The NEDD8 conjugation pathway regulates p53 transcriptional activity and head and neck cancer cell sensitivity to ionizing radiation

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Abstract. Human papillomavirus (HPV)-related oropharyngeal cancer represents a distinct head and neck squamous cell carcinoma (HNSCC) subpopulation, with improved disease-free and overall survival. In general, HPV-positive HNSCCs express wild-type *TP53*, which could explain its increased radiosensitivity. However, the molecular mechanisms underlying this higher sensitivity remain elusive. We have previously shown that HPV-related oropharyngeal carcinomas express decreased levels of the *NEDD8-activating enzyme 1/amyloid β precursor protein-binding protein 1* (*NAE1/APP-BP1*) gene. NAE1/APP-BP1 function is required for the NEDDylation of target proteins, and has been shown to be a negative regulator of p53 transcriptional activity. In this study, we addressed the hypothesis that *NAE1/APP-BP1* expression levels regulate p53 activity and cell survival upon ionizing irradiation. We used the radiosensitive and naturally HPV16-infected UPCI:SCC90 cell line and the radioresistant and HPV-negative SQ20B cell line as the control. *NAE1/APP-BP1* expression levels were modulated with expression constructs and siRNAs. Radiosensitivity was evaluated with clonogenic survival assays. p53 transcriptional activity was measured with a luciferase assay. The overexpression of *NAE1/APP-BP1* in UPCI:SCC90 cells resulted in the increased NEDDylation of p53, inhibition of p53 activity and increased cell resistance to ionizing radiation. Conversely, the inhibition of *NAE1/APP-BP1* expression in SQ20B cells induced p53-dependent cell death after treatment with X-rays. Taken together, these results indicate that *NAE1/APP-BP1* and NEDDylation are invovled in modulating p53 activity and regulating its role in the response of cells to ionizing radiation. Our findings bring new insights in the molecular mechanisms underlying the increased radiosensitivity of HPV-related oropharyngeal tumors. This is of importance, as no reliable and robust predictive biomarkers for tumor response to radiotherapy are currently available. These results also have potential clinical significance, as drugs targeting NAE1/APP-BP1 have recently emerged as a novel therapeutic modality in cancer treatment.

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Introduction

The persistent infection of the head and neck epithelium by high-risk human papillomaviruses (HR-HPVs), mainly HPV type 16, predominantly in the region of the oropharynx, is associated with an increased risk of head and neck squamous cell carcinoma (HNSCC) (1). HR-HPVs define a distinct clinical subpopulation that displays improved disease-free and overall survival (reviewed in ref. 1 and references therein). These observations may be explained by the molecular mechanisms that underlie HPV-driven cell transformation. HR-HPV induces carcinogenesis by interfering with the p53 and pRb signaling pathways (1). The HPV E6 oncoprotein is known to induce p53 ubiquitination and subsequent degradation. The *TP53* gene is not mutated in the majority of HPV-related HNSCCs, and data from cell culture assays suggest that non-degraded p53 persisting in HPV-positive cells may be sufficient to induce the expression of p53 target genes and cell death upon treatment with ionizing radiation (2).

Genomic and transcriptomic analyses have shown that HR-HPV-positive HNSCC display specific chromosomal gains and losses, as well as changes in gene expression relative to their HPV-negative counterparts. HR-HPV-related tumors have fewer genomic aberrations, but display specific imbalances, such as a loss of the 16q region (3,4). Of note, this aberration correlates with improved local control (4). We have previously shown that the expression of the *NEDD8-activating enzyme 1/amyloid β precursor protein-binding protein 1* (*NAE1/APP-BP1*) gene, which is located on chromosome 16q22, is expressed at lower levels in HPV-positive oropharyngeal tumors (3). Together with the UBA3 protein, NAE1/APP-BP1 forms the NEDD8-activating enzyme, which has E1 ubiquitin-ligase activity, and initiates the NEDD8 conjugation pathway. In this complex, NAE1/APP-BP1 plays the role of a regulatory

subunit for NEDD8 E1 enzyme activity (5,6). Post-translational NEDD8 conjugation has mainly been described for members of the cullin family of proteins (7). Previously, a function of the NEDD8 conjugation pathway in the regulation of the p53 transcriptional activity has been unraveled: NAE1/APP-BP1 is required for the NEDD8 conjugation of p53 via MDM2, which results in the inhibition of p53 transcriptional activity without triggering its degradation (8).

In this study, we investigated the possibility that the expression of NAE1/APP-BP1 affects p53-dependent transcriptional activity and sensitivity to ionizing radiation in head and neck cancer cells. We used the naturally HPV16-infected oropharyngeal cell line, UPCI:SCC90, that expresses wild-type p53 (9), and the HPV-negative SQ20B cell line that expresses mutant p53 as the control. UPCI:SCC90 cells are more radiosensitive than SQ20B cells (10). We show that UPCI:SCC90 cells express lower levels of *NAE1/APP-BP1* mRNA, and that the overexpression of *NAE1/APP-BP1* in these cells induces radioresistance. Conversely, SQ20B cells become radiosensitive when wild-type *TP53* is expressed. The inhibition of *NAE1/APP-BP1* potentiates the effect of wild-type p53. These results point to the importance of NEDDylation in the radiosensitivity of tumor cells that express wild-type p53.

Materials and methods

Cell culture and transfections. The HNSCC SQ20B cell line was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). The radioresistant SQ20B cells have been reported to express non-funtional p53 protein (10). The hHNSCC UPCI:SCC90 cell line (9) was a kind gift from Professor Susan Gollin (University of Pittsburgh, Pittsburgh, PA, USA). UPC1:SCC90 cells originate from a HPV16-positive oropharyngeal tumor, and bear wild-type *TP53* (9). UPCI:SCC90 cells have been shown to be more radiosensitive than SQ20B cells (11). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; PAN Biotech GmbH, Dominique Dutscher, Brumath, France) according to standard conditions.

All transfection experiments were carried out using Lipofectamine™ 2000 (Invitrogen, Fisher Scientific, Illkirch, France) according to the manufacturer's instructions. UPCI:SCC90 cells ($5x10^5$) were transfected with 4 μg of the *NAE1/APP-BP1* expression vector (12) (kind gift from Dr Dimitris Xirodimas). Mock transfections with 4 μg of the empty pcDNA3 vector (Invitrogen) or the empty pCI-neo vector (Promega, Charbonnières-les-bains, France) were used as the negative controls. p53 was overexpressed by transfection of $5x10^5$ SQ20B cells with 4 μg of pC53-C1N3 plasmid.

siRNA experiments. The *NAE1/APP-BP1* downregulation experiments were performed using the ON-TARGET plus SMARTpool (L-006401-00-0005; Dharmacon, Fisher Scientific, Illkirch, France). This pool is composed by the combination of 4 single siRNAs: siRNA-1, upgrade siRNA *NAE1/APP-BP1* J-006401-05; siRNA-2, upgrade siRNA *NAE1/APP-BP1* J-006401-06; siRNA-3, upgrade siRNA *NAE1/APP-BP1* J-006401-07; and siRNA-4, upgrade siRNA *NAE1/APP-BP1* J-006401-08. The ON-TARGET plus non-targeting pool (D-001810-10-05) was used as the negative control. siRNAs (40 pmol) were transfected into $4x10^4$ SQ20B cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions.

RNA extraction and quantitative real-time RT-PCR (qRT-PCR). RNA was extracted from $5x10^5$ cells and cDNA synthesis was performed as previously described (3). Cycle threshold (Ct) levels were normalized to the average Ct values of 3 internal controls (housekeeping genes): ubiquitin B (UBB), 18S rRNA and hypoxanthine phospho-ribosyltransferase 1 (HPRT1). Primers specific for the genes of interest were used: *HR-HPV16 E6/E7* mRNA forward, 5'-GTTACTGCGACGTGAGGTA TATG-3' and reverse, 5'-CATTTATCACATACAGCATATGG ATTC-3' (13); *NAE1/APP-BP1* forward, 5'-CTGGAAAGCT GCTCAAGG-3' and reverse, 5'-TCTTCTCCGCTGACCT GATT-3'; *CDKN2A*^{p16} forward, 5'-GCTGCCCAACGCACCGA ATA-3' and reverse, 5'-ACCACCAGCGTGTCCAGGAA-3'; *WAF1/CIP1*^{p21} forward, 5'-ATGAAATTCACCCCTTTCC-3' and reverse, 5'-CCCTAGCTGTGCTCACTTC-3'; *PUMA* forward, 5'-ACGACCTCAACGCACAGTACGA-3' (14) and reverse, 5'-GTAAGGGCAGGAGTCCCATGATGA-3' (14); *MDM2* forward, 5'-GGTGGGAGTGATCAAAAGGA-3' and reverse, 5'-GTGGCGTTTTCTTTGTCGTT-3'; *HPRT1* forward, 5'-TGCTCGAGATGTGATGAAGG-3' and reverse, 5'-GT CCCCTGTTGACTGGTCATT-3'; *UBB* forward, 5'-GCTTTG TTGGGTGAGCTTGT-3' and reverse, 5'-CGAAGATCTGCA TTTTGACCT-3'; *18S rRNA* forward, 5'-TGTGGTGTTGA GGAAAGCAG-3' and reverse, 5'-TCCAGACCATTGGCT AGGAC-3'. Gene expression levels observed in the non-irradiated mock-transfected cells were artificially set to 100, and other expression values were normalized with respect to this level. Differences were considered statistically significant when $p<0.05$ (ANOVA).

Irradiation procedures, clonogenic survival assay and surviving fraction at 2 Gy (SF2) calculation. Cells in the exponential growth phase were detached, counted and plated in 6-well plates in 2 ml of growth medium 24 to 48 h prior to irradiation, in order to allow them to attach and undergo transfection. X-ray irradiation was performed with 6 MV photons at the Department for Radiation Therapy of the Paul Strauss Cancer Centre (Strasbourg, France). Clonogenic assays were performed as previously described (15). In brief, cells were collected 24 h after irradiation, diluted, seeded and allowed to grow for up to 3 to 4 weeks after irradiation. Clones were stained with 0.05% crystal violet (Sigma-Aldrich, Lyon, France) in a 5% ethanol solution, and positive colonies (>64 cells) were counted. The SF2 was calculated by dividing the number of positive colonies by the number of cells that were seeded, multiplied by the plating efficiency. The plating efficiency was calculated by dividing the number of positive colonies that grew in the absence of irradiation by the number of cells that were seeded. Differences were considered statistically significant when $p<0.05$ (ANOVA).

Western blot analyses. Cells ($4x10^4$ to $5x10^6$) were harvested in lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-Cl pH 8.0, 0.5% DOC, 0.1% SDS) and proteins were analyzed by SDS-PAGE according to standard methods. Proteins were detected with the following antibody dilutions: polyclonal mouse AO1 anti-

APP-BP1 (1:1000; Abnova, Tebu-Bio, Le-Perray-en-Yvelines, France), monoclonal mouse DO1 anti-p53 (1:1000; Santa Cruz Biotechnologies, CliniSciences, Montrouge, France), polyclonal rabbit anti-p53 FL-393 (1:200; Santa Cruz Biotechnologies), anti-NEDD8 rabbit polyclonal (1:2000; Alexis Biochemicals, Enzo Life Sciences, Villeurbanne, France), mouse monoclonal CM1 anti-β actin (1:1000; Abnova), anti-β tubulin (1:5000; Sigma-Aldrich), enhanced chemiluminescence (ECL) sheep anti-mouse IgG, HRP-conjugated (1:10000; GE Healthcare, Saclay, France), and anti-rabbit IgG, HRP-linked antibodies (1:20000; Cell Signaling, Ozyme, Saint-Quentin-en-Yveline, France). Proteins were revealed with the ECL western blot analysis system (GE Healthcare) according to the manufacturer's instructions. Quantification of the signals was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (16,17).

p53 transcriptional activity assay. With the use of the Lipofectamine™ 1000 transfection system, $5x10^5$ UPCI:SCC90 cells were co-transfected with 0.75 μg of PG13-Luc plasmid (kind gift from Dr Arnold Levine) or MG15-Luc plasmid, 0.75 μg of RSV-LacZ vector [used as a transfection efficiency control (18)], and increasing amounts (0, 1.0, 1.5, 2.5 μg) of either the *NAE1/APP-BP1* expression vector or of the empty vector (pcDNA3). The total amount of transfected plasmid was made up to 4 μg with pUC19 (New England Biolabs). Cells were harvested 36 h after transfection in lysis buffer (25 mM Tris-phosphate, pH 7.8; 2 mM EDTA; 1 mM DTT; 10% glycerol; 1% Triton X-100), and luciferase activity was evaluated by mixing 50 μl of protein extract with luciferase assay buffer (265 μM ATP; 235 μM luciferin; 135 μM coenzyme A; 20 mM tricine; 1.07 mM $MgCl_2$; 2.70 mM $MgSO_4$; 0.10 mM EDTA; 33.3 mM DTT) using a MikroWin 2000 microplate reader (Mikrotek Laboratories GmbH, Overath, Germany). The luminescence levels were normalized to the β-galactosidase activity from the RSV-LacZ control plasmid that was measured with an *o*-nitrophenyl-β-galactoside (ONPG) colorimetric reaction. In brief, β-galatcosidase activity was evaluated by mixing 25 μl of cell extract with 150 μl of ONPG staining solution (0.666 mg/ml ONPG; 2% β-mercaptoethanol; 60 mM Na_2HPO_4; 40 mM NaH_2PO_4; 10 mM KCl; 1 mM $MgSO_4$), and *o*-nitrophenol was monitored on a Berthold 900 spectrophotometer using Gen5 Data analysis software. Fluorescence observed in cells transfected with 0 μg of *NAE1/APP-BP1* expression vector was set at a value of 100, and other measures were normalized with respect to that point. Differences were considered significant when $p<0.05$ (Student-Newman-Keuls test for pairwise comparisons).

Immunoprecipitations. Cells ($1.5x10^6$) were harvested in denaturing lysis buffer (1% SDS, 5 mM EDTA, 10 mM DTT, 15 U/ml DNase, PMSF). A total of 5 μl of each cell lysate was diluted in 45 μl of non-denaturing buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, PMSF). Diluted cells lysates were pre-incubated with 0.4 μg of anti-p53 antibody (DO1 anti-p53; Santa Cruz Biotechnologies) or with 2 μl of anti-NEDD8 antibody (Alexis Biochemicals). A total of 10 μl of Protein A/G PLUS-Agarose Immunoprecipitation Reagent (sc-2003; Santa Cruz Biotechnologies) was further mixed with extracted protein, pelleted and resuspended in loading buffer and subjected to western blot analysis with an anti-NEDD8 antibody and a polyclonal rabbit anti-p53 FL-393 antibody (Santa Cruz Biotechnologies). Normal mouse IgG-AC (10 μl) (sc-2343; Santa Cruz Biotechnologies) was incubated with protein lysate as the negative control.

Apoptosis detection by Annexin V staining. UPCI:SCC90 cells were transfected and irradiated as described above. Annexin V staining was monitored by flow cytometry on an Agilent Bioanalyzer 2100 (Agilent Technologies Deutschland, Waldbronn, Germany), according to the manufacturer's instructions. In brief, cells were incubated first with Annexin V-Biotin (Annexin V-Biotin apoptosis detection kit, Oncogene Research Product, Merck Chemicals, Nottingham, UK), and later with streptavidin-Cy5 (Amersham, GE Healthcare). In order to discriminate early apoptotic cells from dead cells, samples were counterstained with Calcein-AM (Molecular Probes, Invitrogen, Cergy Pontoise, France). Cell samples were loaded on Cell Fluorescence LabChip® kits, and flow cytrometry data were analyzed with Agilent Cell Fluorescence software. Data were analyzed with one-way ANOVA, together with a Student-Newman-Keuls test of all pairwise possible comparisons, and differences were considered significant when $p<0.05$.

Results

NAE1/APP-BP1 gene and protein expression are lower in the UPCI:SCC90 cells than in SQ20B cells. In order to evaluate the involvement of *NAE1/APP-BP1* gene expression levels in HR-HPV-positive oropharyngeal squamous cell carcinoma radiosensitivity, we selected 2 different cell lines: the HPV-negative SQ20B cell line that originates from a laryngeal tumor, harboring a *TP53* mutant allele and that is radioresistant (10); and the HR-HPV16-positive UPCI:SCC90 cell line that was established from an oropharyngeal squamous cell carcinoma, that bears a wild-type *TP53* locus and is radiosensitive as compared to the SQ20B cell line (11). Using a qRT-PCR approach with 3 independent total RNA extracts from the 2 cell lines, we confirmed that the mRNA that encodes the HPV16 E6 and E7 oncoproteins is expressed in UPCI:SCC90 cells, whereas it was not detectable in RNA extracts from SQ20B cells (Fig. 1A). As expected, the expression of the *CDKN2A*^{p16} gene, which is used as a surrogate marker of HPV infection, was found to be dramatically increased in the UPCI:SCC90 cells as compared to the SQ20B cells (Fig. 1A). These results confirm that HPV16 is biologically active in UPCI:SCC90 cells. We measured the expression of *WAF1/CIP1*^{p21}, whose expression is regulated by p53. In agreement with their respective *TP53* statuses, the expression of *WAF1/CIP1*^{p21} was found to be higher in the UPCI:SCC90 cells as compared to the SQ20B cells (Fig. 1A). Finally, the expression of the *NAE1/APP-BP1* gene was found to be lower in the UPCI:SCC90 cells than in the SQ20B cells. We further evaluated the expression levels of the NEA1/APP-BP1 protein in these 2 cells lines by western blot analysis. Blots were probed with an anti-NEA1/APP-BP1 antibody, and quantification of the signals obtained from total protein extracts confirmed that the NEA1/APP-BP1 protein displays higher expression levels in SQ20B cells than in UPCI:SSC90 cells (Fig. 1B). Thus, these 2 cell lines display NAE1/APP-BP1 expression patterns that are similar to those reported in HR-HPV-positive and HPV-negative HNSCC. They

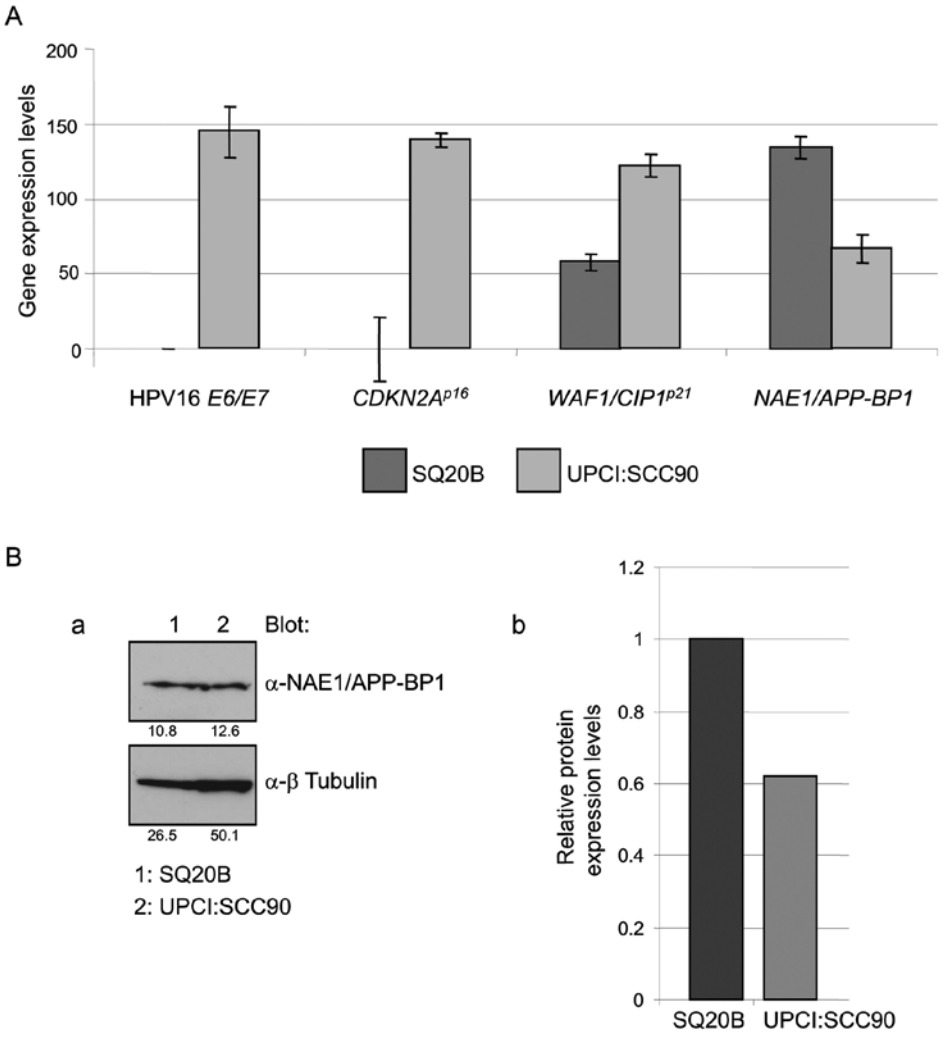

Figure 1. qRT-PCR analysis of gene expression levels in the UPCI:SCC90 and SQ20B cell lines. (A) The expression levels of HPV16 *E6/E7*, *CDKN2A*^{p16}, *WAF1/CIP1*^{p21} and *NAE1/APP-BP1* mRNA were measured in UPCI:SCC90 and SQ20B cell extracts using specific primers and a SYBR-Green-based qRT-PCR approach. Mean expression levels and standard deviations were calculated with the data from 3 independent experiments. Results are presented in arbitrary units. (B-a) NEA1/APP-BP1 protein levels were evaluated by western blot analyses of UPCI:SCC90 and SQ20B cell extracts (upper panel). Protein loads were evaluated by probing the membrane with an anti-β tubulin antibody (lower panel). Numbers under the gel refer to the quantification of the signal. (B-b) Protein expression levels in SQ20B and UPCI:SCC90 cells as calculated with the normalization to the loading control. Value observed in SQ20B cells has been set to 1.

were used to evaluate the influence of the *NAE1/APP-BP1* gene on their response to ionizing radiation.

Forced expression of NAE1/APP-BP1 enhances resistance to ionizing radiation. We examined the effect of modulating *NAE1/APP-BP1* expression levels in UPCI:SCC90 cells on clonogenic survival. *NAE1/APP-BP1* was overexpressed by the transfection of an expression vector. Transfection with the *NAE1/APP-BP1* construct resulted in an increase in *NAE1/APP-BP1* mRNA levels compared to the mock-transfected cells, as measured by qRT-PCR in 3 independent extracts of cells harvested 24 h after transfection (Fig. 2A). A western blot analysis of protein extracts from the same cells, using an anti-NAE1/APP-BP1 antibody, showed that NAE1/APP-BP1 protein levels also increased (Fig. 2B). In a clonogenic survival assay, the cells were irradiated with a single 2-Gy dose of X-rays, and the number of cells that were able to grow colonies was measured. Non-irradiated cells were used as the control to calculate the fraction of cells that survived the treatment. The SF2 of mock-transfected cells and that of cells that over-expressed *NAE1/APP-BP1*, calculated from 6 independent measures, was 0.36 and 0.89, respectively (Table I; $p<0.001$), showing that *NAE1/APP-BP1* overexpression augments resistance to ionizing radiation in UPCI:SCC90 cells.

Downregulation of NAE1/APP-BP1 increases radiosentivity in a p53-dependent manner. We reasoned that if *NAE1/APP-BP1* is involved in cell survival after irradiation, then the response of SQ20B cells to ionizing radiation may be modulated by both restoring functional *TP53* and downregulating *NAE1/APP-BP1* expression. Consequently, SQ20B cells were co-transfected with a wild-type *TP53* expression vector and siRNAs that specifically target *NAE1/APP-BP1*. A non-related siRNA was used as the negative control. SQ20B cells bear a point mutation in the *TP53* gene that results in the production of a non-functional p53 protein, which can be detected by western

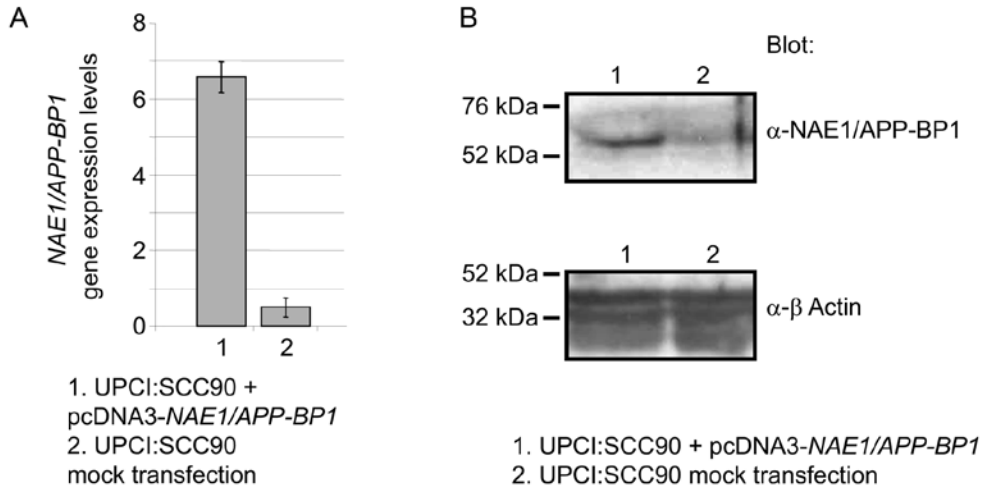

Figure 2. Overexpression of *NAE1/APP-BP1* in UPCI:SCC90 cells. (A) UPCI:SCC90 cells were transfected with a *NAE1/APP-BP1* expression construct or with an empty vector as the negative control. The expression levels of the *NAE1/APP-BP1* gene were measured by qRT-PCR in 3 independent RNA extracts from transfected UPCI:SCC90 cells. Mean expression levels and standard deviations were calculated with the data from 3 independent experiments. Results are presented in arbitrary units. (B) Western blot analysis of the expression of the NAE1/APP-BP1 protein (60 kDa) in extracts of UPCI:SCC90 cells that were transfected with either the *NAE1/APP-BP1* expression construct (lane 1; upper panel) or with the empty vector as a negative control (lane 2; upper panel). Protein loads were evaluated by probing the membrane with an anti-β actin antibody (lanes 1 and 2; lower panel).

Table I. Effects of *NAE1/APP-BP1* and *TP53* expression on the fraction of UPCI:SCC90 and SQ20B cells that survived after irradiation with 2 Gy X-Rays (SF2).

Cell transfection	SF2 Gy	p-value ANOVA
UPCI:SCC90 cells		
Mock transfection	0.36±0.16	<0.001
pcDN3-*NAE1/APP-BP1*	0.89±0.07	
SQ20B cells		
Single transfection		
Mock transfection	0.46±0.01	
Control siRNA	0.64±0.10	NS
NAE1/APP-BP1 siRNA	0.53±0.12	NS
pC53-C1N$_3$	0.35±0.02	0.030
Double transfection		
pC53-C1N$_3$ + control siRNA	0.46±0.14	
pC53-C1N$_3$ + *NAE1/APP-BP1* siRNA	0.26±0.04	0.045

UPCI:SCC90 cells were transfected with the *NAE1/APP-BP1* expression construct or with the empty vector as the negative control (mock transfection). SQ20B cells, used as a radioresistant control cell line, were single-transfected with the following constructs: empty vector (mock transfection), *NAE1/APP-BP1* unrelated control siRNA, anti-*NAE-1/APP-BP1* siRNA, wild-type p53 expression construct. SQ20B cells were doubly-transfected with the wild type p53 expression construct and either the *NAE1/APP-BP1* unrelated control siRNA or the anti-*NAE-1/APP-BP1* siRNA. The cells were irradiated with 2 Gy of X-Rays, and SF2s and standard deviations were calculated with the data from 3 independent experiments, with 6 independent measurements per experiment. Differences were considered as significant when $p<0.05$. NS, not significant.

blot analysis (Fig. 3A, lanes 1 and 3). The transfection of SQ20B cells with an expression vector for wild-type *TP53* further increased p53 levels. Non-irradiated and irradiated transfected cells displayed increased amounts of p53 as compared to the mock-transfected cells (Fig. 3A, lanes 2 and 4). The SF2 of mock-transfected SQ20B cells and that of SQ20B cells that overexpressed p53, was 0.46 and 0.35, respectively (Table I; p=0.030), indicating that exogenous p53 function is functional in SQ20B cells. The expression of the NAE1/APP-BP1 protein was downregulated up to 48 and 72 h after transfecting SQ20B cells with an anti-NAE1/APP-BP1 siRNA pool (Fig. 3B, lane 7). This pool is composed of 4 individual siRNAs, which

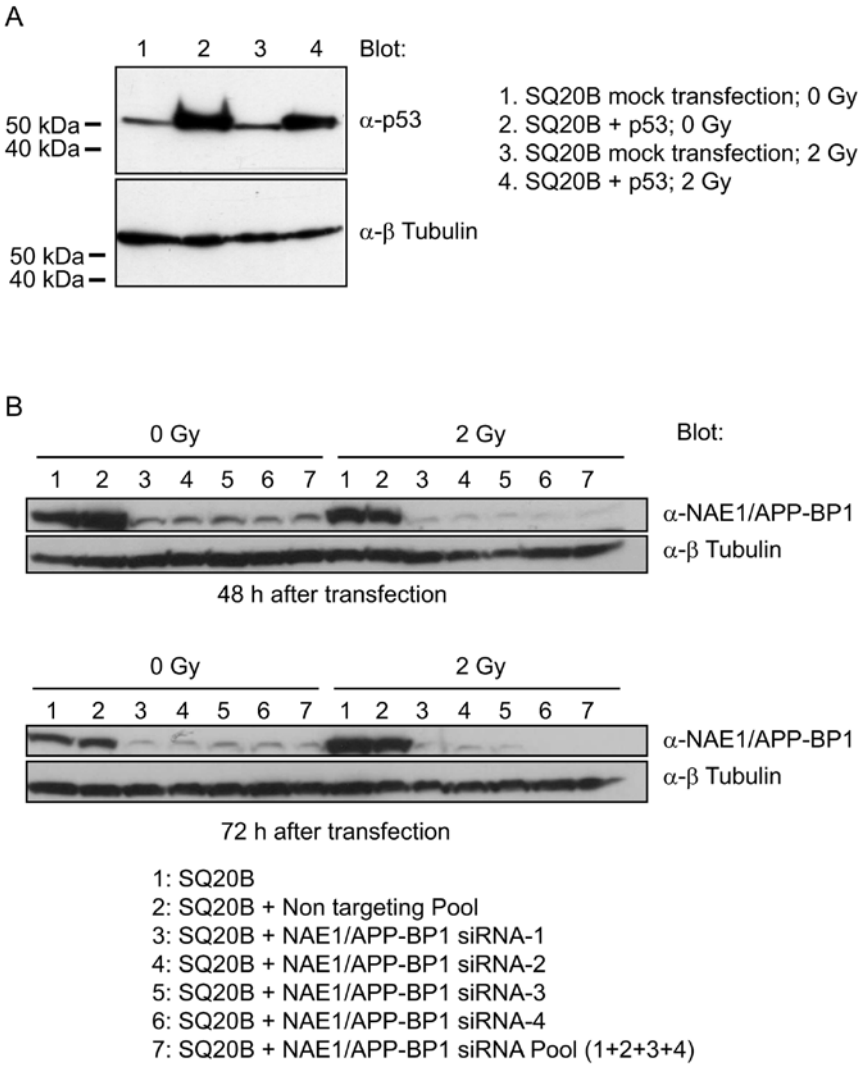

Figure 3. Western blot analysis of p53 and NAE1/APP-BP1 protein expression in SQ20B cells. (A) SQ20B cells were transfected with the p53 expression construct (lanes 2 and 4) or with the empty vector as the negative control (lanes 1 and 3). Cells were either non-irradiated (lanes 1 and 2) or irradiated with 2 Gy X-rays (lanes 3 and 4). p53 protein (53 kDa) levels were evaluated by western blot analysis of SQ20B cells extracts (upper panel). The membranes were probed with an anti-β tubulin antibody to control for loading (lower panel). (B) SQ20B cells were not transfected (negative control, lane 1), or transfected with an anti-*NAE1/APP-BP1* siRNA pool (lane 7), with the deconvoluted siRNA pool (lane 3, anti-NAE1/APP-BP1 siRNA-1; lane 4, siRNA-2; lane 5, siRNA-3; lane 6, siRNA-4; or lane 2, with an unrelated control siRNA. Cells were irradiated with a 2 Gy X-ray dose, or non-irradiated as the control. Cells were harvested 48 and 76 h after transfection. NAE1/APP-BP1 protein levels were detected by western blot analysis of SQ20B cells extracts (upper panel). Loading was controlled by probing the membrane with an anti-β actin antibody (lower panel).

all were found to efficiently downregulate the expression of the *NAE1/APP-BP1* gene (Fig. 3A, lanes 3-6). Transfection with a non-related siRNA, used as the negative control, had no effect on NAE1/APP-BP1 protein levels (Fig. 3B, lane 2). Neither the anti-*NAE1/APP-BP1* siRNA, nor the control siRNA increased cell sensitivity to ionizing radiation. The SF2 in these conditions was 0.53 and 0.64, respectively (Table I). Strikingly, the irradiation of SQ20B cells that were co-transfected with wild-type *TP53* and anti-*NAE1/APP-BP1* siRNA resulted in a drop in the SF2 to 0.26, whereas cells that were co-transfected with *TP53* and the control siRNA displayed a SF2 of 0.46 (Table I; p=0.045). Taken together, these observations suggest that the inhibition of *NAE1/APP-BP1* in SQ20B cells restores cell sensitivity to ionizing radiation in a p53-dependent manner.

Overexpression of NAE1/APP-BP1 in UPCI:SCC90 cells inhibits p53 transcriptional activity and impairs apoptosis. In order to examine whether the modulation of *NAE1/APP-BP1* expression levels in UPCI:SCC90 cells has an effect on p53 transcriptional activity, we used the pG13-Luc reporter, in which the gene that encodes the luciferase protein is under the control of p53-dependent cis-regulatory elements. UPCI:SCC90 cells were co-transfected with equal amounts of pG13-Luc and increasing amounts of either the *NAE1/APP-BP1* expression vector, or the empty control vector. Cells were harvested 36 h after transfection and luciferase activity was measured with 3 independent protein extracts. There was no change in p53 transcriptional activity in the cells that were mock-transfected with various amounts of control vector (Fig. 4A, left histograms). By contrast, p53 transcriptional activity was markedly and significantly repressed in UPCI:SCC90 cells transfected with increasing amounts of the *NAE1/APP-BP1* vector, in a dose-dependent manner (Fig. 4A; 1.5 and 2.5 μg of *NAE1/APP-BP1* vector; $p<0.05$). In the control experiment, we used

the MG15-Luc reporter plasmid, which contains mutated p53-binding elements upstream of the luciferase gene. The fluorescence that we observed was markedly reduced, and no differences were observed between the cells transfected with either the empty plasmid or the NAE1/APP-BP1 expression construct (Fig. 4A, right histograms). The UPCI:SCC90 mock-transfected cells displayed rapid and significant ($p<0.05$) induction of p53 target genes, such as *WAF1/CIP1*^{p21}, *PUMA* and *MDM2*, 30 min after a 2-Gy irradiation (Fig. 4B). This induction was not observed in UPCI:SCC90 cells that overexpressed *NAE1-APP-BP1* (Fig. 4B). In order to evaluate whether this modulation of the p53 dependent transcriptional activity depends on a NAE1/APP-BP1-dependent increase in NEDDylation, we performed a western blot analysis to detect NEDDylated p53 in proteins extracted under denaturating conditions. NEDD8-conjugated proteins were immunoprecipitated with a p53 antibody, and the blots were probed with an anti-NEDD8 antibody (Fig. 4C-a). Conversely, NEDD8 was immunoprecipitated from the protein extracts, and the blots were then probed with an anti-p53 antibody (Fig. 4C-b). NEDDylated proteins were present in similar amounts in the extracts from the mock-transfected cells and from the cells transfected with NAE1/APP-BP1 (Fig. 4C-c). In both cases, we observed a band whose size corresponded to NEDDylated p53 (approximately 63 kDa; Fig. 4C-a and -b; asterisk). This signal was stronger in the UPCI:SCC90 cells that overexpressed NAE1/APP-BP1 than in the mock-transfected UPCI:SCC90 cells (Fig. 4C-a and -b). No detectable signal corresponding to a NEDDylated p53 protein was observed when transfected UPCI:SCC90 cell lysates were immunoprecipitated with an unrelated IgG (Fig. 4C-a). These results indicate that the overexpression of *NAE1/APP-BP1* in UPCI:SCC90 cells results in an increased NEDDylation of p53. These observations are consistent with the decreased transcriptional activity of p53 (Fig. 4A and B).

We evaluated whether the modulation of radiosensitivity by *NAE1/APP-BP1* expression levels and p53 activity is associated with changes in apoptosis in the control and transfected UPCI:SCC90 cells, prior to and 6 h following a 4-Gy irradiation. A flow cytometry approach was used in which living and dead cells were discriminated by staining with a calcein probe, and apoptotic cells by Annexin V. Calcein-positive and -negative cells were cross-gated to cells that displayed high levels of Annexin V ($>10^2$; Fig. 5A), and the proportion of each cell category was counted. The graph represents the statistical analysis of 3 independent experiments (Fig. 5B). The proportion of calcein-negative Annexin V-positive cells was found to be decreased when *NAE1/APP-BP1* was overexpressed in irradiated UPCI:SCC90 cells as compared to irradiated mock-transfected cells ($p<0.05$). Taken together, these observations suggest that *NAE1/APP-BP1* regulates cell sensitivity to ionizing radiation by modulating apoptosis.

Discussion

A hallmark of patients bearing HR-HPV-positive HNSCCs is improved disease-free and overall survival as compared to their HPV-negative counterparts (1), which may be due to increased tumor sensitivity to chemo- and radiation-therapy (19). We have previously described the HPV-positive tumor-specific loss of genetic material in the 16q region (3), which has been reported to be linked to improved local control (4). We have shown that the gene that encodes NAE1/APP-BP1, and which is located on chromosome 16q22, is expressed at lower levels in HPV-positive lesions (3). In the present study, we addressed the role of *NAE1/APP-BP1* in the regulation of the cellular response to ionizing radiation in HPV-positive and -negative cell lines. NAE1/APP-BP1 is a negative regulator of p53 transcriptional activity. The majority of HR-HPV-related oropharyngeal carcinomas express wild-type *TP53* (1) and have a decreased expression of *NAE1/APP-BP1* (3). Thus, we reasoned that the loss of NAE1/APP-BP1 may be responsible for the increased p53 activity and subsequent tumor cell death upon genotoxic stress.

Similar to the data that has previously been presented for HPV-positive oropharyngeal tumors, in the present study, we show that the expression of *NAE1/APP-BP1* is downregulated in radiosensitive UPCI:SCC90 cells (11) as compared to SQ20B radioresistant cells (10). The radiosensitive phenotype of UPCI:SCC90 cells was rescued by the overexpression of *NAE1/APP-BP1*, and conversely, the radioresistance of SQ20B cells was inhibited by the decreasing *NAE1/APP-BP1* expression. This effect appears to be p53-dependent.

The NEDDylation of p53 in other cancer cell lines has been reported. FBXO11, a component of the SCF E3 ubiquitin-ligase complex, has NEDD8-ligase activity. It physically interacts with p53 in the human lung carcinoma H1299 cell line, triggers p53 NEDDylation and inhibits its transcriptional activity (20). *NAE1/APP-BP1* expression levels correlate with cell death in *Drosophila melanogaster*. Clones of homozygous *dAPP-BP1* mutant cells, generated in heterozygous surrounding wild-type tissue, have an unusual small size due to a reduction in the number of cells. This phenotype is due to a dramatic increase of apoptosis in mutant cells (21).

The NEDD8 conjugation pathway has been implicated in the regulation of the activity of the SCF ubiquitin-ligase complex (7,22). Of note, a link between the activity of the SCF complex and sensitivity to ionizing radiation has recently been established (23). The sensitive to apoptosis gene (SAG) protein is a component of the SCF E3 ubiquitine ligase that is overexpressed in certain human cancers. The silencing of SAG in cancer cell lines impairs cell growth and colony formation, and results in increased sensitivity to radiation. Of note, the pro-apoptotic protein, NOXA, has been found to be more abundant in cells in which *SAG* expression is downregulated, and apoptosis is increased, suggesting that SAG is required for the degradation of NOXA. In our study, we show that the NEDD8 conjugation pathway has an influence on p53-dependent radiation-induced apoptosis. However, we cannot rule out the possibility that the effects observed are also partially linked to effects of the SCF complex on the turn-over of anti- or pro-anti-apoptotic factors and cell cycle regulators (22). In addition, it has previously been shown that TAp73 transcriptional activity is inhibited by NEDDylation in the ts41 or wild-type CHO cells (24). Therefore, we cannot exclude that the inhibition of TAp73 in transfected UPCI:SCC90 cells partially accounts for the effect of NAE1/APP-BP1 on luciferase and p53 target gene expression.

The radiosensitivity of cancer cells varies during the cell cycle (25,26). However, we did not observe changes in the G1

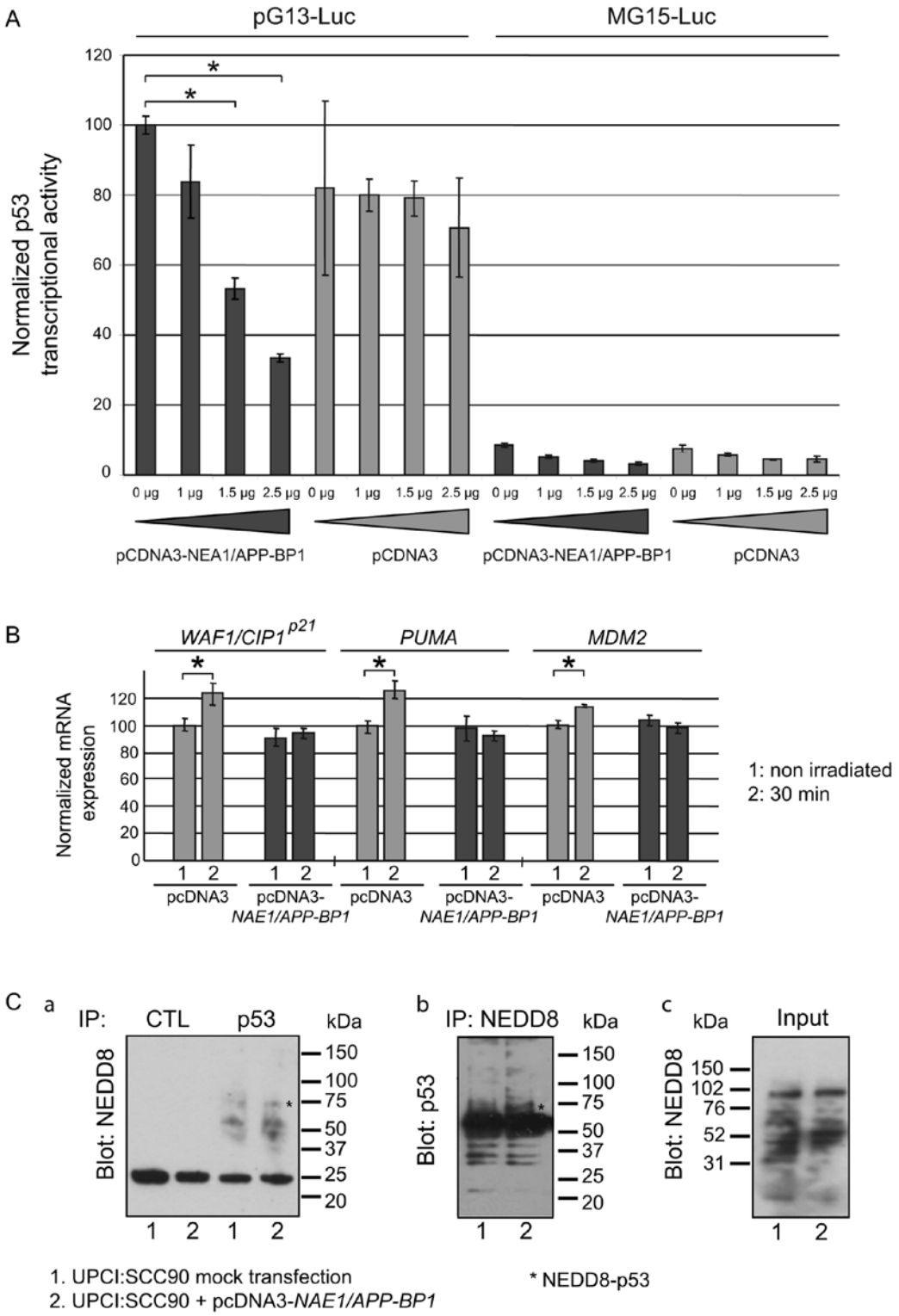

Figure 4. Overexpression of *NAE1/APP-BP1* in UPCI:SCC90 cells inhibits p53 transcriptional activity. (A) UPCI:SCC90 cells were transfected with the pG13-Luc reporter vector and increasing amounts (0, 1, 1.5 and 2 μg) of either the *NAE1/APP-BP1* expression construct (dark grey bars) or with the empty expression construct as the negative control (light grey bars). p53 transcriptional activity was measured using luciferase assays in 3 independent samples. Mean p53 transcriptional activity and standard deviations were calculated for each sample. Results were normalized with respect to the non-transfected UPCI:SCC90 sample, whose value was set to 100. Similar experiments were performed by using a MG15-Luc reporter gene as the negative control. $^{*}p<0.05$ indicates statistically significant differences. (B) The expression levels of the *WAF1/CIP1*^{p21}, *PUMA* and *MDM2* genes were measured by qRT-PCR in UPCI:SCC90 cells transfected with either the empty pCDNA3 vector (light grey bars) or the *NAE1/APP-BP1* expression construct (dark grey bars), in 3 independent experiments. Cells were harvested prior to (1) and 30 min (2) after a 2 Gy irradiation. Mean gene expression and standard deviations were calculated for each sample. Results were normalized with respect to the mock-transfected UPCI:SCC90 sample, whose value was set to 100. $^{*}p<0.05$ indicates statistically significant differences. (C) *In vivo* NEDDylation levels of p53 were evaluated in UPCI:SCC90 cells transfected with either the pCDNA3 empty vector (lane 1) and the *NAE1/APP-BP1* expression construct (lane 2). (a) Protein extracts were immunoprecipitated with either a control IgG (left lanes), or an anti-NEDD8 antibody (right lanes). Purified proteins were resolved by SDS-PAGE and membranes were probed with an anti-p53 antibody. *A band corresponding to NEDD8-p53 (63 kDa) is observed. (b) Protein extracts were immunoprecipitated with an anti-p53 antibody and membranes were probed with an anti-NEDD8 antibody. *A band corresponding to NEDD8-p53 (63 kDa) is observed. (c) The protein input was evaluated by resolving protein extracts by SDS-PAGE and membranes were probed with an anti-NEDD8 antibody.

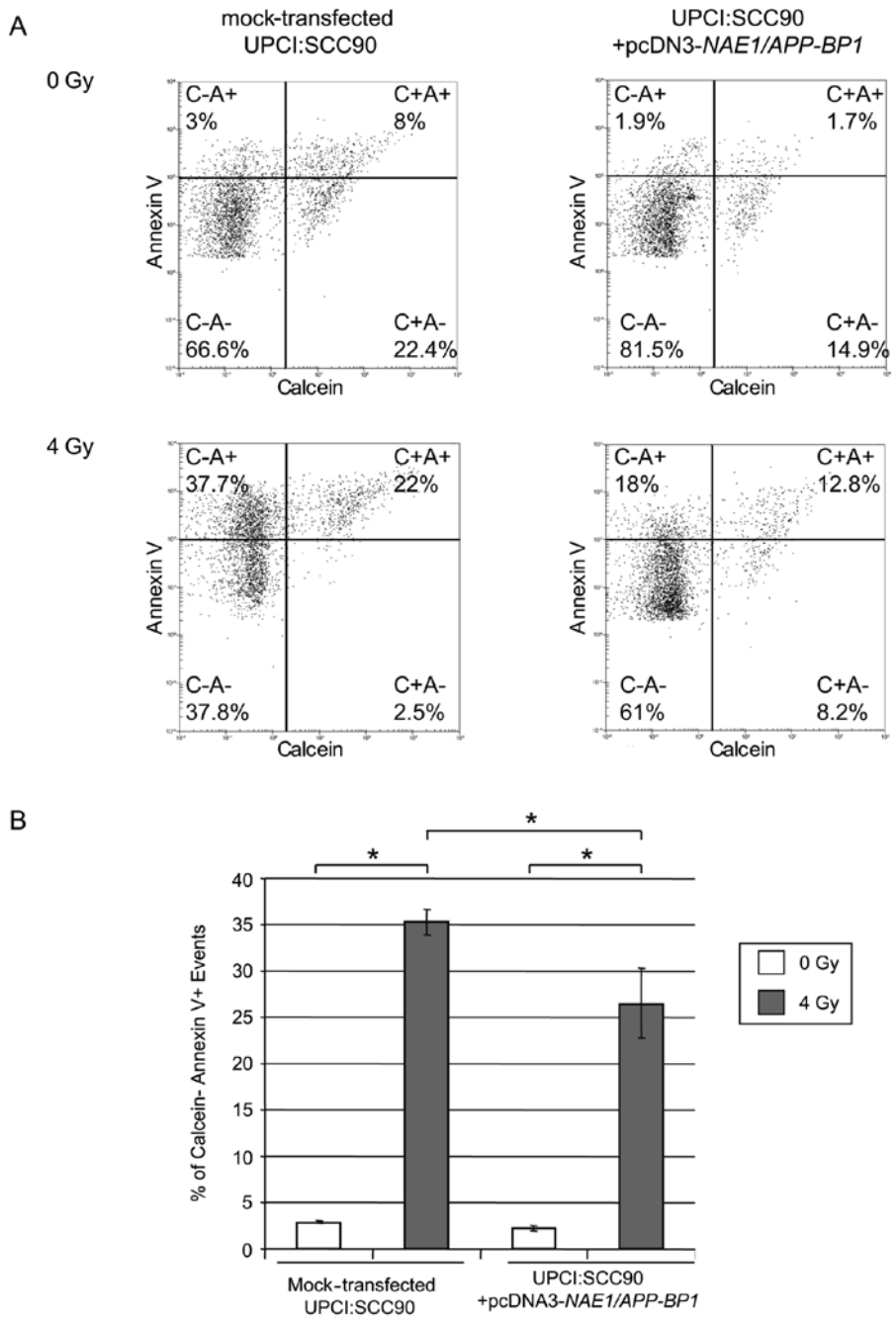

Figure 5. Apoptosis detection by Annexin V staining of transfected and irradiated UPCI:SCC90 cells. (A) Mock-transfected UPCI:SCC90 cells (left panels) and UPCI:SCC90 cells transfected with the *NAE1/APP-BP1* expression construct (right panels) were stained with Annexin V and calcein probes prior to (top panels) and 6 h after a 4 Gy irradiation (bottom panels). The percentage of each cell categories in one representative experiment is shown (C-A-, calcein-negative Annexin V-negative; C+A-, calcein-positive Annexin V-negative; C-A+, calcein-negative Annexin V-positive; C+A+, calcein-positive Annexin V-positive). (B) Statistical analysis and representation of the calcein-negative Annexin V-positive events observed in mock-transfected cells (left bars) and cells that overexpress *NAE1/APP-BP1* (right bars), prior to (white bars) and after irradiation (grey bars). Mean and standard deviation were calculated on 3 independent experiments. P-values were calculated with a Student-Newman-Keuls pairwise comparison. *Differences were considered significant when $p<0.05$.

and G2 phase distribution of UPCI:SCC90 cells that overexpress *NAE1/APP-BP1* (data not shown), suggesting that the effects were not due to changes in the cell cycle.

As a consequence of our findings, the correlation between *NAE/APP-BP1* expression levels and local control and disease-free survival of irradiated patients is currently being investigated. More generally, several studies have unambiguously demonstrated that HR-HPV is a major prognostic factor with respect to both disease-free and overall survival, and this prognosis is independent of treatment modality (1). The question as to whether HPV-positive patients should receive dose de-escalation (decreased irradiation dose delivered to the tumor area, or decreased area that receives the irradiation dose) in order to improve the therapeutic index, while sparing them long-term toxicity, is currently a matter of debate (29). Alternatively, HPV-positive tumors could be managed by modulating existing therapies, in order to potentiate the efficacy of ionizing radiation.

Our data highlight the relevance of the NEDDylation pathway to the cellular response to radiotherapy, and indicate this pathway is a potential drug target to potentiate existing therapies. Recently, a novel class of pharmacological inhibitors of the NAE1/APP-BP1/UBA3 complex has been tested in preclinical and clinical tumor models (27,28). In the light of our findings, it would be of interest to determine whether a functional copy of p53 is required for the efficacy of this novel treatment. In addition, these kind of therapeutic approaches could improve the efficacy of the treatment delivered to wild type *TP53* HPV-negative tumors.

HR-HPV detection (by immunohistochemistry) has been incorporated into an algorithm that could be used to predict overall survival (29). A stratification of patients based on that type of algorithm would be a first step to randomized trials. In HPV-related oropharyngeal cancer, the loss of genetic material in the 16q region is related to improved local control (3,4). It would be of great interest to further dissect this locus, in order to identify molecular markers that predict tumor response to therapy.

Besides increasing our knowledge of the molecular mechanisms of cell sensitivity to ionizing radiation, the identification of relevant biomarkers, combined with already established clinical features, may provide physicians with tools to select patients for tailored and more appropriate treatment strategies.

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