The chromatin-remodeling factor CHD4 coordinates signaling and repair after DNA damage

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I n response to ionizing radiation (IR), cells delay cell cycle progression and activate DNA repair. Both processes are vital for genome integrity, but the mechanisms involved in their coordination are not fully understood. In a mass spectrometry screen, we identified the adenosine triphosphate–dependent chromatin-remodeling protein CHD4 (chromodomain helicase DNA-binding protein 4) as a factor that becomes transiently immobilized on chromatin after IR. Knockdown of CHD4 triggers enhanced Cdc25A degradation and p21^Cip1 accumulation, which lead to more pronounced cyclin-dependent kinase inhibition and extended cell cycle delay. At DNA double-strand breaks, depletion of CHD4 disrupts the chromatin response at the level of the RNF168 ubiquitin ligase, which in turn impairs local ubiquitylation and BRCA1 assembly. These cell cycle and chromatin defects are accompanied by elevated spontaneous and IR-induced DNA breakage, reduced efficiency of DNA repair, and decreased clonogenic survival. Thus, CHD4 emerges as a novel genome caretaker and a factor that facilitates both checkpoint signaling and repair events after DNA damage.

Introduction

DNA double-strand breaks (DSBs) arise as products of stochastic replication failure, reactive oxygen species, or because of environmental clastogens such as ionizing radiation (IR; Löbrich and Jeggo, 2007). DSBs are highly cytotoxic lesions and pose extreme demands on coordinating DNA repair with vital transactions such as transcription, DNA replication, or chromosomal segregation. To safeguard genome integrity challenged by DSBs, cells mobilize repair and signaling pathways, whose activation and coordination involve damaged DNA as well as chromatin composed of histones and histone-binding proteins (Fernandez-Capetillo et al., 2004; Stucki and Jackson, 2006; Jackson and Bartek, 2009; van Attikum and Gasser, 2009).

After DSB generation, the neighboring chromatin undergoes extensive modifications, initiated by the ataxia telangiectasia mutated (ATM)–mediated phosphorylation of the histone H2AX (γ-H2AX) followed by recruitment of the MDC1 adaptor (Stucki et al., 2005) and two ubiquitin ligases, RNF8 and RNF168 (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Wang and Elledge, 2007; Doil et al., 2009; Stewart et al., 2009). The ensuing chromatin ubiquitylation allows amplification of the ATM signaling and local concentration of repair factors including the BRCA1A complex (van Attikum and Gasser, 2009). In parallel, the DSB sites undergo local histone eviction and enzymatic DNA resection, and the resulting single-stranded DNA generates a structural platform for another signaling cascade.

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module triggered by assembly of the ataxia telangiectasia and Rad3 related (ATR) kinase with its coactivators (Bartek and Lukas, 2007). All of these events are essential for timely initiation and amplification of the DNA damage signaling.

The signal generated at the DSBs must be transmitted to the entire nucleus to delay cell cycle progression (Lukas et al., 2003; Bartek et al., 2004). The key signal transducers are the CHK2 and CHK1 kinases, which propagate and amplify the pathways initiated by ATM and ATR, respectively. Among targets of CHK1/CHK2 is the Cdc25A phosphatase, which, when phosphorylated, undergoes a proteasome-mediated degradation (Mailand et al., 2000). This in turn inhibits Cdk2 and Cdk1, the two major kinases governing cell cycle progression. This checkpoint pathway is rapidly implemented and delays cell cycle for several hours, which in most cases, is sufficient to provide time for repair (Bartek et al., 2004). In parallel, S phase progression can be slowed down also by ATM/ATR-mediated phosphorylation of the cohesin SMC1 (Falck et al., 2002; Kitagawa et al., 2004). Finally, cells possess a mechanism to extend checkpoint activity in cases of complex or extensive DNA damage. This branch depends on p53, which is also targeted by ATM/ATR and CHK2/CHK1 (Bartek and Lukas, 2007). Phosphorylation of p53 leads to its stabilization and trans-activation of the p53 targets including the p21Cip1 Cdk inhibitor; p21 then reinforces the cell cycle arrest and can maintain it for an extended period of time (Kastan and Bartek, 2004).

Despite the recent progress in dissecting the pathways involved in DSB repair and signaling, their functional cross-talk and coordination are not understood. To elucidate these issues, we performed an unbiased proteomic screen for factors that become specifically enriched on chromatin after IR and report on identification of CHD4 (chromodomain helicase DNA-binding protein 4) as a new component of the genome surveillance machinery.

Results and discussion

Identification of CHD4 as a factor involved in the DNA damage response (DDR)

By combining stable isotope labeling with amino acids in cell culture (SILAC) labeling (Ong et al., 2002), cellular fractionation, tandem mass spectrometry (Aebersold and Mann, 2003), and statistical analysis, we screened the nuclear proteome for factors that become specifically enriched on chromatin after IR and report on identification of CHD4 (chromodomain helicase DNA-binding protein 4) as a new component of the genome surveillance machinery.

Knockdown of CHD4 sensitizes cells to IR and slows down cell cycle progression

We started by examining the impact of CHD4 on cellular fitness and observed that knockdown of CHD4 reduced colony formation of cells exposed to IR (Fig. 2 A). To gain insight into the impaired survival, we first followed cell cycle progression. Although treating cells with CHD4 siRNAs moderately reduced S and G2 compartments in otherwise unstressed cells, combined irradiation and CHD4 depletion had a much more pronounced impact on cell cycle progression. This was manifested by a marked S phase delay (Fig. 2 B, 12 h) followed by accumulation of cells in G2 (Fig. 2 B, 24 and 30 h). Furthermore, a fraction of CHD4-deficient cells was also clearly arrested in G1, an effect that was virtually absent in a control cell population treated with an identical dose of IR (Fig. 2 B, compare the matching 12- and 24-h time points). Similar consequences of CHD4 knockdown were observed after treating irradiated cells with an independent siRNA (Fig. S1 A). The extended cell cycle checkpoints were further substantiated by a more pronounced inhibition of the cyclin A–associated kinase activity (Fig. S1 B), decreased DNA replication measured by BrdU incorporation (Fig. S1 C), and delayed mitotic entry (Fig. S1 D). Importantly, the impact of CHD4 knockdown on cell cycle progression...
Figure 1. Identification of CHD4 as a factor involved in the DDR. (A) Proteomic screening procedure. GM00130 lymphocytes were grown in heavy or light SILAC media, exposed to 10 Gy of IR, fractionated, and analyzed by tandem mass spectrometry (MS/MS). LC, liquid chromatography. (B) Box plot showing quantitative tandem mass spectrometry data for 53BP1 (positive control). Y axis, normalized ratios [IR peptide/control peptide] showing protein elution by progressive salt fractionation of irradiated lymphocytes relative to control lymphocytes. The box represents the central 50% of the distributions, and the whiskers approximate the 95% interval. (C) Tandem mass spectrometry data for NuRD subunits are shown. Box plots are as in B. (D) Accumulation of GFP-CHD4 at laser-generated DSBs [left] and real-time recruitment of GFP-CHD4 derived from 10 independent cells [right]. Error bars indicate SEM. Bar, 10 μm.
Figure 2. **Knockdown of CHD4 sensitizes cells to IR and deregulates cell cycle progression.** (A) Clonogenic survival assay. U2OS cells were treated with control or CHD4 siRNAs (SMARTpool) for 72 h as indicated, irradiated, and colonies with >50 cells were counted. CHD4 down-regulation was monitored by immunoblotting. SMC1, loading control. (B) U2OS cells were treated with control or CHD4 siRNA (#2) for 72 h, irradiated [6 Gy], and analyzed at the indicated time points by flow cytometry. (C) U2OS cells were treated with control or CHD4 siRNA (SMARTpool), treated with ATM inhibitor for 1.5 h, irradiated, and analyzed by flow cytometry. The efficiency of CHD4 siRNAs in B and C is shown in Fig. S3B. (D) U2OS cell lines conditionally expressing GFP or GFP-CHD4 resistant to siRNA (#3) were treated with control or CHD4 siRNA (#3) as indicated. After 48 h, the transgenes were induced by addition of doxycycline after an additional 24 h, irradiated, and analyzed by flow cytometry. To compensate for minor differences in the starting S phase content in the two cell lines, the data were normalized and are presented as the ratios between the S phase content measured 10 h after IR ($T_10$) and that in unirradiated cells ($T_0$). The GFP-CHD4 cell line and the efficiency of siRNA [#3] are characterized in Fig. S1E. Error bars indicate SEM.
involved bona fide DNA damage signaling, demonstrated by the reversal of the S phase accumulation by treating the cells with a specific inhibitor of ATM (Fig. 2 C). Finally, reintroducing siRNA-resistant GFP-CHD4 into cells depleted of endogenous CHD4 reversed the S phase accumulation, arguing against off-target effects of the siRNA treatment (Fig. 2 D and Fig. S1 E).

**Elevated checkpoint signaling in the absence of CHD4**

To understand the reasons for these cell cycle aberrations, we examined the critical components of the genome surveillance pathways. Consistent with the aforementioned cell cycle delay, we observed that the Cdc25A phosphatase, the key effector of rapidly deployed cell cycle checkpoints, became degraded and remained low in the absence of CHD4, even at stages when Cdc25A in control cells recovered to predamage levels (Fig. 3 A). We could confirm that the absence of Cdc25A recovery in CHD4-deficient cells was caused by ongoing protein degradation and not by reduced mRNA expression (Fig. S2, A and B). Together, these data indicate that in the absence of CHD4, the rapid checkpoint response lasts longer than in CHD4-proficient cells.

Because CHD4-depleted cells responded to IR also by a G1 and G2 accumulation (Fig. 2 B), we examined the p53–p21 axis that plays an important role in sustaining the cell cycle arrest at these crucial transitions. Interestingly, knocking down CHD4 triggered p21 accumulation already in unstressed cells, and this further increased after exposing the cells to IR (Fig. 3 B and Fig. S2 C). Such p21 elevation was associated with a progressive dephosphorylation of pRb, an established surrogate of Cdk inhibition (Fig. 3 B). In addition, the IR-induced induction of p21 was p53 dependent (Fig. S2 D), and it was consistently more robust and occurred earlier in CHD4-depleted cells (Fig. 3 B and Fig. S2 C). We conclude that the sustained branch of the DNA damage–induced checkpoint signaling is more robust in CHD4-depleted cells and that it is partially activated already before exposing cells to external genotoxic stress.

**CHD4-deficient cells transiently hyperactivate ATM and ATR**

Consistent with the extended Cdc25A degradation, both ATM (ATM<sup>S1981-P</sup>, γ-H2AX, and SMC1<sup>S966-P</sup>) and ATR-mediated (CHK1<sup>S317-P</sup> and CHK1<sup>S345-P</sup>) signaling were elevated to supraphysiological levels in the absence of CHD4, an effect that was most evident on increased γ-H2AX (Fig. 3 A and Fig. S2 C). This spike of ATM/ATR phosphorylations was restricted to the early stages of DDR (≤1 h after IR) and was consistently observed after knocking down CHD4 with independent siRNAs targeting distinct regions of the CHD4 transcript (Fig. 3 A and Fig. S2 C). Two scenarios could explain the enhanced ATM/ATR activities. First, depletion of CHD4 may cause local chromatin changes that increase the signaling from a similar amount of DNA breaks as generated in control cells. Second, CHD4 depletion may result in more widespread chromatin modifications that poise it to accumulate more breaks when exposed to IR. Although these scenarios are not mutually exclusive, we obtained evidence that the latter might represent the source of the transient spike of the ATM/ATR-mediated phosphorylations in CHD4-deficient conditions. Thus, using pulsed field gel electrophoresis (PFGE; Hanada et al., 2007), we detected an increased amount of DSBs in CHD4-deficient cells immediately after IR exposure (Fig. 3 C, compare lane 2 with lane 7; and Fig. S2 E). Of note, the elevated amount of DSBs in CHD4-depleted cells remained apparent during the first 2 h after IR (Fig. 3 C, compare...
Figure 4. Impaired ubiquitylation and delayed accumulation of BRCA1 at the site of DSBs in the absence of CHD4. (A–D) U2OS cells were treated with control (CTR) or CHD4 siRNAs (SMARTpool) for 72 h, microirradiated by the laser, and immunostained with antibodies to BRCA1 (A), RNF8 (B), RNF168 (C), and conjugated ubiquitin (FK2 antibody; D). Cells were coimmunostained with antibodies to γ-H2AX (A–C) or MDC1 (D) to mark the DSB-containing tracks. (left) Representative fields for each DSB regulator (A–C, acquired 8 min after microirradiation; D, acquired 15 min after microirradiation) are shown. (right) Graphs show quantification of relative fluorescence intensities in the microirradiated areas subtracted by the background fluorescence in the undamaged parts of the nucleus. The efficiency of CHD4 siRNAs in A–D is shown in Fig. S3 B. RFU, relative fluorescence units. Error bars indicate SD. Bar, 10 µm.

lanes 3 and 4 with lanes 8 and 9, respectively), indicating that CHD4-deficient cells have reduced ability to repair DSBs. Finally, the PFGE assay revealed reproducible DSB generation even in nonirradiated CHD4-depleted cells, an effect evident especially after quantitatively knocking down CHD4 by the most efficient siRNA (#2; Fig. S2 E). This is consistent with
a recent study reporting that the physiological loss of the NuRD complex in senescent cells is accompanied by a progressive increase of spontaneous DNA damage (Pegoraro et al., 2009).

Knockdown of CHD4 impairs retention of repair proteins at the sites of DNA damage

To gain more insight into mechanisms that might be subverted by NuRD disruption, we turned to the earlier observation that a fraction of CHD4 accumulates directly at the DSB sites (Fig. 1 D) and asked whether this influences accumulation of proteins in this compartment. Interestingly, accumulation of BRCA1, a key genome caretaker involved in DSB signaling and repair, was consistently impaired in early phases of the DSB response in CHD4-depleted cells (Fig. 4 A). This was unexpected because the extent of H2AX phosphorylation (an upstream prerequisite for BRCA1 retention on damaged chromatin) was more pronounced in CHD4-deficient cells (Fig. 3 A and Fig. S2 C). Importantly, these observations were not restricted to the laser-induced DNA lesions; the transient impairment of BRCA1 focus formation was also observed in irradiated cells treated with two independent siRNAs against CHD4 (Fig. S3 A).

Because the BRCA1 requires binding to conjugated ubiquitin for its accumulation at DSBs, we tested the impact of CHD4 knockdown on RNF8 and RNF168, the two key ubiquitin ligases involved in this process. Strikingly, although RNF8 remained stable and robustly accumulated at DSBs regardless of the CHD4 status (Fig. 4 B and Fig. S3 B), RNF168 was partially destabilized, and its retention of RNF168 was attenuated in CHD4-depleted cells (Fig. 4 C and Fig. S3 B). Correspondingly, generation of ubiquitin conjugates at the microlaser-generated DSBs was reduced (Fig. 4 D). Together, these data suggest that the transition from the RNF8- to RNF168-controlled step in the DSB-induced chromatin response might be more complex than originally thought (Doi et al., 2009; Stewart et al., 2009). For instance, we can envisage that the transient recruitment of CHD4 to the DSB sites (and the ensuing chromatin remodeling) allows more efficient recognition of the nascent ubiquitin chains (generated by RNF8) by the ubiquitin-binding domains of RNF168, which would in turn facilitate RNF168 recruitment and amplification of the local ubiquitin reaction.

Model of the CHD4 involvement in protecting the genome against chromosomal breakage

Large-scale genetic and biochemical surveys suggested a role of the NuRD chromatin-remodeling complex in the DDR (van Haaften et al., 2006; Matsuoka et al., 2007). In this study, we provide additional and complementary evidence from an unbiased proteomic screen in which we identified four distinct subunits of the NuRD complex among proteins that gain affinity to chromatin damaged by IR. In addition, our study and an accompanying paper in this issue by Smeenk et al. report the first set of functional analyses that delineate the consequences of CHD4 disruption for genome surveillance. Among the salient alterations in CHD4-deficient cells are decreased clonogenic survival, supraphysiological increase of ATM/ATR signaling, protracted cell cycle checkpoints after IR, and delayed assembly of a subset of repair factors at the sites of DNA damage. In addition, we consistently observed that reduction of cellular levels of CHD4 is accompanied by spontaneous DNA damage. Molecular explanations of these diverse defects are likely complex, and our data indicate that disruption of CHD4 may deregulate DDR at multiple levels (Fig. 5).

First, the decreased stability and impaired accumulation of RNF168 at DSBs, the reduced local chromatin ubiquitylation, and the impaired accumulation of BRCA1 on damaged chromosomes likely attenuate the DNA repair efficiency and thereby contribute to the increased IR sensitivity in CHD4-deficient cells. The compromised DNA repair under such conditions might also contribute to the extended cell cycle checkpoints caused by continuous presence of unrepaired DNA and chromatin intermediates that amplify ATR and ATM signaling. Such a protracted cell cycle arrest, when extended over certain threshold, may undermine viability for instance by inducing cell death or allowing checkpoint adaptation followed by mitotic entry with unrepaired DSBs (Syljuåsen et al., 2006).

However, recent development in the field and results in this study indicate that the local events at the DSB sites cannot explain the entire complexity of phenotypes observed in CHD4-deficient cells. Most notably in this regard, the prolonged S phase, transient spike of ATM/ATR signaling after IR, and spontaneous DNA damage were not readily observed in experiments analyzing the currently known factors associated with the
DSB-flanking chromatin. Based on our PFGE results, we propose that the latter consequences of CHD4 depletion reflect at least in part more global alterations of higher-order chromatin structure. This in turn can render chromatin vulnerable and prone to accumulate more breaks. In support of such a scenario, a recent study (Pegoraro et al., 2009) showed that loss of NuRD components during premature and physiological ageing induced alterations of the higher order chromatin structure accompanied by increased spontaneous DNA damage. Moreover, the genome-protective role of heterochromatin seems to be conserved throughout evolution, indicated by another recent study showing that loss of heterochromatin-associated histone methylations in *Drosophila melanogaster* also leads to spontaneous DNA damage, checkpoint activation, and chromosomal instability (Peng and Karpen, 2009). Interestingly, the NuRD complex (specifically its MBD3 subunit) was shown to facilitate deposition and stability of epigenetic marks including the heterochromatin-associated histone methylations (Morey et al., 2008). Thus, in addition to facilitating local assembly of repair factors directly at DSBs (and thereby directly contributing to repair efficiency), the NuRD complex may contribute to genome maintenance by organizing potentially vulnerable segments of eukaryotic genomes (such as the heterochromatin-associated repetitive sequences; Peng and Karpen, 2009) to a state that makes them more resilient to adverse effects of stochastic or castastrophe-induced DNA breakage.

**Data analysis**

Raw individual peptide ratios were normalized by dividing by the median value, calculated across all three chromatin fractions; the final value is therefore the abundance of a peptide in the treated samples, relative to the untreated, in which a value of 1 corresponds to equivalent amounts. For each individual protein, ANOVA was used as a large-scale screening technique to identify proteins that showed a statistically significant variation in the elution profile, i.e., under the null hypothesis of each chromatin fraction to the elution profile, having the same mean. Normalized peptide ratios were assumed to be log-normally distributed about the mean, and were therefore log (ln)-transformed before analysis: applying the Shapiro-Wilks test for normality on the log-transformed ratios showed that this assumption could not be rejected at the 95% level for the majority (92%) of proteins. The ANCOVA was then performed using the R statistical package (http://www.r-project.org). Proteins in which the null hypothesis could be rejected at the 99% level were identified as candidates.

**Plasmids and transfections**

The expression plasmids for CHD4 were generated by inserting PCR-amplified CHD4 CDNA in frame into either pcDNA3.1-HA or pcDNA4TO-GFP. siRNA-resistant form of CHD4 was generated using the QuickChange site-directed mutagenesis kit (Agilent Technologies) with the oligonucleotides (forward) 5′-CCGGCCAGCCGCTTTTCTGGTGAATATGCCAAGGC-3′ and (reverse) 5′-GCCTGGCATTCAACAAAAATGGCCCCTGCGCCG-3′. Plasmid transfections were performed using FuGENE 6 according to the manufacturer’s recommendations (Roche).

**RNA interference**

The siRNA oligonucleotides were obtained from Thermo Fisher Scientific (SMARTpool of four oligonucleotides) and MWG Biotech (individual siRNAs). The annotations and sequences of the siRNA oligonucleotides were as follows (sense strands): (#1) 5′-GAAUAAAUUCUUCGUGAAU-3′; (#2) 5′-GGGCUUAUUGUCUUGAUUUUCG-3′; (#3) 5′-GAAGAGCCGACUUCUUAGUAU-3′; and (#4) 5′-AAGAGUUAUCGCGCAUUU-3′. All siRNA transfections were performed with 100 nM siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen). Cells were transfected by siRNAs 24 h after plating. Transfection reagents and remaining oligonucleotides were washed off 6 h after treatment. Samples were harvested 72 h after initiation of transfection unless stated otherwise. Control siRNA (5′-GGGAGGCAACAGGUGUUA-3′) was against Hst/70B (Leung et al., 1990), a variant of the human heat shock protein that is not expressed in U2OS cells. For complementation assays, oligonucleotide duplex #3 was used by transient transfection.

**Cell culture**

Human U2OS osteosarcoma cells and 293T human embryonic kidney cells were grown in DME containing 10% fetal bovine serum (Invitrogen), 100 U penicillin, and 100 µg/ml streptomycin. GM00130 (Coriell Cell Repositories) Epstein-Barr virus–transformed B-lymphocytes were cultured in RPMI1640 and GlutaMAX (Invitrogen) containing 15% serum, 100 U penicillin, and 100 µg/ml streptomycin. Where indicated, the culture medium was supplied with 5 µM of the proteasome inhibitor MG132 (EMD) or 100 nM ATM inhibitor KU55933 (Kudos Pharmaceuticals). For SILAC experiments, cells were grown for at least five cell divisions in -lysine– and -arginine–deficient RPMI1640 and GlutaMAX containing 15% dialyzed serum and either normal isotope or 15N lysine/15C arginine, also referred to as light and heavy, respectively. U2OS derivative cell lines expressing GFP/CHD4 protein in a doxycycline-responsive manner were isolated by cotransfecting U2OS cells with pcDNA6/TR (Invitrogen) and pcDNA4/TO-GFP/CHD4 constructs and selecting productively transfected cells with 400 µg/ml zeocin and 5 µg/ml blasticidin S (Invitrogen).

**Generation of DNA damage**

IR was delivered by an x-ray generator (150 kV; 15 mA; 2.18 Gy/min; 100 U penicillin, 100 µg/ml streptomycin). Laser microirradiation and real-time recording was performed as described previously (Lukas et al., 2003, 2004). Typically, a mean of 150 cells were microirradiated for each experiment.

**BrDU incorporation**

Cells were labeled for 10 min with 25 µM BrdU (Sigma-Aldrich), fixed in 4% formaldehyde solution (VWR) and permeabilized with 0.2% Triton X-100. Samples were treated with DNase (Roche) for 30 min at room temperature and immunostained according to the manufacturer’s instruction.

**Antibodies**

Rabbit polyclonal antibodies used in this study included CHK1 phospho-S317 (2344; Cell Signaling Technology), CHK1 phospho-S345 (2341;
Cell Signaling Technology), SMCL1 (Ab29622; Abcam), phospho--SMCL1-566 (ab1272; Abcam), phospho-ATM S1981 (200-301-400; Rockland), γ-H2AX (2577; Cell Signaling Technology), cyclin A (sc-751; Santa Cruz Biotechnology, Inc.), HA (sc-7392; Santa Cruz Biotechnology, Inc.), histone 3 phospho--Ser10 (ab65-757; Millipore), p53 (sc-6243; Santa Cruz Biotechnology, Inc.), and RFNB4 (Milliard et al., 2007). Mouse monoclonal antibodies included CHD4 (H0001108MO1; Abnova), Rb (#5543-136; BD), Cdc25A (sc-339P; Santa Cruz), + full inhibitor range). Cells were spun for 5 min at 1,500 g, and soluble nuclear proteins were collected. The pellet was washed twice in no-salt buffer C (20 mM Hepes, pH 7.9, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1 mM NaF). Cells were incubated for 15 min at 4°C and spun at 2,000 g for 5 min. Extracted proteins were collected in the supernatant. This was repeated with an NaCl concentration of 210 mM, and finally, samples were resuspended in loading sample buffer (50 mM Tris, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), boiled for 5 min, and sonicated.

Kinase assay After standard immunoprecipitation, beads were washed twice in kinase buffer (50 mM Hepes, pH 7.5, 10 mM MgCl2, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM DTT, and 0.1 mM Na3VO4). 10% SDS-SB solution (18 μl kinase buffer, 75 μM cold ATP, 2 μg protein H1, and 10 μCi γ[32P]ATP) was added and incubated for 30 min at 30°C with interval shaking. Samples were analyzed using SDS-PAGE followed by Coomassie staining and drying, and the signal was quantified using a phosphoimager.

Chromogenic survival assay U2OS cells were untreated or transfected with control or CHD4 siRNA. 2 d after transfection, between 100 and 2,000 cells (depending on radiation dose to yield 30–200 colonies per dish) were seeded to 6-cm-diameter dishes, incubated for 20–24 h, and treated with IR (0, 2, 3, or 4 Gy). Subsequently, cells were incubated for an additional 10 d and stained with crystal violet. Colonies containing >50 cells were scored as survivors. Survival fractions were calculated in each experiment as the mean cloning efficiency (from at least two parallel dishes) after treatment corrected for plating efficiency.

PFGE Optimized PFGE protocol allowing quantitative detection of DSBs already in a range of 10 Gy of IR was performed as described previously (Hanada et al., 2007). In brief, U2OS cells were treated with indicated siRNAs on two sequential days. After 72 h, cells were either irradiated or left untreated, trypanized, and agarose plugs 5 × 104 cells were prepared with a CHEF disposable plug mold (Bio-Rad Laboratories). Plugs were incubated in lysis buffer (100 mM EDTA, 0.2% sodium deoxycholate, 1% sodium laurylsarcosine, and 1 mg/ml protease K) for ~40 h at 37°C. After washing with TE buffer (10 mM Tris, pH 8.0, and 100 mM EDTA), plugs were embedded into a 0.9% agarose gel. Electrophoresis was performed in TBE buffer with the following parameters: [block I] 9 h, 120° angle, 1.5 V/cm voltage, 30–18-s interval; [block II] 6 h, 117° angle, 4.5 V/cm voltage, 19–8-s interval; [block III] 6 h, 112° angle, 4 V/cm voltage, 9–5-s interval. The gel was stained with ethidium bromide.

Real-time PCR Real-time PCR was performed as described previously (Lüffler et al., 2003) with the following primer sequences: Cdc25A, (sense) 5′-ACCGTCACTAGGACCAAGC-3′ and (antisense) 5′-TTCAGAGCTGAGTACATCC-3′, and phosphoribosylpyrophosphate aminase, (sense) 5′-TCCAACGGGACCCAGCCTGTC-3′ and (antisense) 5′-AGAAAATGTTGCCCTTGTTGGTGA-3′.

Online supplemental material Fig. S1 provides additional evidence for extended cell cycle delay in CHD4-depleted cells exposed to IR. Fig. S2 shows impact of CHD4 depletion on checkpoint signaling and DNA repair. Fig. S3 shows impaired BRCA1 retention at IR-induced nuclear foci and partial destabilization of RNFI68 in CHD4-deficient cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200912135/DC1.

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References

Aebischer, R., and M. Mann. 2003. Mass spectrometry-based proteomics. Nature. 422:198–207. doi:10.1038/nature01511

Bartek, J., and J. Lukas. 2007. DNA damage checkpoints: from initiation to recovery or adaptation.Curr. Opin. Cell Biol. 19:238–245. doi:10.1016/j.ceb.2007.02.009

Bartek, J., C. Lukas, and J. Lukas. 2004. Checking on DNA damage in S phase. Nat. Rev. Mol. Cell Biol. 5:792–804. doi:10.1038/nrm1493
Bekker-Jensen, S., C. Lukas, R. Kitagawa, F. Melander, M.B. Kastan, J. Bartek, and J. Lukas. 2006. Spatial organization of the mammalian genome sur-veillance machinery in response to DNA strand breaks. J. Cell Biol. 173:195–206. doi: 10.1083/jcb.200510130

Bowen, N.J., N. Fujita, M. Kajita, and P.A. Wade. 2004. Mi-2NuRD: multiple complexes for many purposes. Biochem. Biophys. Acta. 1677:52–57.

Doil, C., N. Mailand, S. Bekker-Jensen, P. Menard, D.H. Larsen, R. Peperkok, J. Ellenberg, S. Panier, D. Durocher, J. Bartek, et al. 2009. RNFL68 binds and amplifies ubiquitin conjugates on damaged chromosomes to allow accumulation of repair proteins. Cell. 136:435–446. doi:10.1016/j.cell.2009.02.041

Falck, J., J.H.J. Petriti, B.R. Williams, J. Lukas, and J. Bartek. 2002. The DNA damage-dependent intra-S phase checkpoint is regulated by parallel pathways. Nat. Genet. 30:290–294. doi:10.1038/ng845

Fernandez-Capetillo, O., A. Lee, M. Nussenzeig, and A. Nussenzeig. 2004. H2AX: the histone guardian of the genome. DNA Repair (Amst.). 3:959–967. doi:10.1016/j.dnarep.2004.03.024

Hanada, K., M. Budzowska, S.L. Davies, E. van Drunen, H. Onizawa, H.B. Beerovero, A. Maas, J. Essers, L.D. Hickson, and R. Kanaar. 2007. The structure-specific endonuclease Mus81 contributes to replication restart by generating double-strand DNA breaks. Nat. Struct. Mol. Biol. 14:1096–1104. doi:10.1038/nsmb1313

Huen, M.S.Y., R. Grant, I. Manke, K.M. Minn, X.C. Yu, M.B. Yaffe, and J.J. Chen. 2007. RNFL8 transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly. Cell. 131:901–914. doi:10.1016/j.cell.2007.09.041

Jackson, S.P., and J. Bartek. 2009. The DNA-damage response in human biology and disease. Nature. 461:1071–1078. doi:10.1038/nature8467

Kastan, M.B., and J. Bartek. 2004. Cell-cycle checkpoints and cancer. Nature. 432:316–323. doi:10.1038/nature03097

Kitagawa, R., C.J. Bakkenist, P.J. McKinnon, and M.B. Kastan. 2004. Phosphorylation of SMCl is a critical downstream event in the ATM-NBS1-BRCA1 pathway. Genes Dev. 18:1423–1438. doi:10.1101/gad.1209304

Kolas, N.K., J.R. Chapman, S. Nakada, J. Ylanko, R. Chahwan, F.D. Sweeney, S. Minucci, F. Fuks, and L. Di Croce. 2008. MBD3, a component of the NuRD complex, facilitates chromatin alteration and deposition of epigenetic marks. Mol. Cell. Biol. 28:5912–5923. doi:10.1128/MCB.00467-08

Ong, S.E., B. Blagoev, I. Kratchmarova, D.B. Kristensen, H. Steen, A. Pandey, and M. Mann. 2002. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol. Cell. Proteomics. 1:376–386. doi:10.1074/mcp.M200025-MCP200

Pegoraro, G., N. Kebben, U. Wickert, H. Höhger, K. Hoffmann, and T. Mistelli. 2009. Ageing-related chromatin defects through loss of the NuRD complex. Nat. Cell Biol. 11:1261–1267. doi:10.1038/ncb1971

Peng, J.C., and G.H. Karpen. 2009. Heterochromatin genome stability requires regulators of histone H3 K9 methylation. PLoS Genet. 5:e1000435. doi:10.1371/journal.pgen.1000435

Schmidt, D.R., and S.L. Schreiber. 1999. Molecular association between ATR and two components of the nucleosome remodeling and deacetylating complex, HDAC2 and CHD4. Biochemistry. 38:1417–1417. doi:10.1021/bi991614n

Smeenk, G.W, W.W. Vlieg, H. Vrolijk, A.P. Solari, A. Pastink, and H. van Attikum. 2010. The NuRD chromatin-remodeling complex regulates signaling and repair of DNA damage. J. Cell Biol. 190:741–749. doi:10.1083/jcb.201001048

Stewart, G.S., S. Panier, K. Townsend, A.K. Al-Hakim, N.K. Kolas, E.S. Miller, S. Nakada, Y. Ylanoki, S. Olivierius, M. Mendez, et al. 2009. The RIDDLE syndrome protein mediates a ubiquitin-dependent signaling cascade at sites of DNA damage. Cell. 136:420–434. doi:10.1016/j.cell.2008.12.042

Stucki, M., and S.P. Jackson. 2006. gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes. DNA Repair (Amst.). 5:534–543. doi:10.1016/j.dnarep.2006.01.012

Stucki, M., J.A. Clapperton, D. Mohammad, M.B. Yaffe, S.J. Smerdon, and S.P. Jackson. 2005. MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. Cell. 123:1213–1226. doi:10.1016/j.cell.2005.09.038

Syljuåsen, R.G., C.S. Sørensen, J. Nylundsted, C. Lukas, J. Lukas, and J. Bartek. 2004. Inhibition of Chk1 by CEP-3891 accelerates mitotic nuclear fragmentation in response to ionizing radiation. Cancer Res. 64:9035–9040. doi:10.1158/0008-5472.CAN-04-2343

Syljuåsen, R.G., S. Jensen, J. Bartek, and J. Lukas. 2006. Adaptation to the ionizing radiation-induced G2 checkpoint occurs in human cells and depends on checkpoint kinase 1 and Polo-like kinase 1 kinases. Cancer Res. 66:10253–10257. doi:10.1158/0008-5472.CAN-06-2144

Tong, J.K., C.A. Hassig, G.R. Schnitzler, R.E. Kingston, and S.L. Schreiber. 1998. Chromatin deacetylation by an ATP-dependent nucleosome remodeling complex. Nature. 395:917–921. doi:10.1038/27699

van Attikum, H., and S.M. Gasser. 2009. Crosstalk between histone modifications during the DNA damage response. Trends Cell Biol. 19:207–217. doi:10.1016/j.tcb.2009.03.001

van Haften, G., R. Romeijn, J. Pothof, W. Koole, L.H. Mullenders, A. Pastink, R.H. Plasterk, and M. Tijsterman. 2006. Identification of conserved pathways of DNA-damage response and radiation protection by genome-wide RNAi. Curr. Biol. 16:1344–1350. doi:10.1016/j.cub.2006.05.047

Wade, P.A., P.L. Jones, D. Vermaak, and A.P. Wolffe. 1998. A multiple subunit NuRD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. Nucleic Acids Res. 26:1160–1166. doi:10.1093/nar/26.3.1160

Xue, Y., J. Wong, G.T. Moreno, M.K. Young, J. Côté, and W. Wang. 1998. NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. Mol. Cell. 2:851–861. doi:10.1016/S1097-2765(00)82899-3

Zhang, Y., G. LeRoy, H.P. Seelig, W.S. Lane, and D. Reinberg. 1998. The dermatomyositis-specific autoantigen M2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. Cell. 95:279–289. doi:10.1016/S0007-8215(00)81758-4