NFκB mediates cisplatin resistance through histone modifications in head and neck squamous cell carcinoma (HNSCC)☆

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ABSTRACT

Cisplatin-based chemotherapy is the standard treatment of choice for head and neck squamous cell carcinoma (HNSCC). The efficiency of platinum-based therapies is directly influenced by the development of tumor resistance. Multiple signaling pathways have been linked to tumor resistance, including activation of nuclear factor kappa B (NFκB). We explore a novel mechanism by which NFκB drives HNSCC resistance through histone modifications. Post-translational modification of histones alters chromatin structure, facilitating the binding of nuclear factors that mediate DNA repair, transcription, and other processes. We found that chemoresistant HNSCC cells with active NFκB signaling respond to chemotherapy by reducing nuclear BRCA1 levels and by promoting histone deacetylation (chromatin compaction). Activation of this molecular signature resulted in impaired DNA damage repair, prolonged accumulation of histone γH2AX and increased genomic instability. We found that pharmacological induction of histone acetylation using HDAC inhibitors prevented NFκB-induced cisplatin resistance. Furthermore, silencing NFκB in HNSCC induced acetylation of tumor histones, resulting in reduced chemoresistance and increased cytotoxicity following cisplatin treatment. Collectively, these findings suggest that epigenetic modifications of HNSCC resulting from NFκB-induced histone modifications constitute a novel molecular mechanism responsible for chemoresistance in HNSCC. Therefore, targeted inhibition of HDAC may be used as a viable therapeutic strategy for disrupting tumor resistance caused by NFκB.

Introduction

Chromatin is the state in which DNA is packaged within the cell. The nucleosome is the fundamental unit of chromatin, and it is composed of an octamer of histones (H2A, H2B, H3 and H4) around which 147 base pairs of DNA are wrapped. In eukaryotes, the chromatin structure profoundly affects replication, transcription, and repair by interfering with the accessibility of DNA to enzymes that carry out these processes [1]. Changes in DNA folding driven by histone acetylation and deacetylation dynamically regulate gene expression, resulting in alterations of the cellular response to environmental cues. Histones are considered molecular markers of epigenetic changes and, in combination with their modifiers, modulate tumor behavior and cellular phenotype [2,3]. However, little is known about how histones are modified in response to genotoxic agents.

Cisplatin (cis-Diamminedichloroplatinum II) is a chemotherapeutic agent used in the treatment of a variety of human cancers. The mechanism of cisplatin action involves forming covalent platinum-DNA adducts that cause DNA damage [4]. Cisplatin incorporation in the genome triggers various cellular responses, including DNA repair, inhibition of transcription, cell cycle arrest, and apoptosis, all processes that require remodeling of the structural and dynamic properties of chromatin [5]. Despite new surgical techniques and chemotherapy protocols, tumor resistance to cisplatin remains a significant challenge for head and neck squamous cell carcinoma HNSCC patients. Numerous mechanisms underlie chemoresistance, including genetic and epigenetic alterations in the cancer cell that may be acquired

during the treatment cycles [6,7]. Additionally, cancer cells may circumvent treatment by increasing drug inactivation or efflux [8–10], disrupting tumor suppressor genes [11], altering DNA damage repair (DDR) [12,13], and activating mitogenic signaling pathways, such as nuclear factor kappa B (NFκB) signaling, that result in reduced apoptosis (reviewed in Ben-Neriah et al. [14]).

The mammalian NFκB family of proteins is comprised of RelA/p65, NFκB1, NFκB2, c-Rel, and RelB subunits that form a variety of dimers to control gene expression downstream of signals elicited by cytokines, bacterial products, viral expression, growth factors, and stress stimuli. NFκB is negatively regulated by interacting with the IκB family of proteins, which prevent DNA binding and promote cytoplasmic accumulation of NFκB family members. NFκB is positively regulated by the IκB kinase (IKK) complex that phosphorylates IκB proteins and induces their degradation, thereby permitting NFκB to translocate to the nucleus. Once in the nucleus, NFκB binds to target DNA sequences and regulates the expression of genes involved in the immune response, cell growth, and cell survival [15].

We identified a novel mechanism by which NFκB signaling drives chemoresistance in HNSCC. We found that NFκB acts as a novel modulator of chromatin modifications following cisplatin treatment by preventing histone acetylation and inducing condensation of tumor chromatin. Furthermore, NFκB-induced cisplatin resistance was prevented following histone acetylation achieved through the administration of HDAC inhibitors or by silencing the NFκB signaling pathway.

Results

Chemoresistant HNSCC cell lines have reduced nuclear size and active NFκB signaling

Patients suffering from advanced stage head and neck tumors are often treated with a combination of surgery and chemotherapy. Platinum based therapies are considered the standard of care for many solid tumors, including HNSCC, but the development of chemoresistance is common [16]. Tumor resistance to chemotherapy is a major problem during therapy, especially for patients presenting tumor spread beyond the primary site. For these patients, chemotherapy represents the basis of their treatment and is critical in determining their survival [10]. We used a heterogeneous panel of HNSCC cell lines that have varying degrees of sensitivity to cisplatin. In response to cisplatin, HN13, UMSCC74A and Cal27 had an IC50 of more than 7 μg/ml and were deemed chemoresistant. HN6, UMSCC17B and the control normal oral keratinocyte (NOK-SI) cell line had an IC50 under 5 μg/ml and were deemed chemosensitive (Fig. 1A). Interestingly, the active subunit of NFκB, as identified by the phospho-p65 antibody, was localized in the cytoplasm of cisplatin-sensitive tumor cells and in the nucleus of cisplatin-resistant cells (Fig. 1B). NFκB is activated following translocation from the cytoplasm to the nucleus where is functions as a transcription factor and as a pro-survival factor [17]. Active NFκB promotes HNSCC resistance to chemotherapy and radiotherapy by preventing tumor cells from undergoing apoptosis [18]. We found that treating HNSCC cells with cisplatin (5 μg/ml) for 24 h caused noticeable morphological changes in the nucleus. To characterize this phenotype, we stained tumor cells with Hoechst 33342 and analyzed the nucleus by morphometry. We measured fifty nuclei from each cell line. Unexpectedly, we found that chemosensitive cells and normal oral epithelial cells that show a dramatic increase in nuclear size following cisplatin treatment (UMSCC 17B, HN6 and NOK-SI [***], $p < 0.001$) in contrast to chemoresistant tumor cells that respond to cisplatin either by reducing the size of their nucleus (UMSCC74A and HN13 [***], $p < 0.001$) or maintaining its original size (Cal27) (Fig. 1C). These findings suggest that tumor cells respond to chemotherapy through alterations in chromatin condensation/decondensation and organization. Notably, changes in chromatin condensation correlate

to decreases or increases in nuclear diameter [19,20]. These findings encouraged us to examine the effect of chemotherapy on histones given that acetylation disrupts the electrostatic interactions between histone and DNA, resulting in a decrease in chromatin condensation [21].

Deacetylation of tumor histones is accompanied by reduced levels of BRCA1 and enhanced genomic instability in chemoresistant tumor cells

Chromatin structure is involved in a range of well-characterized DNA metabolism processes, including replication [22], transcription [23] and DNA damage repair [24]; however, the role of chromatin organization in chemoresistance is unknown. To assess whether changes in nuclear size are associated with chromatin condensation, we treated HNSCC and control cells with several concentrations of cisplatin (1, 2.5 or 5 μg/ml) for 24 h and analyzed acetyl histone H3 (Lys9) protein levels (Fig. 2A). Dynamic variations in the organization of chromatin structure are mediated by histone acetylation and deacetylation. Following cisplatin exposure, we found that chemoresistant tumor cells had reduced nuclear size and deacetylation of histone H3 (Lys9), a known marker of chromatin relaxation (Fig. 2A_Ac.H3) [25,26]. Histone deacetylation is involved in enhanced histone–DNA interactions and chromatin condensation [27–29]. Interestingly, cisplatin had no effect on histone H3 levels in chemosensitive and control cells, as evidenced by the similarities to vehicle control. Of interest to our analysis, functional acetylation of histone 3 at Lys 9 is associated with histone deposition, chromatin assembly, and gene activation [30–32].

Chromatin decondensation, also known as decondensation kinetics, is a key mechanism that ensures proper gene transcription and repair of the genome in which histone acetylation plays a central role in unwinding of DNA [33]. Activation of the DNA damage response machinery triggers chromatin decondensation and access to DNA repair proteins [12]. Because chromatin organization is essential for the accessibility of DNA repair molecules, we examined whether cisplatin affects BRCA1 accumulation and cell death in chemoresistant tumor cells. Cisplatin (1, 2.5 or 5 μg/ml) treatment for 24 h (Fig. 2A) did not prevent BRCA1 accumulation in HN6 and UMSCC17B chemosensitive cells. In contrast, chemoresistant cells treated with cisplatin had compacted chromatin evident by reduced levels of acetylated histone 3 (Fig. 2A_Ac.H3) and reduced BRCA1 levels (Fig. 2A_BRCA1). The protein encoded by the BRCA1 breast cancer susceptibility gene protects the genome by activating cell cycle checkpoints and participating in DNA repair [13]. Following extensive DNA damage, BRCA1 translocates to the nucleus to induce cell cycle arrest and cell death; however, dysfunctional BRCA1 enhances genomic instability [14,34–36]. We found that BRCA1 is localized to the nucleus in chemosensitive tumor cells compared to its cytoplasmic localization in chemoresistant tumor cells (Supplementary Fig. S1 – arrow head). Reduced BRCA1 in the nucleus of HNSCC cells correlated with increased genomic instability following 24 h of cisplatin (5 μg/ml) treatment and 24 h of recovery (Fig. 2B). As determined by Comet assay, cisplatin produced more DNA damage in HN6 cells ([***] $p < 0.001$) compared to HN13 cells ([**] $p < 0.01$). Although HN6 cells are chemosensitive, they recovered from DNA damage more efficiently than HN13 chemoresistant cells ([***] $p < 0.001$ and NS respectively). To confirm these results, we assessed recovery of DNA damage in HN6 and HN13 cells using immunofluorescence and the anti-γH2AX antibody. Histone γH2AX plays an important role in assembling the DNA damage response complex by identifying DNA double strand breaks (DSB) in the chromatin. Upon DSB, ATM induces phosphorylation of γH2AX at serine 139, resulting in recruitment of BRCA1, BRCA2, Rad51, Mre11, NBS1, FANCD2 and p53 repair proteins to sites of DNA damage [37,38]. Prolonged maintenance of phosphorylated γH2AX foci in the chromatin represents unrepaired DSB, making this molecule a suitable marker for assessing DNA repair activity and length of time for repair [37,38]. We
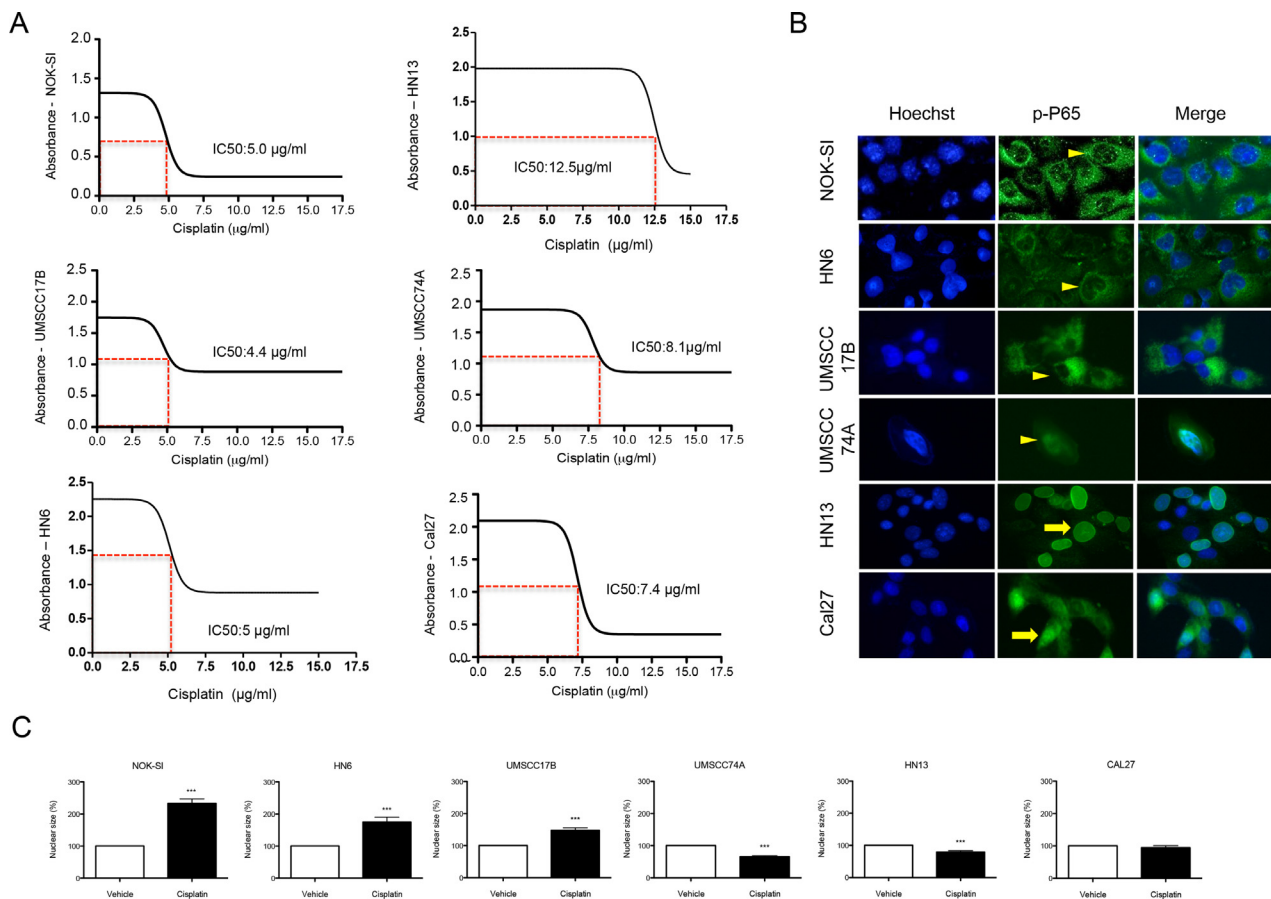
Fig. 1. *Cisplatin resistant HNSCC cell lines have NFkB activity and decreased nuclear size.* (A) Graphic determination of the inhibitory concentration (IC50) of cisplatin using a cell viability assay. NOK-SI, UMSCC17B, HN6, HN13, UMSCC74A and Cal27 cell lines were treated with several concentrations of cisplatin (2.5, 5, 7.5, 10, 12.5, 15, 17.5 and 20 μg/ml) for 24 h. (B) Immunofluorescence staining localization of phospho-p65 (p-P65) in NOK-SI, UMSCC 17B, HN6, HN13, UMSCC 74A and Cal27 cells. Arrowheads indicate nuclei without p-P65 staining. Note nuclear localization of p-P65 in HN13 and Cal27 cells (arrow). Nuclei were stained with Hoechst 33342 (blue). (C) Graphics depict nuclear size in NOK-SI, UMSCC 17B, HN6, HN13, UMSCC 74A and Cal27 cells treated with vehicle or cisplatin (5 μg/ml) for 24 h ($n = 150$ cells/condition). Reduced nuclear size was observed in HN13 ($^{***}p < 0.001$), UMSCC 74A ($^{***}p < 0.001$) and Cal27 cells. Increased nuclear size was observed in NOK-SI ($^{***}p < 0.001$), UMSCC 17B ($^{***}p < 0.001$) and HN6. ($^{***}p < 0.001$) (For interpretation of the references to color in this figure legend, the reader is reffered to the web version of this article.)

quantified γH2AX foci at different time points during DNA damage recovery (5, 10, 15 and 60 min and 6, 12 and 24 h) (Supplementary Fig. S2). After 5 min, both cell lines had reduced accumulation of γH2AX foci, indicating that nearly half of the DSBs were repaired. In contrast, HN6 cells with BRCA1 localized to the nucleus were still in the process of DNA repair (reduced levels of γH2AX_mean 1.15), which had completely failed in HN13 cells (stable levels of γH2AX_mean 2.27). Collectively, these findings support the notion that tumor cells resistant to chemotherapy respond to cisplatin through chromatin condensation that prevents access of BRCA1 to sites of DNA damage. We still do not know if decondensation of tumor chromatin by histone acetylation will improve the cytotoxicity of cisplatin.

Pharmacological acetylation of histones sensitizes tumor cells to cisplatin

The acetylation process is determined by histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzyme activities. HATs transfer acetyl groups to lysines in the tail of histones, promoting the expansion of chromatin and increasing accessibility of the DNA. In contrast, HDACs catalyze the removal of acetyl groups, leading to chromatin condensation [39]. Because chemoresistant cells have a smaller nucleus and reduced histone acetylation following cisplatin treatment compared to chemosensitive cells, we decided to induce

histone acetylation pharmacologically. We treated HN6 (chemosensitive) and HN13 (chemoresistant) cells with the trichostatin A (TSA) HDAC inhibitor and cisplatin for 24 h and analyzed cell viability by MTS. TSA increased cisplatin-induced cytotoxicity in both tumor cell lines (Fig. 3A). Maximum cytotoxicity was achieved in both cells at low doses of cisplatin (5 μg/ml) which become indistinguishable from cellular viability achieved by high doses of cisplatin (12.5 μg/ml) (ns $p > 0.05$) compared to HN6 and HN13 tumor cells receiving vehicle plus cisplatin ($^{***}p < 0.001$ and $^{**}p < 0.01$ respectively). Nuclear staining using Hoechst 33342 revealed increased nuclear size in both cell lines in response to TSA (Fig. 3A_ Hoechst 33342).

We next wanted to determine whether NFκB plays a role in chromatin condensation given that NFκB was activated (nuclear accumulation) in chemoresistant HN13 cells and inactivated (cytoplasmic accumulation) in HN6 cells (chemosensitive). Numerous signaling proteins can activate NFκB, including TNF-α, which triggers rapid degradation of IκBα that is mediated by TNFR1 and results in nuclear accumulation of NFκB [27,40] (Supplementary Fig. S3). We activated NFκB in chemosensitive HN6 cells, which normally have low NFκB, using TNF-α and treated with cisplatin before staining nuclei with Hoechst 33342 (Fig. 3B upper panel). Interestingly, activation of NFκB alone did not significantly affect HN6 chromatin organization, as determined by nuclear size. However, induction of the NFκB pathway using TNF-α partially blocked cisplatin-induced nuclear decondensation (Fig. 3B, cisplatin + TNF-α; $^{**}p < 0.01$) compared to cisplatin
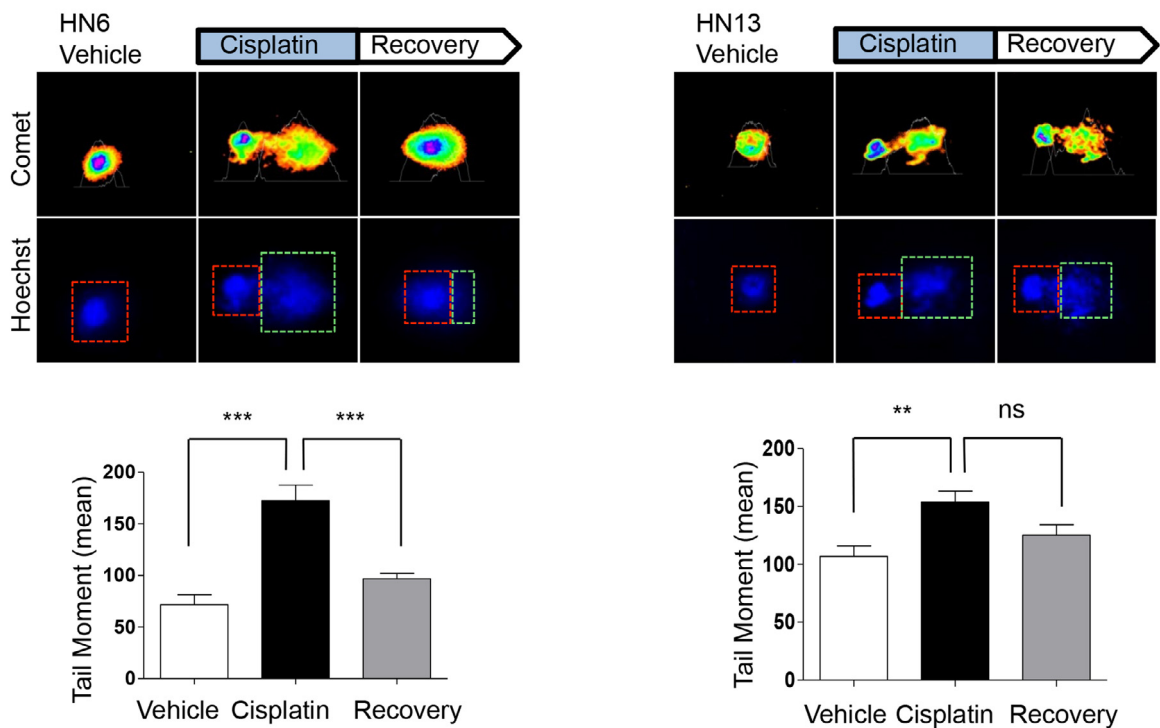
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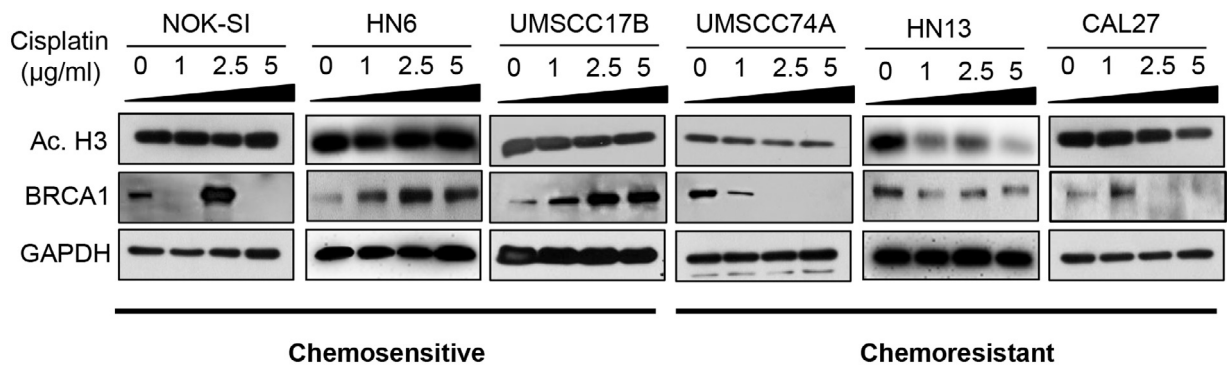
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Fig. 2. *HNSCC chemoresistance to cisplatin is associated with loss of BRCA1 and chromatin acetylation and failure of the DNA repair machinery.* (A) Western blot assay of NOK-SI, UMSCC 17B, HN6, HN13, UMSCC 74A and Cal27 cells treated with cisplatin (1, 2.5 and 5 μg/ml) for 24 h and probed with anti-acetyl-H3 and anti-BRCA1 antibodies. GAPDH served as a loading control. Note that chemosensitive cells have increased BRCA1 and acetyl-H3, and chemoresistant cells have reduced BRCA1 and acetyl-H3. (B) Representative microphotographs of the alkaline comet assay depict DNA fragmentation in response to vehicle and cisplatin treatment. The DNA damage recovery phase was established after cisplatin was removed for 24 h. Note the undamaged HN6 and HN13 cells (vehicle; comet head only) and fragmentation of DNA following cisplatin treatment (cisplatin; tail formation of the comet). HN6 displayed DNA damage repair (HN6 recovery; comet head only) in contrast to HN13 (HN13 recovery – maintenance of the tail). Comet assay quantification showed DNA damage in HN6 ($^{***}p < 0.001$) and HN13 ($^{**}p < 0.01$) after cisplatin treatment. Note that HN6 lack DNA fragmentation (no comet tail) after 24 h of cisplatin withdrawal ($^{***}p < 0.001$) and HN13 DNA remain fragmented during the entire recovery period (NS $p > 0.05$). ($n = 200$ cell/condition; error bar: mean $\pm$ SEM).

alone.

An active NFκB pathway is often associated with tumor survival and chemoresistance in many tumors, including HNSCC. However, the mechanism by which NFκB drives and sustains tumor survival and enhances tumor resistance remains largely unknown (reviewed in Karin et al. [41]). To determine the role of NFκB in cisplatin resistance, we treated chemosensitive HN6 cells with TNF-α and cisplatin and assessed cell viability. Interestingly, TNF-α-induced upregulation of NFκB increased cell viability and abrogated cisplatin toxicity in HN6 cells (Fig. 3B – lower panel, $^{***}p < 0.001$).

NFκB regulates tumor resistance to chemotherapy by controlling chromatin folding and nuclear influx of BRCA1

We next examined whether disruption of NFκB would affect the chromatin condensation phenotype observed in chemoresistant cells. It is well established that NFκB activity is the result of IκB kinase alpha (IKKα)- and beta (IKKβ)-induced phosphorylation of IκB that permits nuclear transport of NFκB [28,29]. Thus, by interfering with IKKα and IKKβ, IκBα promotes NFκB ubiquitinization and degradation [41]. We used siRNA directed against IKKα and IKKβ to enable IκB to re-press NFκB in chemoresistant HN13 cells (Supplementary Fig. S4).
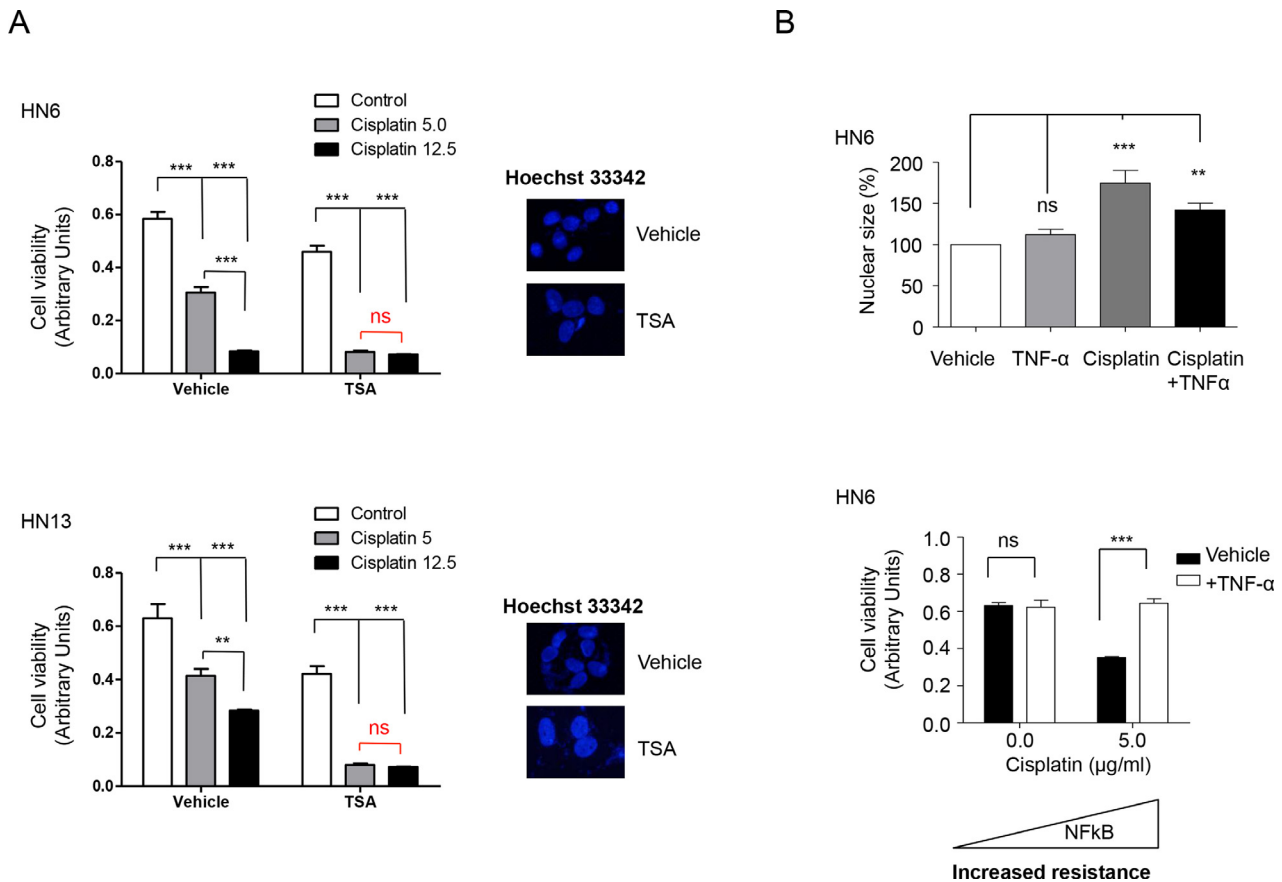
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Fig. 3. *Chromatin acetylation sensitizes cells to cisplatin and loss of NFκB results in increased nuclear size.* (A) HN6 and HN13 cells were treated with Trichostatin A (TSA) for 1 h prior to cisplatin administration. Immunofluorescence with Hoechst staining showed that TSA treatment resulted in increased nuclear size in both cell lines (immunofluorescence). MTS assay for cellular viability demonstrates enhanced cytotoxicity to cisplatin at 5 and 12.5 μg/ml following co-administration of TSA for 24 h compared to vehicle. Low doses of cisplatin (5 μg/ml) accompanied by TSA is sufficient to reduce cellular viability of HN6 and HN13 cells to similar levels observed during administration of 12.5 μg/ml of cisplatin. Note increased nuclear size of tumor cells receiving TSA (Hoechst 33342). (B) HN6 cells were treated with cisplatin (5 μg/ml), TNF-α (10 ng/ml), and cisplatin + TNF-α. An immunofluorescence assay was performed using Hoechst 33342 to define nuclear morphometry and cytokeratin 14 delineated cellular morphology. Chemosensitive HN6 cells respond to cisplatin treatment through chromatin decondensation ($^{***}p < 0.001$ cisplatin). Chromatin decondensation was impaired by co-administration of TNF-α ($^{**}p < 0.01$). Lower panel, HN6 cells, treated with cisplatin (5 μg/ml), TNF-α (10 ng/ml), and cisplatin + TNF-α, were evaluated for cellular viability using MTS assay. HN6 became resistant to cisplatin when exposed to TNF-α ($^{***}p < 0.001$). TNF-α (in the absence of cisplatin) did not alter cell viability.

Following knockdown of IKKα and IKKβ, HN13 cells were treated with cisplatin (5 μg/ml) and stained with Hoechst 33342. Surprisingly, suppression of IKKα and IKKβ resulted in increased nuclear size after treatment with cisplatin (Fig. 4A upper panel, $^{***}p < 0.001$). These results suggest that pharmacological acetylation of histones can act as chemical sensitizers in HNSCC, and that active NFκB signaling restricts chromatin decondensation in response to cisplatin.

We then examined whether suppression of NFκB would prevent chemoresistance in HN13 cells. We suppressed IKKα and IKKβ expression in HN13 cells using siRNA, treated cells for 24 h with cisplatin (5 and 12.5 μg/ml) and analyzed cell viability. HN13 cells were sensitized to 5 μg/ml of cisplatin following suppression of IKKα and IKKβ compared to 12.5 μg/ml of cisplatin when transfected with scramble siRNA (Fig. 4A lower panel). We next examined whether disruption of NFκB signaling alters histone acetylation and induces expression of BRCA1. Following suppression of IKKα and IKKβ with siRNA, HN13 cells were treated with TSA, cisplatin, or vehicle and lysed. Proteins were separated by Western blot and membranes probed for anti-Ac.H3 and anti-BRCA1. GAPDH served as a loading control. Suppression of IKKα and IKKβ resulted in accumulation of acetyl-histone 3 and increased BRCA1 compared to control cells (Fig. 4B). Furthermore, we analyzed whether NFκB signaling selectively regulate nuclear levels of BRCA1 during cisplatin induced DNA damage. Interestingly, although cisplatin does not increase accumulation of BRCA1 foci upon

administration of scramble siRNA in HN13 chemoresistant cells, disruption of NFκB signaling through silencing of IKKα resulted in a dramatic accumulation of nuclear BRCA1 (Fig. 4C, $^{**}p < 0.01$; and Supplementary Fig. S5). Aligned with these results, overexpression of NFκB signaling through administration of TNF-α resulted in reduced accumulation of BRCA1 in tumor cells receiving cisplatin while no statistical significance was observed in the control group with vehicle (Supplementary Fig. S6, cisplatin treated cells: $^{***}p < 0.001$; vehicle treated cells: ns, $p > 0.05$).

Collectively, our results indicate that NFκB plays a role in HNSCC tumor resistance by modulating chromatin organization through deacetylation of histones and DNA compaction (Fig. 5 – ↑NFκB). Changes in chromatin organization disrupt BRCA1 translocation to the nucleus, resulting in resistance to cisplatin. Disruption of NFκB signaling is sufficient to chemosensitize cells to cisplatin by acetylating chromatin and increasing BRCA1 levels (Fig. 5 – ↓NFκB).

Discussion

Resistance to anticancer drugs represents a major problem for the long-term effectiveness of chemotherapy. Deregulation of various genetic pathways drives drug resistance in cancer cells. Thus, novel therapeutic interventions that prevent drug resistance are needed. We found that cisplatin resistant cells have active NFκB signaling. NFκB
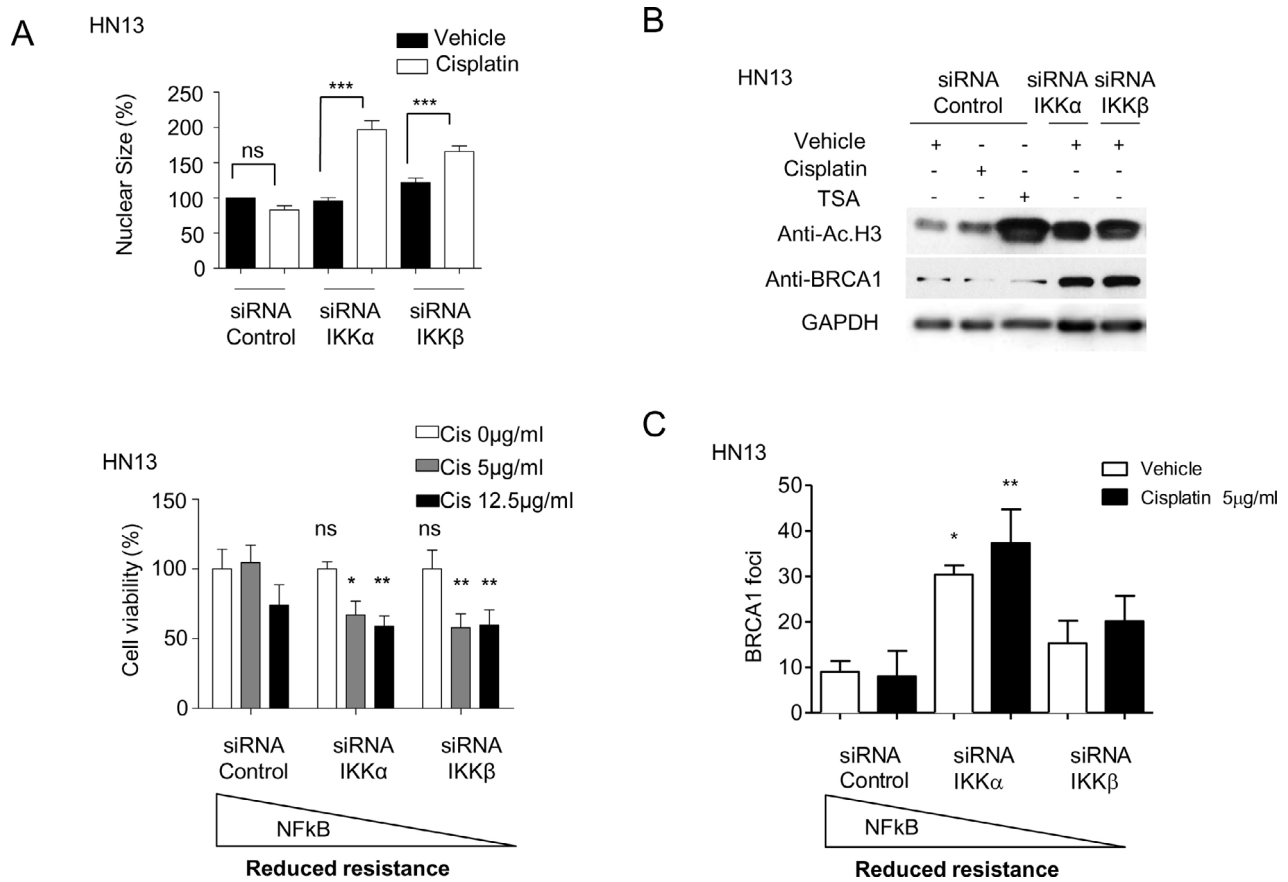
Fig. 4. *NFκB signaling induces tumor resistance and chromatin remodeling in HNSCC.* (A) HN13 cells were treated with cisplatin (5 μg/ml) after suppressing NFκB using small interference RNA for IKKα and IKKβ. Cellular morphometry denotes increased nuclear size of chemoresistant HN13 cells following siRNA interference. Note that maintenance of chromatin deacetylation and condensation, observed in HN13 cells treated with cisplatin, are overcome by small interference RNA for IKKα (siRNA IKKα, [***]p < 0.001) and IKKβ (siRNA IKKβ, [***]p < 0.001). After suppressing NFκB using small interference RNA for IKKα and IKKβ in HN13, cells were treated with cisplatin (5 and 12.5 μg/ml) (lower panel). IKKα and IKKβ knockdown alone did not alter cell viability. However, silencing NFκB sensitized the cells to the cisplatin treatment, as evidenced by reductions in viability in response to 5 μg/ml cisplatin (siRNA IKKα [*]p < 0.05 and siRNA IKKβ [**]p < 0.01). Note that chemoresistant HN13 cells respond to low doses of cisplatin (5 μg/ml), which is equivalent to the IC50 concentration observed initially in HNSCC cells sensitive to cisplatin. (B) Immunoblot analysis of HN13 cells transfected with siRNA Control and siRNA for IKKα and IKKβ. Cells were treated with vehicle, cisplatin (5 μg/ml), and TSA (100 ng/ml). BRCA1 levels were unchanged following vehicle, cisplatin or TSA treatments. BRCA1 was increased following NFκB blockage (siRNA IKKα and siRNA IKKβ). Additionally, HN13 cells with blocked NFκB (siRNA for IKKα and IKKβ) had abrupt H3 acetylation of chromatin. TSA treatment was used as a positive control for H3 acetylation, and GAPDH was used as a loading control. (C) BRCA1 foci formation was accessed upon disruption of NFκB signaling. Silencing of IKKα resulted in increased accumulation of BRCA1 foci compared to scramble siRNA treated cells ([**]p < 0.01). Administration of cisplatin resulted in further accumulation of BRCA1 foci.

is retained in the cytoplasm through interaction with its inhibitors (IκBs). In response to a variety of stimuli, IκBs are phosphorylated by IκB kinases (IKK) and degraded. This permits NFκB to translocate to the nucleus where it stimulates the expression of target genes [42,43]. Constitutive activation of NFκB is commonly observed in a variety of tumor types. Increased NFκB activity is often associated with the development of chemoresistance [16,41,44]; however, the mechanism underlying the chemoresistant phenotype is not understood. Recent advances in tumor biology have identified nuclear translocation of NFκB as a suppressive mechanism for programmed cell death. Additionally, inhibition of NFκB nuclear transport enhances apoptosis [45–47].

Chromatin compaction, gene expression, apoptosis and cellular division influence nuclear size [20]. Chromatin compaction is regulated via DNA binding proteins and histone modifications [48]. Deacetylation of histones promotes chromatin compaction, and acetylation results in chromatin decondensation, permitting accessibility to interacting proteins [49]. Collectively, changes in nuclear morphology correlate with global changes in histone acetylation [20]. We found that chemoresistant HNSCC cell lines respond to cisplatin treatment through reduction in nuclear size and histone deacetylation. These data prompted us to explore the molecular mechanism involved in

histone deacetylation and chromatin compaction given this process reduces the association of DNA with larger molecules, such as cisplatin and DNA repair complexes [50]. We showed that chemoresistant HNSCC cells treated with cisplatin had accumulation of DSB, but BRCA1 did not translocate to the nucleus, resulting in increased genomic instability. These data suggest an epigenetic mechanism by which tumor cells control chromatin accessibility through histone modifications. It has been proposed that HDAC proteins regulate NFκB activity through deacetylase and that chromatin acetylation using TSA is sufficient to inhibit this activity [51]. Therefore, it is conceivable that chromatin acetylation plays a role in chemoresistance in a NFκB-dependent manner. Yet, enhanced NFκB signaling may restrict its interaction with HDAC proteins, suggesting that highly active NFκB signaling often found in head and neck tumors may influence chromatin remodeling. Indeed, we found that activation of NFκB through co-administration of TNF-α and cisplatin in tumor cells resulted in a substantial reduction in the size of the nucleus, and that inhibition of NFκB by suppression of IKKα and IKKβ resulted in increased nuclear size and increased acetyl histone 3. In this case, directly targeting NFκB signaling in head and neck tumors influences chromatin acetylation and gene transcription.

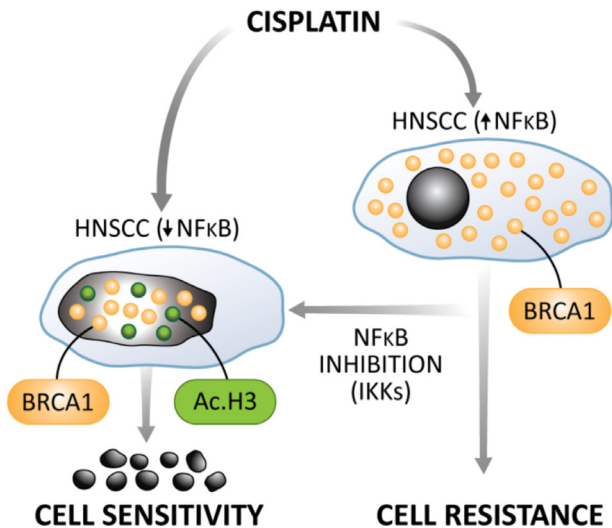The effect of NFκB on chromatin remodeling was a very exciting

Fig. 5. *Proposed mechanism of NFκB-induced chemoresistance in HNSCC.* Genotoxic therapy driven by cisplatin induces chromatin acetylation (Ac.H3) and increased nuclear influx of BRCA1. HNSCC cells with low levels of NFκB have reduced cellular viability and respond to cisplatin treatment. In contrast, tumor cells with high levels of NFκB fail to undergo chromatin acetylation, which is reflected in the lack of BRCA1 nuclear translocation and resistance to cisplatin chemotherapy. When NFκB is inhibited, cisplatin induces chromatin acetylation and nuclear translocation of BRCA1, resulting in chemosensitive HNSCC cells.

finding. Interfering with NFκB signaling resulted in chromatin decondensation and accumulation of BRCA1, potentially due to increased availability of DSBs to protein complexes from the DDR machinery. Indeed these findings led to another exciting result, which was that inhibition of NFκB sensitized HNSCC to cytotoxic chemotherapy. We do not know if the increased sensitivity to cisplatin following suppression of IKKα and IKKβ was a direct effect of low levels of NFκB or a consequence of chromatin acetylation induced by siRNA interference of the NFκB signaling. Regardless, the effect of NFκB signaling on chromatin remodeling is clear and indicates a new therapeutic opportunity for patients who have acquired resistance to cisplatin.

Our findings demonstrate that NFκB acts as a regulator of chromatin remodeling in HNSCC. This mechanism is triggered by genotoxic insults that result in chromatin deacetylation, increased genomic instability, and reduced levels of nuclear BRCA1; collectively, these factors confer resistance to HNSCC. Pharmacological inducton of chromatin acetylation by HDAC inhibitors, or interference with NFκB signaling, completely ablated tumor resistance to cisplatin. Nonetheless, reestablishment of BRCA1 was dependent on NFκB silencing. These findings provide new insights into the role of NFκB in chemoresistance in HNSCC.

Materials and methods

Cell lineages and reagents

HNSCC cell lines (HN6, HN12, HN13, Cal27, UMSCC17B, and UMSCC74A) [52–56] and NOK-SI were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. Cells were maintained in a 5% CO_2-humidified incubator at 37 °C. Cells were treated with Trichostatin A (TSA; Sigma), cisplatin (Sigma), and TNF-α (PeproTech) at the concentrations indicated. HN6, HN12, HN13, UMSCC17B, and UMSCC74A HNSCC cell lines were kindly provided by Dr. J. Silvio Gutkind (National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD) after authentication by PCR amplification of short tandem repeats to ensure cell identity. Cal27 cells were from the American Type Culture Collection.

IκB kinase (IKK) knockdown

Knockdown of IKKα and IKKβ was performed in HNSCC cell lines as previously described [40]. Briefly, cells were seeded in 24-well plates and transfected with 12.5 nM double-stranded RNA oligonucleotides directed against human IKKα (NM_001278; forward: 5′-CAG GAG AAG UUC GGU UUA GUAdTdT-3′ and reverse: 5′- UAC UAA ACC GAA CUU CUC CUGdTdT-3′) and 25 nM double-stranded RNA oligonucleotides against IKKβ (NM_001556; forward: 5′- CUG GAG AAG UAC AGC GAG CAAdTdT-3′ and reverse: 5′- UUG CUC GCU GUA CUU CUC CAGdTdT-3′) (Invitrogen). Optimal concentrations and time points were determined by dilution curves of siRNA for each target and immunoblot analysis [40]. The sequences of the control negative siRNA (Invitrogen) oligonucleotides were as follows: 5′-UUC UCC GAA CGU GUC ACG UdTdT-3′ and 5′-ACG UGA CAC GUU CGG AGA AdTdT-3′ [40].

IC50 determination

We used the CellTiter 96TM AQueous non-radioactive cell proliferation kit (Promega) to determine the cisplatin concentration that inhibited cell proliferation by 50% (IC50). Cell proliferation was determined by reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) following the manufacturer's protocol. In brief, 2500 cells were plated into 96-well plates and treated in triplicate with control (vehicle) or cisplatin for 24 h. Cells were incubated with MTS at 37 °C for 4 h, and the results were assessed by absorbance (Bio-Tek EL-311, Bio-Tek Instruments) at 490 nm. Chemosensitivity or chemoresistance of HNSCC was determined by comparison with normal oral keratinocytes IC50. Tumor cell lines presenting higher IC50 then NOK-SI (5 μg/ml) were considered chemoresistant, while similar or lower IC50 were considered chemosensitive.

Cell viability

The Rapid Colorimetric MTS assay (Promega) was used to measure HNSCC cell viability and proliferation following treatment. The assay detects metabolically active viable cells and was performed according to manufacturer's instructions.

Comet assay

After treatment, HNSCC cells were embedded in 0.75% low-melting point agarose and allowed to solidify on glass slides. Cells were then placed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris; pH 10.0–10.5) containing fresh 1% (v/v) Triton X-100 and 10% (v/v) dimethyl sulfoxide (DMSO) for 1 h. Following exposure to alkaline buffer (300 mM NaOH and 1 mM EDTA; pH > 13) for 20 min, DNA was electrophoresed using 25 V (0.90 V/cm) and 300 mA. The slides were neutralized in 400 mM Tris (pH 7.5) and stained with Hoechst 33342 (Invitrogen). Cells were analyzed using the TriTek CometScore TM software (TriTek). Three output parameters were measured, including the percentage of tail DNA, tail length, and the tail moment. Tail moment was used for statistical analysis [57,58].

Immunofluorescence and morphometric assay

Cells were placed on glass coverslips in 12-well plates and fixed with absolute methanol at −20 °C for 5 min. Cells were blocked with 0.5% (v/v) Triton X-100 in PBS and 3% (w/v) bovine serum albumin (BSA) and incubated with anti-Acetyl-Histone H3 (Lys9) (Cell Signaling), anti-BRCA1 (C-20) (Santa Cruz Biotechnology), anti-NFκB p65 (Santa Cruz Biotechnology), anti-phospho-NFκB p65 (Ser536) (Cell

Signaling) or anti-phospho-Histone H2A.X (Ser139) (Millipore) antibodies as indicated. Cells were then washed three times and incubated with FITC or TRITC-conjugated secondary antibody and stained with Hoechst 33342 for visualization of DNA content. Images were taken using a QImaging ExiAqua monochrome digital camera attached to a Nikon Eclipse 80i Microscope (Nikon, Melville, NY) and visualized with QCapturePro software. For the morphometric assay, cells were incubated with Hoechst 33342 and anti-Keratin 14 antibody (Covance) and measured using the AxioVision 4.8.1 software measuring tools (Carl Zeiss, Thornwood, NY). Grayscale images were captured separately after fluorescence excitation using FITC_HYQ and TRITC_HYQ filters. The total number of cells was quantified using grayscale images captured from Hoechst 33342, γH2AX, BRCA1, or acetyl-histone H3 staining.

Immunoblotting

Cells were harvested at indicated times, treated with RIPA buffer, and briefly sonicated. Protein lysates (30 µg) were separated by 10% to 15% SDS–PAGE and transferred to a polyvinyl difluoride membrane (Immobilon) (Millipore). Membranes were blocked in 0.1 M Tris (pH 7.5), 0.9% NaCl and 0.05% Tween-20 (TBS-T) with 5% nonfat dry milk. Membranes were incubated with anti-Acethyl-Histone H3 (Lys9) (Cell Signaling) and anti-BRCA1 (C-20) (Santa Cruz Biotechnology) primary antibodies and secondary antibodies conjugated to horseradish peroxidase. GAPDH (Calbiochem) served as a loading control. The reaction was visualized using ECL SuperSignal West Pico Substrate (Pierce Biotechnology).

Statistical analysis

All statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical analysis of the Comet assay, γH2AX and BRCA1 stains were performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Nuclear morphometry analyses were performed by one-way ANOVA followed by Newman-Keuls multiple comparison test. Cellular viability was assessed by two-way ANOVA followed by the Bonferroni posttest. BRCA1 nuclear staining was performed by Student's t-test. Asterisks denote statistical significance ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; and NS $p > 0.05$).

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2013.12.003.

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