異常、中山書店
258-259頁、2010

H．知的財産権の出願・登録状況

1．特許取得　なし
2．実用新案登録　なし
3.その他　なし

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

難治性神経芽腫の幹細胞性とトランスクリプトーム解析

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研究要旨：難治性神経芽腫の新規治療戦略の開発を目指し、神経芽腫細胞株ならびに臨床サンプルを用いた神経芽腫の幹細胞性に関わるゲノム異常やトランスクリプトームなどの分子的特性の同定を進めている。本年度は幹細胞様神経芽腫細胞株についてアレイ CGH 解析と non-coding RNA を含む網羅的遺伝子発現解析を行ない、これまでに報告された難治性神経芽腫に特徴的な遺伝子発現プロファイルとの関連性を検討した。幹細胞様神経芽腫細胞株で特異的に高発現する遺伝子群には複数の予後不良関連遺伝子群が同定されたことから、神経芽腫の幹細胞性維持に強く関与する遺伝子の絞り込みとそれらが関与する分子パスウェイの絞り込みに有用であると期待される。

A.　研究目的

小児の代表的な腹部固形腫瘍のひとつである神経芽腫の進行例は依然として非常に予後不良であり、新たな治療戦略の構築が緊急の課題となっている。そこで本研究課題では、難治性神経芽腫の分子的背景を幹細胞性の観点から網羅的に解析し、新規治療標的の同定につなげることを目的とする。具体的には複数の幹細胞様神経芽腫細胞株の詳細な解析に加え、Neurospere 形成前後、幹細胞性制御因子候補の導入前後の網羅的遺伝子発現（ mRNA ならびに ncRNA ）を解析し、本研究班の成果から得られるエピゲノムデータとの比較を行なうことにより、神経芽腫におけるがん幹細胞性維持などに関与する分子パスウェイとその機構の解明を目指す。

B.　研究方法

幹細胞様神経芽腫細胞株：同一の神経芽腫細胞株から派生し、神経分化タイプ(N-type neuroblastic/neuroendocrine precursors)、Schwannian タイプ(S-type Schwannian/melanoblastic precursors)、そして N-type、S-type 両方への分化能を有することから中間タイプあるいは幹細胞様タイプ(I-type stem cells)と呼ばれる 3 つの形態的に分離されたサブラ イン (Ross RA et al, *Cancer Lett.* 197:35-9, 2003) 2 種類について、アレイ CGH 解析と遺伝子発現解析を行なった。今回は SK-N-BE ならびに SH-SY5Y 由来のサブライン間の比較を行なった。

神経芽腫組織の選択：全国の小児がん治療施設において検査後の残余サンプルの研究使用についての文書による説明と同意取得ののち、千葉県がんセンターの神経芽腫組織バンクに寄託された腫瘍検体 48 例を対象とした。神経芽腫は病期、診断時年齢、*MYCN* 遺伝子増幅の有無など既知の予後マーカーにより、予後良好群、予後不良群、中間予後群に分類されるが、本研究では予後良好例と予後不良例の各 8 例、合計 16 例の遺伝子発現解析を行った。

アレイ CGH 解析： 500ng のゲノム DNA を対象とし、直接標識法により DNA の蛍光標識を行ないヒトオリゴアレイ（アジレント社 Whole Human Genome oligo DNA microarray、244k フォーマット）を用いてゲノムコピー数異常解析を行った。

遺伝子発現解析：神経芽腫細胞株ならびに神経芽腫の凍結腫瘍組織から調製した small RNA 分画を含む total RNA を調製

し、そのうち 5μg を用いて蛍光標識を行ない、小児がん特化型 DNA チップならびに市販遺伝子発現解析用 DNA チップ（アジレント社 Whole Human oligo DNA microarray、4x44k フォーマット）へのハイブリダイゼーションを行った。マイクロ RNA 発現レベルの解析には miRNA 解析用マイクロアレイ（アジレントテクノロジー社）を用いて既知の miRNA の発現レベルを網羅的に解析した。神経芽腫細胞株については N-type と I-type の比較を、腫瘍組織由来 RNA の解析には進行例と予後良好例についてそれぞれ発現パターンを比較検討した。

（倫理面への配慮）

本研究で行った臨床検体を用いた実験は、関連法規を遵守し、倫理委員会ならびに実施機関長の承認を経た上で、検体提供者の人権の擁護、個人情報保護に細心の注意を払って実施した。

C. 研究結果

神経芽腫がん幹細胞の分離・同定と新規マーカーの発見を目的とした基礎データの取得：

同一の神経芽腫細胞株から派生した神経分化タイプ(N-type)、Schwannian タイプ(S-type)、そして N-type、S-type 両方への分化能を有する中間タイプ(I-type)と呼ばれる 3 つの形態的に分離されたサブライン 2 種類について、アレイ CGH 解析と遺伝子発現解析を行なった。I-type の細胞株はこれまでにヌードマウスへの移植実験における高い腫瘍形成能、軟寒天培地における足場非依存性増殖能の亢進などの結果から幹細胞様の性質を持つことが報告されており(Ross RA et al, 2003)、その特徴的な発現を明らかにすることは今後の実験系、ならびに臨床検体の結果の解釈において重要なヒントとなると考えられる。アレイ CGH 解析から SK-N-BE 株、SH-SY5Y 株それぞれで I-type で獲得されたゲノム異常が 1p 欠失、2p 欠失や増加など各染色体に散見されたが、これまでに両細胞株の I-type に共通に獲得されたゲノム異常は見いだされなかった。一方、遺伝子発現プロファイリングからは N-type 特異的遺伝子群、I-type 特異的遺伝子群など特徴的な遺伝子候補が合計 36 種類同定された。I-type 細胞株において特に高発現が見られた遺伝子群には、これまでに進行神経芽腫において特異的に高発現していることが報告されている神経増殖関連転写因子群、転写因子複合体に含まれるアダプタータンパク質、MAP キナーゼ群などが候補として含まれていたことから、今後 RT-PCR 法などによる検証は必要であるが、これらのプロファイルが I-type 細胞株の強い増殖性に強く関連していることが示唆された。

予後良好例及び予後不良例の神経芽腫検体の遺伝子発現解析：

これまでに複数のグループから神経芽腫予後良好例と予後不良例の間で遺伝子発現レベルが有意に異なる遺伝子群の報告がなされている。これらは予後診断マーカーとして有効に利用されつつあるが、難治性神経芽腫の幹細胞性の維持に関与する遺伝子群の同定は未だ進んではいない。本研究ではまず基礎的データとして、これまでにまだ十分なデータが揃っていない miRNA の遺伝子発現レベルについて今回解析を行ない、これまでに得られた遺伝子発現プロファイルや幹細胞性維持に関与する遺伝子群との関連性について検討を行った。神経芽腫予後良好例と予後不良例各 8 例、合計 16 例について既知の miRNA の発現レベルを解析した。両群の間で Fold Change>2.0、かつ t-test で有意差($p<0.05$)を示す miRNA を 70 種類ピックアップし、TargetScan データベースに公開されている各 miRNA の予想ターゲット遺伝子のリストと、進行神経芽腫における遺伝子発現レベルとの相関を検討したところ、mRNA 発現レベルが有意に予後と相関する遺伝子群が複数含まれていた。さらに、上記 I-type 幹細胞様神経芽腫細胞株と N-type 細胞株との

miRNA 発現プロファイルの比較も並行して進めており、これらに共通した挙動を示す miRNA ならびにそのターゲット遺伝子の統合的解析を行なうことにより、幹細胞性維持に関わる分子パスウェイの検索を今後進めていく計画である。

考察：

今回解析を行なった複数の N-type および I-type 神経芽腫細胞株の間に共通のゲノム異常が見られなかったことから、両者の差は *MYCN* 増幅の有無によって、そのメカニズムが異なる可能性があること、あるいは N-type 細胞株の性質がゲノムコピーの変化としては現れないエピジェネティックなプロファイルの特徴に起因するものである可能性が考えられる。この点に関しては、さらに多くの *MYCN* 増幅、非増幅の神経芽腫細胞株ならびに臨床サンプルを用いた Neurospere 形成、iPS 化時のゲノムならびにトランスクリプトーム解析を行なうことにより、詳細が明らかになると期待される。本研究班の他の分担研究において進められている神経芽腫細胞株のゲノムワイドなヒストン修飾解析とも連携しながらこれらについて明らかにしていきたい。

D. 研究発表

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E. 知的財産権の出願・登録状況

1. 特許取得
   無し

2. 実用新案登録
   無し

3. その他

厚生労働科学研究費補助金（第3次対がん総合戦略研究事業）
分担研究報告書

遺伝子改変マウスを用いた神経芽腫発がんに関わる新規遺伝子の解析

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研究要旨
脱ユビキチン化酵素であるUSP7/HAUSPが、ポリコム群、Ink4a、p53、MDM2などを介した細胞増殖や細胞死を制御することが考えられている。このメカニズムを解明するために、本年度はUSP7/HAUSPをコンディショナルに欠損しうるマウス系統を作成し、コンディショナルKO-ES細胞を樹立した。

A．研究目的

ポリコム群複合体（PRC１）を精製したところ、そこにユビキチン特異的分解酵素７（USP7/HAUSP）を見出した。USP7/HAUSPは、p53とMDM2を脱ユビキチン化し、それらを機能的にバランスさせて、がん細胞の増殖を制御する。一方、PRC1とMBLR複合体とをバランスさせることで、ホメオボックス遺伝子の転写抑制を制御することを我々は最近明らかにした。本研究では、USP7/HAUSPがどのようにがん抑制遺伝子であるInk4aに作用するかを明らかにする。これにより、USP7/HAUSPを介した制御が、Ink4a、p53、MDM2をどのようにバランスさせ、正常細胞あるいはがん細胞の増殖や細胞死をバランスさせているのかを明らかにしうる。

B．研究方法

・USP7/HAUSPを薬剤依存的に欠損するマウスから、神経芽細胞腫などの腫瘍を実験的に誘導するシステムを樹立し、生体内あるいは試験管内において、USP7/HAUSPの腫瘍増殖への作用を解析する。

・USP7/HAUSPコンディショナル変異マウスとRing1Bコンディショナル変異、p５３変異、Ink4a変異等を交配して、２重変異MEFを作製し、表現型、遺伝子発現プロファイルを明らかにする。

C．研究結果

USP7/HAUSPコンディショナル欠損マウスを作製し、そこからタモキフェン依存的にUSP7/HAUSPを欠損するマウス系統を作出した。胎生６日目に経胎盤的にタモキシフェン処理を行ったところ、胎生１２日ごろに致死となることが明らかになった。USP7/HAUSPをタモキシフェン誘導的に欠損する

ES 細胞を作製したところ、USP7/HAUSP は ES 細胞の生存や増殖には必須ではないことが示された。USP7/HAUSP 欠損 ES 細胞における PRC1 の発現をウェスタンブロッティングによって調べたところ顕著な減少が見られた。しかし、PRC1 の標的遺伝子への結合には大きな変化が見られなかった。

D. 考察

USP7/HAUS は、PRC1 の発現制御に寄与することが明らかになり，その経路を介して ink4a の発現制御に寄与する可能性が示された。特に，PRC1 のクロマチンに結合していない分画をその標的としていることが考えられた。

E. 結論

新たな PRC1 発現の制御因子の候補として USP7/HAUS を同定することに成功した。

G. 研究発表

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厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
平成２２年度 分担研究報告書

難治性神経芽腫における特異的ヒストン修飾の解析とその臨床応用

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研究要旨

神経芽腫の癌幹細胞に近い状態におけるゲノムワイドなヒストン修飾を解析中であり、神経芽腫の癌幹細胞特異的なヒストン修飾と遺伝子発現制御を明らかにし、難治性神経芽腫の治療に有効なエピジェネティクス治療の探索につなげる予定である。

A．研究目的

遺伝子異常に加えて、エピジェネティックな転写制御の異常が癌の悪性化に関わることが明らかになりつつある。難治性神経芽腫におけるエピジェネティクス異常はまた解析されておらず、基盤となる情報が必要である。本研究では難治性神経芽腫におけるヒストン修飾のゲノムワイドな解析を通して難治性神経芽腫におけるエピジェネティクス異常の一端を明らかにするとともに、得られた知見をもとに、難治性神経芽腫の治療に有効なエピジェネティクス治療の可能性を探る。

B．研究方法

神経芽腫細胞株IMR32におけるゲノムワイドなヒストン修飾状態をクロマチン免疫沈降とDNAマイクロアレイを組み合わせたChIP-chip法により検証する。

（倫理面への配慮）

特になし。

C．研究結果

神経芽腫細胞株IMR32はスフェアを形成させることにより未分化状態を維持し、神経芽腫の癌幹細胞に近い状態を模倣することが可能である。このスフェアと通常の接着培養による分化度の高い細胞の２群を用いてゲノムワイドなヒストン修飾（H3K4me23, H3K27me3, H2AK119ub1, H3K9Ac）を解析中である。

D．考察

ゲノムワイドなヒストン修飾解析データと既に解析済みのトランスクリプトームを比較検討することにより、神経芽腫の癌幹細胞特異的なヒストン修飾と遺伝子発現制御を明らかにできるものと期待される。

E．結論

神経芽腫の癌幹細胞に近い状態におけるゲノムワイドなヒストン修飾を解析中であり、神経芽腫の癌幹細胞特異的なヒストン修飾と遺伝子発現制御を明らかにし、難治性神経芽腫の治療に有効なエピジェネティクス治療の探索につなげる予定である。

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Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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Ⅳ. 研究成果の刊行物・別刷

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 22, pp. 16693–16703, May 28, 2010
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RUNX3 Modulates DNA Damage-mediated Phosphorylation of Tumor Suppressor p53 at Ser-15 and Acts as a Co-activator for p53*

Received for publication, August 14, 2009, and in revised form, March 7, 2010 Published, JBC Papers in Press, March 30, 2010, DOI 10.1074/jbc.M109.055525

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Although it has been shown that the gastric tumor suppressor RUNX3 has a growth inhibitory activity, the precise molecular mechanisms behind RUNX3-mediated tumor suppression remained unclear. In this study, we found that RUNX3 is closely involved in DNA damage-dependent phosphorylation of tumor suppressor p53 at Ser-15 and acts as a co-activator for p53. The small interference RNA-mediated knockdown of RUNX3 inhibited adriamycin (ADR)-dependent apoptosis in *p53*-proficient cells but not in *p53*-deficient cells in association with a significant reduction of p53-target gene expression as well as phosphorylation of p53 at Ser-15. In response to ADR, RUNX3 was induced to accumulate in the cell nucleus and co-localized with p53. Immunoprecipitation experiments demonstrated that RUNX3 forms a complex with p53 in cells. *In vitro* pulldown assays revealed that the COOH-terminal portion of p53 is required for the interaction with RUNX3. Forced expression of RUNX3 enhanced p53-mediated transcriptional activation. Additionally, RUNX3 had an ability to induce the phosphorylation of p53 at Ser-15, thereby promoting p53-dependent apoptosis. Intriguingly, RUNX3 interacted with phosphorylated forms of ataxia telangiectasia-mutated in response to ADR; however, it did not affect the extent of DNA damage. From the clinical point of view, coordinated *p53* mutation and decreased expression of *RUNX3* in 105 human lung adenocarcinomas were significantly associated with the poor outcome of patients (p = 0.0203). Thus, our present results strongly suggest that RUNX3 acts as a novel co-activator for p53 through regulating its DNA damage-induced phosphorylation at Ser-15 and also provide a clue to understanding the molecular mechanisms underlying RUNX3-mediated tumor suppression.

RUNX3, which is mapped to human chromosome 1p36, is one of the RUNX family of transcription factors, including RUNX1–3 (1). The RUNX family contains the well conserved 128-amino acid region (Runt domain) and forms a stable complex with PEBP2β/CBFβ to exert its transactivation ability. Extensive studies demonstrated that RUNX1 plays an important role in the regulation of hematopoiesis (2, 3), whereas RUNX2 contributes to the generation and maturation of osteoblasts (4, 5). In contrast to RUNX1 and RUNX2, it has been shown that RUNX3 acts as a candidate tumor suppressor for human gastric cancers (6). According to their results, the *RUNX3* gene was rarely mutated in primary gastric cancers; however, its expression levels were significantly down-regulated in primary gastric cancers and gastric cancer-derived cell lines, which might be due to the combination of its hemizygous deletion and the hypermethylation of its promoter region. Additionally, a mutation (R122C) found within the Runt domain of RUNX3 resulted in a complete lack of its tumor suppressive activity.

Subsequent studies revealed that the frequent reduction of *RUNX3* expression levels is also observed in several human cancers such as lung cancer, breast cancer, colon cancer, pancreatic cancer, and prostate cancer, which might be attributed to promoter hypermethylation (7–13), indicating that the down-regulation of *RUNX3* is not restricted to gastric cancer. Intriguingly, Yano *et al.* (15) demonstrated that, during transforming growth factor-β-mediated apoptotic cell death, RUNX3 has an ability to transactivate pro-apoptotic *Bim* (Bcl-2-interacting mediator of cell death) (14) in gastric cancer-derived cell lines. Based on their observations, RUNX3 was induced to translocate into the cell nucleus in response to TGF-β[3] in association with a significant up-regulation of *Bim*, suggesting that RUNX3 has a critical role in the regulation of TGF-β-mediated apoptotic cell death. In support of this notion, Yamamura *et al.* (16)

* This work was supported in part by a grant-in-aid from the Ministry of Health, Labor and Welfare for Third Term Comprehensive Control Research for Cancer, a grant-in-aid for scientific research on priority areas from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and a grant-in-aid for scientific research from Japan Society for the Promotion of Science.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

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[3] The abbreviations used are: TGF-β, transforming growth factor β; ADR, adriamycin; DAPI, 4, 6-diamidino-2-phenylindole; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; RT, reverse transcription; siRNA, small interference RNA; ATM, ataxia telangiectasia-mutated.

described that RUNX3 cooperates with Forkhead transcription factor FoxO3a/FKHRL1 to induce apoptotic cell death through transcriptional activation of *Bim*. In addition, the gastric epithelium of *RUNX3*-knockout mice exhibited a hyperplasia, a reduced rate of apoptotic cell death, and a lower sensitivity to TGF-β (6). Recently, it has been shown that, in addition to the hemizygous deletions and promoter hypermethylation, protein mislocalization of RUNX3 to cytoplasm is an alternative molecular mechanism behind the dysfunction of RUNX3 in gastric and breast cancers (17, 18). Jin *et al.* (19) found that p300 with histone acetyltransferase activity acetylates RUNX3 to protect its proteolytic degradation mediated by the E3 ubiquitin protein ligase Smurf.

p53 is a founding member of the p53 tumor suppressor family of sequence-specific nuclear transcription factors, including p53, p73, and p63 (20, 21). In response to DNA damage, p53 is induced to stabilize and exert its pro-apoptotic function. DNA damage-induced post-translational modifications of p53, such as phosphorylation and acetylation, play a critical role in the regulation of p53. The activated form of p53 has an ability to transactivate its direct target genes implicated in cell cycle arrest and/or apoptotic cell death, including $p21^{WAF1}$, *BAX*, *PUMA*, *NOXA*, and *p53AIP1* (20). Thus, the sequence-specific transactivation activity of p53 is tightly linked to its pro-apoptotic function (22). In a sharp contrast to *p73* and *p63*, *p53* is frequently mutated within its sequence-specific DNA-binding domain in primary human cancers (23–25). Indeed, *p53*-deficient mice developed spontaneous tumors (26).

In this study, we found for the first time that there exists a functional relationship between RUNX3 and p53. Based on our present results, RUNX3 is closely involved in the regulation of DNA damage-mediated phosphorylation of p53 at Ser-15 and acts as its co-activator.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—African green monkey embryonic kidney COS7, human cervical carcinoma HeLa, human osteosarcoma U2OS, and SAOS-2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% of heat-inactivated fetal bovine serum (Invitrogen), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Human lung carcinoma H1299 cells were cultivated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotic mixture. Cells were grown at 37 °C in a water-saturated atmosphere of 95% air and 5% CO_2. Where indicated, cells were exposed to adriamycin (ADR). Transient transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions.

RT-PCR—U2OS cells were treated with the indicated concentrations of ADR. At the indicated time after ADR treatment, total RNA was prepared using an RNeasy mini kit (Qiagen, Valencia, CA). One microgram of total RNA was used to synthesize the first-strand cDNA by using random primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The resultant cDNA was subjected to the PCR-based amplification. Oligonucleotide primer sets used were as follows: *RUNX3*, 5′-CAGAAGCTGGAGGACCAGAC-3′ (sense) and 5′-TCGGAGAATGGGTTCAGTTC-3′ (antisense); *p53*, 5′-ATTTGATGCTGTCCCCGGACGATATTGA-3′ (sense) and 5′-ACCCTTTTTGGACTTCAGGTGGCTGGAGT-3′ (antisense); *BAX*, 5′-AGAGGATGATTGCCGCCGT-3′ (sense) and 5′-CAACCACCCTGGTCTTGGAT-3′ (antisense); $p21^{WAF1}$, 5′-GACACCACTGGAGGGTGACT-3′ (sense) and 5′-GGCGTTTGGAGTGGTAGAAA-3′ (antisense); *PUMA*, 5′-GCCCAGACTGTGAATCCTGT-3′ (sense) and 5′-TCCTCCCTCTTCCGAGATTT-3′ (antisense); *p53AIP1*, 5′-ACCAGAACCTCTCGGTGATG-3′ (sense) and 5′-AAGGAAAGGCCTGGAGAGAC-3′ (antisense); and *GAPDH*, 5′-ACCTGACCTGCCGTCTAGAA-3′ (sense) and 5′-TCCACCACCCTGTTGCTGTA-3′ (antisense). The expression of *GAPDH* was measured as an internal control. The PCR products were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining.

Construction of the Deletion Mutants of RUNX3—RUNX3(1–198) and RUNX3(1–67) were amplified by PCR with the following primer sets: 5′-CG**GAATTC**CGATGGCATCGAACAGCATCTT-3′ (sense) and 5′-GAGCCCAGACGGCACCGGTAACGG**CTCGAG**CGG-3′ (antisense); 5′-CG**GAATTC**CGATGGCATCGAACAGCATCTT-3′ (sense) and 5′-GCCCGGCCCGAGGTGCGCTAACCG**CTCGAG**CGG-3′ (antisense), respectively. PCR primers included 5′-EcoRI and 3′-XhoI restriction sites (boldface) to aid cloning. PCR products were digested completely with EcoRI and XhoI, gel-purified, and inserted into the identical sites of pcDNA3 to give pcDNA3-RUNX3(1–198) and pcDNA3-RUNX3(1–67). The nucleotide sequences of these expression plasmids were verified by DNA sequencing.

Immunoblotting and Immunoprecipitation—For immunoblotting, cells were lysed in a lysis buffer containing 25 mM Tris-HCl, pH 7.5, 137 mM NaCl, 2.7 mM KCl, 1% Triton X-100, and protease inhibitor mixture (Sigma), and spun to separate insoluble debris from the clear lysates. Equal amounts of cell lysates were separated by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). The transferred membranes were incubated with monoclonal anti-$p21^{WAF1}$ (Ab-1, Oncogene Research Products, Cambridge, MA), monoclonal anti-p53 (DO-1, Oncogene Research Products), monoclonal anti-BAX (6A7, eBioscience, San Diego, CA), monoclonal anti-PARP (F-2, Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-γH2AX (2F3, BioLegend, San Diego), polyclonal anti-RUNX3 (Active Motif, Carlsbad, CA), polyclonal anti-phosphorylated p53 at Ser-15 (Cell Signaling, Beverly, MA), polyclonal anti-ATM (Ab-3, Calbiochem), polyclonal anti-PUMA (Ab9643, Abcam, Cambridge, UK), or with polyclonal anti-actin (20-33, Sigma) antibody followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Bound antibodies were visualized by the ECL system (Amersham Biosciences). For immunoprecipitation, 1 mg of protein was incubated with 25 μl of protein G-Sepharose beads (Amersham Biosciences). The pre-cleaned lysates were incubated with polyclonal anti-RUNX3 antibody for 2 h at 4 °C, and immunocomplexes were precipitated with protein G-Sepharose beads for additional 1 h at 4 °C. The immunocomplexes were washed three times with the lysis buffer, eluted from beads