life Technologies), penicillin (50 u/ml) and streptomycin (50 μg/ml) (Pen Strep; Gibco-Life Technologies). Cells were collected at 75 % confluence for passage or lysis for experiments. Cells were rinsed with PBS and detached using trypsin-EDTA (TrypLE Express; Gibco-Life Technologies-Invitrogen). Cell lysates were prepared by suspending cells in mild lysis buffer (50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, and 1 % triton X-100), phosphatases inhibitors (1 mM NaF and 1 mM sodium orthovanadate) and proteases inhibitors (1 mM PMFS, 10 μg/mL aprotinin and 10 μg/mL leupeptin) [22].

Plasmid transfections were performed as previously reported [16,22,57]. Briefly, transfections were performed mixing plasmids with Polyethylenimine (PEI) reagent (Polysciences Inc.) [42,53]. All experiments with transfections were performed three times.

2.4. Induction of DNA damage

DNA damage was induced by treatment with doxorubicin, which was added to cell cultures with the dose and time indicated in individual experiments. For induction of DNA damage by doxorubicin treatment [22,57], cells were serum-deprived (0.5 % FBS) for forty-eight hours before doxorubicin addition to the culture. Serum deprivation was used to prevent cell proliferation and to accumulate cells in G0/G1, which was checked by flow cytometry and the lack of cyclin D1 and phosho-Rb [30,35]. In time course experiments, the starting time point (0 min) is the reference value, and all time points were normalized by the levels of endogenous and transfected protein, where applicable. In experiments using inhibitors, these were added ten hours before the addition of doxorubicin. All experiments were performed three times.

2.5. Depletion of VRK1 or Tip60 by siRNA

The depletion of VRK1 by siRNA has been previously reported for cell lines A549 [16,37,42] and HT-144 [37,45] used in this work. Briefly, depletion of endogenous VRK1 or Tip60 was performed using the corresponding siRNA at a final concentration of 200 nM using Lipotransfectine reagent (Solmeglas, Madrid, Spain). All the siRNA used for depletion were obtained from Dharmacon (Lafayette, CO). VRK1 was depleted with siVRK1-02 (target sequence: CAAGGAACCUGGUGUUGAA) or siVRK1-03 (target sequence: GGAAUGGAAAGUAGGAUUA). Tip60 was depleted with siTip60 obtained from Dharmacon-Horizon Discovery (Ref: J-006301-08-00200). As negative controls, we used the "ON-TARGETplus siCONTROL Non-targeting siRNA" (siCt) (Dharmacon). The efficiency of RNAi transfection was determined with "siGLO RISC-free siRNA" (Dharmacon) [22,57].

2.6. Cell lysates, nucleoplasm and chromatin fractionation

Cells were lysed with mild-lysis buffer (50 mM Tris-HCl, pH 8,0, 150 mM NaCl, 1 % Triton X-100, 1 mM EDTA) supplemented with protease inhibitors, (1 mM PMSF, 10 μg/mL leupeptin and 10 μg/mL aprotinin), and phosphatase inhibitors (1 mM NaF y 1 mM Na_3VO_4). The suspension was incubated at 4 °C for 20 min followed by centrifugation at 13.200 rpm in an Eppendorf-5415R centrifuge for 20 min at 4 °C to remove cellular debris [22].

Nuclear and cytoplasmic fractions were prepared suspending cells in lysis buffer composed of: 10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 mM sacarose, 10 % glycerol, 20 μM MG132, 0,1 % Triton X-100, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM NaF, 1 mM Na_3VO_4, and incubated at 4 °C for 8 min [50]. The cell lysate was centrifuged at 1.300 *g* for 5 min to separate nuclei from cytoplasm (supernatant). The nuclear pellet was resuspended in nucleoplasmic buffer (400 mM NaCl, 3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 20 μM MG132, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM NaF, 1 mM Na_3VO_4), incubated at 4 °C for 20 min, and centrifuged at 1000 *g* for 2 min to separate chromatin from nucleoplasm. The chromatin pellet was resuspended in chromatin buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5 % SDS, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM NaF, 1 mM Na_3VO_4), and sonicated at 4 °C with 5 × 30 s pulses, spaced 30 s, in a Sonics Vibra Cell VCX-500 (Newtown, CT) [50]. Quantification of protein concentrations was determined by the Bradford method using the Bio-Rad Protein Assay (Bio-Rad) kit. Protein concentration in lysates were within the linear response of the Bradford method.

2.7. Immunoprecipitations and immunoblots

Proteins from cell lysates with a concentration of 1–2 mg/ml were used for immunoprecipitations. Table 3 lists the primary antibodies and Table 4 lists the secondary antibodies. The corresponding primary antibody, are indicated in individual experiments, was added to these extracts and were incubated for 12 h at 4 °C with gentle shaking. Next, 60 μL of the Protein G Agarose Resin 4 Rapid Run (Agarose Bead Technologies) equilibrated with mild lysis buffer (50 mM Tris-HCl, pH 8,0, 150 mM NaCl, 1 % Triton X-100, 1 mM EDTA) was added. Next, it was incubated for 8 h at 4 °C, collected by centrifugation at 2200 rpm for 2 min, and washed three times. The immunoprecipitated protein was resuspended in loading buffer (62.5 mM Tris-HCl, pH 6.8, 10 % glycerol, 2.3 % SDS, 0.1 % bromophenol blue and 5 % β-mercaptoethanol), boiled and loaded in a polyacrylamide gel [22,57]. The proteins in the gel were transferred to a PVDF Immobilon-FL membrane (Millipore) that was blocked with 5 % defatted milk in TBS-T buffer (25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2.5 mM KCl, 0,1 % Tween-20 (*Sigma-Aldrich*), or alternatively with 5 % bovine serum albumin in TBS-T buffer. The membranes were incubated with primary antibodies for 8 h, washed three times in TBS-T for 10 min, followed by the addition of secondary antibodies and

Table 3
Primary antibodies.

Antibody	Type	Dilution (WB/IF)	Clone/ reference	Supplier
VRK1	Mouse monoclonal	1:1000 1:200	1F6	[87]
VRK1	Mouse monoclonal	1:1000 1:200	1B5	[87]
VRK1	Rabbit polyclonal	1:1000 –	VC	[87]
Anti-VRK1 (N-term)	Rabbit polyclonal	1:1000 1:200	– HPA000660	Sigma-Aldrich
Tip60	Rabbit polyclonal	1:500 1:200	– ab151432	Abcam
V5 Tag	Mouse monoclonal	1:1000 1:300	V5–10 V8012	Sigma-Aldrich
V5 Tag	Rabbit polyclonal	1:1000 –	– V8137	Sigma-Aldrich
Phosphoserine	Mouse monoclonal	1:500 –	4A4 05–1000	Merck Millipore
β-Actin	Mouse monoclonal	1:1000 –	AC15 A5441	Sigma-Aldrich
H4K16ac	Rabbit monoclonal	1:1000 1:300	EPR1004 ab109463	Abcam
H3	Rabbit polyclonal	1:1000 –	– 9715	Cell Signaling
53BP1	Rabbit polyclonal	– 1:200	– NB100–304	Novus Biologicals
PARP	Mouse monoclonal	1:1000 –	C2–10 556,362	BD Pharmigen
HA Tag	Rabbit polyclonal	1:1000 –	– H6908	Sigma-Aldrich
His Tag	Mouse monoclonal	1:1000 –	H-3 sc-8036	Santa Cruz Biotech.
GST	Mouse monoclonal	1:1000 –	B-14 sc-138	Santa Cruz Biotech.
p53-T18ph	Rabbit polyclonal	1:1000 –	– 2529	Cell Signaling
p53-K120ac	Rabbit polyclonal	1:500 –	– HW186	Signaling Antibody
Ubiquitin	Rabbit polyclonal	1:1000 –	– ab19247	Abcam
ATM-S1981ph	Mouse monoclonal	1:300 1:200	10H11.E12 Q2669086	Merck Millipore
ATM	Rabbit polyclonal	1:500 –	– 819–844	Calbiochem
CHEK1	Mouse monoclonal		2G1D5 2360	Cell Signaling
CHEK1-S317ph	Rabbit polyclonal	1:500 –	– PA5–99338	Invitrogen
DNA-PKcs-S2056ph	Rabbit polyclonal	1:500 –	Ab18192	Abcam
CENP-C	Rabbit monoclonal	1:500 –	EPR15939 Ab193666	Abcam
Acetyl-lysine	Rabbit polyclonal	1:500 –	– 9441S	Cell Signaling
JNK1/3	Rabbit polyclonal	1:1000 –	– sc474	Santa Cruz Biotech.
c-Jun	Rabbit monoclonal	1:1000 –	60A8 9165	Cell Signaling
c-Jun-S63ph	Mouse monoclonal	1:1000 –	J.973.7 MA5–15115	Invitrogen

incubated for an additional hour, which were washed in a similar way. The fluorescence on the membranes was scanned with a LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences), and images were analyzed using the ImageJ software version 1.53 (https://imagej.nih.gov) [22,34]. In addition, immunoblots were quantified using the Quantity One software from BioRad.

2.8. Acetyl transferase activity assays

The in vitro acetyl transferase activity of the different KAT5/Tip60 mutants was determined using the HAT activity colorimetric assay (Sigma-Aldrich, Ref.: EPI001). Briefly, 20 μg of Tip60 dissolved in 40 μl of water were mixed with 50 μl of HAT buffer, 5 μl of substrate 1, 5 μl of substrate 2 and 8 μl of enzyme generating NADH according to manufacturer instructions. The mix was placed in 96 well plates and incubated for 2 h at 37 °C. Absorbance was determined at a wavelength of 440 nm in an Infinite 200 PRO plate reader (Tecan Life Sciences, Männedorf, Switzerland).

Table 4
Secondary antibodies.

Antibody	Use	Dilution	Reference	Supplier
Goat *Anti*-Rabbit *IgG, DyLight 800*	*WB*	1:10000	35571	ThermoFisher Scientific
Goat *Anti*-Mouse *IgG, DyLight 680*	*WB*	1:10000	35518	ThermoFisher Scientific
Cy2-Goat *Anti*-Rabbit	IF	1:1000	111-225-144	Jackson ImmunoResearch
Cy2-Goat *Anti*-Mouse	IF	1:1000	115-225-146	Jackson ImmunoResearch
Cy3-Goat *Anti*-Rabbit	IF	1:1000	111-165-144	Jackson ImmunoResearch
Cy3-Goat *Anti*-Mouse	IF	1:1000	115-165-146	Jackson ImmunoResearch
Cy5-Goat *Anti*-Rabbit	IF	1:1000	111-175-144	Jackson ImmunoResearch
Cy5-Goat *Anti*-Mouse	IF	1:1000	115-175-146	Jackson ImmunoResearch

2.9. Protein stability

The stability of the different Tip60 phosphomutants was determined by transfecting A549 cells with the specific Tip60 mutant to express the protein. Forty-eight hours after the transfection, cycloheximide (50 μg/mL) was added to cell cultures to block the novo protein synthesis and is considered the starting point [58]. Cells were collected at different time points after cycloheximide addition. Cells were lysed in a mild-buffer (50 mM Tris-HCl, pH 8,0, 150 mM NaCl, 1 % Triton X-100, 1 mM EDTA). Proteins were fractionated in PAGE and transferred to an Immobilon-FL membrane (Millipore). The proteins were detected in western blots with an anti-tag antibody [22,59].

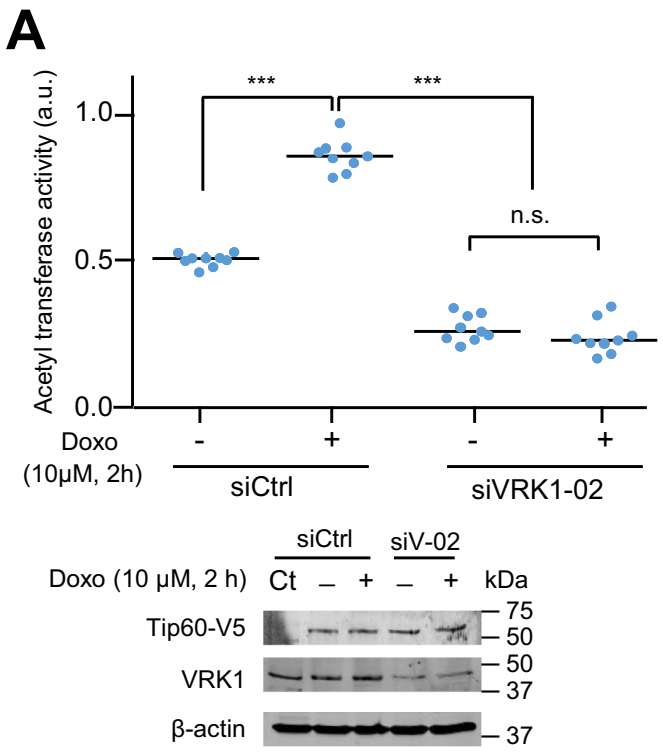
2.10. Statistical analysis

Data was analyzed using one-way ANOVA with Tukey-Kramer post-hoc test with the GraphPad Prism 7 program. All experiments were performed in triplicate with three determinations in each individual experiment. For statistical analysis the recommendation of the American Society for Cell Biology [60] and Cold Spring Harbor Laboratory [61] were followed.

3. Results

3.1. DNA damage activates the acetyl transferase activity of Tip60 that is impaired by VRK1 depletion

VRK1 directly interacts with and phosphorylates Tip60 in vitro [22], and in cells. VRK1 phosphorylates Tip60 in response to DNA damage [22]. Therefore, we studied whether this phosphorylation regulates the acetyl transferase activity of Tip60. For this aim, VRK1-depleted A549 cells were transfected with a V5-tagged Tip60 plasmid, and treated with doxorubicin. The transfected Tip60/KAT5 was immunoprecipitated with an anti-V5 antibody, and the acetyl-transferase activity of Tip60 was determined in a chemical colorimetric assay. DNA damage induced a significant increase in the Tip60 acetylase activity, which was lost by VRK1 depletion (Fig. 1A). Next, the effect of VRK1 on Tip60 phosphorylation and its autoacetylation was determined. Doxorubicin treatment caused an increase in both Tip60 phosphorylation and autoacetylation, and VRK1 depletion impaired both (Fig. 1B). In this



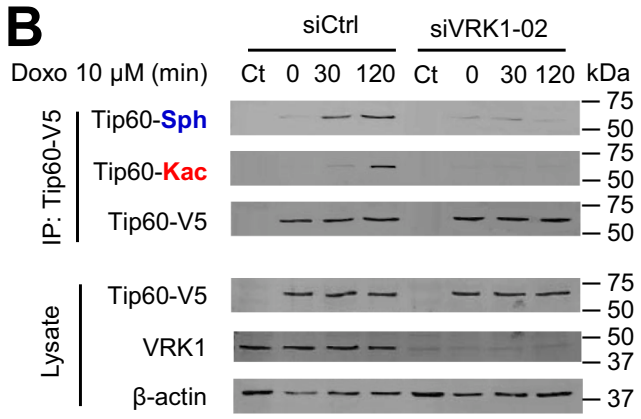


Fig. 1. VRK1 depletion impairs Tip60/KAT5 acetyl transferase activity induced by DNA damage. A. The acetyl transferase activity of Tip60 induced by DNA damage was detected by a colorimetric method using immunoprecipitated protein. The experiments were independently performed three times in triplicate, and analyzed using one-way ANOVA with Tukey-Kramer correction. *** $p < 0.001$. n.s.: not significant. In the western blot are shown the protein levels. B. Effect of VRK1 depletion on the phosphorylation and autoacetylation of Tip60 induced by DNA damage. Cells were treated with doxorucin (10 μM) for ten minutes. Ct: control with empty plasmid.

response to DNA damage, the Tip60 residue phosphorylated by VRK1 is unknown.

3.2. DNA damage induces the phosphorylation of two residues in Tip60

Next, we aimed to identify the KAT5/Tip60 residues phosphorylated during the response to DNA damage caused by doxorubicin. The Tip60 protein has several residues in its regulatory domain that has a disordered structure that allows for alternative conformation, which has several phosphorylation targets (S86, S90, T155, T158, S190 and S199), based on Phosphosite data. All these amino acid residues were mutated to either alanine or aspartic acid, and A549 cells were transfected with different Tip60 phosphorylation mutants, followed by treatment with doxorubicin to induce an alteration of chromatin because of the DNA damage. The phosphorylation of the Tip60 mutants was detected with an anti phospho-serine antibody in the immunoprecipitated proteins. All of them were phosphorylated except two of the Tip60 phosphonull mutants, T158A and S199A, that were not phosphorylated (Fig. 2A) suggesting that both residues are potential phosphorylation targets of VRK1. Next, we compared the response of these two residues, mutated to phosphomimetic Asp. When one of them was mutated to Asp, the other available residue was always phosphorylated in response to DNA damage (Fig. 2B). However, Tip60 phosphorylation was lost when both

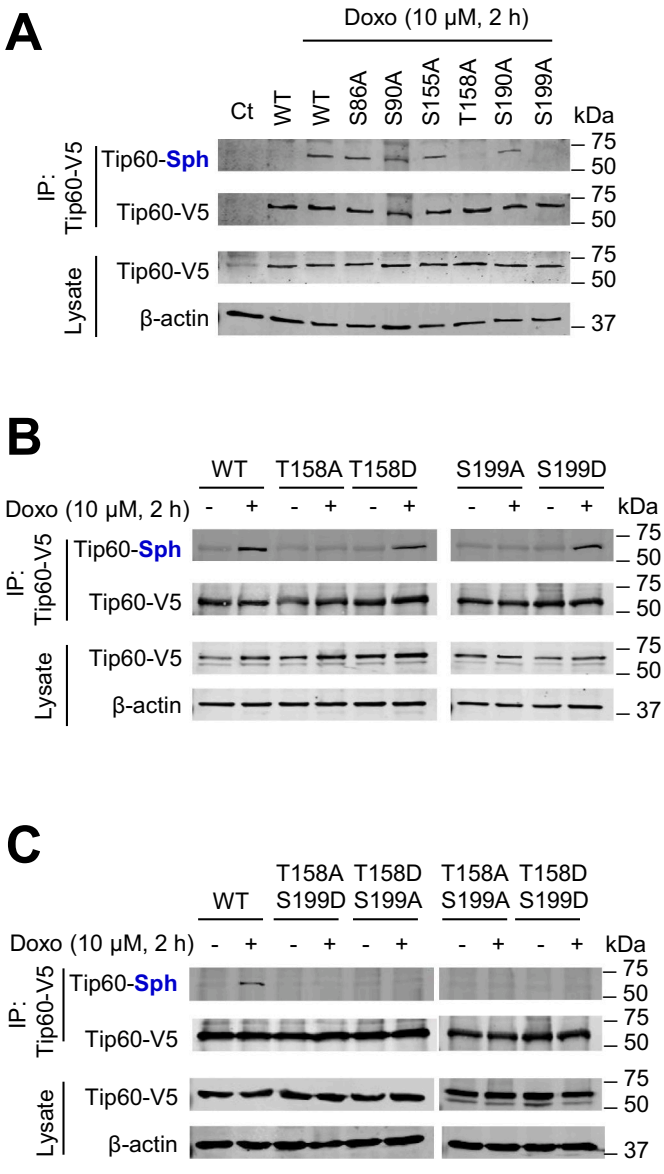


Fig. 2. Phosphorylation of Tip60 mutants. A. Phosphorylation of different phosphomutant of Tip60 induced by doxorubicin treatment indicated at the top. B. Effect of doxorubicin (10 μM, 2 h) on phosphomimetic and phosphonull mutants of residues T158 and S199. C. Effect of doxorubicin (10 μM, 2 h) on different combinations of T158/S199 phosphomutants. The different Tip60 mutants were expressed from Tip60-V5 constructs transfected in A549 cells. The phosphorylation of Tip60 was detected with an anti-phosphoserine antibody in immunoprecipitated tagged Tip60 proteins. The experiments were performed three times. Sph: phosphoserine, Kac: acetylated lysine. The transfected Tip60 was detected with an anti-V5 antibody.

residues were simultaneously mutated (Fig. 2C). These data suggested that both residues, T158 and S199, are potential phosphorylation targets in this response to DNA damage, but their temporal order and functional role is unknown.

Together with the identified T158 and S199, residues S86 and S90 are the most well characterized phosphorylated residues of Tip60. In order to confirm that S86 and S90 were not phosphorylated in response to doxorubicin, we analyzed the S86A, S90A and S86A/S90A mutants [47,51]. The VRK1 kinase phosphorylated the S86A, S90A and S86A/S90A Tip60 phosphomutants in vitro. Therefore, these mutated residues were ruled out as targets (Fig. S1A). The S86A and S90A phosphonull mutants were also phosphorylated like the wild type, in a different residue, in response to doxorubicin in A549 cells (Fig. S1B), and their phosphorylation in these other residues was lost by VRK1 depletion (Fig. S1C), suggesting that the VRK1 chromatin kinase can play a role in the regulation of KAT5/Tip60.

3.3. *Tip60 phosphorylation of T158 precedes that of S199 in response to doxorubicin*

Next, we determined the order of the Tip60 phosphorylation in T158 and S199 residues during the response to doxorubicin treatment. For this, we studied the phosphorylation of the alanine or aspartic acid phosphomutants over time. First, we determined the phosphorylation of V5-tagged Tip60 as reference. The phosphorylation of wild-type V5-Tip60 (Fig. 3A) has a similar phosphorylation pattern to that of the endogenous Tip60 in response to doxorubicin [22]. Therefore, we next analyzed the effect of different Tip60 phosphonull and phosphomimetic mutants, individually or in combination. The available T158 residue was phosphorylated in the S199A mutant (Fig. 3B). This T158

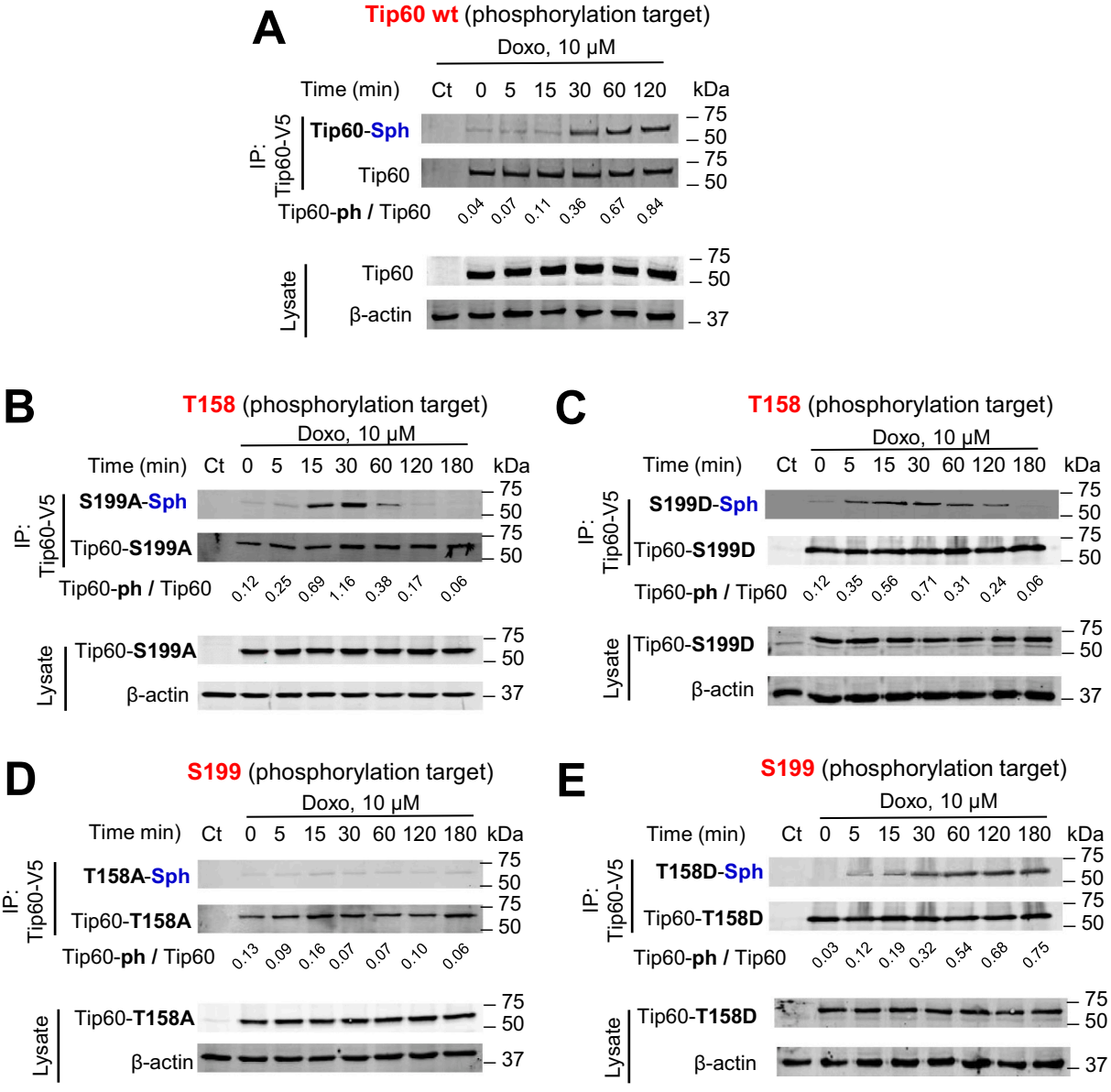


Fig. 3. Time course of Tip60 phosphorylation of the T158 or S199 mutants to alanine or aspartic acid after treatment with doxorubicin. Effect of doxorubicin treatment (10 μM) on phosphorylation of Tip60 wild-type (A), Effect of doxorubicin treatment (10 μM) on T158 phosphorylation using the S199A phosphonull mutant (B), or the S199D phosphomimetic mutant (C). Effect of doxorubicin (10 μM) treatment on the phosphorylation of S199 using the phosphonull T158A (D) or the phosphomimetic T158D (E). The corresponding V5-tagged Tip60 construct transfected in A549 cells, immunoprecipitated at different time points and its phosphorylation detected in an immunoblot. The experiment was performed three times. Sph: phosphoserine, Kac: acetylated lysine. The ratio between phosphorylated and non-phosphorylated immunoprecipitated Tip60 is shown at the bottom of the gels. Ct: control with empty plasmid. Immunoblot images were quantified using the Image J (NIH) and Quantity One (BioRad) programs. The transfected Tip60 was detected with an anti-V5 antibody.

phosphorylation is transient, peaks within the 15 to 60 min range, and does not require S199 phosphorylation (Fig. 3B). Moreover, the available T158 is phosphorylated reaching its transient peak signal at 30 min in the case of the phosphomimetic S199D (Fig. 3C), and behaved similarly to the S199A mutant (Fig. 3B). Therefore, we concluded that S199 phosphorylation is not necessary for T158 phosphorylation.

The available S199 residue was not phosphorylated in the T158A phosphonull mutant in response to doxorubicin, indicating that the available S199 in this context requires the previous phosphorylation in T158 (Fig. 3D). This was confirmed in the case of the phosphomimetic T158D (Fig. 3E). In this case, the phosphorylation of the available S199 residue is progressive with time, reaching its peak at 3 h after DNA damage (Fig. 3E). This indicated that for the phosphorylation of S199, a previous phosphorylation of T158, or its phosphomimetic mutant, is necessary, and in this case the phosphorylation of the available T199 is stable in time (Fig. 3E). These results indicated that in the response to DNA damage, the phosphorylation of Tip60 in T158 precedes and is necessary for the phosphorylation of S199, and these two residues individually have a different, timing in the response to DNA damage, but are stable and overlap in time when combined. This reflects that each phosphorylation probably play a different role between the initial chromatin/nucleosome alteration caused by DNA damage, which activates the kinase activity of VRK1 [35] and mediated by the VRK1-Tip60 complex, and later during the progression of the specific DNA repair mechanism through its different sequential steps.

3.4. VRK1 depletion prevents the phosphorylation of Tip60 in T158

In order to identify which of these two Tip60 residues is the direct target of VRK1, the effect of VRK1 depletion with two different siRNA was determined on the phosphorylation of either T158 or S199 changed to either Ala or Asp. In the case of the phosphomimetic T158D mutant, the available residue S199 was phosphorylated independently of VRK1, since S199 is not phosphorylated in the case of the T158A mutant that cannot be phosphorylated by VRK1 (Fig. 4A). However, in both the S199D and S199A mutants, the T158 residue is available for phosphorylation in response to doxorubicin. VRK1 depletion resulted in the loss of this phosphorylation (Fig. 4A). These data indicated that the direct target of VRK1 is the Tip60-T158 residue.

Next, we performed a time course of the phosphorylation of T158D and S199D to confirm the effect of VRK1 depletion. In the case of T158D that mimics its phosphorylation by VRK1, there is no effect of VRK1 depletion on S199 phosphorylation (Fig. 4B top). However, in the case of the S199D, depletion of VRK1 impairs the phosphorylation of its available target T158, independently of the time of treatment (Fig. 4B, bottom). Therefore, we concluded that although both residues are phosphorylated in response to DNA damage, only the T158 residue is the direct target of VRK1, and this specific phosphorylation is required for the additional Tip60 phosphorylation in S199 by another kinase that should be implicated in the response to DNA damage. This will connect phosphorylation changes associated with the initial chromatin alteration caused by DNA damage with those occurring in pathways implicated in DNA damage responses, which should be sequential in time.

3.5. Tip60 phosphorylation of S199 is mediated by DNA-PK

To identify the kinase involved in the phosphorylation of Tip60-S199, we performed a kinase assay in the presence of inhibitors targeting specific kinases. VRK1 is an activator of PI3K kinases involved in DDR [35]. Therefore, we tested the effect of inhibitors targeting members of this kinase family. The ATM role in the phosphorylation of S199 in the T158D mutant was ruled out, since this Tip60-S199 phosphorylation was insensitive to the ATM inhibitor KU55933 (Fig. 5A), in which the phosphorylation of ATM in S1981 was used as control. Furthermore, this Tip60 phosphorylation in the available S199 also occurred in HT-144 (ATM−/−) cells (Fig. 5B). However, the phosphorylation of S199

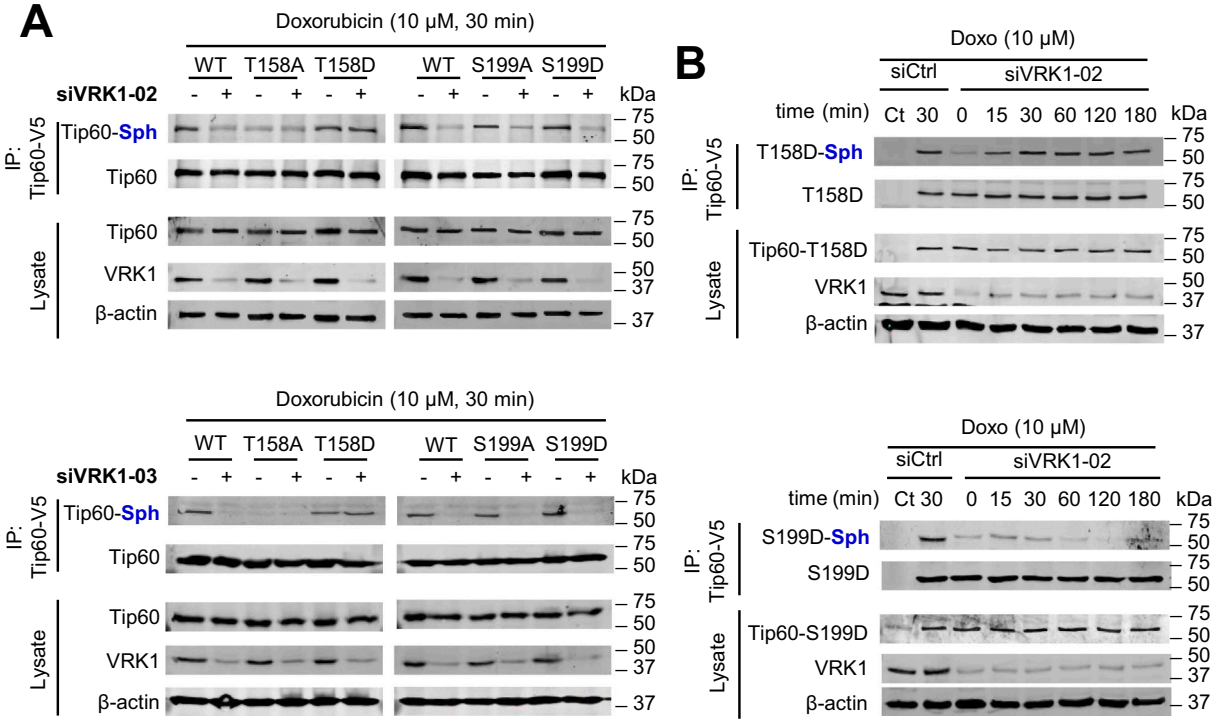


Fig. 4. Effect of VRK1 depletion on the phosphorylation of Tip60 phospho-mutants in response to doxorubicin. A. Effect of two different siRNA targeting endogenous VRK1 on the phosphorylation of Tip60 T158 and S199 phosphonull and phosphomimetic mutants. B. Time course of the phosphorylation of Tip60 phosphomimetic mutants in response to doxorubicin and effect of VRK1 depletion. A549 cells transfected with the corresponding V5-tagged Tip60 constructs, and the protein was immunoprecipitated at different time points and its phosphorylation detected in an immunoblot. The experiment were performed three times. Sph: phosphoserine, Kac: acetylated lysine. Ct: control with empty plasmid. The transfected Tip60 was detected with an anti-V5 antibody.

in the T158D phosphomimetic mutant was inhibited by NU7026 [62], an inhibitor of DNA-PK (Fig. 5C), and in which DNA-PK phosphorylation in S2056 was used as control. VE-821 [63,64], an ATR inhibitor, has no effect on Tip60 phosphorylation, in which CHEK1-S317 phosphorylation was used as control (Fig. 5D). The phosphorylation of Tip60-T158D on S199 was inhibited by caffeine, an inhibitor of the PI3K kinase family (Fig. S2A), but has no effect on the phosphorylation of T158 in the S199D mutant in response to doxorubicin (Fig. S2B).

Inhibitors of other kinases known to phosphorylate Tip60 in other residues, such as CDK, p38 or JNK did not prevent S199 phosphorylation in response to DNA damage induced by doxorubicin (Fig. S2C, D, E).

3.6. *T158 phosphorylation by VRK1 protects Tip60 from ubiquitin mediated-degradation*

VRK1 could affect the stability of Tip60, as it does on some of its known direct phosphorylation targets such as p53 [46,55,65], NBS1 [45] or coilin [53]. Depletion of VRK1 led to the loss of Tip60 protein that was rescued by the use of MG132, a proteasome inhibitor (Fig. 6A), indicating that VRK1 protects Tip60 from ubiquitin-mediated degradation. Therefore, we tested the effect of overexpressing mdm2 (Fig. 6B) or RNF8 (Fig. 6C) on Tip60, two ubiquitin ligases associated with DNA damage response [66] and chromatin remodeling [67]. Overexpression of both ubiquitin ligases led to downregulation of Tip60, which was prevented by MG132 (Fig. 6B, C). The ubiquitination of Tip60 by RNF8 was confirmed and it increased in the presence of MG132 (Fig. 6D). Finally, we tested whether the ubiquitination of Tip60 was prevented by treatment with doxorubicin, an activator of VRK1 activity [35]., The knockdown of VRK1 resulted in an increase of Tip60 ubiquitination under this condition of DNA damage (Fig. 6E).

Next, we studied whether the phosphorylation of Tip60 can contribute to its stability. For this aim, we tested the protein stability of individual T158 or S199 Tip60 phosphomutants in the presence or absence of VRK1 in the response to doxorubicin, which implies that the non-mutated site is available for phosphorylation. Single T158A and S199A phosphonull mutants were unstable in the presence and absence of VRK1 (Fig. S3A, top). However, the T158D protect Tip60 from degradation and its level is stable for twenty-four hours independently of VRK1 (Fig. S3A bottom). The S199D phosphomimetic is stable, in the presence of VRK1 (Fig. S3A, bottom), but after depletion of VRK1 it

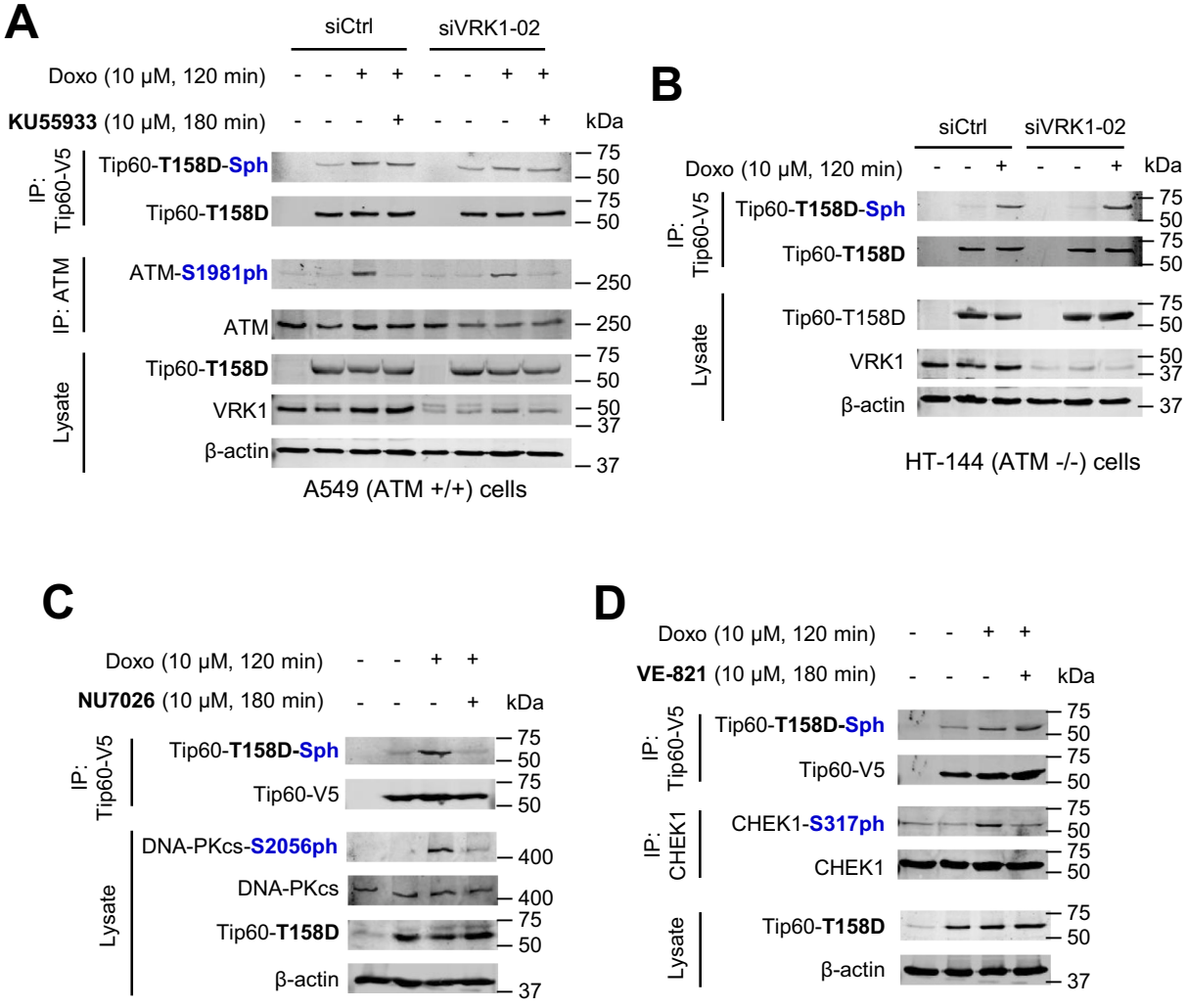


Fig. 5. Effect of different kinase inhibitors on the phosphorylation of Tip60-S199 residue using the phospho-mutant T158D. A. The ATM inhibitor KU55933 had no effect on the phosphorylation of the T158D mutant, although ATM phosphorylation in S1981 as control was inhibited. The phosphorylation was not affected by VRK1 depletion. B. The phosphorylation of S199 in the T158D mutant induced by DNA damage is insensitive to VRK1 depletion or DNA damage in ATM−/− cells. C. Inhibition of Tip60-T158D phosphorylation on the available S199 residue by NU7026, inhibitor of DNA-PK. As control, the inhibition of the activating phosphorylation of DNA-PK in response to doxorubicin is shown. D. Inhibition of Tip60-T158D phosphorylation on the available residue S199 by VE-821, inhibitor of ATR. As control, the inhibition of the activating phosphorylation Chek1 in response to doxorubicin is shown. The experiments were performed three times. Sph: phosphoserine, Kac: acetylated lysine. The transfected Tip60 was detected with an anti-V5 antibody.

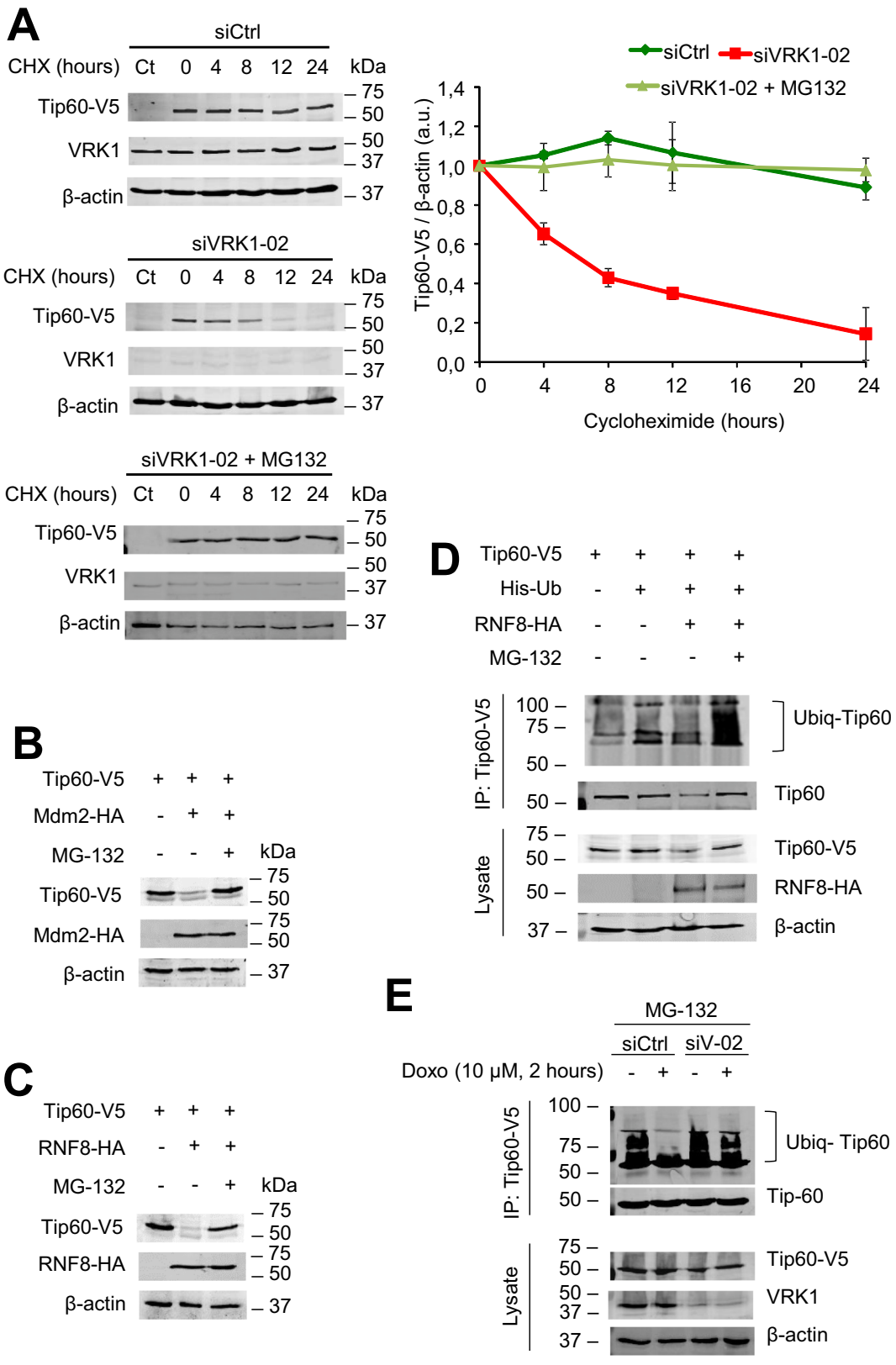


Fig. 6. Effect of VRK1 on Tip60 protein stability. A. VRK1 depletion facilitates Tip60 degradation that is prevented by the proteasome inhibitor MG132. Ct: control with empty plasmid. B. Tip60 degradation is promoted by Mdm2 and prevented by the proteasome inhibitor MG132. C. Tip60 is degraded by RNF8 that is blocked by the proteasome inhibitor MG132. D. Tip60 ubiquitination increases by RNF8 overexpression. E. Tip60 ubiquitination is reduced after DNA damage induced by doxorubicin treatment. This protection from ubiquitination is lost by depletion of VRK1. Prior to doxorubicin treatment, cells were treated with MG-132 (25 μM, 2 h) to prevent Tip60 degradation and to facilitate analysis of ubiquitination levels. Experiments were performed three times. The transfected Tip60 was detected with an anti-V5 antibody.

becomes more unstable after 4 h, probably in part due the early phosphorylation of the available T158 residue, since the timing of S199D stability is similar to its pattern of phosphorylation T158 in the S99D (Fig. 3) up to 3 h. However, S199 phosphorylation, or its phosphomimetic mutant, might delay the initiation of the degradation. After depletion of VRK1, the T158D remains stable, but the S199D becomes unstable, although its degradation is somewhat delayed.

To clarify the role of each Tip60 individual mutant, we determined the sensitivity of different Tip60 phosphomutants combinations to ubiquitin-mediated degradation by RNF8, which is a ubiquitin ligase associated with chromatin remodeling and DNA repair [67], and the effect of the proteasome inhibitor MG132. T158A and S199A were both

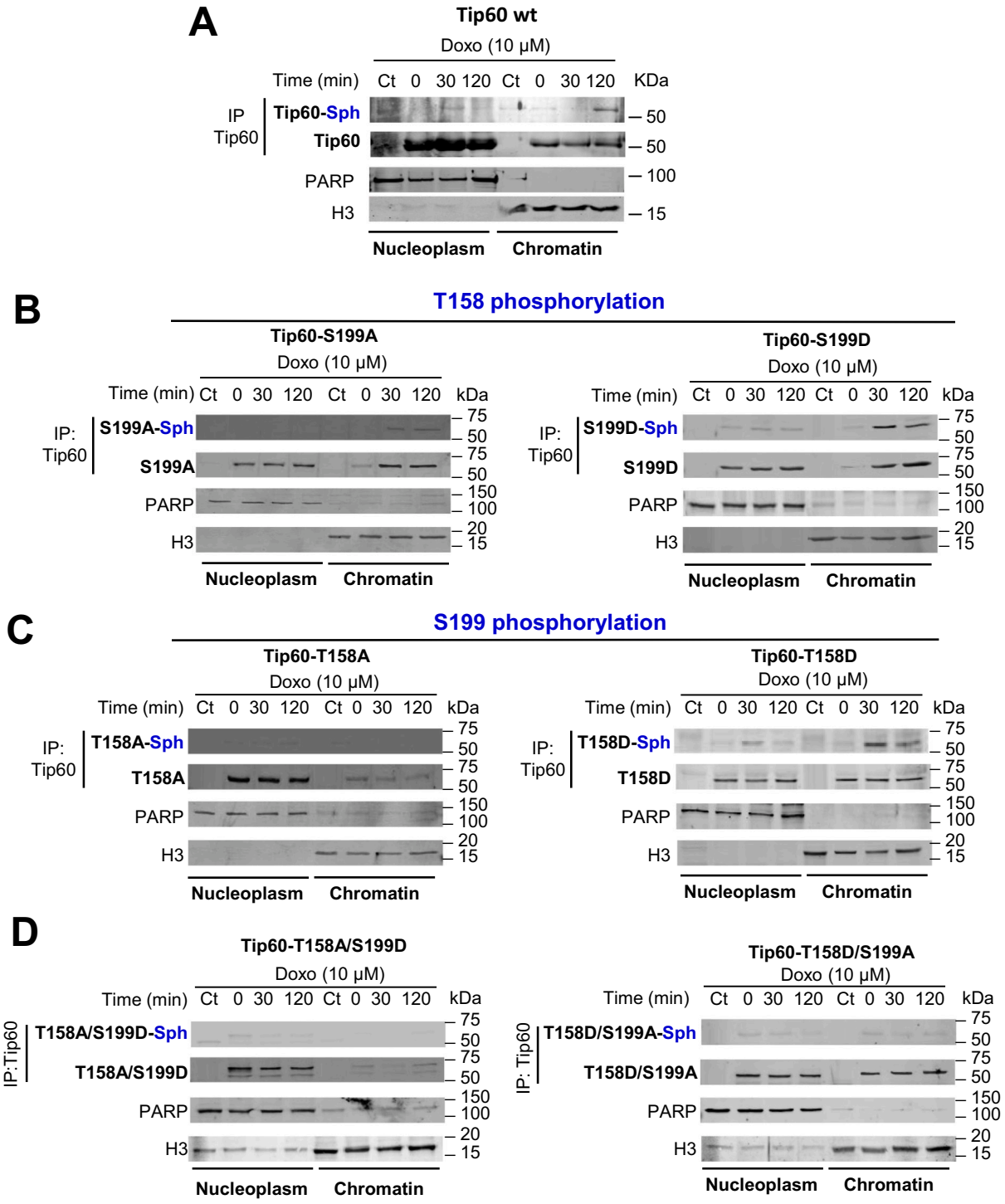


Fig. 7. T158 phosphorylation induced by DNA damage is necessary for the translocation of Tip60 to chromatin. A. Distribution between nucleoplasm and chromatin of the Tip60 wild-type and its phosphorylation at different times of doxorubicin treatment. B. Effect of doxorubicin treatment on the distribution between nucleoplasm and chromatin on T158 using the Tip60-S199A (left) and Tip60-S199D mutants (right). C. Effect of doxorubicin treatment on the distribution between nucleoplasm and chromatin on S199 using the e Tip60-T158A (left) and Tip60-T158D phosphomutants (right) at different times of doxorubicin treatment. D. Distribution between nucleoplasm and chromatin of the Tip60-T158A/S199D (left) and Tip60-T158D/S199A mutant (right). Sph: phosphoserine. Ct: control transfected with empty plasmid. The transfected Tip60 was detected with an anti-V5 antibody.

sensitive to RNF8 overexpression and MG132 protected them from degradation (Fig. S3B). In the case of the phosphomimetic Tip60 S158D and S199D mutants, the phosphomimetic mutation protects them from RNF8-induced degradation in the response to doxorubicin at 2 h, in agreement with the timing in which the phosphorylation of wildtype T158 is induced (Fig. S3B). However, in double mutant combinations, the T158A/S199D and T158A/S199A mutants were degraded by RNF8 since they cannot be phosphorylated in T158, and protected by MG132 (Fig. S3C). The T158D/S199A and the T158D/S199D were equally protected from RNF8 mediated degradation (Fig. S3C).

These results indicated that the phosphorylation of T158 residue is necessary for Tip60 stability, since the phosphomimetic effect on this residue always prevents Tip60 degradation, while the T158A Tip60 is always unstable. On the other hand, the phosphomimetic effect on S199 only prevents Tip60 degradation when phosphorylation in T158 is feasible in the single S199D mutant, in which T158 is available for phosphorylation by DNA damage induction, or mimicked by the T158D/S199D mutant, but not when T158 phosphorylation is impaired in the T158A/S199D mutant. Therefore, these data suggest that S199 phosphorylation does not contribute to the early stabilization of Tip60 by itself, but might contribute to delay its degradation during the response to DNA damage, and perhaps participate in later steps of the process.

3.7. Phosphorylation of Tip60 in T158 induced by DNA damage is necessary for its translocation to chromatin

Previously, we have demonstrated that phosphorylation of Tip60 in an unknown residue, and mediated by VRK1, is required the for the translocation of Tip60 to chromatin in response to DNA damage [22]. Therefore, we tested the effect that the two phosphorylated residues identified in Tip60 have on this translocation to chromatin. For these experiments, we used different Tip60 phosphonull or phosphomimetic mutants either individually or in combination. First, we determined the localization of the Tip60 wild-type. In response to doxorubicin, phosphorylated Tip60 is translocated to chromatin (Fig. 7A), confirming the response of the endogenous Tip60 [22]. To study the effect on T158, the S199A and S199D mutants were used. The mutants were translocated to chromatin in response to DNA damage, and both were phosphorylated in T158 when this Tip60 translocation occurs (Fig. 7B). Next, we studied the effect on S199 by using the T158 mutants. The T158A mutant is always located in nucleoplasm is not translocated to chromatin in response to DNA damage, and is not phosphorylated in S199 (Fig. 7C left). However, the phosphomimetic T158D is mostly located in chromatin and phosphorylated in S199, which indicates that the previous phosphorylation in T158 or its phosphomimetic is required for S199 phosphorylation. (Fig. 7C, right). Using mutant combinations, the

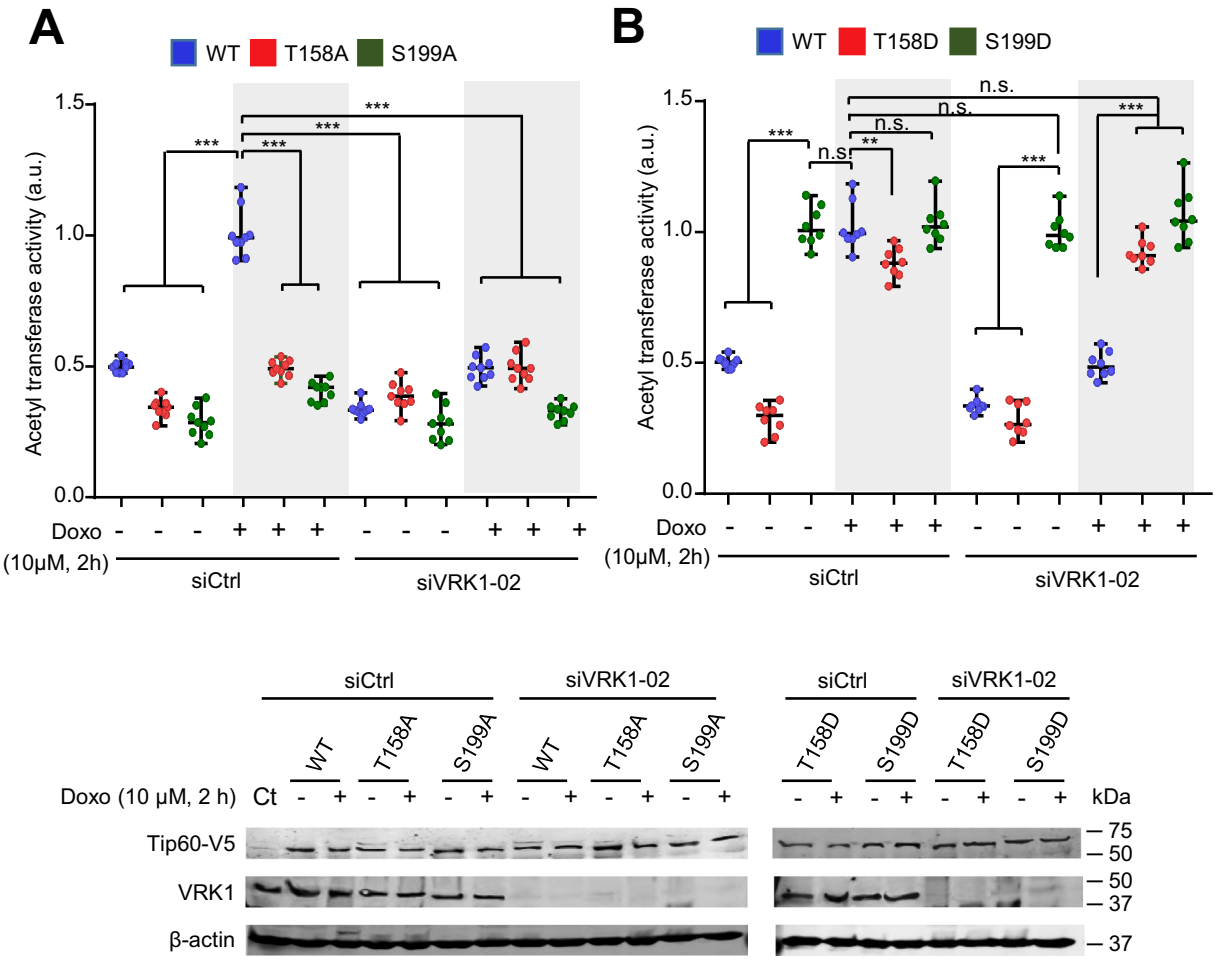


Fig. 8. Effect of Tip60 phosphomutants on acetyltransferase activity depending on VRK1 expression and DNA damage induction. A. Effect of the phospho-null Tip60 mutants on the acetyltransferase activity determined by an in vitro colorimetric assay. B. Effect of the phospho-mimetic Tip60 mutants on the acetyltransferase activity. The experiment was independently performed three times in triplicate, and analyzed using one-way ANOVA text with Tukey-Kramer correction. *** $p <$ 0.001. n.s.: not significant. The control is the same as the one shown in Fig. 1A, because the three different proteins, wild-type and two mutants, were studied simultaneously. The effect of VRK1 depletion on its protein level is at the bottom for each of the two parts (A, B) of the figure. Ct: control transfected with empty plasmid. The transfected Tip60 was detected with an anti-V5 antibody.

T158A/S199D, which cannot be phosphorylated in T158, remained in the nucleoplasm (Fig. 7D, left), but the T158D/S199A (Fig. 7D, right), is located in both nucleoplasm and chromatin, supporting the role of T158 phosphorylation for its chromatin translocation. These results indicated that in the DNA damage response, T158 phosphorylation promotes and is necessary for translocation of Tip60 to chromatin.

3.8. S199 phosphorylation is necessary for Tip60 basal acetylase activity

An additional effect of VRK1-mediated phosphorylation of Tip60 is the regulation of its acetyl transferase activity on chemical substrates. To study this effect, the basic acetyl transferase activity of different Tip60 phosphomutants was determined using an in vitro colorimetric assay. The mutation of T158 or S199 to alanine resulted in the loss of the in vitro acetyl transferase activity of Tip60. However, the Tip60 wild type was acetylase active, an effect that was lost by VRK1 depletion in the response to DNA damage (Fig. 8A).

The T158D and S199D Tip60 phosphomimetic mutants behaved differently. All were tested for their in vitro acetylase activity in response to doxorubicin, but the S199D mutant was acetylase active in the absence of DNA damage and was not affected by VRK1 depletion, a phenomenon that was not observed in the T158D phosphomimetic mutant (Fig. 8B).

These results suggested that phosphorylation of S199 catalyzed by DNA-PK, or alternatively, the mimetic effect of this phosphorylation is enough to induce Tip60 basal acetylase activity, but not the trans-acetylase activity. Therefore, doxorubicin activated the T158D mutant due to its phosphorylation in S199, independent of VRK1. On the other hand, the loss of activity in the T158A mutant, despite the availability of S199 for phosphorylation, can be explained due to the previous requirement of T158 phosphorylation by VRK1 (Fig. 4A and B) that is impaired in this mutant.

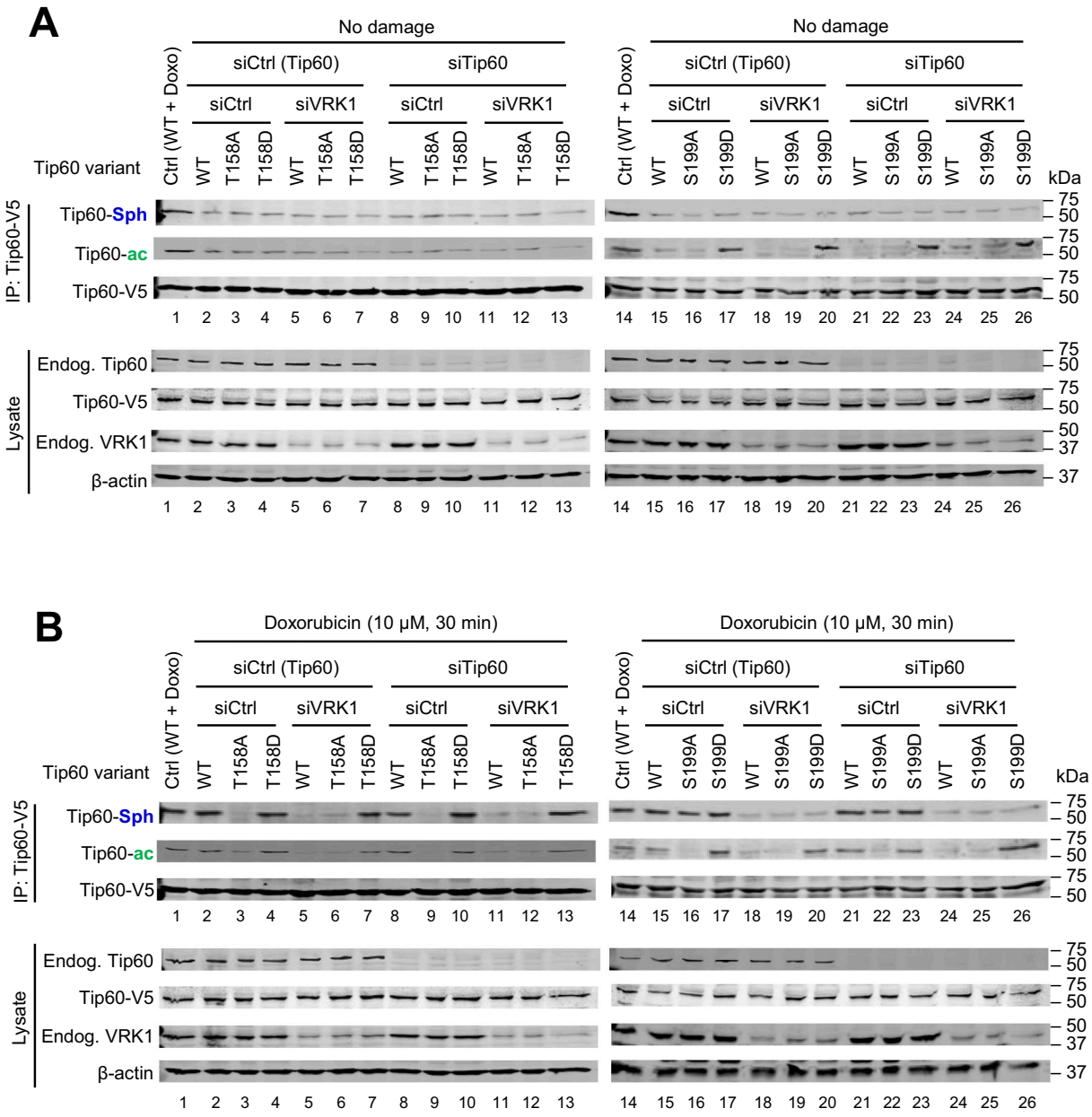


Fig. 9. Effect of Tip60 phosphomutants on the activating autoacetylation of Tip60 and the effect of VRK1 depletion. A. Effect of the phosphonull and phospho mimetic Tip60 mutants on their autoacetylation in basal conditions without DNA damage. B. Effect of the phosphonull and phospho mimetic Tip60 mutants on the their autoacetylation. The experiment was performed three times. Sph: phosphoserine, Kac: acetylated lysine.

The basal autoacetylation of Tip60 is necessary for its activation and subsequent acetylation of other protein targets. Therefore, we first studied the autoacetylation of the different Tip60 phosphomutants in response to DNA damage induced by doxorubicin in order to determine the contribution of the two identified phosphorylated residues to this activating modification (Fig. 9), in which depletion of the endogenous Tip60 permitted to rule out its potential contribution to the acetylation of Tip60-V5 mutants.

Mutants T158A and T158D were not acetylated in absence of DNA damage (Fig. 9A, lanes 1–13). These data indicate that a second kinase is involved, targeting Tip60 in S199 to induce its auto-acetylase activity, once it has been stabilized by the previous T158 phosphorylation mediated by VRK1. Regarding the S199 mutants, autoacetylation of Tip60 in the absence of DNA damage and of VRK1 was only detected in the S199D phosphomimetic (Fig. 9A, lanes 23 and 26) but not in the S199A mutant (Fig. 9A, lanes 17 and 20), suggesting that the activating Tip60 autoacetylation required S199 phosphorylation.

In the response to DNA damage induced by doxorubicin, Tip60-T158D, but not T158A, was acetylated, confirming that T158 phosphorylation (or its mimetic effect) is necessary and permits the subsequent S199 phosphorylation, which is ultimately responsible for this autoacetylation (Fig. 9B, lanes: 4, 7, 10, 13). Finally, we observed that, in response to DNA damage, only S199D mutant (Fig. 9B, lanes 17, 20, 23, 26), but not S199A (Fig. 9B, lanes 16, 19, 22, 25), was acetylated, as

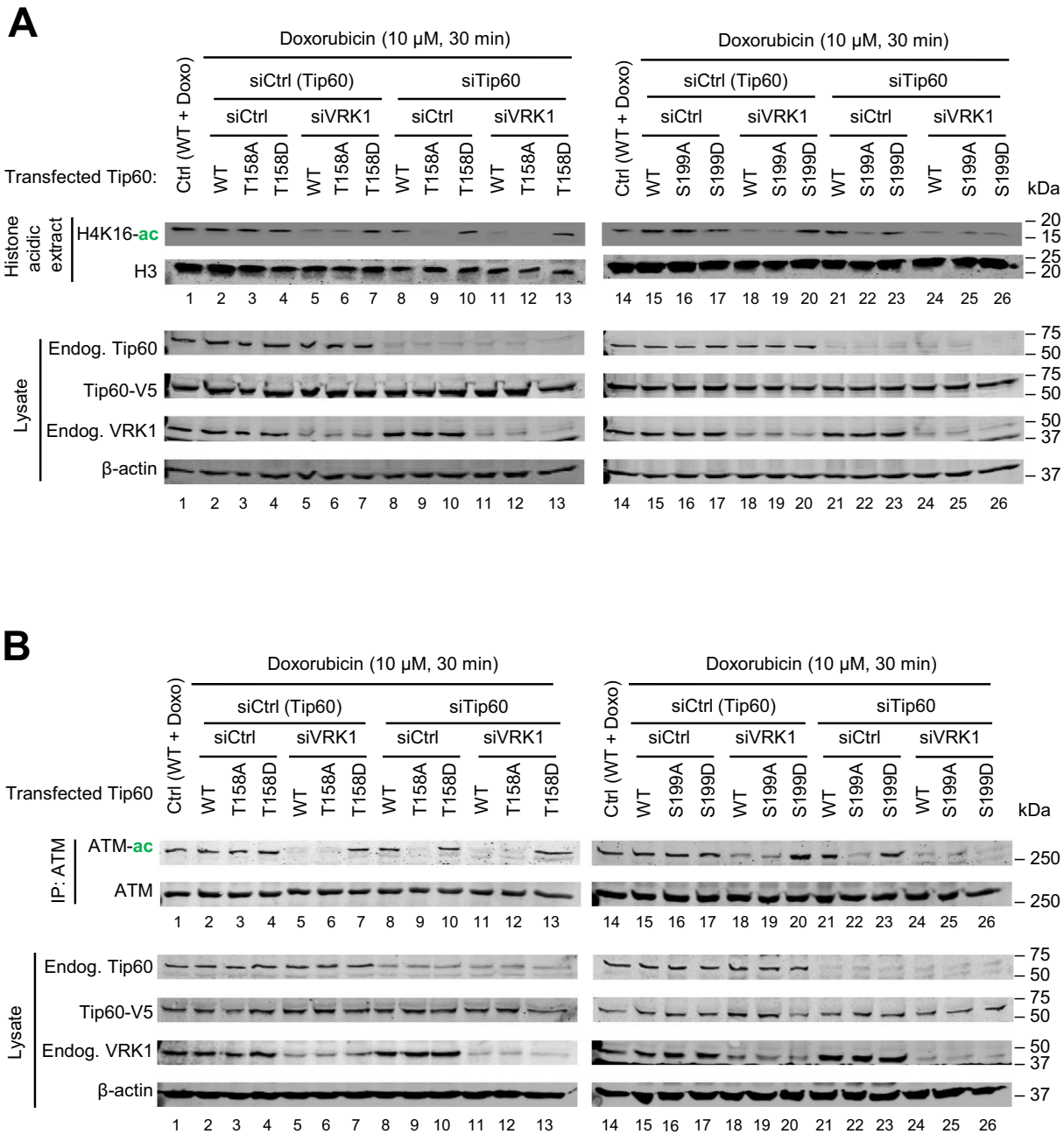


Fig. 10. Effect of Tip60-T158 and Tip60-S199 phosphorylations on the acetylation of H4K16 and ATM in the DNA damage response and effect of VRK1 depletion. A. Effect of Tip60 phosphomutants on the K16 acetylation of histone H4 in cells treated with doxorubicin. The experiments in the absence of DNA damage are shown in Fig. S4. B. Effect of Tip60 phosphomutants on the acetylation of ATM in cells treated with doxorubicin. sCt: si-control. siV-02: si-VRK1. The experiments in the absence of DNA damage are shown in Fig. S4. The experiment was performed three times. Sph: phosphoserine, Kac: acetylated lysine. The transfected Tip60 was detected with an anti-V5 antibody.

occurred in absence of DNA damage. These results confirm that once the S199 phosphorylation occurs, it facilitates the autoacetylation of Tip60.

3.9. Role of the Tip60 T158 and S199 residues on the trans-acetylation of H4K16 and ATM in the response to doxorubicin treatment

Next we studied the role of the Tip60 in the *trans*-acetylation of specific protein targets such as different substrates, including ATM and histone H4 [22]. The acetylation of ATM is necessary to promote the activation of its kinase activity [68–70]. In the absence of DNA damage, none of the Tip60, T158 or S199 mutants were able to acetylate H4K16 (Fig. S4A) or ATM (Fig. S4B). This point to a significant difference regarding the S199D phosphomimetic mutant that is able to promote its own autoacetylation (Fig. 9), but not the *trans*-acetylation of H4 in K16 or ATM as substrates (Fig. S4A, B). This suggests that an additional Tip60 phosphorylation is required for acetylation of Tip60 substrates, such as the one that occurs in T158 mediated by VRK1.

Therefore, we studied the effect of the different Tip60 phosphomutants, T158 and S199, on the acetylation of histone H4 in K16 (Fig. 10A), as well as on the acetylation of ATM (Fig. 10B) in response to DNA damage. In the response to DNA damage induced by doxorubicin, and in which the endogenous *TIP60* gene is silenced, the acetylation of H4 or ATM will depend on the Tip60 phosphomutants used. Tip60 phospho-null mutants, T158A (Fig. 10A, B, lanes 9, 12) and S199A (Fig. 10A, B, lanes 22, 25), were unable to acetylate H4 in K16 or ATM, which can be explained by their inability to activate their own autoacetylation (Fig. 9). The mutant T158D, phosphorylated in S199 in response to DNA damage, was able to acetylate H4K16 and ATM independently of VRK1 depletion (Fig. 10A, B, lanes 4, 7, 10, 13). However, the S199D, which is constitutively autoacetylated (Fig. 9), was only able to acetylate H4K16 and ATM in the presence of VRK1 (Fig. 10A, B, lane 23), but not when VRK1 was depleted (Fig. 10A, B, lanes 26).

These results indicate that the autocatalytic activity of Tip60 depends on the phosphorylation of S199 by DNA-PK. However, for the acetylation of chromatin proteins as substrates, such as histone H4 or ATM, the phosphorylation of Tip60 in T158, mediated by VRK1, is necessary. Therefore, the Tip60 double phosphorylation in T158 and S199 residues mediates histone H4 and ATM *trans*-acetylations.

4. Discussion

The process of DNA repair entails many sequential steps, all of them requiring different changes in the organization of chromatin. Therefore, understanding the regulation of chromatin remodeling is fundamental in order to develop new, and specific, therapeutic strategies aiming to the treatment of different diseases in which alterations of DDR pathways are very frequently altered, such as cancer [7].

In this context, the local chromatin relaxation of the damaged region is one of the first events during DDR, since it facilitates the access to and the incorporation of different components implicated in the DNA repair (55). This response requires specific epigenetic modifications, such as histone H4K16 acetylation (16, 54) and, consequently, the regulation of their corresponding modifiers, such as specific lysine acetyl transferases. Kinases are likely candidates to play a major role in coordinating these epigenetic modulators through regulation of their enzyme activity. In this report, we have studied the sequential modifications and activation of the histone acetyl transferase Tip60/KAT5 that regulates the acetylation of histone H4 in K16, main marker of chromatin relaxation. The chromatin alteration caused by DNA damage causes the activation of the kinase activity of VRK1 and thus by the phosphorylation and stabilization of Tip60 this is relocated to chromatin. VRK1 is an intermediate step between the initial chromatin alteration, and the initiation of specific DDR pathway. Subsequent steps in the specific response to DNA damage require acetylation by Tip60, not only of histone H4 associated with chromatin relaxation at the site of DNA damage but also to facilitate its opening and accessibility to subsequent steps in DDR. Among these is the acetylation of ATM that is necessary for its autophosphorylation and subsequent kinase activation [71], as well as for DNA-PK activation [72].

VRK1 is able to regulate the acetylation of H4K16 in response to DNA damage by phosphorylation of Tip60 in an up to now unidentified residue (25). We have identified the phosphorylation of two different residues in Tip60 in the early response to DNA damage. Interestingly, the phosphorylation of these two residues has a different temporal pattern. This might indicate that they have sequential roles during the response. The initial phosphorylation of Tip60 in T158 is early and transient, while phosphorylation in S199 increases steadily with time. Therefore, the specific histone acetylation associated to Tip60 activity may have different roles depending on its phosphorylation state and resultant stage of the DDR sequential process. In this context, it is very likely that, together with VRK1, additional and different kinases can participate in the regulation of Tip60, such as ATM-CHEK2, ATR-CHEK1 or DNA-PK, each contributing to specific effects. Notably, during DDR, the activation of VRK1 and DNA-PK have a different time scale based on the phosphorylation and activation of Tip60. VRK1 is an early kinase, directly activated after the generation of DNA damage, while DNA-PK activity is stimulated in later stages of the response in a VRK1-dependent manner. Thus, in the progression of DDR that involves a dynamic remodeling of chromatin, VRK1 is likely to be relevant in the earlier stages of Tip60 regulation, facilitating its stabilization, translocation to chromatin, where DNA-PK is located (56), and facilitating its transacetylase activity. This suggests that DNA-PK could be participating at this stage of the response, becoming a candidate to have a modulatory effect on the regulation of the acetyltransferase, which remains to be identified during later stages of DDR, and in which several sequential proteins complexes, with different roles, are implicated and dynamically remodeled.

In this work, we have shown that the VRK1 chromatin kinase is able to first phosphorylate Tip60 in T158 as a consequence of local chromatin alteration by DNA damage. This phosphorylation modulates the response by controlling several steps of its sequential activation such as Tip60 protein stability and its recruitment to chromatin, which bridge the aberrant chromatin alteration, caused by DNA damage, with the recruitment and progression of specific DDR pathways. A diagram summarizing the sequential order of events associated to VRK1-dependent Tip60 activation is shown in Fig. 11. We have shown that the effect of VRK1 on the Tip60 protein stability is a consequence of the effect that the phosphorylation exerts by preventing ubiquitin-mediated degradation. This protective effect of VRK1 has also been detected in some of its direct targets, such as p53 (44), coilin (50) or NBS1 (43). In addition, we have demonstrated that this phosphorylation cooperates with an additional phosphorylation in S199, mediated by DNA-PK. At this point, this phosphorylation of Tip60 by DNA-PK induces the autoacetylation and activation of Tip60, which in turns, could cooperate with MOF in the activating acetylation of DNA-PK (26). The sequential phosphorylation of T158 and S199 in response to DNA damage indicates that T158 phosphorylation is an early event that stabilizes and translocate Tip60 to chromatin, while the S199 phosphorylation is a later event in the repair process that also requires acetylation to restore chromatin back to its normal state. Moreover, beyond Tip60 participation on chromatin remodeling, its phosphorylation in these two different residues might be involved in its interactions with other protein forming part of the different complexes assembled on the initially altered chromatin and during the sequential steps along the progression of the DNA repair process, which need to be identified.

Phosphorylation of T158 and S199 in DDR were detected independently of the known phosphorylation of Tip60 in S86 or S90, since they also occur when using phosphor-null mutants (S86A, S90A or S86A/S90A). Therefore, these other residues are associated with additional roles of Tip60 in transcription (21), G2/M cell cycle progression (58) or apoptosis (19), but not in chromatin remodeling at early stages of DDR. Moreover, our results suggest the existence of a non-phosphorylated

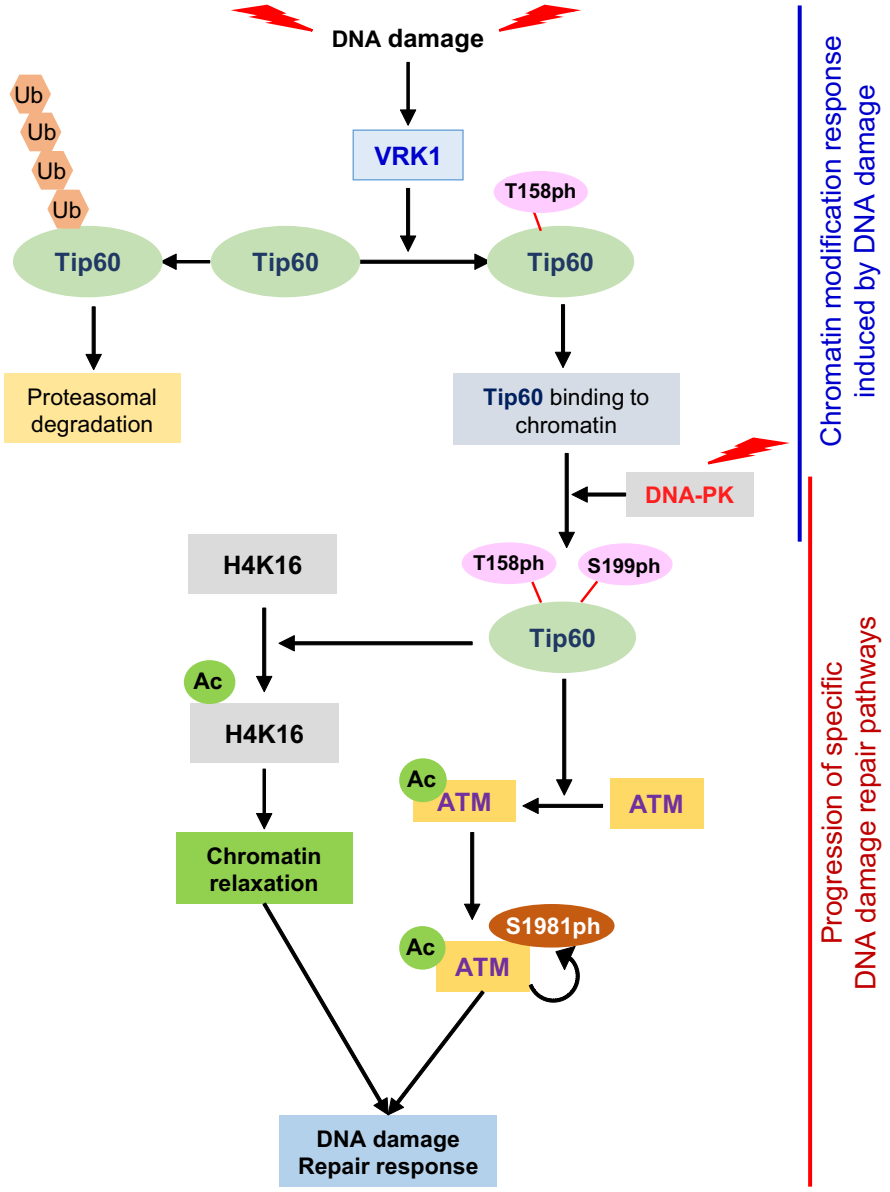


Fig. 11. Sequential cooperation of VRK1 and DNA-PK in the activation of Tip60 in the response to DNA damage. The process includes two sequential phases, the initial response to altered nucleosome/chromatin caused by DNA damage that activates VRK1/Tip60, which is followed in time by the specific DNA damage response based on its type and implicating DNA-PK associated with the NHEJ (non-homologous end-joining) pathway.

Tip60 pool in nucleoplasm, which is likely to have different roles that are not yet known and might cooperate with p53 in other functions such as apoptosis in response to DNA damage when repair fails [51]. In this context, it is also remarkable the existence of other kinase involved in the phosphorylation of Tip60 in T158 by p38α in a different context [51]. This phosphorylation by p38α occurs during later stage of the DDR and contributes to the induction of apoptosis when DNA lesions are unrepaired [51]. Therefore, a single phosphorylation in Tip60 can trigger different cellular responses depending on the kinases involved in the process and its individual stage. However, how the coordination of different kinases targeting different Tip60 residues throughout the following sequential steps in DDR needs to be addressed in further studies. It is likely that the combinatorial pattern of phosphorylated residues in the regulatory, and low complexity, domain of Tip60 will determine the type of process to which they are associated with, as well as determine the sequential protein interactions that occur as specific steps of the DNA repair and chromatin restoration processes progress. Thus, in DDR the local protein complexes ranging from damage detection, recruitment of specific repair proteins to restoration back to a normal chromatin organization, which require different components whose participation may be determined by the sequential protein interactions that are selected by specific patterns of phosphorylation. However, currently there is no data available on the combination patterns of phosphorylated residues in Tip60, but it is an issue that has to be studied in future studies.

Oxidative stress is the main cause of DNA damage in neural cells. Mutations and rare variants of the *VRK1* gene are associated, in homozygosis or compound heterozygosis, with motor neuron diseases such as spinal muscular atrophy [73–76], amyotrophic lateral sclerosis [77–80], distal motor neuropathy [81–84] and spastic paraplegia [59]. Therefore, it is likely that *VRK1* mutations can alter the neurodevelopmental pathway and contribute to the pathogenesis of these neurological syndromes by altering chromatin remodeling required in the progression of the DNA damage response.

This early role of VRK1 on the regulation of H416ac further confirms that VRK1 overexpression is associated to resistance to DNA-based

cancer treatments [16,22,57,85]. The consequence of VRK1 depletion on histone H4K16 by impairing this its acetylation and chromatin remodeling, associated with a defective DDR, is consistent with its effect on the sensitization of cells to DNA damage induced by doxorubicin or radiation [35,37,45]. Therefore, the pharmacological manipulation by using VRK1 or Tip60 inhibitors can be useful for development of synthetic lethality strategies [16,57,86]. Furthermore, the level of H4K16 acetylation is a consequence of the balance between acetylase and deacetylase activities. To reach a certain level of acetylation, not yet identified regulators should inhibit the deacetylating enzyme. In the context of DNA damage responses, VRK1 is a likely candidate to be involved in the modulation, not only of Tip60, but also of other epigenetic modifiers in order to control and coordinate the evolving changes in the epigenetic pattern during the DDR, and in other processes requiring a dynamic chromatin remodeling. These regulators of both activities could potentially become targets for novel drug development, as a novel synthetic lethality strategy, aiming to manipulate the functional organization of chromatin and facilitating the sensitization to genotoxic drugs that will permit a reduction in toxic doses and its side effects, thus improving patient quality of life or survival.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagrm.2022.194887.

CRediT authorship contribution statement

All authors contributed to generation of information included in this report. R. G-G., E-M-S., E. N-C and P. M-G. performed the experiments. PAL coordinated and supervised the work, and wrote the manuscript.

Declaration of competing interest

The authors declare no conflict of interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data availability statement

All data generated or analyzed during this study are included in this published article. There are no datasets.

Acknowledgements

This work was supported by grants from Agencia Estatal de Investigación (doi: 10.13039/501100011033) - Ministerio de Ciencia e Innovación-FEDER (RED2018-102801-T, PID2019-105610RB-I00), Consejería de Educación de la Junta de Castilla y León-ERDF (CSI264P20, CLC-2017-01) to P.A.L. P. M.-G. was supported by Ministerio de Educación predoctoral fellowship (FPU16/01883). R. G-G and E. M-S. were supported (Consejería de Educación-Junta de Castilla y León-Fondo Social Europeo (CSI004-18), predoctoral fellowships respectively. E. N-C was supported by Agencia Estatal de Investigación-MINECO predoctoral fellowship (BES-2017-080543).

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