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A protein phosphatase feedback mechanism regulates the basal phosphorylation of Chk2 kinase in the absence of DNA damage

Luigi Carlessi, Giacomo Buscemi, Enrico Fontanella, Domenico Delia *

Dept of Experimental Oncology, Fondazione IRCCS Istituto Nazionale Tumori, Via Amadeo 42, 20133 Milano, Italy

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ABSTRACT

The checkpoint kinase Chk2 is an effector component of the ATM-dependent DNA damage response (DDR) pathway. The activation of Chk2 by genotoxic stress involves its phosphorylation on T68 by ATM and additional auto/transphosphorylations. Here we demonstrate that in unperturbed cells, chemical inhibition of Chk2 by VRX0466617 (VRX) enhances the phosphorylation of Chk2-T68 throughout the cell cycle phases. This event, dependent on the presence of ATM and catalytically functional Chk2, is not consequential to DNA damage, as neither γ-H2AX nuclear foci nor increased ATM activation is detected in VRX-treated cells, suggesting the involvement of other regulatory proteins. As serine/threonine protein phosphatases (PPs) regulate the phosphorylation and deactivation of proteins of the DDR pathway, we analyzed their role in phospho-T68-Chk2 regulation. We found that intracellular inhibition of PP1 and PP2A-like activities by okadaic acid markedly raised the accumulation of Chk2-pT68 without DNA damage induction, and this phenomenon was also seen when PP1-C, PP2A-C, and Wip1/PPM1D were simultaneously knockdown by siRNA. Altogether, these data indicate a novel mechanism in undamaged cells where PPs function to maintain the balance between ATM and its direct substrate Chk2 through a regulatory circuit.

1. Introduction

Chk2 is a serine/threonine kinase with an effector function in the DNA damage response (DDR) pathway elicited by double-strand breaks (DSBs). By coordinating DNA replication and repair and cell cycle checkpoint progression, the DDR ensures proper repair of lesions normally arising from replication errors, metabolic by-products, and environmental mutagens [1,2], thereby preventing genomic instability and ultimately cancer. Upon DNA damage, Chk2 is phosphorylated within the N-terminus S/TQ cluster domain on T68 residue by the upstream kinase ATM [3], a product of the gene mutated in the human ataxia telangiectasia (A-T) and master regulator of the DDR, leading to Chk2 homodimerization and transactivating autophosphorylation on T383 and T387, and *cis*-autophosphorylation on S516 [4,5]. Once activated, Chk2 acts as a diffusible signal transducer, phosphorylating a multitude of substrates involved in cell cycle control, transcription, and apoptosis. The phosphorylation of Cdc25C-Ser216 by Chk2 inhibits this phosphatase, thereby preventing the activation of Cdc2 and imposing a checkpoint arrest at multiple phases. Chk2 also regulates the transcriptional activity and degradation of p53 by targeting p53 on S20 and Hdmx-Ser367 [6,7], hence controlling p53-dependent apoptosis and p21waf1-mediated cell cycle arrest. By phosphorylating the transcription factor E2F-1 and of the transcription regulator Che1, Chk2 enhances the stability of these proteins and modulates the apoptotic response to genotoxic agents [8,9]. Other known Chk2 substrates include BRCA1 and PML with their functions implicated in DNA repair and apoptosis [10,11]. Very recently, the interplay between Chk2 and TRF2 has been established, whereby Chk2 phosphorylates TRF2 on telomeres while the latter inhibits the activity of Chk2 [12]. A connection between Chk2 and the RNA binding protein HuR has recently defined a role for Chk2 in regulating translation of SIRT1 and c-Myc [13,14].

A number of evidences highlight the role of Chk2 as tumor suppressor functioning in preneoplastic lesions as a barrier against overt tumor progression by driving incipient cells to apoptosis or senescence [15]. In line with this, inherited mutations in *CHEK2* gene confer multiorgan cancer susceptibility in various epidemiological studies [16]. It should be noted, however, that, on its own, Chk2 manifests a weak antitumor activity based on the modest increase in the incidence and early onset of DMBA-induced skin carcinogenesis in Chk2 deficient mice [17]. Interestingly, Chk2 modulates the response to genotoxic chemotherapy depending on the p53 genetic background since, in the Chk2-deficient setting, disruption of p53 sensitizes cells, whereas functional p53 confers resistance [18].

As genetic alterations in DDR genes can modulate the survival of tumor cells to genotoxic treatments that cause DNA damage, such as ionizing radiation and chemotherapy, this pathway has emerged as an attractive pharmacologic target of therapeutic potential, prompting the development of selective inhibitors. Accordingly, a number of small molecule inhibitors of ATP-binding pocket of Chk2 have been

* Corresponding author. Tel.: +39 02 23902641.
E-mail address: domenico.delia@istitutotumori.mi.it (D. Delia).

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identified by high-throughput screening of compound libraries and optimization of analogues based on structure–activity relationships and X-ray structure analysis of Chk2 in complex with inhibitors [19–21]. We have previously reported the identification and characterization of a new ATP-competitive small molecule inhibitor of Chk2, VRX0466617 (hereafter denoted VRX) [22] and shown that it inhibits the enzymatic activity of Chk2, both *in vitro* and *in vivo*, in cells. VRX does not modify the cell cycle phase distribution of normal and cancer cells after IR, but it markedly attenuates the radiation–apoptosis of thymocytes [22]. The serine/threonine phosphatases are a conserved family of proteins able to regulate the reversible phosphorylation status of the DDR protein. In particular, Wip1/PPM1D, PP1, and PP2A are directly connected to the checkpoint regulation through the dephosphorylatation of multiple phospho-S/TQ sites targeted [23]. In this work, we show that, in undamaged cells, the chemical inhibition of Chk2 kinase activity significantly boosts the accumulation of Chk2 phosphorylated on Thr68 (Chk2-pT68), the key residue phosphorylated by ATM kinase following DNA damage. Besides, we demonstrate the regulatory role of protein phosphatases in the maintaining Chk2 unphosphorylated in the absence of DNA damage.

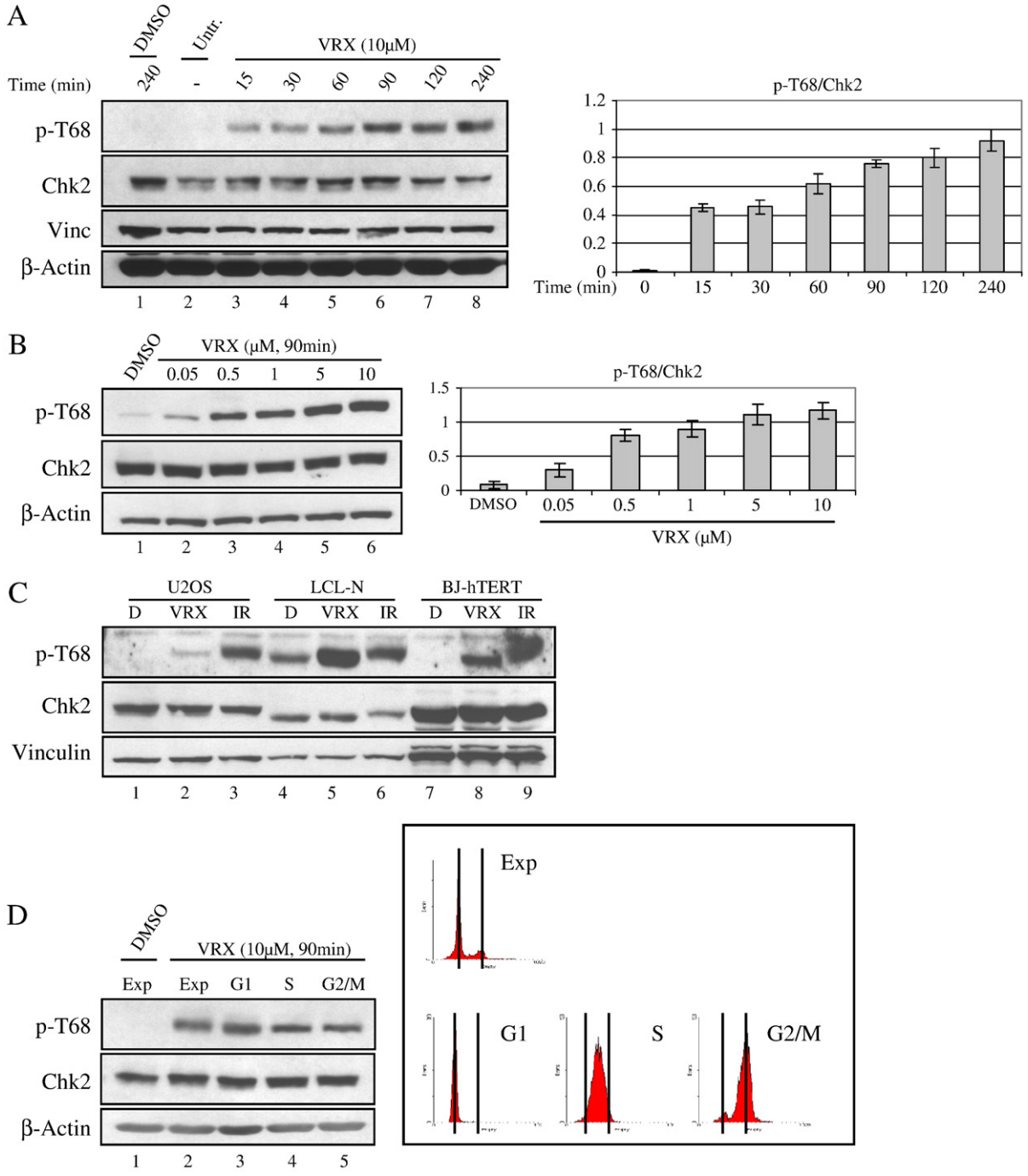


Fig. 1. VRX induces Chk2-T68 phosphorylation in a time- and dose-dependent manner *in vivo*. (A) LCL-N cells were incubated for the indicated times with 10 μM VRX or DMSO (1:1000, vehicle) and analyzed by Western blot for Chk2 phosphorylation. (B) LCL-N cells were collected 90 min after treatment with escalating doses of VRX and analyzed for Chk2 phosphorylation. Graphs A and B show the levels of Chk2-pT68 obtained from the densitometric analysis of the bands normalized for the total amount of Chk2 (data from three independent experiments). (C) Chk2-pT68 in LCL-N, BJ-hTERT, and U2OS cells collected 90 and 45 min after treatment with VRX or 10 Gy IR, respectively. (D) LCL-N cells treated for 90 min with VRX were separated by FACS according to DNA content, and the individual fractions were analyzed for Chk2-pT68 by Western blot (left). The purity of the G_1-, S-, and G_2/M-sorted fractions was verified by FACS analysis (right). exp, exponential growing.

2. Materials and methods

2.1. Cell lines and treatments

The EBV-immortalized human lymphoblastoid cell line LCL-N was from a normal individual, whereas GM07078A and AT52 (kindly obtained from Dr Luciana Chessa) were from patients with Nijmegen breakage syndrome (NBS) and ataxia telangiectasia (AT), respectively. The cells were cultured in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum (FCS). U2OS human osteosarcoma cell lines, MCF-7 breast adenocarcinoma transfected with shGFP or shChk2 [24], the Chk2-defective HCT15 stable transfected with HA-Chk2 WT or KD [24], and 293FT were grown in DMEM with 10% FCS. Immortalized normal human foreskin fibroblasts BJ-hTERT were cultured in DMEM plus M199 (4:1 ratio) with 10% FCS. Culture media contained penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (2 mmol/l). Cells were cultured at 37 °C in a 5% CO_2 incubator. The Chk2 and ATM inhibitors VRX046617 (Valeant

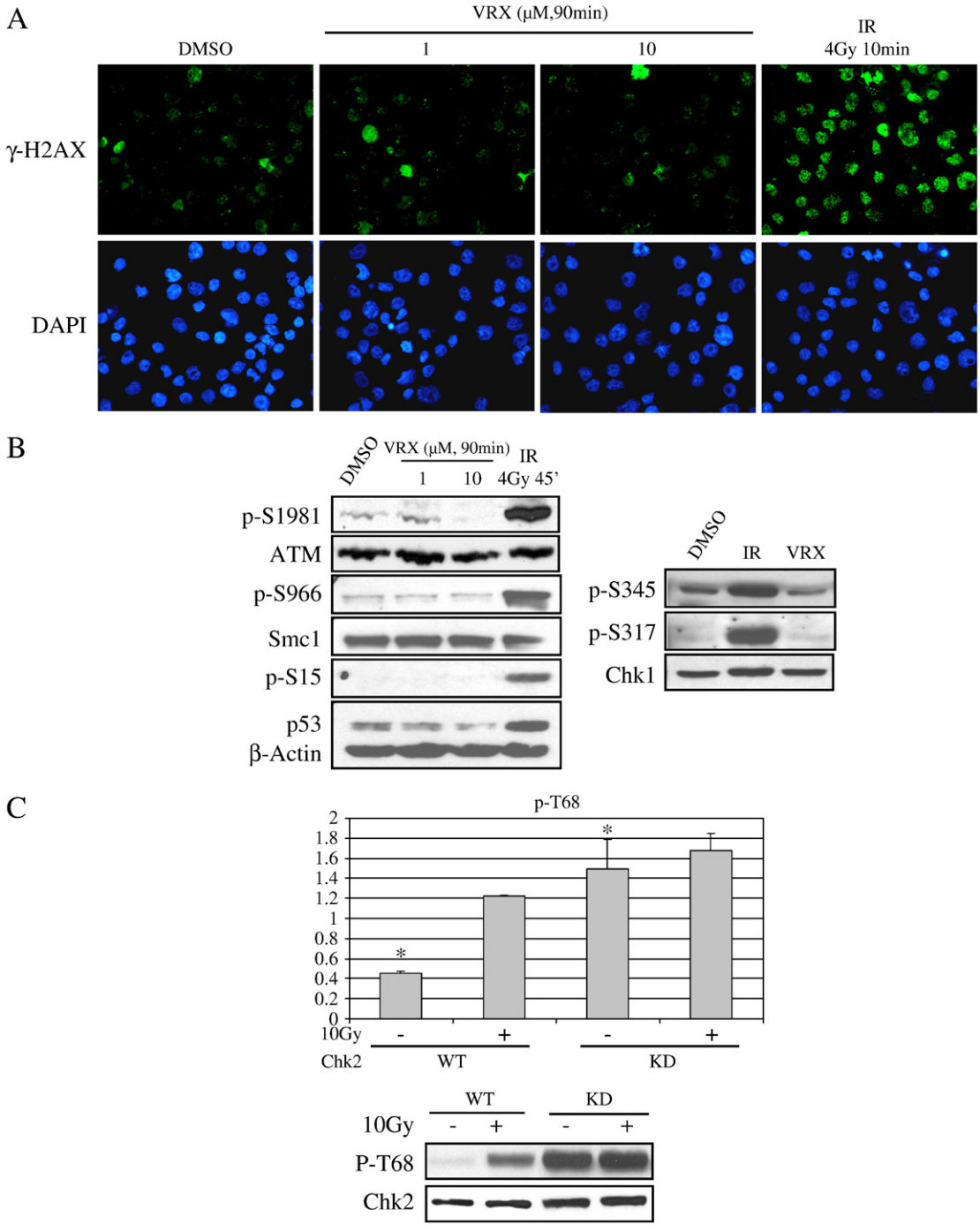


Fig. 2. VRX does not induce DNA damage or phosphorylation of ATM substrates. (A) Immunofluorescence microscopy of γ-H2AX (upper panel) in LCL-N cells treated with DMSO (1:1000, control), VRX or IR. Nuclei were stained with DAPI (lower panel). (B) LCL-N cells were treated with VRX or IR and analyzed by Western blot with antibodies phosphospecific for ATM-S1981, Smc1-S966, and p53-S15 (left) and Chk1-S317 and Chk1-S345 (right) or recognizing the total forms of these proteins. (C) The Chk2-defective HCT15 cell line was transiently transfected with Chk2 wild type (HA-Chk2^{wt}) or kinase dead (HA-Chk2^{kd}) and collected 45 min after treatment with 0 or 10 Gy IR and levels of total Chk2 and Chk2-pT68 examined by Western blot. The histogram shows the densitometric analysis of Chk2-pT68 from a different experiment, after normalization for the total amount of Chk2. The difference between Chk2^{wt} and Chk2^{kd} was statistically significant (*P<0.01, analysis performed by the Student's t-test).

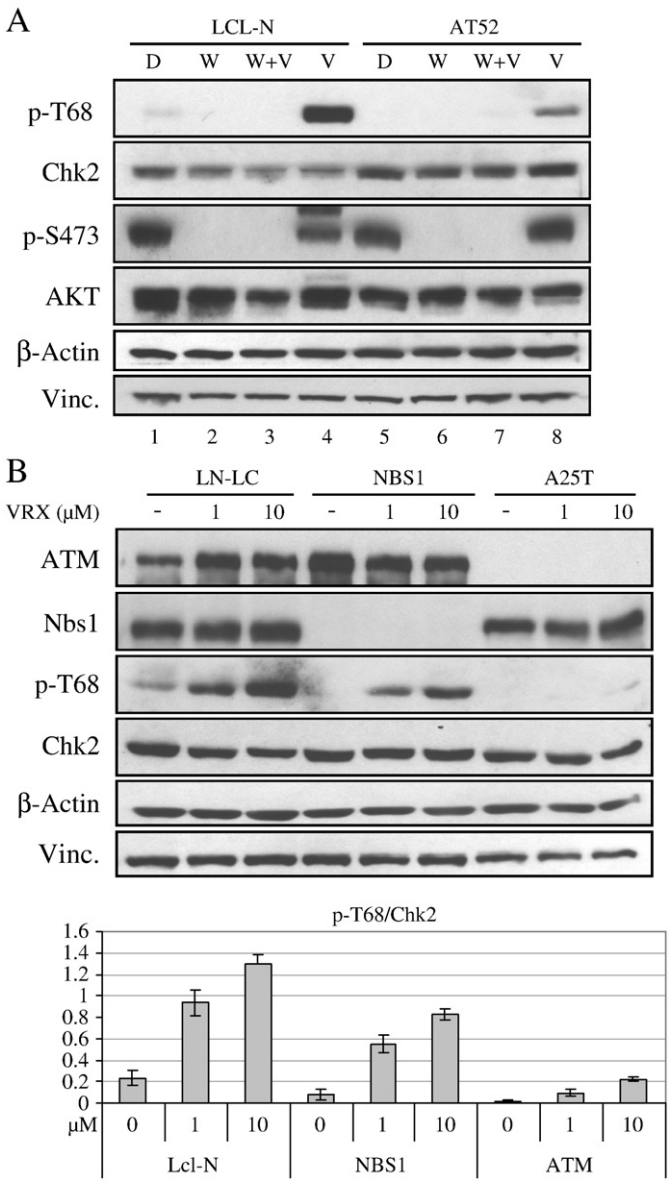


Fig. 3. VRX induces the phosphorylation of Chk2-T68 in an ATM-dependent manner. (A) LCL-N cells and the ATM-defective AT52 cell line were incubated with DMSO (0.1%), VRX (10 μM, 90 min), wortmannin (W, 50 μM, 2 hours), or wortmannin followed by VRX. Cells were collected at the indicated times and analyzed by Western blot. (B) LCL-N, AT52, and the NBS1-defective cell line GM07078A were incubated with VRX, collected 90 min later, and analyzed by Western blot (top). The histogram (bottom) shows the densitometric analysis of the Chk2 p-T68 bands normalized for the total levels of Chk2 per lane (data from three different experiments).

Pharmaceuticals Intl, Aliso Viejo, CA) [22] and KU-55933 (KuDOS Pharmaceuticals, Cambridge, UK) [25], respectively, were prepared as 10 mM stock solutions in DMSO and stored at −20 °C. Inhibitors were diluted to give a maximal final 0.1% DMSO concentration. Okadaic acid and wortmannin were purchased from Sigma Aldrich. Irradiations were performed with an IBL437CO instrument (Oris Industries, France) equipped with a ^{137}Cs source providing 675 cGy/min.

2.2. Western blot analysis

Untreated or treated cells were washed with PBS plus 0.1 mM Na_3VO_4 (Sigma), pelleted, and lysed in Laemmli buffer (0.125 M Tris–HCl pH 6.8, 5% SDS) containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], pepstatin [10 μg/ml], aprotinin [100 kIU/ml], leupeptin [10 μg/ml] [all from Calbiochem, San Diego, CA], and 1 mM Na_3VO_4). After boiling for 5 min and sonication, lysates were quantitated by micro-BCA assay (Pierce, Rockford, IL). Aliquots containing 50 μg of protein plus 5% beta-mercaptoethanol were size-fractionated on SDS–PAGE and were electroblotted onto PVDF membranes (Millipore, Bedford, MA). After blocking with 4% nonfat dried milk in PBS plus 0.1% Tween 20 (Sigma), membranes were incubated in sealed plastic bags with an X-Blot hybridization equipment (Integrated Systems Engineering, Milan, Italy; www.isenet.it) as in the study of Carlessi et al. [26], with monoclonal antibodies for Chk2 (clone 44D4/21) [24], ATM (clone 9E6) [22], p53 (clone DO-7), β-actin (Sigma, Italy), vinculin (Sigma), PP2A-C (a kind gift from David Pim, ICGEB, Trieste, Italy) [27], His-tag (Invitrogen), and with rabbit antibodies specific for the phosphorylated Chk2-Thr387, Chk2-Thr68, Chk2-Ser19, Chk2-Ser33-35, p53-Ser15, Chk1-Ser345, AKT-Ser473, and Erk1/2-Thr202/Tyr204 (p44/p42 MAPK) (all from Cell Signaling Technology, Beverly, MA). Rabbit antibodies were against PP1-C (a kind gift from Emma Villa-Moruzzi, University of Pisa, Italy) [28], PPM1D/Wip1 (clone H300,;Santa Cruz Biotechnology, Inc, Santa Cruz, CA), total Erk1/2 and AKT (from Cell Signaling), Smc1-pSer966, total Smc1, Chk1-pSer317 (Bethyl Laboratories, Inc, Montgomery, TX), and ATM-pSer1981 (R&D Systems, Minneapolis, MN). Binding of antibodies to membranes was detected with peroxidase-conjugated secondary antibodies and ECL (Pierce, Rockford, IL) on autoradiographic films. Bands were acquired with a digital scanner and were quantified by ImageQuant software (Molecular Dynamics).

2.3. Expression of dominant-negative PP2A-C

Exponentially growing 293FT and U2OS cells seeded on 35-mm plates were transfected when 70–80% confluent with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The two plasmids pcDNA4-His-PP2A-C wt or dominant-negative (L199P) were a kind gift from Stefanie Dimmeler, University of Frankfurt, Germany [29]. The cells were then harvested and processed for immunoblotting at the indicated time after transfection.

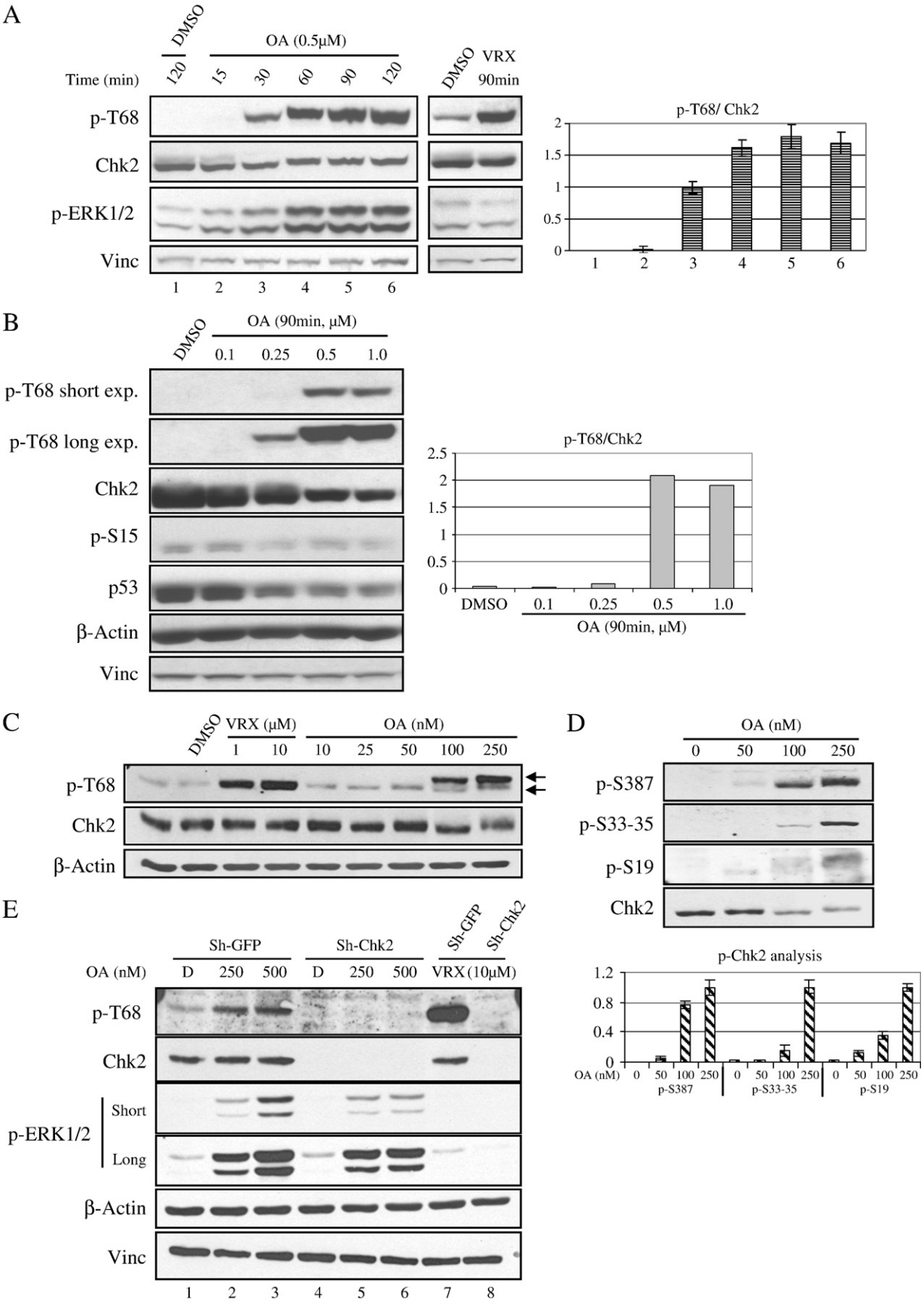
2.4. Knock down of PP2A-C, PP1-C, and Wip1 by siRNA

293FT cells were seeded in 12-well dishes and transfected 18 hours later with SiGenome Smartpool siRNA against PP1 (M00892701), PP2A (M00359801) and Wip1 (PPM1D) (M00455400), and a scrambled control sequence (D00121002) (all from Dharmacon) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At the indicated times, cells were harvested and analyzed by immunoblotting.

2.5. Immunofluorescence labelling

γ-H2AX nuclear foci were analyzed on treated or untreated LCL-N cells deposited onto glass slides using a cytocentrifuge (Shandon), air-dried, and fixed in ice-cold methanol (20 min at −20 °C) and then in acetone (2 min at −20 °C). After washing with PBS and blocking for

Fig. 4. Okadaic acid (OA) induces Chk2-pT68 in a time- and dose-dependent manner. (A) LCL-N cells were treated with DMSO (1:1000), 0.5 μM OA, or 10 μM VRX for the indicated times (left). Whole-cell extracts were analyzed by Western blot with antibodies specific for Chk2-pT68, total Chk2, and phospho-p42/p44 (Erk1/2 and p-T202/T204, respectively) as shown. (B) LCL-N cells were incubated with increasing concentrations of OA and analyzed as in panel A. LCL-N cells were treated with the indicated doses of OA and VRX for 90 min and analyzed by Western blot for Chk2-pT68 (C) or for Chk2-pS387, Chk2-pS33-35, and Chk2-pS19 (D). Densitometric analysis of Chk2 phosphorylation bands are summarized in the relative graphs, after normalization for the total amount of Chk2. (E) MCF-7 cells stably transfected with shGFP and shChk2 were incubated for 90 min with DMSO (abbreviated D), OA, and VRX and were analyzed by Western blot for phosphorylation of Chk2-T68 and pERK 1/2.


A
DMSO
OA (0.5μM)
Time (min)
120
15
30
60
90
120
DMSO
VRX
90min
p-T68
Chk2
p-ERK1/2
Vinc
1 2 3 4 5 6
p-T68/ Chk2
B
DMSO
OA (90min, μM)
0.1
0.25
0.5
1.0
p-T68 short exp.
p-T68 long exp.
Chk2
p-S15
p53
β-Actin
Vinc
p-T68/Chk2
DMSO
OA (90min, μM)
C
DMSO
VRX (μM)
OA (nM)
p-T68
Chk2
β-Actin
D
OA (nM)
p-S387
p-S33-35
p-S19
Chk2
p-Chk2 analysis
p-S387
p-S33-35
p-S19
E
Sh-GFP
Sh-Chk2
OA (nM)
D
250
500
VRX (10μM)
p-T68
Chk2
p-ERK1/2
Short
Long
β-Actin
Vinc
1 2 3 4 5 6 7 8

1 hour with 3% BSA, the slides were incubated for 2 hours with 1:450 dilution of the anti H2AX-pS139 antibody clone JBW301 (Upstate Biotechnology) and thereafter with an $F(ab)_2$ fragment of a FITC-conjugated secondary antibody for 1 hour (Jackson Laboratories). After three washes in PBS, slides were counterstained with DAPI and mounted with an antifade solution. Images were collected on a Nikon fluorescence microscope equipped with a CCD camera. Experiments were performed three times independently on duplicate slides.

2.6. Cell sorting

LCL-N cells were incubated with VRX, treated with 10 mg/ml of the fluorescent DNA-specific permeable dye Hoechst 33342 (Calbiochem, La Jolla, CA), and harvested 20 min later. Cells were fractionated according to the cell cycle phases with a FACS-Vantage cell sorter (BD Biosciences) equipped with a UV argon ion laser and a sample refrigeration system.

2.7. Statistical analysis

Statistical analyses were performed with Student's *t*-test as indicated in the figure legends.

3. Results

3.1. Chk2 kinase inhibition raises its phosphorylation on Thr 68

While studying the regulation of Chk2, we found in unperturbed cells that selective inhibition of its catalytic activity with VRX0466617 (VRX) [22] promotes a rapid and striking accumulation of Chk2 phosphorylated on Thr68 (Chk2-pT68), the residue specifically targeted by ATM kinase in response to genotoxic stress. As shown in Fig. 1A in human LCL-N cells, Chk2-pT68 signal was detectable within 15 min of exposure to 10 μM VRX, becoming maximal between 90 and 120 min, and was induced by as low as 0.05 μM VRX (Fig. 1B). The VRX-induced accumulation of Chk2-pT68 was not cell line-specific because it was also seen in U2OS and BJ-hTERT, although with significant differences among these cells (Fig. 1C, compare lanes 2, 5, and 8). Although Chk2 is variably phosphorylated in the cell cycle [24], analysis of cells treated with VRX and enriched by FACS according to DNA content (Fig. 1D, right) showed no differences in the levels of Chk2-pT68 among the cell cycle phases (Fig. 1D, left).

3.2. VRX induces neither DNA DSBs nor ATM-dependent checkpoint activation

The phosphorylation of Chk2-Thr68 by ATM is a primary event in cells treated with genotoxic agents [30]. To determine whether the accumulation of Chk2-pT68 was the consequence of a DNA damage inflicted by VRX and which would activate ATM, we assessed in LCL-N cells the formation of γ-H2AX nuclear foci, an index of DNA damage. In contrast to IR, VRX treatment induced neither any foci (Fig. 2A) nor the activation of ATM, as assessed by the lack of ATM autophosphorylation on Ser1981 and phosphorylation of its substrates Smc1-S966 and p53-S15 (Fig. 2B, left), altogether suggesting that VRX does not elicit a classical DNA damage. VRX was also unable to induce the phosphorylation of Chk1 on S317 or S345 (Fig. 2B, right), two ATR phosphoresidues [31–33]. Hence, neither ATM nor ATR was activated by VRX. These findings would thus suggest that the Chk2-pT68 accumulation reflects a response to the inhibition of Chk2 catalytic activity but not to DNA damage. This is further supported by findings in the Chk2-deficient HCT15 cells in which the ectopic pT68 levels are much greater in the case of kinase-dead than wild-type Chk2 (Fig. 2C) especially before irradiation. Thus, the accumulation of pT68 in undamaged cells is associated with the catalytic status of Chk2, suggesting that this activity positively regulates the dephosphorylation of Chk2.

3.3. Chk2-T68 phosphorylation is prevalently ATM-dependent

Since, besides ATM, ATR and DNA-PKcs can phosphorylate Chk2 T68, we assessed in normal LCL-N and ATM-deficient AT52 cells the effect of wortmannin at a dose that inhibits all three kinases [34,35] on VRX-induced Chk2-pT68. When cells were preincubated with 50 μM wortmannin for 2 hours before treatment with 10 μM VRX, Chk2-T68 phosphorylation was totally ablated (Fig. 3A, compare lanes 3 and 4). However, since in AT52 cells the pT68 signal was still present, although at significantly lower level than in LCL-N cells (Fig. 3A, compare lanes 4 and 8), these results underscore the contribution of ATM kinase in this phenomenon. The phosphorylation of AKT-S473 was abolished by wortmannin, verifying that the dose used here is actually inhibiting all PIK3 members [34–36]. The activity of ATM is partly dependent on Nbs1 [37–39]. To demonstrate the role of ATM basal activity on VRX-induced pT68, we compared the response of LCL-N and AT52 with those of an NBS-derived cell line deficient in Nbs1 expression. It can be seen (Fig. 3B) that the phosphorylation of Chk2-T68 is reduced compared to LCL-N, albeit not to the same extent as in AT52, again indicating the role of ATM in this response.

3.4. Okadaic acid treatment induces Chk2-pT68 accumulation

Protein serine/theronine phosphatases are implicated in the regulation of Chk2 [40,41]. Moreover, the autophosphorylation of ATM-S1981 and phosphorylation of Chk1-S317 and Chk1-S345 in unirradiated cells is upregulated by concentrations of okadaic acid (OA) that inhibit protein phosphatase 2A-like activities [42,43]. Importantly, OA induces the phosphorylation of ATM-S1981 without causing detectable DNA damage or increase in ATM activity [42]. OA also increases the autophosphorylation of DNA-PK [44]. Given these evidences, we sought to determine the involvement of protein phosphatases in the negative regulation of Chk2-T68 phosphorylation. In LCL-N cells incubated with 0.5 μM OA, detectable levels of Chk2-pT68 became apparent at 30 min and increased significantly up to 120 min (Fig. 4A, left). As a positive control of the PP2A inhibition, we assessed the MAP kinase pathway members ERK1/2 (p42/p44 MAPK) whose phosphorylation is regulated by PP2A [45,46]. In agreement with previous reports [47,48], PP2A inhibition by OA raised the phosphorylation of ERK1/2 (Fig. 4A), while conversely, VRX had no effect on ERK1/2 (Fig. 4A, right), excluding this agent as an inhibitor of PP2A. The intensity of Chk2-pT68 was OA dose-dependent, being undetectable below 0.25 μM OA (Fig. 4B). In a subsequent experiment, we found that even 100 nM OA is able to induce Chk2-pT68 (Fig. 4C). Of note, OA did not induce the phosphorylation of p53-S15, a direct substrate of ATM (Fig. 4B), concordant with the fact that OA promotes the autophosphorylation but not full enzymatic activity of ATM [42]. In contrast to VRX, OA induced a phosphorylation-dependent mobility shift of Chk2 (Fig. 4C, arrows) associated with Chk2 autophosphorylation on S387 and phosphorylation on S19 and S33-35 (Fig. 4D) [24]. Of note, VRX does not induce the phosphorylation of these additional residues [22]. As expected, no pT68 signal was seen in cells depleted of Chk2 (Fig. 4E), validating the anti-pT68 for its target substrate.

3.5. PP2A, PP1, and Wip1/PPM1D phosphatases cooperatively maintain Chk2-T68 unphosphorylated in undamaged cells

We further investigated the role of serine/threonine protein phosphatases on Chk2 pT68 accumulation by two different approaches. In a set of experiments, cells were incubated with DMSO alone and 10 μM VRX for 45 min or were transiently transfected with PP2A-C^{wt} or PP2A-C^{L199P}, the latter containing an amino acid

substitution in the catalytic subunit and acting as dominant negative [42,50,51]. The expression of PP2A-C^{L199P} did not induce Chk2-pT68 accumulation in U2OS (Fig. 5A) or 292FT cells (Fig. 5B), suggesting that PP2A-C alone does not contribute to T68 dephosphorylation. In another set of experiments, we investigated the effects of knocking down by siRNA the levels of PP2A-C, PP1-C, and the PP2C-member

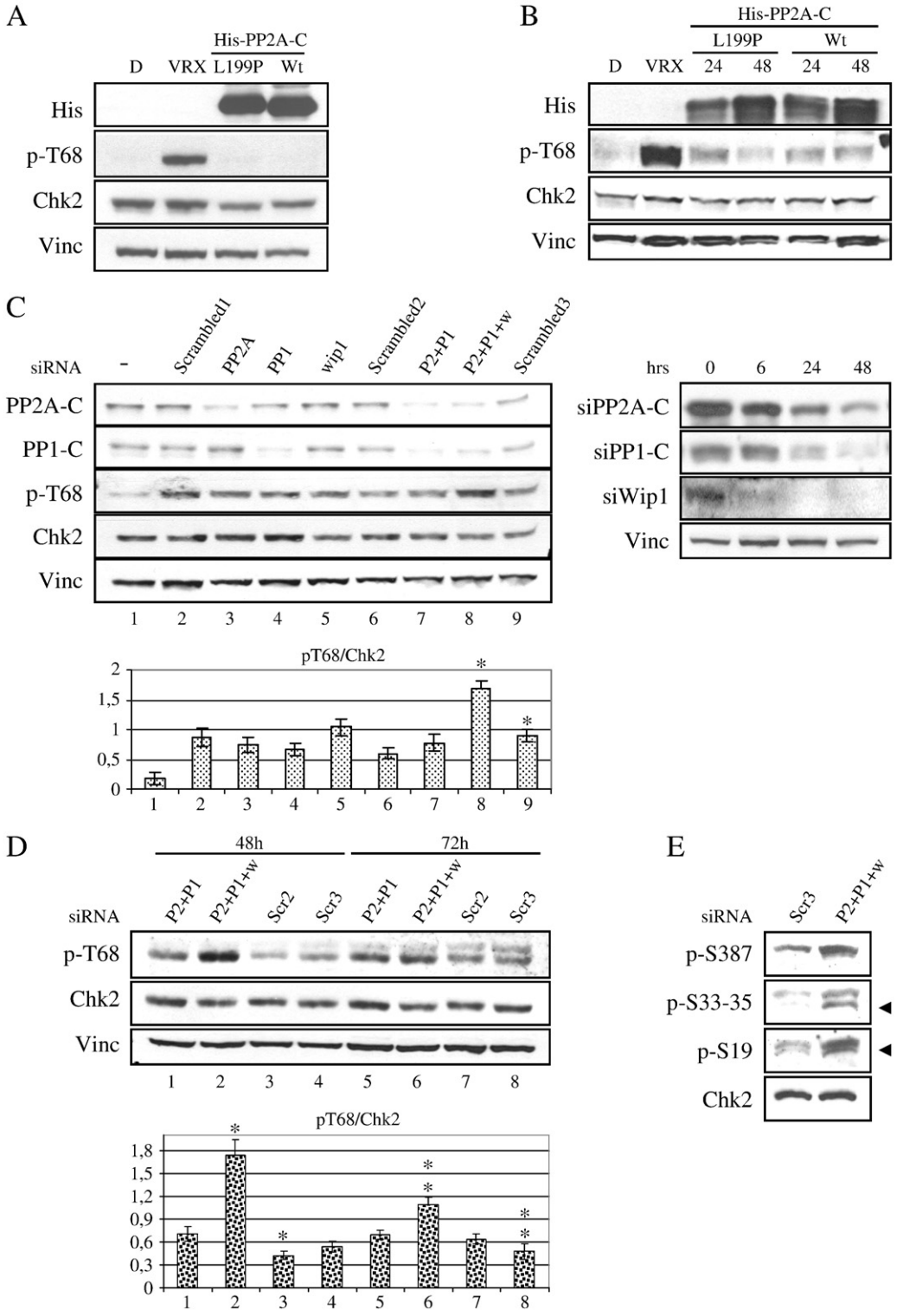


Fig. 5. Protein phosphatases cooperate to maintain Chk2-T68 unphosphorylated. U2OS (A) and 293FT (B) cells were incubated with 10 μM VRX for 90 min or transiently transfected with either His-PP2A-C or dominant-negative His-PP2A-C^{L199P}. Cell extracts were immunoblotted for Chk2-pT68, total Chk2, and His-tag. (C and D) Knock down of PP2A, PP1, and PPM1D/Wip1 phosphatases in 293FT cells was achieved by transfection with siRNA (scrambled, Scr2 and Scr3 are a negative control for the single, double, or triple silencing) and validated by Western blot (C, right). After 48 hours (C, left) or 48 and 72 hours (D), cells were tested for Chk2-pT68, total Chk2, PP1, and PP2A. Abbreviations to panel C: P2 = PP2A-C, P1 = PP1-C, W = Wip1, scrambled = P2, P1, and W control, scrambled 2 = P2 + P1 control, scrambled 3 = P2 + P1 + W control. Legend for panel D is the same as in C. Densitometric analysis of Chk2-pT68 from C and D immunoblots are shown in the bottom graphs, after normalization for total Chk2. (E) The same lysates as in D, lanes 2 and 4, were tested for Chk2-pT68 by Western blot (arrows indicate the antibody-specific band). The statistical significance of the difference between siScr and siP2 + P1 + W is indicated by * ($P<0.01$) or ** ($P<0.05$). All other differences were not statistically significant (analysis performed by the Student's *t*-test).

Wip1/PPM1D [40,41,52,53]. The siRNA depletion of these PPs is shown in Fig. 5C, right. Although the accumulation of Chk2-pT68 in undamaged cells was unaffected by these phosphatases when individually ablated, it was markedly enhanced when all three were simultaneously knocked down (Fig. 5C, lane 8 and graph). Similar results were observed when cells were harvested at two different time points after siRNA transfection (Fig. 5D and graph). Furthermore, siRNA depletion of all three phosphatases significantly raised the levels of Chk2 phosphorylated on S387, S19, and S33-35 (Fig. 5E).

3.6. pT68 accumulation after OA treatment is only partially independent of ATM

To determine if the induced phosphorylation of Chk2-T68 by OA and VRX arises from the effects on a common pathway, we analyzed the role of ATM kinase, which directly phosphorylates T68. LCL-N cells were incubated for 90 min with VRX, OA, and KU (a selective inhibitor of ATM) [54] singly or in combination (in the latter case KU was added 45 min before the other agents) and assessed Chk2 status. The results revealed that the phosphorylation of Chk2-T68 was ATM-dependent in response to VRX since it was abrogated by KU (Fig. 6A, compare lane 2 with lane 5) but was only partially dependent on ATM in response to OA (Fig. 6A, compare lanes 3 and 6). Concordant with this, the levels of Chk2-pT68 induced by OA in the ATM-deficient AT52 cells were greatly reduced compared to LCL-N cells (Fig. 6B). Interestingly, pretreatment of LCL-N cells with KU repressed ERK1/2 phosphorylation in response to OA (Fig. 6A, compare lanes 3 and 6), indicating an ATM dependence of this phenomenon. Concordant with this, the levels of OA-induced phospho-ERK1/2 in the ATM-null AT52 cells were greatly reduced relative to LCL-N (Fig. 6B, compare lanes 2 and 3 and lanes 5 and 6). Hence, these results extend the link between the ATM activity and ERK1/2 phosphorylation not only in relation to DNA damage [55] but also in an undamaged setting. The absence of ATM only slightly affected OA-induced phosphorylation of T68. To

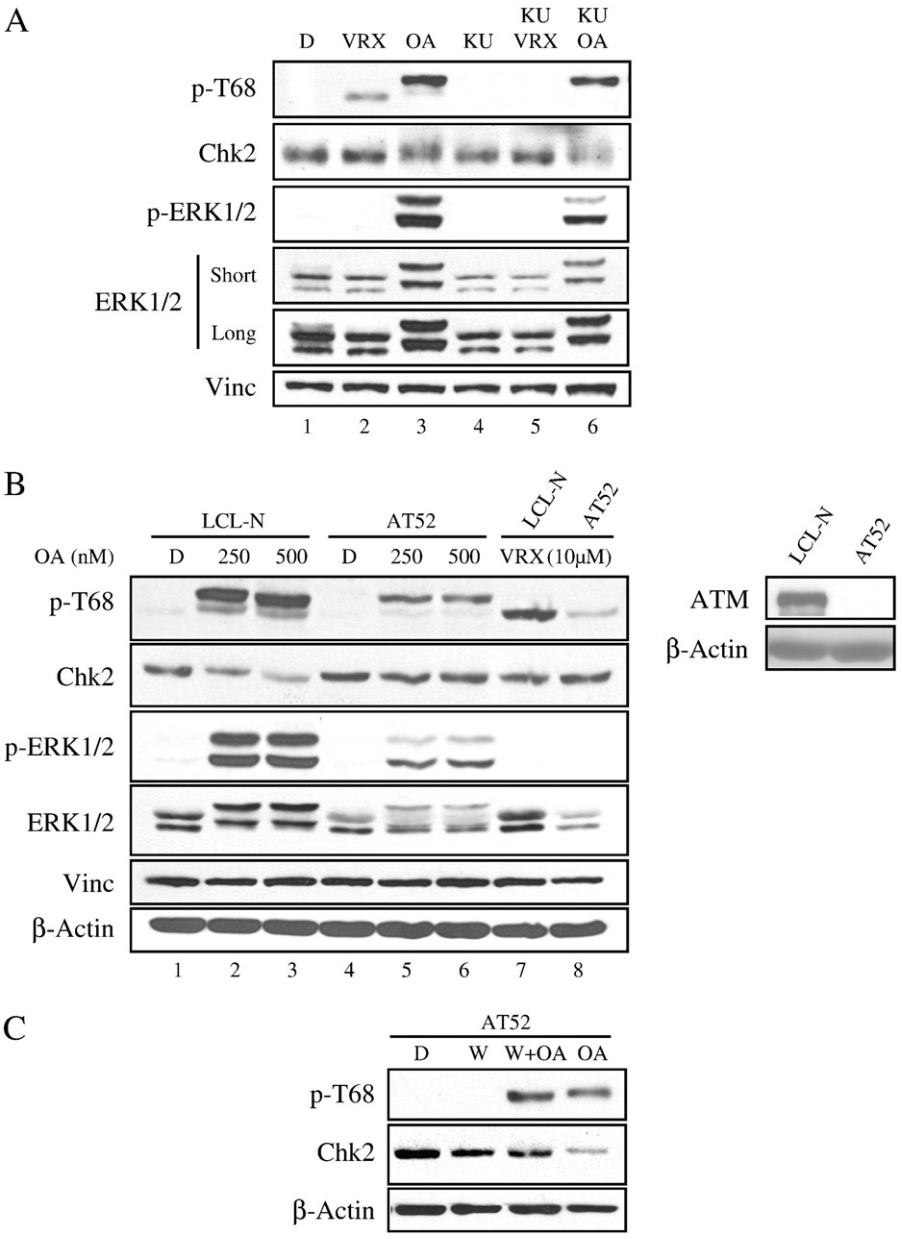


Fig. 6. OA-induced Chk2-T68 phosphorylation is partially dependent on ATM. (A) LCL-N cells were treated for 90 min with 10 μM VRX and 250 nM OA, 0.1% DMSO (D, control), for 45 min with 10 μM KU, lysed, and immunoblotted with the indicated antibodies. (B) LCL-N and AT52 were treated with OA and VRX for 90 min and processed as in panel A. In panel C, AT52 cells were incubated with wortmannin (W; 50 μM for 2 hours), OA (250 nM for 90 min), wortmannin followed by OA (W + OA), or DMSO (D; 0.1%).

determine whether this was due to other PI3Ks other than ATM, AT cells were treated with a concentration of wortmannin that inhibits all PI3Ks before incubating with OA. Under this condition, the OA-induced Chk2-pT68 was not prevented by wortmannin (Fig. 6C), excluding the dependence of this event by PI3Ks.

4. Discussion

In this work, we demonstrate that in unperturbed cells, the catalytic inhibition of Chk2 induces a hyperphosphorylation of this kinase on T68 [22]. The phosphorylation of Chk2-T68 is a key event in cells responding to genotoxic stress, initiated by ATM and leading to the autophosphorylation and full activation of Chk2 [24,30,56]. However, the phosphorylation of Chk2-T68 following its inhibition by VRX is DNA damage-independent since, as shown here, it was not accompanied by the formation of γ-H2AX nuclear foci, an index of genomic DSBs, or by ATM activation as inferred from the absence of phosphorylation of known substrates of this kinase (e.g., p53-S15 and SMC1-S966).

In unperturbed cells, protein serine/threonine phosphatases play a role in dephosphorylating checkpoint kinases, as in the case of PP2A which maintains ATM and Chk1 in a hypophosphorylated state [42,43]. To investigate the interplay of PPs with Chk2, we first assessed the phosphorylation of Chk2 on T68, S19, S33-35, and T387 autophosphorylation site, upon treatment of cells with OA, a chemical inhibitor of PP2A-like activities [42,43,49], and shown a marked phosphorylation of Chk2 on these residues. Furthermore, we have assessed the effects of knocking down by RNA interference the expression of the PP2A, Wip1, and PP1 catalytic subunit previously implicated in Chk2 phosphoregulation after DNA damage [40,41,52,53,57]. Like the effects of OA, the simultaneous depletion of all three phosphatases induced the accumulation of Chk2-pT68 and other phosphosites involved in its full activation, suggesting a cooperating activity of PPs.

The intracellular accumulation of Chk2-pThr68 appeared ATM-dependent in response to VRX but only partially dependent on ATM in response to OA. The noncomplete ATM-dependent phosphorylation of Chk2 suggests the contribution of an OA-sensitive kinase, as shown in the case of Chk1 [43]. Furthermore, we excluded an off-target effect of VRX against PPs in this response since it did not promote the accumulation of phospho-ERK1/2 (p42-p44 MAPK), unlike OA [47,48].

Taken together, these results suggest a regulatory model for Chk2 similar to that recently proposed for ATM and Chk1 [42,43]. We propose that, in unperturbed cells, Chk2 regulation is under control of both kinase and the phosphatase proteins. A low basal activity of ATM on Chk2, perhaps due to endogenous stress, is counterbalanced by the dephosphorylating activity of PP2A, PP2C, and Wip1. The pT68 accumulation owing to PPs repression or Chk2 kinase inhibition, suggests a role for Chk2 activity in a negative loop through which it restrains and finely tunes Chk2 kinase, also to avoid an inappropriate Chk2 activation in the presence of minimal amounts of DNA lesions (Fig. 7). In perturbed cells, this negative feedback loop destabilizes, allowing the build up of an optimal DNA damage response, but how this occurs remains unclear. Given that the activation of Chk2 involves transient dimerization partly through intermolecular pT68–FHA domain interactions, one possibility is that this might impair the affinity of PPs for Chk2 and/or enhance their dissociation [57], allowing the kinase to become fully active. Our results concord with others showing a role for PPs in the dephosphorylation of targets involved in DDR and checkpoint recovery, like PP2A, which regulates the autophosphorylation of ATM on Ser1981 without promoting its kinase activity [42,58], Chk1 [43], p53 [59], and γ-H2AX [60]. Moreover, Wip1/PPM1D dephosphorylates Chk1, Chk2, γ-H2AX, and p53 [53,61,62], while ATM-regulated PP1 activity [63] acts on p53 [64] and on Chk2-dependent phosphorylation of Brca1 [65].

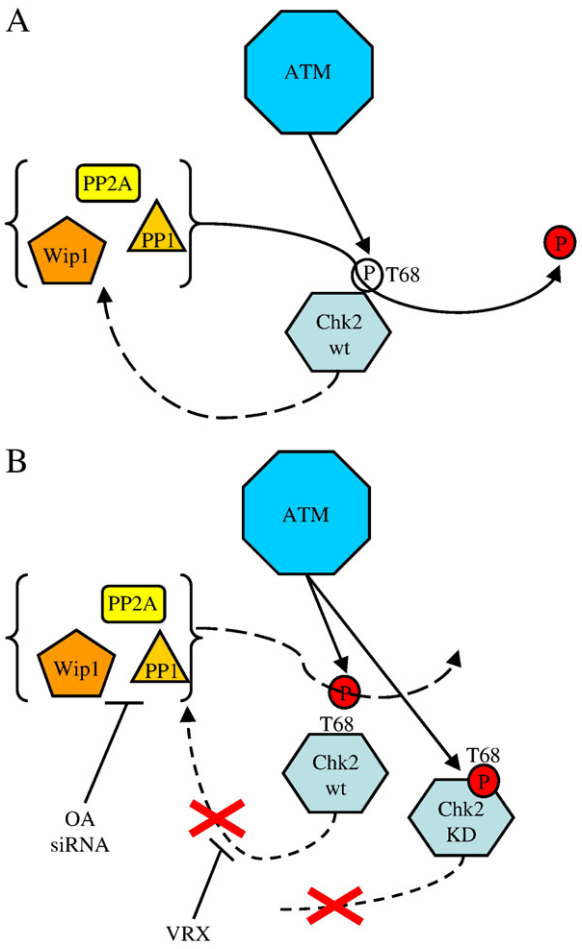


Fig. 7. Model for the regulation of Chk2 by protein phosphatases. (A) In undamaged cells, owing to the basal constitutive activity of ATM, Chk2 is sufficiently phosphorylated on T68 to elevate its catalytic activity. This, in turn might stimulate PP2A, PP1, and Wip1 to keep in check the levels of Chk2-pT68. (B) The block of Chk2 activity by VRX or the suppression of protein phosphatases' expression or activity by OA enhances the accumulation of Chk2-pT68 *in vivo*. Thus, in the absence of DNA lesions or cell cycle perturbations, Chk2 maintains a dephosphorylated status through the interplay with phosphatases.

In conclusion, our work provides a detailed characterization of the phosphorylative regulation of Chk2 in unperturbed cells and underscores the role of PPs in keeping, through feedback mechanisms, the ground state of activation of the ATM/Chk2 circuit, whose inappropriate deregulation can induce growth arrest, apoptosis, premature ageing, and senescence.

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