Evidence of centromeric histone 3 chaperone involved in DNA damage repair pathway

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Abstract

The centromeric protein-A (CENP-A) is an evolutionary conserved histone H3 variant that marks the identity of the centromeres. Several mechanisms regulate the centromeric deposition of CENP-A as its mislocalization causes erroneous chromosome segregation, leading to aneuploidy-based diseases, including cancers. The most crucial deposition factor is a CENP-A specific chaperone, HJURP (Scm3 in budding yeast), which specifically binds to CENP-A. However, the discovery of HJURP as a DDR (DNA damage repair) protein and evidence of its binding to Holliday junctions in vitro indicate a CENP-A-deposition-independent role of these chaperones. In this study, using budding yeast, we demonstrate that Scm3 is crucial for the DDR pathway as *scm3* cells are sensitive to DNA damage. We further observe that the *scm3* mutant interacts with the *rad52* DDR mutant and is compromised in activating DDR-mediated arrest. We demonstrate that Scm3 associates with the DNA damage sites and undergoes posttranslational modifications upon DNA damage. Overall, from this report and earlier studies on HJURP, we conclude that DDR functions of CENP-A chaperones are conserved across eukaryotes. Thus, the revelation that these chaperones confer genome stability in more than one pathway has clinical significance.

Introduction

The faithful replication and partitioning of the replicated chromatin as chromosomal units from mother to daughter cell is important for cell survival. New nucleosomes, the units of chromatin, are assembled on DNA during replication that requires deposition of the histones within the nucleosomes in a highly regulated step-by-step process that involves the deposition of $(H3\text{-}H4)_2$ tetramer followed by two H2A-H2B dimers (Hammond et al., 2017; Ransom et al., 2010). The deposition of the histones to their specific positions within the nucleosome occurs in a temporal manner assisted by a group of proteins called histone chaperones. For this, the chaperones physically interact with the histones in a pre-nucleosomal complex and thus prevent premature histone-DNA interaction or nucleosome assembly (Burgess and Zhang, 2013; Ransom et al., 2010). The deposition of the canonical histones at the gross chromatin takes place mostly during DNA replication via a replication-coupled (RC) pathway using the chaperones dedicated to the RC pathway (Serra-Cardona & Zhang, 2018). For instance, Asf1 and CAF-1 deposit $(H3\text{-}H4)_2$ tetramer (Burgess and Zhang, 2013; De Koning et al., 2007) and Nap1 facilitates $(H2A\text{-}H2B)_2$ assembly (De Koning et al., 2007; Eitoku et al., 2008) into the nucleosomes. On the other hand, the variant histones that earmark specialized chromatin domains committed to do specific biological functions are deposited at specific sites throughout the cell cycle using replication-independent (RI) pathway using variant-specific chaperones dedicated to RI pathway (Smith, 2002). For Instance, deposition of the variant histones H2A.Z, centromeric H3 (CenH3), MacroH2A signifying transcriptional activation (Giaimo et al., 2019), kinetochore formation (Earnshaw and Rothfield, 1985; Earnshaw and Tomkiel, 1992; McKinley and Cheeseman, 2016) and X chromosome inactivation (Sun and Bernstein, 2019) occurs via chromatin remodeler (Fan et al., 2022), HJURP chaperone (Dunleavy et al., 2009; Foltz et al., 2009) and acidic nuclear phosphoprotein 32B (ANP32B) (Mandemaker et al., 2023), respectively.

Almost all the eukaryotes harbor CenH3 at the centromeres, which promotes the formation of kinetochore at those loci (Blower and Karpen, 2001; Buchwitz et al., 1999; Howman et al., 2000; Stoler et al., 1995; Van Hooser et al., 2001). Since the kinetochores bind to the microtubules and drive faithful chromosome segregation, deposition of CenH3 at the centromeres is essential for cell survival. In metazoans, CENP-A (Centromeric protein- A) is the CenH3 (Earnshaw and Rothfield, 1985), and HJURP (Holliday junction recognition protein) is the cognate chaperone (Dunleavy et al., 2009; Foltz et al., 2006). The CENP-A binding domain within HJURP present at its N-terminal is highly conserved (Aravind et al., 2007; Sanchez-Pulido et al., 2009; Shuaib et al., 2010). Despite the well-established function of HJURP in CENP-A deposition at the centromeres, HJURP was initially identified as a

protein involved in DNA damage response (DDR) due to the following reasons (Kato et al., 2007). HJURP was so named because of its ability to bind in vitro to Holliday junction DNA substrates that are in vivo generated during DNA damage repair through homologous recombination. In addition, HJURP was found to interact physically with DNA damage sensing complex MRN (Mre11-Rad50-Nbs1), and the interaction was found to increase significantly upon induction of double-strand breaks (DSBs). As a corollary, the level and subcellular localization of HJURP change in the cells treated with DNA-damaging agents such as Infrared Radiation (IR), Hydroxyurea (HU), cisplatin, and camptothecin (CPT). Finally, the expression of HJURP was found to be regulated by ATM, a DDR kinase, as no expression was observed in ATM-deficient cancerous cell lines. Perhaps due to genome wide function on DDR, HJURP has been visualized to be diffusely present throughout the nucleus (Dunleavy et al., 2009; Kato et al., 2007).

Given the functions of HJURP in CENP-A deposition and DDR pathway, it is not surprising that misregulation of HJURP is involved in disease states. HJURP is considered an oncogene as its overexpression has been associated with several cancers (Chen et al., 2018; Kang et al., 2020) including breast cancer (Coates et al., 2010; Hu et al., 2010; Montes de Oca et al., 2015), lung cancer (Kato et al., 2007), ovarian cancer (Dou et al., 2022), glioblastoma (Valente et al., 2013, 2009), hepatocellular carcinoma (Chen et al., 2018; Hu et al., 2017; Luo et al., 2022), colorectal cancer (Kang et al., 2020) and in pancreatic cancer (Wang et al., 2020). Consequently, the inhibition of HJURP attenuates the properties of the cancer cells and increases the survival rate of the patients, and thus, HJURP acts as a prognostic marker for several cancers (Kang et al., 2020; Lai et al., 2021).

In budding yeast, Cse4 and Scm3 are the homologs of CENP-A and HJURP, respectively. Scm3 deposits Cse4 at the centromeres during the S phase through its physical interaction with Ndc10 that binds to the centromeric DNA as a part of the CBF3 complex (Camahort et al., 2007; Cho and Harrison, 2012, 2011). It has been shown that following the deposition of Cse4, Scm3 remains associated with the centromeres throughout the cell cycle (Wisniewski et al., 2014; Xiao et al., 2011). Like HJURP, Scm3 has been found on the gross chromatin besides its localization at the centromeres (Mizuguchi et al., 2007). Furthermore, a comparison of the domain organization between HJURP and Scm3 suggests that besides the highly conserved CENP-A binding domain, both HJURP and Scm3 also harbour homologous DNA binding domain (Figure S1), which is predicted to be evolutionarily conserved in all vertebrates and fission yeast (Müller et al., 2014). Notably, overexpressed Scm3, like HJURP, shows chromosome instability, and it is believed that Scm3, when excess, binds to the centromeric DNA independently and leads to chromosome missegregation (Choy et al., 2012;

103 Mishra et al., 2011). Both the proteins dimerizes and in *in-vitro* assay, they can bind even to
104 non-*CEN* DNA (Kato et al., 2007; Xiao et al., 2011). While there is no mammalian homolog of
105 budding yeast Ndc10 and no yeast homolog of mammalian Mis18, both these proteins are
106 critical for CENP-A deposition as they bind to their respective CENP-A chaperones through
107 regions independent of CENP-A or DNA binding domain. Therefore, despite the large
108 difference in the polypeptide length between HJURP and Scm3 (748 vs. 229), most of the
109 functions are highly conserved from humans to yeast. Given the physical interaction of HJURP
110 with the MRN complex, it is highly plausible that Scm3 might also interact with DDR proteins
111 in yeast. Altogether, from the above observations, we hypothesize that Scm3, like HJURP,
112 may also be involved in the DDR pathway in budding yeast. In this work, we report that the
113 cells lacking Scm3 are sensitive to the DNA damaging agents and are compromised both in
114 DNA damage repair. In addition to the known functions of HJURP in DDR pathway, in this
115 report we show, using Scm3, that these chaperones are required for mounting DDR-
116 dependent checkpoint activation. Importantly, we demonstrate for the first time that they
117 indeed associate with the DNA damage sites upon induction of DNA damage and persists
118 there perhaps to facilitate damage repair. Our results suggest that the phosphorylation of
119 Scm3 may be attributed to the DDR functions of Scm3. Taken together, this study reveals an
120 evolutionary kinship amongst CenH3 chaperones in conferring genome stability through
121 efficient DNA damage repair, besides their canonical role in CenH3 deposition.

122 **Results**

123 **Scm3 depleted cells are sensitive to MMS mediated DNA damage.**
124 The yeast cells compromised in DNA damage repair (DDR) function show sensitivity to DNA
125 damaging agents such as methyl methane sulfonate (MMS), HU and CPT. To investigate the
126 role of Scm3 in DDR, we constructed an auxin-inducible (Nishimura et al., 2009) Scm3
127 conditional mutant since the protein is essential for growth (Camahort et al., 2007; Mizuguchi
128 et al., 2007; Stoler et al., 2007), and assayed the behaviour of the mutant in the presence of
129 the DNA damaging agents. Since the cells devoid of the homologous recombination protein,
130 Rad52, are known to show extreme sensitivity to such drugs, *rad52*Δ cells were taken as a
131 positive control in our assays (Hallwyl et al., 2008; Kaytor and Livingston, 1994). Mid-log grown
132 cells were harvested, serially diluted, and spotted on plates supplemented with auxin and DNA
133 damaging agents (MMS, HU, CPT) in different combinations and found similar results for all
134 the drugs (Figure 1A, Figure S2A). We observed that *scm3* mutants (three independent
135 transformants) were more sensitive in the presence of MMS (plate supplemented with both
136 auxin and MMS) as compared to those without MMS (plate supplemented with only auxin),
137 whereas *rad52*Δ cells, as expected, were sensitive to MMS but not to auxin (Figure 1A). To

138 quantify the levels of sensitivity towards MMS, we performed a CFU count assay where *SCM3-*
139 *AID* cells were pregrown in rich media without auxin till the mid-log phase before an equal
140 number of cells were plated on media supplemented with auxin and MMS in different
141 combinations as indicated in Figure 1A. The number of colonies formed after 36 hrs were
142 counted and are quantified as follows. The number of colonies that appeared on the plates
143 free of auxin and MMS were taken as 100% cell viability; with respect to this, the number of
144 colonies that appeared on other plates (conditions) was used to calculate the viability
145 percentage for those conditions. We observed a statistically significant drop in cell viability in
146 the plate supplemented with both auxin and MMS compared to auxin alone (Figure 1B).
147 Since the presence of auxin in the plate can hinder the growth of the *SCM3-AID* cells,
148 in another approach to examine the sensitivity of *scm3* mutant to MMS, we pre-treated the
149 cells with 0.75 mM auxin for 2 hrs to deplete Scm3 before they were washed and spotted on
150 plates containing different concentrations of MMS, but without auxin. The depletion of Scm3
151 was verified by observing higher percentage of G2/M arrested cells (Figure S2B). Here also
152 we found that Scm3-depleted cells were more sensitive to MMS as compared to the wild-type
153 cells (Figure S2C). To quantify the sensitivity observed by this approach, we performed a CFU
154 count assay as above. Following auxin pretreatment, an equal number of wild-type, *SCM3-*
155 *AID,* and *rad52*Δ cells were spread on the YPD plate or YPD plate containing 0.01% MMS. By
156 calculating the viability percentage as described above, we again found a statistically
157 significant drop in cell viability on the plate where Scm3 was predepleted (Figure S2D). The
158 *rad52*Δ cells, as the positive control, showed high sensitivity, as expected.
159 The increased sensitivity of *scm3*-depleted cells to DNA-damaging agents could be
160 due to weakening of the kinetochores as Scm3-mediated deposition of Cse4 promotes
161 kinetochore assembly (Camahort et al., 2007; Cho and Harrison, 2011). If this holds true,
162 perturbation of the kinetochore by degradation of other kinetochore proteins must show a
163 similar sensitivity to MMS. In budding yeast, Ndc10 is recruited to the centromeres upstream
164 of Scm3 (Lang et al., 2018), whereas the centromeric localization of Mif2, another essential
165 inner kinetochore protein, depends on Scm3 and Cse4 (Xiao et al., 2017). We constructed
166 *NDC10-AID* and *MIF2-AID* strains and used them for our assay to represent the proteins
167 independent or dependent on Scm3 for centromeric localization, respectively. We also
168 included one non-essential kinetochore protein, Ctf19, a protein of the COMA complex, to
169 remove any possible mis-judgement in distinguishing cell-growth-arrest phenotype occurring
170 due to drug-sensitivity vs. auxin-mediated degradation of essential proteins. The COMA
171 complex is directly recruited to the centromeres through interaction with the N terminal tail of
172 Cse4, hence dependent on Scm3 (Chen et al., 2000; Fischböck-Halwachs et al., 2019). Mid-
173 log grown cells were harvested and spotted on the indicated plates, however, we did not
174 observe any increased sensitivity of such cells to MMS (Figure S3). In fact, Mif2 depleted cells

175 showed a slight resistance to MMS due to unknown reasons. Therefore, the increased
176 sensitivity to MMS in *scm3* mutant but not in other kinetochore mutant cells indicates that
177 Scm3 possesses an additional function in genome stability besides its role in kinetochore
178 assembly.

179 Since HJURP is involved in DDR through the HR pathway (Kato et al., 2007) and If
180 Scm3 mimics HJURP to repair damaged DNA, then the *scm3* mutant is expected to interact
181 genetically with the HR mutants. We tested that possibility by analyzing the genetic interaction
182 between *SCM3* and *RAD52,* as Rad52 is essential for efficient homologous recombination in
183 *S. cerevisiae* (Barlow & Rothstein, 2010; Lee et al., 2003). We deleted *RAD52* in the *SCM3-*
184 *AID* strain, and the mid-log grown cells were spotted on plates supplemented with increasing
185 concentrations of auxin (Figure 1C). Interestingly, the double mutant grew slowly as compared
186 to the single mutants and the wild-type strain. To further validate the genetic interaction
187 between the two genes, the mid-log grown cells were pre-treated with a 0.75 mM auxin to
188 deplete Scm3 for the indicated duration before the cells were spotted on YPD plates. Again,
189 we observed that the double mutant grew slower than the wild-type and single mutants (Figure
190 1D). Next, we wished to investigate how the double mutant behaves when challenged with
191 DNA damaging agent. As expected, when the double mutant was spotted in the presence of
192 0.005% MMS, all the single mutants grew better than the double mutant (Figure S2E). These
193 results indicate a genetic interaction between *SCM3* and *RAD52,* which becomes pronounced
194 in the presence of DNA-damaging agents.

195 Further, we wanted to identify the regions in Scm3 whose loss confers sensitivity to
196 DNA damage. The domain organization of Scm3 reveals two essential conserved regions,
197 namely NES (nuclear export signal) and HR (heptad repeat), having functions in DNA and
198 Cse4 binding, respectively (Figure S1A). Scm3 has a non-essential D/E-rich region, which is
199 important for protein stability but not involved in Cse4 binding (Stoler et al., 2007). Scm3 also
200 possesses two BR (bromodomain) regions, also referred to as the NLS (nuclear localization
201 signal). The deletion of the C-terminal 25 amino acids or the bromodomain regions does not
202 cause cell lethality (Shivaraju et al., 2011; Stoler et al., 2007). We used the versions of Scm3
203 mutants used elsewhere (Shivaraju et al., 2011) harbouring deletion in one or the other non-
204 essential region as the sole source of Scm3 and challenged these mutants (two independent
205 transformants) to MMS and CPT along with wild-type Scm3 as a control. We found no growth
206 defect in response to MMS or CPT (Figure S1B) suggesting that at least the nonessential
207 regions are not important for the DDR function of Scm3. Although this indicates that the
208 essential NES and/or HR might be involved but that is technically difficult to test using growth
209 based assays.

210 From these results, we conclude that the loss of Scm3 confers sensitivity to DNA
211 damaging agents, indicating that Scm3, much like its mammalian homolog HJURP, may play
212 a role in the DDR pathway.
213
214 **The loss of Scm3 generates more Rad52 foci**

215 Having shown that the *scm3* mutant is sensitive to DNA damage, we hypothesized that the
216 mutant cells might be deficient in repairing intrinsic and extrinsic DNA damages. To examine
217 this, we wished to visualize the Rad52-GFP foci in these cells with or without MMS-induced
218 DNA damage. Since in budding yeast, the primary pathway to repair DNA damage is through
219 homologous recombination (HR) mediated by Rad52, deficiency in repair will cause
220 accumulation of DNA lesions and hence Rad52 (Dornfeld and Livingston, 1991; Lisby et al.,
221 2003, 2001). The mid-log grown *RAD52-GFP SCM3-AID* cells were split into auxin- and
222 DMSO-treated halves. Following 2 hrs of treatment, the cells were again split and were mock-
223 treated or treated with 0.02% MMS for 90 mins (Figure 2A). We visualized the formation of
224 Rad52-GFP foci in these cells (Figure 2B). Interestingly, we observed that 18% of the Scm3
225 depleted (+auxin) cells showed Rad52-GFP foci in contrast to only 5% in the wild type (Figure
226 2C, untreated). In accord with this, when these cells were challenged with MMS, a significant
227 increase of cells with Rad52-GFP foci was observed in the mutant (70%) compared to the wild
228 type (45%) (Figure 2C, 0.02% MMS). Moreover, while the mutant cells harbored an average
229 of ~ 3 foci, the wild type showed ~2 foci per cell (Figure 2D) in response to MMS treatment.
230 These results suggest that both intrinsically and extrinsically generated DNA lesions remain
231 unrepaired in the absence of Scm3.

232 To understand the dynamics of Rad52 foci formation in *scm3* cells, we used a strategy
233 as shown in Figure S4A. Briefly, the *bar1Δ RAD52-GFP SCM3-AID* cells were arrested in the
234 G1 phase of the cell cycle by adding alpha factor. 1 hour before release from the G1 phase,
235 cells were treated with auxin to deplete Scm3 or with DMSO control. Subsequently, the cells
236 were washed several times and released into alpha factor free media supplemented with or
237 without MMS but in the presence of nocodazole in order to have all the cells eventually
238 arrested at metaphase. Following release, the cells were harvested every 30 mins and the
239 percentage of cells containing Rad52-GFP foci were visualized (Figure S4B). Scm3 depleted
240 cells, but not the wild type, with or without MMS, showed a peak of Rad52-GFP accumulation
241 at around 60 mins from G1 release (Figure S4C, D). Notably, this time point possibly coincides
242 with the timing of DNA replication (judged by bud morphology) when DNA is most susceptible
243 to intrinsic and extrinsic damages. Taken together, Scm3-depleted cells exhibit more Rad52
244 foci, indicating a compromised DDR pathway in these cells.

245 If the DNA lesions remain unrepaired, the cells might undergo an apoptotic pathway
246 leading to cell death. To determine the percentage of cell death in the *scm3* mutant culture,
247 the cells harvested at 90 mins of MMS or mock treatment (after auxin treatment), as shown in
248 Figure 2A were stained with propidium iodide (PI), which does not permeate the live cells and
249 stains only the dead cells (Figure S4E, arrow). We observed that while ~15% of the Scm3
250 depleted (+auxin, -MMS) cells were PI positive, it was only ~4% for the wild type (DMSO, -
251 MMS) (Figure S4E, untreated). The PI positive cell population increased after MMS treatment
252 in both the cultures with significant difference between them (35% vs 27%) (Figure S4E, 0.02%
253 MMS). These results suggest that unrepaired intrinsic and extrinsic DNA lesions in the
254 absence of *scm3* lead to compromised cell viability.

255 **Scm3 associates with non-centromeric sites in response to DNA damage**

256

257 Having observed that Scm3 depleted cells are compromised in DNA damage repair and *scm3*
258 interacts genetically with *rad52Δ*, it is reasonable to argue that Scm3 goes to the DNA damage
259 sites to promote repair. To examine this, we first wanted to see if Scm3 alters its localization
260 pattern in response to MMS-mediated DNA damage. Since Scm3 is also expected to localize
261 at the kinetochores (Mizuguchi et al., 2007), we visualized Scm3 along with the kinetochore
262 marker, Ndc10, on chromatin spreads. The cells harboring *NDC10-6HA SCM3-13MYC* were
263 mock-treated or treated with 0.02% MMS for 90 mins. Ndc10 showed bright foci representing
264 the kinetochore cluster, whereas Scm3 was localized to multiple locations across chromatin,
265 including its colocalization with Ndc10 (Figure 3A, arrow). Similar localization of Scm3 has
266 been observed earlier in live cells where Scm3-GFP showed a wider nuclear localization,
267 which might argue for its genome-wide functions (Luconi et al., 2011; Wisniewski et al., 2014).
268 We also observed a significant enrichment of Scm3 at the rDNA loops irrespective of the
269 extrinsic DNA damage (Figure 3A, arrowhead). The enrichment of Scm3 at the rDNA loops is
270 perhaps to maintain the highly repetitive organization of rDNA. HJURP has also been shown
271 to localize rDNA arrays in mammalian systems (Kato et al., 2007). Additionally, upon
272 quantifying the total intensity of Scm3-13Myc and Ndc10-6HA and normalizing that with the
273 background intensity, we observed a statistically significant increase in the Scm3-13Myc, but
274 not Ndc10-6HA intensity in response to MMS treatment (Figure 3B). No change in the intensity
275 of Ndc10-6HA post-MMS treatment justifies the absence of increased sensitivity of Ndc10
276 depleted cells in response to MMS (Figure S3A).
277 Having observed an increased chromatin localization of Scm3 in response to MMS on
278 chromatin spreads, we wished to verify this by determining genome wide association of Scm3
279 using ChIP-Seq. To negate any effect on Scm3's binding due to cell cycle progression we
280 first arrested the cells harboring *SCM3-13MYC* at metaphase by nocodazole. The arrested

281 cells were further mock treated or treated with 0.05% MMS for an additional 90 mins before
282 they were harvested for ChIP. The Scm3 ChIP-seq signal was normalized with the input
283 signal, and genome-wide plots were generated to see Scm3 binding along the entire
284 chromosomes. We also performed ChIP-seq from a no-tag control strain that provided the
285 background signal due to the antibody or the beads treatment. We could observe an
286 enrichment of Scm3 in both MMS treated and untreated cells at the centromeres, while no
287 enrichment in the no-tag sample was observed (Figures 3C, 3E, S5). Further, we could
288 observe the localization of Scm3 at the 9.1 kb rDNA region on ChrXII, validating our chromatin
289 spread data (Figure 3D). Interestingly, in MMS treated cells, we could see peaks of Scm3
290 binding to the non-centromeric sites as well (Figures 3C 3E, S5, arrow). Such non-
291 centromeric chromatin enrichment of Scm3 was observed for several chromosomes and
292 corresponds to the increased chromatin localization of Scm3 in response to MMS, as
293 visualized on chromatin spreads (Figure 3B).
294
295 **Scm3 partially co-localizes with Rad52, perhaps at the DNA damage sites**
296
297 Given the increased chromatin association of Scm3 in response to DNA damage we wished
298 to investigate if this occurs due to its binding to the DNA damage sites. For this, we examined
299 the colocalization of Scm3 with Rad52 upon DNA damage, as the latter is known to be targeted
300 to the damage sites (Lisby et al., 2003; Miyazaki et al., 2004). To visualize both Rad52 and
301 Scm3 simultaneously on chromatin spreads, cells harboring *RAD52-6HA* and *SCM3-13MYC*
302 were mock-treated or treated with 0.02% MMS for 90 mins. We did not observe any significant
303 co-localization between Scm3-13Myc and Rad52-6HA in untreated cells as judged by low
304 Pearson's Correlation Coefficient value (PCC < 0.4) (Figure 4A-B). However, in the cells
305 treated with MMS, we could observe a significant increase in the PCC value (PCC > 0.5)
306 (Figure 4B). This indicates a partial co-localization between Scm3 and Rad52 in the presence
307 of the DNA damage and implicates that Scm3 may localize to the damage sites in response
308 to DNA damage.
309 Next, we wished to examine the behavior of Scm3 with respect to another DNA
310 damage marker. In mammals, the DSB sites are marked by γ-H2Ax due to phosphorylation of
311 the H2A variant, H2Ax by ATM/ATR kinases (Burma et al., 2001; Lee et al., 2014) In yeast
312 that lacks H2Ax, the DSBs are marked by γ-H2A due to phosphorylation of histone H2A by
313 Mec1 and Tel1 kinases (Lee et al., 2014). To be noted, γ-H2A is also found at the
314 heterochromatin region and co-localizes with yeast heterochromatin protein Sir3 (Javaheri et
315 al., 2006; Kirkland et al., 2015; Kitada et al., 2011). We performed chromatin spread assay
316 using antibodies specific to γ-H2A and observed a drastic increase in the intensity of γ-H2A

317 when the cells were subjected to DNA damage, which is expected (Figure S6 A, B). To verify
318 that the phenomenon is DNA damage associated, we observed a significant increase in the
319 colocalization between Rad52 and γ-H2A in response to DNA damage (Figure S6 C-D). To
320 further correlate the localization of Scm3 with DNA damage, we then visualized both Scm3-
321 13Myc and γ-H2A simultaneously on chromatin spreads from *SCM3-13MYC* cells mock-
322 treated or treated with 0.02% MMS for 90 mins before they were harvested for the assay. We
323 did not observe any significant co-localization between Scm3-13Myc and γ-H2A in untreated
324 cells as judged by low Pearson's Correlation Coefficient value (PCC < 0.3) but found a
325 significant increase in the value (PCC > 0.6) in MMS treated cells (Figure 4C-D). Taken
326 together, we conclude that the association of Scm3 with chromatin and its co-localization with
327 DDR proteins increases with DNA damage.
328
329 **Scm3 is recruited to a site-specific DSB**
330 The overall increased association of Scm3 with the chromatin and its colocalization with
331 Rad52 and γ-H2A in response to MMS-mediated DNA damage prompted us to examine if
332 Scm3 is recruited specifically to DNA damage sites. For this we utilized the chromatin
333 immunoprecipitation (ChIP) assay to visualize the dynamics of Scm3 recruitment at an
334 induced DSB site. We used a yeast strain (NA14) harboring an HO cut site integrated at the
335 mutant *ura3* locus, which is cleavable by HO endonuclease upon galactose induction (Agmon
336 et al., 2009; Fangaria et al., 2022). The strain also contains a distant *URA3* locus, which is
337 utilized to repair the cut *ura3* locus post-DSB induction. The cleavage by HO endonuclease
338 was first verified by PCR-based analysis using primers complementary to the DNA sequence
339 flanking the HO cut site (Figure 5A). The DNA damage was maximal at 2 hrs of galactose
340 induction, and the damage was majorly repaired by 4 hrs of galactose induction (Figure S7 A,
341 B).
342 We monitored the recruitment of Scm3 near DSB and at sites distal to that up to -3 kb
343 (Figure 5A) by performing ChIP of Scm3-6HA from the samples collected at every hr up to 4
344 hrs following galactose induction. As a positive control for Scm3 ChIP, we found significant
345 enrichment of Scm3 at the centromere (*CEN3)* (Figure S7C). We observed a significant
346 increase in the Scm3 binding near DSB at 2 hrs of galactose induction and a decrease in the
347 enrichment at 4 hrs, possibly due to the gradual repair of the damage (Figure 5B, C). We also
348 observed an increase in the enrichment of Scm3 up to -3 kb sites distal to the cut site, which
349 decreased gradually with damage repair (Figure 5C) and no significant enrichment at the
350 *TUB2* locus, used as negative control (Figure 5C) or in the no antibody control (Figure 5D).
351 This suggests that Scm3 is maximally recruited to the cleavage sites at the time of DNA
352 damage and gradually dissociates as and when the DNA is repaired. To verify that HO-

353 induced DSB is able to recruit the DNA repair proteins, we also investigated the enrichment
354 of such a protein, Rad51, that is known to bind the DSB sites (Fangaria et al., 2022) and found
355 it is maximally enriched at these sites during 2-3 hrs of post-galactose induction (Figure S7D).
356 We conclude that upon DNA damage Scm3 associates with the damage sites and may act in
357 *cis* to facilitate damage repair.
358
359 **Activation of the DDR checkpoint is perturbed in the *scm3* mutant.**
360
361 Having shown the recruitment of Scm3 at the DNA damage sites, we wished to address the
362 role of Scm3 at these sites in case of DNA damage. Since we found that Scm3 depleted cells
363 are compromised in repairing DNA damage, it is possible that being at the damage sites Scm3
364 activates the DDR checkpoint, which is required for the damage repair. In the DDR pathway,
365 Rad53 is an effector kinase that becomes phosphorylated in a Mec1 sensor kinase-dependent
366 manner in response to DNA damage (Ciccia and Elledge, 2010; Melo and Toczyski, 2002;
367 Zhou and Elledge, 2000). Phosphorylation of Rad53 is essential to halt the cell cycle and
368 provide cells time to repair the damaged DNA. To examine whether Scm3 depleted cells have
369 defects in the activation of DDR checkpoint and in halting the cell cycle, we measured Rad53
370 phosphorylation status in the wild type and the Scm3 depleted cells after treating them with
371 0.02% MMS for 90 mins (Figure 6A). Anti-Rad53 antibodies that recognize both
372 phosphorylated and unphosphorylated forms of Rad53 were used. We observed lesser
373 phosphorylation of Rad53 in the *scm3* than in the wild-type cells treated with MMS, but no
374 difference was found in the untreated cells (Figure 6B).
375 Since Scm3-depleted cells, unlike the wild type, arrest at the G2/M stage due to
376 activation of spindle assembly checkpoint (SAC) (Camahort et al., 2007; Stoler et al., 2007),
377 the observed difference in Rad53 phosphorylation could be due to the difference in cell cycle
378 stages. To circumvent this issue, we grew *CDC20-AID* and *SCM3-AID* cells for 3 hrs before
379 treating them with 0.75 mM auxin for 2 hrs to arrest them first at G2/M by depleting Cdc20 and
380 Scm3, respectively (Figure 6C). The cells were then treated with 0.02% MMS and harvested
381 at indicated time points for total protein extraction. The *CDC20-AID* and *SCM3-AID* cultures
382 showed around ~80% metaphase arrest cells, in 2 hrs of auxin treatment (0 mins in Figure
383 S8A). Notably, while the Cdc20-depleted cells maintained the arrest even with a longer
384 incubation in auxin, the Scm3-depleted cells tend to bypass the arrest after a certain time and
385 showed accumulation of multibudded cells with fragmented DAPI, an arrest-release
386 phenotype (Figure S8B, Figure 6E) Consistent with our previous results (Figure 6C), we
387 observed that in Scm3 depleted cells, Rad53 is less phosphorylated than in Cdc20-depleted
388 cells (Figure 6D), in particular, while comparing the bands at 120 mins when the percentage
389 of metaphase cells was found similar in two cultures (Figure S8B). These results indicate that

390 in absence of Scm3 when there is DNA damage the activation of the DDR checkpoint is
391 improper and consequently the phosphorylation of Rad53 is compromised.
392 If the loss of Scm3 fails to activate the damage checkpoint properly, then it is expected
393 that the cell cycle arrest phenotype in response to DNA damage would also be defective and
394 would produce multibudded cells. We wished to examine this possibility. However, Scm3-
395 depleted cells arrest by spindle assembly checkpoint (SAC) at G2/M due to improper
396 kinetochore-microtubule attachment because of failure in Cse4 deposition (Camahort et al.,
397 2007; Stoler et al., 2007). To exclude the effect of SAC-mediated arrest, we deleted the SAC
398 component *MAD2* in *SCM3-AID* cells. These cells were first depleted of Scm3 by treatment
399 with auxin and then, along with *mad2Δ* control cells, were challenged with or without MMS for
400 90 mins before they were analyzed for percentage of multi budded cells (Figure 6E). While
401 the *mad2Δ* cells in the presence of MMS showed nominal multi budded cells (<5%), the same
402 cells upon depletion of Scm3 showed a steep rise in the phenotype (>40%) suggesting that
403 Scm3 depleted cells fail to activate DNA damage checkpoint and to arrest the cell cycle in the
404 presence of DNA damage.
405 Since Scm3 depleted cells showed reduced Rad53 phosphorylation, we wished to
406 examine if the lack of Scm3 perturbs Mec1 kinase function that phosphorylates Rad53. Mec1
407 phosphorylates histone H2A at S129 position upon DNA damage (Lee et al., 2014). However,
408 we failed to observe any significant difference in the phosphorylation status of H2AS129
409 between Scm3 or Cdc20 depleted cells (Figure S8C), indicating that Mec1 function is not
410 perturbed in absence of Scm3.
411
412 **Phosphorylation of Scm3 in response to DNA damage**
413 The function of Scm3 in DDR could be mediated by a damage-induced post-translational
414 modification of this protein. To examine if Scm3 becomes modified upon DNA damage, we
415 treated *SCM3-6HA* cells with increasing concentrations of MMS or with 0.02% MMS with
416 increasing duration. We observed slower migrating bands of Scm3 (Scm3*) that became
417 brighter with increasing concentrations of MMS and present very faintly even in the untreated
418 cells (Figure 7A, left panel). We observed an increase in the normalized intensity of Scm3*
419 and Scm3*/Scm3 ratio with increasing MMS concentration (Figure 7A, middle and right
420 panels). A similar observation was also made when the cells were treated with 0.02% MMS
421 for increasing time (Figure S9A). Since we could detect Scm3* bands in the untreated cells as
422 well, its enrichment in response to MMS could be due to a cell cycle arrest caused by MMS.
423 To understand the dynamics of the appearance of Scm3* bands over different cell cycle stages
424 and in response to MMS, we released the *bar1Δ SCM3-6HA* cells from alpha-factor mediated
425 G1 arrest into the medium in the absence or presence of MMS, as shown in Figure 7B. We

426 harvested the cells at indicated time points and evaluated the cell cycle synchrony using bud
427 morphology and DAPI staining (Figure 7C, Figure S9 B, C), which depicts that the majority of
428 the cells at 0, 30, 60, 90, and at 120 mins are in G1, S, metaphase, metaphase/anaphase and
429 anaphase, respectively. We could faintly observe the Scm3* bands after release from the G1
430 phase in MMS-free (untreated) media, and the band intensity did not change over the time
431 period. However, when the cells were released in the presence of MMS, we observed an
432 increase in the intensity of the Scm3* bands over time (Figure 7D). These results suggest that
433 the accumulation of Scm3* in the MMS treated cells is due to DNA damage rather than an
434 effect of the pace of the cell cycle. It is possible that Scm3* bands are phosphorylated forms
435 of Scm3 since the protein has several serine/threonine residues. To test this, we challenged
436 the Scm3-6HA cells with MMS, as in Figure 7A, and resolved the proteins on SDS-PAGE with
437 or without Phos-Tag reagent. The presence of Phos-Tag in the gel enables slow mobility of
438 the phosphorylated proteins and thus further differentiates them from their unphosphorylated
439 forms. Figures S9D and E show the migration of the proteins isolated from *SCM3-6HA* cells
440 challenged with different concentrations of MMS in the absence or presence of Phos-Tag
441 reagent, respectively. The mobility of the Scm3* bands was further reduced with respect to
442 the Scm3 band in Phos-Tag gel compared to the normal gel, suggesting that the modification
443 is likely due to phosphorylation. Notably, in several high throughput studies, Scm3 has been
444 shown to be phosphorylated at S39, S64, and S165 residues (Lanz et al., 2021; MacGilvray
445 et al., 2020; Zhou et al., 2021). The S64 phosphorylation of Scm3 was found to be enriched
446 in cells after MMS treatment for 2 hrs as compared to G1 arrested cells, although the authors
447 could find S64 phosphorylation in only 2 out of 4 replicates used in their study (Lanz et al
448 2021). However, in the absence of further validation in those studies, our observation of a
449 gradual increase in the Scm3* band intensity with increasing dose of MMS convincingly
450 demonstrate DNA damage dependent phosphorylation of Scm3.
451 Since, Mec1 kinase majorly contributes to the activation of DDR through protein
452 phosphorylation, we tested if Scm3 undergoes a Mec1-dependent phosphorylation in
453 response to MMS. We constructed the *sml1*Δ *mec1*Δ *SCM3-6HA* strain as *mec1*Δ cells are
454 viable in the absence of Sml1 (Zhao et al., 2001, 1998) and challenged this and other control
455 strains with MMS. Briefly, wild-type, *SCM3-6HA*, *sml1*Δ*SCM3-6HA*, and *sml1*Δ *mec1*Δ *SCM3-*
456 *6HA* cells were grown till mid-log and spotted on the plates containing MMS (Figure S9F). As
457 expected, the *sml1*Δ *mec1*Δ *SCM3-6HA* cells showed extreme sensitivity to MMS. However,
458 we could not observe any loss of Scm3 phosphorylation in the *sml1*Δ *mec1*Δ background in
459 presence of MMS, suggesting that Scm3 phosphorylation in response to DNA damage is not
460 dependent on Mec1 kinase activity (Figure S9G).
461
462 **Discussion**

463 The bonafide function of the Cse4 chaperone, Scm3, is to deposit Cse4 at the centromeres
464 during the S phase in budding yeast to facilitate kinetochore formation. The observation that
465 Scm3 persists not only at the centromeres throughout the cell cycle but also associates with
466 the gross chromatin argues for additional kinetochore-independent functions of Scm3 in
467 maintaining genome stability. While in metazoan, Scm3 homolog HJURP was initially
468 identified as a protein involved in DNA damage response (DDR), how HJURP regulates the
469 DDR pathway besides its role in CENP-A deposition is not well understood. The DDR role of
470 Scm3 has never been reported in budding yeast. Whether Scm3 also contributes to DDR
471 pathway in budding yeast is important to address since this organism is distinct from
472 metazoans in terms of harboring small 125 bp point centromeres contrasting megabase pair
473 large regional centromeres in metazoans and having lesser epigenetic control on biological
474 functions, including DNA damage repair. Here, for the first time, we report that Scm3, perhaps
475 upon phosphorylation, promotes DNA damage repair by contributing to the activation of the
476 DNA damage checkpoint, and in the process, it associates with the DDR proteins at the DNA
477 damage sites (Figure 8). Our findings have a significant impact on advancing knowledge of
478 how CENP-A chaperones can be involved in the DDR pathway.
479
480 **Lack of Scm3 increases DNA damage sensitivity**
481 It is technically challenging to perform a growth-based sensitivity assay using cells depleted
482 for an essential gene. To circumvent this issue with Scm3, which is essential for cell survival,
483 we used partial depletion of Scm3 to assess the sensitivity of the partially depleted cells to the
484 genotoxic agents, including MMS, HU, and CPT and observed that such cells were sensitive
485 to those agents, (Figure 1, Figure S2A). However, since the primary function of Scm3 is to
486 promote kinetochore formation by depositing Cse4 at the centromeres, it is important to
487 address if the observed sensitivity is due to perturbation in kinetochores. Therefore, we
488 similarly partially depleted two essential kinetochore proteins, Ndc10 and Mif2, and deleted
489 one non-essential kinetochore protein, Ctf19, in separate cells but failed to detect any
490 increased sensitivity to DNA damage stress (Figure S3). These results indicate that the drug
491 sensitivity phenotype of Scm3 depleted cells is not due to weakly formed kinetochores. The
492 arguments favoring the possible centromere-independent DDR functions of Scm3 can be
493 gleaned as follows. From comparing the domain organization of Scm3 with HJURP (Figure
494 S1A), we noticed that besides the CENP-A/Cse4 binding domain, both HJURP and Scm3
495 have a common DNA binding domain, which is dispensable for CENP-A/Cse4 binding and
496 also for Ndc10 binding in budding yeast. Both in vitro and in vivo evidence are there
497 demonstrating binding of the DNA binding domain to the DNA (Kato et al., 2007; Mizuguchi et
498 al., 2007), suggesting this domain can indeed recruit Scm3 also at the non-centromeric DNA
499 damage sites. In support of this, Scm3 has recently been shown to be phosphorylated in an

500 MMS-dependent manner at S64 (Lanz et al., 2021), which, interestingly, lies within the DNA
501 binding domain. Among other non-essential regions of Scm3, neither the NLS region nor the
502 C-terminal region is involved in DDR (Figure S1B). On the other hand, the D/E-rich region,
503 which is important for Scm3 stability (Stoler et al., 2007), forms a disordered domain as per
504 alpha-fold prediction and hence is believed to be capable of interacting with multiple proteins
505 or can act as a scaffold for engaging several protein-protein interactions which is required in
506 DDR.

507 MMS halts replication forks due to formation of DNA adducts, which eventually
508 generate recombinogenic lesions (Conde and San-Segundo, 2008). In yeast, these lesions
509 are repaired primarily through homologous recombination (HR), facilitated by the Rad52
510 epistasis group of proteins (Pizzul et al., 2022; Sirbu and Cortez, 2013). Rad52, the central
511 component of the HR pathway, deposits Rad51 onto the broken DNA strand, which then
512 searches for the homologous template and undergoes strand exchange to repair the broken
513 DNA (Haber, 2018). In the presence of any DNA damage, Rad52 relocalizes to form bright
514 foci, which mark the repair centres. Strikingly, we observed that the Rad52 foci are increased
515 drastically in the absence of Scm3, even without any external DNA damage (Figure 2C, Left).
516 The maximal accumulation of Rad52-GFP foci was noticed during the 60 mins post-G1
517 release, which coincides with the timing of DNA replication (Figure S4C), indicating that Scm3
518 might be required to avoid generating DNA lesions during replication, which itself is genotoxic.
519 In support of this, previously, it was observed that a significant cell population arrests during
520 the S phase in the absence of Scm3 (Camahort et al., 2007). As expected, the accumulation
521 of Rad52-GFP foci was significantly higher in the *scm3* mutant as compared to the wild-type
522 cells when they were treated with MMS, suggesting that cells become more vulnerable to DNA
523 damage in the absence of Scm3 (Figure 2C, Right).
524

525 **Association of Scm3 with non-centromeric loci**

526

527 In addition to the localization of Scm3 at the kinetochores, we also found it at other loci,
528 including at the ribosomal DNA (rDNA) loops (Figure 3A, arrowhead). In budding yeast,
529 chromosome XII, harbors rDNA with around 150 repetitive loci (Dammann et al., 1993; Smith,
530 2022). Recombination among these repeats excise rDNA circles, and therefore, efficient DNA
531 damage repair through HR at these loci is crucial for preserving genomic stability. Notably,
532 HJURP has been identified in mammals to bind to the rDNA region and maintain its stability
533 (Kato et al., 2007). The degradation of HJURP using siRNA resulted in increased rDNA
534 recombination events and chromosome instability (Kato et al., 2007). The presence of Scm3
535 at the rDNA loops suggests that Scm3, like HJURP, is also important in regulating the
536 recombination and repair processes at those loci (Figure 3D).

537 Both Rad51 and Rad52 have been shown to mark the site of recombination and repair
538 (Lee et al., 2003; Lisby et al., 2001; Miyazaki et al., 2004). In our assay, we observed a
539 statistically significant co-localization of Scm3 with Rad52 in response to DNA damage (Figure
540 4A). Since during DSB repair, multiple lesions are recruited to a few repair centers, which are
541 marked by Rad51 and Rad52, the colocalization of these proteins with Scm3 implicates the
542 recruitment of Scm3 at the repair centers. Moreover, an overall increase in the chromatin
543 association of Scm3 in response to MMS also suggests that Scm3 is recruited to several repair
544 centers (Figure 3B, C, E, S5). However, as expected, no such increase in the chromatin
545 association was observed for an inner kinetochore protein, Ndc10. It is possible that Scm3
546 might physically interact with Rad51/Rad52 through the HR or DNA binding domain or the D/E
547 rich region present in the protein (Figure S1A). However, we failed to observe any interaction
548 of Scm3 with Rad52 using co-IP assay.
549 DNA damage sites are epigenetically marked by phosphorylation of H2AS129 (γ-H2A)
550 through ATM/ATR kinase pathway (Burma et al., 2001; Lee et al., 2014) However, in budding
551 yeast, γ-H2A is also recruited at the heterochromatin regions (Kitada et al., 2011). In
552 unperturbed cells, we could visualize γ-H2A foci (Figure 4C), possibly due to its presence at
553 the stalled replication forks, fragile sites, telomeric regions, or at the heterochromatin regions
554 (Kirkland et al., 2015; Kitada et al., 2011; Rg Bewersdorf et al., 2006). The increase in intensity
555 of γ-H2A upon MMS treatment (Figure S6A, B) indicates the presence of γ-H2A at multiple
556 DNA damage sites. The significantly high co-localization of Scm3 (PCC > 0.6) with γ-H2A in
557 the MMS treated cells, therefore, indicates that Scm3 is indeed recruited to the DNA damage
558 sites (Figure 4D). This was further verified by using an ectopic DSB system, where we showed
559 the recruitment of Scm3 to the induced DSB sites (Figure 5B). Notably, Scm3's presence was
560 found not only limited to the regions very close to the cut site but also up to 3 kb distal from
561 there. This may be the reason for observing a high co-localization of Scm3 with γ-H2A in
562 response to MMS, as the latter also spreads distal to the cut site (Lee et al., 2014). Although
563 Scm3 binding at the cut site peaked at 2 hrs following HO induction, the time of maximal DNA
564 cleavage, it persisted for at least 3-4 hrs post induction even when ~60% of the cut DNA was
565 repaired. This suggests that Scm3 is recruited to the sites of DNA damage and persists there
566 during the repair process.
567
568 **Scm3 regulates DNA damage checkpoint**
569 We wanted to address the mechanism by which Scm3 might be involved in DDR. The DNA
570 damage checkpoint arrests the cell cycle in response to DNA damage, allowing cells to repair
571 the damage and maintain cell viability. The serine/threonine kinases Mec1/Tel1 in yeast are
572 the sensor kinases that are activated and recruited to the sites of DNA damage. These kinases

573 relay the signal downstream by phosphorylating and activating the effector kinases, including
574 Chk1 and Rad53 (Pizzul et al., 2022; Zhou and Elledge, 2000). The activation of the effector
575 kinases is critical for activating the further downstream substrates that eventually halts the cell
576 cycle. We observed a defect in the Rad53 phosphorylation in response to MMS-mediated DNA
577 damage in the absence of Scm3 (Figure 6B). Failure to activate Rad53 in the presence of
578 damage leads to improper G2/M cell cycle arrest, which can produce the multi-bud phenotype.
579 In the Scm3 depleted cells deleted for Mad2 to remove the ability of SAC mediated arrest (due
580 to kinetochore perturbation), we observed a significant population of multibudded cells (Figure
581 6E) when they were challenged with MMS. Only *mad2*Δ cells challenged with MMS did not
582 show such multi-bud phenotype, suggesting that Scm3 is important to arrest the cells in
583 response to DNA damage. Next, we wished to know why the Scm3 depleted cells are
584 compromised to phosphorylate Rad53. It is known that Rad9 acts as an adaptor upon
585 phosphorylation by upstream Mec1 kinase to phosphorylate Rad53 (Gilbert et al., 2001;
586 Pellicioli and Foiani, 2005). To test if lack of Scm3 attenuates Mec1 activity, we tested the
587 level of γ-H2A in the Scm3 depleted cells as Mec1 activity is also responsible to generate γ-
588 H2A (Burma et al., 2001; Lowndes and Toh, 2005). However, the level of γ-H2A was not
589 perturbed in the *scm3* mutant, suggesting that Mec1 activity is intact (Figure S8C). Therefore,
590 currently, we are uncertain about the cause of improper Rad53 phosphorylation; a defect in
591 its auto-activation property or in the action of some other mediator kinases, e.g., Tel1, may be
592 responsible, which can be tested. Nevertheless, given the increased phosphorylation of Scm3
593 in response to DNA damage, it is possible that the p-Scm3 is required to activate Rad53
594 (Figure 7A). Although we found that Scm3 phosphorylation is Mec1 independent (Figure S9G),
595 it is possible that other kinases are involved. While the involvement of CDKs in the DNA
596 damage checkpoint is not well understood, it is known that CDKs contribute to the
597 phosphorylation of both Rad52 and Rad51 to promote repair (Lim et al., 2020). Given that the
598 centromeric association of Scm3 varies with the cell cycle stage (Mishra et al., 2011), probably
599 due to oscillating levels of CDK activity, the DNA damage-dependent phosphorylation of Scm3
600 might also be regulated by CDKs. However, this does not rule out the involvement of other
601 kinases including Rad53 and Tel1, which are closely associated with the DNA damage
602 checkpoint pathway. Further investigation is required to precisely determine the kinase
603 involved in the Scm3 phosphorylation.
604 In summary, we report here that, like HJURP, Scm3 in budding yeast also possesses
605 functions in DDR, indicating that the centromere independent functions of the CenH3
606 chaperone are evolutionarily conserved. Although the chromatin landscape of budding yeast
607 is far more 'open' with lesser involvement of epigenetic determinants than in metazoans, the
608 inherent similarity in the DNA damage repair pathway may necessitate the utilization of these

609 chaperones in a similar fashion in both cell types. Not much has been studied about how
610 HJURP is involved in the DDR pathway, and as we reveal that Scm3 is involved in the proper
611 activation of the DNA damage checkpoint in the presence of DNA lesions (Figure 8), the same
612 can be tested for HJURP in metazoans as abrogation of such surveillance function in humans
613 can lead to aneuploidy and disease states. Our findings on Scm3 will pave the way for future
614 work using yeast as a model to decipher more genome wide functions of the CenH3
615 chaperone, which is crucial given its genome wide localization and evidence of its modulation
616 in several patients.

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622 Author contributions: PA performed experiments. PA and SKG designed experiments,
623 analyzed and interpreted the data. PA and SKG wrote the manuscript.

624 **Materials and Methods:**
625
626 **Yeast strains**
627
628 All *Saccharomyces cerevisiae* strains used in this study are derived from W303 genetic
629 background and are mentioned in Table S1. Standard growth conditions were used to grow
630 and harvest yeast for all the experiments. C-terminal tagging (AID, HA, Myc, GFP) or gene
631 deletions were performed using PCR-based integration at endogenous locations as described
632 before (Janke et al., 2004; Longtine et al., 1998; Wach, 1996). Standard lithium acetate-based
633 yeast transformation methods were employed for all genetic manipulations (Gietz and Woods,
634 2002).
635
636 **Media, reagents and growth conditions**
637 Yeast strains were grown using standard growth conditions (Sherman, 2002). For plates
638 containing MMS, HU and CPT (Sigma Chemicals, Switzerland), the drugs were top-plated on
639 YPD plates at appropriate concentrations. To arrest the cells at the G1 or metaphase stage of
640 the cell cycle, alpha factor (Sigma Chemicals, Switzerland) or nocodazole (Sigma Chemicals,
641 Switzerland) was added to the cells at indicated concentrations. For HO endonuclease
642 induction, strains were grown on a rich medium containing 3% glycerol prior to the addition of
643 3% galactose. For western blotting, immunoprecipitation, or immunofluorescence studies the

644 primary antibodies used were rat anti-HA (3F10, Roche), mouse anti-HA (12CA5, Roche),
645 mouse anti-Myc (9E10, Roche), rat anti-tubulin (MCA78G, Serotec), rabbit anti-Rad53
646 (ab104232, Abcam), rabbit anti-Rad51 (PA5-34905, Invitrogen) and rabbit anti-Histone H2A
647 (phospho-S129) (ab15083, Abcam). The secondary antibodies used were Rhodamine
648 (TRITC)-conjugated goat anti-Rat (Jackson), Alexa Fluor 488 conjugated goat anti-Mouse IgG
649 (Jackson), Rhodamine (TRITC)-conjugated goat anti-Rabbit, HRP-conjugated goat anti-
650 mouse IgG (Jackson), HRP-conjugated goat anti-rat (Jackson), HRP- conjugated goat anti-
651 rabbit (Jackson) at appropriate dilutions.
652
653 **Cell spotting and viability assay**
654
655 The cells were grown in standard media conditions till the mid-log phase. The cultures were
656 then 10-fold serially diluted and spotted on the plates containing auxin (dissolved in DMSO)
657 or other DNA-damaging agents. All the reagents were top-plated onto YPD or specific dropout
658 plates. After spotting, the plates were incubated at 30°C for 2-3 days before they were
659 photographed, depending on the growth rate.
660 For treatment of the cells in liquid culture, mid-log grown cells were treated with auxin
661 (dissolved in DMSO) or MMS for the indicated time points before they were harvested and
662 spotted on YPD plates or plates containing DNA damaging agents. To measure cell viability,
663 an equal number of cells were plated on drug free or drug containing YPD plates. The cells
664 were grown at 30°C for 2 days, and the number of viable colonies was counted. The colonies
665 formed on the drug free plate were taken as 100% viability, and accordingly the viability
666 percentage was calculated and plotted for drug containing plates.
667
668 **G1 arrest and release**
669
670 Cells were arrested in the G1 phase by adding 0.5 µg/mL α-factor to early log phase cells in
671 liquid culture (Amon, 2002). The cells were incubated for 2-3 hours or until at least more than
672 90% of cells showed shmoo morphology. For the release of the cells from α-factor arrest, the
673 cells were harvested and washed twice with pre-warmed water at 30°C which was followed
674 by washing twice with pre-warmed α-factor free media at 30°C with at least 10 times the
675 volume of the initial culture. The cells were then released in α-factor free media, and samples
676 were collected at different time points.
677
678
679 **Fluorescence imaging**
680

681 For live cell imaging, the samples were harvested at indicated time points and washed twice
682 in 0.1 M phosphate buffer before imaging. The cells were processed for DAPI staining, as
683 mentioned elsewhere (Mittal et al., 2020; Shah et al., 2023). Typically, the cells were fixed with
684 4% formaldehyde at RT for 5 mins. The fixed cells were washed twice with 0.1M phosphate
685 buffer (pH 7.5), vortexed in freshly prepared 50% ethanol for 30 seconds, and rewashed with
686 0.1M phosphate buffer. Before imaging, the cells were resuspended in freshly prepared DAPI
687 (1 µg/ml, Invitrogen) solution for 20 mins. The images were acquired through the Zeiss Axio
688 Observer Z1 fluorescence microscope (63× or 100×) in z-stack mode (0.2-0.5 µm spacing).
689
690
691 **Chromatin spread**
692
693 Chromatin spread was performed, as mentioned previously (Ma et al., 2023; Mehta et al.,
694 2014; Shah et al., 2023). Briefly, mid-log ($O.D._{600}$ = ~1.0) grown cells were harvested and
695 washed once with spheroplasting buffer (1.2 M sorbitol in 0.1 M phosphate buffer). The cells
696 were resuspended in 0.5 ml of the same buffer supplemented with 5 µl of β-mercaptoethanol
697 and 12.5 µl of zymolyase 20T (10 mg/ml, MP Biomedicals, USA) and incubated at 30ºC for 1
698 h. Once 80-90% of the cells were spheroplasted (by observing under the microscope), the
699 spheroplasting was stopped by adding 1 ml of ice-cold stop solution (0.1 M MES- pH 6.4, 1
700 mM EDTA, 0.5 mM $MgCl_2$, 1 M Sorbitol). The spheroplasts were centrifuged at 2000 rpm for
701 2 mins and resuspended in 120 µl of ice-cold stop solution. About 60 µl of spheroplasts were
702 mounted on an acid-washed clean glass slide. The cells were treated with freshly made
703 paraformaldehyde solution (4% paraformaldehyde, 3.4% sucrose), followed by 1% Lipsol (LIP
704 Equipment and services) solution for cell lysis. The lysed cells were homogenously spread
705 over the slide and air-dried at RT. After overnight drying, the slides were treated with 0.4%
706 Kodak Photoflow-200 to avoid photobleaching, followed by 5% skim milk as a blocking
707 solution, which was covered with coverslips. The coverslips were removed, and the slides
708 were incubated with primary and secondary antibodies (1:200) for 60 mins with three times
709 PBS washing between antibody treatments. The slides were then incubated with 100 µl of
710 DAPI (1 µg/ml, Invitrogen) for 30 mins. After PBS wash for 5 mins, 100 µl of mounting solution
711 (90% glycerol supplemented with 1 mg/ml p-phenylenediamine) was added over the slides. A
712 clean coverslip was placed over a slide and was sealed with transparent nail paint.
713
714 **Microscopic image analysis**
715
716 The images acquired through the Zeiss Axio (Observer Z1) fluorescence microscope were
717 processed using Zeiss Zen 3.1 software. The z-stacks with the best signal for a particular

718 channel were extracted and merged separately. For intensity calculation, a Region Of Interest
719 (ROI) was drawn around the signal of interest, and the integrated density was determined to
720 get the 'signal intensity' for both channels. An ROI of the same size was put elsewhere in the
721 background, and integrated density was determined to get the 'background intensity.' Each
722 signal intensity value was normalized with the background intensity values to get the
723 'normalized signal intensity' which was used to plot the graphs (Shah et al., 2023).
724 For Pearson's correlation coefficient (PCC) values to determine the extent of co-
725 localization, the merged z-stacks were extracted, and the 'Coloc' tool from the 'Imaris 8.0.2'
726 software was used. The 'automatic thresholding option' in the 'Coloc' tool was used to
727 calculate the threshold for each fluorescence emission channel. The PCC values were
728 recorded separately for each individual spread. The colocalization was described as no (PCC
729 <0.1), partial (PCC = 0.3-0.5) and complete (PCC>0.5) (Ma et al., 2023; Prajapati et al., 2018;
730 Zinchuk and Grossenbacher-Zinchuk, 2014).
731
732 **Induction of a site-specific DSB**
733
734 NA14 (Fangaria et al., 2022) cells were grown till 0.3 OD in the presence of 3% glycerol. 5 OD
735 of the cells were harvested (0 h), and the remaining cells were treated with 3% galactose for
736 4 hrs. 5 OD cells were harvested every hour till 4 hrs. The cells were lysed using glass beads
737 in lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8, and 1 mM EDTA
738 pH 8.0), and total DNA was isolated as described before ((Hoffman, 1997) and was solvent
739 extracted using PCI. The genomic DNA was precipitated and finally resuspended in 30 µl 1X
740 TE. The concentration of DNA was measured using a nano spectrophotometer. About 30 ng
741 of DNA was used for PCR to detect the DNA break using primers OSB289 and kanB1
742 (Fangaria et al., 2022). Primers against *TUB2* ORF were taken as a control.
743
744 **ChIP assay and qPCR quantification**
745
746 ChIP assay was performed as mentioned previously (Fangaria et al., 2022; Kumar et al., 2021;
747 Makrantoni et al., 2019; Mehta et al., 2014). First, cells were grown in the presence of 3%
748 glycerol till 0.3 OD. A total of 60 OD cells were harvested (0 h), and the remaining cells were
749 treated with 3% galactose. A total of 60 OD of cells were harvested every hr till 4 hrs. The cells
750 were then fixed with 1% formaldehyde for 30 mins at 25°C at 100 rpm, followed by quenching
751 by the addition of glycine at a final concentration of 125 mM. The cells were then harvested
752 and washed twice in ice-cold TBS buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl) and once
753 with 10 ml of FA lysis buffer (50 mM Hepes–KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1%
754 Triton X-100, 0.1% Na-deoxycholate) supplemented with 0.1 % SDS. The pellet was

755 resuspended in 500 µl of 1X FA lysis buffer supplemented with 0.5% SDS, 1 mM PMSF, 1X
756 PIC. The cells were lysed using 0.5 mm glass beads using a mini-bead beater (BIOSPEC,
757 India) for 7 cycles (1 min ON, 2 mins OFF on ice). The lysate was collected in a pre-chilled
758 1.5 ml eppendorf tube and centrifuged twice at 16,000 x g for 15 mins at 4°C. The pellet was
759 resuspended in 300 µl ice-cold 1X FA lysis buffer supplemented with 0.1% SDS, 1 mM PMSF,
760 and 1X PIC. The chromatin was then sheared to 200-600 bps using a water bath sonicator
761 (Diagenode SA, Picoruptor, BC 100,27 LAUDA Germany) for 30 cycles of 30 s ON / 30 s OFF
762 at 4°C. After clarification of the lysate by centrifugation at 16,000 x g for 10 mins at 4°C, 3-5
763 µg of appropriate antibodies (were added and incubated at 4°C overnight with gentle rotation.
764 Subsequently, 50 µl Protein-A conjugated Sepharose beads were added and incubated for 2
765 h at 4°C with rotation. The beads were washed twice with IP wash buffer I (1X FA lysis buffer,
766 0.1%SDS, 275 mM NaCl), followed by one wash in IP wash buffer II (1X FA lysis buffer,
767 0.1%SDS, 500 mM NaCl, III (10 mM Tris pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium
768 deoxycholate, 1 mM EDTA), and 1X TE at room temperature The beads were resuspended
769 in elution buffer and the chromatin was eluted by boiling at 65°C. The eluate was then
770 decrosslinked overnight at 65°C, followed by Proteinase K (SRL, India) treatment for 2 hrs at
771 45°C. The DNA was purified by PCI-based purification and precipitated overnight in chilled
772 ethanol at -80°C. The enrichment of obtained chromatin fragments was estimated using qPCR
773 (BioRad CFX96, USA) using specific primers targeting specific and non-specific (negative
774 control) chromatin loci, listed in Table S2. As mentioned before (Kumar et al., 2021; Shah et
775 al., 2023) the following equation was used to estimate the percentage of chromatin
776 enrichment. ChIP efficiency = Enrichment/Input X 100; Enrichment/Input = E^-ΔCT;
777 ΔCT = CT(ChIP) − [CT(Input) − LogEX(D)]; E = primer efficiency value, CT = Threshold values
778 obtained from qPCR, D = Input dilution factor. E was estimated as {[10^(-1/slope)]– 1} from
779 standardization graphs of CT values against dilutions of the input DNA.
780
781 **Chromatin immunoprecipitation (ChIP) and sequencing**
782
783 ChIP assay was performed as mentioned above with certain modifications. The cells were
784 grown till mid-log and treated with nocodazole (20 ug/ml) for 90 mins to arrest cells at
785 metaphase and were subsequently treated with 0.05% MMS for additional 90 mins. The cells
786 were harvested and processed for immunoprecipitation, as mentioned above except Protein-
787 A conjugated dyna beads were used instead of sepharose beads. 10 ng of DNA eluate was
788 taken to prepare the NGS library using a TruSeq ChIP sample preparation kit from Illumina.
789 The library was prepared as per the manufacturer's protocol. Library QC was performed by
790 measuring the concentration and size of the libraries by using the 4150 Tapestation system
791 from Agilent and Qubit dsDNA HS assay kit (Thermo Fisher Scientific). The libraries were

792 sequenced using the Illumina platform in paired-end mode with a read length of 151 bp by
793 Macrogen Asia Pacific Pte. Ltd, Singapore.
794
795 **Bioinformatics analysis**
796 The ChIP-Seq analysis was performed by Genotypic (Bengaluru,India), The raw reads were
797 processed using FastQC (Andrews S., 2010) for quality assessment and pre-processing,
798 which includes removing the adapter sequences and low quality bases (<q30) using
799 TrimGalore (Krueger F., 2015). The pre-processed high quality read data was aligned to
800 reference *Saccharomyces cerevisiae* genome data base using Bowtie**,** which is an ultrafast,
801 memory-efficient short read aligner geared towards quickly aligning large sets of short DNA
802 sequences (reads) to large genomes. The aligned files (BAM files) were used for peak calling
803 using MACS2 software. The peaks were called with respect to the input sample. The .bed files
804 generated were further converted to .bigwig files and the plots were prepared in the IGV
805 browser (Robinson et al., 2011).
806
807 **Protein extraction, gel running and western blotting**
808
809 Yeast cells harvested at the mentioned time points were washed once with ice-cold water and
810 once with ice-cold 20% TCA. To preserve the phosphorylation moieties, the cells were lysed
811 in the presence of 1X PhosSTOP (Roche). The pellet was resuspended in 200 µl of 20% TCA,
812 and an equal volume of glass beads was added, and cells were lysed using a mini bead beater
813 (BIOSPEC, India) for 7 cycles (1 min ON, 2 mins OFF on ice). The supernatant was collected
814 after puncturing the O-ring tube and centrifugating at 2800 rpm for 2 mins at 4°C. 400 µl of 5%
815 TCA was added to the beads and spun again to collect the supernatant. The total supernatant
816 was spun at maximum speed for 10 mins at 4°C. The precipitate was kept for air-drying for
817 10-15 mins before resuspending in 2X SDS sample buffer and 1X Tris (pH 8.0) for
818 neutralization. Before loading, the protein was boiled for 5 mins at 95°C and spun at maximum
819 speed for 30 seconds. Standard procedure was followed for SDS-PAGE and western blot
820 transfer.
821 For the gel containing the PhosTag reagent, 10 mM $MnCl_2$ was added to the resolving
822 gel along with 50 µM of the PhosTag reagent (AAL-107, FUJIFILM, JAPAN). Before western
823 blotting, the gel was washed three times with 1 mM EDTA for 10 mins, followed by 2 washes
824 in transfer buffer. The blot was further incubated with primary antibodies at a dilution of 1:2500,
825 while the secondary antibodies were used at 1:5000 dilution.
826
827 **Statistical analyses**
828

The data presented in the main figures and the supplementary figures were obtained from two to three independent experiments. The error bars in the individual bar graphs represent the standard error (SE) derived from the standard deviation (SD). The statistical significance (*p*) was determined by two-tailed Student's t-test, or one-way ANOVA test, as appropriate. When comparing more than one group, a one-way ANOVA test was used; otherwise, Student's t-test was used. The 'N' values denote the total number of cells analyzed from the combined 'n' number of replicates of the individual assays. The *p* values less than or equal to 0.05 are categorized as significant differences. The SD, SE, and statistical significance (*p*) values were calculated using automated modules of GraphPad Prism 9.0 (Version 9.4.1) software.

Figures

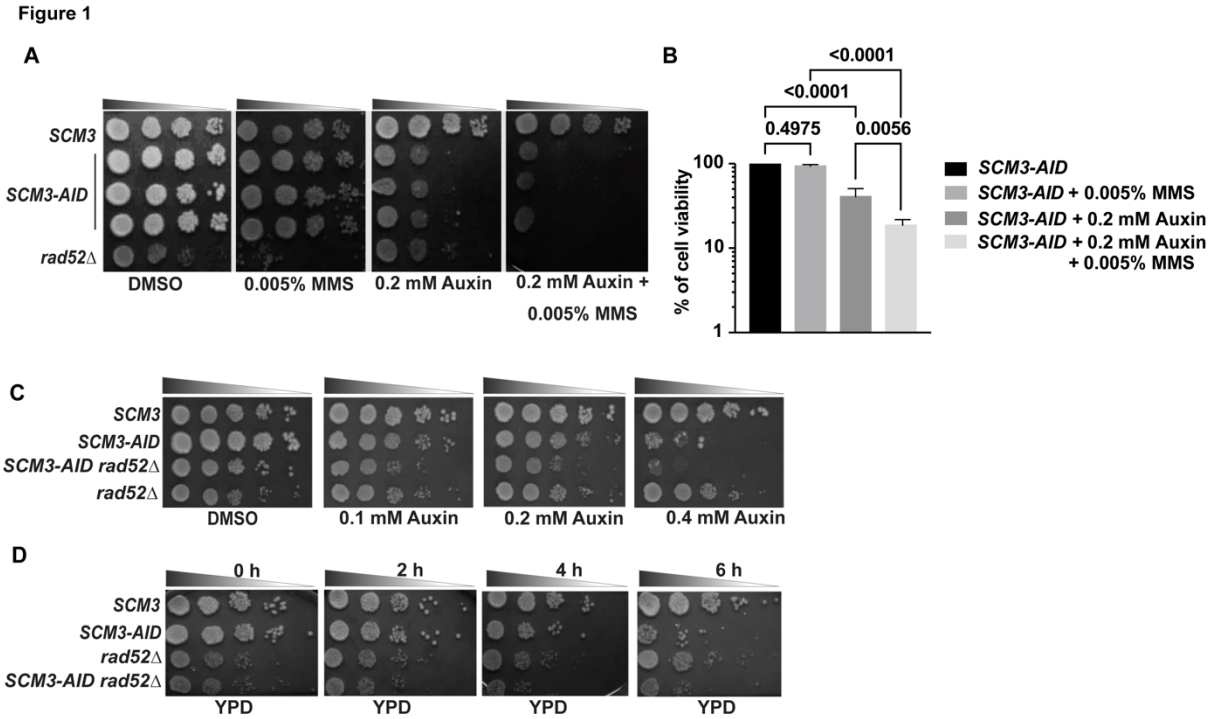
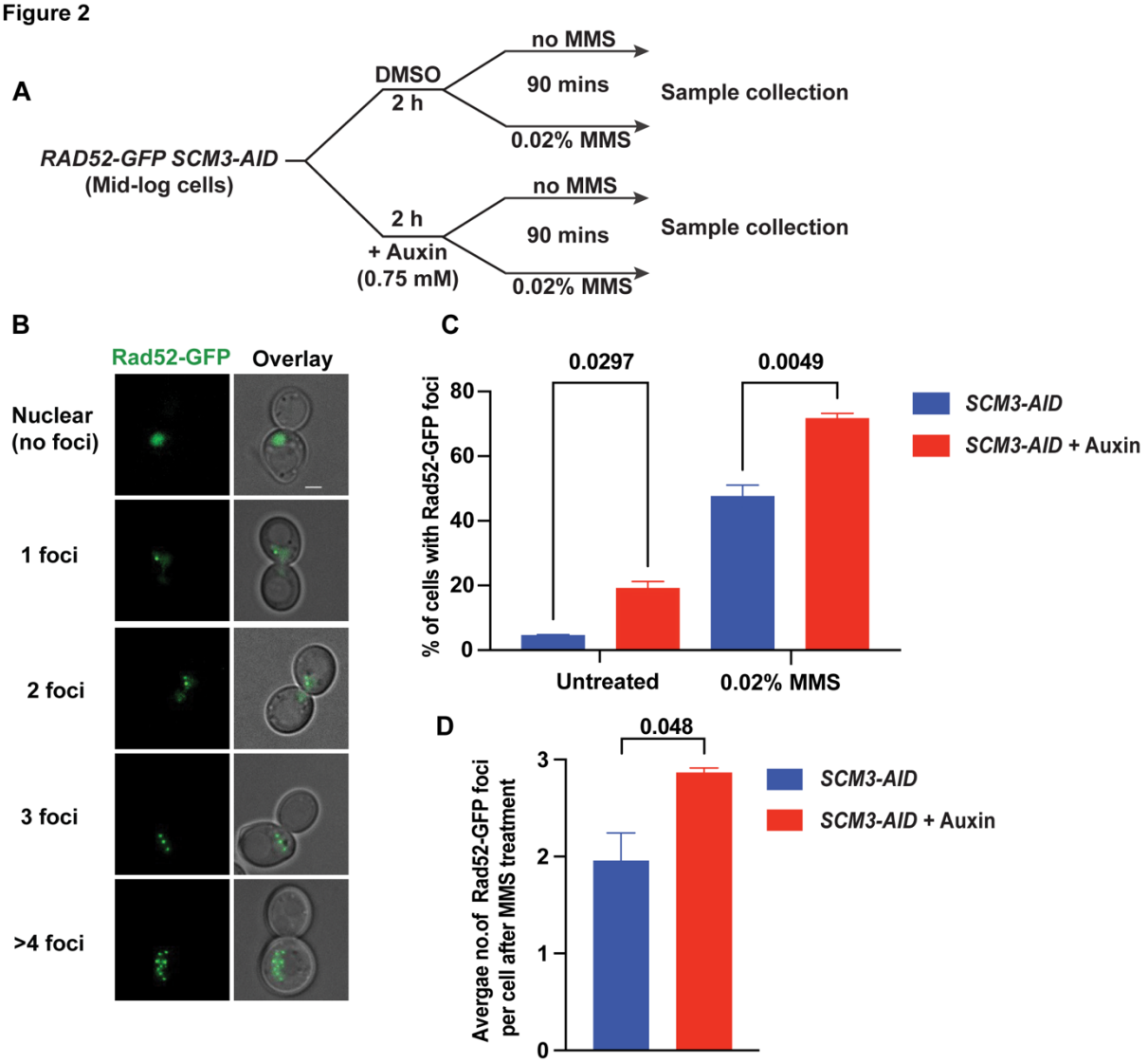


Figure 1. Loss of Scm3 causes sensitivity to MMS-mediated DNA damage. (A) Wild-type (*SCM3*), *SCM3-AID* (three independent transformants)*, and rad52*Δ cells grown till mid-log were serially diluted and spotted on the indicated plates. The plates were incubated at 30°C for 24-48 hrs before imaging. **(B)** An equal number of mid-log grown *SCM3-AID* cells were spread on plates containing indicated supplements. From the number of colonies that appeared after incubation at 30°C for 2 days, the cell viability was estimated (see materials and methods) and plotted. Colonies formed on the plate without auxin and MMS were taken as 100% viability, and accordingly, viability percentage was calculated for other plates. The *p* values were estimated by one-way ANOVA test from three independent experiments. **(C)** Wild-type (*SCM3)*, *SCM3-AID, SCM3-AID rad52*Δ*,* and *rad52*Δ cells grown till mid-log were serially diluted and spotted in the presence of indicated auxin concentrations. The plates were incubated at 30°C for 24-48 hrs before imaging. **(D)** The cells of the strains used in (C) were

854 grown till mid-log and treated with 0.75 mM auxin for the indicated duration before they were
855 washed, serially diluted and spotted on YPD plates that were incubated at 30°C for 24-48 hrs
856 before imaging.

857

Figure 2



858

859 **Figure 2. Loss of Scm3 causes increased Rad52 foci in the presence or absence of**
860 **MMS. (A)** The schematic of the experimental strategy that was followed for visualization of
861 Rad52-GFP foci in the *RAD52-GFP SCM3-AID* cells either in the presence of DMSO (wild-
862 type) or auxin (Scm3 depleted) and each either treated with MMS or left untreated. **(B)**
863 Representative images showing different patterns of Rad52-GFP foci. Rad52-GFP produces
864 a nuclear signal (row 1) in the absence of any DNA damage, but with the damage, it coalesces
865 to form bright foci (rows 2-5) representing the DNA repair sites. **(C)** The percentage of
866 indicated cells containing Rad52-GFP foci with or without MMS treatment for 90 mins. **(D)** The
867 average number of Rad52-GFP foci per MMS-treated cell from the experiment described in
868 (C). At least 300 cells were analyzed from three independent experiments for each set in **(C)**
869 and **(D)**. The *p* values were estimated by unpaired two-tailed student's t-test. Scale bar = 2
870 µm.

Figure 3

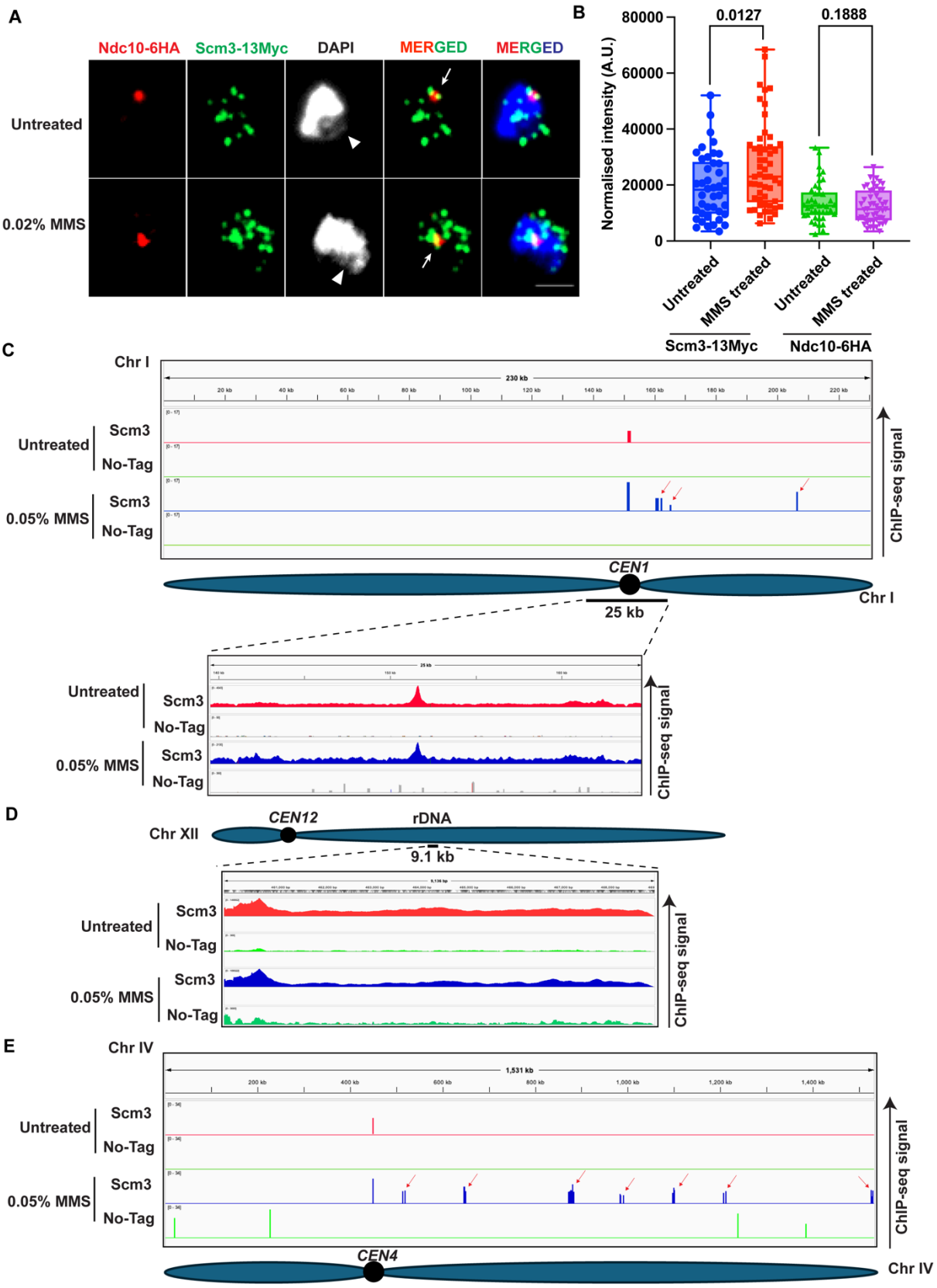
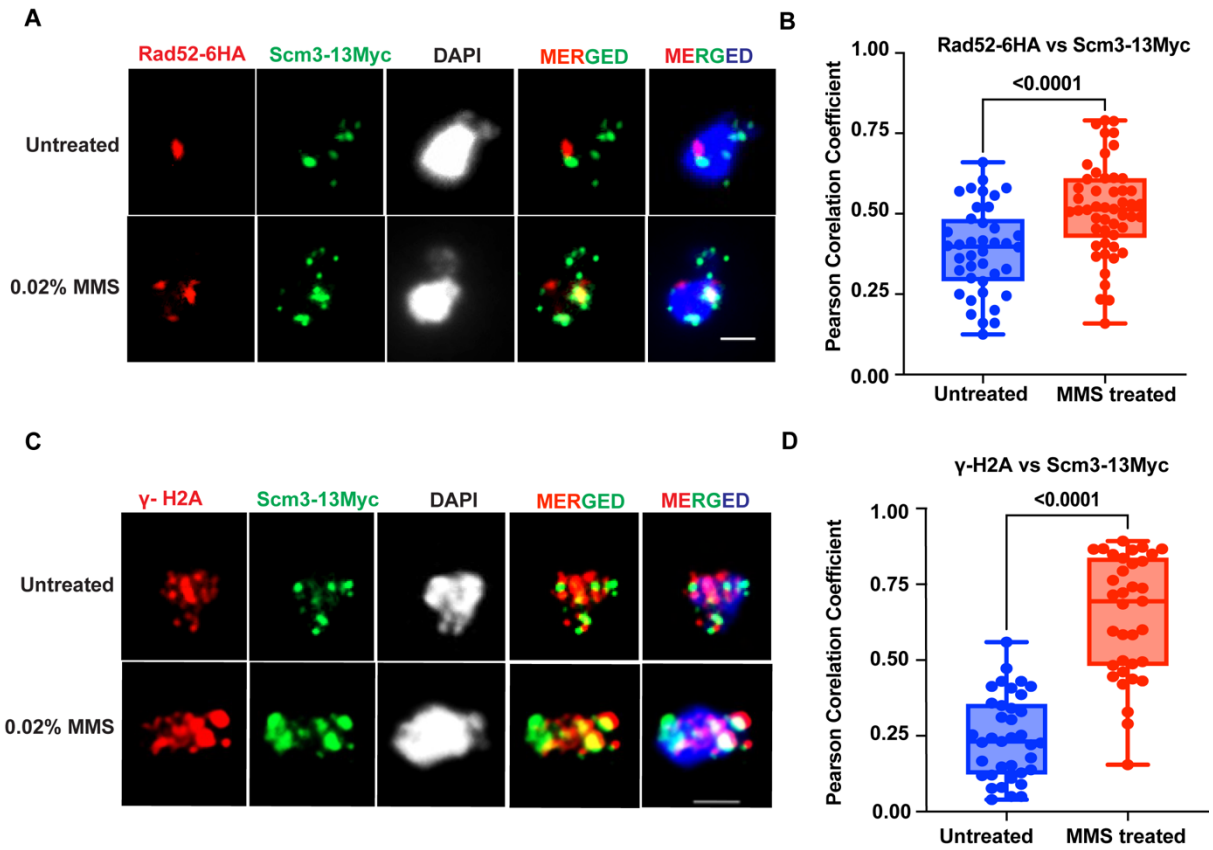


Figure 3. The localization of Scm3 in response to DNA damage. (A) Representative images showing the localizations of Ndc10-6HA and Scm3-13Myc using chromatin spread. The mid-log grown *NDC10-6HA SCM3-13MYC* cells were treated with 0.02% MMS or mock-treated for 90 mins for the assay. The arrow shows the co-localization of Ndc10-6HA and

876 Scm3-13Myc at the kinetochore cluster, and the arrowhead shows the rDNA loop. **(B)** The
877 intensities of Scm3-13Myc and Ndc10-6HA normalized with the background intensity were
878 quantified using ImageJ software and were plotted. At least 30 spreads from 2 independent
879 experiments were analyzed for each set in **(B).** The *p* values were estimated by unpaired two-
880 tailed student's t-test. Scale bar = 2 µm. **(C) The** ChIP-seq signal of Scm3 (Scm3-13Myc) from
881 cells either untreated or treated with 0.05% MMS for the entire chromosome I ; no-tag strain
882 was used as a control. The plot shows the binding of Scm3 across chromosome I. The inset
883 shows the magnified view of the pericentromeric region (25 kb) flanking *CEN1*. The inset
884 image is not input-corrected. The same is shown for the **(D)** 9.1 kb rDNA repeat from a portion
885 of chromosome XII and**(E)** entire chromosome IV. The non-centromeric localization of Scm3
886 in MMS treated cells is marked by the red arrow.
887
888
889

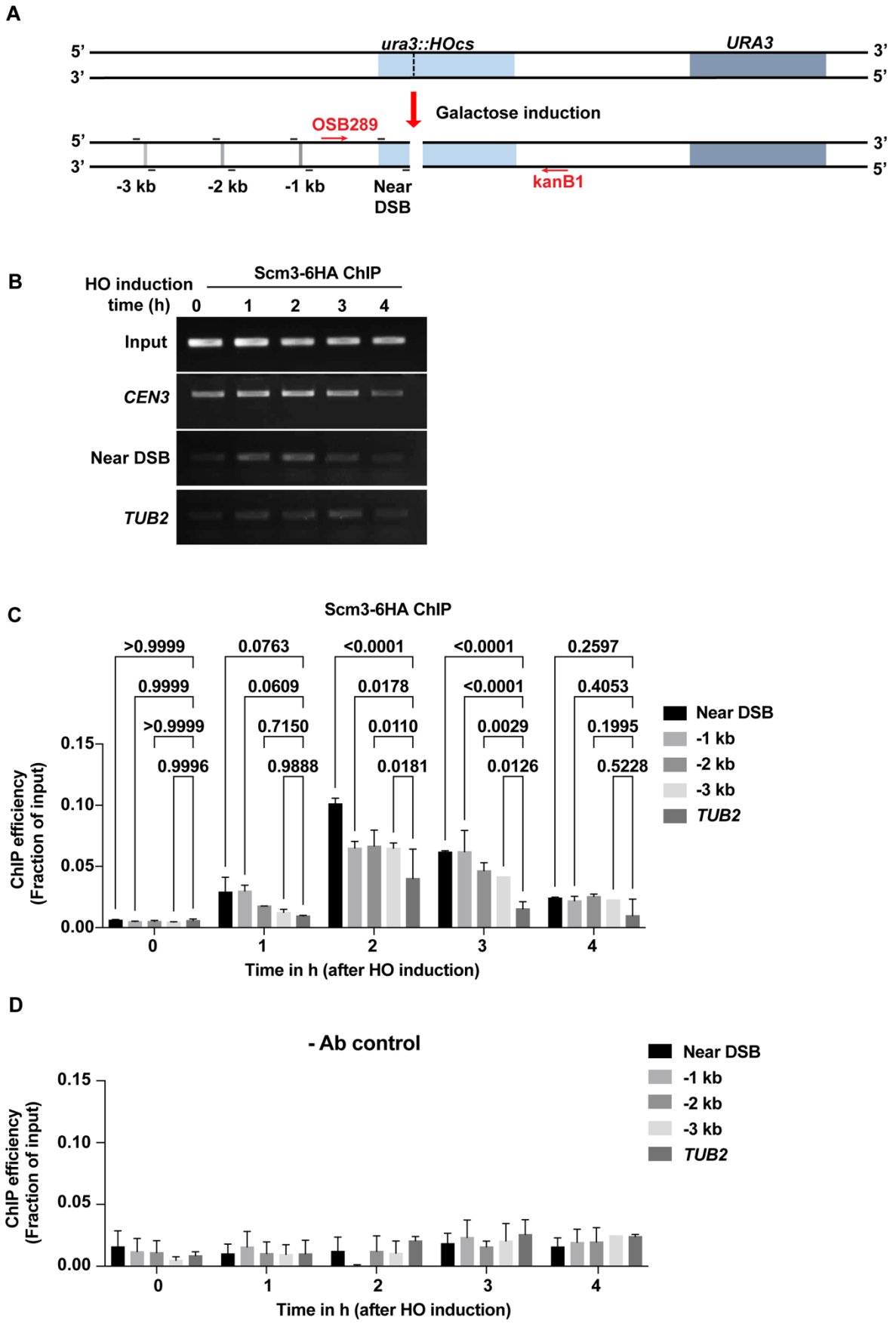
Figure 4



890
891
892 **Figure 4. Scm3 colocalizes with DDR proteins in response to DNA damage. (A)**
893 Representative images showing the localizations of Rad52-6HA and Scm3-13Myc using
894 chromatin spread. The mid-log grown *RAD52-6HA SCM3-13MYC* cells were treated with
895 0.02% MMS or mock-treated for 90 mins for the assay. **(B)** The quantification of the
896 colocalization between Rad52-6HA and Scm3-13Myc was calculated using Pearson's
897 correlation coefficient (PCC). **(C)** Representative images showing the localizations of γ-H2A
898 and Scm3-13Myc using chromatin spread. The mid-log grown *SCM3-MYC* cells were treated
899 with 0.02% MMS or mock-treated for 90 mins for the assay. **(D)** The quantification of the
900 colocalization between γ-H2A and Scm3-13Myc was calculated using PCC. Anti-HA (12CA5,

901 Roche), anti-Myc (9E10, Roche), and anti-H2A (ab15083, Abcam) antibodies were used to
902 detect HA, Myc, and H2A-S129, respectively. At least 30 spreads from 2 independent
903 experiments were analyzed for each set in **(B),** and **(D)**. The *p* values were estimated by
904 unpaired two-tailed student's t-test. Scale bar = 2 μm.
905

Figure 5


A
ura3::HOcs
URA3
5'
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3'
5'
Galactose induction
OSB289
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-1 kb
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kanB1
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HO induction time (h)
Scm3-6HA ChIP
0
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Input
CEN3
Near DSB
TUB2
C
Scm3-6HA ChIP
>0.9999
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0.9996
0.0763
0.0609
0.7150
0.9888
<0.0001
0.0178
0.0110
0.0181
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Near DSB
-1 kb
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TUB2
ChIP efficiency (Fraction of input)
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Time in h (after HO induction)
D
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Near DSB
-1 kb
-2 kb
-3 kb
TUB2
ChIP efficiency (Fraction of input)
0.15
0.10
0.05
0.00
0
1
2
3
4
Time in h (after HO induction)

906

907 **Figure 5. Scm3 is recruited to a site-specific double-stranded break (DSB). (A)**
908 Schematic representation of a cut site of the *HO* endonuclease (*HOcs*) integrated at the *URA3*
909 locus in the NA14 strain. Upon galactose induction, the *HO* endonuclease cleaves the DNA at
910 the specific site generating DSB. The positions of the primers (OSB289 and kanB1) are shown
911 that were used to detect the DSB, and the binding of Scm3 near and distal (-1 to -3 kb) to the
912 DSB **(B)** ChIP analyses were performed to quantify the association of Scm3-6HA in NA14
913 strain harboring *SCM3-6HA* with the indicated loci at the indicated time points. The PCR
914 amplified DNA bands following ChIP were run on agarose gel and stained with ethidium
915 bromide. **(C)** ChIP analyses for measuring the association of Scm3-6HA with the indicated loci
916 were performed at the indicated time points of HO induction using anti-HA (12CA5, Roche)
917 antibodies. The ChIP efficiency as percentage enrichment per input obtained by qPCR
918 analysis of the immunoprecipitated DNA was plotted. **(D)** Same analysis as in (C) using no
919 antibody (-Ab) control. The *p* values in **(C)** and **(D)** were estimated from two independent
920 experiments.
921
922

Figure 6

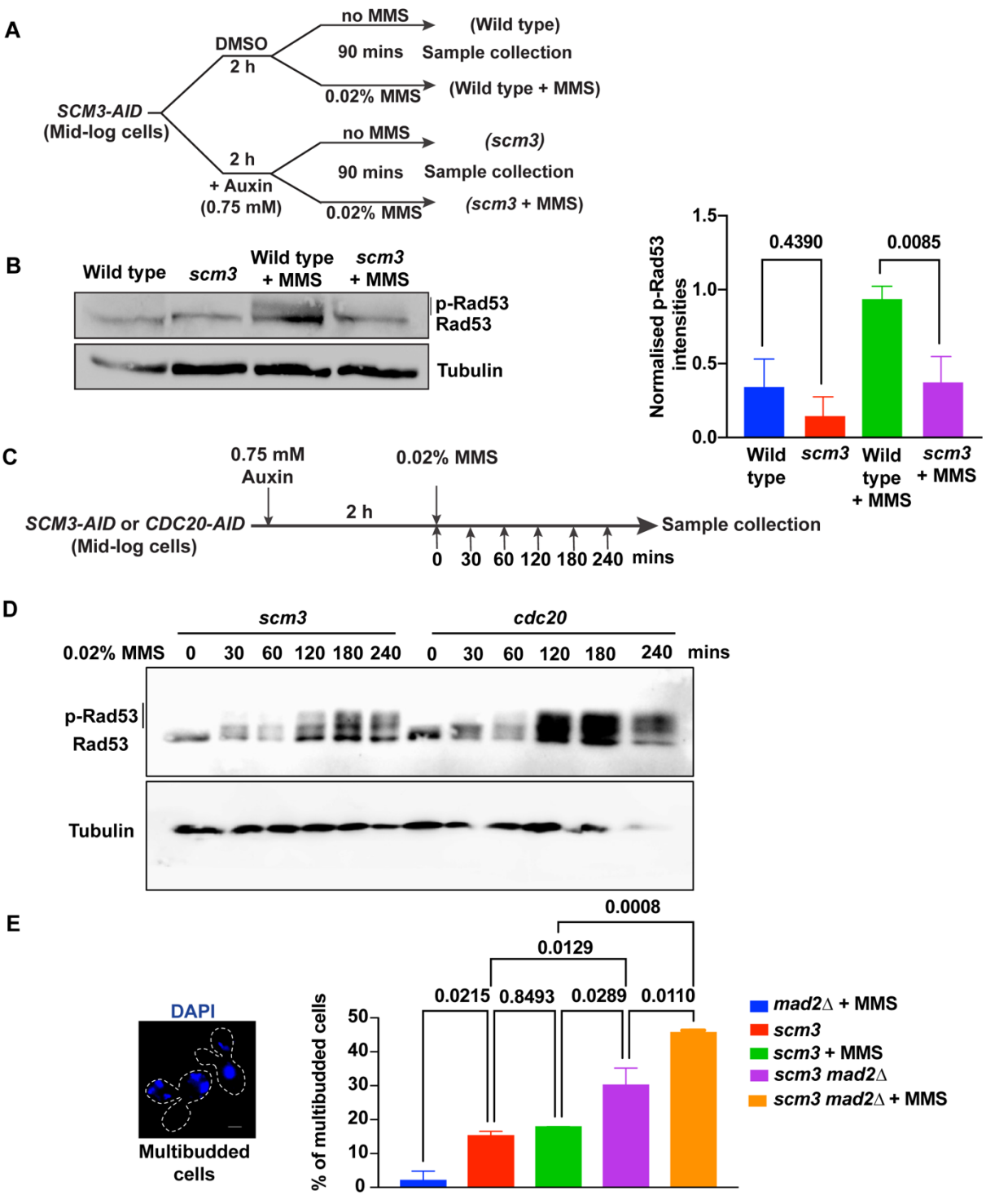

Figure 6. Evidence of improper activation of DNA damage checkpoint in absence of Scm3. (A) The schematic of the experimental strategy that was followed for detecting Rad53 in *SCM3-AID* cells in the presence of DMSO (wild-type) or auxin (Scm3 depleted) and each either treated with MMS or left untreated. **(B)** Left**,** western blots showing p-Rad53/Rad53 and tubulin (as loading control) using anti-Rad53 (ab104232, Abcam) and anti-tubulin (MCA78G, Serotec) antibodies, respectively, from indicated cells obtained from (A). Right, the intensities of the p-Rad53 measured by ImageJ were plotted after normalizing with the corresponding loading control. **(C)** The schematic of the experimental strategy to detect Rad53 in Scm3 or

932 Cdc20 depleted cells treated with MMS for different time points. **(D)** Western blots developed
933 as in **(**B) from indicated cells obtained from (**C**). **(E)** *mad2*Δ*, SCM3-AID,* and *SCM3-AID mad2*Δ
934 cells were first treated with 0.75 mM auxin for 2 hrs to deplete Scm3 and then either treated
935 with 0.02% MMS for 90 mins or left untreated. The cells were harvested and were scored for
936 the presence of multibudded cells (shown on the left). At least 150 cells from three
937 independent experiments were analyzed for each set**.** The *p* values were estimated by one-
938 way ANOVA test. Scale bar = 2 µm.

939

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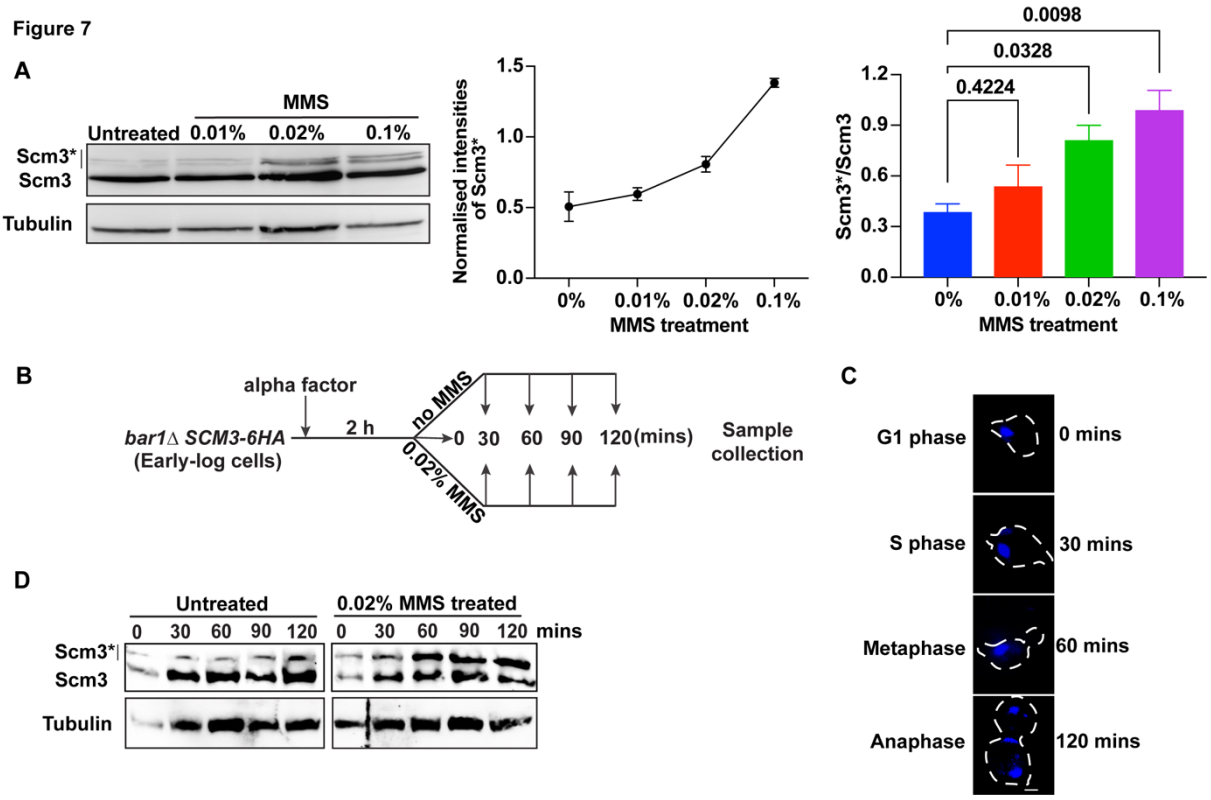

941

942

943 **Figure 7. Post-translational modification of Scm3 in response to DNA damage (A)** Left,
944 western blots show Scm3-6HA and its modified form (Scm3*) along with tubulin as loading
945 control from the cells untreated or treated with indicated concentrations of MMS for 90 mins.
946 The cell lysates were probed using anti-HA (12CA5, Roche) or anti-tubulin (MCA78G, Serotec)
947 antibodies. Middle, the graph shows the intensity of Scm3* with increasing MMS
948 concentrations, normalized with respect to tubulin using ImageJ software. Right, the graph
949 represents the ratio of the modified to unmodified forms of Scm3. **(B)** The schematic of the
950 experimental strategy that was followed for detecting Scm3-6HA in the G1 arrested cells and
951 following their release from the arrest for the indicated time in the absence or presence of
952 MMS. **(C)** Representative images showing the cell cycle stages by DAPI staining and bud
953 morphology at indicated time points since their release from G1 arrest. **(D)** Western blot
954 showing Scm3-6HA along with tubulin as a loading control from the cells harvested, as shown

in (B). The cell lysates were probed using anti-HA and anti-tubulin antibodies to detect Scm3-6HA and tubulin, respectively. Error bars represent the standard deviation from mean values obtained from two independent experiments. Scale bar = 2 µm.

Figure 8

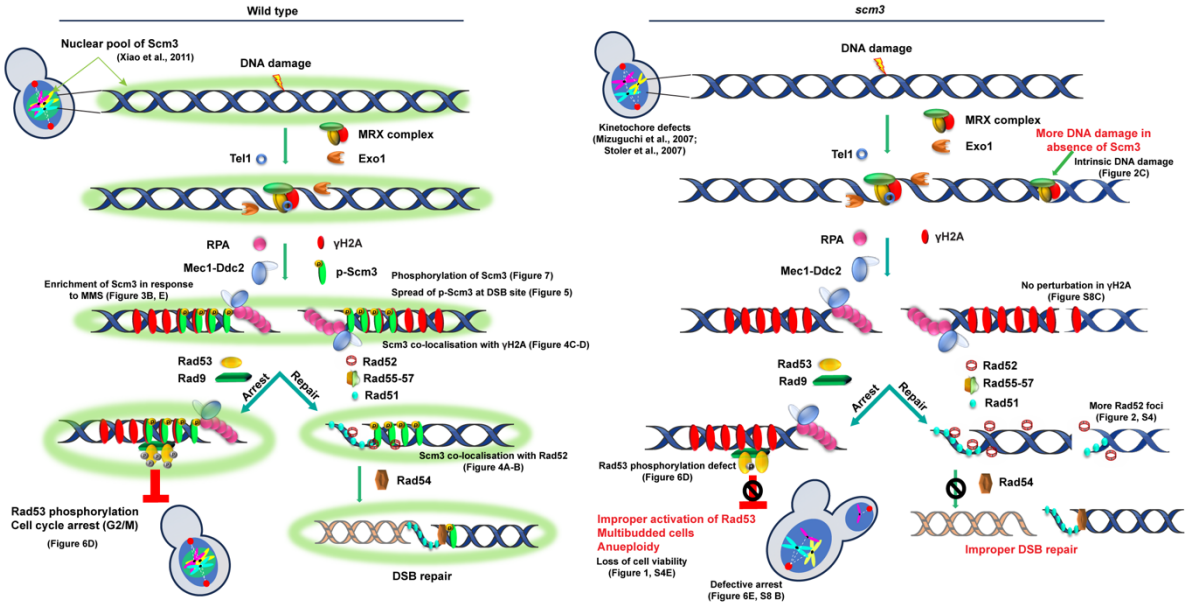

Figure 8: A working model summarizing the role of Scm3 in DNA damage response (DDR). Left, in wild type cells, a subset of the Scm3 molecules present as a nuclear pool are phosphorylated upon DNA damage. The phosphorylated Scm3 (p-Scm3) associates with double-strand break (DSB) sites and colocalizes with yH2A, which spreads to the chromatin surrounding the DSB site. Possibly, the p-Scm3 facilitates the proper activation of Rad53 through its phosphorylation, leading to cell cycle arrest. Additionally, p-Scm3 co-localizes with Rad52 and assists in DSB repair. Right, in cells lacking Scm3, there is also an increase in intrinsic DNA damage, corresponding to more Rad52 foci formation, and due to the inability to repair DSBs, the cell viability drops. Furthermore, reduced Rad53 activation causes improper DDR-mediated cell cycle arrest and generates aneuploids and multibudded cells.

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