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Inhibition of ATM kinase activity enhanced TRAIL-mediated apoptosis in human melanoma cells

Vladimir N. Ivanov[1,*], **Hongning Zhou**[1], **Michael A. Partridge**[1], and **Tom K. Hei**[1,2]

[1]Center for Radiological Research, College of Physicians and Surgeons, Columbia University, New York, NY 10032

[2]Department of Environmental Health Sciences, Columbia University, New York, NY 10032

Abstract

The aim of the present study was to elucidate the effects of ATM kinase on the regulation of the extrinsic TRAIL-R2/DR5-mediated death pathway in human melanoma cells. We revealed that total ATM protein levels were high in some human melanoma lines compared to normal cells. The basal levels of active form ATM-Phospho-(Ser1981) were also detectable in many melanoma lines and could be further upregulated by γ-irradiation. Pretreatment of several melanoma lines just before γ-irradiation with inhibitor of ATM kinase, KU-55933, suppressed p53 and NF-κB activation, but notably increased radiation-induced DR5 surface expression, downregulated cFLIP (caspase-8 inhibitor) levels and substantially enhanced exogenous TRAIL-induced apoptosis. Furthermore, γ-irradiation in the presence of KU-55933 rendered TRAIL-resistant HHMSX melanoma cells susceptible to TRAIL-mediated apoptosis. Furthermore, suppression of ATM expression by the specific shRNA also resulted in downregulation of cFLIP levels, upregulation of surface DR5 expression and TRAIL-mediated apoptosis in melanoma cells. Besides p53 and NF-κB, crucial regulators of DR5 expression, transcription factor STAT3 is known to negatively regulate DR5 expression. Suppression of Ser727 and Tyr705 phosphorylation of STAT3 by KU-55933 reduced STAT3 transacting activity accompanied by elevation in DR5 expression. Dominant negative STAT3β also efficiently upregulated the DR5 surface expression and downregulated cFLIP levels in melanoma cells in culture and *in vivo*. Taken together, our data demonstrate the existence of an ATM-dependent STAT3-mediated antiapoptotic pathway, which upon suppression sensitizes human melanoma cells to TRAIL-mediated apoptosis.

Introduction

The nuclear protein kinase Ataxia Telangiectasia Mutated (ATM) plays an important role in signaling the presence of DNA double-strand breaks to the cell cycle checkpoint machinery and to the DNA repair system (1). Furthermore, *ATM* deficiency results in neurodegeneration, immunodeficiency and enhanced radiosensitivity, suggesting a crucial role for this gene in the regulation of cell proliferation and cell death (2). ATM, together

[*]Correspondence to: Vladimir Ivanov, Columbia University, VC11-236, 630 W 168th Street, New York, NY 10032, USA., Phone: (212) 305-9667; Fax: (212) 305-3229; vni3@columbia.edu.

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with ATR, controls extensive protein networks encompassing over 700 proteins that are responsive to DNA damage (3). The important targets of the ATM signaling pathways are transcription factors p53, NF-κB, ATF2 and STAT3, which are ultimately linked to regulation of the cell cycle and apoptosis (1, 4–6). Ionizing radiation and chemotherapy are the two main modalities for cancer treatment. The cytotoxic effects of many anticancer drugs and ionizing radiation are mediated through DNA damage resulting in the activation of DNA damage-induced cell signaling pathways, including activation of the ATM networks. In this scenario, ATM kinase plays a crucial role by balancing cell cycle arrest versus cell death (1).

The incidence of melanoma has substantially increased worldwide over the last 40 years. Most advanced melanomas respond poorly to radio- and chemotherapy and no effective therapy exists to inhibit the metastatic spread of this cancer (7). Alternative therapies that have been suggested for inducing apoptosis in cancer cells are based on the direct activation of the extrinsic death signaling pathways using recombinant death ligands of the TNF superfamily, such as TNF-related apoptosis-inducing ligand (TRAIL), or agonistic monoclonal antibodies to TRAIL-Receptor-2 (TRAIL-R2/DR5) and TRAIL-R1/DR4. TRAIL-R2 could often be found on the surface of different types of cancer cells, including melanoma cells, at higher expression levels compared to TRAIL-R1 (8). In contrast to normal cells, the expression of the decoy receptors, TRAIL-R3 and TRAIL-R2, rarely occurred in human melanomas. Targeting TRAIL-Receptor mediated signaling pathways for induction of apoptosis is currently being evaluated in multiple clinical trials for several cancer types (9, 10). Finally, combined modality treatments, which may include γ-irradiation and stimulation of the TRAIL-R-mediated pathways, appear to be promising potential treatments to suppress cancer development (11–13). Since ATM activity is strongly upregulated by γ-irradiation, we address the role of ATM in the regulation of apoptosis in human melanomas. The main aim of the present study was to resolve the question of how inhibition of ATM activity affected TRAIL-mediated extrinsic death pathway in human melanoma cells.

Materials and Methods

Materials

Human *Killer*-TRAIL was purchased from Alexis. JNK inhibitor SP600125 was obtained from Biomol; MEK inhibitor U0126, PI3K inhibitor LY294002, IKK inhibitor BMS-345541, JAK2 inhibitor AG490 and ATM Kinase inhibitor KU-55933 were purchased from Calbiochem.

Cell lines

Human melanoma cell lines LU1205 (also known as 1205lu), WM9, WM35 (14), HHMSX and normal human fibroblasts TIG3 were maintained in a DMEM medium supplemented with 10% fetal bovine serum (FBS), L-glutamine and antibiotics. Normal human lung fibroblasts (MRC-5) and human ATM deficient fibroblasts (GM02052) were obtained from Coriell Cell Repository. These cells were maintained in an Eagle’s minimal essential medium supplemented with 15% FBS, vitamins, amino acids and antibiotics.

Irradiation procedures

To determine sensitivity to γ-rays, the plates with melanoma cells were exposed to radiation from a Gammacell 40 ^{137}Cs irradiator.

FACS analysis of DR5 and Fas levels

Surface levels of DR5 and Fas were determined by staining with the PE-labeled mAbs from eBioscience and from BD Biosciences. A FACS Calibur flow cytometer (Becton Dickinson) combined with the CellQuest program was used to perform flow cytometric analysis.

Transfection and luciferase assay

The NF-κB luciferase reporter containing two κB binding sites, Jun2-Luc reporter (15) and the STAT-Luc reporter containing three repeats of GAS sites were used to determine NF-κB, AP-1 and STAT transactivation, respectively. Additional reporter constructs used included: DR5/TRAIL-R2-full-Luc, which contained 1.6 kb upstream of the ATG site through intron 2 in the DR5 genomic locus (16), a 1 kb cFLIP-promoter-Luc (17, 18) and p53RE-Luc (19). Transient transfection of reporter constructs (1 μg) together with pCMV-β gal (0.25μg) into 5×10^5 melanoma cells was performed using Lipofectamine (Life Technologies). Luciferase activity was determined using the Luciferase assay system (Promega) and was normalized based on β-galactosidase levels.

Mutational analysis of DR5 promoter

Mutations within the STAT-binding site of the human TRAIL-R2/DR5 promoter and within the κB-site of the intron 2 were generated using DR5/TRAIL-R2-full-Luc construct (16) and the QuikChange kit (Stratagene).

Suppression of ATM expression with shRNA

ATM expression in melanoma cells was stably knocked down using pRetroSuper constructs with shRNA sequences corresponding to position 912 (ATM#1) and 8538 (ATM#3) of the ATM transcript. GFP shRNA served as a mock control. These constructs have been kindly provided by Dr. J. Shiloh (20). LU1205 melanoma cells were transfected by shRNA constructs using Lipofectamine and selected with 0.5 μg/ml puromycin.

Apoptosis studies

Cells were exposed to soluble TRAIL (50 ng/ml) alone or in combination with cycloheximide (2 μg/ml). Different variants of combined treatment were used, including γ-irradiation (5 Gy) in the presence or in the absence of specific inhibitors of signaling pathways followed by TRAIL treatment. Apoptosis was then assessed by quantifying the percentage of hypodiploid nuclei using FACS analysis.

Western blot analysis

Total cell lysates (50 μg protein) were resolved on SDS-PAGE, and processed according to standard protocols. The monoclonal antibodies used for Western blotting included: anti-β-Actin (Sigma); anti-FLIP (NF6) (Axxora); anti-caspase-3 and anti-caspase-8; anti-ATM (D2E2) and anti-phospho-ATM (Ser1981); anti-phospho-p53 (Ser15) (Cell Signaling); anti-

XIAP (clone 48, BD Biosciences). The polyclonal antibodies used included: anti-TRAIL and anti-DR5 (Axxora); anti-phospho-p53 (Ser20), and anti-total p53; anti-phospho-SAPK/JNK (Thr183/Tyr185) and anti-JNK; anti-phospho-p44/p42 MAP kinase (Thr202/Tyr204) and anti-p44/p42 MAP kinase; anti-phospho-AKT (Ser473) and anti-AKT; anti-PARP; anti-STAT3, anti-phospho-STAT3 (Tyr705) and anti-phospho-STAT3 (Ser727); anti-Chk2 and anti-phospho-Chk2 (Thr68) (Cell Signaling). The secondary Abs were conjugated to horseradish peroxidase; signals were detected using the ECL system (Amersham).

EMSA

Electrophoretic mobility shift assay (EMSA) was performed for the detection of NF-κB DNA-binding activity as previously described. Ubiquitous NF-Y DNA-binding activity was used as an internal control (21).

Human melanoma transplant in nude mice

LU1205 cells (1×10^6) stably transfected with control empty vector pBabe-puro or with STAT3β expression construct were injected subcutaneously into 7-weeks old athymic nude mice BALB/c nu/nu (six mice per group) obtained from Jackson Laboratories. Tumor growth was monitored every two days. Three weeks after beginning of the experiments, the tumor were excised, weighted and subject to histopathological and immunochemical examination.

Statistical analysis

Data were calculated as means and standard deviations. Comparisons of results between treated and control groups were made by the Students' *t*-tests. A *P*-value of 0.05 or less between groups was considered significant.

Results

ATM levels and DR5 surface expression in human melanoma cell lines

Two primary human fibroblast lines, MRC-5 *(ATM +/+)* and GM2052 *(ATM −/−)*, were used as positive and negative control, respectively, for comparing the expression levels of ATM protein and its signaling pathways in human melanoma cells (Fig. 1A and B). Autophosphorylation of ATM after γ-irradiation (22), with the subsequent ATM-Chk2-dependent phosphorylation of p53-Ser20 (23) that are characteristic features of the ATM signaling pathway, are clearly deficient in *ATM −/−* fibroblast line (Fig. 1A). In contrast to normal fibroblasts MRC-5, human melanoma lines were characterized by increased protein levels of both total ATM and its active form, phospho-Ser1981-ATM (Fig. 1B). Basal p53 protein levels in melanoma lines were also correlated to ATM protein levels. ATM levels in human melanocytes were similar to levels in human fibroblasts (data not shown).

Numerous human melanoma lines express TRAIL-R2/DR5 on their surface (12, 24). In contrast, surface expression of TRAIL-R1/DR4 was found at marginal levels in many melanoma lines (Suppl Fig. 1). The remarkable exceptions were the early melanomas WM1552C and SBcl2 (25), as well as ocular melanoma OM431 (26), which exhibited the

increased levels of surface DR4. Interestingly, surface expression of DR5 in WM9 and LU1205 melanoma cells was substantially higher than in HHMSX melanoma cells, demonstrating an inverse correlation between the basal ATM levels and DR5 surface expression (Fig. 1B and D). Both MRC-5 *(ATM +/+)* and GM02052 *(ATM −/−)* fibroblasts contained two cell subpopulations with zero and moderate levels of DR5 on the cell surface, respectively. Although GM02052 cells had a modest increase in the percentage of DR5-positive cells (58%) compared to MRC-5 (35%) (Fig. 1C), the different genetic background of these cell lines did not allow us to make a definite conclusion concerning quantitative effects of ATM on the DR5 surface expression in fibroblasts. However, these data demonstrate that in the case of ATM deficiency, moderate levels of the DR5 surface expression are observed in fibroblasts. Interestingly, ATM deficiency did not change the levels of surface FAS in fibroblasts (Fig. 1C). Next we used pRetroSuper constructs expressing ATM#1, ATM#3 shRNA for stable suppression of ATM levels and GFP shRNA as a mock control (20) in MRC5 cells. ATM#1 shRNA caused almost 70% decrease in total ATM protein levels of MRC5 cells that was accompanied by modest increase in DR5 surface expression (Fig. 1D) indicating a negative role of ATM in regulation of DR5 expression.

TRAIL-R2/DR5 gene expression is a target for positive regulation by γ-irradiation in many cancer cell lines (27). Indeed, DR5 total and surface levels and activity of the *DR5* promoter were further increased 6–16 h after irradiation of LU1205 cells, while for MRC-5 and GMO2052 fibroblasts, upregulation of the surface DR5 expression was not pronounced (Fig. 1C, D and 2B, C). Since activation of ATM kinase by DNA damage after γ-irradiation is involved in the upregulation of the p53 and NF-κB pathways (28), a role for ATM kinase in the regulation of *DR5* gene expression, the levels of NF-κB-dependent antiapoptotic proteins and TRAIL-mediated apoptosis in melanoma cells was further investigated in this study.

Inhibition of ATM kinase in γ-irradiated melanoma cells reduced p53 and NF-κB activities and cFLIP levels

γ-Irradiation of LU1205 cells induced autophosphorylation and upregulation of ATM kinase activity in a dose-dependent manner (Fig. 2A) that was accompanied by further activation of Chk2 and Chk2-mediated Ser20-phosphorylation of p53. On the other hand, direct ATM-mediated Ser15-phosphorylation of p53 was observed at low levels. KU-55933 (10 μM), a specific inhibitor of ATM kinase activity (29), suppressed ATM autophosphorylation after irradiation, caused downregulation of p53-phospho-(Ser20) levels (Fig. 2B) and NF-κB DNA-binding activity (Fig. 2C), which was accompanied by a decrease of general p53- and NF-κB-dependent gene expression in the irradiated cells (Fig. 2C). KU-55933 was less effective for inhibition of the basal NF-κB activity, compared to BMS-345541 (20 μM), a specific inhibitor of IKK-NF-κB. Both inhibitors were equally effective in suppression of the radiation-induced NF-κB activity (Fig. 2C). Luciferase assay also demonstrated downregulation of the cFLIP promoter activity with KU-55933 (Fig. 2C) that was accompanied by decrease in cFLIP-S and cFLIP-L protein levels detected by Western blotting 8 h after treatment with KU-55933 in both nonirradiated and γ-irradiated cells (Fig. 2B). In contrast, the DR5 promoter activity and total DR5 protein levels modestly increased

4 – 6 h after irradiation, especially in the presence of KU-55933 (Fig. 2B and C). The observed decrease in p53 and NF-κB activities following inhibition of ATM kinase activity by KU-55933 in γ-irradiated cells correlated with a substantial upregulation of G2/M arrest of the cell cycle. This was likely, due to a failure to arrest in S phase (29, 30), a characteristic feature of ATM deficiency in both LU1205 melanoma cells and in TIG3 human fibroblasts. KU-55933-upregulated levels of G2/M arrest in irradiated normal TIG3 fibroblasts were higher compared to treated cancer cells (Fig. 2D).

Inhibition of ATM activity increased the sensitivity of melanoma cells to TRAIL-mediated apoptosis

DNA repair mechanisms might permit the cells to overcome the consequences of the DNA damage. However, suppression of ATM activity after γ-irradiation in the presence of KU-55933 could exert an alternative effect on the apoptotic machinery in the cells via regulation of expression of the components of the extrinsic apoptotic pathway. KU-55933 efficiently upregulated TRAIL-mediated apoptosis of LU1205 and WM35 cells (Fig. 3A and D), probably, due to downregulation of cFLIP levels, that was quite similar to cFLIP-RNAi mediated suppression of cFLIP and the subsequent upregulation of TRAIL-induced apoptosis in melanoma cells (24). Downregulation of basal cFLIP protein levels by KU-55933 was dependent on downregulation of *cFLIP* gene expression (Fig. 2C) followed by proteasome-dependent degradation of cFLIP and could be blocked with the proteasome inhibitor, MG132 (data not shown). On the other hand, cleavage of procaspase-8, procaspase-3 and the caspase-3-mediated cleavage of PARP and XIAP p57 were well pronounced 6 h after treatment by a combination of KU-55933 and TRAIL. XIAP p57 and cFLIP levels were also effectively downregulated by treatment with TRAIL alone via caspase cascade (Fig. 3A). A detailed analysis of cFLIP-L levels after treatment with KU55933 or TRAIL in the presence or absence of a universal caspase inhibitor, zVAD-fmk, and a specific inhibitor of caspase-8, Ac-IETD-CHO), was also performed (Suppl Fig. 2). Results obtained clearly demonstrated a caspase-independent downregulation of cFLIP levels 2–4 h after treatment with KU55933 and caspase-dependent downregulation 6 h after treatment. Hence, the initial decrease in cFLIP-L levels after treatment with KU55933 was not a result of apoptosis, while the late decrease in cFLIP-L levels was indeed a consequence of apoptosis. Levels of apoptosis determined 48 h after treatment were substantially higher for a combination of TRAIL and KU-55933, than for TRAIL alone (Fig. 3D).

Efficacy of TRAIL-mediated apoptosis could be further increased through radiation-induced upregulation of the total and surface DR5 expression (12), which was positively controlled by p53 and NF-κB (16, 31). As expected, BMS-345541, a specific inhibitor of the IKK-NF-κB pathway, suppressed NF-κB activation in melanoma cells (Fig. 2C) and prevented radiation-induced upregulation of DR5 surface expression in WM35 and LU1205 cells (Fig. 3B). Given the positive role for NF-κB and p53 in regulation of *DR5* transcription (16, 31, 32), it was quite unexpected, however, that instead of suppression of *DR5* expression, we observed an additional upregulation of the *DR5* promoter activity (Fig. 2C) and DR5 surface expression after γ-irradiation in the presence of KU-55933 (10 μM) in four melanoma lines, WM35, LU1205, WM9 and HHMSX (Fig. 3B and data not shown).

Hence, pretreatment of LU1205 and WM35 cells with γ-irradiation that upregulated the total and surface expression of DR5 (Fig. 3B and C), especially in the presence of KU-55933, further increased levels of TRAIL-mediated apoptosis (Fig. 3D). On the other hand, IKK-NF-κB inhibitor BMS-345541 decreased apoptotic levels after irradiation of melanoma cells (Fig. 3D), correlating with decreased levels of NF-κB-dependent proapoptotic DR5 after irradiation in the presence of BMS-345541. In contrast to DR5, radiation-induced surface FAS expression in LU1205 cells was not further increased in the presence of KU-55933 (Fig. 3C).

KU-55933 sensitizes resistant HHMSX melanoma cells to TRAIL-mediated apoptosis

Additional experiments demonstrated that upregulation of DR5 surface levels and downregulation of cFLIP protein levels after irradiation in the presence of KU-55933 (data not shown) was accompanied by accelerated TRAIL-mediated apoptosis in WM9 melanoma cells. In contrast, TRAIL-mediated apoptosis of TIG3 normal fibroblasts was substantially lower (Fig 4A) based, probably, on a protective mechanism for TRAIL-mediated apoptosis in normal cells. We used the similar treatment for upregulation of TRAIL-mediated apoptosis in resistant HHMSX melanoma cells (Fig. 4A). Besides low surface expression of DR5 (Fig. 1D), these cells were characterized by notably suppressed mitochondrial function (24). KU-55933 alone, but not TRAIL caused only modest downregulation of cFLIP-L levels in these cells (Fig. 4B). The sequential treatment of these cells initially with a combination of KU-55933 and γ-radiation that upregulated DR5 surface levels (Fig. 4B) and then with recombinant TRAIL (50 ng/ml) in the presence of KU-55933 (10 μM) that additionally decreased cFLIP levels (Fig. 4B) induced pronounced apoptosis demonstrating a sensitization to TRAIL.

ATM suppression by shRNA

We used pRetroSuper constructs expressing ATM#1, ATM#3 shRNA for stable suppression of ATM and GFP shRNA as a mock control (20) in melanoma cells. After selection in the presence of puromycin, LU1205 mass cultures expressing ATM#1, ATM#3 and GFP shRNA were obtained. ATM#3 and especially ATM#1 shRNA were effective in the suppression of total ATM levels. ATM#1 shRNA caused almost 75% decrease in total ATM protein levels of LU1205 cells (Fig. 4C). ATM downregulation was accompanied by an increase in the percentage of G2/M arrested cells 48 h after irradiation (5 Gy) of the ATM-deficient culture in comparison with the percentage in the irradiated control culture (from 18% to 34%; data not shown). Furthermore, we observed in ATM deficient cells a partial suppression of the basal cFLIP-L, but not XIAP protein levels and a notable upregulation of DR5 surface expression, which did not further increase after γ-irradiation (Fig. 4D). TRAIL-induced apoptosis was maximal in LU1205/ATM#1 cells, compared to the control cells (Fig. 4D). Results obtained with suppression of ATM expression by specific shRNA generally confirmed our data based on an inhibition of ATM activity by its inhibitor KU-55933 (see Fig. 3). However, certain differences between these two approaches have been also observed. Irradiation had no additional effect on DR5 surface expression in ATM deficient cells compared to untreated ATM deficient cells with already increased DR5 expression (Fig. 4D), while irradiated and KU-55933-treated cells showed higher DR5 expression than KU-55933-treated non-irradiated cells (Fig. 4A). Consequently, there was

no additional increase in levels of TRAIL-mediated apoptosis in ATM-deficient irradiated cells compared to already high levels of TRAIL-mediated apoptosis in non-irradiated ATM deficient cells (Fig. 4D). Such differences are probably results of a substantial change in the basal characteristics of ATM-deficient cells. However, the critical feature, pronounced upregulation of TRAIL-mediated apoptosis in melanoma cells, was observed for both approaches.

A role of STAT3 Tyr705- and Ser727-phosphorylation in ATM-dependent regulation of DR5 expression

Several signaling pathways and the correspondent transcription factors may be involved in the regulation of radiation-induced DR5 expression: ATM-Chk2-p53, IKK-NF-κB, MEK-ERK-Elk1, MKK4/7-JNK-cJun and JAK2-STAT3. Since suppression of p53 and NF-κB activities by KU-55933 was not accompanied by a decrease in the DR5 surface expression, it might indicate the existence of an alternative ATM-dependent signaling pathway, characterized by the activation of a transcription factor that could negatively control DR5 expression.

We used specific inhibitors of signaling pathways to further resolve the mechanism of regulation of DR5 expression. Similarly to the inhibitor of IKK-NF-κB (Fig. 3A), JNK inhibitor SP600125 (10 μM) blocked an increase in DR5 surface expression (Fig. 5A, B). As expected, this inhibitor downregulated cJun phosphorylation (Fig. 5C) after γ-irradiation of LU1205 cells. These results indicated a positive role of JNK in the regulation of radiation-induced DR5 expression (Fig. 5A and B) that further confirmed observations on the significance of JNK-SP1 for enhancing expression of DR5 (33). U0126 (10 μM), a MEK-ERK inhibitor, substantially decreased DR5 surface expression in both nonirradiated and irradiated cells (Fig. 5A, B). This inhibitor suppressed constitutive ERK1/2 phosphorylation in LU1205 cells while KU-55933 had no effects (Fig. 5C). Only AG490 (50 μM), a JAK2-STAT3 inhibitor, resembled the positive effects of KU-55933 on DR5 levels in melanoma cells (Fig. 5A and B). STAT3 Tyr705-phoshorylation mediated by JAK2 is involved in STAT3 dimerization and its subsequent translocation to the nucleus and interaction with genomic DNA (34). Ser727-phosphorylation of STAT3 further increases STAT3 transacting functions in the gene promoters.

KU-55933 treatment, as expected, suppressed radiation-induced ATM activation and notably reduced basal and radiation-induced levels of both STAT3 Tyr705-phosphorylation and STAT3 Ser727-phosphorylation (Fig. 5C). A luciferase assay confirmed the summary negative effects of KU-55933 on STAT3-dependent reporter activity in γ-irradiated cells that was quite similar to STAT3-Luc inhibition by AG490 (Fig. 5D). A role of ATM, as well as the MAPK and mTOR pathways in Ser727-phoshorylation of STAT3 was previously established (6, 34), while the ATM-dependent pathway that affects Tyr-phosphorylation of STAT3 is currently unknown. Interestingly, KU-55933 also partially suppressed JNK-1, but not p38 phosphorylation, which might be a reason of the decrease in Ser727-phosphorylation of STAT3 via JNK-1 (Fig. 5C). Our data indicated an upregulation of DR5 total and surface expression upon suppression of phospho-Tyr705- and phospho-Ser727-dependent STAT3 functions, which was consistent with suggestion on STAT3 to be a

negative regulator of DR5 expression and TRAIL-mediated apoptosis (35, 36). To further confirm a negative regulation of DR5 expression by STAT3, we mutated the STAT3-binding site in the DR5 promoter using a site-directed mutagenesis kit (QuikChange, Stratagene). This resulted in pronounced upregulation of the DR5-luciferase activity before and after γ-irradiation, while mutations in the intronic NF-κB binding site resulted in a downregulation of DR5-luciferase activity (31) in melanoma cells (Fig. 5D).

Dominant negative STAT3β substantially increased DR5 surface expression on melanoma cells both in cell culture and *in vivo*

Next we used previously established melanoma lines, LU1205 stably transfected with the empty vector (pBabe-puro) or by STAT3β, a dominant negative form of STAT3 with the 55-aa C-terminal deletion, including Ser727, which strongly suppressed STAT3 transcriptional activity (37, 38). STAT3β overexpression moderately decreased cell growth in culture and strongly increased surface expression of DR5 in both nontreated and irradiated LU1205 melanoma cells (Fig. 6A and B). Furthermore, LU1205/STAT3β cells contained a decreased basal level of cFLIP-L and cFLIP-S compared to the control cells (Fig. 6A). Taken together, this correlated with increased TRAIL-mediated apoptosis in STAT3β-transfected LU1205 cells (Fig. 6C). Then we examined whether STAT3β-dependent upregulation of the DR5 surface expression was maintained *in vivo*. Interestingly, the LU1205-STAT3β melanoma xenotransplant exhibited a dramatic decrease in tumor volume compared to the control LU1205-puro transplant in athymic nude mice 3 weeks after injection of melanoma cells (Fig. 6D). The DR5 surface expression was maintained notably higher in LU1205-STAT3β tumor cells *in vivo* than in the control LU1205-puro tumors (Fig. 6D). Taken together, these data confirmed that suppression of ATM-dependent STAT3 activation enhanced TRAIL-mediated apoptosis through upreguation of surface DR5 expression while suppression of both STAT3 and NF-κB appeared to be involved in downregulation of cFLIP (see Fig. 2B, 3A and 6A) accompanied by additional increase in apoptotic levels.

In general, the ATM inhibitor KU-55933 affected TRAIL-mediated apoptosis more strongly than the JAK2 inhibitor, AG490, or overexpression of STAT3β (Fig. 6C). Finally, LY294002 (50 μM), a universal inhibitor of PI3K and PI3K-like kinases, including ATM, induced the strongest acceleration of TRAIL-mediated apoptosis in melanoma cells (Fig. 6C) indicating for a role of other members of this family in suppression of TRAIL-mediated apoptosis. A combination of KU-55933 and LY294002 did not additionally increase levels of TRAIL-induced apoptosis, compared to apoptosis in the presence of LY294002 alone. Hence, the ATM pathway regulated antiapoptotic function in melanoma cells that substantially suppressed TRAIL-mediated apoptosis.

Discussion

A role for the suppression of ATM activity in sensitizing cancer cells to radiation-induced death was already described (39, 40). Consequently, targeted cancer therapy based on the inhibition of DNA strand break repair is currently being undertaken in numerous clinical trials (41). As most metastatic melanomas are radioresistant, ionizing radiation alone is rarely used for treatment of this disease. We investigated in the present study an alternative

approach, involving a combined treatment of γ-irradiation and an inhibitor of ATM kinase followed by induction of recombinant TRAIL-mediated apoptosis. Using inhibition of ATM activity by KU-55933 or a partial suppression of ATM expression by specific ATM shRNA stably transfected in melanoma cells, we observed a substantial upregulation of TRAIL-mediated apoptosis.

A previous report described ATM-dependent activation of STAT3 transcriptional activity (6) that might negatively control DR5 gene expression, as we observed in the present study. Consequently, partial reduction of Ser727- and Tyr705-phosphorylation of STAT3 by KU-55933 reversed DR5 downregulation, which was directed by ATM. A role for ATM, mTOR and the MAPK pathways in Ser727 phosphorylation of STAT3 was described previously (6, 34), while an ATM-dependent signaling pathway resulting in upregulation of Tyr-phosphorylation of STAT3 needs to be investigated. Further, dominant negative STAT3β with deleted Ser727 was effective in several actions, such as suppression of melanoma xenotransplant growth, probably via inhibition of STAT3-dependent expression of cyclin D1 (42), and an upregulation of DR5 and Fas (37) surface expression in cell culture and *in vivo*. A substantial inhibition of melanoma xenotransplant growth by STAT3β might also indicate a role for DR5- and Fas-mediated apoptosis in this process.

A negative role for STAT3 was previously demonstrated for the regulation of *FAS* (37) and *p53* transcription (43), while STAT3 together with NF-κB were involved in the positive control of antiapoptotic *Bcl-xL* and *cFLIP/CFLAR* gene expression (44–47). Coordinated upregulation of DR5 expression (through suppression of ATM-STAT3 activity) and downregulation of cFLIP protein levels (through suppression of ATM-NF-κB- and ATM-STAT3-dependent expression) may represent an efficient combination that can dramatically accelerate TRAIL-mediated apoptosis in several cancer cell systems (26, 46).

One of the critical factors that determine the efficacy of anticancer therapy is the ability to selectively kill cancer cells while minimally affecting normal cells. In this context, TRAIL via interaction with the correspondent TRAIL-R1/R2 on the surface of cancer cells may induce a fatal signaling cascade in cancer cells and has only minimal cytotoxic effects in normal cells (10). Numerous attempts have been performed for increasing sensitivity of cancer cells to TRAIL using different types of combined treatment. Based on the results of the present study, combined treatment with γ-irradiation and an ATM inhibitor that is followed by treatment with exogenous TRAIL represents a potentially effective approach for cancer cell killing. Our findings provide a basis for further investigation of TRAIL-mediated target treatment to overcome apoptotic resistance of metastatic melanomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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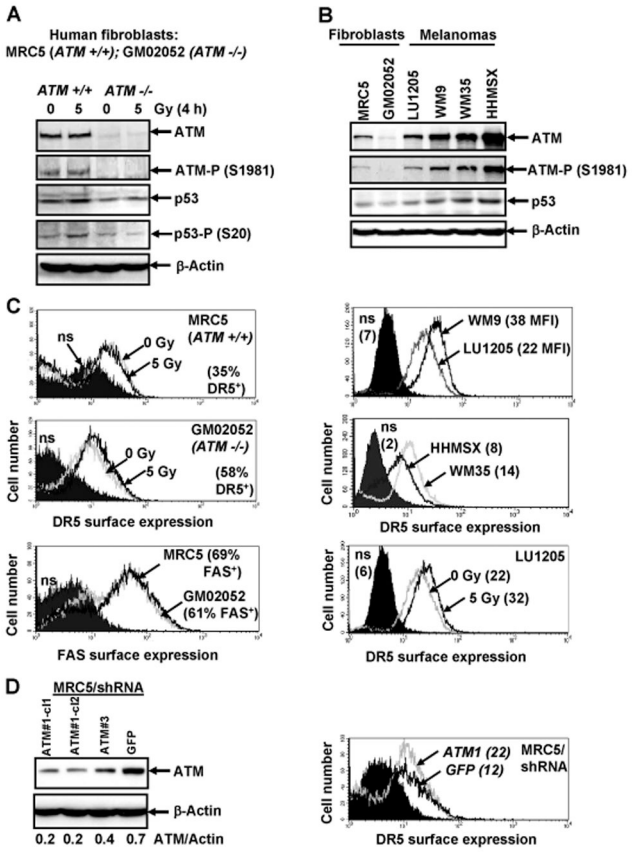

Figure 1. ATM levels and TRAIL-R2/DR5 surface expression in human fibroblast and melanoma lines

(A) Western blot analysis of total ATM, ATM-P (Ser1981), total p53 and p53-P (Ser20) in MRC5 *(ATM +/+)* and GM02052 *(ATM−/−)* human fibroblasts before and 4 h after γ-irradiation (5 Gy). **(B)** Western blotting of indicated proteins in fibroblast and melanoma lines. Actin served as a control of protein loading. **(C)** Surface expression of DR5 and FAS in fibroblast and melanoma lines was determined before and 16 h after γ-irradiation using FACS analysis. Medium fluorescent intensity (MFI) is indicated for melanoma cells. **(D)** MRC5 cultures permanently transfected with pRetroSuper constructs expressing shRNA for GFP (mock control), ATM#1, ATM#3 were established. Western blotting demonstrated ATM and β-Actin levels. Surface DR5 expression (MFI) increased in ATM-deficient cells.

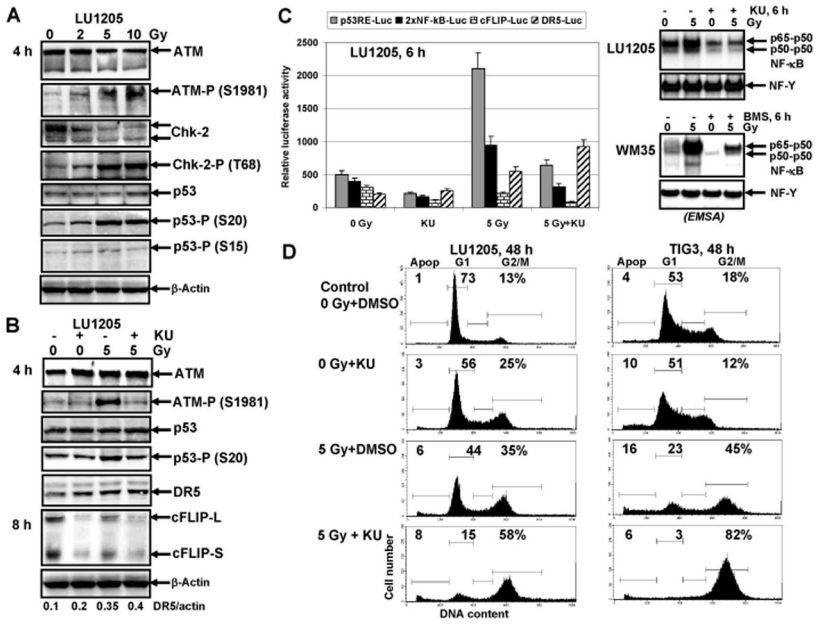

Figure 2. Suppression of ATM activity by KU-55933 increased G2/M cell cycle arrest after γ-irradiation

(A, B) Western blotting of indicated proteins in LU1205 cells 4 or 8 h after γ-irradiation in the presence or absence of KU-55933 (10 μM). Actin served as a protein loading control. **(C)** Effects of γ-irradiation (5 Gy) and KU-55933 (10 μM) on NF-κB-, p53-dependent luciferase reporter activities and on cFLIP- and DR5-promoter activities 6 h after treatment. *Error bars* represent mean ± S.D. (Student's *t* test, $P < 0.05$). EMSA of nuclear NF-κB activity in LU1205 and WM35 cells 6 h after γ-irradiation in the presence or absence of KU-55933 (10 μM), BMS-345541 (20 μM). Positions of two NF-κB DNA-binding complexes are indicated. Ubiquitous NF-Y DNA-binding complex was used as an internal control. **(D)** Cell cycle analysis of LU1205 melanoma cells and TIG3 fibroblasts 48 h after treatment using flow cytometry. KU-55933 in 0.1% DMSO that was added 0.5 h before γ-irradiation.

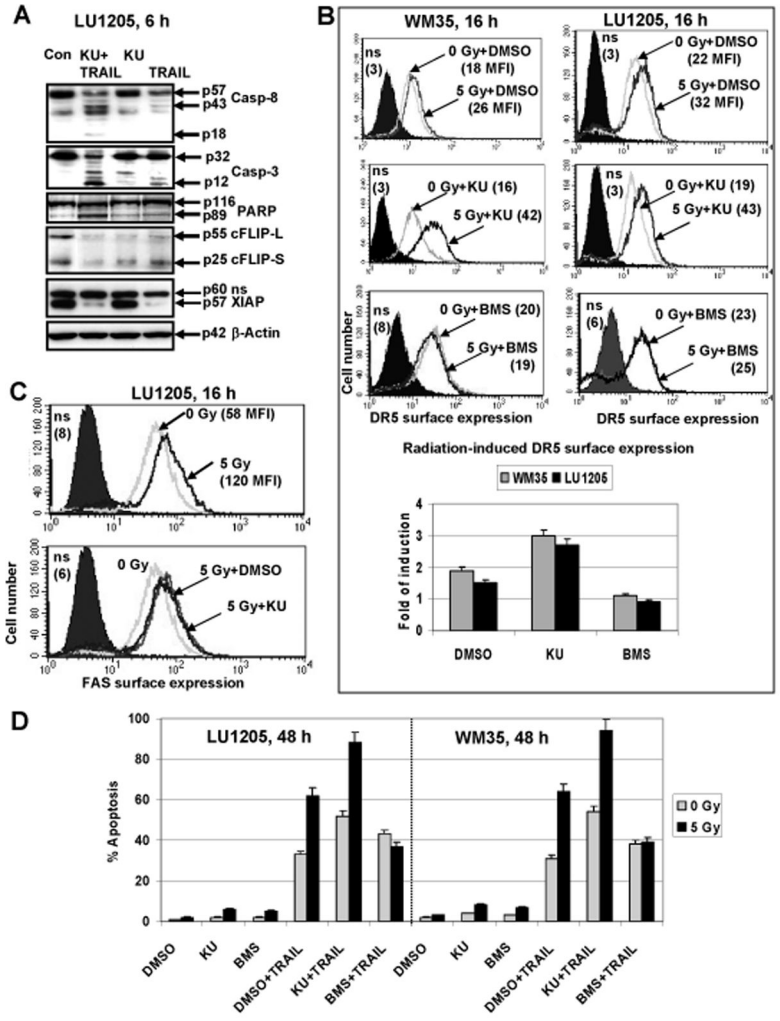

Figure 3. KU-55933 increased DR5 surface expression and TRAIL-mediated apoptosis of γ-irradiated LU1205 and WM35 melanoma cells

(A) Western blot analysis of caspase-8, caspase-3 and PARP cleavage, XIAP, cFLIP-L, sFLIP-S and β-Actin levels following treatment of LU1205 cells by TRAIL (50 ng/ml), KU-55933 (10 μM) or their combination. Band p60 determined by anti-XIAP mAb (clone 48) was nonspecific. **(B, C)** Surface expression of DR5 and FAS in LU1205 and WM35 cells 16 h after irradiation (5 Gy). KU-55933 and BMS-345541 were added to media 0.5 h before irradiation. Results of a typical experiment with WM35 and LU1205 cells are shown in the upper part of panel B. Radiation-induced levels of DR5 surface expression in the presence DMSO, KU-55933 or BMS-345541 normalized to DR5 levels before irradiation are shown in the bottom of the panel B. *Error bars* represent mean ± S.D. **(D)** LU1205 and WM35 cells were γ-irradiated in the presence of DMSO, KU-55933 or BMS-345541. 16 h after irradiation, cells were treated with TRAIL (50 ng/ml) for an additional 32 h. Apoptosis levels were determined as the percentage of cells with hypodiploid content of DNA using flow cytometry. *Error bars* represent mean ± S.D. from four independent experiments (Student's *t* test, $P < 0.05$).

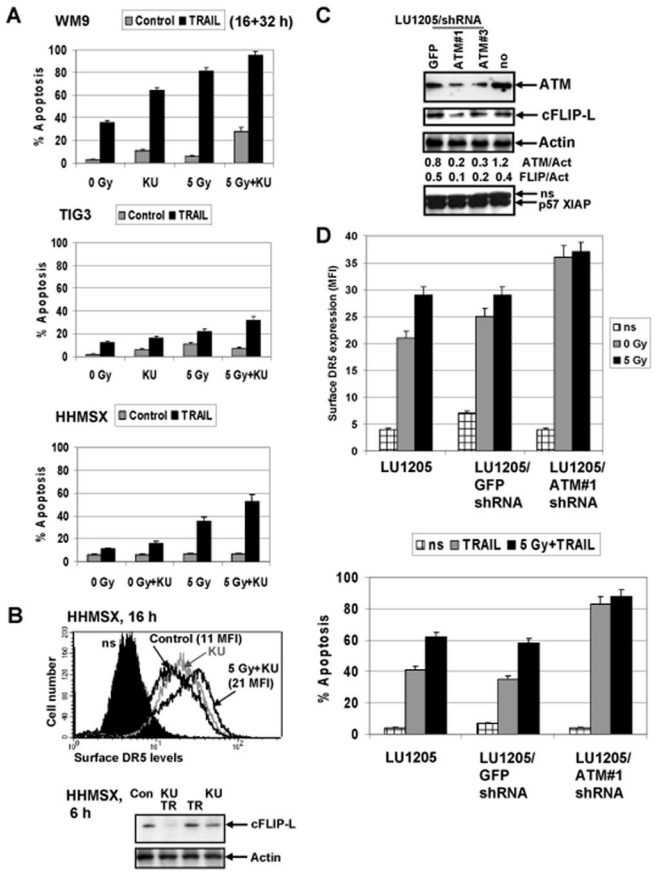

Figure 4. Inhibition of ATM activity by KU-55933 or downregulation of ATM levels by specific shRNA increased DR5 surface expression and TRAIL-mediated apoptosis in melanoma cells

(A) Indicated cell lines were γ-irradiated in the presence or in the absence of KU-55933. Eighteen h after irradiation, cells were treated with TRAIL (50 ng/ml) for an additional 32 h. Apoptosis levels were determined as the percentage of cells with hypodiploid content of DNA. *Error bars* represent mean ± S.D. **(B)** DR5 surface levels of HHMSX cells after treatments with DMSO, KU-55933 or 5 Gy+KU-55933 were determined by flow cytometry. Western blotting demonstrated cFLIP levels 6 h after treatment of HHMSX cells with TRAIL (TR) and KU-55933. **(C)** LU1205 mass cultures permanently transfected with pRetroSuper constructs expressing shRNA for GFP (mock control), ATM#1, ATM#3 were established. Western blotting demonstrated ATM, cFLIP-L and XIAP levels in these cultures. **(D)** Surface DR5 expression (MFI) and apoptosis levels in indicated cultures. *Error bars* represent mean ± S.D. (Student's *t* test, $P < 0.05$).

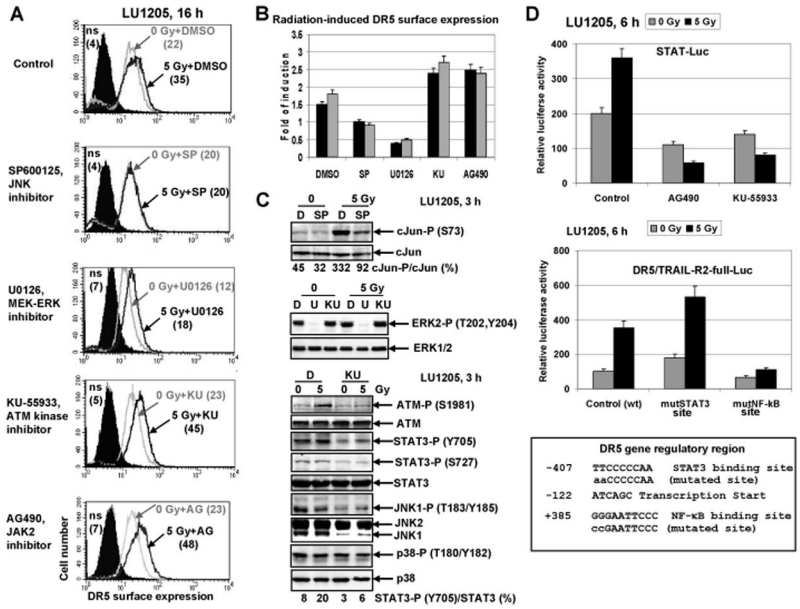

Figure 5. Effects of specific inhibition of signaling pathways on DR5 surface expression in LU1205 cells

(A, B) LU1205 cells were γ-irradiated in the presence of DMSO, SP600125 (10 μM), U0126 (10 μM), AG418 (50 μM) or KU-55933 (10 μM). Surface expression of DR5 in melanoma cells was determined 16 h after irradiation (5 Gy). Results of a typical experiment are presented in the panel A. Radiation-induced levels of DR5 surface expression in the presence DMSO, SP600125, U0126, KU-55933 or AG490 normalized to DR5 levels before treatment are shown in the panel B. *Error bars* represent mean ± S.D. (Student's *t* test, $P < 0.05$). **(C)** Western blotting of total and phosphorylated protein levels of cJun, ERK, ATM, STAT3, JNK and p38 in LU1205 cells after indicated treatment. **D**MSO, **U**0126 and **KU**-55933 were added 30 min before irradiation. A ratio STAT3-P (Y705)/total STAT3 (%) is indicated. **(D)** Effects of AG490 and KU-55933 on STAT-dependent reporter activity before and after γ-irradiation (5 Gy). A role of STAT3-binding site for the *DR5* promoter activity. Positions of the transcription start, the STAT3 and NF-κB binding sites are indicated. Transient transfection of the control (w/t) DR5/TRAIL-R2-full-Luc reporter, as well as constructs with the mutated STAT3- or NF-κB binding site was performed using LU1205 cells. Sixteen h after transfection, cells were irradiated and normalized luciferase activity was determined 6 h after treatment.

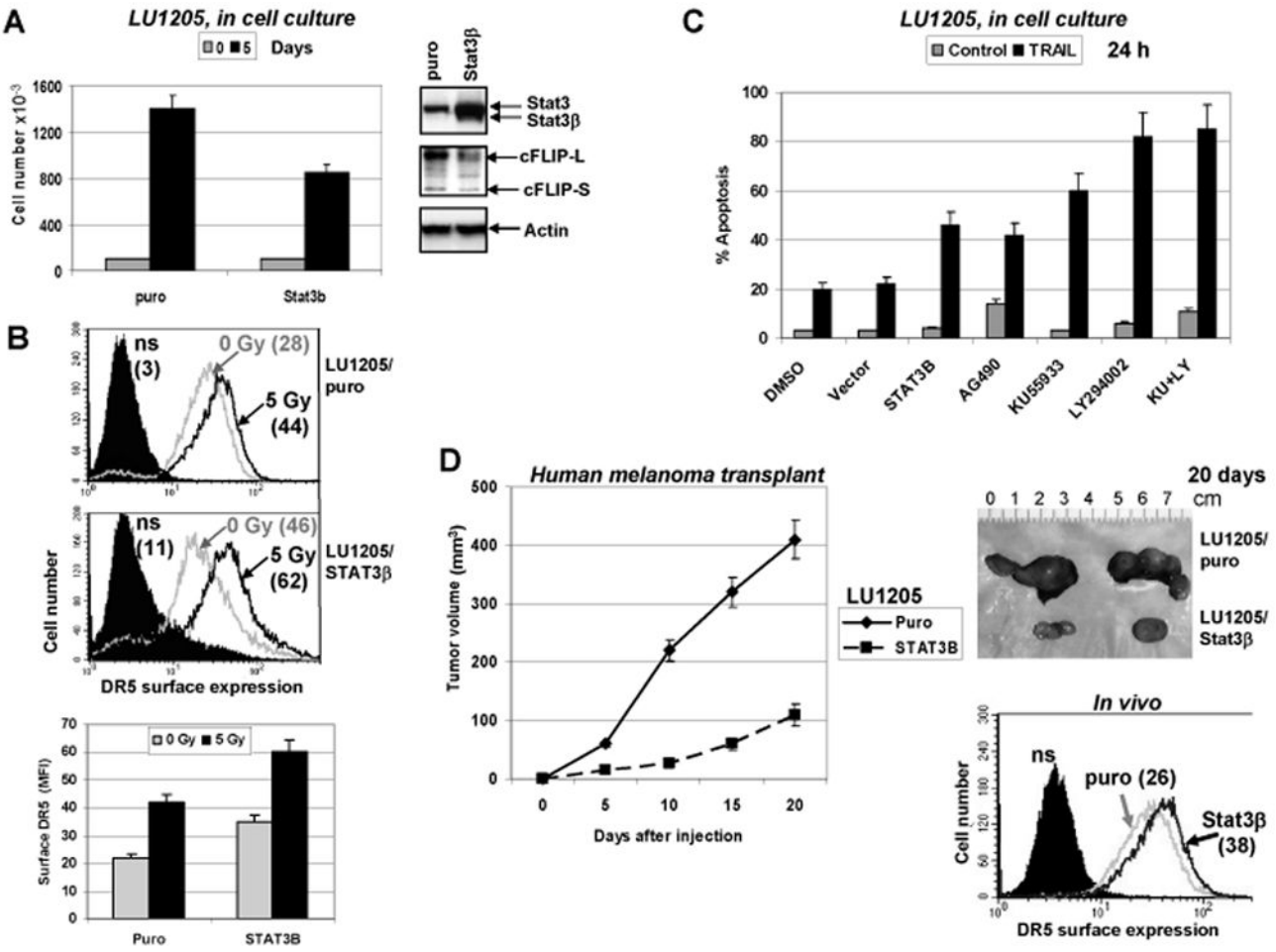

Figure 6. Effects of dominant-negative STAT3β on DR5 surface expression in cell culture and in melanoma transplant in nude mice

(A) Cell culture growth of LU1205-puro and LU1205-STAT3β cells transfected with the empty vector pBabe-puro or with STAT3β construct. Western blotting demonstrated levels of STAT3β. **(B)** Surface expression of DR5 on LU1205-puro and LU1205-STAT3β cells in culture before and after γ-irradiation. Results of typical experiment are shown on the top of the panel. *Error bars* represent mean ± S.D. **(C)** Apoptosis induced by TRAIL (50 ng/ml) in the control LU1205-puro and LU1205-STAT3β cells, as well as by TRAIL in combination with AG490 (50 μM), KU-55933 (10 μM) or LY294002 (50 μM) in LU1205 cells. *Error bars* represent mean ± S.D. (Student's *t* test, $P < 0.05$). **(D)** LU1205-puro and LU1205-STAT3β melanoma transplant growth in nude mice. Melanoma cells were injected subcutaneously into atymic nude mice, and tumor volume was monitored during the next three weeks. Surface expression of DR5 on LU1205-puro and LU1205-STAT3β cells *in vivo* was determined by FACS analysis.