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INT6/EIF3E controls the RNF8-dependent ubiquitylation pathway and facilitates DNA double-strand break repair in human cells

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Abstract

Unrepaired DNA double-strand breaks (DSBs) are the most destructive chromosomal lesions driving genomic instability, a core hallmark of cancer. Here, we identify the anti-oncogenic breast cancer factor INT6/EIF3E as an essential regulator of DSB repair that promotes homologous recombination (HR)-mediated repair and, to a lesser extent, non-homologous end joining repair. INT6 silencing impaired the accrual of the ubiquitin ligase RNF8 at DSBs and the formation of ubiquitin conjugates at DSB sites, especially Lys63-linked polyubiquitin chains, resulting in impaired recruitment of BRCA1, BRCA2 and RAD51, which are all involved in HR repair. In contrast, INT6 deficiency did not affect the accumulation of RNF168, 53BP1, or RPA at DSBs. In INT6-silenced cells, there was also an alteration in DNA damage-induced localization of MDC1, a key target for ATM phosphorylation, which is a pre-requisite for RNF8 recruitment. The attenuated DNA damage-localization of RNF8 resulting from INT6 depletion could be attributed to the defective retention of ATM previously reported by us. Our findings deepen insights into how INT6 protects against breast cancer by showing how it functions in DSB repair, with potential clinical implications for cancer therapy.

Keywords

INT6-EIF3E; DNA repair; RNF8; ATM; breast cancer

Introduction

Mammalian cells have evolved two major systems to repair DNA double-strand breaks (DSBs): non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ ligates broken DNA ends after minimal processing but is potentially an error-prone repair system operating in all cell cycle phases. HR, in contrast, functions in an error-free manner but occurs only during S/G2 phases, when a newly synthesized sister chromatid is available

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as a template for repair. Complex regulatory mechanisms exist to determine whether a DSB will be repaired by HR or NHEJ (1).

Proper recruitment of DNA repair proteins to sites of DSBs involves a coordinated series of events that depends on numerous post-translational modifications including phosphorylation and non-proteolytic ubiquitylation in particular. The first event in this signaling cascade is the activation through autophosphorylation of the ATM kinase (2). Once activated and relocalized at DSB, ATM phosphorylates the histone variant H2AX at Ser139 (referred to as γ H2AX) (3). The MDC1 protein subsequently binds to γ H2AX and recruits additional ATM protein (4). MDC1 is then phosphorylated by ATM at multiple sites, leading to recruitment of RNF8. This E3 ubiquitin ligase can form Lys48-branched ubiquitin chains destined for proteasome-mediated degradation and also non-degradative Lys63-linked ubiquitin chains required for DSB repair (5). Current evidence suggests that RNF8 catalyzes the formation of Lys63-linked ubiquitin chains mainly on linker histone H1 (6). RNF168, another major ubiquitin ligase in the DSB response, subsequently binds to Lys63-ubiquitylated H1 (6) and monoubiquitylates histones H2A and H2AX at Lys13/Lys15 (7,8). These RNF8 and RNF168-mediated chromatin changes in the vicinity of DSBs are critical for efficient assembly of the repair factors BRCA1 and 53BP1 (9,10).

In a previous project, we established that human INT6/EIF3E, a component of the multi-subunit eIF3 translation initiation factor, is required for proper execution of the cellular response to DSBs (11). Specifically, we observed that sustained accumulation of ATM at DSBs was defective in INT6-deficient cells, while γ H2AX recruitment was preserved. Supporting this, we found that INT6 could interact with ATM and was partially relocalized at DSB sites. Several lines of evidence suggest that loss of normal INT6 expression is an important event in breast cancer formation and progression (12-16). However, the clinical significance and function of INT6 in breast cancer remain largely unclear. Thus, further exploring how INT6 influences DNA damage signaling and repair would be particularly important for clarifying the role of this protein in cancer prevention.

In this study, we found that INT6 promotes HR-mediated repair and, to a lesser extent, NHEJ repair. We then focused on understanding the underlying mechanism, and found that INT6 depletion strongly impairs the loading of the RAD51 recombinase on resected DNA, a result consistent with the fact that focal enrichment of BRCA1 and BRCA2, two factors involved in RAD51 assembly, is reduced in INT6-depleted cells. Mechanistically, this could be due to defective formation of ubiquitin conjugates at DSB sites, especially Lys63-linked polyubiquitin chains needed for the ensuing accrual of repair proteins. Consistent with this defect, the ubiquitin ligase RNF8 did not accumulate efficiently at DSBs in INT6-depleted cells whereas RNF168 recruitment persisted. In sum, our data support a novel and important role for INT6 in DSB repair that might account in part for the protective effect of INT6 on breast cancer risk.

Materials and Methods

Cells culture

HeLa cells were obtained from ECACC and U2OS cells from ATCC. Cells were repeatedly screened for mycoplasma and maintained in culture for less than 6 months after receipt. RNA interference experiments were performed within 20 passages of cells. RG37 cells harboring the HR reporter were derived from GM639 human fibroblasts (17). GCS5 cells harboring the NHEJ reporter were derived from the GC92 cell line (18). RG37 and GCS5 cells were obtained from Bernard Lopez (Institut Gustave Roussy, Villejuif, France). Cells were maintained in D-MEM supplemented with 10% fetal calf serum and antibiotics.

RNA interference

Cells were transfected with siRNAs using INTERFERin (Polyplus-Transfection) according to manufacturer's instructions. The siRNAs are listed in Supplementary Materials.

Plasmids

Plasmid transfections were performed using the jetPRIME reagent (Polyplus-Transfection) according to manufacturer's specifications. The HA-I-SceI expression vector was provided by Bernard Lopez (18). The GFP-RNF8 plasmid was obtained from Jiri Lukas (19).

Primary antibodies

Antibodies to INT6 (C-20 for immunoblotting and C-169 for immunofluorescence) have been described previously (20). The RNF8 antibody was obtained from Michael Huen (University of Hong Kong). Commercial antibodies are listed in Supplementary Materials.

Cell irradiation and laser micro-irradiation

Cells were irradiated with gamma rays using a ^{137}Cs source (CIS BIO international, IBL 637). For micro-irradiation, U2OS cells were pre-sensitized by overnight incubation with BrdU ($10 \mu\text{g ml}^{-1}$) and micro-irradiated with a pulsed nitrogen laser (365 nm, 10Hz; Spectra-Physics), as previously described (21). For live-cell imaging combined with micro-irradiation, U2OS cells transfected with the GFP-RNF8 construct were seeded on glass bottom culture dishes (MatTek Cultureware) and maintained at 37°C in CO_2 -independent medium (Invitrogen) during micro-irradiation and image acquisition. Time-lapse images were captured and fluorescence intensities of micro-irradiated areas relative to non-irradiated areas were calculated using AxioVision software (Carl Zeiss).

Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 10 min, incubated in 100 mM glycine for 10 min, permeabilized with 0.5% Triton X-100 for 5 min, and blocked with 1% BSA for 30 min. Primary antibodies were incubated for 2 h at room temperature or overnight at 4°C and secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 555 (Cell Signaling Technology) were incubated for 1 h. Nuclei were counterstained with DAPI and slides were mounted in Fluoromount-G medium (Electron Microscopy Sciences). To visualize RAD51

foci, cells were pre-extracted for 30 sec in 0.2% Triton X-100 before fixation with paraformaldehyde.

Microscope image acquisition

Microscope images were acquired using a LSM 710 confocal microscope (Carl Zeiss) mounted on an Axio Observer Z1 microscope (Carl Zeiss) equipped with a Plan-Apochromat 63x/1.4 NA oil-immersion objective. Image acquisition and analysis were performed using LSM ZEN software (Carl Zeiss). Quantitative analysis of immunofluorescence experiments was done manually on ~100-200 cells acquired with a Zeiss Axio Imager Z1 microscope equipped with a CoolSNAP camera (Photometrics) and a 63x/1.4 NA oil-immersion objective. Acquisition software and image processing used the MetaMorph software (Molecular Devices). Cells with more than five foci were considered positive. Automated analysis of images was done with ImageJ software macros (Supplementary Materials).

Immunoblotting

Cell extracts were prepared in Laemmli sample buffer. Protein concentrations were determined using Bradford assay. Proteins were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked with 5% dry milk in 0.1% Tween-20 in PBS, probed with the primary antibodies, followed by HRP-labeled secondary antibodies. Blots were developed using ECL Prime reagent, scanned with an ImageQuant LAS500 imaging system, and analyzed using ImageQuant TL software (GE Healthcare).

HR and NHEJ assays

RG37 cells (HR assay) and GCS5 cells (NHEJ) were seeded into six-well plates. The next day, cells were treated with siRNAs and, 48 h later, transfected again with siRNAs and 1 µg of an I-SceI vector. The culture medium was changed 24 h after. Cells were harvested by trypsinization 24 h (HR assay) or 36 h (NHEJ) after medium change. The GFP signal arising from DNA repair events was acquired using a MACSQuant VYB cytometer (Milenyi Biotec) with GFP fluorescence detected in the FL1-H channel (logarithmic scale). Data were analyzed using the FlowJo v10 software (Treestar).

Statistical analysis

Statistical analyses were performed using Microsoft Excel 2010. Two-tailed Student's t-test was used to determine statistical significance (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Error bars represent standard error of the mean for all plots.

Results

INT6 facilitates foci formation by BRCA1 but not by 53BP1

Our previous study suggested that siRNA-mediated depletion of INT6 led to decreased amounts of BRCA1 at DSBs (11). We re-evaluated this point in more detail by asking whether INT6 could differentially control the recruitment of BRCA1 and 53BP1 to DNA lesions. The rationale for this is that these factors influence HR-mediated repair in opposite

ways. While 53BP1 restricts DNA end resection and hence HR, BRCA1 conversely promotes HR and is thought to relieve a barrier to resection posed by 53BP1 (1). We therefore wanted to determine whether the low accumulation of BRCA1 at DSBs in INT6-silenced cells could be a consequence of increased 53BP1 accrual. Immunofluorescence experiments were performed on siRNA-treated HeLa cells subjected to irradiation (IR). As expected, BRCA1 IR-induced foci were observed in some control cells that are likely in S or G2 since these foci are not formed in G1 (Fig. 1A and B). BRCA1 foci were also visible in a large proportion of INT6-depleted cells (Fig. 1A and B), but with a significant reduced intensity as compared to control cells, particularly at the 4 h time point after IR (Fig. 1C). Also, control cells showed an increase in intensity of BRCA1 foci between 1 and 4 h post-IR which was not seen in the INT6-deficient cells. However, INT6 knockdown did not alter foci formation by 53BP1 (Fig. 1A and B), suggesting that reduced focal accumulation of BRCA1 upon INT6 depletion is not caused by an antagonism between BRCA1 and 53BP1 with regard to DNA damage localization. Recruitment of BRCA1 to DSB sites was also attenuated in U2OS cells deficient for INT6 (Supplementary Fig. S1A). We verified by immunoblotting analysis using HeLa cell lysates that INT6 downregulation did not change BRCA1 and 53BP1 protein levels (Supplementary Fig. S1B). Collectively, our results indicate that INT6 is necessary for correct foci formation by BRCA1 but has no obvious effect on 53BP1 accumulation at DSB sites.

INT6 promotes DSB repair by both HR and NHEJ

We next asked whether INT6 has a role in repair of DSBs through the two major pathways, HR and NHEJ. To this end, we utilized human fibroblast cell lines harboring stably integrated reporters for HR (Fig. 2A) or NHEJ (Fig. 2D) repair pathways (17,18). We used siRNA-mediated knockdown of RAD51 as a control for HR, and expectedly found a ~90 % decrease in HR efficiency (Fig. 2B and C). Silencing of INT6 with two distinct siRNAs also lowered the frequency of recombination events by ~65 % (Fig. 2B and C). An even stronger effect was obtained using the siRNA pool from Dharmacon (Supplementary Fig. S2A and S2B), likely because of higher RNA interference as shown by immunoblot analysis (Supplementary Fig. S2B). This observation argues against an off-target effect of INT6 silencing. Importantly, the HR defect observed upon INT6 downregulation cannot be explained by inefficient I-SceI expression (Fig. 2C and Supplementary Fig. S2B) or by an indirect consequence on cell cycle distribution, which is essentially similar to that of control cells with a slight increase in G2 and no accumulation in G1 (Supplementary Fig. S2C). For NHEJ-mediated repair, we found that depletion of INT6 and 53BP1, used here as control, led to a reduction in the frequency of joining events by 40 % and 20 %, respectively (Fig. 2E and F). Taken together, these findings indicate that INT6 promotes the two main pathways employed to repair DSB, especially HR.

INT6 is required for BRCA2-mediated RAD51 loading onto damaged DNA

To provide mechanistic insights into how INT6 influences HR levels, we evaluated the impact of INT6 depletion on early stages of HR, specifically on RAD51 nucleation onto resected DNA. HR repair starts with an essential DNA end resection step that generates a long single-stranded DNA (ssDNA) tail immediately coated by the RPA protein. As a surrogate of efficient DNA end resection (22), we monitored the phosphorylation of the

RPA2 subunit in HeLa cells treated with camptothecin (CPT) to induce DSBs in S phase. Immunoblotting analysis showed similar kinetics and extent of RPA2 phosphorylation in INT6-silenced cells compared with control cells (Fig. 3A). Consistent with this, accumulation of RPA at DSB was unaffected in INT6-depleted U2OS cells subjected to laser micro-irradiation (Fig. 3B and C). Thus, these data showing intact RPA2 phosphorylation together with unaltered RPA loading upon INT6 knockdown strongly suggest that DNA end resection proceeds properly in the absence of INT6.

We next asked whether INT6 knockdown might affect the subsequent replacement of RPA from resected DNA by the RAD51 recombinase. Strikingly, RAD51 was mostly absent at laser stripes in INT6-depleted cells at the two time points tested (Fig. 4A). Graphs in Fig. 4B indicate a 3 to 5-fold decrease in the proportion of INT6-depleted cells showing co-localization of RAD51 with γ H2AX on the laser tracks. Formation of RAD51 IR-induced foci was also monitored in INT6-depleted HeLa cells and, again, we observed a strong reduction in the recruitment of RAD51 at DSBs regardless of the time after IR, in striking contrast to the situation for RPA (Fig. 4C). Quantitative measurements indicated a ~6-fold decrease in the percentage of INT6-silenced cells with RAD51 foci compared to control cells (Fig. 4D). Impairment of RAD51 foci formation could not be explained by marked changes in cell cycle distribution (Supplementary Fig. S2C), nor by reduced RAD51 protein levels (Fig 4E). Although the INT6#3 siRNA used above caused a slight decrease in RAD51 protein amounts, the siRNAs INT6#1 and INT6#4 recapitulated RAD51 foci abrogation (Supplementary Fig. S3) without affecting RAD51 abundance (Fig. 4E). To exclude any off-target effect, we performed a rescue assay and found that expression of an INT6 cDNA resistant to degradation by the siRNA INT6#4 restored the formation of RAD51 foci (Supplementary Fig. S4).

Given that RAD51 binding on resected DNA is controlled by several mediators including BRCA2 (23), we reasoned that poor recruitment of BRCA2 could contribute to the inefficient accrual of RAD51 at repair sites. Indeed, we observed that INT6 depletion caused a significant decrease in BRCA2 focal accumulation at DSBs (Supplementary Fig. S5A), without affecting BRCA2 abundance (Supplementary Fig. S5B). Collectively, our findings indicated that INT6-depleted cells can resect DSB but cannot replace RPA with RAD51 and BRCA2 at repair sites.

INT6 controls proper ubiquitylation at DNA damage sites

Next, we asked whether INT6 could facilitate DSB-triggered local ubiquitylations that promote assembly of DNA repair factors. In brief, RNF8 and RNF168 are the major E3 ubiquitin ligases that act sequentially at DNA damage sites (5). RNF8 catalyzes the formation of Lys48- and Lys63-linked ubiquitin chains on chromatin and chromatin-binding proteins (6), and RNF168 monoubiquitylates H2A-type histones (7,8), thus triggering recruitment of BRCA1 and 53BP1. Specifically, BRCA1 re-localizes at DSBs via the RAP80 protein that binds Lys 63-linked ubiquitin chains (24-27). Recruitment of 53BP1 requires prior removal of chromatin-bound proteins through the cooperative action between RNF8, which synthesizes Lys48-ubiquitin chains, and the VCP/p97 segregase, which extracts Lys48-polyubiquitylated substrates (28,29). Thereafter, 53BP1 binds to methylated

histones and monoubiquitylated H2A-type histones (10). We first assessed the formation of IR-induced ubiquitin conjugates at DSBs, by using the FK2 antibody that recognizes different ubiquitin chain types. A pronounced reduction in ubiquitin foci was observed in INT6-depleted cells, to an extent almost comparable to that achieved by RNF8 depletion (Supplementary Fig. S6A and S6B). Because INT6 can interact with VCP (30) and with proteasome subunits (31,32), we considered that INT6 downregulation could impact degradation of Lys48-polyubiquitylated proteins at DSB sites. Hence, we looked for formation of Lys48- and Lys63-linked ubiquitin conjugates using specific chain type antibodies, at predetermined time points (33). Lys48-ubiquitin chains did not form microscopically detectable foci, and instead were pan-nuclear in control cells (Supplementary Fig. S6C). There was no significant change in INT6-depleted cells, in contrast with VCP-knockdown cells which showed an increased signal, as expected, with intense cytoplasmic foci in some of the cells. Importantly, formation of IR-induced Lys63-ubiquitin foci was severely impaired in INT6-depleted cells, similarly to RNF8-silenced cells (Fig. 5A). This effect was observed at both time points tested (Fig. 5A and Supplementary Fig. S6D). There was no marked change upon VCP downregulation (Fig. 5A). Furthermore, laser micro-irradiation experiments were performed and confirmed that accumulation of Lys63-ubiquitin conjugates, but not Lys48-linked chains, were strongly abrogated at laser stripes upon INT6 depletion (Fig. 5B and C). Together, these results do not support a role of INT6 with VCP to mediate proteasome degradation of Lys48-branched substrates, but suggest that INT6 is crucial for accrual of Lys63-ubiquitin conjugates at DSBs.

INT6 is required for RNF8-mediated ubiquitylation in response to DNA damage

To further characterize the role of INT6 in accumulation of Lys63-ubiquitin chains at DNA breaks, we next assessed whether INT6 could facilitate the recruitment of RNF8 to lesion sites. Formation of RNF8 IR-induced foci was strongly compromised in INT6-knockdown cells (Fig. 6A) and was not caused by reduced RNF8 protein levels (Fig. 6B). Notably, the faint residual signal observed in cells depleted for INT6 or RNF8 (Fig. 6A) might be ascribed to a non-specific band detected on immunoblots below the RNF8 specific one (Fig. 6B). Next, we performed live-cell imaging of GFP-tagged RNF8 in U2OS cells in which DNA lesions were induced focally by laser micro-irradiation. We observed that GFP-RNF8 redistribution to micro-irradiated regions was strongly attenuated in INT6-depleted cells (Fig. 6C and 6D). Taken together, these findings demonstrate that INT6 is required for the proper recruitment and functioning of RNF8 upon DNA damage.

Because RNF8 triggers the recruitment of RNF168 at DNA lesions (5,6), we then addressed the impact of INT6 depletion on RNF168 localization. Surprisingly, we found that depletion of INT6, which impairs RNF8 foci assembly (Fig. 6A), did not compromise formation of RNF168 IR-induced foci (Supplementary Fig. S7A). To ensure the reliability of this finding, we performed immunoblots that confirmed the specificity of the antibody and normal RNF168 protein pattern upon INT6 depletion (Supplementary Fig. S7B). Interestingly, a study showed that RNF168, but not RNF8, interacts with 53BP1 before their relocalization at DSB and RNF168 catalyzes Lys63-linked ubiquitylation of 53BP1, which is required for the initial recruitment of 53BP1 to DSB sites (34). Our data showing that RNF168 and

53BP1, but not RNF8 or BRCA1, were normally enriched at DSBs following INT6 silencing are in agreement with this work. Furthermore, we tested the effect of INT6 knockdown on monoubiquitylation of H2A-type histones, a modification catalyzed by RNF168 and required for accumulation of 53BP1 at DSB (7,8,10). No marked changes were observed upon INT6 downregulation (Supplementary Fig. S7C). Collectively, our findings suggest that INT6 selectively impacts one of the two branches of the chromatin ubiquitylation pathway, the one controlled by RNF8 that promotes enrichment of the RAP80-BRCA1 complex at DSBs. By contrast, INT6 does not seem to be important for the RNF168-dependent branch that regulates 53BP1 function.

INT6 depletion leads to mislocalization of MDC1 phosphorylated by ATM

Finally, we addressed the mechanism by which INT6 promotes RNF8 recruitment to DSB. We reasoned that attenuated localization of RNF8 at DSBs in INT6-depleted cells could be due to the defective retention of ATM that we previously reported (11). Upon DNA damage, ATM relocates rapidly to DNA breaks and phosphorylates many proteins including H2AX and MDC1. γ -H2AX provides a binding site for MDC1 which, once phosphorylated by ATM, recruits RNF8 (19,35,36). Our previous results showed that INT6 was dispensable for γ -H2AX or MDC1 recruitment at DSBs, but we did not examine at that time MDC1 phosphorylation status (11). Several sites on MDC1 are phosphorylated by ATM following DNA damage. Because RNF8 localization at DSBs depends on its interaction with phosphorylated MDC1, we assessed the impact of INT6 depletion on the localization of phosphorylated MDC1. We employed a phosphospecific antibody recognizing the Thr4 residue of MDC1. This site is phosphorylated primarily by ATM and required for MDC1 dimerization (37). When cells were either irradiated or treated with neocarzinostatin to induce DSBs, p-Thr4-MDC1 was clearly detected in nuclear foci in control cells; however, in INT6-depleted cells we observed pan-nuclear phosphorylation of MDC1 aggregates (Fig. 7 and Supplementary Fig. S8). The specificity of the antibody towards p-Thr4-MDC1 was demonstrated by attenuated signal in cells with siRNA-mediated knockdown of ATM or MDC1 (Fig. 7) and in non-irradiated cells (Supplementary Fig. S8). One possible explanation for these observations is that ATM, which is itself improperly retained at DSBs in the absence of INT6 (11), might carry out phosphorylation of MDC1 in a pan-nuclear (rather than focal) pattern. Collectively, our data support the notion that loss of RNF8 accrual at DNA damage sites in cells depleted for INT6 might be ascribed to a defect in the nature of MDC1 phosphorylation (pan-nuclear rather than focal at DSB sites), due to improper retention of ATM at DSBs and altered ATM signaling.

Discussion

Our previous publication establishing a link between INT6 depletion and defective ATM recruitment to DSB sites (11) provided a mechanistic basis for the causal connection between INT6 alteration and risk of breast cancer development that was put forward from various experimental and clinical observations (12-16). Our current work extends our knowledge about INT6 function in DNA damage signaling and repair. First, we show that INT6 is important for DSB repair by HR and, to a lesser degree, by NHEJ. Second, we provide mechanistic insights into how INT6 influences HR-mediated repair. Persistence of

RPA loading on ssDNA in INT6-depleted cells indicates that INT6 is dispensable for the initial end-resection step of HR. In contrast, INT6 is crucial for the subsequent HR steps, as indicated by impaired BRCA2 and RAD51 binding on resected DNA upon INT6 depletion. Mechanistically, the ssDNA is first bound by RPA, and then RAD51 replaces RPA to trigger DNA strand invasion. BRCA2 is a key player in this exchange step, as it interacts with RAD51 and promotes RAD51 loading onto RPA-bound ssDNA (23). Our observations suggest that INT6 knockdown affects BRCA2-mediated replacement of RPA by RAD51. These findings raise important questions regarding the fate of DSBs induced in an INT6-null background and subjected to extensive resection. Although NHEJ is only partially inhibited by INT6 downregulation, repair of such DNA lesions will no longer proceed by NHEJ because resection triggers an irreversible commitment to HR-mediated repair. In INT6-depleted cells that can neither proceed with HR nor with NHEJ, harmful unrepairable DSB might accumulate, thus leading potentially to complex cancer-promoting genomic rearrangements or cell death.

Additionally, we show that INT6 downregulation impairs the accumulation of BRCA1 at DSBs but preserves 53BP1 accrual. Whereas BRCA1 promotes resection and hence HR, 53BP1, in contrast, is a NHEJ-promoting factor that restricts resection (38,39). Localization of BRCA1 at DNA lesions is mediated by RAP80 that binds Lys63-linked ubiquitin chains (24). Recruitment of 53BP1 occurs through its binding to histones H2A/H2AX monoubiquitylated on Lys15 by RNF168 and to Lys20-dimethylated histone H4 (10). Also, binding of 53BP1 to DSBs needs prior protein removal that requires Lys48-ubiquitin chains catalyzed by RNF8 and extraction of Lys48-polyubiquitylated substrates by VCP (28,29). In this study, we provide evidence that INT6 is crucial for Lys63-branched ubiquitylation at DSBs, but not for Lys48-linked ubiquitylation. These findings were unexpected in light of evidence indicating that INT6 can interact with VCP (30) as well as proteasome subunits (31,32), and ubiquitylation via Lys63 linkages is a nonproteolytic-triggering modification.

Another unexpected observation was that in INT6-depleted cells, recruitment of RNF168 is preserved despite the abrogated accumulation of RNF8 at DSBs. Further validation of this result comes from our data showing that two RNF168-dependent events, namely H2A-type histone ubiquitylation and subsequent binding of 53BP1 at DNA lesions, are unaffected in INT6-deficient cells, thus suggesting that RNF168 is present and active at DSBs. A recent study reported that formation of Lys63-linked ubiquitin chains at DNA lesions is predominantly mediated by RNF8 but not RNF168 (6). The authors provide further evidence that RNF8 adds Lys63-branched ubiquitin chains mainly on histone H1, which in turn recruits RNF168. However, such a mechanism cannot explain our findings that RNF168 assembly persists at DSBs although RNF8 and Lys63 ubiquitin chains are strikingly reduced in INT6-depleted cells. Insights provided by previous studies may help in solving this apparent paradox. Bohgaki et al. (2013) showed that RNF168 interacts with 53BP1 before their assembly at DSB and RNF168 catalyzes Lys63-linked ubiquitylation of 53BP1 required for the initial localization of 53BP1 at damage sites (34). Additionally, Thorslund et al. (2015) reported that overexpression of RNF168 is sufficient to restore 53BP1 recruitment at DSBs in cells that can no longer synthesize Lys63-linked ubiquitin chains (6). Also, RNF8 may accumulate sufficiently at DSBs in INT6-knockdown cells, although to a much lesser extent than in control cells, to initiate RNF168 assembly. This possibility is suggested

by our experiments monitoring GFP-RNF8 real-time recruitment, with a less pronounced defect seen during the first five minutes after micro-irradiation compared with later time points. Notably, the relative kinetics of GFP-RNF8 accumulation is reminiscent of the one we observed previously for GFP-ATM (11). Several studies have shown that retention of RNF8 at DSBs depends on phosphorylation of MDC1 by ATM (19,35,36). From our data showing that INT6 depletion affects proper localization of phosphorylated MDC1 at DSBs, it is tempting to speculate that RNF8 dysfunction in INT6-depleted cells might result from defective ATM signaling.

Finally, our findings, establishing a new activity of INT6 in DSB repair, strengthen the notion that reduced INT6 protein levels or protein alteration can contribute to onset of breast cancer, similar to BRCA1 or BRCA2 defects. Furthermore, our findings underscore the importance of INT6 as a novel therapeutic target to potentiate the cytotoxic effect of DSB-inducing chemotherapeutic drugs during cancer therapy. Moreover, because cells with defective HR-mediated repair are especially sensitive to inhibition of poly(ADP-ribose) polymerase (PARP) (40-42), our data raise the possibility that tumors deficient for INT6 might be successfully treated with PARP inhibitors similar to BRCA-mutated breast and ovarian cancers and HR-defective prostate cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

INT6 controls BRCA1 but not 53BP1 foci formation after irradiation. A, HeLa cells transfected with control or INT6 siRNAs for 72 h were irradiated (6 Gy) and immunostained 1 h or 4 h post-treatment with antibodies to BRCA1 and 53BP1. Representative confocal images are shown. The merged red and green channels show colocalization in yellow. Scale bar, 10 μ m. B, Quantification of experiment in A. Results are expressed as the percentage of cells displaying BRCA1 and 53BP1 foci. C, Measurements of BRCA1 foci intensity 1 h and 4 h post-IR. The intensity of all foci for a total of 25 cells was estimated using ImageJ

software (see macro in supplemental data). Graphs show the mean intensity of the 20 most intense foci for each cell.

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Figure 2.

INT6 promotes DSB repair by HR and NHEJ. A, Schematic of the HR reporter assay. The DR-GFP plasmid consists of two inactive cassettes coding for GFP, with one containing a cleavage site for the *I-SceI* endonuclease. Transient expression of *I-SceI* generates a DSB within the GFP sequence. Repair by HR recreates a functional GFP protein. HR efficiency is measured by quantifying the percentage of GFP positive cells using flow cytometry. B, RG37 human fibroblasts with the stably integrated HR reporter were transfected with control or two different INT6 or RAD51 siRNAs and after 48 h transfected with an HA-I-SceI

expression vector or the empty plasmid control. Cells were harvested 48 h later and GFP intensity was quantified. Shown are results obtained from a representative experiment and numbers on graphs correspond to the percentage of GFP-positive cells. C, HR efficiencies in RG37 cells treated with the indicated siRNAs. The values correspond to HR efficiency relative to the control set to 100% and represent the mean $\pm$ SD of three independent experiments performed in duplicates. Immunoblots of a representative experiment which monitor HA-I-SceI expression, efficiency of INT6 or RAD51 depletion, and equal protein loading are shown below the graph. D, Schematic of the NHEJ reporter assay. In this intrachromosomal NHEJ substrate, translation of the *GFP* gene is suppressed by an upstream out-of-frame translation start site (Koz-ATG) flanked by two *I-SceI* cleavage sites separated by 34 bp. Transient expression of the endonuclease generates DSB that induce the release of the Koz-ATG sequence, and religation of the closely adjacent DNA ends allows translation of GFP in the correct frame, with or without conservation of an I-SceI site. E, The GCS5 human fibroblast cell line containing the NHEJ reporter was transfected with control or 53BP1 siRNAs or with the INT6-specific ON-TARGET plus SMART pool siRNA from Dharmacon or a non-targeting control siRNA (si-NT#1). Forty-eight hours later, cells were transfected with an HA-I-SceI vector or the empty plasmid. Cells were collected after 60 h and NHEJ efficiency was monitored as in Fig. 2B. F, Efficiency of NHEJ repair in GCS5 cells treated with the indicated siRNAs. The values correspond to NHEJ efficiency relative to the controls set to 100% and represent the mean $\pm$ SD of four independent experiments. Immunoblots monitoring HA-I-SceI expression, efficiency of 53BP1 or INT6 depletion, and equal protein loading are shown below the graph.

Figure 3. RPA phosphorylation and recruitment to DSBs are unaffected by INT6 knockdown. A, Phosphorylation of the subunit 2 of RPA occurs normally upon INT6 depletion. HeLa cells were transfected with control or two INT6 siRNAs and 72 h later treated with 1 μ M camptothecin (CPT). Cells were harvested at the indicated time points and immunoblots were performed with antibodies recognizing RPA2 phosphorylated on Ser4/Ser8 or total RPA2. Efficiency of INT6 depletion and equal protein loading were determined by detection of INT6 and β -actin, respectively. B and C, U2OS cells transfected with control or INT6

siRNAs for 72 h were laser micro-irradiated, fixed at indicated times, immunostained with antibodies to RPA2 and 53BP1, and counterstained for the nucleus with DAPI. Representative images (B) and quantification of data (C) are shown. The values correspond to the percentage of cells displaying RPA2 along the laser track. Scale bar, 10 μ M.

Figure 4.

INT6 expression is important for BRCA2-mediated RAD51 loading onto damaged DNA. A, U2OS cells transfected with control or INT6 siRNAs for 72 h were laser micro-irradiated, fixed at indicated times, immunostained with antibodies to RAD51 and γ H2AX, and nuclei were stained with DAPI. Representative confocal images are shown. Scale bar, 10 μ M. B, Quantification of experiment in A. The values correspond to the percentage of cells with RAD51 along the laser track. C, HeLa cells were transfected with control or INT6 siRNAs for 72 h, irradiated (6Gy), and immunostained 1 h or 6 h later with antibodies to RAD51 and

RPA2. Representative confocal images are shown. The merged red and green channels show colocalization in yellow. Scale bar, 10 μ m. D, Quantification of experiment in C. Results are expressed as the percentage of cells displaying RAD51 or RPA foci. E, Immunoblotting to monitor RAD51 protein levels in HeLa cells treated with the indicated siRNAs and to verify the efficiency of INT6 depletion. β -actin was used as loading control.

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Figure 5.

INT6 controls Lys63-linked ubiquitylation at DSBs. A, HeLa cells were transfected with control siRNAs or siRNAs targeting INT6, RNF8 or VCP for 72 h, then exposed to 6 Gy of irradiation and immunostained after 3 h with antibodies to Lys63-linked ubiquitin chains and to BRCA1. Representative confocal images are shown. Scale bar, 10 μ M. B, U2OS cells transfected with control or INT6 siRNAs for 72 h were laser micro-irradiated, fixed 1 h later, immunostained with antibodies to Lys63 or Lys48-linked ubiquitin chains and γ H2AX, and nuclei were stained with DAPI. Representative confocal images for Lys63-ubiquitin are

shown. Scale bar, 10 μ M. C, Quantification of experiment in B. The values correspond to the proportion of cells showing an accumulation of Lys63 or Lys48-ubiquitin chains along the laser track out of γ H2AX-positive cells.

Figure 6.

INT6 controls RNF8-mediated ubiquitylation. A, HeLa cells were transfected with control siRNAs or siRNAs targeting INT6 or RNF8 for 72 h, then irradiated (6 Gy) and immunostained 1 h later with antibodies to RNF8 and BRCA1. Representative confocal images are shown. Scale bar, 10 μM. B, Immunoblotting to monitor RNF8 protein levels and to verify INT6 depletion in HeLa cells treated with the indicated siRNAs. β-actin was used as loading control. C, U2OS cells expressing GFP-RNF8 were treated with control or INT6 siRNAs for 72 h before micro-irradiation. Accumulation of GFP-RNF8 at laser-induced

DNA damage was monitored by time-lapse imaging. White arrows on left images indicate micro-irradiated areas. Scale bar, 10 μ M. D, Relative kinetics of GFP-RNF8 accumulation at laser-irradiated area in control and INT6-depleted cells. Mean value of fluorescence intensities for each time point was calculated from at least 2 independent measurements.

Figure 7.

INT6 expression is important for proper localization of phosphorylated MDC1. A, HeLa cells transfected for 72 h with control siRNAs control or siRNAs targeting INT6, ATM, or MDC1 were irradiated (6 Gy) and immunostained 1 h later with an antibody recognizing MDC1 phosphorylated at Thr4. DNA was stained with DAPI. Representative confocal images are shown. Scale bar, 10 μm . B, Quantitation of experiment in A. Results are

expressed as the number of phospho-MDC1 foci per 100 μm^2 of nucleus for a total of 50-65 cells using ImageJ software.

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