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Chapter 14

The Use of Laser Microirradiation to Investigate the Roles of Cohesins in DNA Repair

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Abstract

In addition to their mitotic and transcriptional functions, cohesin plays critical roles in DNA damage response (DDR) and repair. Specifically, cohesin promotes homologous recombination (HR) repair of DNA double-strand breaks (DSBs), which is conserved from yeast to humans, and is a critical effector of ATM/ATR DDR kinase-mediated checkpoint control in mammalian cells. Optical laser microirradiation has been instrumental in revealing the damage site-specific functions of cohesin and, more recently, uncovering the unique role of cohesin-SA2, one of the two cohesin complexes uniquely present in higher eukaryotes, in DNA repair in human cells. In this review, we briefly describe what we know about cohesin function and regulation in response to DNA damage, and discuss the optimized laser microirradiation conditions used to analyze cohesin responses to DNA damage in vivo.

Key words Cohesin, DNA double-strand breaks (DSBs), Condensin I, Cohesin-SA2, Sister chromatid homologous recombination (HR), Near-infrared (NIR) laser, Microirradiation, SUMOylation, MMS21

1 Introduction

Genome integrity is continually threatened by endogenous metabolic products generated during normal cellular respiration, by errors that arise during DNA replication and recombination, and by exogenous exposure to DNA damaging agents. The resulting DNA lesions, if not faithfully repaired, can accumulate as mutations ranging from single nucleotide changes to chromosomal rearrangements and loss. Different insults to DNA are recognized by lesion-specific repair factors, which invoke distinct repair pathways including base excision repair (BER) and double-strand break (DSB) repair, among others [1]. DSB damage is addressed primarily by two major repair pathways, designated nonhomologous endjoining (NHEJ) and homologous recombination (HR), which involve distinct sets of factors [1]. The NHEJ pathway simply re-ligates the DSB ends with nonspecific nucleotide addition or deletion, thereby presenting the

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risk of introducing errors. In mammalian cells, HR repair mainly utilizes the sister chromatid for copying and restoring the damaged region accurately, and thus it primarily takes place after DNA replication in S and G2 phases. The factors essential for DNA repair and the major players in the DNA damage response (DDR) have been largely identified [1]. Significant technical advances have been made in recent years to study DDR protein assembly dynamics at damage sites, including the development of systems to induce damage at specific nuclear or genomic regions (e.g., by laser microirradiation or DSB induction by sequence-specific endonucleases) and biochemical and optical methods for detection of factor recruitment and modification (e.g., chromatin immunoprecipitation (ChIP) and live-cell imaging of fluorescently labeled factors by time-lapse microscopy, respectively) [2, 3]. These approaches have led to further understanding of DDR and repair processes as they happen in vivo and have allowed us to better evaluate the activities of new factors at damage sites in combination with appropriate functional assays (i.e., pathway-specific DNA repair assays). Facilitated by these tools, recent studies have uncovered different aspects of cohesin regulation and function in DDR and repair.

1.1 Induction of Strand Breaks (SSBs and DSBs) and Complex DNA Damage by Laser Microirradiation and Simple DSBs by Endonucleases

Laser microirradiation can induce DNA damage at a specific submicron region in the cell nucleus, and has become a standard technique to study DSB site factor recruitment or modification in vivo [2, 4–7]. The method is particularly useful to monitor the recruitment of those factors that do not form large irradiation-induced foci (IRIF), such as NHEJ factors and cohesin [6, 8]. It is also an ideal approach to examine the spatiotemporal dynamics of chromatin structural changes at damage sites. We extensively analyzed DNA damage induced by different laser systems and dosages and evaluated their physiological relevance.

We confirmed that (1) laser-microirradiated cells (even after high input-power irradiation) are viable (>48 h) and are subject to damage checkpoint control [9, 10], and (2) repair factor recruitment/modifications faithfully mirror those observed with conventional DNA damaging agents and endonuclease-induced DSB sites, consistent with the type and amount of DNA lesions [6, 8–13]. We found that compared to relatively simple single-strand breaks (SSBs) and DSBs induced by low input-power laser irradiation, high input-power laser irradiation generates complex DNA damage that includes high concentrations of strand breaks as well as cross-linking and base damage [10, 12, 14, 15]. This type of damage is accompanied by robust poly(ADP-ribose) polymerase (PARP) activation and recruitment of BER proteins such as DNA glycosylases (e.g., Neil2) (Fig. 1) [10]. Consistent with this, another major SMC protein-containing complex, condensin I, which interacts with PARP1 and a subset of BER factors and participates in BER/SSB repair in human cells [15], is also preferentially recruited to high input-power

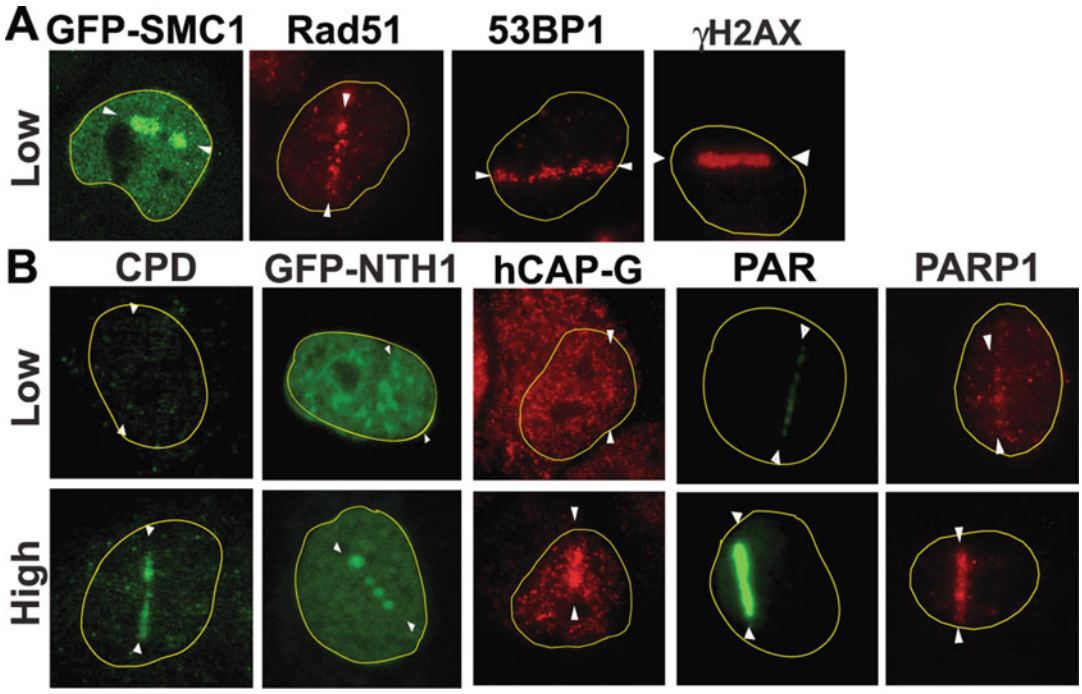

Fig. 1 Low and high input-power lasers induce strand breaks and complex damage, respectively. (**a**) Cohesin and Rad51 recruitment at low input-power (20 %) damage sites. (**b**) High input-power (30 %)-specific recruitment of factors involved in base damage/SSB repair, DNA glycosylases, such as NTH1, and condensin I (hCAP-G)

damage sites [12] (Fig. 1). In contrast, the efficient accumulation of DSB repair factors such as Rad51, cohesin, and 53BP1 preferentially occurs at low input-power laser damage sites (Fig. 1). Previously, high input-power laser damage was found to suppress 53BP1 recruitment, which was thought to be due to the "unphysiological" nature of damage [16]. We found, however, that suppression of PARP significantly restores 53BP1 recruitment, indicating that robust PARP activation in response to complex DNA damage induced by high input-power laser specifically inhibits 53BP1 recruitment [10]. Together, these data provide strong evidence that laser microirradiation with defined conditions can be used effectively to study DDR and repair of DNA lesions enriched for strand breaks or complex damage. This enables us to study damage-specific dynamics and functions of cohesin and condensin I in vivo.

An alternative strategy to specifically examine the DSB response entails the use of sequence-specific endonuclease systems such as I-SceI, FokI, AsiSI, and I-PpoI, which generate simple DSBs. While the I-SceI target sequence needs to be provided exogenously by transgene integration, multiple AsiSI and I-PpoI target sites exist in human genome and FokI can be engineered to target any endogenous locus [17–22]. Approximately 1000 AsiSI target sites are present in the human genome, although cutting efficiency varies due to differential DNA methylation [22–24]. I-PpoI mainly

cuts ribosomal DNA (rDNA) sequences and some additional sites elsewhere in the genome, corresponding to approximately 30 DSBs (10% of 200–300 potential target sites) [21, 25]. Factor recruitment or modifications can be assessed by ChIP followed by either PCR using region-specific PCR primers or genome-wide high-throughput sequencing [21–24, 26]. This allows detection of those factors that do not form IRIF and offers high spatial resolution in terms of the nucleotide distance that damage signals (factor binding or modification) spread from the DSB site.

The major difference between laser and endonuclease systems is not only the type and amount of damage induced, but also the ways that the associated factors or modifications are analyzed; that is, data collection from a single cell (laser) versus a pooled cell population (ChIP). Tagging of the endonuclease with a steroid hormone receptor enables rapid translocation of the fusion endonuclease into the nucleus upon addition of ligand, allowing induction of DSBs in a relatively synchronous fashion [21–24, 26]. Even so, there is cell-to-cell variability in terms of the efficiency of endonuclease expression and cutting at each target site with the ongoing repair. Thus, different states of DSB induction and processing are captured and averaged by the ChIP analysis. In contrast, laser irradiation offers the highest possible temporal resolution (milliseconds) of damage response dynamics as well as spatial resolution in the cell nucleus at the single-cell level. For I-PpoI, it is also possible to visualize factor recruitment using fluorescence microscopy due to the presence of multiple copies of rDNA repeats that cluster at the perinucleolar areas in the cell nucleus [11]. The system, thus, provides the opportunity to compare cytological and biochemical methods (i.e., single-cell fluorescence microscopy analyses and pooled population-based ChIP assays).

1.2 Cohesin Promotes Sister Chromatid HR and Suppresses NHEJ and Other Types of HR

Although cohesin may not be essential for HR repair, it does specifically promote sister chromatid HR [11, 13, 27]. Importantly, depletion of cohesin not only decreases sister chromatid HR frequency but significantly stimulates other DSB repair pathways, including other types of HR repair and NHEJ, indicating that the presence of cohesin significantly dictates repair pathway choice in vivo [11, 27]. Although it was unclear how NHEJ is upregulated by cohesin depletion, a recent study demonstrated that cohesin suppresses end joining of distant DNA ends in a sororin-dependent and S/G2-specific manner, which is critical for suppressing chromosomal rearrangements [28]. Cohesin depletion was shown to increase chromatin mobility (both at damage site and undamaged chromatin region) [29]. Taken together, these studies raise the possibility that cohesin restricts chromosome movement to suppress distant NHEJ repair. Whether this is primarily mediated by cohesin recruited to damage sites, or by cohesin distributed genome-wide remains to be determined.

1.3 Cohesin Recruitment to Damage Sites Does Not Occur in Early G1 Phase

Although we previously demonstrated that cohesin clustering to green laser-induced damage sites is S/G2-specific [14], a recent study using an AsiSI endonuclease-ChIP analysis approach suggested that cohesin can be recruited to damage sites in G1 phase [24]. Based on our analysis of individual cells followed out of mitosis and into G1 phase prior to damage induction, we established that cohesin recruitment does not happen in G1 phase. This same cell cycle specificity was confirmed for both SMC subunits [14] and non-SMC subunits [11]. However, since the synchronization method used for ChIP analysis was serum starvation and release for ~10 h [24], we performed a longer time course analysis and found that some of cells in late G1 phase did begin to accumulate cohesin at DNA damage sites (data not shown). This is consistent with a gradual shift from NHEJ to HR during the G1-S transition, and some DSBs induced in late G1 can be repaired by HR as cells progress into S phase [30]. Thus, it is plausible that the "G1" ChIP signal of cohesin at damage sites might have been skewed by the subpopulation of cells in late G1 phase.

1.4 Cohesin-SA2, But Not Cohesin-SA1, Is Specifically Recruited to DSB Sites and Is Involved in DSB Repair Pathway Choice

In somatic vertebrate cells, there are two different cohesin complexes (cohesin-SA1 and cohesin-SA2) (Fig. 3) [31, 32] in contrast to a single Scc3 (SA homolog) in yeast, and their functional redundancies and distinctions have just begun to be investigated. While both cohesin-SA1 and cohesin-SA2 contribute to genome-wide sister chromatid cohesion, SA1 is particularly important for telomeric sister chromatid cohesion in mammalian cells and has distinct transcriptional effects compared to SA2 [31, 33–35]. We found that only cohesin-SA2 stably associates not only with laser-induced damage sites, but also I-PpoI endonuclease-induced DSB sites (Fig. 3a) [11]. Furthermore, depletion of SA2 (but not SA1) results in inhibition of sister chromatid HR and stimulation of NHEJ [11]. Thus, cohesin-SA2 is primarily recruited to damage sites and is responsible for dictating DSB repair pathway choice (Fig. 3b).

Interestingly, ChIP analysis of AsiSI cut sites indicated no difference between the accumulation of SA1 and SA2 [24]. There are several possibilities: (1) differences in the ChIP efficiencies of the SA1 and SA2 antibodies and/or (2) potentially transient recruitment of both SA1- and SA2-containing cohesins to damage sites, which may be efficiently captured by cross-linking. To address the first possibility, we examined the effect of SA1 or SA2 siRNA depletion on Rad21 (Scc1/Mcd1) binding to damage sites by I-PpoI ChIP-PCR, and observed that depletion of SA2, but not SA1, affected Rad21 binding to damage sites [11]. To address the second scenario, we performed kinetic analyses using GFP-SA1, GFP-SA2 and GFP-SA1NMSA2C chimera recombinant constructs (Fig. 3c) [11]. We observed that both SA1 and SA2 start to accumulate at damage sites but the SA1 signal peaks at a much lower level than SA2. A chimeric mutant of SA1 containing SA2's C-terminal domain

accumulates at damage sites at a level comparable to wild type SA2, indicating that the diverged C-terminal domains of SA1 and SA2 are responsible for this difference (Fig. 3c) [11]. Thus, it is possible that chemical cross-linking may artificially stabilize the transient and unstable recruitment of cohesin-SA1 for the ChIP analysis. In addition, SA1 depletion had no significant effects on the association of the other cohesin subunits and the loading factor NIPBL at damage sites, and also on the repair activity of cohesin [11]. Thus, while ChIP has its advantages, its value for quantitative and comparative assessment of factor recruitment may be undermined by its detection of signals from heterogeneous cell populations, cross-linking effects, and variable ChIP efficiencies of the chosen antibodies. Thus, these two studies highlighted the different characteristics of population-based ChIP and single-cell fluorescence imaging methods [11, 24].

1.5 Cohesin SUMO Response and Relationship to the SMC5-SMC6 Complex

Cohesin was shown to be SUMOylated by the MMS21 subunit of the SMC5-SMC6 complex [13, 27]. MMS21's catalytic activity as a SUMO E3 ligase is critical for the repair activity of the complex, and MMS21 localizes to the damage sites as part of the complex [36]. MMS21 SUMOylates subunits of the SMC5-SMC6 complex as well as cohesin's SA proteins and Rad21 [27, 37]. Mutational analysis indicated that Rad21 SUMOylation is not required for mitotic sister chromatid cohesion but is critical for the sister chromatid HR repair activity of cohesin in human cells [13]. Specifically, SUMOylation was found to antagonize the cohesin destabilizer Wapl because Wapl depletion alleviates the sister chromatid HR defect caused by SUMO target site mutation in Scc1 (Rad21) [13]. Interestingly, the SUMOylation sites overlap with SA protein binding sites, which are also found to be mutated in a developmental disorder related to Cornelia de Lange Syndrome (CdLS), a type of cohesinopathy (diseases caused by dysregulation of cohesin and related factors) [38]. Although the exact function of SUMOylation remains unclear, these results provide important clues as to how cohesin is regulated under damage conditions to enable efficient sister chromatid HR.

Highly concentrated damage in a small area in the nucleus induced by laser microirradiation enables detection of low-abundance repair factor assemblies and modifications. While biochemical detection of cohesin SUMOylation in human cells requires overexpression of SUMO and MMS21, the endogenous SUMO response is readily detectable at laser-induced damage sites [13]. This implies that only a small subpopulation of cohesin is modified in human cells, which may represent cohesins that are selectively involved in repair. No clear induction of SUMOylation was observed in response to DNA damage [13], suggesting that the SUMOylated population of cohesin may preexist, and may specifically accumulate at damage sites. Whether cohesin-SA2 that selectively accumulates at damage sites is the primary target of

SUMOylation by MMS21 remains to be determined. Among many repair factors identified to be SUMOylated and multiple E3 ligases found to mediate damage-associated SUMO responses [37, 39–45], MMS21 and cohesin depletion significantly compromised the SUMO signal at damage sites in S/G2 phase, indicating that the MMS21-cohesin pathway plays a major role in the SUMO response at damage sites [13].

Unlike in human cells, SUMOylation of Mcd1 (Scc1/Rad21), Smc1, and Smc3 was shown to be robustly induced in response to DNA damage and even to a single DSB site in *S. cerevisiae* [46]. This suggests that the significant population of cohesin is SUMOylated in response to damage in yeast, possibly reflecting genome-wide post-replicative reactivation of the cohesion function of cohesin [47–51]. It would be interesting to speculate that as cohesin functions diversified during evolution and a significant fraction of cohesin was required for higher-order chromatin organization important for cell type and differentiation stage-specific gene regulation, a division of labor by different subpopulations of cohesin (i.e., cohesin-SA1 as opposed to cohesin-SA2) became necessary in higher eukaryotes. Interestingly, damage site recruitment appeared to be compromised with the SUMOylation-defective mutant of Mcd1, indicating that SUMOylation is important for efficient damage site recruitment of cohesin in yeast [46]. This is in contrast to the human Scc1 (Mcd1 homolog) whose SUMOylation is not required for damage site accumulation [13].

1.6 Genome-Wide Cohesin in Checkpoint Signaling

In addition to sister chromatid HR repair, cohesin plays a critical role in DNA damage checkpoint control. SMC1 and SMC3 are the major targets of ATM for the intra-S checkpoint (inhibition of DNA synthesis) in mammalian cells [52–54]. Several lines of evidence suggest that cohesin at its preexisting binding sites throughout the genome are involved in this signaling. Cohesin phosphorylation by ATM occurs even in G1 phase [55], when no significant clustering of cohesin is observed at damage sites [11, 14]. Furthermore, SMC1 in both cohesin-SA1 and cohesin-SA2 is phosphorylated by ATM at a comparable level, and depletion of either SA1 or SA2 compromises the intra-S checkpoint (inhibition of DNA replication) resulting in radio-resistant DNA synthesis (RDS), and decreases cell survival following DNA damage (Fig. 3b) [11]. In addition, genome-wide ChIP-seq analysis revealed that cohesin binding was bolstered at preexisting binding sites, rather than being redistributed to new locations, in response to irradiation [56]. Phosphorylation of SMC1 by ATM (critical for the intra-S checkpoint) also plays an important role in this process [56]. Interestingly, preexisting cohesin binding sites appear to limit the spreading of γH2AX [24]. Thus, cohesin distributed genome-wide and important for chromatin domain organization and gene regulation may also play a pivotal role in DDR-associated

chromatin domain organization, possibly serving as a roadblock/boundary element to inhibit DNA replication as well as DDR signal spreading in response to damage. Further investigation is necessary to understand how preexisting cohesin distributed genome-wide functions in DDR signaling.

1.7 Multiple Ways That Cohesin Contributes to the Maintenance of Genome Integrity

Cohesin subunit mutations have been found in many types of human cancers. In particular, recent studies demonstrated mutations of STAG2 (SA2) in myeloid malignancies, bladder cancers, glioblastomas, Ewing sarcomas, and melanomas [57]. Interestingly, not all cases exhibit chromosome segregation defects [58]. In addition to the canonical role of cohesin in mitotic sister chromatid cohesion important for chromosome segregation during cell division, cohesin was shown to play a role in centrosome integrity, spindle assembly, gene regulation, and efficient DNA replication [59, 60]. Thus, it is not surprising that the nature of these mutations and the genetic background will determine how these cohesin mutations contribute to carcinogenesis. In terms of the repair function of cohesin, since cohesin-SA2 is preferentially involved in DNA repair pathway choice (promoting sister chromatid HR and suppressing other repair pathways including error-prone NHEJ), it would be interesting to speculate that mutations of STAG2/SA2 may tip the balance to hyperactivate NHEJ, contributing to chromosome translocation and accumulation of mutations without significantly affecting mitotic sister chromatid cohesion.

2 Materials

1. Adherent human cells (e.g., HeLa cells). Cells may be stably expressing various fluorescently tagged fusion proteins (e.g., hSMC1-GFP, GFP-SA1, and GFP-SA2) [11].
2. Various mammalian expression plasmids for fluorescently tagged fusion proteins (e.g., GFP-Neil2) and siRNAs (e.g., specific for cohesin subunits and the loading factor NIPBL) [11].
3. Cell culture dish (35 mm) with a gridded coverslip (MatTek) or handmade with a 35 mm × 10 mm petri dish (BD Biosciences), gridded coverslip (Bellco Biotechnology), and silicone glue (*see* **Note 1**) (Fig. 2a).
4. Biosafety cabinet equipped with UV lamp.
5. Fixation buffer, 4% paraformaldehyde in PBS.
6. SNBP buffer (0.02% saponin, 0.05% NaN3, 1% BSA in PBS).
7. Permeabilization buffer (0.5% Triton X-100 in PBS).
8. DNA/RNA transfection reagents (e.g., Lipofectamine 2000 (Thermo Fisher Scientific) and HiPerFect (Qiagen).
9. LSM 510 laser scanning microscope system.

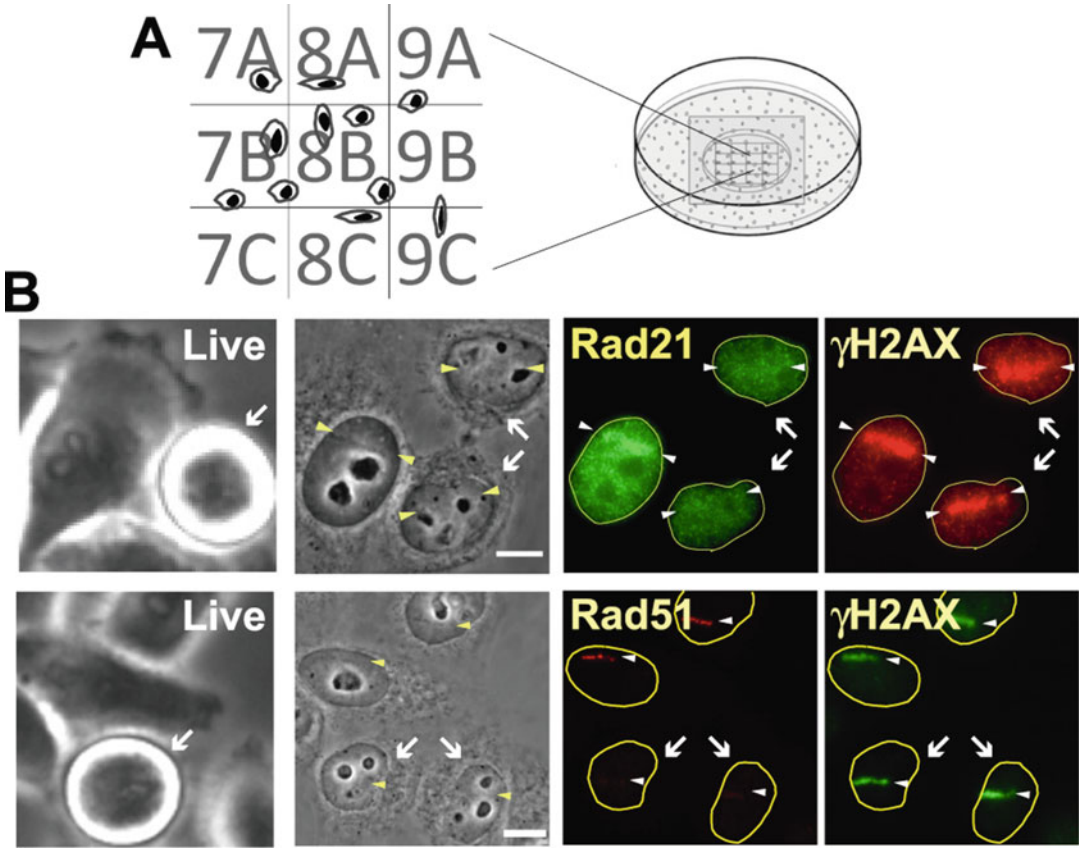

Fig. 2 (**a**) Identification of cells seeded on a gridded coverslip. (**b**) Damage-site recruitment of cohesin and Rad51 in S/G2, but not in G1, cells. Cells in metaphase identified in the *left panel* (live cells, *white arrows*) were divided after 3 h and stained with indicated antibodies. Scale bar = 10 μm

3 Methods

3.1 Sample Cell Preparation

3.1.1 Untransfected Cells and Stable Cell Lines

Seed appropriate number of adherent cells (for example, 1.2×10^5 HeLa cells) in a 35 mm culture dish with a gridded coverslip bottom (see above) 36–48 h before DNA damage induction (*see* **Note 2**) (Fig. 2a). To be able to read the number/letter on the gridded coverslip, it is better that the final cell confluency is no greater than 40–60 %.

3.1.2 Cell Preparation for Transient Transfection

Transient transfection of tagged proteins is widely used to visualize their localization at laser-induced damage sites. HeLa cells are used here as an example (*see* **Note 3**).

1. Day 1, seed $6–8 \times 10^4$ HeLa cells in a well of a 24-well plate.
2. Day 2, perform DNA transfection with Lipofectamine 2000 (or other transfection agent) following manufacturer's instructions.

3. Two to six hours later, trypsinize the cells and seed them onto a 35 mm gridded coverslip dish.
4. Day 4, proceed with laser microirradiation.

*3.1.3 siRNA Transfection (See **Note 4**)*

1. Day 1, seed $6–8 \times 10^4$ HeLa cells in a well of a 24-well plate.
2. Day 2,first siRNA transfection with HiPerFect. Change medium to remove transfection complexes after 6 h.
3. Day 3, second siRNA transfection. Six hours later, seed 60–100% of the cells from one well into a 35 mm gridded coverslip dish.
4. Day 5, proceed with laser microirradiation.

3.1.4 Cell Cycle Synchronization

The cell cycle phase can influence DNA damage repair pathway choice. DSB site recruitment of repair factors may be different depending on the cell cycle stage. Here, HeLa cells are used as an example.

1. Preparation of S/G2 phase cells.

   Both untransfected and transfected HeLa cells can be synchronized to S/G2 phase with a double thymidine-block protocol [11]. Briefly, 4–6 h after seeding cells in a gridded coverslip dish, thymidine is add to a final concentration of 2.5 mM for 17 h, followed by washing and releasing cells into fresh medium for 9 h (or longer if transfection delays DNA replication), and then incubating with 2.5 mM thymidine for another 15 h. Release cells into fresh medium and induce DNA damage at the appropriate time point. Cells in S/G2 phase can be identified by cell cycle-specific cyclins (A and B1 for S and G2, respectively), S/G2 phase specific DSB repair factor (i.e., Rad51), or IdU/EdU incorporation (for S phase cells).
2. Preparation of G1 phase cells.

   Identify metaphase cells under the microscope on a gridded coverslip and note their position on the grid. After 3 h, look at the same position to find each cell's two daughter cells, which are in G1 phase, and induce DNA damage. For transfected cells, some M phase cells don't divide properly after 3 h, and it may help to enrich for M phase cells by single thymidine block for 15 h and releasing for 10 h.

3.2 Laser Microirradiation Using the LSM 510 Laser Scanning Microscope System (See Note 5)

The Zeiss LSM 510 META NLO laser-scanning microscope has been the primary apparatus used in our study, but it is possible to use other near-infrared (NIR) laser systems. The system contains a Zeiss Axiovert 200 M microscope and combines standard fluorescence confocal imaging at six different excitation wavelengths with multi-photon fluorescence/second harmonic generation. DNA damage is induced with a NIR laser (780 nm) which, coupled to

the microscope as the irradiation source, is a mode-locked Ti:Sapphire pulsed femtosecond laser (Chameleon Ultra, Coherent Inc.) tunable in a wavelength range of 690–1040 nm. The NIR laser pulse width and repetition rate are 140 fs and 80 MHz, respectively. The laser beam was collimated, expanded, and steered by a series of mirrors into the microscope, and passed through an objective (100×/1.3 NA) to a diffraction limited spot with calculated diameter of ~732 nm. The laser power was controlled by changing the laser power transmission percentage parameter through the user interface software provided by the manufacturer.

The advantages of this system are: (1) it does not require presensitization of DNA with nucleotide analogs (e.g., BrdU) or DNA-intercalating dyes (e.g., Hoechst) as required for the UV laser system, thus exerting no effect on chromatin packing; (2) NIR laser beam can penetrate into the nucleus without being absorbed by the cell membranes or cytoplasm; (3) it is easier to induce different types or amounts of DNA damage and detect different repair factor (e.g., cohesin and condensin) recruitment at the DNA damage sites by adjusting the laser input-powers; (4) as a laser-scanning confocal microscope system, it can start to record high-quality time-lapse imaging of fluorescent protein dynamics immediately and perform further analyses, such as fluorescence recovery after photobleaching (FRAP).

3.3 Image Analysis

3.3.1 Real-Time Analysis

The fluorescently tagged DNA repair factor of interest can be expressed by transient transfection or in a stable cell line, and damage site recruitment can be analyzed in real time in live cells. As for cohesin, it is necessary to make stable cell lines in order to avoid overexpression and ensure efficient incorporation of the recombinant protein into the cohesin complex in a stoichiometric manner. This is required because only the holo-cohesin complex is stably loaded at damage sites [11]. Furthermore, cohesin recruitment to damage sites is restricted to (late G1)/S/G2, it is necessary to synchronize or identify cells in these stages (Fig. 2b) (*see* Subheadings 1.3 and 3.4.2). The use of a HeLa cell line stably expressing GFP-SA2 is described here as an example (Fig. 3c).

1. Use a cell line that stably expresses EGFP alone as a control to confirm that the damage induction does not result in nonspecific protein clustering at the damage site.
2. Microirradiate cells with the 780 nm NIR laser with an optimal input power as determined by laser power titration [10] (in our case, 20% input power; *see* Subheading 3.4.1).
3. Following damage induction, perform time-lapse fluorescent-imaging analysis at selected time intervals.
4. If necessary, quantify the fluorescence intensity change, at each time point for kinetics analysis [11].

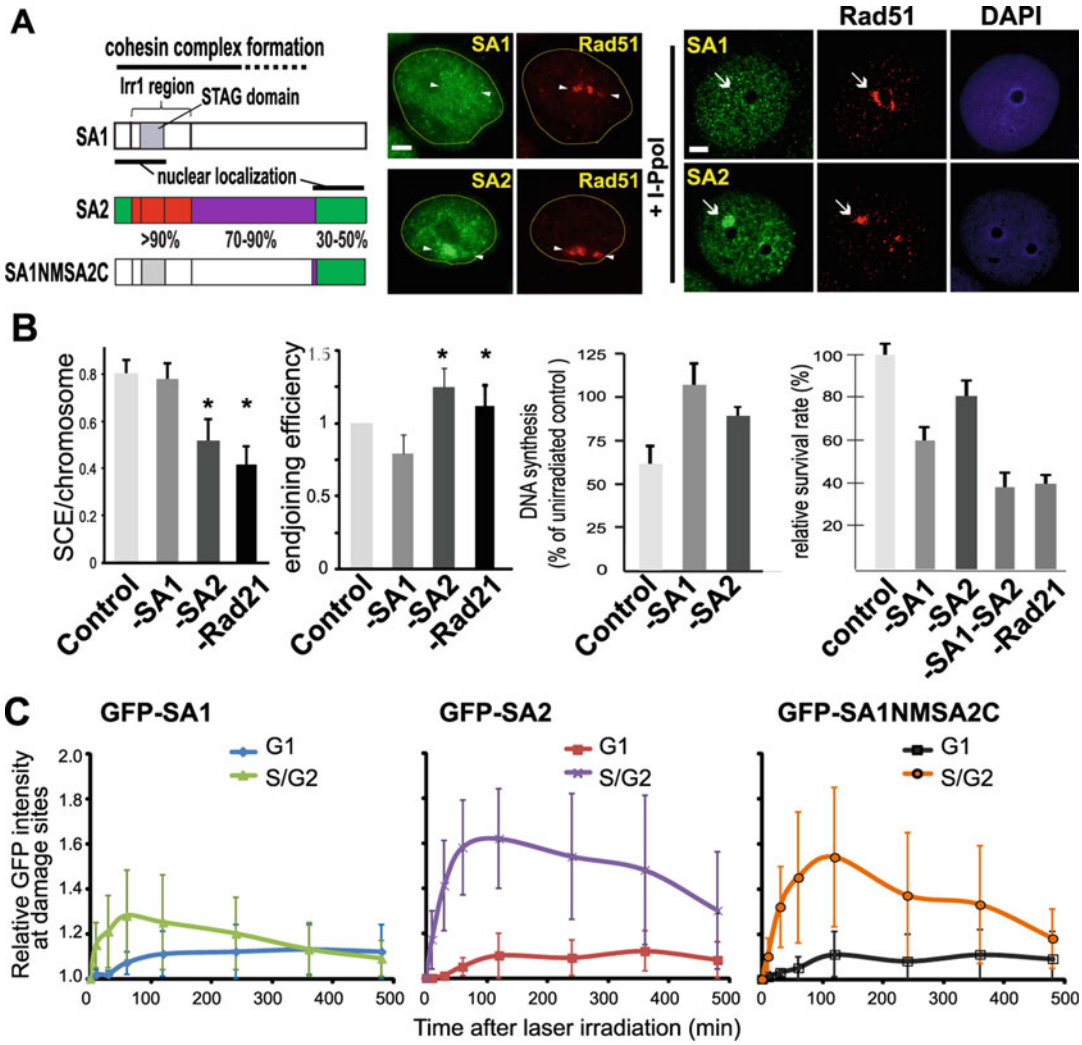

Fig. 3 Cohesin-SA2 is preferentially recruited to damage sites and dictates repair pathway choice. (**a**) *Left*: Diagrams of SA1 and SA2 with known domains and homology comparison as well as the chimeric mutant of SA1NMSA2C are shown. *Middle*: Immunostaining of the endogenous SA1 and SA2 in laser-damaged cells. Rad51 is used as an S/G2-specific damage marker. *Right*: Similar analysis at I-PpoI endonuclease-induced DSB sites in the ribosomal DNA (rDNA) loci. (**b**) Functional analyses of SA1 and SA2 depletion. *Left two panels*: Decrease of sister chromatid HR frequency (sister chromatid exchange assay) and increase of NHEJ (endjoining assay) in SA2-, but not SA1-, depleted cells. *Right two panels*: Radioresistant DNA synthesis (RDS) and decreased cell survival following damage in both SA1- and SA2-depleted cells. (**c**) G1 and S/G2 phase cells expressing GFP-SA1, GFP-SA2 or GFP-SA1NMSA2C were damaged with 780 nm laser microirradiation and accumulation of the GFP signal at the damage sites was measured at the indicated time in live cells. Relative GFP signals were calculated using GFP signal at the same area before damage induction in each cell as "1" [11]

3.3.2 Immunofluorescence Staining Analysis

1. Fix cells at the appropriate time point after DNA damage induction with 4% PFA for 30 min at 4 °C, or with an alternative fixative.
2. Permeabilize the fixed cells with 0.5% Triton X-100 for 5 min at 4 °C.
3. Keep cells in SNBP buffer and finish Immunostaining within 1 week.
4. Incubate in blocking solution (SNBP buffer with 4% serum and 0.1% fish gelatin) at 37 °C for 15 min (Note that appropriate serum must be selected based on the species of animal from which the secondary antibody is derived.).
5. Remove blocking solution, and incubate with primary antibody diluted in SNBP buffer with 1% serum and 0.05% fish gelatin at 37 °C for 30 min.
6. Wash three times with SNBP buffer at room temperature; 10 min for each wash.
7. Incubate with fluorochrome-conjugated secondary antibody solution at 37 °C for 30 min.
8. Wash three times with SNBP buffer, at room temperature; 10 min for each wash.
9. Visualize under microscope (*Note*: If planning to do double or triple staining and one signal is particularly strong (especially at the DNA damage site), it is better to do sequential staining. Perform immunostaining with the weaker antibody first, take pictures under the microscope and then repeat steps 5–8 for the stronger antibody staining.

3.4 Analysis of Cohesin Recruitment at DNA Damage Sites

3.4.1 Visualization of Cohesin Recruitment at DNA Damage Sites Induced with NIR Laser

Using 532 nm green laser microirradiation, we demonstrated previously that human cohesin is recruited to DNA damage sites in an S/G2-specific manner [14]. Similar results were obtained with the 780 nm NIR laser [11]. One hour after damage induction, significant cohesin recruitment was observed at DNA damage sites in S/G2-synchronized HeLa cells using 20%, but not 10 or 30%, input-power. γH2AX was barely detectable at 10%, suggesting that an insufficient number of DSBs was induced for detection of cohesin, which does not form IRIF. At 30% input power, complex damage was induced, which appears to inhibit cohesin and Rad51 recruitment (*see* Subheading 1.1). Cohesin can be detected by antibodies specific for SMC1, SMC3, Rad21, and SA2 (Figs. 2b and 3a) or by using stable cell lines expressing hSMC1-GFP, myc-Scc1, or GFP-SA2 (Fig. 3c) [11, 13, 14].

3.4.2 Cell Cycle Specific Recruitment of Cohesin

In order to observe robust cohesin recruitment, it is necessary to synchronize and examine cells in S/G2 phase (Fig. 2b). This is similar to Rad51 (Figs. 2b and 3a) [11]. Similar results can be obtained at the I-PpoI endonuclease-induced DSB clusters in the perinucleolar region (Fig. 3a) [11].

4 Notes

1. Preparation of cell culture dish.
   (a) Machine an opening (18- to 20-mm diameter) at the bottom of a 35 mm × 10 mm tissue culture dish.
   (b) Rinse the coverslips with double-distilled water (ddH_2O) and dry well.
   (c) Attach a cleaned gridded coverslip with silicone glue over the opening at the bottom.
   (d) Wait 1–2 days for silicone hardening, clean the dish with ethanol to get rid of residual silicone glue on the coverslip surface, and wash dish thoroughly with sterile ddH_2O.
   (e) Dry and sterilize dishes for 15 min in a biosafety cabinet with a UV light source.
2. A shorter cell seeding incubation time may be sufficient for some cells (e.g., U2OS and PtK2 cells).
3. (a) Performing transfection in a well of 24-well plate saves plasmid and reagents. If the transfected cells are used for other experiments (e.g., Western blot) as well, transfection can be done on a larger scale.
   (b) Since transfected cells do not adhere well to the gridded coverslip, minimize cell exposure to transfection reagent, and seed at a higher density than untransfected cells.
   (c) If the transient expression of the recombinant protein is harmful to the cells, transfection can be done in an alternative way: Seed $1.5–2 \times 10^5$ HeLa cells in a gridded coverslip dish; 30 h later, perform DNA transfection with the selected transfection reagent; Change medium 2–6 h after transfection; Proceed with laser microirradiation 12–20 h later if protein expression is good and cells are healthy. The transfection protocol needs to be optimized depending on cell type and the level of recombinant protein expression.
4. The second siRNA transfection can be omitted if depletion efficiency is high and/or too much depletion is toxic to the cell.
5. Laser power may not be stable. Turn the NIR laser on for 30 min before each use. Measure laser input power periodically [10].

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