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# CDC42 Gtpase Activation Affects Hela Cell DNA Repair and Proliferation Following UV Radiation-Induced Genotoxic Stress

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## ABSTRACT

Cell division control protein 42 (CDC42) homolog is a small Rho GTPase enzyme that participates in such processes as cell cycle progression, migration, polarity, adhesion, and transcription. Recent studies suggest that CDC42 is a potent tumor suppressor in different tissues and is related to aging processes. Although DNA damage is crucial in aging, a potential role for CDC42 in genotoxic stress remains to be explored. Migration, survival/proliferation and DNA damage/repair experiments were performed to demonstrate CDC42 involvement in the recovery of HeLa cells exposed to ultraviolet radiation-induced stress. Sub-lines of HeLa cells ectopically expressing the constitutively active CDC42-V12 mutant were generated to examine whether different CDC42-GTP backgrounds might reflect different sensitivities to UV radiation. Our results show that CDC42 constitutive activation does not interfere with HeLa cell migration after UV radiation. However, the minor DNA damage exhibited by the CDC42-V12 mutant exposed to UV radiation most likely results in cell cycle arrest at the G2/M checkpoint and reduced proliferation and survival. HeLa cells and Mock clones, which express endogenous wild-type CDC42 and show normal activity, are more resistant to UV radiation. None of these effects are altered by pharmacological CDC42 inhibition. Finally, the phosphorylation status of the DNA damage response proteins γ-H2AX and p-Chk1 was found to be delayed and attenuated, respectively, in CDC42-V12 clones. In conclusion, the sensitivity of HeLa cells to ultraviolet radiation increases with CDC42 over-activation due to inadequate DNA repair signaling, culminating in G2/M cell accumulation, which is translated into reduced cellular proliferation and survival. J. Cell. Biochem. 116: 2086–2097, 2015. © 2015 Wiley Periodicals, Inc.

**KEY WORDS:** CDC42 GTPASE; CONSTITUTIVELY ACTIVE CDC42-V12 MUTANT; DNA DAMAGE AND REPAIR; PROLIFERATION AND CELL CYCLE; MIGRATION; ULTRAVIOLET RADIATION

The Cell Division Cycle 42 (CDC42) protein was first purified from placental membranes and called Gp due to its homology with Gs and Gq proteins [Evans et al., 1986]; after its cloning in 1990, CDC42 was termed G25K [Munemitsu et al., 1990; Shinjo et al., 1990]. The human CDC42 gene has been mapped to chromosome 1p36.1, and the protein structure showed similarities with Ras and other Rho GTPases. The CDC42 protein comprises five α-helices, six β-strands, and two highly mobile switch regions, which are able to

Abbreviations: CDC42, cell division control protein 42 homolog; CDC42-T17N, dominant-negative mutant; CDC42-G12V, constitutively active mutant; DMEM, Dulbecco's modified Eagle's medium; DNA, deoxyribonucleic acid; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; GAP, GTPase-activating protein; GDP, guanosine diphosphate; GEF, guanine nucleotide exchange factor; GPCR, G-protein-coupled receptor; GTP, guanosine-5′-triphosphate; N-WASP, neuronal Wiskott–Aldrich syndrome protein; PAK, p21-activated kinase; PBS, phosphate-buffered saline; PI, propidium iodide; Rho GTPase, Ras homolog of small GTPase; ROS, reactive oxygen species.

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All authors contributed equally to this work.

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recognize its ligand after structural modification. Two mutants important to the study of CDC42 are CDC42-G12V (hereafter referred to as CDC42-V12), a constitutively active protein that is bound to GTP and that cannot cycle to its inactive form, and CDC42-T17N, which, conversely, confers an inactivated protein bound to GDP that is unable to cycle to its active form. These mutations do not alter the CDC42 structure itself but are precisely located in switch regions I and II, which are the primary regions responsible for GDP–GTP binding and exchange. Two other relevant mutants exist: CDC42-F28L, a mutation that enables CDC42 to cycle from its inactive to active form faster than the wild type, and the C188S mutation positioned in the anchorage motif CAAX, which is present in all GTPases and confers prenylation deficiency, thereby inducing a CDC42 isoform that is exclusively cytosolic [Johnson, 1999].

CDC42, which was originally discovered in *S. cerevisiae*, was shown to regulate the actin cytoskeletal architecture [Johnson and Pringle, 1990]; its human homolog is highly conserved, indicating an important role in mammalian biology [Rathinam et al., 2011]. Later genetic studies showed that CDC42 is critical for cell polarity in yeast and *C. elegans*. In mammalian systems, CDC42 has roles in cytoskeleton architecture, transcription, cell cycle progression, vesicle trafficking, and other cellular pathways. To be able to perform so many functions, CDC42 is stimulated by receptors activated by a variety of extracellular signals and can respond to these signals by linking to different effectors. The most important signals for CDC42 activation are transmitted by GPCRs, RTKs, and integrins [Lin et al., 2006; Deevi et al., 2011]; in addition to receptors, CDC42 can be activated by cross-talk between different intracellular signaling cascades [Ponimaskin et al., 2007]. CDC42 is considered to induce entry into S phase by activating p38 [Philips et al., 2000]; however, the mechanism responsible is unknown to date.

The importance of CDC42 is evident when studying CDC42-knockout mice. The knockout of this gene is lethal and leads to embryonic death before 7.5 days [Chen et al., 2000]. Some reviews have listed several important functions regulated by CDC42, including filopodia formation, which leads to neurite extension formation, axonal growth, axonal myelinization, focal complex formation at the plasma membrane, chemotaxis, polarity, and cell differentiation determination [Melendez et al., 2011]. For these functions, CDC42 interacts with several proteins, some of which have already been identified and include scaffold proteins such as WASP and Par6 and protein kinases such as PAKs and MRCKs [Cerione, 2004].

Accordingly, the enormous interest in studying the role of Rho GTPases in cancer is due to the important roles that these proteins play in the cell cycle, cell-cell interactions, and migration [Nobes and Hall, 1995], and Rho GTPases have been found to be dramatically up-regulated in different types of cancer. For instance, breast, colorectal and testicular cancers have been shown to present higher levels of CDC42 compared with normal cells [Kamai et al., 2004; Bray et al., 2011; Rathinam et al., 2011]. Two types of tumor migration are dependent on CDC42 activity: in amoeboid tumor motility, CDC42 is activated, and its effectors N-WASP and PAK2 are important for movement maintenance; whereas in mesenchymal tumor motility, activated CDC42 is required to regulate directional movement [Gadea et al., 2008].

Some more recent studies show that CDC42 may be related to aging [Wang et al., 2007; Kerber et al., 2009]. In mice, CDC42 activation promotes premature senescence, an effect that is dependent on p53 [Wang et al., 2007]. A compelling theory is that DNA damage is crucial to aging [Freitas and de Magalhaes, 2011] and that proteins involved in DNA damage repair might be directly involved in aging. The C-terminal portion of CDC42 was identified as a possible nuclear localization signal sequence (NLS) [Williams, 2003], and it was recently reported that CDC42 translocates to the nuclear membrane of photoreceptor cells after stress due to excessive exposure to light [Heynen et al., 2011]. These data suggest that CDC42 may play a role in the DNA damage response by interacting with possible repair pathway components, with proteins involved in senescence or with pro- or anti-apoptotic partners. Additionally, as discussed above, CDC42 participates in a myriad of cellular signaling pathways, though little is known concerning its activity in cervix adenocarcinoma HeLa cells. The aim of this study was to investigate the effects of the constitutively active CDC42-V12 mutant in a new, unexplored area of DNA damage and repair after genotoxic stress triggered by UV radiation.

## MATERIALS & METHODS

### CELL TRANSFECTION

Packing ψNX-Ampho (Phoenix) cells, which were kindly provided by Prof. Garry P. Nolan (Stanford University, San Francisco, CA), were transfected with empty pCM plasmid (Mock) or with pCM plasmid containing the constitutively active CDC42-V12 mutant (kindly provided by Dr. Nigel Carter, The Salk Institute, La Jolla, CA), also harboring a geneticin-resistance gene, using Lipofectamine. The supernatant, which contained retroviral particles, was collected at 24, 48, and 72 h after transfection, filtered through a 45-μm membrane and frozen at −80°C. HeLa cells were subsequently infected or transduced with the viral supernatant. After weeks of 100 μg/ml geneticin selection, individual clones were collected using cloning iron rings and transferred to a 24-well plate until confluence was reached. The transduced clones were trypsinized, transferred to 5 $cm^2$ flasks after culture stabilization, the cells were trypsinized and frozen in liquid nitrogen for further analysis (adapted from [Forti and Armelin, 2007]). Two HeLa-Mock clones and four CDC42-V12 clones were isolated, and the migration ability of these cells was tested using scratch wound healing assays in the presence and absence of serum. Additionally, their proliferative and survival capacities were assessed using clonogenic assays with different cell densities (results not shown). We selected the two best representative clones from each condition and assayed the basal levels of CDC42 and CDC42-GTP to examine functionality. We demonstrated that the CDC42-V12 clones exhibit the highest levels of CDC42 activity, as expected, compared with the parental HeLa cells or HeLa-Mock control cells (Supplementary Fig. S1).

### CELL CULTURE, IRRADIATION, AND INHIBITOR TREATMENTS

HeLa cells were cultured as monolayers in Dulbecco's modified Eagle's medium (Invitrogen, Walthan, MA) supplemented with 10% fetal calf serum (FCS) (Cultilab, Campinas, SP, Brazil). The

CDC42-V12 clones were cultured as monolayers in DMEM supplemented with 10% FCS and 100 μg/ml geneticin. The cells were incubated at 37°C in a 5% $CO_2$ atmosphere; for all the experiments, the cells were plated 24 h prior to the exposure to UVC irradiation at 4 kJ/$m^2$ for 6 s. For the CDC42 inhibitor treatments, at 24 h after plating, the cells were treated with two different concentrations of inhibitors (10 or 20 μM for ML141; 50 or 100 μM for ZCL278) for 24 h prior to UV irradiation. These inhibitors were reconstituted in DMSO; thus, we used DMSO as a control, as necessary. In relation to other members of the Rho GTPase family, including Rac1, Rab2, and Rab7 [Hong et al., 2013], ML141 is a selective competitive inhibitor of CDC42. ZCL278 selectively inhibits CDC42 because it binds to the GEF binding sites on CDC42, thereby inhibiting its cellular effects without inducing cytotoxic effects [Friesland et al., 2013; Hong et al., 2013].

### PREPARATION OF PBD-GST FUSION PROTEINS

The plasmid PAK1-PBD (PAK1-Binding Domain Glutathione-S Transferase; a kind donation from Gary M. Bokoch of The Scripps Research Institute, La Jolla, CA), which contains a sequence encoding the fusion protein PBD-GST, was transformed into *E. coli* (BL21) via thermal shock. The transformed bacteria were then plated on LA medium containing 100 μg/ml ampicillin and incubated at 37°C. A colony of *E. coli* (BL21) that had been transformed with the PAK1-PBD plasmid was inoculated into 200 ml of LB medium and incubated for approximately 18 h at 37°C under constant agitation (250 rpm). This culture was then inoculated into 2 l of LB medium and maintained at 37°C under constant agitation until reaching an optical density (absorbance) of approximately 0.6. The expression inducer isopropyl β-D thiogalactopyranoside (IPTG; 0.5 mM) was added to this culture and incubated at 37°C for 2 h, after which the cells were recovered via centrifugation (8,000 rpm for 10 min at 4°C). The pellet was resuspended in 20 ml of lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton ×-100, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF) and sonicated on ice, applying eight cycles of 2 min at a 50% amplitude, with the pulse on for 15 s and off for 30 s. After lysis, this suspension was centrifuged for 30 min at 14,000 rpm at 4°C, and the soluble fraction containing the PAK1-PBD fusion protein was collected. Approximately 12 ml of this soluble fraction was incubated with 500 μl of glutathione-Sepharose 4B resin (GE Healthcare, Cleveland, OH) for 90 min at 4°C with constant mixing. The resin-bound fusion protein was washed (3,000 rpm for 3 min) six times with wash buffer (50 mM Tris pH 7.5, 0.5% Triton ×-100, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 0.1 mM PMSF), and the beads were then resuspended in 5 ml of wash buffer containing 10% glycerol, aliquoted and stored at −80°C [Ren et al., 1999].

### MEASUREMENTS OF CDC42 GTPASE ACTIVITY BY PULL-DOWN ASSAY

Cellular protein lysates were obtained from 10-mm dishes at approximately 60% confluence by washing the cells twice with ice-cold PBS and disrupting the cells with RIPA lysis buffer (50 mM Tris, pH 7.2, 1% Triton ×-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 1 mM $Na_3Vo_4$, 1 mM NaF, 10 μg/ml each of aprotinin and leupeptin, and 1 mM PMSF). The lysates were then stored at −20°C, and protein quantification was performed using the Bradford (Bio-Rad, Hercules, CA) colorimetric method. A 500-mg sample of the total lysate was subsequently incubated with 100 μg of PAK-PBD-GST at 4°C for 90 min. The beads were centrifuged (at 4°C for 3 min), washed three times with buffer B (Tris buffer containing 1% Triton ×-100, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $Na_3Vo_4$, 1 mM NaF, 10 μg/ml each of aprotinin and leupeptin, and 1 mM PMSF) and collected via centrifugation at 3,000 rpm for 3 min in a cold room. The active CDC42 protein (CDC42-GTP) bound to the glutathione-Sepharose beads containing the PAK-PBD-GST fusion protein was detected via western blotting [Ren et al., 1999].

### WESTERN BLOTTING FOR CDC42, PHOSPHO-H2AX, PHOSPHO-CHK1, AND TUBULIN

For the analysis of selected proteins, electrophoresis was performed at a constant voltage of 120 V under denaturing conditions using polyacrylamide gels with an acrylamide concentration of 5% in the stacking gel and 13% in the separating gel. The proteins separated by SDS-PAGE were then transferred for 90 min to a nitrocellulose membrane (Millipore, Billerica, MA) using a dry system (Bio-Rad, Hercules, CA) at a constant amperage (300 mA). The membrane was blocked with 5% milk in TBS-T (20 mM Tris pH 7.6; 137 mM NaCl; 0.1% Tween) for 1 h with stirring at room temperature and then washed three times with TBS-T. Next, the membrane was incubated overnight in a cold room with a monoclonal primary antibody against CDC42 (BD Biosciences, Franklin Lakes, NJ), phospho-H2AX-Ser-139 (R&D Systems, Minneapolis, MN), phospho-Chk1-Ser-296 (Cell Signaling, Danvers, MA), or tubulin (Santa Cruz Biotechnologies, Santa Cruz, CA), all diluted at 1:1500 in TBS-T. Finally, the membrane was incubated with the fluorescent secondary antibody IRDye 680CW for 1 h and revealed using the Odyssey Infrared Image System (LI-COR, Lincoln, NE); the bands were quantified using Odyssey V3.0 software (LI-COR).

### FACS CELL CYCLE ANALYSIS

In total, $35 \times 10^4$ cells were plated in 35-mm Petri dishes (Corning, New York, NY) for 24 h prior to treatment. After UV irradiation, the cells were collected after being detached by trypsinization. The cells were then washed with PBS, fixed with ethanol 80% in PBS (9:1) and stored at 4°C until measurement. Before the measurements, the cells were stained with 10 μg/ml propidium iodide (PI), and the analysis was performed using a Beckman Coulter FC500 MPL cytometer. The obtained data were analyzed using WinMDI 2.8 software (Pardue University Cytometry Laboratories, West Lafayette, IN).

### ALKALINE COMET ASSAY

In total, $25 \times 10^4$ cells were plated in 35-mm Petri dishes for 24 h prior to treatment. After UV irradiation, the cells were collected after being detached by trypsinization. The cells were then washed with comet PBS (0.003 M KCl, 0.0015 M $KH_2PO_4$, 0.14 M NaCl, and 0.008 M $Na_2HPO_4$) and mixed with 0.5% low melting point agarose (InLab, Sao Paulo-SP, Brazil). The mixture was dropped onto a slide containing a layer of 1.5% normal agarose (InLab, Sao Paulo, SP, Brazil) and covered with a glass coverslip. After the slides were refrigerated at 4°C

for 15 min, the coverslips were removed, and the slides were submerged in lysis buffer (0.22 M NaCl, 8.9 mM EDTA, 0.89 mM Tris, 1 ml Triton ×-100, and 10 ml DMSO for 100 ml of buffer) overnight; electrophoresis was performed the next day. The slides were washed three times in neutralization buffer (0.4 M Tris for 1 l of buffer) for 5 min before being fixed with absolute ethanol for another 5 min. The analysis was performed using Andor Komet software (Andor Technology, Belfast, BT, UK); 60 μl (20 μg/ml) of ethidium bromide (Invitrogen) was added to the surface of the slide, and the sample was covered with a rectangular glass coverslip. Single cell nuclei were observed and photographed using a fluorescence microscope Olympus IX51 (Olympus, Shinjuku, Tokyo, Japan).

### MIGRATION ASSAY

In total, $1 \times 10^6$ cells were plated in 35-mm Petri dishes for 24 h prior to treatment. After reaching confluence, the cells were UV irradiated, and a scratch was made in the center of the Petri dish with a pipette tip; many different regions of the same scratch were photographed at 0 and 24 h after treatment using an inverted microscope Olympus IX51 (Olympus). These micrographs were obtained at 200× magnification in triplicate plates, and migration or invasion measurements were performed using the appropriate tools in Cell-F software (Olympus). In the micrographs, the dark red dotted lines indicate the edges of the wound at time zero, and the light red dotted lines indicate the leading edge of migration; the quantification was based on the boundary of migrated cells at the desired time point, which was 24 h in our case. Thus, the % of wound closure after 24 h is equivalent to the % of invaded area or the % of migration [Liang et al., 2007].

### CLONOGENIC ASSAY (COLONY FORMATION ASSAY)

In total, $2 \times 10^3$ cells were plated in 60-mm Petri dishes for 24 h prior to treatment with UV irradiation. At 10–14 days after treatment, colonies were fixed with 10% formaldehyde in PBS, stained with 0.5% crystal violet stain solution, manually counted and photographed.

### STATISTICAL ANALYSIS

The results were analyzed using Prism (GraphPad 5 Software, San Diego, CA). The standard errors of the means (SEM) are shown using error bars, and significance values were calculated via two-way ANOVA of the mutant clone versus the parental HeLa cell line or the Mock clone for each treatment. Statistical significance was assumed at $P < 0.05$.

## RESULTS AND DISCUSSION

### ACTIVE CDC42-V12 HELA CELL CLONES SHOW ALTERED MORPHOLOGY AND MIGRATION BUT WERE NOT AFFECTED BY UV TREATMENT

The most elementary characteristic of HeLa CDC42 mutant cells is the observed difference in morphology when compared with wild-type HeLa cells. In a 100% confluence 25 $cm^2$ culture flask, we were able to estimate approximately $5 \times 10^6$ wild-type cells, which corresponded to approximately $2 \times 10^6$ cells for the CDC42-V12 mutant in the same area. This difference in cell density/$cm^2$ can clearly be observed in Figure 1A, with the constitutively active cells showing increased size, as they are more spread out and elongated in a direction exhibiting increased polarity, which is expected for cells presenting high CDC42 activity. This result is consistent with other reports, indicating that Rho GTPases are involved in cytoskeleton architecture, adhesion, and polarity. Specifically, CDC42 is involved because changing the quantities of active CDC42 protein in these cells causes observed morphology changes [Heasman and Ridley, 2008]. Additionally, CDC42 overexpression has been reported to culminate in cytokinesis failure and cell aneuploidy, resulting in cells that typically exhibit larger sizes and slower growth (Zhu X, Wang J et al. & Murata-Hori M, Exp Cell Res, 2011). This phenotype is quite similar to what we observed for HeLa-CDC42-V12 cells; according to our photomicrographs and cell counting results, approximately 1–2% of the constitutively active Cdc42-V12 cell population is polynucleated (with three or more nuclei), a morphological feature not observed in HeLa or HeLa-Mock cells (Fig. 1B). To complete this CDC42 clonal characterization, total and active levels of CDC42 were assessed by pull-down activity assays (Fig. 1C and Supplementary Fig. S1). The constitutively active clone showed the highest basal level of GTP-bound CDC42 in the asynchronous condition among the three studied cells (Fig. 1C). Additionally, after 48 h of serum starvation and subsequent stimulation with 30% serum, the CDC42-V12 clone was the most responsive (Supplementary Fig. S1). Following UV treatment, the CDC42-V12 clone again presented higher levels of GTPase activation (Fig. 1C) compared with the control cells.

CDC42 in is involved in filopodia formation and functions in normal and tumor cell migration [Rathinam et al., 2011]. Thus, we investigated the role of CDC42 in the migration of HeLa control cells or the CDC42-V12 mutant on fully confluent cell monolayers; in Figure 2A, the dark red dotted lines indicate the edge of the wound at time zero, and the light red dotted lines indicate the leading edge of migration. Our quantification was based on the boundary of migrating cells at 24 h after treatment using the Cell-F software (Olympus) tool and was conducted according to methodologies recently published in the literature [Liang et al., 2007]. The % of wound closure after 24 h is equivalent to the % of invaded area or the % of migration. As observed in Figure 2, the scratch created was partially closed 24 h later in the untreated Mock and HeLa cell cultures (~60%) and almost filled in the CDC42-V12 clonal cell culture (~80%). The constitutively active clones migrated slightly faster (~20%) than the wild-type CDC42 cells, as expected for active Rho GTPase cells. Furthermore, UV treatments did not affect their migration abilities. Unpublished results from our laboratory have demonstrated that other small GTPases, such as RhoA and Rac1, present completely opposite migration profiles. Among other products, UV radiation generates reactive oxygen species (ROS), and an accumulation of ROS can increase motility activity, resulting in augmented cell migration [Gadea et al., 2008]. In our experiments, the constitutively active CDC42-V12 clones migrated faster than the cells expressing normal levels of CDC42-GTP, and this migration, in contrast to proliferation (shown in the following section), may not require CDC42 overactivation to allow different cell behaviors in response to UV radiation-induced genotoxic stress.

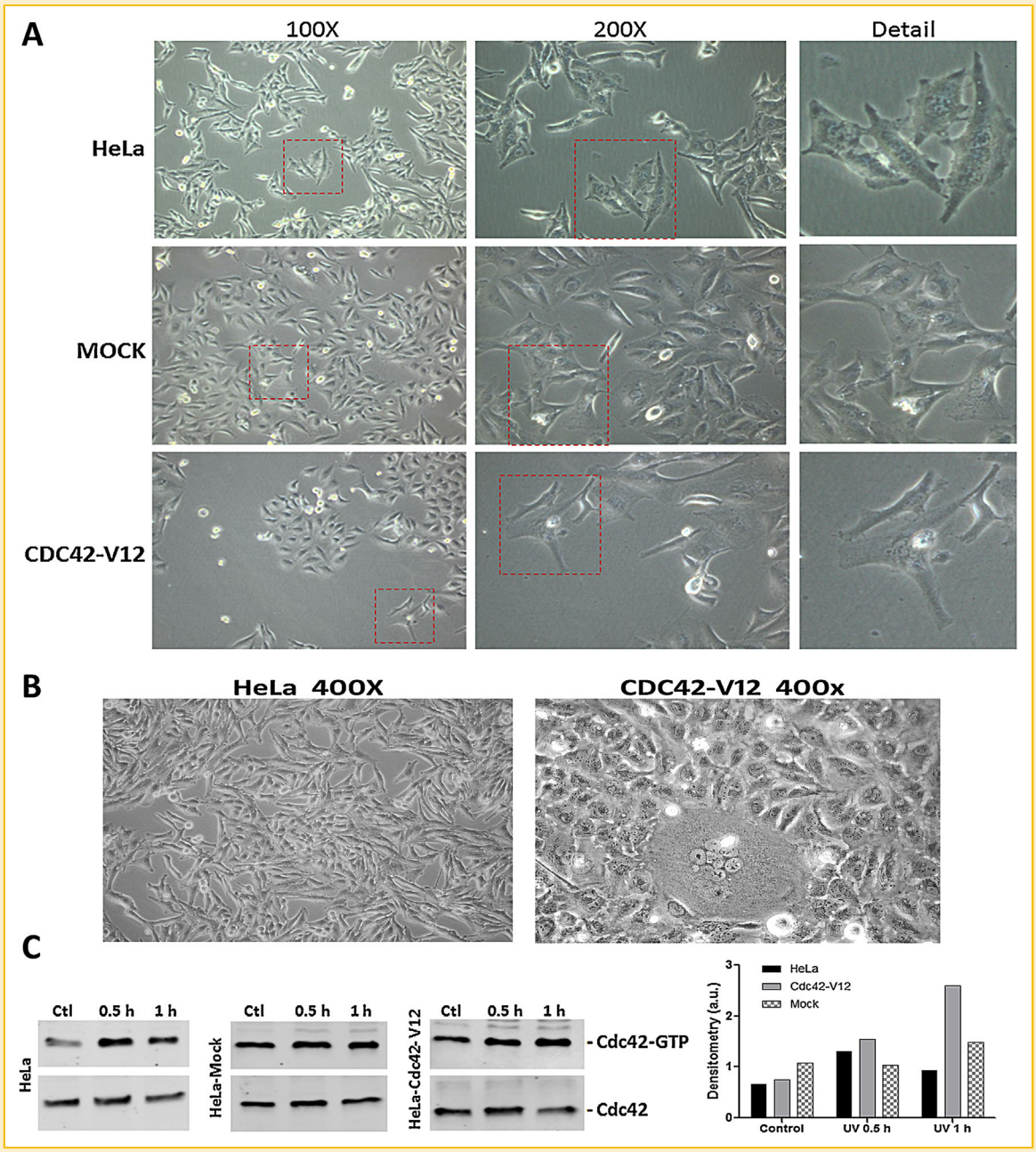


**Fig. 1.** Morphological evaluation of HeLa cell and clone cultures, viewed by bright field optic microscopy, as associated with CDC42 activity. (A) The constitutively active cells (CDC42-V12) show increased size, greater spreading, and an elongated morphology compared with the control cells (HeLa and Mock). (B) In CDC42-V12 clone cultures, approximately one percent of the cell population is found to be polynucleated, which is not observed in the control cells. All micrograph images were obtained using an inverted Olympus microscope, model IX51. (C) Pull-downs of HeLa cells and clones (Mock and CDC42-V12) show differences in CDC42 activity under basal and UV-treated conditions. Immunoblots are representative of two independent and equivalent experiments (at least), performed in pairs of HeLa cells and clones on the same gel; the data were quantified and shown as the average in a bar graph.

### THE REDUCED SURVIVAL OF HELA CELLS UPON UV TREATMENT REMAINS AUGMENTED IN ACTIVE CDC42 CLONES AND IS NOT AFFECTED BY CDC42 INHIBITORS

Colony assays were performed to evaluate the survival ability of the CDC42-V12 mutants compared with control cells under UV treatment, as based on their colony forming capability at low cellular densities of 2,000 cells/plate (Fig. 3). As observed in Figure 3B, when compared with wild-type Mock and HeLa cells, the CDC42-V12 mutant exhibited a reduced number of colonies under untreated conditions. A similar result was reported in fibroblasts and indicated the inhibition of focus proliferation even in the presence of constitutively active CDC42 [Vanni et al., 2005]. To analyze this hypothesis, the authors evaluated CDC42-F28L, a fast-cycling mutant that behaves differently from the CDC42-V12

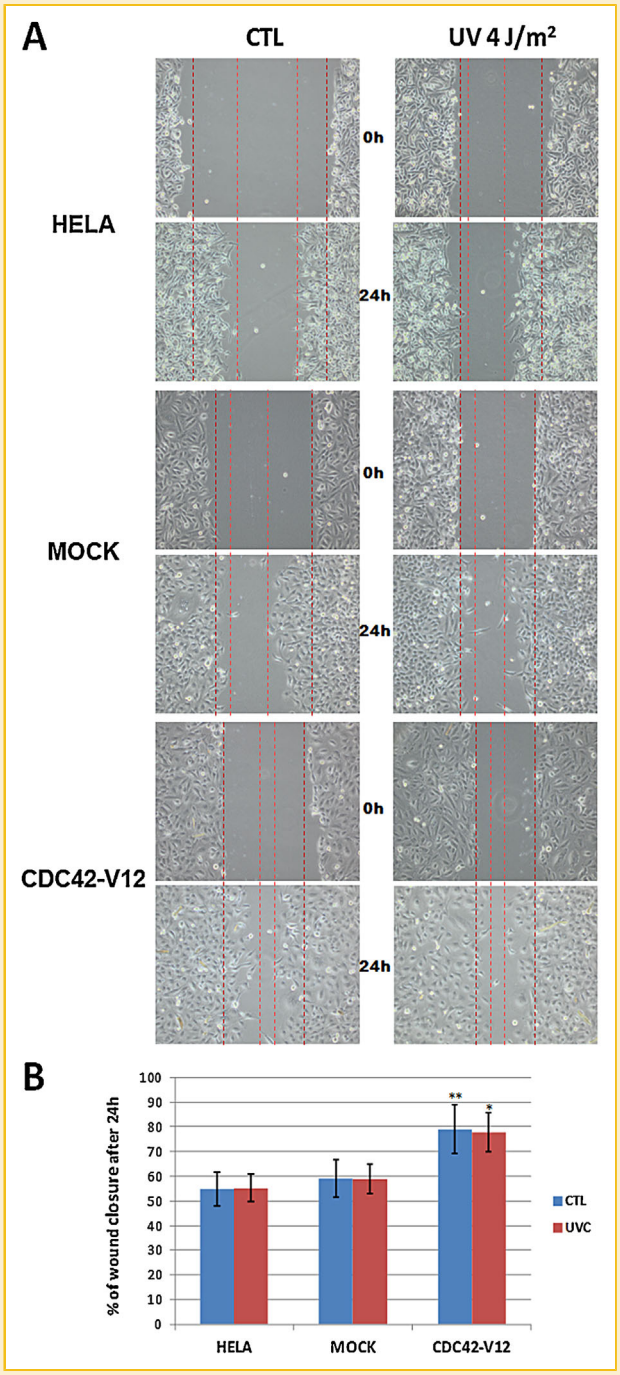


**Figure 2.** CDC42V12 mutant clones migrate further than control cells even after UV radiation exposure. (A) Light photomicrographs of scratches of HeLa cells and clones at 0 (dark dotted red lines) and 24 h (light dotted red lines), with or without 4 J/m$^2$ UV treatment. Representative micrographs were selected from an average of 4 different experiments in triplicate. B) Histograms representing the percentage of invaded area on the scratches or % of wound closure (corresponding to % migration) by the cells at 24 h after creating the monolayer wound. The results represent the average and standard deviation from three independent experiments, and statistical significance was assessed by two-way ANOVA between the CDC42-V12 clones versus the two other control cells, paired by specific similar treatment conditions. *$P = 0.005$ and **$P = 0.001$.

mutant and is able to form much more foci than wild type, showing that rapid GDP-GTP cycling from inactive to active states is required for proper CDC42 function in survival. We suggest that CDC42 may function similarly in both HeLa cells and fibroblasts and that the presence of a constitutively active CDC42 mutant does not confer a proliferation advantage. However, after UV treatment, we found a stronger reduction in the number CDC42-V12 mutant colonies when compared with the untreated condition, particularly compared with UV-treated Mock and HeLa cells (Fig. 3A). UV radiation is absorbed by DNA due to its chemical structure and leads to CPD and 6-4 photoproduct formation [Palomera-Sanchez and Zurita, 2011]. These products inhibit DNA replication and cell proliferation by blocking cell cycle checkpoints, during which DNA integrity is checked, allowing the repair of DNA or the elimination of damaged cells [Yang, 2011]. This inhibition most likely caused the observed smaller number of colonies after UV treatment in all cells but primarily in the CDC42-V12 mutant, which strongly sensitizes HeLa cells. As expected, the formation and the morphology of colonies were also different: we observed much smaller CDC42-V12 mutant colonies that contained fewer cells compared with the wild-type CDC42 cells, even under untreated conditions, because these mutant colonies present an elongated and more spread-out morphology (Fig. 3C).

In the experiments shown in Figure 3, we also investigated the possibility of reversion of the CDC42-overactivation phenotype by using two different pharmacological inhibitors of CDC42 (ML141 and ZCL278), which were added 24 h after plating the cells and 24 h before the UV irradiation treatment. No differences were observed when using two different concentrations of the two different inhibitors (10 or 20 μM for ML141; 50–100 μM for ZCL278), which act through different mechanisms: ML141 is a competitive inhibitor of CDC42, and ZCL278 inhibits CDC42 by blocking its GEF binding sites [Friesland et al., 2013; Hong et al., 2013]. However, these CDC42 inhibitor treatments were not able to promote rescue of the UV-sensitive phenotype of the CDC42-V12 clone, and the individual inhibitor treatments evoked a significant perturbation in the colony formation capability of all three cell lines investigated (Fig. 3). Thus, the clonogenic survival assay results shown in Figure 3 and the results previously published for fibroblasts [Vanni et al., 2005] corroborate preliminary and parallel experiments performed in Hek293 cells in which we transiently transfected similar and different CDC42 constructs and then treated with (or without) UV radiation under identical conditions as described above for the clonogenic assay. Once again, the CDC42-V12 mutants exhibited reduced survival compared with control cells (exhibiting normal CDC42 expression and activity) and compared with the fast-cycling CDC42-F28 mutants in the completely different Hek293 cell background (Supplementary Fig. S2).

## CDC42-V12 CLONES ACCUMULATE IN G2/M AFTER UV IRRADIATION

Among the cellular pathways in which CDC42 is implicated, the cell cycle may be one of the most important [Philips et al., 2000; Kamai et al., 2004]. Essential for budding yeast formation and for *Drosophila melanogaster* development [Johnson and Pringle, 1990], CDC42 may strongly mediate S-phase entry in mammals [Gjoerup et al., 1998; Yasuda et al., 2006]. Therefore, we decided to

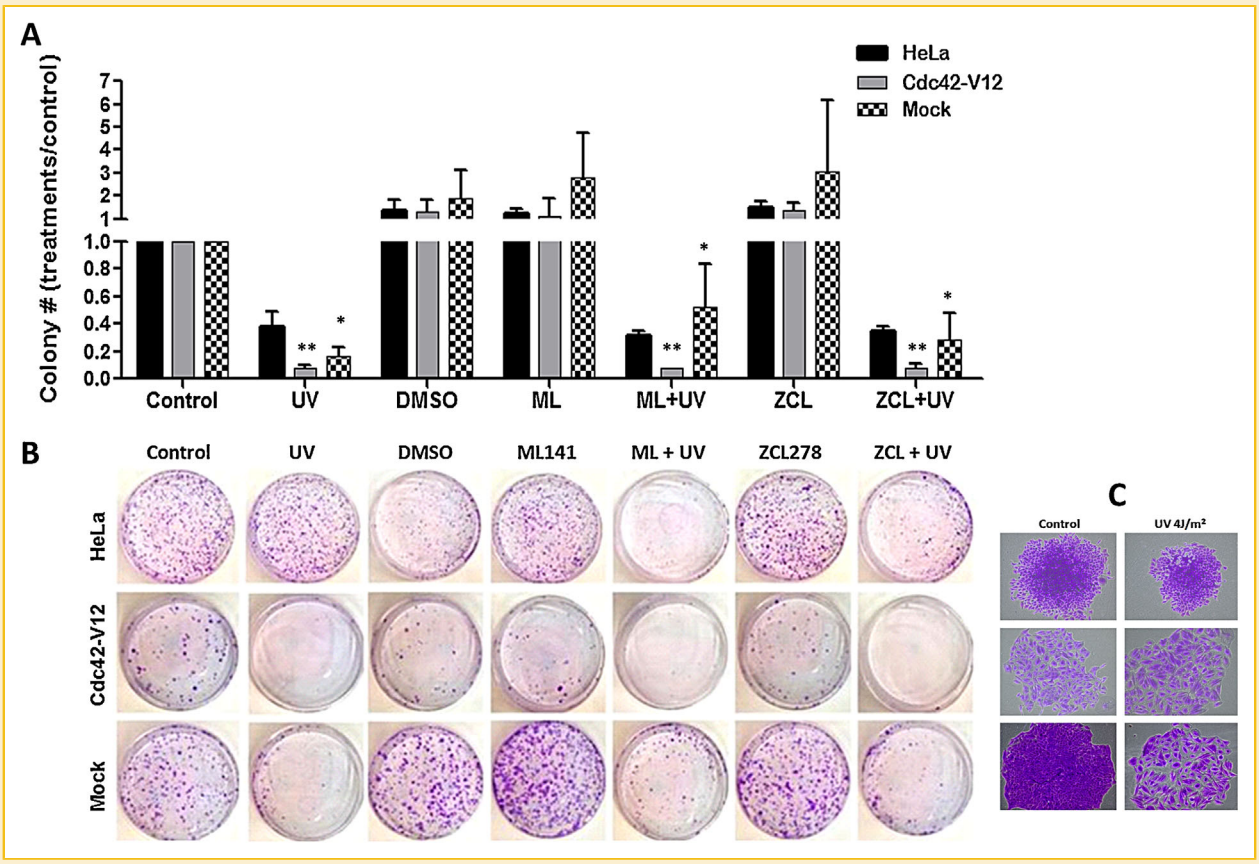


**Figure 3.** Clonogenic assays showing the reduced survival or increased sensitivity to UV radiation of HeLa cells expressing the constitutively active CDC42-V12 mutant. (A) Histogram showing the fold of reduction/increase in the number of colonies (in relation to untreated conditions) after UV treatment with or without the prior addition of two CDC42 inhibitors, 10 μM ML141 or 50 μM ZCL278 for 24 h. The bars represent the average and standard deviation of three independent experiments. Two-way ANOVA was performed for clones (CDC42-V12 or Mock) versus HeLa cells, paired based on the specific treatment condition, to evaluate the significance of differences. $^{*}P < 0.01$; $^{**}P < 0.005$. (B) Scanned 60-mm Petri dishes with fixed colonies subjected to crystal violet staining assays of HeLa cells and clones after 14 days of culture, treated with or without 4 J/m$^2$ UV plus or minus the CDC42 inhibitors. (C) Representative photomicrographs of zoomed-in (200×) colonies, showing their morphology and cell numbers after colony formation was stopped.

verify the role of CDC42 in the cell cycle of HeLa cells and clones using overactivated GTPase with and without UV treatment by performing time course measurements (at 0, 6, 12, 24, and 48 h) of cell cycle distribution by flow cytometry with PI staining (Fig. 4). The cycle analysis shown in Figure 4A indicates that all cell lines behave similarly without any treatment, independent of CDC42 activity level, and exhibit comparable levels of populations distributed in G1, S, and G2/M phases. Major changes were observed, with a G1 increase associated with a G2/M decrease over time, which was completely expected, whereas the opposite was observed when the cells were exposed to UV radiation-induced genotoxic stress (Fig. 4B). However, surprisingly, CDC42 activity was associated with greater G2/M arrest after UV exposition, which was associated with a substantial reduction in S and G1 cell populations compared with cells having normal GTPase activity. The G2/M arrest in CDC42-V12 mutant clones appeared to be more pronounced after 48 h compared with the Mock clones, with a cumulative effect.

In particular, the cell death analysis, which was performed by examining sub-G1 populations based on flow cytometry histograms, lacked relevance at 48 h when comparing untreated or UV-treated cells, independent of the CDC42 activity status. However, an increasing trend in sub-G1 cell populations of the Mock and CDC42-V12 clones should appear after 48 h (Fig. 4B); this expectation appears to be true according to the clonogenic assays shown in Figure 3A. The simultaneously decreasing G1 population and increasing G2/M population indicates that the cells are most likely stopping at the G2/M checkpoint to repair DNA damage. The increased population of dead cells at 48 h suggests that the cells attempted to repair the damage but that those cells that could not return to the cell cycle died, most likely via apoptosis. We suggest that CDC42 has an active role in the G1/S checkpoint, allowing healthy cells to enter S phase, as suggested by Molnar [Molnar et al., 1997]. However, endogenous normal CDC42, which is associated with exogenous overactiveCDC42-V12, may allow damaged cells to pass through the first checkpoint without repairing damage and may stop cells at the G2/M checkpoint. Cells that cannot repair this damage begin to die, as observed at 48 h after UV exposure and in the clonogenic assays (Fig. 3A); these cells likely harbor a DNA repair deficiency, as suggested by comet assays (Fig. 5).

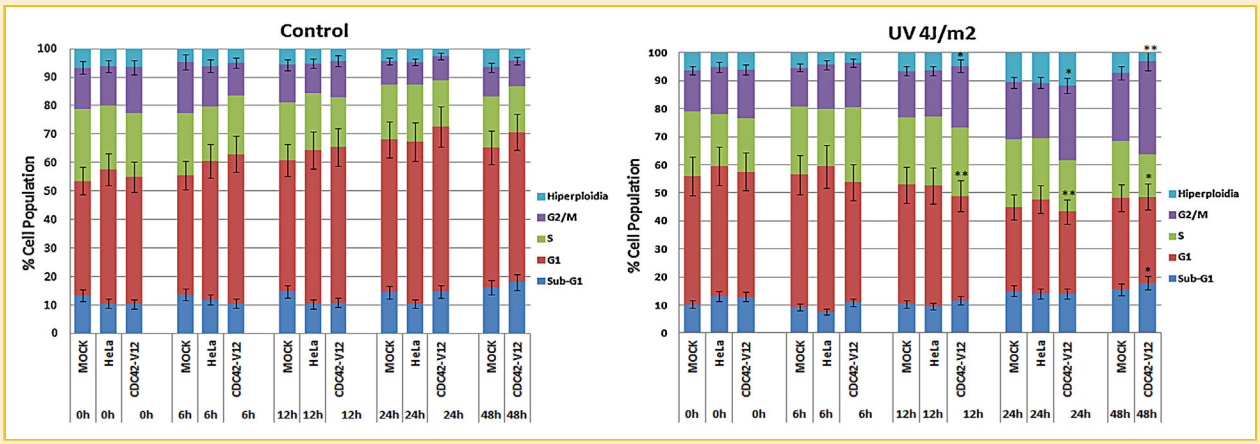


**Figure 4.** Cell cycle distribution of asynchronous HeLa cells and clones with (right) or without UV (left) treatment. The results are the average of three independent experiments, and error bars (SD) are shown only for sub-G1, G1, and G2/M phases; the statistical significance was assessed by comparing UV treatments of CDC42-V12 cells versus untreated Mock clones or HeLa cells using two-way ANOVA. $*P < 0.01$ and $**P < 0.001$.

## THE REDUCED DNA DAMAGE RESPONSE AND REPAIR CAPACITY OF CDC42-V12 CLONES IS VERY LIKELY THE TRIGGER OF G2/M ARREST

The single-cell gel electrophoresis assay (SCGE), also known as the comet assay, is a method used to evaluate single nuclei exhibiting different levels of strand breaks (damage) and the ability of cells to repair their lesioned DNA. In this investigation, we analyzed HeLa cells with normal levels of CDC42 and the constitutively active CDC42-V12 clone upon treatment with or without UV and with or without CDC42 inhibitors (Fig. 5). At 0.5 h after UV treatment, the nuclei from both cell lines showed higher damage compared with the control conditions. The CDC42-V12 clone was the least susceptible to DNA damage, which is represented by Olive tail moment (OTM Index) data, showing approximately two- to threefold less damage than the parental cells. The same cellular responses were observed at 3 h after treatment and continued for up to 6 h following the beginning of the DNA repair process, leading to decreases in the OTM index for both cell types. Additionally, the other notable result indicated by these comet assays is that the CDC42-V12 clones did not reach basal levels of damage within 6 h after UV irradiation, leading to a greater accumulation of fragmented DNA compared with that in HeLa cells. On the one hand, the apparent failure of the DNA repair mechanisms might be associated with an inability to detect UV-damaged DNA; on the other hand, the DNA repair systems that manage UV-promoted damage might be positively affected by the constitutively active expression of CDC42. If this is the case, the latter effect most likely overrides the cellular capacity to cope with repair in a timely manner during the cell cycle or results in escape from the correct repair, which, in both cases, could cause the G2/M arrest observed for the CDC42-V12 clone (Fig. 4). More interestingly, in the presence of prior CDC42 inhibition, both HeLa and CDC42-V12 cells presented an overall reduction in DNA damage levels, but the HeLa cells were unable to recover to the basal levels of damage, and fragmented DNA remained; in contrast, the constitutively active CDC42-V12 clone exhibited a slight reversion of its phenotype by reaching the basal levels of DNA damage. However, even in the presence of CDC42 inhibitors, the CDC42-V12 clone maintained approximately two- to threefold less DNA damage than the parental HeLa cells (Fig. 4); that is, these clones remained more sensitive to UV radiation.

To expand on our results and to confirm that CDC42 over-activation in HeLa cells (CDC42-V12) leads to reduced susceptibility to UV-induced DNA damage (decreased sensitivity of cells to UV irradiation), we performed immunoblotting to detect the status of two DNA damage response proteins (H2AX and Chk1). In kinetic experiments for the phosphorylated form of the histone variant H2AX (at serine 139), the constitutively active CDC42-V12 clone presented a delayed response (6 h) compared with that of HeLa cells (3 h) after UV treatment (Fig. 6A), showing impairment of the DNA damage response (DDR) under cellular conditions (backgrounds) of high CDC42 activity. A lower responsiveness of the HeLa-CDC42-V12 clone was also observed for the levels of Chk1 phosphorylation (at serine 296) compared with the control Mock clonal cells (Fig. 6B), again showing that the overactivation of CDC42 negatively affects the DDR under conditions of UV irradiation-promoted genotoxic stress, which is known to occur through the activation of the ATR-Chk1 pathway [Bartek and Lukas, 2001].

Thus, a possible explanation for the decreased sensitivity of HeLa-CDC42-V12 cells to UV irradiation-promoted lesions is a lower efficiency of specific DNA repair mechanisms triggered by UV irradiation. As this type of radiation (wavelength 100–280 nm) may also induce other downstream or parallel events in addition to DNA damage, we initiated some preliminary host cell reactivation (HCR) assays comparing HeLa-Mock and HeLa-CDC42-V12 clones. Our preliminary results showed that in CDC42-V12 cells, the repair machinery is negatively affected such that exogenous UV-lesioned DNA is repaired to a lesser extent than that in control cells (Supplementary Fig. S3). This tentative orthogonal assay to measure the repair of UV-induced DNA damage can be firmly considered as corroboration of all the results shown in this study.

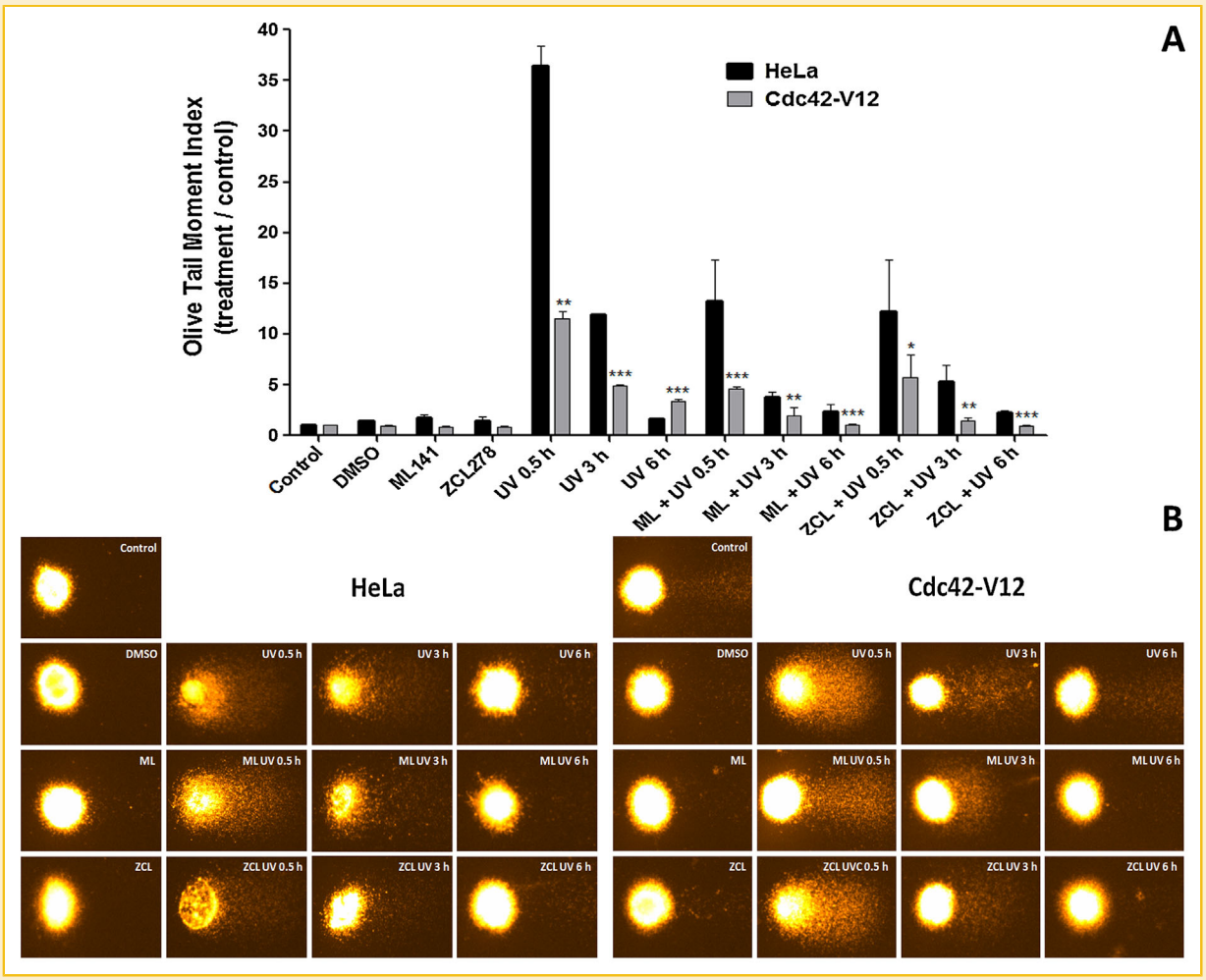


**Figure 5.** Alkaline comet assay results of UV-irradiated HeLa cells and clones in the presence or absence of CDC42 inhibitors (10 μM ML141 and 50 μM ZCL278). (A) Quantification of damaged and repaired DNA by the Olive tail moment (OTM), which is defined as the product of the tail length and the fraction of total DNA in the tail (OTM incorporates a measure of both the smallest detectable size of migrating DNA, which is reflected in the comet tail length, and the number of relaxed/broken pieces, which is represented by the intensity of the DNA in the tail). The graphs represent the average and standard deviation of three independent experiments, and statistical significance was assessed after comparing different treatments in HeLa cells versus clones using two-way ANOVA. $^{*}P < 0.01$, $^{**}P < 0.001$, and $^{***}P < 0.0001$. (B) Caption of representative cells with a single nucleus stained with ethidium bromide before and after 0.5, 3, and 6 h of UV radiation treatment, with or without previous treatment with CDC42 inhibitors.

## DISCUSSION

CDC42 is a Rho GTPase known to participate in a variety of cellular processes that are primarily related to the cytoskeleton, including adhesion, migration, and invasion [Nobes and Hall, 1995; Kaibuchi et al., 1999], and also participates in cell cycle progression and aging [Yeh et al., 2005; Yasuda et al., 2006; Wang et al., 2007; Stengel and Zheng, 2011]. In the first case, CDC42 functions through interactions with scaffold proteins such as WASP and Par6 and with certain protein kinases, including PAKs and MRCKs [Cerione, 2004]. In contrast, the effects of CDC42 on cell cycle progression and aging depend on unclear mechanisms involving cell cycle regulators such as p53, p16, p21, CDC42 GAPs/GEFs [Wang et al., 2007], and PKCζ [Yeh et al., 2005] as well as p38 MAPK [Molnar et al., 1997], which was shown to be activated by UV stress in a CDC42-dependent manner in cellular models of COS-1 and HaCat keratinocytes [Seo et al., 2004]. Additionally, CDC42 roles in mediating apoptosis were also described for colorectal epithelial cells [Deevi et al., 2011], thymocytes [Na et al., 1999], and others and are dependent on both the canonical PTEN/AKT/GSK3 pathway and the intrinsic apoptotic pathway [Deevi et al., 2011; Melendez et al., 2011].

The general involvement of CDC42 in tumorigenesis (cell cycle, proliferation, apoptosis, and senescence) and in aging has been attributed to the overactivation of this GTPase for different reasons, including increases in specific GEFs, decreases in specific GAPs, overexpression of CDC42, or a mutation in GTP binding/hydrolysis residues, such as G12V, which leads to CDC42 oversignaling [Gjoerup et al., 1998; Muris et al., 2002; Vanni et al., 2005; Wang et al., 2007; Chen et al., 2012; Florian et al., 2012]. Thus, our work, which was performed in HeLa cells, intended to examine whether CDC42 overactivation is also required for cell survival and proliferation under conditions of

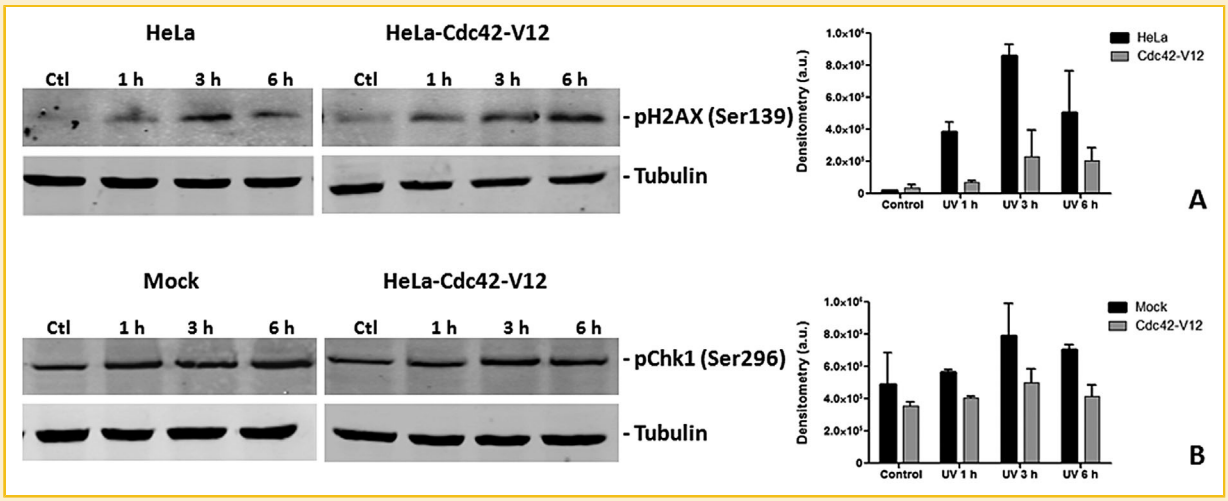


**Figure 6.** Histone H2AX and Chk1 phosphorylation are poorly induced by UV radiation in CDC42-V12 clones but not in control cells. HeLa, HeLa-Mock, and HeLa-CDC42-V12 cells were treated with UV radiation as previously described, and cellular lysates were obtained up to 6 h after irradiation. Western blots were performed using a rabbit monoclonal anti-phospho-H2AX-Ser139 (R&D Systems) antibody (A), a rabbit monoclonal anti-phospho-Chk1-Ser296 (Cell Signaling) antibody (B), or an anti-tubulin (Santa Cruz Biotechnologies) antibody, as a control; the blots were detected using Odyssey-Licor infrared scanning with specific Alexa 680- or Alexa 800-conjugated secondary antibodies (Licor). The immunoblots are representative of two independent and reproducible experiments (at least), which were performed with pairs of cells (HeLa and Cdc42-V12 clone) on the same gel; the quantified blots are shown as the average plus or minus the standard deviation.

UV-induced oxidative stress and to examine how DNA damage could be affected within this context.

Our results showed a direct correlation between G2/M phase cell cycle arrest (Fig. 4) and a reduced amount of DNA damage associated with possibly exaggerated and/or accelerated DNA strand break repair, as measured by alkaline comet assays (Fig. 5) in HeLa cell sublines ectopically expressing the CDC42-V12 mutant compared with empty-vector transfected (Mock) or parental HeLa cells. This behavior exhibited by the constitutively active CDC42 mutant after UV treatment may cause the cells to override the G1/S checkpoint, with a shortened G1 phase and accumulation during the G2/M transition; these findings suggest defects in this checkpoint as CDC42 activity increases, for example, with stimulation by UV radiation (Fig. 1C). Normal levels of CDC42 activity in HeLa cells and Mock clones indicate that their G2/M checkpoints are more effective than those in the CDC42-V12 mutant, in which incomplete repair and accumulated damage were observed after 6 h of UV treatment (Fig. 5). There are two possible explanations for the systematic low levels of DNA damage in CDC42-overactivation clones: exaggerated and accelerated DNA repair (which does not explain the accumulation of UV-promoted damage by 6 h) or failure of the DNA damage sensing and repair systems. In assessing these possibilities, our data for the phosphorylation status of the DDR proteins H2AX and Chk1 showed a delay in H2AX phosphorylation up to 6 h after damage and attenuated Chk1 phosphorylation compared with control cells exhibiting normal CDC42 activity (Fig. 6). These results indicated that the CDC42-V12 clonal cells are deficient in sensing DNA damage and triggering DNA repair. The results from clonogenic assays (Fig. 3) are consistent with inefficiency of the DNA repair mechanism, as the CDC42-V12 mutants displayed greater sensitivity to UV treatment than Mock and HeLa cells, with consequently reduced survival. Similar CDC42 activity-dependent effects were first shown in *S. pombe*, in which caffeine was used to stress the cells and to promote an uncontrolled G2-M checkpoint [Wang et al., 1999]; however, such effects were also found in several normal mammalian cells as a key mechanism inducing apoptosis or malignant transformation due to checkpoint defects [Bartek and Lukas, 2001]. To summarize the most relevant results from the comet and clonogenic assays (Figs. 3 and 5) in this study, previous CDC42 inhibition using pharmacological inhibitors was interestingly not sufficient to rescue the higher UV sensitivity associated with CDC42 overactivation. This was very likely because the inhibitors only efficiently act on endogenous, wild-type CDC42 and not on the ectopically expressed CDC42-V12 mutant protein.

Many plausible mechanisms could underlie the findings in this study: (i) it was reported in the literature that the disruption of CDC42GAP, a GTPase-activating protein, acts as a negative regulator of CDC42 [Wang et al., 2007]; and (ii) other forms of CDC42 activation [Muris et al., 2002; Vanni et al., 2005; Kerber et al., 2009; Florian et al., 2012] lead to premature senescence and aging in mice and humans via p53 activation, culminating in increased expression of p16 and p21 proteins, which are known senescence markers and act by inhibiting Cyclin/Cdk complexes and cell cycle progression. Regarding the p53 status in the HeLa cells used in this study, a correlation can be made between our CDC42 overactivation results and p53 function. This is because the HeLa cells used in the laboratory are not entirely p53 deficient: the cells still express this protein at relatively normal levels and are additionally still responsive to ionizing gamma radiation for serine 15 phosphorylation, which is a marker of p53 functionality (Osaki JH and Forti FL, unpublished results). Thus, all of these possibilities could underlie our results; that is, the overactivation of CDC42 leads to reduced DDR

and less repair, along with the accumulation of genomic defects, in turn activating p53 and culminating in cell cycle arrest followed by cellular senescence and/or cell death.

In contrast, our two-dimensional migration assays of the three studied cell lines were augmented by constitutively active CDC42-V12 cells, which migrated faster than the other two cell lines (Fig. 2) and presented a more stretched morphology with higher polarity (Fig. 1A and 1B). These results appear to be independent of UV-induced stress because this treatment did not affect these responses in a significant manner; hence, we consider these results insufficient to prove that UV does not regulate cytoskeleton remodeling and related biological functions. Nonetheless, p53 could again play a role because morphological alterations are relevant characteristics of the senescent phenotype, which also involves CDC42 activation, contributing to this morphology [Cho et al., 2004]. Additionally, in other reports, p53 was shown to regulate CDC42-dependent cellular actions, controlling actin cytoskeleton dynamics and cell movement by regulating filopodia formation, cell attachment, and spreading into matrix substrates, and finally preventing cell polarity during migration [Gadea et al., 2002].

In conclusion, in this study, we demonstrated that the over-activation of CDC42 by ectopically expressing the CDC42-V12 mutant in HeLa cells causes increased radiosensitivity to UV, with cells arresting in G2/M and showing reduced survival compared to cells expressing normal levels of the wild-type CDC42 GTPase. These results are a step toward a complete understanding of the signaling roles of CDC42 in maintaining genomic stability and integrity.

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