Checkpoint inhibition of origin firing prevents inappropriate replication outside of S-phase

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Abstract

Across eukaryotes, checkpoints maintain the order of cell cycle events in the face of DNA damage or incomplete replication. Although a wide array of DNA lesions activates the checkpoint kinases, whether and how this response differs in different phases of the cell cycle remains poorly understood. The S-phase checkpoint for example results in the slowing of replication, which in the budding yeast *Saccharomyces cerevisiae* is caused by Rad53 kinase-dependent inhibition of the initiation factors Sld3 and Dbf4. Despite this, we show here that Rad53 phosphorylates both of these substrates throughout the cell cycle at the same sites as in S-phase, suggesting roles for this pathway beyond S-phase. Indeed we show that Rad53-dependent inhibition of Sld3 and Dbf4 limits re-replication in G2/M phase, preventing inappropriate gene amplification events. In addition we show that inhibition of Sld3 and Dbf4 after DNA damage in G1 phase prevents premature replication initiation at all origins at the G1/S transition. This study redefines the scope and specificity of the 'S-phase checkpoint' with implications for understanding the roles of this checkpoint in the majority of cancers that lack proper cell cycle controls.

Introduction

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32   It is vitally important that in every cell division the entire genome is replicated once

33   and only once. In eukaryotes this is achieved by linking DNA replication control to the

34   cell cycle (Siddiqui et al., 2013). The first step in replication is the formation of the

35   pre-replicative complex (pre-RC) at origins – a process called 'licensing'. Licensing

36   involves the Orc1-6 and Cdc6-dependent loading of double hexamers of the Mcm2-7

37   helicase on double stranded DNA. Licensing is restricted to late mitosis/early G1

38   phase by the activity of the APC/C, which eliminates licensing inhibitors such as

39   cyclin-dependent kinase (CDK) and geminin in this window of the cell cycle. In the

40   budding yeast *Saccharomyces cerevisiae,* which lacks geminin*,* CDK inhibits

41   licensing from late G1 phase until mitosis by multiple mechanisms including direct

42   phosphorylation of Orc2/Orc6, nuclear exclusion of the Mcm2-7 complex and by

43   mediating SCF^{CDC4}-dependent degradation of Cdc6 (Blow and Dutta, 2005; Nguyen

44   et al., 2001).

45   Importantly, Mcm2-7 double hexamers loaded in late M/early G1 phase are inactive

46   and replication initiation can only occur after the inactivation of the APC/C at the

47   G1/S transition. APC/C inactivation allows the accumulation of S-phase CDK and

48   Dbf4-dependent (DDK) kinase activities (Labib, 2010). DDK directly phosphorylates

49   the inactive Mcm2-7 double hexamers, generating a binding site for firing factors

50   including Sld3/Sld7 and Cdc45, while CDK phosphorylates Sld3 and an additional

51   initiation factor Sld2, which via phospho-interactions with Dpb11, results in replisome

52   assembly by poorly understood mechanisms (Riera et al., 2017). This duality of

53   function of CDK, both as an inhibitor of licensing and as an activator of the replisome

54   is critical to ensure once per cell cycle replication (Diffley, 2004).

55   In light of the importance of the linkage between DNA replication control and cell

56   cycle progression, multiple checkpoints exist to regulate DNA synthesis and genome

57   integrity before (G1 checkpoint), during (S-phase checkpoint) and after S-phase

58   (G2/M checkpoint, Hartwell and Weinert, 1989; Kastan and Bartek, 2004). These

59   checkpoints are mediated by the PI3 kinase superfamily checkpoint kinases

60   ATM/ATR (Tel1/Mec1 in budding yeast) and the effector checkpoint kinases

61   Chk1/Chk2 (Chk1/Rad53 in budding yeast).

62   In G1 phase, DNA damage such as UV photoproducts causes checkpoint-dependent

63   delays in the onset of DNA replication by inhibition of G1/S cyclins (Lanz et al., 2019;

64   Shaltiel et al., 2015). In budding yeast this occurs in part by Rad53-dependent

65   phosphorylation and inhibition of the Swi6 subunit of the transcriptional activator SBF

66   (SCB binding factor) leading to reduced cyclin transcription (Sidorova and Breeden,

67   1997) and in humans by ATM-Chk2 mediated stabilisation of p53, as well as by

68   checkpoint-dependent degradation of cyclin D and Cdc25A (Lanz et al., 2019;

69   Shaltiel et al., 2015).

70   Although CDK and DDK are activated at the G1/S transition, normally origin firing

71   occurs as a continuum throughout S-phase, with some origins firing in the first half of

72   S-phase (early origins) and others in the second half (late origins). When replication

73   forks emanating from early firing origins stall, for example due to DNA lesions,

74   activation of the S-phase checkpoint kinase response results in the dramatic slowing

75   of replication rates (Painter and Young, 1980; Paulovich and Hartwell, 1995), which

76   occurs in large part through inhibition of late firing origins (Yekezare et al., 2013). In

77   budding yeast, Rad53 blocks late origin firing by directly inhibiting two replication

78   initiation factors; the DDK subunit Dbf4 and the CDK target Sld3 (Lopez-Mosqueda et

79   al., 2010; Zegerman and Diffley, 2010). The checkpoint-mediated inhibition of origin

80   firing likely occurs by similar mechanisms in human cells as the checkpoint kinases

81   also bind to and inhibit the Sld3 orthologue Treslin (Boos et al., 2011; Guo et al.,

82   2015) and inhibit DDK (Costanzo et al., 2003; Lee et al., 2012). One function of

83   inhibiting origin firing during S-phase in the presence of DNA lesions is to prevent the

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84   exhaustion of essential factors, such as topoisomerase activities, by excessive

85   numbers of replisomes (Morafraile et al., 2019; Toledo et al., 2017).

86   A key proposed feature of the DNA damage checkpoints is that the response is

87   tailored to the cell cycle phase in which the DNA damage occurred. Despite this,

88   there is very little evidence to suggest that substrate specificity of the checkpoint

89   kinases changes during the cell cycle. Indeed, in budding yeast most forms of DNA

90   damage and replication stress converge on the single effector kinase Rad53, but how

91   different checkpoint responses in different cell cycle phases can be mediated by a

92   single kinase is not known. In this study we set out to explore the specificity of Rad53

93   towards the replication substrates Sld3 and Dbf4 across the cell cycle in the budding

94   yeast *Saccharomyces cerevisiae*. We show that Rad53 phosphorylates both of these

95   substrates throughout the cell cycle at the same sites as in S-phase. From this we

96   hypothesised that although these substrates are deemed to be targets of the 'S-

97   phase checkpoint', Rad53 may also prevent aberrant origin firing outside of S-phase.

98   Indeed we show that Rad53-dependent inhibition of Sld3 and Dbf4 limits re-initiation

99   of replication in G2/M phase and also prevents premature firing of all origins, not just

100   late origins, at the G1/S transition. This study overhauls our understanding of the cell

101   cycle phase specificity of the 'S-phase checkpoint' and provides a novel mechanism

102   that restricts replication initiation to a specific window of the cell cycle after DNA

103   damage.

Results and Discussion

Sld3 and Dbf4 are phosphorylated by Rad53 outside of S-phase

Since the DNA damage checkpoint response can be activated in all phases of the cell cycle, we addressed whether the replication factors Sld3 and Dbf4 could be targeted by Rad53 outside of S-phase *in vivo* in budding yeast. To test this, we first analysed the consequences of DNA damage in G1 phase cells arrested with the mating pheromone alpha factor (Figure 1A). These experiments were conducted with strains containing a null mutation in the alpha factor protease, *bar1∆*, to ensure that cells were fully arrested in G1 phase and had not started DNA replication. Addition of the UV mimetic drug 4-NQO to G1 phase cells resulted in robust Rad53 activation, as determined by the accumulation of the phospho-shifted forms of the kinase (Figure 1B, 1C). Importantly, we observed a dramatic increase in lower mobility forms of Sld3 when Rad53 was activated in G1 phase (Figure 1B), which was indeed Rad53-dependent (Supplementary Figure 1). For Dbf4, which is an APC/C substrate and partially degraded in alpha factor arrested cells (Ferreira et al., 2000), we also observed a mobility shift in G1 phase coincident with Rad53 activation (Figure 1C).

To test whether Sld3 and Dbf4 could also be phosphorylated by Rad53 after DNA replication is complete we performed the same experiment as in Figure 1A-C, except in cells arrested in G2/M phase with nocodazole (Figure 1D). Significantly, we observed a Rad53-dependent mobility shift in Sld3 and Dbf4, even in G2/M arrested cells (Figure 1E and 1F). Sld3 and Dbf4 are phosphorylated by other kinases in G2/M, such as by CDK (Holt et al., 2009), giving rise to additional isoforms of Sld3/Dbf4 proteins even in Rad53 null cells (* Figure 1E and 1F). Note that the CDK phosphorylated form of Sld3 is visible in Figure 1E as this is a phos-tag gel.

Previously we have identified the serine and threonine residues in Sld3 and Dbf4 that are directly phosphorylated by Rad53 in S-phase (Zegerman and Diffley, 2010). We

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130   mapped 38 such phospho-sites in Sld3 and 19 sites in Dbf4, of which 4 were critical

131   for the Rad53-dependent inhibition of Dbf4. Mutation of these serine/threonine

132   residues to alanine in Sld3 and Dbf4 generated alleles that are refractory to Rad53

133   phosphorylation in S-phase (Zegerman and Diffley, 2010) and are hereafter referred

134   to as *sld3-A* and *dbf4-A* respectively. We reasoned that if the same sites in Sld3 and

135   Dbf4 are phosphorylated by Rad53 throughout the cell cycle, then *sld3-A* and *dbf4-A*

136   should be defective in Rad53-dependent phosphorylation in G1 and G2/M as well. In

137   G1 phase the Sld3-A protein demonstrated a dramatic loss of Rad53 phosphorylation

138   (Figure 2A), consistent with direct phosphorylation of Sld3 by Rad53 at the same

139   sites as in S-phase. We also observed a similar result with the Dbf4-A protein in G1

140   phase after Rad53 activation (Figure 2B). As in G1 phase, both Sld3-A and Dbf4-A

141   showed greatly reduced phosphorylation during Rad53 activation in G2/M phase

142   (Figure 2C and 2D). Together, Figures 1 and 2 show that although Sld3 and Dbf4 are

143   considered to be 'S-phase checkpoint' substrates of Rad53, they are phosphorylated

144   at the same sites as in S-phase after DNA damage in G1 and G2 phase.

145   **Rad53-dependent phosphorylation of Sld3 and Dbf4 reduces re-replication in**

146   **G2 phase**

147   We have previously shown that Rad53 phosphorylates Sld3 and Dbf4 in S-phase to

148   inhibit origin firing (Lopez-Mosqueda et al., 2010; Zegerman and Diffley, 2010). As

149   Sld3 and Dbf4 are phosphorylated at the same sites by Rad53 after DNA damage in

150   both G1 and G2 phase (Figure 1 and 2) we wondered whether this phosphorylation

151   could also be required to inhibit replication initiation outside of S-phase. DNA

152   replication is tightly restricted to S-phase in large part by the action of CDK, which

153   prevents licensing outside of late M/early G1 phase. As a result, transient reduction

154   of CDK-activity in G2/M phase is sufficient to induce re-replication (Dahmann et al.,

155   1995). To test a role for Rad53 phosphorylation of Sld3/Dbf4 in re-replication control

156   we first combined the *sld3-A/dbf4-A* alleles, which cannot be inhibited by Rad53

157   (Zegerman and Diffley, 2010), with a hypomorphic mutant of the CDK catalytic

158   subunit Cdc28 (*cdc28-as1*). This allele of Cdc28 is analogue sensitive (as) and is

159   inhibited by the addition of the ATP competitive inhibitor 1-NM-PP1. Interestingly we

160   observed that the *sld3-A/dbf4-A* alleles are synthetically sick with *cdc28-as1* in the

161   presence of sub-lethal doses of 1-NM-PP1 (Figure 3A), suggesting that inhibition of

162   Sld3 and Dbf4 by Rad53 is important in cells that have reduced CDK activity.

163   To specifically test whether the Rad53-dependent inhibition of origin firing is

164   important to prevent re-replication, we combined the *sld3-A/dbf4-A* alleles with

165   mutants that circumvent the CDK-dependent inhibition of licensing. Over-expression

166   of Cdc6, forcing the nuclear localisation of the Mcm2-7 complex (through an Mcm7-

167   2xNLS fusion) and mutation of the CDK phosphorylation sites in ORC is sufficient to

168   induce re-replication in G2/M phase (Finn and Li, 2013; Nguyen et al., 2001) and has

169   been shown to induce Rad53 activation (Archambault et al., 2005; Green and Li,

170   2005). Importantly, conditional over-expression of licensing mutants that cannot be

171   inhibited by CDK combined with *sld3-A* and *dbf4-A* led to an increase in the total re-

172   replication in nocodazole arrested cells (Figure 3B – compare FACS overlay red vs

173   black). This suggests that Rad53-dependent inhibition of replication initiation can

174   reduce inappropriate replication in G2 phase.

175   One of the consequences of re-replication is the generation of head-to-tail tandem

176   gene amplifications, a process termed RRIGA (re-replication induced gene

177   amplification, Green et al., 2010). To examine whether the Rad53-dependent

178   inhibition of origin firing helps to prevent RRIGA we adapted an assay to

179   quantitatively assess gene amplification events in G2/M arrested cells (Finn and Li,

180   2013). Briefly a marker gene (in this case LEU2, which allows growth on media

181   lacking leucine) was split with some remaining homology across an origin that re-

182   initiates when licensing control is lost (ARS317). Re-initiation at ARS317 followed by

183   fork-breakage and strand annealing at the regions of LEU2 homology results in gene

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184   amplification and the generation of a functional LEU2 gene (Figure 3C and

185   Supplementary Figure 2). In this assay, as in Figure 3B, the re-replication mutants

186   were induced only in G2/M arrested cells. In contrast to wild type yeast or the *sld3-*

187   *A/dbf4-A* strain alone, expression of the licensing mutants by the addition of

188   galactose resulted in a large increase in RRIGA events, as expected (Figure 3D).

189   Importantly RRIGA events were even greater when the mutants that allow licensing

190   in the presence of CDK were combined with the *sld3-A/dbf4-A* alleles (Figure 3D).

191   This assay demonstrates that the checkpoint kinase Rad53 indeed reduces gene

192   amplification events after re-replication through inhibition of Sld3 and Dbf4, even in

193   G2/M arrested cells.

194   **Rad53 prevents precocious origin firing after DNA damage in G1 phase**

195   DNA damage in G1 phase delays the G1/S transition, which from humans to yeast,

196   involves the checkpoint kinase-dependent down-regulation of G1/S cyclins, delaying

197   cell cycle entry (Bertoli et al., 2013; Lanz et al., 2019; Shaltiel et al., 2015; Sidorova

198   and Breeden, 1997). Here we have shown that DNA damage in G1 phase also

199   results in the inhibitory checkpoint phosphorylation of two replication initiation factors,

200   Sld3 and Dbf4 (Figure 1 and 2), suggesting that this might be an additional

201   mechanism to prevent premature DNA replication at the G1/S transition (Figure 4A).

202   To specifically analyse the consequences of DNA damage in G1 phase we added 4-

203   NQO to G1 arrested yeast cells and then released cells into S-phase in fresh medium

204   without 4-NQO. Crucially, this approach resulted in robust Rad53 activation in G1

205   phase, such that cells enter S-phase with an already active checkpoint (Figure 4D

206   and Supplementary Figure 3C). Rad53 activation in G1 phase resulted in the slowing

207   of the G1/S transition as detected by the delay in budding (a G1 cyclin mediated

208   event) and delay in DNA synthesis (Supplementary Figure 3A-B). Despite this the

209   *sld3-A dbf4-A* alleles caused little difference in S-phase progression after DNA

210   damage in G1 phase, compared to the wild type strain (Supplementary Figure 3A).

211   As Rad53 is known to inhibit CDK activation through phosphorylation of the Swi6

212   subunit of the transcriptional activator SBF (Sidorova and Breeden, 1997), we

213   wondered whether Rad53-dependent inhibition of both origin firing and G1/S

214   transcription might prevent precocious DNA replication after damage in G1 phase

215   (Figure 4A). To test this we over-expressed a truncated form of the SBF transcription

216   factor Swi4 (Swi4-t), which lacks the C-terminus required for interaction with Swi6

217   and thus cannot be inhibited by Rad53 (Sidorova and Breeden, 1997). Over-

218   expression of Swi4-t indeed resulted in faster progression through the G1/S transition

219   in the presence of 4-NQO (Supplementary Figure 3D), as expected (Sidorova and

220   Breeden, 1997). Importantly the combination of expression of Swi4-t together with

221   *sld3-A dbf4-A* resulted in much faster S-phase progression after DNA damage in G1

222   phase compared to Swi4-t expression alone (Figure 4B). These differences in the

223   onset of DNA replication between *swi4-t* with and without *sld3-A/dbf4-A* were not due

224   to differences in the G1/S transition as these strains budded at the same time (Figure

225   4C) and both strains also exhibited similar levels of Rad53 activation (Figure 4D).

226   Together this suggests that Rad53 activation in G1 phase prevents precocious DNA

227   replication initiation by not only inhibiting G1/S transcription, but also by inhibiting

228   Sld3 and Dbf4.

229   Activation of Rad53 during S-phase caused by fork stalling/DNA damage at early

230   replicons results in inhibition of subsequent (late) origin firing, which in yeast is

231   mediated by inhibition of Sld3 and Dbf4 (Lopez-Mosqueda et al., 2010; Zegerman

232   and Diffley, 2010). We therefore wondered whether the accelerated S-phase we

233   observe when we combine *swi4-t* with *sld3-A dbf4-A*, is simply due to the canonical

234   S-phase checkpoint inhibition of late origin firing or whether by activating Rad53 in

235   G1 phase we are actually causing a delay in genome duplication from all origins. To

236   assess this we analysed the replication dynamics of the time-course in Figure 4B-D

237   by high-throughput sequencing and copy number analysis. At the earliest time-point

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238   (20mins), while the *swi4-t* over-expressing strain alone had barely begun to replicate

239   (Figure 4E, chromosome VII as an example), the *swi4-t sld3-A dbf4-A* strain showed

240   peaks of replication initiation at the earliest firing origins (for example see *, Figure

241   4E), even though Rad53 is highly activated (Figure 4D). By analysis of initiation at all

242   origins, split into quintiles according to their normal firing time, we observe much

243   greater firing of early origins in the *swi4-t sld3-A dbf4-A* strain compared to swi4-t

244   alone throughout the time-course (Figure 4F and Supplementary Figure 4). Over

245   time, we also observe an increase in later firing origins in the *swi4-t sld3-A dbf4-A*

246   strain (arrows Figure 4F and Supplementary Figure 4), suggesting that the relative

247   timing of origin firing is not affected. Together this demonstrates that activation of

248   Rad53 and inhibition of Sld3 and Dbf4 in G1 phase contributes to the mechanism

249   preventing the onset of DNA replication from all origins, not just late firing origins, in

250   the presence of DNA damage (Figure 4A).

251   If the checkpoint-mediated inhibition of G1/S transcription and Sld3/Dbf4 both

252   contribute to prevent precocious S-phase entry then we hypothesised that loss of

253   both pathways should show synthetic lethality in the presence of DNA damage. We

254   have previously conducted genetic interaction analysis of the *sld3-A/dbf4-A* alleles

255   with the yeast whole genome gene knock-out collection in the presence of the DNA

256   damaging agent phleomycin (Morafraile et al., 2019). Significantly loss of function of

257   genes that result in a delay in the G1/S transition, such as *CLN2*, *SWI4* and *BCK2*

258   (Di Como et al., 1995) improved the growth of *sld3-A/dbf4-A* in the presence of

259   phleomycin (suppressors, Supplementary Figure 5), whereas loss of function of

260   genes that would result in the acceleration of G1/S, such as *WHI5* and *SIC1* (Bertoli

261   et al., 2013) were synthetic sick with the *sld3-A/dbf4-A* alleles (enhancers,

262   Supplementary Figure 5). These genetic interactions are consistent with an important

263   role for Rad53-dependent inhibition of origin firing in preventing precocious

264   replication after DNA damage in G1 phase.

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265   Here we show that the two critical targets of the S-phase checkpoint mediated

266   inhibition of origin firing, Sld3 and Dbf4, are actually regulated by Rad53 after DNA

267   damage throughout the cell cycle (Figures 1 and 2). This has important implications

268   for understanding the consequences of inappropriate re-replication in human cells,

269   where the role of the checkpoint differs depending on the cell cycle phase in which

270   re-replication occurs (Klotz-Noack et al., 2012; Liu et al., 2007). By combining tight

271   cell cycle arrests with the separation-of-function mutants, *sld3-A dbf4-A*, we show

272   specifically that the checkpoint-dependent inhibition of origin firing limits further re-

273   replication and gene amplifications in G2 phase when licensing control is

274   compromised (Figure 3). This pathway is likely to be evolutionarily conserved, as

275   checkpoint activation in S-phase in human cells also appears to limit re-replication

276   through inhibition of Dbf4 (Lee et al., 2012). As tandem head-to-tail duplications are a

277   prominent feature of many cancers (Menghi et al., 2018), knowledge of the pathways

278   that prevent this form of structural variation may be important for understanding

279   oncogenesis.

280   In addition to preventing re-replication in G2, we show that the checkpoint also

281   inhibits the replication initiation factors Sld3 and Dbf4 to delay origin firing after DNA

282   damage in G1 phase (Figure 4). This likely increases the time for DNA repair to occur

283   before replication begins and may also serve to increase the window of time where

284   origin firing and licensing are mutually exclusive, preventing re-replication. It is

285   interesting that failure to inhibit Sld3 and Dbf4 alone has little effect on the G1/S

286   transition (Supplementary Figure 3A), probably because Sld3 (and Sld2) act

287   downstream of CDK activation (Figure 4A). Inhibition of origin firing may therefore be

288   a failsafe mechanism when inhibition of G1/S CDK activity is incomplete. Mutations in

289   genes such as Rb and p53 that control the G1/S transition and the G1 checkpoint

290   response respectively are amongst the most common mutations in cancers

291   (Malumbres and Barbacid, 2001; Massague, 2004). Work from yeast to humans has

292   shown that defects in the G1/S transition results in increased dependence on the

293   checkpoint kinases for survival (Rundle et al., 2017; Sidorova and Breeden, 2002).

294   The checkpoint inhibition of all origin firing as a failsafe to prevent precocious S-

295   phase entry described in this study (Figure 4) may provide a potential mechanistic

296   rationale for the selective targeting of p53/Rb mutant cancers using Chk1 and ATR

297   inhibitors, which are currently in clinical trials (Bradbury et al., 2020).

298

299   **Figure legends**

300   **Figure 1.** Dbf4 and Sld3 are phosphorylated by Rad53 after DNA damage in G1 and

301   G2 phase

302   A) Flow cytometry of strains arrested in G1 phase with the mating pheromone alpha

303   factor. Strains were held in G1 phase, with or without the addition of 10µg/ml 4-NQO

304   for the indicated times. All strains are bar1Δ to maintain G1 arrest.

305   B) Western blot of Sld3 (anti-myc) and Rad53 phosphorylation from the experiment

306   outlined in A. Sld3 was resolved on a phos-tag SDS PAGE gel.

307   C) As B, but for Dbf4. Both blots are from SDS-PAGE.

308   D) As A, except strains were arrested in G2/M in nocodazole before the addition of 4-

309   NQO. All strains are sml1Δ.

310   E) Western blot of Sld3 (anti-myc) and Rad53 phosphorylation as in B from the

311   experiment outlined in D. Sld3 was resolved on a phos-tag SDS PAGE gel, * is CDK

312   phosphorylated Sld3.

313   F) As E, but for Dbf4. Dbf4 is phosphorylated by other kinases in G2/M, resulting in

314   residual phosphorylated forms remaining in *rad53Δ* cells *.

315

316 **Figure 2.** Rad53 phosphorylates Sld3 and Dbf4 in G1 and G2 phase at the same

317 residues as in S-phase

318 A) and B) as Figure 1 B/C. *sld3-A* and *dbf4-A* refers to mutant alleles with Rad53

319 phosphorylation sites mutated to alanine (38 sites for Sld3 and 19 sites for Dbf4). All

320 strains are bar1Δ.

321 C) as Figure 1E, except this is Western blot from an SDS-PAGE gel.

322 D) as Figure 1F.

323

324 **Figure 3.** Checkpoint-dependent inhibition of origin firing prevents re-replication in

325 G2 phase

326 A) Growth assay of the indicated strains

327 B) Flow cytometry of the indicated strains grown overnight in YPraffinose, then

328 arrested in G2/M with nocodazole. After addition of fresh nocodazole, 2% galactose

329 was added for the indicated times to express the licensing mutants. Right, overlay

330 between the 0 and 2 hour timepoints for the licensing mutant strain with (red) or

331 without (black) the *sld3-A/dbf4-A* alleles.

332 C) Schematic diagram of RRIGA assay for gene amplification events. Re-replication

333 of the split LEU2 gene from origin ARS317 results in tandem head to tail gene

334 duplications, leading to a functional LEU2 gene.

335 D) RRIGA assay in C) was performed with the indicated strains. Strains were grown

336 overnight in YPraffinose then arrested in G2/M with nocodazole (pre-induction, blue

337 timepoint). After addition of fresh nocodazole, 2% galactose was added for 3 hours to

338 express the licensing mutants (red timepoint). Cells were plated on YPD (viable cell

339 count) and SC-leu plates (LEU+ count) and the % of LEU+ colonies out of the viable

14

340   cell population was plotted. N=3, error bars are SD and P value was calculated using

341   an unpaired t-test.

342

343   **Figure 4.** Checkpoint-dependent inhibition of origin firing prevents premature

344   replication initiation at all origins at the G1-S transition.

345   A) Activation of Rad53 in G1 phase can delay genome duplication by at least 2

346   mechanisms; by inhibition of the transcriptional activator Swi6, which is required for

347   G1- and subsequently S-phase cyclin transcription and by inhibition of the origin firing

348   factors Sld3 and Dbf4.

349   B) Flow cytometry of the indicated strains grown overnight in YPraffinose, then

350   arrested in G1 phase with alpha factor. Cells were held in fresh alpha factor, while

351   2% galactose and 0.5μg/ml 4-NQO was added for 30 minutes (0 timepoint) before

352   washing and release from alpha factor arrest into fresh YPgal medium without 4-

353   NQO.

354   C) Budding index from the experiment in B. Timepoint 0 refers to cells held in alpha

355   factor + galactose + 0.5μg/ml 4-NQO for 30mins.

356   D) Rad53 Western blot from experiment in B.

357   E) Copy number analysis of chromosome VII of the indicated strains 20 mins after

358   release as in B-D. The y-axis ratio refers to the amount of DNA at the 20 mins

359   timepoint divided by the DNA copy number in G1 phase. Known origins are

360   annotated above the replication profile and coloured according to their normal

361   median replication time (T_{rep}).

362   F) Box plots of the amount of replication at all origins, split into equal quintiles

363   depending on their normal median firing time (T_{rep}). Arrows in time-point 40 mins

364   indicate that later firing origins also initiate by 40mins in the *swi4-t sld3-A dbf4-A*

365   strain. For example the yellow and green quintiles are significantly different at

366   20mins, but non-significantly different (ns) at 40mins. P-values are from t-tests.

367 **Methods**

368 **Strains and Growth Conditions**

369 Cell growth, arrests, flow cytometry and yeast protein extracts were as previously

370 described (Zegerman and Diffley, 2010). All the strains used in this work are derived

371 from W303 (*ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100, rad5-535)*

Strain	Genotype	Source
yPZ 1223	*MATa Sld3-10his13myc::KanMX bar1∆::hisG*	this work
yPZ 1319	*MATa sld3-38A-10his13myc::KanMX bar1∆::hisG*	this work
yPZ 1317	*MATa Dbf4-13myc::KanMX bar1∆::hisG*	this work
yPZ 4018	*MATa YCLWdelta5::HphMX-EU2-ARS317-LEU::NFS1 Mcm7-2xNLS::AbA ura3::P$_{GAL}$-Cdc6-13myc::URA3 orc6-4A*	this work
yPZ 705	*MATa*	PZ lab
yPZ 4076	*MATa trp1::P$_{GAL}$-swi4-t::TRP1*	this work
yPZ 4137	*MATa dbf4-4A::HIS3 sld3-38A-10his13myc::KanMX trp1::P$_{GAL}$-swi4-t::TRP1*	this work
yPZ 917	*MATa dbf4-4A::HIS3 sld3-38A-10his13myc::KanMX*	PZ lab
yPZ 1523	*MATa sml1∆::URA3 rad53∆::LEU2 Sld3-10his13myc::KanMX bar1∆::hisG*	this work
yPZ 1522	*MATa sml1∆::URA3 Sld3-10his13myc::KanMX bar1∆::hisG*	this work
yPZ 1198	*MATa cdc28-as1 (F88G)*	this work
yPZ 1767	*MATa cdc28-as1 (F88G) dbf4-4A::HIS3 sld3-38A-10his13myc::KanMX*	this work
yPZ 4085	*MATa YCLWdelta5::HphMX-EU2-ARS317-LEU::NFS1 trp1::orc6-4A-P$_{GAL1-10}$-Mcm7-2xNLS::TRP1 ura3::P$_{GAL}$-Cdc6-13myc::URA3*	this work
yPZ 4089	*YCLWdelta5::HphMX-EU2-ARS317-LEU::NFS1 trp1::orc6-4A-P$_{GAL1-10}$-Mcm7-2xNLS::TRP1 sld3-38A-10his13myc::KanMX dbf4-4A::HIS3 ura3::P$_{Gal}$-Cdc6-13myc::URA3*	this work
yPZ 125	*MATa Dbf4-13myc::KanMX*	PZ lab
yPZ 170	*MATa dbf4∆::TRP1 his3::P$_{DBF4}$-dbf4-19A-13myc::KanMX::HIS3*	PZ lab
yPZ 2	*MATa sld3-38A-10his13myc::KanMX*	PZ lab
yPZ 52	*MATa Sld3-10his13myc::KanMX*	PZ lab
yPZ 1471	*MATa sml1∆::URA3 rad53∆::HphNT1 Dbf4-13myc::KanMX bar1∆::hisG*	this work
yPZ 1473	*MATa sml1∆::URA3 Dbf4-13myc::KanMX bar1∆::hisG*	this work
yPZ 520	*MATa sml1∆::URA3 Sld3-13myc::KanMX*	PZ lab
yPZ 89	*MATa sml1∆::URA3 rad53∆::LEU2 Sld3-13myc::KanMX*	PZ lab
yPZ 519	*MATa sml1∆::URA3 Dbf4-13myc::KanMX*	PZ lab
yPZ 228	*MATa sml1∆::URA3 rad53∆::LEU2 Dbf4-13myc::KanMX*	PZ lab

372

373   *dbf4-4A* refers to the rad53 site mutant. It has serine/threonine to alanine mutations

374   at amino acids: 518, 521, 526, 528.

375   *dbf4-19A* refers to the rad53 site mutant. It has serine/threonine to alanine mutations

376   at amino acids 53, 59, 188, 192, 203, 222, 224, 226, 228, 318, 319, 328, 374, 375,

377   377, 518, 521, 526, 528.

378   *sld3-38A* refers to the rad53 site mutant. It contains serine/threonine to alanine

379   mutations at amino acids 306, 310, 421, 434, 435, 438, 442, 445 , 450, 451, 452,

380   456, 458, 459, 479, 482, 507 , 509, 514, 519,521,524, 540, 541, 546, 547, 548, 550,

381   556, 558, 559, 565, 569, 582, 607, 653 and 654. 539 is mutated to arginine.

382   *Orc6-4A* refers to CDK sites 106, 116, 123 and 146 mutated to alanine*.*

383   **Western blot**

384   Western blots were performed as previously described (Can et al., 2019). Rad53 was

385   detected with ab104232 (Abcam, dilution 1:5000).

386   **Replication profiles**

387   Yeast genomic DNA was extracted using the smash & grab method (https://fangman-

388   brewer.genetics.washington.edu/smash-n-grab.html). DNA was sonicated using the

389   Bioruptor Pico sonicator (Diagenode) and the libraries were prepared according to

390   the TruSeq Nano sample preparation guide from Illumina. To generate replication

391   timing profiles, the ratio of uniquely mapped reads in the replicating samples to the

392   non-replicating samples was calculated following (Batrakou et al., 2020). Replication

393   profiles were generated using custom R scripts and smoothed using a moving

394   average. The values of Trep were taken from OriDB (Siow et al., 2012).

395   **RRIGA assay using split LEU2 marker**

396   Cells were pre-grown under permissive conditions in YPraff overnight 30°C. At 1×10^7

397   cells/ml, nocodazole (2mg/ml in DMSO) was added to a final concentration of

398   10µg/ml. Cells were arrested for 90mins at 30°C (uninduced timepoint) and then

399   galactose was added to a final concentration of 2% + fresh nocodazole for 3 hours

400   (induced timepoint).

401   For each timepoint a 0.5ml sample was spun at 3.2K for 1min in benchtop centrifuge,

402   cells were washed with 1ml sterile water to remove YPD, respun and resuspended in

403   0.5ml sterile water. Cells were sonicated briefly to ensure cells are separated then a

404   serial dilution was made into sterile water as follows:

405   dilution 1: 10µl cells + 990µl water = approx 1×10^5 cells/ml = 1×10^2 cells/µl

406   dilution 2: 10µl dilution 1 + 990µl water = approx 1×10^3 cells/ml = 1 cell/µl

407   100µl of dilution 2 was plated on YPD plates in triplicate for the viability calculation.

408   100µl and 10µl of undiluted cells were plated in triplicate on SC-leu plates to obtain

409   the fraction of viable cells that are LEU+ before and after induction of re-replication.

410   Plates were incubated at 25°C for 48hrs before colonies were counted. To calculate

411   the percentage of cells in the population that were LEU+ the number of LEU+

412   colonies per ml was divided by the number of viable cells per ml.

Acknowledgments

421   **Author Contribution**

422   All authors performed and designed the experiments. PZ wrote the paper.

423   **Declaration of Interests.** The authors declare no conflicts of interest.

424   **Supplementary Figure legends**

425   **Supplementary Figure 1.** Sld3 phosphorylation in G1 phase after DNA damage is

426   Rad53-dependent.

427   A) Flow cytometry of strains arrested in G1 phase with the mating pheromone alpha

428   factor. Strains were held in G1 phase, with the addition of 10μg/ml 4-NQO for the

429   indicated times. All strains are *bar1Δ* to maintain G1 arrest and also *sml1Δ*.

430   B) Western blot of Sld3 and Rad53 phosphorylation from the experiment outlined in

431   A. Sld3 was resolved on a phos-tag SDS PAGE gel.

432

433   **Supplementary Figure 2.** RRIGA assay using split LEU2 marker

434   A. Schematic diagram of the RRIGA assay. Endogenous LEU2 was replaced with a

435   split LEU2 marker, separated by the re-replication origin ARS317. The two non-

436   functional halves contain 417bp of identity *. Re-initiation at ARS317 followed by fork-

437   breakage and strand annealing at the regions of LEU2 homology results in gene

438   amplification and the generation of a functional LEU2 gene.

439   B. Verification of RRIGA by PCR using the amplicons as depicted in A. The parental

440   strain (which is leu-) was induced to re-replicate in the presence of galactose and

441   cells were plated on SC-leu plates. 10 independent colonies were assayed for the

442   presence of the duplication and all were positive.

443

444   **Supplementary Figure 3.** 4-NQO addition in G1 phase delays the G1/S transition

445   A. Flow cytometry of the indicated strains, which were grown overnight in YPraffinose

446   and arrested in G1 phase with alpha factor (G1 time point). Strains were then held in

447   G1 phase for an extra 30 minutes by the addition of fresh alpha factor plus galactose,

20

448   with or without 0.5μg/ml 4-NQO. Cells were washed into fresh YPgalactose medium

449   to release into S-phase. Note that YPraffinose and galactose medium was used in

450   this experiment to match the exact conditions that were used in the main Figure 4B

451   with the P_{GAL}-swi4-t strains, allowing a direct comparison, as in D.

452   B. Budding index (a G1 cyclin mediated event) from experiment in A.

453   C. Rad53 western blot from the 4-NQO treated samples in A.

454   D. Overlay of budding profiles (from Figure 4C and this Figure B) between strains

455   expressing or not expressing Swi4-t. Swi4-t, which cannot be inhibited by Rad53,

456   causes earlier activation of G1 cyclin/CDK as shown here by budding.

457

458   **Supplementary Figure 4.** Rad53 activation in G1 phase inhibits all origin firing

459   through phosphorylation of Sld3 and Dbf4.

460   Replication profile of Chromosome VII as an example at 20, 30 and 40mins from the

461   experiment in Figure 4B. Green * are examples of very early firing origins, which

462   initiate at the 20min timepoint in the P_{GAL}-swi4-t sld3-A dbf4-A strain. Purple * are

463   examples of late firing origins which do not initiate at the 20min timepoint, but are

464   clearly firing by the 40 min timepoint in the P_{GAL}-swi4-t sld3-A dbf4-A strain. This

465   shows that the relative timing of origin firing is not affected in the P_{GAL}-swi4-t sld3-A

466   dbf4-A strain.

467

468   **Supplementary Figure 5.** Genetic interactions of the sld3-A dbf4-A alleles with

469   mutants that either accelerate or delay the G1/S transition.

470   Scatter plot of the fitness of the yeast genome knock out collection grown in 0.5μg/ml

471   phleomycin with (y-axis) or without (x-axis) the sld3-A dbf4-A alleles. Each dot

472   corresponds to a different gene deletion. The top 25% of gene deletions (yfg = your

473   favourite gene) that significantly enhance (green) or suppress (red) the fitness of

474   *sld3-A dbf4-A* are indicated. This data was originally published in (Morafraile et al.,

475   2019).

476

477

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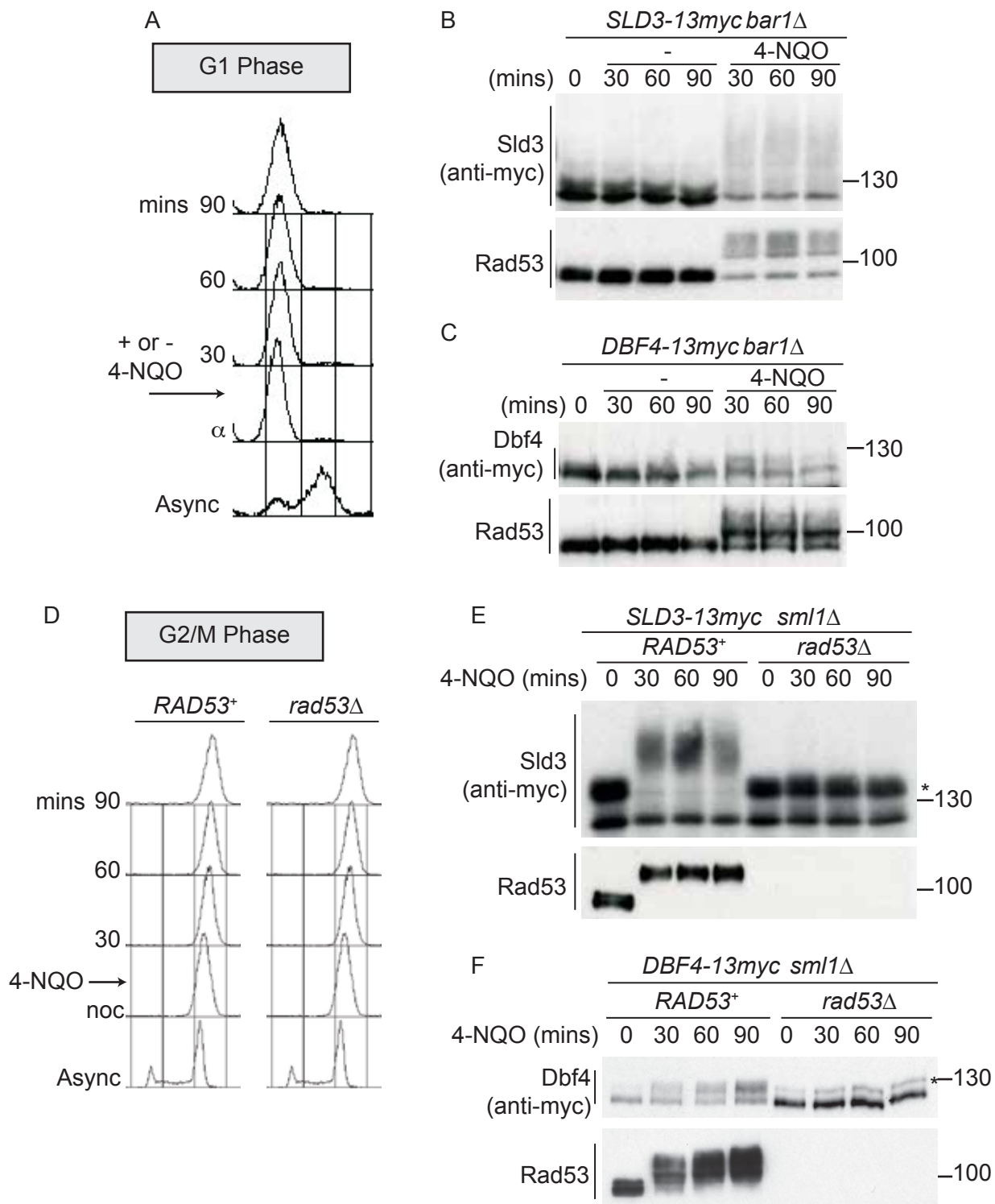
596

Figure 1

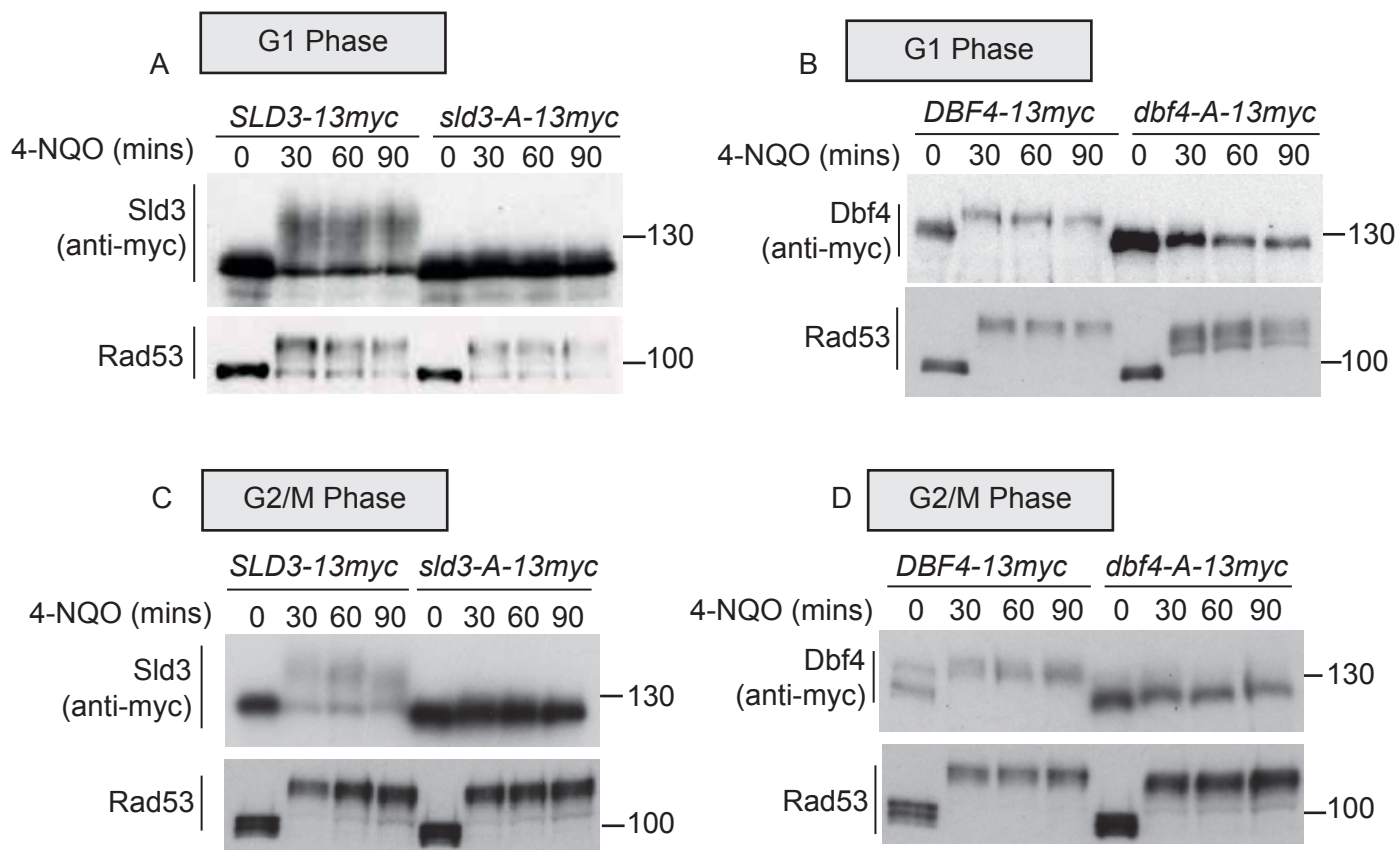Figure 2. Rad53 phosphorylates Sld3 and Dbf4 in G1 and G2 phase at the same residues as in S-phase

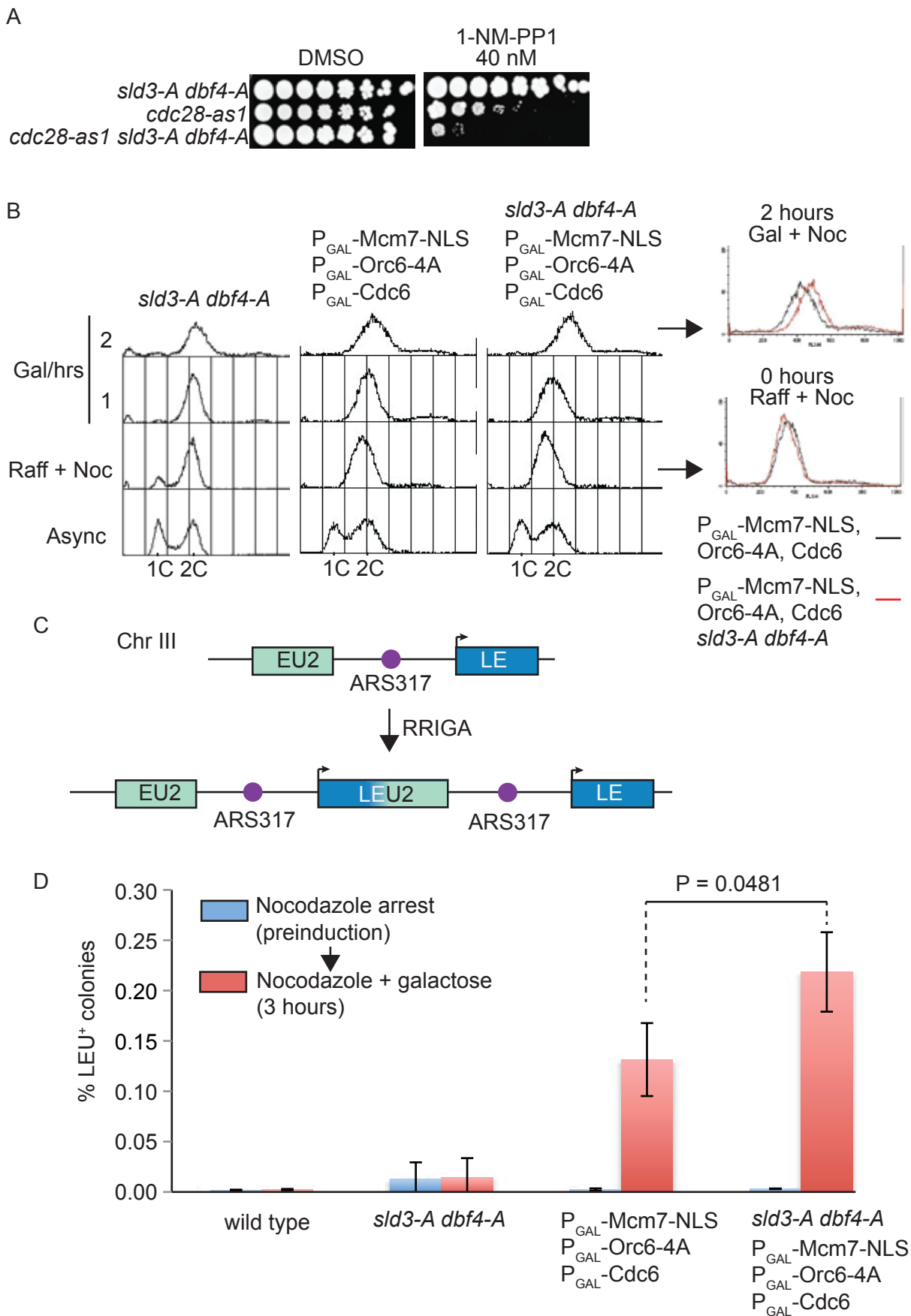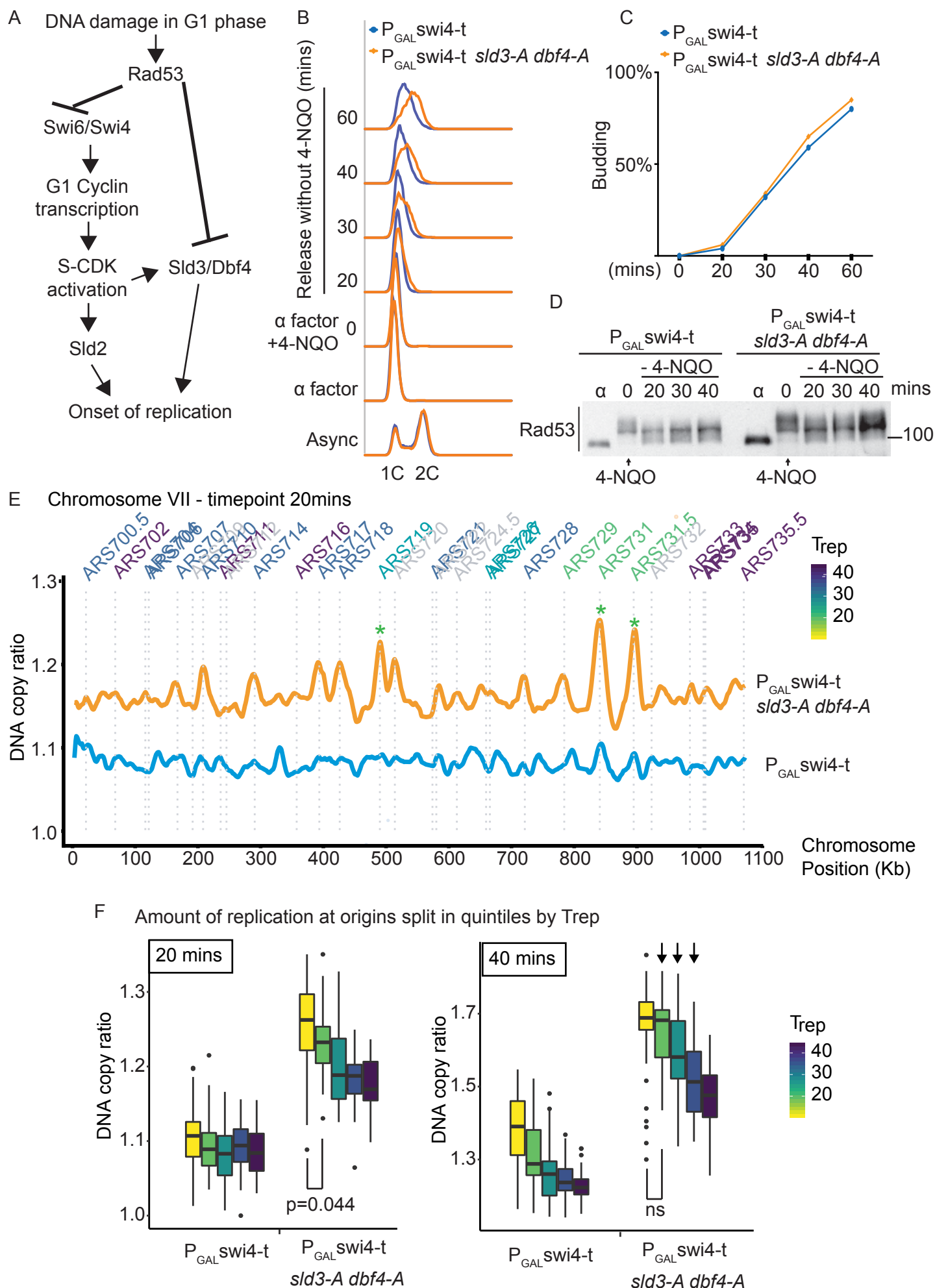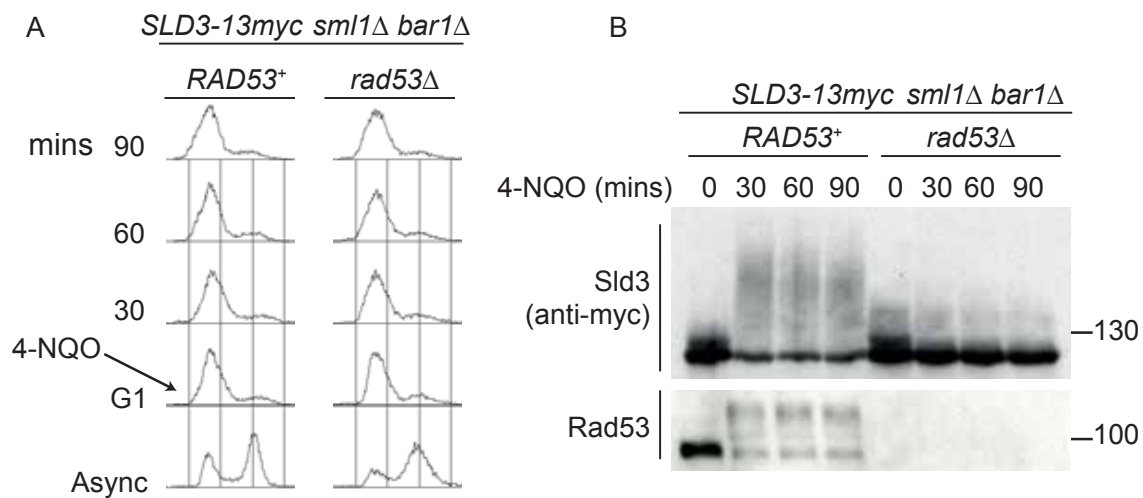Figure 3. Rad53 inhibition of Sld3 and Dbf4 prevents re-replication in G2/M

Figure 4. Checkpoint inhibition of origin firing prevents premature replication at the G1-S transition
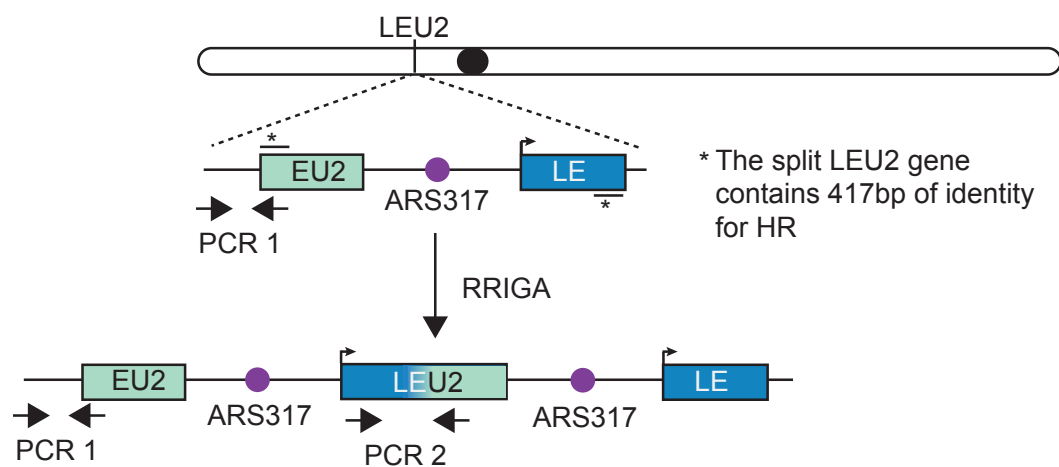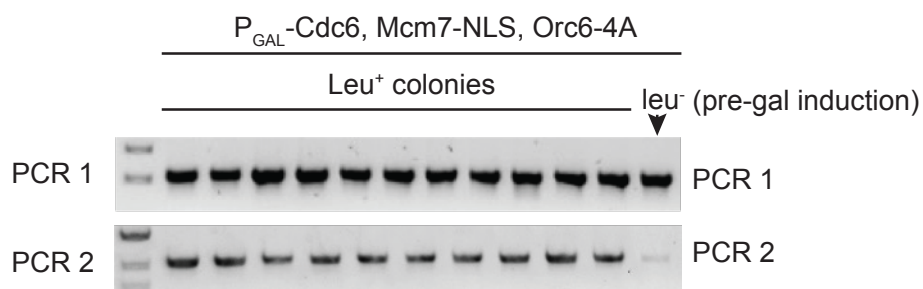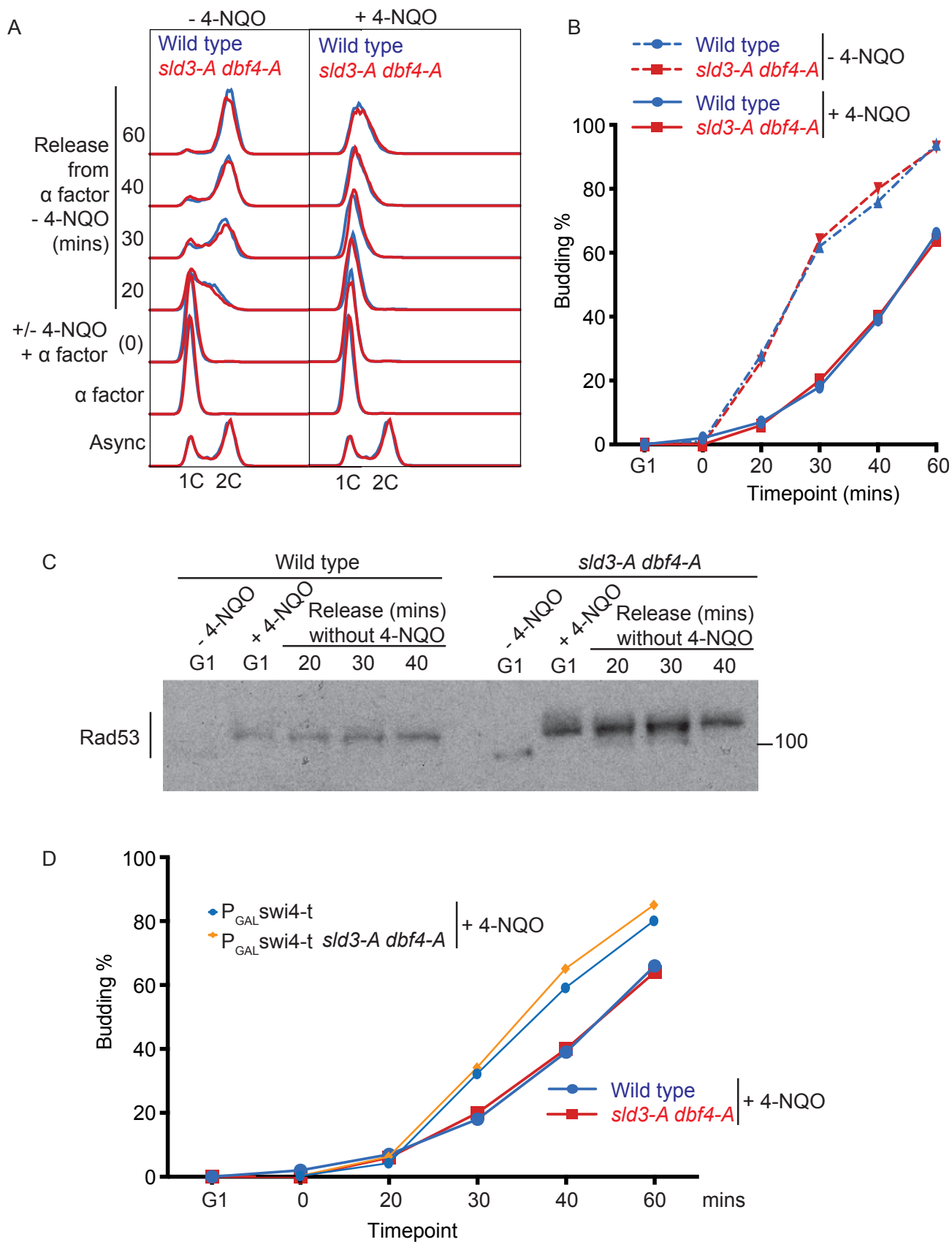
Supplementary Figure 1

Supplementary Figure 2

A   Chr III (replaces endogenous LEU2 gene)

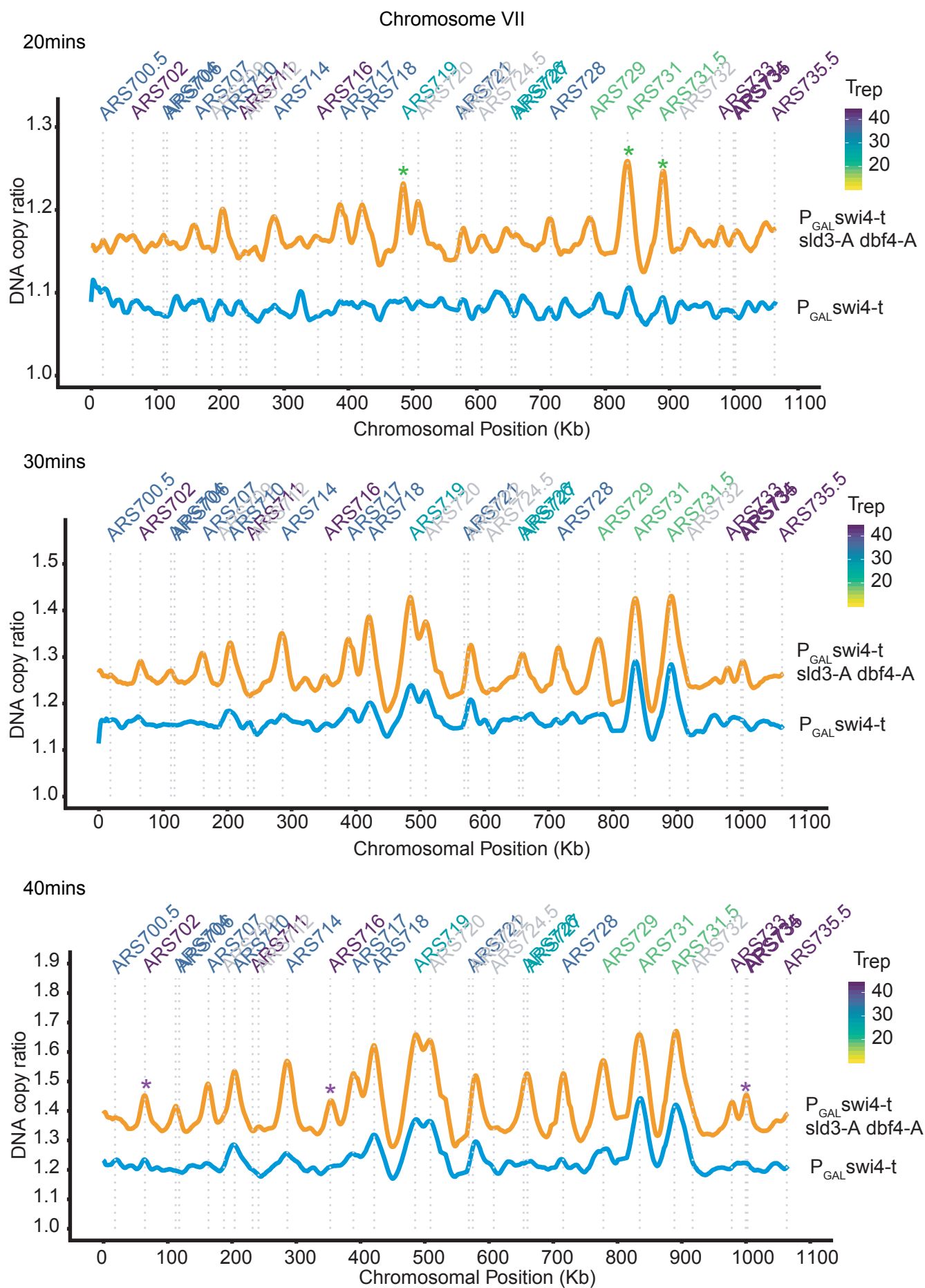* The split LEU2 gene contains 417bp of identity for HR

B

Supplementary Figure 3

Supplementary Figure 4

Supplementary Figure 5