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CDC28, *NET1*, AND *HFI1* ARE REQUIRED FOR CHECKPOINTS IN *SACCHAROMYCES CEREVISIAE*

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Колтовая Н. А. и др. E19-2009-95

Гены *CDC28*, *NET1* и *HFI1* необходимы для чекпойнт-контроля
у дрожжей *Saccharomyces cerevisiae*

Изучено участие генов *SRM*, выделенных по их влиянию на генетическую стабильность и радиочувствительность, в остановке клеточного цикла под действием повреждающих агентов. Показано, что мутации *srm5/cdc28-srm*, *srm8/net1-srm*, *srm12/hfi1-srm* сокращают остановку клеточного цикла при повреждении ДНК и влияют на чекпойнт-остановку в фазах G_0/S (*srm5, srm8*), G_1/S (*srm5*, *srm8, srm12*), S (*srm5*, *srm12*) и S/G_2 (*srm5*). По-видимому, у почкующихся дрожжей гены *CDC28*, *HFI1/ADA1* и *NET1* участвуют в формировании ответа клетки на повреждения ДНК, в частности, в чекпойнт-регуляции. В отношении чувствительности к γ-излучению обнаружено, что известные чекпойнт-гены *RAD9*, *RAD17*, *RAD24* и *RAD53* и исследуемые гены *CDC28* и *NET1* принадлежат к одной эпистатической группе генов, названной *RAD9*-группой. Анализ радиочувствительности двойных мутантов показал, что мутация *cdc28-srm* гипостатична мутациям *rad9*Δ и *rad24*Δ, но аддитивна *rad17*Δ. Мутация *net1-srm* гипостатична мутации *rad9*Δ, но аддитивна мутациям *rad17*Δ, *rad24*Δ и *rad53*. Мутация *hfi1-srm* имеет аддитивный эффект в компаунде с мутациями *rad24*Δ и *rad9*Δ. Таким образом, анализ эпистатических взаимодействий продемонстрировал разветвленность *RAD9*-зависимого пути. Анализируемые гены могут также участвовать в минорном механизме, участвующем в детерминации радиочувствительности клеток независимо от вышеупомянутого *RAD9*-зависимого пути.

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CDC28, *NET1*, and *HFI1* Are Required
for Checkpoints in *Saccharomyces cerevisiae*

The involvement of *SRM* genes selected as genes affecting genetic stability and radiosensitivity in a cell cycle arrest under the action of damaging agents was studied. It was shown that the *srm5/cdc28-srm*, *srm8/net1-srm*, and *srm12/hfi1-srm* mutations prevent checkpoint activation by DNA damage, particularly the G_0/S (*srm5, srm8*), G_1/S (*srm5, srm8, srm12*), S (*srm8, srm12*), and S/G_2 (*srm5*) checkpoints. It seems that in budding yeast the *CDC28*, *HFI1/ADA1*, and *NET1* genes mediate cellular response induced by DNA damage with checkpoint control. The well-known checkpoint-genes *RAD9, RAD17, RAD24*, and *RAD53*, and the genes *CDC28*, and *NET1* have been found to belong to one epistasis group named *RAD9*-group as regards cell sensitivity to γ radiation. An analysis of the radiosensitivity of double mutants has revealed that the mutation *cdc28-srm* is hypostatic to each of mutations *rad9*Δ, and *rad24*Δ, and additive to *rad17*Δ. The mutation *net1-srm* is hypostatic to the mutations *rad9*Δ but additive to *rad17*Δ, *rad24*Δ, and *rad53*. The mutation *hfi1-srm* has an additive effect in compound with the mutations *rad24*Δ and *rad9*Δ. So, investigations of epistatic interactions have demonstrated a branched *RAD9*-dependent pathway. The analyzed genes can also participate in a minor mechanism involved in determining cell radiation sensitivity independently of the mentioned *RAD9*-dependent pathway.

The investigation has been performed at the Laboratory of Radiation Biology, JINR.

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1. INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* is a suitable eukaryotic model and the first for which the concept of checkpoint regulation was elaborated [1]. It is still used in numerous investigations of this mechanism [2]. Yeast and other eukaryotic organisms have several checkpoints at which the cell cycle progression is controlled in response to DNA damage or aberrant structures when DNA replication is blocked. The activation of checkpoints by DNA damage in G_1 or G_2 causes a cell cycle arrest in these phases [3]. DNA damage in the S phase leads to the inhibition of DNA replication and to its block in G_2. DNA replication inhibition is a consequence of a combined effect of the checkpoint-independent inhibition of the replication fork progression and the checkpoint-dependent inhibition of late-firing *ori*. The DNA replication delay is controlled by intra-S checkpoint by suppressing the activation of late-firing *ori* [4]. The replicative S checkpoint causes a cell cycle arrest in response to the DNA replication block, for example, by hydroxyurea (HU), as a result of the depletion of nucleotides [5]. These signaling pathways check the completion of DNA replication and prevents mitosis until replication is finished. Checkpoints are critical for preventing genomic instability induced by DNA damage, because checkpoints delay or arrest the cell cycle progression in response to DNA damage to allow its repair [6–8].

Although the cell cycle regulation is best studied in *S. cerevisiae*, many components of this process are still unknown. We demonstrated earlier that mutations in the *CDC28*, *NET1*, and *HFI1* genes are accompanied by a decrease in the mitotic stability of natural and recombinant nuclear genetic structures and by an increase in sensitivity to the lethal effect of γ irradiation [9–11], properties characteristic for checkpoint genes. In this work, the involvement of the *CDC28*, *NET1*, and *HFI1* genes in checkpoint regulation is shown by the results of a direct analysis of a cell cycle arrest after exposure to damaging agents. Morphological studies had shown the involvement of these genes in practically all checkpoints. An analysis of the epistatic interaction of mutations in determining radiosensitivity revealed that *CDC28* and *NET1* belong to the *RAD9* group of checkpoint genes [12].

2. MATERIALS AND METHODS

2.1. Yeast strains. The used genotypes of *Saccharomyces cerevisiae* strains are given in Table 1. The yeast strains were constructed by genetic methods using a micro-manipulator in the 71a background [10]. The yeast strains constructed in this work carry the mutations *srm1* and *srm5/cdc28-srm* [9]; *srm8/net1-srm* and *srm12/hfi1-srm* [11]; *rad9*Δ, *rad17*Δ, *rad24*Δ, and *rad53* [12]. The *rad52-1* mutation was derived from the strain g160/2b (*rad52-1*) of the Yeast Genetic Stock Center in Berkeley. As sources of the *cdc6-1* and *cdc9-1* mutations, strains 327A (*MAT*a *cdc6-1 ade1 ade2 ura1 his7 lys2 tyr1 gal1*) and STX435-1-3B (*MAT*α *cdc9-1 ade1 ade2 lys2 ura1 his7 leu1 gal1*) were kindly offered by Dr. Yu. Pavlov (St. Petersburg State University).

2.2. Media. YPD [13] and complete medium (CM) [9] were used.

2.3. Synchronization in G_1 was obtained by *S. cerevisiae* α-mating factor (Sigma) [14].

2.4. Irradiation by γ rays and UV light was performed as was described before [12].

2.5. Determination of rho$^-$-mutant frequencies has already been described [11].

3. RESULTS

3.1. G_1- and G_0-arrests after UV irradiation. Cell synchronization in G_1 was obtained by a treatment of exponentially growing YPD cell cultures with 5 μg/ml α-factor [14] followed by their release into YPD. The appearance of small-budded cells is commonly used as an indicator of the progression of yeast cells from G_1 to S and the initiation of DNA replication. We calculated the budding delay by subtracting the time of appearance of small-budded cells in untreated cultures from that in treated cultures (Table 2). When synchronized cultures were irradiated with UV light and immediately released from the α-factor arrest, we observed a delay in the emergence of small-budded cells in a wild-type strain (Fig. 1, *a*). In the checkpoint mutant *rad9*Δ, no UV light inhibition of cell cycle is observed according to literature data [14]. In the *srm5*, *srm8*, and *srm12* mutants, both irradiated and unirradiated cells restart division practically simultaneously. Time of generation is increased in the *srm5*, *srm8*, and *srm12* mutants [10, 11], *rad9*Δ did not alter it. Budding kinetic is delayed by irradiation, and this difference is more pronounced in the mutants (Fig. 1, *a*).

Budding delay was also observed after UV irradiation of stationary-phase cultures (Fig. 1, *b*). Cultures were grown in a liquid medium for 36–48 h with intensive agitation. Unirradiated cultures of the wild type began budding within $\sim$ 50 min after their replacing into a fresh medium; irradiation of stationary

Table 1. Strains used in the study

Strain	Genotype	Source of origin
327A	*MAT*a *cdc6-1 ade1 ade2 ura1 his7 lys2 tyr1 gal1*	Yu. Pavlov (University of St. Petersburg, St. Petersburg)
STX435-1-3B	*MAT*α *cdc9-1 ade1 ade2 lys2 ura1 his7 leu1 gal1*	Yu. Pavlov (University of St. Petersburg, St. Petersburg)
7859-7-4a	*MAT*a *rad9::LEU2 leu2-3,112 trp1-289 ura3-52 his7*	L. H. Hartwell (University of Washington, Seattle)
SX46A rad24Δ	*MAT*a *rad24::URA3 ade2 his3-532 trp1-289 ura3-52*	W. Siede (University of Texas, Dallas)
SX46A rad17Δ	*MAT*a *rad17::URA3 ade2 his3-532 trp1-289 ura3-52*	”
CRY1	*MAT*a *rad53* (=*sad1-1*) *ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 can1-100*	“
g160/2b	*MAT*a *rad52-1 ade2-1 arg4 arg9 trp1 his5 lys1-1 ilv3 leu pet*	*YGSC**
711a	*MAT*a *SRM*$^+$ *ade2*	Constructed by authors [9]
M1a	*MAT*a *srm1 ade1*	‘
M5a	*MAT*a *srm5 ade1*	’
C3	*MAT*a *srm8 leu2 ade2*	’
9a	*MAT*a *srm12 ade2 leu2 ura3 trp1*	’
3C	*MAT*a *rad9::LEU2 ade2*	’
1D	*MAT*α *rad17::URA3 ade2*	’
16B	*MAT*a *rad24::URA3 ade2*	’
14α	*MAT*α *srm12 ade2 leu2 ura3*	’
12C	*MAT*a *rad53 ade2*	‘
71a	*MAT*a *SRM+ ade1*	‘
71α	*MAT*α *SRM+ ade1*	”
R8a	*MAT*a *net1-srm (=srm8) ade2 trp1 ura3*	“
R8α	*MAT*α *net1-srm ade2 trp1 ura3*	“
R12a	*MAT*a *hfi1-srm (=srm12) ade2 trp1 ura3*	“
R12α	*MAT*α *hfi1-srm ade2 trp1 ura3*	“

Table 1. Continuation

Strain	Genotype	Source of origin
		Constructed by authors
Series 5/52:		
C50/1-C50/4	*MATa/MATα cdc28-srm/cdc28-srm ade1/ade1*	”
C40/1, C40/2	*MATa/MATα rad52-1/rad52-1 ade1/ade1 cyh2/+ leu1/+ ade6/+*	“
C54/1, C54/2	*MATa/MATα rad52-1/rad52-1 cdc28-srm/cdc28-srm cyh2/+ leu1/+ ade6/+ ade1/ade1*	“
C3/1-C3/2	*MATa/MATα rad52-1/rad52-1 cdc28-srm/cdc28-srm rad53/rad53 ade2/ade2*	”
Series 5/9:		
C59/1-C59/4	*MATa/MATα cdc28-srm/cdc28-srm rad9::LEU2/rad9::LEU2 ade1/ade1 leu2-3,112/leu2-3,112*	“
C09/1-C09/4	*MATa/MATα rad9::LEU2/rad9::LEU2 ade1/ade1 leu2-3,112/leu2-3,112*	“
C05/4-C05/7	*MATa/MATα cdc28-srm/cdc28-srm ade1/ade1 leu2-3,112/leu2-3,112*	“
C00/5-C05/8	*MATa/MATα ade1/ade1 leu2-3,112/leu2-3,112*	“
Series 5/17:		
5/+ 17/+ (1-3)	*MATa/MATα CDC28/cdc28-srm RAD17/rad17::URA3*	“
5/5 17/+ (1-3)	*MATa/MATα cdc28-srm/cdc28-srm RAD17/rad17::URA3*	“
5/+ 17/17 (1-3)	*MATa/MATα CDC28/cdc28-srm rad17::URA3/rad17::URA3*	“
5/5 17/17 (1-3)	*MATa/MATα cdc28-srm/cdc28-srm rad17::URA3/rad17::URA3*	“
Series 5/24:		
5/+ 24/+ (1-3)	*MATa/MATα CDC28/cdc28-srm RAD24/rad24::URA3*	“
5/5 24/+ (1-3)	*MATa/MATα cdc28-srm/cdc28-srm RAD24/rad24::URA3*	“
5/+ 24/24 (1-3)	*MATa/MATα CDC28/cdc28-srm rad24::URA3/rad24::URA3*	“
5/5 24/24 (1-3)	*MATa/MATα cdc28-srm/cdc28-srm rad24::URA3/rad24::URA3*	“
Series 5/53:		
5/+ 53/+ (1-2)	*MATa/MATαCDC28/cdc28-srm RAD53/rad53*	“
5/5 53/+ (1,2)	*MATa/MATα cdc28-srm/cdc28-srm RAD53/rad53*	“
5/+ 53/53 (1,2,3)	*MATa/MATα CDC28/cdc28-srm rad53/rad53*	“
5/5 53/53 (1,2,3)	*MATa/MATα cdc28-srm/cdc28-srm rad53/rad53*	“

Table 1. Continuation

Strain	Genotype	Source of origin
		Constructed by authors
Series 8/9:		
+/8 +/9 (1-4)	*MATa/MATα NET1/net1-srm RAD9/rad9::URA3*	“
9/9 8/+ (1-4)	*MATa/MATα NET1/net1-srm rad9::URA3/rad9::URA3*	“
9/+ 8/8 (1-4)	*MATa/MATα net1-srm/net1-srm RAD9/rad9::URA3*	“
9/9 8/8 (1-4)	*MATa/MATα net1-srm/net1-srm rad9::URA3/rad9::URA3*	‘
Series 8/17:		
8/+ 17/+ (1-4)	*MATa/MATα NET1/net1-srm RAD17/rad17::URA3*	“
8/8 17/+ (1-4)	*MATa/MATα net1-srm/net1-srm RAD17/rad17::URA3*	“
8/+ 17/17 (1-4)	*MATa/MATα NET1/net1-srm rad17::URA3/rad17::URA3*	“
8/8 17/17 (1-4)	*MATa/MATα net1-srm/net1-srm rad17::URA3/rad17::URA3*	“
Series 8/24:		
8/+ 24/+ (1-3)	*MATa/MATα NET1/net1-srm RAD24/rad24::URA3*	“
8/+ 24/24 (1-3)	*MATa/MATα NET1/net1-srm rad24::URA3/rad24::URA3*	“
8/8 24/+ (1-4)	*MATa/MATα net1-srm/net1-srm RAD24/rad24::URA3*	“
8/8 24/24 (1-3)	*MATa/MATα net1-srm/net1-srm rad24::URA3/rad24::URA3*	“
Series 8/53:		
8/+ 53/+(1-3)	*MATa/MATα NET1/net1-srm RAD53/rad53*	“
8/8 53/+ (1-3)	*MATa/MATα net1-srm/net1-srm RAD53/rad53*	“
8/+ 53/53 (1-3)	*MATa/MATα NET1/net1-srm rad53/rad53*	“
8/8 53/53 (1-3)	*MATa/MATα net1-srm /net1-srm rad53/rad53*	”

Table 1. Continuation

Strain	Genotype	Source of origin
		Constructed by authors
Series 12/5:		
5/+ 12/+ (1-4)	*MATa/MATα CDC28/cdc28-srm HFI1/hfi1-srm*	“
5/5 12/+ (1-4)	*MATa/MATα cdc28-srm/cdc28-srm HFI1/hfi1-srm*	“
5/+ 12/12 (1-3)	*MATa/MATα CDC28/cdc28-srm hfi1-srm/hfi1-srm*	“
5/5 12/12 (1-4)	*MATa/MATα cdc28-srm/cdc28-srm hfi1-srm/hfi1-srm*	“
Series 12/9:		
12/+ 9/+ (1,2)	*MATa/MATα HFI1/hfi1-srm RAD9/rad9::LEU2*	”
12/+ 9/9 (1)	*MATa/MATα HFI1/hfi1-srm rad9::LEU2/rad9::LEU2*	‘
12/12 9/+ (1-4)	*MATa/MATα hfi1-srm/hfi1-srm RAD9/rad9::LEU2*	‘
12/12 9/9 (1-4)	*MATa/MATα hfi1-srm/hfi1-srm rad9::LEU2/rad9::LEU2*	‘
Series 12/24:		
12/+ 24/+ (1-4)	*MATa/MATα HFI1/hfi1-srm RAD24/rad24::URA3*	“
12/12 24/+ (1-3)	*MATa/MATα hfi1-srm/hfi1-srm RAD24/rad24::URA3*	“
12/+ 24/24 (1-4)	*MATa/MATα HFI1/hfi1-srm rad24::URA3/rad24::URA3*	“
12/12 24/24 (1-4)	*MATa/MATα hfi1-srm/hfi1-srm rad24::URA3/rad24::URA3*	“
Series 12/53:		
12/+ 53/+ (1,2)	*MATa/MATα HFI1/hfi1-srm RAD53/rad53*	“
12/+ 53/53 (1)	*MATa/MATα HFI1/hfi1-srm rad53/rad53*	“
12/12 53/+ (1-3)	*MATa/MATα hfi1-srm/hfi1-srm RAD53/rad53*	“
12/12 53/53 (1-3)	*MATa/MATα hfi1-srm/hfi1-srm rad53/rad53*	“

*Yeast Genetic Stock Center, Berkeley, USA.

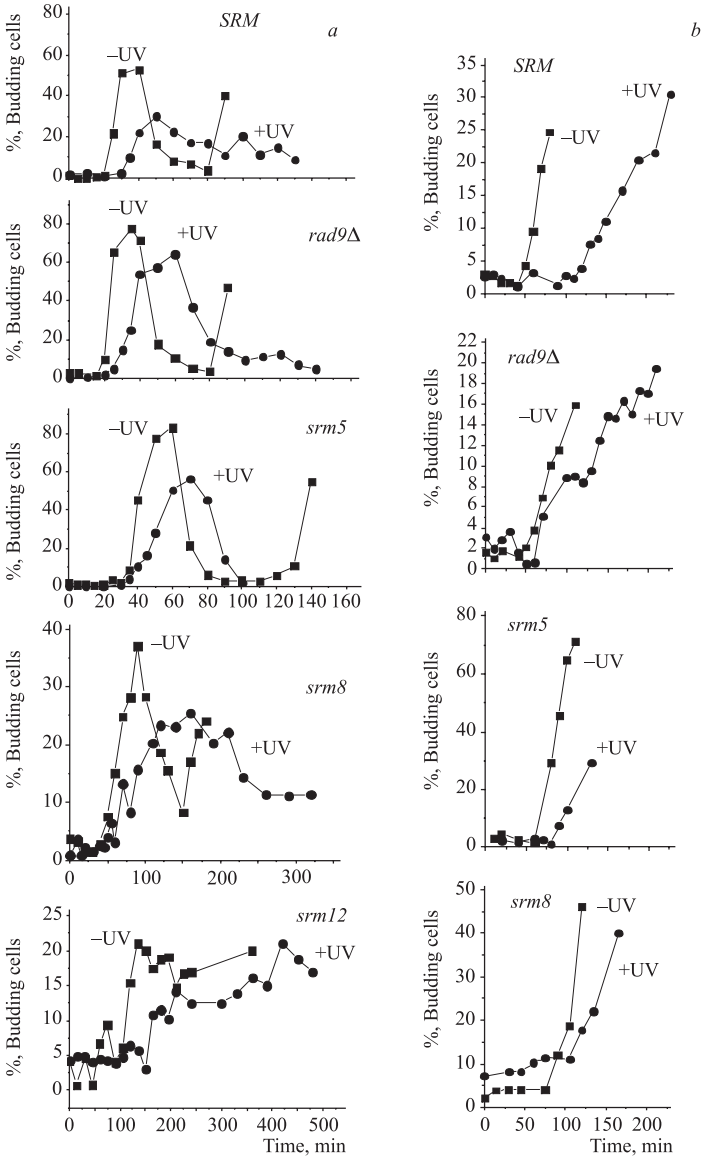

Fig. 1. Percentage of small budding cells as a function of time after their release from an α-factor arrest (*a*) or G_0 in untreated (–UV) or UV-irradiated (+UV) strains with different genotypes: 711a (*SRM*$^+$), M5a (*srm5*), C3 (*srm8*), 9a (*srm12*), and 3C (*rad9*Δ). No less than 200 cells were examined for each point

Table 2. Effect of exposure to UV radiation (10 J/m^2) on the kinetics of early bud formation in wild type and mutant cells

Genotype	Synchronization method	Time of emergence of budding cells, min		DNA damage-induced emergence delay, min
		–UV	+UV	
SRM$^+$	G_0	52.0 ± 3.7	102.5 ± 8.5	50
rad9	G_0	53.3 ± 6.7	70 ± 10	17
srm5	G_0	56.7 ± 3.3	75 ± 5	18
srm8	G_0	75.0 ± 0	75 ± 0	0
srm12	G_0	90.0 ± 0	141.7 ± 4.4	52
SRM$^+$	α factor	15	30	15
rad9	α factor	15	20	5
srm5	α factor	30	30	0
srm8	α factor	40	60	20
srm12	α factor	40	50	10

cultures causes a long delay ($\sim$ 100 min). Practically no delay was observed in the *rad9*Δ mutant, while in *srm5* and *srm8* the delay was shorter than in the wild-type cells. Thus, UV irradiation decreases the arrest time in G_1 in the *srm5*, *srm8*, and *srm12* mutants and in G_0 in the *srm5* and *srm8* mutants (Table 2).

3.2. Cell-cycle response to HU (S checkpoint). HU inhibits DNA replication by depleting cells of dNTP precursors, presumably by inhibiting ribonucleotide reductase, and causes wild-type cells to arrest cell division in the phase S [15]. Using a first cycle arrest assay showed that wild-type cells plated onto a HU-containing medium arrested in the first cycle (forming micro-colonies of large-budded and two adjacent large-budded cells) and eventually recovered to resume cell division. For comparison, we also used the well-known HU-sensitive *rad53* mutation. The *rad53* cells plated onto a HU-containing medium also appeared to arrest in the first cycle as large-budded cells but never resumed cell division because cells attempt mitosis before the completion of DNA replication, die. The *srm5* and *srm12* cells divide in a similar way irrespective of HU presence, and the growth of micro-colonies is blocked irreversibly. Probably, mutations disturb the division restart in the presence of HU and thus lead to the enhancement of the lethal effect. The lethal effect of the *srm5* and *srm12* mutations is lower than that of the *rad53* mutation.

The mutations *srm8* and *srm12* did not affect the arrest of division and sensitivity (Fig. 2, *a*) to incubation with 0.2 M HU during some limited time (2–8 h). However, cell synchronization by α factor in G_1 and an increase in the HU concentration to 0.5 M revealed the influence of the *srm12* mutation on the sensitivity to the lethal action of HU (Fig. 2, *b*).

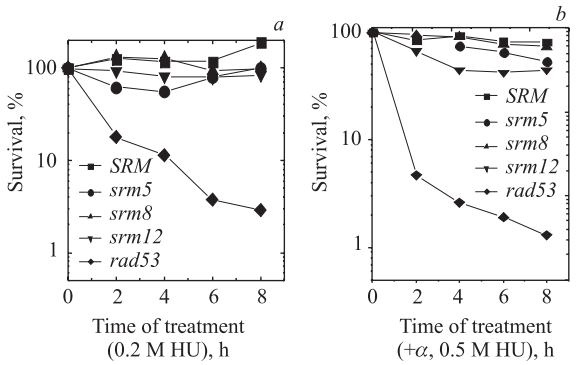

Fig. 2. Viability of strains in HU. Mid-log phase overnight cultures (*a*) or α factor-arrested mid-log phase overnight cultures (*b*) were spun down and resuspended in a fresh medium containing 200 mM (*a*) or 500 mM (*b*) HU. Aliquots were withdrawn at 0, 2, 4, 6, and 8 h and plated on a synthetic complete medium. The graph shows the percentage of cells that could form macroscopic colonies after 4 d of incubation at 30 °C. Typical curves are shown

The next and most dramatic result that indicates an S-phase arrest defect in *rad53* mutant cells was their response to constant incubation with 0.2 M HU (data not shown). HU-treated *rad53* mutant cells failed to actuate a cell cycle arrest and died rapidly, whereas wild-type cells arrested and remained viable. The checkpoint *rad* and *srm* mutations can be arranged by increasing sensitivity to HU:

$$SRM^+/RAD^+ < srm5 < rad9\Delta < srm1 < rad24\Delta < srm8 < \\ < rad17\Delta < srm12 < rad53.$$

Since UV-induced thymine dimers also block replication [16], we studied the capacity of the *srm* mutations to maintain survival in response to UV irradiation. The *srm5* and *srm8* mutations increase the sensitivity of cells to UV light as compared to that of the wild-type cells (Fig. 3). The *srm12* mutation has no influence on the UV sensitivity in haploid strains (data not shown). These data indirectly confirm that *SRM5/CDC28* and *SRM8/NET1* are important for survival under replicative stress.

3.3. Interactions of the *cdc28/srm5* mutation with replicative *cdc* mutations. There is a class of thermosensitive *cdc* mutations arresting cell division in G_2 at nonpermissive temperature through the checkpoint-dependent pathway [17]. To analyze the role of *srm5* in the S/G_2 checkpoint, we used two mutations: *cdc9-1* and *cdc6-1*. Corresponding genes encode the products involved in DNA metabolism, e.g., the *CDC9* gene of DNA ligase and the *CDC6* gene of a compo-

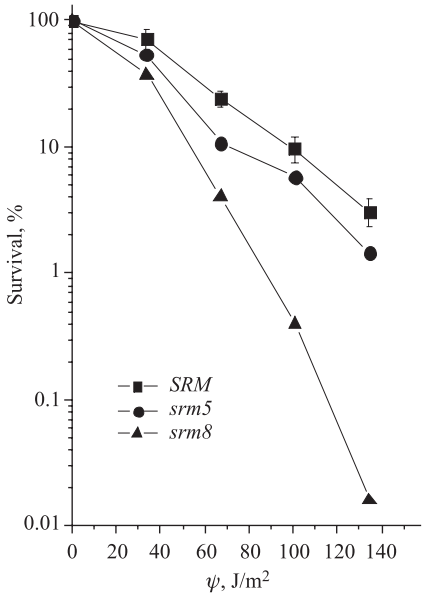

Fig. 3. Viability of strains after exposure to UV. The yeast strains were grown overnight to an early logarithmic phase ($2-5 \cdot 10^6$ cells/ml) in YPD at 30 °C. The cells were harvested and resuspended in water. Suspensions were plated on CM and irradiated with an UV lamp (0.28 J/m$^2\cdot$ s.). The graph shows the percentage of cells that could form macroscopic colonies after 4 d of incubation at 30 °C

nent of the prereplicative complex. Thus, cells of the *cdc9* mutant are arrested in G_2 with a completed DNA synthesis, but with unlinked Okazaki fragments [18]. Temporary inactivation of DNA ligase is not lethal for most cells. The *cdc9* cells survive after a short incubation at nonpermissive temperature. In the absence of

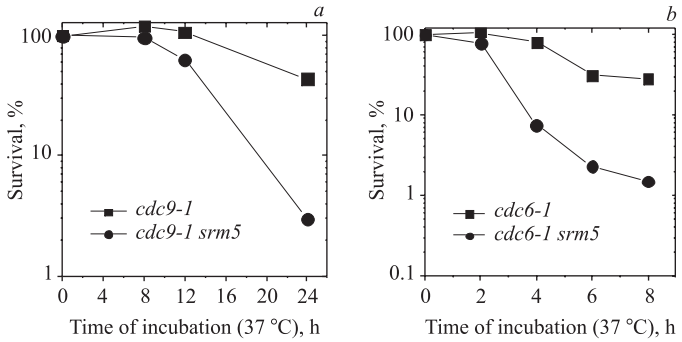

Fig. 4. Thermoinactivation of cells. Cells grown at a permissive temperature were shifted to the restrictive temperature of 37 °C in a liquid culture; at various times, cells were plated at a permissive themperature (28 °C); and cell viability was determined by colony formation after 5-d incubation. The *srm5* cells are not thermosensitive and are not arrested at a restrictive temperature

Table 3. Checkpoint mutants fail to arrest the cell cycle after γ irradiation

Genotypes	Dose, Gy	Arrest[a], %	Lethality[b], %	Arrest/Lethality[c]
SRM$^+$	20	33.3	33.3	1.01 ± 0.04
	40	52.0	60.7	0.86 ± 0.06
srm5	20	29.7	31.3	0.94 ± 0.06
	40	48.8	56.3	0.89 ± 0.07
srm8	20	36.7	49.0	0.74 ± 0.04
	40	64.0	77.7	0.83 ± 0.06
srm12	20	32.0	34.0	0.95 ± 0.07
	40	61.3	61.7	0.99 ± 0.07
rad9	20	9.3	59.7	0.16 ± 0.01
	40	15.0	83.5	0.18 ± 0.03

[a] Arrest was determined as the proportion of microcolonies consisting of two (one cell with a large bud) or four cells (two cells with large buds) within 10 h after irradiation and plating.
[b] Lethality was determined as the proportion of microcolonies within 24 h after irradiation. Results are averaged over three experiments.
[c] The metric characteristic of the cell cycle arrest was the arrested cells/cell lethality ratio. The cells were grown in a rich liquid medium to a mid-log phase, sonicated, plated on a solid medium, and treated with a low dose of γ rays (2 and 4 Gy).

RAD9, however, DNA ligase-defective cells quickly die at nonpermissive temperature because they enter mitosis with damaged DNA. The *cdc6* mutation arrests cells in late S/G_2 [17].

Both mutations were introduced in the genotype of the strain 71a. Then the *cdc9-1 srm5* and *cdc6-1 srm5* double mutants were constructed. The qualitative analysis of the lethal effect of temperature on the cultures of single and double mutants showed that the *cdc9-1 srm5* (Fig. 4, *a*) and *cdc6-1 srm5* (Fig. 4, *b*) double mutants are more sensitive to higher temperature than the single mutants *cdc9-1* and *cdc6-1*, and that *cdc6-1 srm5* cells are inactivated more rapidly than *cdc9-1 srm5*. In the double mutants, as compared to the single mutants, most of the cells die at a higher temperature at the stage of several cells probably due to a checkpoint defect. These results demonstrate that *SRM5/CDC28* is necessary for an arrest in S/G_2 induced by unreplicated DNA, i.e., it participates in the S checkpoint.

3.4. G_2/M checkpoint. It is known that γ irradiation induces a long-term arrest in G_2 for DNA damage repair. Since the *srm5*, *srm8*, and *srm12* mutations increase the sensitivity to γ irradiation [10, 12], it was interesting to find out whether this increase is associated with a defect in the G_2 checkpoint. The *rad9*Δ mutation, which failed the G_2 checkpoint induced by DNA damage, was

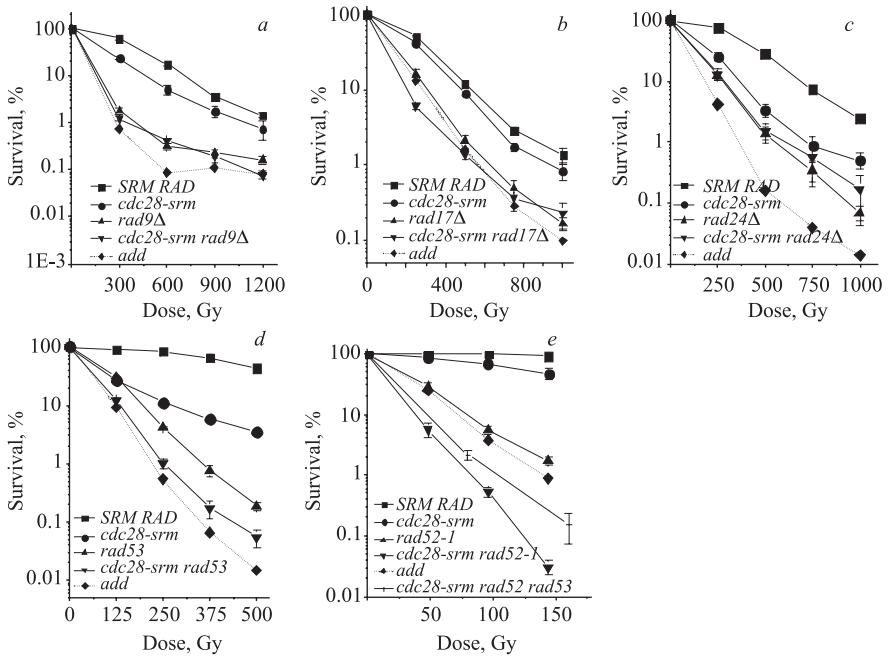

Fig. 5. Survival curves obtained following γ irradiation of diploid single and double mutants homozygous for the mutations *cdc28-srm* and *rad9*Δ (*a*); *cdc28-srm* and *rad17*Δ (*b*); *cdc28-srm* and *rad24*Δ (*c*); *cdc28-srm* and *rad53* (*d*); single (*cdc28-srm*, *rad52*), double (*rad52 cdc28-srm*), and triple (*rad52 rad53 cdc28-srm*) mutants (*e*). Strains were irradiated using a «Svet» γ installation (^{137}Cs, 25 Gy/min), and the triple mutant *rad52 rad53 cdc28-srm* was irradiated using a «Materialovedcheskaya» γ installation (^{60}Co, 180 Gy/min). Each curve corresponds to the data averaged over three or four strains of the same genotype; standard errors are given. For comparison, a curve expected upon an additive effect of two mutations on cell radiosensitivity is presented

also used [1]. The fraction of arrested cells/cell lethality provides a convenient metric of the cell cycle arrest. Haploid strains with different genotypes were exposed to the doses of 20 and 40 Gy, which induce a small number of double-strand breaks per cell [1], and the size of micro-colonies was analyzed depending on the postirradiation incubation time (Table 3). It is seen that only the *rad9*Δ mutation influences the cell cycle arrest in G_2 after γ irradiation. In the *srm5*, *srm8*, and *srm12* mutants this method revealed no defect in the G_2 checkpoint.

3.5. The *SRM* genes belong to the *RAD9* epistasis group. To determine the epistatic interactions between *SRM* and checkpoint genes, we generated a

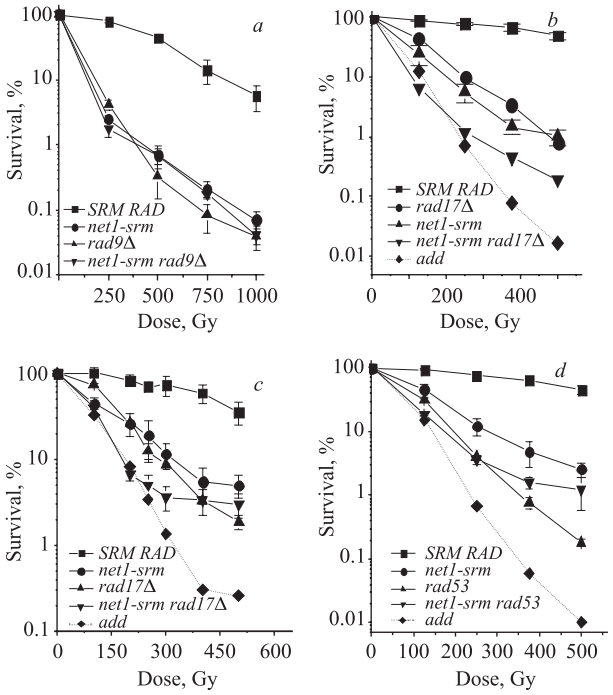

Fig. 6. Interaction of *srm8/net1-srm* with *rad9*Δ (*a*), *rad17*Δ (*b*), *rad24*Δ (*c*), *rad53* (*d*)

panel of closely related single and double mutants (three or four strains of the same genotype) (Table 1). The diploid strains were constructed with the aid of a micromanipulator in the 71a genetic background by crossing the initial strains bearing mutations in the checkpoint genes with mutually isogenic normal strains 71a and 71α and then repeatedly backcrossing (at least four times) the progeny with 71a and 71α parents.

Strains carrying the single *rad9*Δ alleles showed very similar sensitivity to γ irradiation, which was indistinguishable from that of a *cdc28-srm rad9*Δ and *net1-srm rad9*Δ double mutant, indicating that the *CDC28* and *NET1* genes belong to the same *RAD9* epistasis group (Figs. 5, *a* and 6, *a*). The *rad24*Δ mutations were also epistatic to *cdc28-srm* (Fig. 5, *c*). Others double mutants had additive effects, for example, the *cdc28 rad53* double mutants were more sensitive than each single mutant (Fig. 5, *d*) and so protein kinases *CDC28* and *RAD53* mediate the control of different pathways. *CDC28* and *RAD52* also control different pathways, but *rad52-1 rad53 cdc28-srm* triple mutant is not more sensitive than the *rad52-1 cdc28-srm* double mutant (Fig. 5, *e*). Therefore, *SRM5/CDC28* and

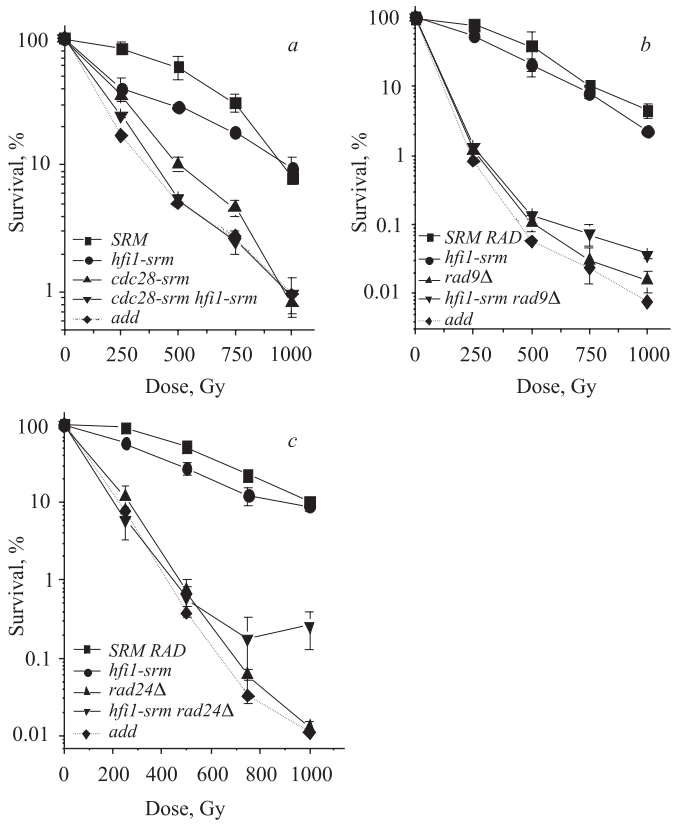

Fig. 7. Interaction of *srm12/hfi1-srm* with *srm5/cdc28-srm* (*a*), *rad9*Δ (*b*), *rad24*Δ (*c*)

SRM8/NET1 belong to the branched *RAD9* epistasis group that also includes the *RAD17*, *RAD24*, and *RAD53* genes [12], while *SRM12/HFI1* represents a different group (Fig. 7, *b*). Some double mutants are nonviable, in particularly, *cdc28-srm net1-srm* and *hfi1-srm rad53*.

3.6. Checkpoint genes and mutability of mitochondrial genome. The phenotypic manifestations of mutations in the *CDC28*, *NET1*, and *HFI1* genes are similar to the phenotypic manifestations of checkpoint gene mutations responsible for genetic instability [6, 8]. *CDC28*, *NET1*, and *HFI1* participate in the stabilization of various genetic structures, such as natural chromosomes and recombinant structures, and in the maintenance of mitochondrial genome [9, 11]. We analyzed mitochondrial *rho*$^-$-mutability in the cultures of segregants from two tetrads of postmeiotic products of heterozygotes for *rad9*Δ, *rad17*Δ, *rad24*Δ, and *rad53*

mutations. It is seen from Table 4 that in contrast to the *srm* mutations these mutations increased the frequency of the mitochondrial *rho*$^-$-mutants.

4. DISCUSSION

It has been demonstrated that the *SRM5/CDC28*, *SRM8/NET1*, and *SRM12/HFI1* genes participate in the regulation of the DNA damage-induced arrest in budding yeast. The cyclin-dependent kinase CDC28 is an attractive target for the G_1 and G_2 checkpoints, as it is involved in the regulation of the cell cycle progression and is necessary for DNA replication and mitosis [19]. However, the mechanism of CDC28 inactivation and involvement in the regulation of the checkpoints is not yet clear. In early G_1, the kinases CDC28/CLN1,2 activate the Swi6/Mbp1 factor of the transcription of the CLB5 and CLB6 cyclins involved in the assembly of a prereplicative complex and DNA replication genes. DNA damages activate the kinase Rad53, which phosphorylates and inactivates the Swi6 coactivator of transcription of the G_1 cyclins [20]. Hyperexpression of cyclins suppresses the checkpoint defect. Mutations of the catalytic subunit also abrogate the cell cycle arrest. The mutation *cdc28-5M* not only affects the arrest caused by UV-induced damage, but also decreases the kinase activity, including that associated with CLB5 and CLB6 [21]. We observed a decrease in the cell cycle delay in G_0 or G_1 upon UV light irradiation in the *cdc28-srm* mutant, but the activity of the kinase complexes in the *cdc28-srm* mutant was not studied. It can be assumed that the G_1 checkpoint controls the activity of some of kinase complexes by regulating cyclin transcription. The inhibition of cyclin transcription or the mutations of the catalytic subunit decreasing affinity to G_1 cyclins inactivate the G_1checkpoint.

The involvement of CDC28 in the S and intra-S checkpoints was not revealed. The mutations *cdc28-srm* (this work), *cdc28-5M* [21], and *cdc28*-Y19F [22], or the inhibition of CDC28 kinase activity [22] had no influence on the S checkpoint and on the sensitivity to HU. But the *cdc28-srm* mutation influences the checkpoint-dependent arrest of the replicative mutants *cdc6-1* and *cdc9-1* in the late S/G_2 and G_2, respectively. Moreover, *cdc28-srm* increased the frequency of conversion, crossing-over and chromosome loss [9, 10]. It is known that defects in the replicative S checkpoint and in the intra-S checkpoint, rather than in the G_1 or G_2 checkpoints, cause an increase in the level of spontaneous rearrangements in the genome [8]. It is possible that the influence of CDC28 on the checkpoint in the phase S is not yet determined.

CDC28 is involved in the arrest in the phase G_2. Posttranslational modifications play an important role in this process. Bud emergence required a stable polarization of the actine cytoskeleton beginning at the end of G_1 and its control by cyclins CLN1,2. Phosphorylation of Y19-CDC28 by the kinase Swe1 was

Table 4. Proportion of *rho*$^-$ mutants in haploid segregants of different genotypes*

Genotypes	Number of strains tested	Number of colonies screened	Proportion of *rho*$^-$, mutants, %
RAD9	8	3441	39.5 ± 4.9
*rad9*Δ	8	8215	55.1 ± 8.8
RAD17	7	5279	23.5 ± 5.9
*rad17*Δ	8	7576	43.1 ± 7.8
RAD24	8	4462	19.8 ± 6.8
*rad24*Δ	8	4420	23.2 ± 4.9
RAD53	8	6504	22.2 ± 3.4
rad53	8	5989	32.2 ± 6.6

*The colonies were obtained after 5-day growth in a nonselective CM medium. Five colonies were suspended in water, and diluted cell suspensions were plated on CM. After 4-day incubation, *rho*$^-$ colonies were scored. The colonies of *rho*$^-$ mutants are easily distinguishable due to their small size and altered pigmentation in the *ade1,2*-strains.

removed by the phosphatase Mih1 causing an increase in kinase activity in G_2. In the case of bud emergence disruption, phosphorylation of Y19 is critical for the morphological checkpoint, which delays nuclear division in G_2 and prevents occurrence of binuclear cells. A delay of nuclear division is completely eliminated by *cdc28*-Y19F preventing tyrosine phosphorylation or by overexpression of *MIH1* [22]. Thus, the direct targets of the morphological checkpoint are the kinase Swe1 and phosphatase Mih1.

However, this mechanism does not function in the case of the spindle body checkpoint [23] or DNA damage-inducible checkpoint [24], which also induces an arrest in G_2. Mitosis in budding yeast is regulated at several points. The spindle assembly is controlled in G_1 soon after the START, and the mitotic segregation of chromosomes is controlled later in the metaphase/anaphase; in addition, the exit from mitosis is also controlled. Different stages of mitosis are orchestrated by two or more forms of CDC28. Some of these forms (CDC28/CLB3 and CDC28/CLB4) are required for the initiation of mitosis and for the formation of the mitotic spindle, and other forms (CDC28/CLB1 and CDC28/CLB2) are necessary for the exit from mitosis and for the completion of the cell cycle.

For transition metaphase/anaphase degradation of the inhibitor of the separation of sister chromatids, securine Pds1, are required. The mitotic form of the kinase CDC28 takes part in the activation of the proteasome APC/C^{Cdc20} by phosphorylating the regulatory subunit Cdc20 and following the ubiquitin-dependent degradation of the anaphase inhibitor Pds1 and the cyclins CLB5 and CLB3.

The DNA damage checkpoint causes a delay in mitosis through the inhibition of Pds1p ubiquitinization due to phosphorylation of securine by CDC28 [25]. Phosphorylated securine Pds1 mediates binding and nuclear localization of separase Esp1 [26] which cleave one of the subunits of the cohesine complex (Med1/Scc1) responsible for the cohesion of chromatids. The second pathway includes the Rad53-dependent phosphorylation of Cdc20p that inhibits the interaction between the Cdc20p and Pds1p [27] and also mediates the inducible phosphorylation of the subunit of the cohesine complex Scc1 [20]. The exit from mitosis is associated with the inactivation of CDC28/CLB2. With the arrest of the cell cycle in the G_2/M, the kinase Rad53 maintains the high activity of CDC28 through the inhibition of the kinase Cdc5 involved in the degradation of cyclins and the exit from mitosis [25].

In our experiments with *cdc28-srm,* no defects in the DNA damage-induced G_2 checkpoint and replication block (HU) were detected, but disturbances in the G_2-arrest were observed in the replicative *cdc6* and *cdc9* mutants at nonpermissive temperature. However, using the *cdc28-as1* [F88G] mutation it was shown that CDC28 is required for activation by a double-strand DNA break of Mec1p-dependent arrest in G_2 [28]. Moreover, in the mutant *cdc28-M* abrogating cell cycle arrest induced by DNA-damage a decrease of the CLB2-associated kinase activity required for the exit from mitosis, is observed [21]. Thus, the different mutant kinase subunit alleles evoke different consequences of the dysfunction of CDC28 and phenotypic properties. It is very interesting to study the specificity of kinase structural rearrangements and their functional role.

We demonstrate for the first time the involvement of *NET1* and *HFI1* in the G_1/S checkpoints and *HFI1* in S checkpoint. Net1p is localized in the nucleolus, where it sequesters the deacetylase Sir2 and the phosphatase Cdc14 [29]. Net1p is a substrate of CDC28 and its phosphorylation is probably necessary for the release of Cdc14p from the nucleolus. One of the Cdc14p substrates is Nbp1p required for the duplication of spindle polar bodies [30]. Hfi1p/Ada1p is a component of the transcription coactivators SAGA and GCN5/ADA [31]. Another component of these complexes, acetylase Gcn5, mediates the global genome acetylation of histones [32]. Acetylation/deacetylation of histones plays the regulatory role both in repair and checkpoint processes.

It was shown that the loss of acetylated lysines 5, 8, 12, and 16 of the histone H4 or a defect of the acetylase Esa1 in NuA4 activates the *RAD9*-dependent G_2/M checkpoint [33–35]. The loss of the deacetylase Sin3 restores the G_2/M arrest of the cell cycle after UV irradiation in checkpoint-deficient strains [36]. This restoration depends on the spindle checkpoint. The Sin3p also regulates the late-firing *ori* and intra-S checkpoint [37]. There is evidence of the role of the acetylation/deacetylation of nonhistone proteins in the control of the apoptosis and radiosensitivity of human cells [38–42]. The component of acetylase complexes hADA3 is a cofactor of the activation of the transcription factor p53 [43]

involved in the G_1-arrest and in apoptosis. The p53 is acetylated by the acetylase PCAF/yGCN5 in response to DNA damage [38–40]. The p53-dependent pathway is also mediated by Sir2α deacetylation of histones [41, 42].

We have investigated the interactions between checkpoint genes in the determination of yeast radioresistance [12]. *CDC28* and *NET1* genes belong to a single, though branched, *RAD9* epistasis group; for instance, kinases *RAD53* and *CDC28* are attributed to different branches of the *RAD9*-dependent pathway. The *cdc28-srm* is epistatic to *rad24*Δ, the others analyzed interactions are additive. *CDC28* does not belong to the *RAD6* and *RAD52* epistasis groups mediating the repair of a major portion of DNA radiation lesions via postreplication repair and HR. Checkpoint genes participate not only in checkpoints but in repair. Mutations *rad9*Δ, *rad17*Δ, and *rad24*Δ decrease the efficacy of NHEJ [44], *rad53* — NHEJ [44] and HR [45], *cdc28* — HR [10], NHEJ [28], and BIR [46]. Activation of DNA repair and checkpoint control are coupled with changes in chromatin structure related, in particular, to chromatin modification of histones. The impact of the nucleolar protein Net1 on radioresistance is likely mediated by deacetylase Sir2 localization. Deacetylase Sir2 or acetylase Gcn5 (with Hfi1p — components of SAGA complex) modifying the histones H3 and H4 are shown to recruit in the DSB region in HR process [47]. The acetylation of N-tails of histone H4 is essential also for minor pathways of DNA repair, NHEJ and BIR [48]. Genetic data testify to the interaction between *CDC28*, *NET1*, and *HFI1*. Double mutants *cdc28-srm net1-srm*, *net1-srm hfi1-srm* [11] and *hfi1-srm rad53* are nonviable. Mutations *hfi1-srm* and *cdc28-srm* manifest additive effect following exposure to γ ray radiation.

In this study, we have shown that *RAD9*, *RAD17*, *RAD24*, and *RAD53* genes increase (but *SRM* genes decrease [9, 12]) stability of mitochondrial genome. It is shown that the Mec1p/Rad53p pathway can influence mtDNA copy number [49]. The study of the role of the *CDC28*, *NET1*, and *HFI1* in checkpoints and checkpoint genes in nuclear and mitochondrial genome maintenance arouses great interest and should be continued.

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