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Research paper

Uncharacterized ORF *HUR1* influences the efficiency of non-homologous end-joining repair in *Saccharomyces cerevisiae*

Katayoun Omidi[a,b], Matthew Jessulat[c], Mohsen Hooshyar[a,b], Daniel Burnside[a,b], Andrew Schoenrock[d], Tom Kazmirchuk[a,b], Maryam Hajikarimlou[a,b], Mary Daniel[a,b], Houman Moteshareie[a,b], Urvi Bhojoo[a,b], Megan Sanders[a,b], Dindial Ramotar[e], Frank Dehne[d], Bahram Samanfar[f], Mohan Babu[c], Ashkan Golshani[a,b,*]

[a] *Department of Biology, Carleton University, Ottawa, Ontario, Canada*
[b] *Ottawa Institute of Systems Biology, University of Ottawa, Ottawa, Ontario, Canada*
[c] *Department of Biochemistry, Research and Innovation Centre, University of Regina, Regina, Saskatchewan, Canada*
[d] *School of Computer Science, Carleton University, Ottawa, Ontario, Canada*
[e] *Maisonneuve-Rosemont Hospital Research Center, University of Montreal, Montreal, Quebec, Canada*
[f] *Agriculture and Agri-Food Canada, Ottawa Research and Development Centre (ORDC), Ottawa, Ontario, Canada*

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ABSTRACT

Non-Homologous End Joining (NHEJ) is a highly conserved pathway that repairs Double-Strand Breaks (DSBs) within DNA. Here we show that the deletion of yeast uncharacterized ORF *HUR1*, Hydroxyurea Resistance1 affects the efficiency of NHEJ. Our findings are supported by Protein-Protein Interaction (PPI), genetic interaction and drug sensitivity analyses. To assess the activity of *HUR1* in DSB repair, we deleted its non-overlapping region with *PMR1*, referred to as *HUR1-A*. We observed that similar to deletion of *TPK1* and *NEJ1*, and unlike *YKU70* (important for NHEJ of DNA with overhang and not blunt end), deletion of *HUR1-A* reduced the efficiency of NHEJ in both overhang and blunt end plasmid repair assays. Similarly, a chromosomal repair assay showed a reduction for repair efficiency when *HUR1-A* was deleted. In agreement with a functional connection for Hur1p with Tpk1p and NEJ1p, double mutant strains *Δhur1-A/Δtpk1*, and *Δhur1-A/Δnej1* showed the same reduction in the efficiency of plasmid repair, compared to both single deletion strains. Also, using a Homologous Recombination (HR) specific plasmid-based DSB repair assay we observed that deletion of *HUR1-A* influenced the efficiency of HR repair, suggesting that *HUR1* might also play additional roles in other DNA repair pathways.

1. Introduction

DNA Double-Strand Breaks (DSBs) are the most severe form of DNA damage. In the event of DSBs, there are two independent repair pathways: Homologous Recombination (HR), and Non-Homologous End Joining (NHEJ). When the break occurs within a sequence that has a homologous region elsewhere in the genome, the cell may repair the damage through HR. HR uses a break region's homology as a template to coordinate efficient repair (reviewed in Dudas and Chovanec, 2004). HR is the primary DSB repair system in the baker's yeast, *Saccharomyces cerevisiae*, and is considered to be less error-prone than its alternative.

A more versatile alternative to HR is classical Non-Homologous End joining (c-NHEJ) in *S. cerevisiae* (Dudásová et al., 2004; Daley et al., 2005). NHEJ is used to repair any DSB regardless of sequence homolog availability. In *S. cerevisiae*, the key protein complexes associated with NHEJ pathway are the MRX (Mre11p, Rad50p, and Xrs2p), YKU (Yku70p/Yku80p) and Lif1p/Dnl4p complexes (Daley et al., 2005). Initial recognition of the DNA lesion in NHEJ is through Yku70p-Yku80p heterodimer (YKU complex), which binds to DNA ends (Milne et al., 1996; Siede et al., 1996). Recognition of the DSB and recruitment of the YKU complex to the site of damage involves a cascade of DNA damage checkpoints in which Tel1p, Mec1p and Rad53p play central roles (Ataian and Kerbs, 2006). The proteins Mre11p, Rad50p, Xrs2p form a complex termed MRX, which is then recruited to the broken ends

Abbreviations: DDA, DNA damage array; DSBs, DNA double stranded breaks; GI, genetic interaction; HR, homologous recombination; HU, hydroxyurea; MMEJ, micro-homology mediated end joining; NHEJ, non-homologous end joining; PPI, protein-protein interaction; c-NHEJ, classical non-homologous end joining; alt-NHEJ, alternative NHEJ; SGA, synthetic genetic array

* Corresponding author at: Department of Biology, Carleton University, Ottawa, Ontario, Canada.
E-mail address: ashkan_golshani@carleton.ca (A. Golshani).

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of DNA. The exact contribution of MRX in NHEJ pathway is not fully understood, because of the involvement of this complex in other cellular pathways (Emerson and Bertuch, 2016). MRX complex strongly stimulates DNA ligation. Ligase activity in NHEJ is the result of the Dnl4p/Lif1p complex (Chen et al., 2001). Nej1p binds to Dnl4/Lif1 complex through an interaction with Lif1, but Nej1 is not critical for Dnl4/Lif1 complex formation (Ooi et al., 2001; Emerson and Bertuch, 2016). Although details of Nej1p's role is still unclear, recent papers suggest it recruits to the site of the break, interacts with DNA, and may function as a regulatory protein (Mahaney et al., 2014). Plasmid repair assays have displayed a role of Nej1p in NHEJ (Deshpande and Wilson, 2007; Emerson and Bertuch, 2016). Nej1p is also shown to affect NHEJ via YKU independent pathways such as micro-homology mediated end joining (MMEJ), which is a subset of alternative NHEJ (alt-NHEJ) (Lee and Lee, 2007; McVey and Lee, 2008). MMEJ, which requires Nej1, plays a significant role in the repair of DNA breaks with blunt ends (Lee and Lee, 2007). The process of alt-NHEJ is less well-characterized, but there is agreement that the MRX complex is necessary for that process (Lee and Lee, 2007; Emerson and Bertuch, 2016).

In addition to key NHEJ genes, recent literature as well as studies in our laboratory have identified a growing number of genes with novel roles in the process of DNA damage repair and NHEJ, such as Rtt109p, Sub1p, Pph3p, Psy2p, Tpk1p etc., which suggests existence of other uncharacterized proteins in the NHEJ pathway (Shim et al., 2005, Jessulat et al., 2008, Yu and Volkert, 2013, Omidi et al., 2014, Jessulat et al., 2015, Hooshyar et al., 2017). For example, Jessulat et al. proposed the novel function for the Rtt109p-Vps75p on the efficiency of NHEJ in yeast *S. cerevisiae* (Jessulat et al., 2008). *SUB1* was found to be required for NHEJ repair of DSBs in plasmid DNA, but not in chromosomal DNA. It also may play a role in the fidelity of NHEJ (Yu and Volkert, 2013). Deletion of *PPH3* and *PSY2* was shown to reduce the efficiency of NHEJ in yeast through cell cycle regulation (Omidi et al., 2014).

Here, we are interested in studying the uncharacterized ORF *HUR1*. *HUR1*, Hydroxyurea Resistance1, is a functionally uncharacterized ORF, which is reported in the Saccharomyces Genome Database (http://www.yeastgenome.org) as a protein of unknown function with no known biological activity. It is a small protein 110 amino acid in length and 12.5 kDa in size. Its expression is reported to be upregulated under different DNA damage conditions (Fry et al., 2003; Takagi et al., 2005; Borde et al., 2009). Its locus partially overlaps with *PMR1*, a calcium import gene associated with Golgi and it was previously reported that this overlap might be the reason for the sensitivity of *HUR1* deletion mutant strain to the DNA damage inducing drug HydroxyUrea (HU) (Jordan et al., 2007). The knowledge gap in the activity of this ORF along with its inferred connection to DNA damage prompt us to further investigate its activity. In this report, we investigated the activity of *HUR1* using high throughput protein-protein interaction (PPI) and genetic interaction (GI) analysis, and observed a possible role for this protein in DSB repair. In follow-up investigations, we deleted the first 70 amino acids of *HUR1* (*HUR1-A* ORF) without affecting its overlap with *PMR1* and reported that *HUR1* influences the efficiency of NHEJ in yeast.

2. Materials and methods

2.1. Yeast strains and plasmid

The yeast strain *S. cerevisiae* deletion library in BY4741 background (MATa orfΔ::KanMX4 *his3Δ leu2Δ met15Δ ura3Δ*) (Winzeler et al., 1999) was utilized unless stated otherwise. Since *HUR1* partially overlaps with *PMR1*, the first 70 amino acids of *HUR1* were deleted (*Hur1-A*) without affecting its overlap with *PMR1* and minimally affecting the 3′ UTR content of *PMR1* mRNA, in BY4741 and Y7092 (MATa can1Δ::STE2pr-HIS3 lyp11Δ ura31Δ leu21Δ his31Δ met151Δ) background strains by PCR transformation containing NAT selection gene (Tong and Boone, 2007).

The yeast gene deletion strains and plasmids were used to perform the experiments as in (Omidi et al., 2014). Plasmids p416 that carries a URA3 marker and Ampr gene (Jessulat et al., 2008) and Ycplac111 and pMV1328 with Leu2 marker and Ampr, were used for plasmid repair assays for overhang and blunt end repairs.

2.2. Protein-protein interaction prediction

Prediction of Protein-Protein Interactions (PPIs) was based on the co-occurring polypeptide regions (Pitre et al., 2008). An updated list of high confidence PPIs, identified in at least two different investigations, was gathered from published data (BioGRID: www.thebiogrid.org and DIP: www.dip.doe-mbi.ucla.edu). This set of PPIs was used by the algorithm to predict the likelihood of an interaction for a target pair of proteins. High confidence interactions were predicted by adjusting the specificity, which measures our confidence for a hit, for the algorithm to 99.55%. In this way Hur1p potential interaction was examined against all other yeast proteins. The regions that mediate PPIs were predicted using PIPE-site algorithm (Amos-Binks et al., 2011).

2.3. Genetic interaction analysis

The Genetic Interaction (GI) with the DNA damage array (DDA) was performed using the principles of synthetic genetic array (SGA) analysis (Tong et al., 2001) as in (Alamgir et al., 2010). Conditional GI analyses were performed in the presence of mild DNA damage conditions induced by low concentrations of bleomycin and HU. Fitness was scored by colony size measurement as in (Memarian et al., 2007, Jessulat et al., 2008, Samanfar et al., 2014). Phenotypic rescue analysis was performed using overexpression plasmids were transformed into the above deletion arrays as in (Alamgir et al., 2010). Each experiment was repeated three times. Double mutant strains with relative reduced fitness of 20% or more in at least two experiments were considered positive hits and were further confirmed using spot test analysis.

2.4. Drug sensitivity spot test

Yeast strains deletions and wild-type were grown in YPD at 30 °C to saturation then 15 μl of each spotted on the YPD media containing 60 mM HU or 4 μg/ml bleomycin and drug free media. Series of cell dilutions 10^{-2} to 10^{-5} were used as explained in (Jessulat et al., 2008, Hooshyar et al., 2017).

2.5. Plasmid repair assay

p416, Ycplac111 and pMV1328 were digested at their unique *Xba*I, Pst1-*Hin*dII-*Sma*I and *Nru*I restriction sites within regions with no homology to yeast chromosomes, respectively. The assay was performed in this study as in (Jessulat et al., 2008; Omidi et al., 2014). Each experiment was repeated at least five times. At least 250 colonies were counted for strains with low NHEJ efficiency. To calculate the *p*-values two-tailed distribution *t*-test was used.

2.6. Chromosomal repair assay

A JKM139-based chromosomal DSB repair was done by knocking-out target genes in JKM139 strain (Moore and Haber, 1996; Omidi et al., 2014). This strain carries a GAL promoter in front of an endonuclease specific to HO site. Different serial dilutions between 10^{-2} to 10^{-5} for mutant and wild-type strains grown to OD 1, were plated on media containing galactose or glucose (as a control). Colony growth differences were used as a measure of survival and related to the ability of the cell to repair induced DSBs. For phenotype compensation experiments, gene overexpression in an individual mutant background was used as in (Jessulat et al., 2008). Each experiment was repeated

five times. To calculate the *p*-values two-tailed distribution *t*-test was used.

2.7. Homologous recombination assay

In the homologous recombination assay, plasmid pGV-256-dead is digested at its non-functional *LacZ* gene, with *Bgl*II restriction enzyme. PCR product containing functional *LacZ* was obtained using pGV-256-live plasmid as a template. Linearized plasmid and PCR products were co-transformed into mutant and wild-type strains as in (Erdimir et al., 2002; Jessulat et al., 2008). A minimum of 50 colonies were transferred to a new plate and grown for 1 day. A β-galactosidase lift assay on transferred colonies was performed to measure recombination repair. Recombination efficiency was calculated based on the ratio of blue (carrying functional *LacZ* gene) to white (carrying non-functional *LacZ* gene) colonies for mutant strain, which is normalized to the wild-type.

3. Results and discussion

3.1. HUR1: a candidate gene involved in DNA damage repair

3.1.1. Protein-Protein interaction analysis

Protein-Protein Interactions (PPIs) display overall profiles and essential aspects of all biological pathways and mechanisms within a cell (Dittrich et al., 2008). As a general rule, proteins with similar functions physically interact with one another within a cell. Consequently, PPIs have been used as a mean to study protein functions (Butland et al., 2007; Wood et al., 2003) and to uncover novel activities for proteins in different organisms (Hu et al., 2009; Krogan et al., 2003; Jin et al., 2007). For example, if protein X is found to physically interact to proteins involved in the process of transcription, it is generally thought that protein X may also play a role in this process. PPIs can be studied using various biochemical and computational approaches with each having inherent advantages and disadvantages. One such computational tool predicts PPIs on the basis of co-occurring short polypeptide regions (Pitre et al., 2008; Pitre et al., 2012). It has one of the highest specificities (low false positives) and sensitivities (low false negatives) among the computational tools and has been used to study novel protein functions for different yeast proteins (Pitre et al., 2012; Schoenrock et al., 2014). To study Hur1p function, we used the principles of co-occurring peptides as a mean of interactions (Jessulat et al., 2011) to study its proteome wide PPI map. In this way, the updated database of high confidence PPIs were searched for pairs of small overlapping windows of 20 amino acids long that co-occur within interacting partners only. To this end, 3 high confidence interactions were found for Hur1p protein with Tpk1p, Tpk2p and Tpk3p proteins (Fig. 1A). TPK family of proteins constitutes cAMP-dependent protein kinases that promote vegetative growth in response to nutrients. We have recently shown that their deletion reduces the efficiency of NHEJ and that this activity for *TPK1* is connected to *NEJ1* function in a YKU independent manner. In agreement with this data, a physical interaction between *HUR1* and *TPK1* has been previously reported (Ptacek et al., 2005). Besides this one interaction for Hur1 no other PPIs are reported in literature.

3.1.2. Genetic interaction analysis

Genetic Interaction (GI) analysis often shows functional redundancy of genes in different pathways and higher order pathway association (Bandyopadhyay et al., 2010; Costanzo et al., 2011). They are determined by comparison of a double mutant's phenotype(s) to individual phenotypes of single mutants, by the deletion of an individual gene with deletion or overexpression of a second gene. A GI is formed if the presented phenotype cannot be explained by observing the phenotype of the single gene deletion or overexpression alone (Boone et al., 2007). Negative interactions or synthetic sickness refer to the double mutant phenotypes, which is more intense (worse) than expected compared to single deletion mutants. Positive or alleviating interaction describes the interactions where a second mutation compensates for the deletion of the first gene, so the phenotype of the double mutant is less severe (Bandyopadhyay et al., 2010).

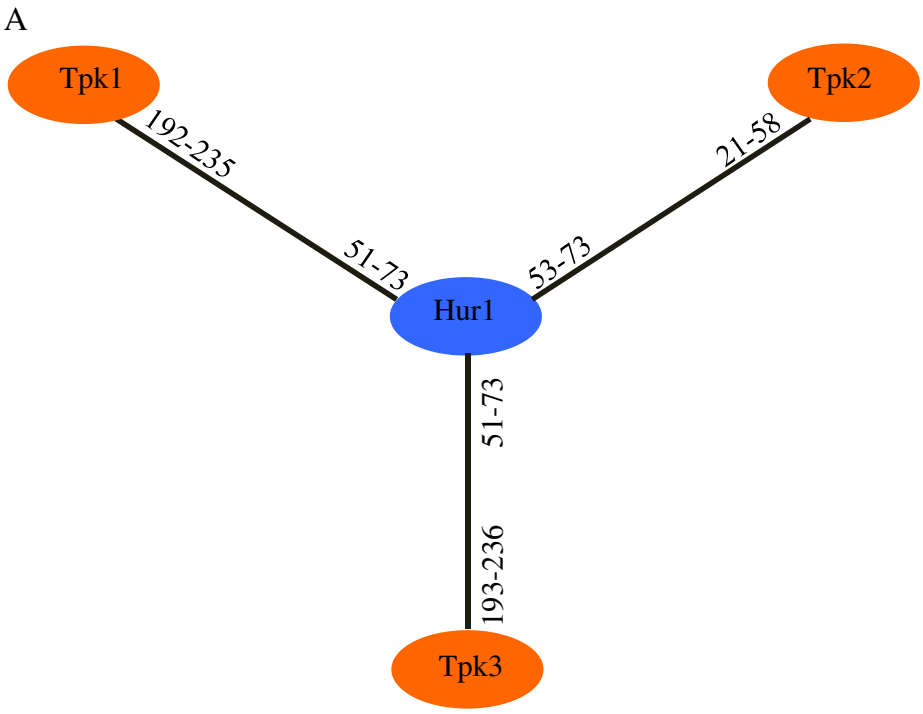
To investigate the activity of Hur1p, the part of *HUR1* (amino acid 1–70) which does not overlap with *PMR1* was deleted forming a partial deletion for the *HUR1* gene (*HUR1-A*). We used a modified Synthetic Genetic Array (SGA) method to explore GIs and networking for *HUR1-A* (Tong et al., 2001; Alamgir et al., 2010). In this way, SGA analysis was performed for *HUR1-A* with two sets of 384 gene deletion strains. One of these arrays contains 384 deletion strains that play a role in DNA damage response, cell cycle progression, checkpoints, DNA replication, and chromatin modifications. The second set contains a collection of 384 random deletion strains, used as a control.

Illustrated in Fig. 1B, we observed that *HUR1-A* deletion negatively interacts with a number of DNA damage repair and cell cycle progression genes including *RAD52*, *RAD18*, *RAD4*, and *BUB1* (Fig. 1B), *RAD52* is a part of HR pathway; *RAD18* is involved in post-replication repair; *RAD4* protein product binds to damaged DNA during nucleotide excision repair; and *BUB1* codes for a checkpoint protein that affects NHEJ. *HUR1-A* did not form negative interactions with key NHEJ genes. This pattern of negative interactions is very similar to those for well-defined NHEJ genes such as *YKU70*, *YKU80*, *DNL4* and *TPK1* (Koh et al., 2010). Negative GIs are often formed between genes involved in parallel pathways but not in the same pathway (Boone et al., 2007). When DNA damage was induced in the presence of sub-inhibitory concentrations of HU (45 mM) and bleomycin (3 μg/ml) a few additional negative GIs were formed. For example, *RTT107* is involved in recruitment of DNA repair complex SMC5/6 to DSBs and *RAD16* participates in nucleotide excision repair pathway.

Another type of genetic interaction is dosage suppression where overexpression of a target gene compensates for a phenotype caused by the deletion of a second gene. This type of phenotypic compensation is termed dosage suppression (Magtanong et al., 2011). Unlike negative GIs explained above, this type of interaction often happens between genes within the same pathway. In the absence of DNA damage drugs, overexpression of *HUR1* did not form any GIs. However, overexpression of *HUR1* compensated for deletion of several key NHEJ genes in the presence of HU or bleomycin. Overexpression of *HUR1* formed GIs with *RAD50* and *XRS2* in the presence of both HU or bleomycin. It also compensates for lack of other NHEJ genes such as *YKU80*, *DNL4* and *LIF1* in the presence of HU (Fig. 1C). These phenotypic suppression interactions are in the agreement with an involvement for *HUR1* in NHEJ pathway.

3.2. Drug sensitivity analysis

It is expected that deletion of genes involved in DNA repair pathways might change the sensitivity of yeast to different DNA damage-inducing drugs (Birrell et al., 2001). To this end, we used bleomycin and HU to study drug sensitivity. Bleomycin uses a free-radical-based mechanism resulting in DSBs, and HU generates DNA replication errors through the depletion of dNTPs that can lead to DSB (Bradley and Kohn, 1979; Rittberg and Wright, 1989; Koç et al., 2004). We observed a mild sensitivity for *Δhur1-A* strain in the presence of 4 μg/ml bleomycin and a more pronounced sensitivity to 60 mM HU (Fig. 2). Jordan et al. (2007) used 100 mM HU to investigate the drug sensitivity of their *Δhur1-A*-like mutant strain and reported no increased sensitivity. They attributed the sensitivity of full length *HUR1* deletion strain to its overlap with *PMR1* gene. Interestingly, when we increased the concentration of HU to 100 mM, we observed that the sensitivity of *Δhur1-A* to HU was reduced. One explanation for this is that in *Δhur1-A* strain, a higher concentration of HU may trigger a compensating pathway that reduces the overall sensitivity to HU. It should also be noted that *Δhur1-A*-like mutant used by Jordan et al. (2007) contained a 27 amino acid deletion region (81 base pair) in contrast to a 70 amino acid deletion



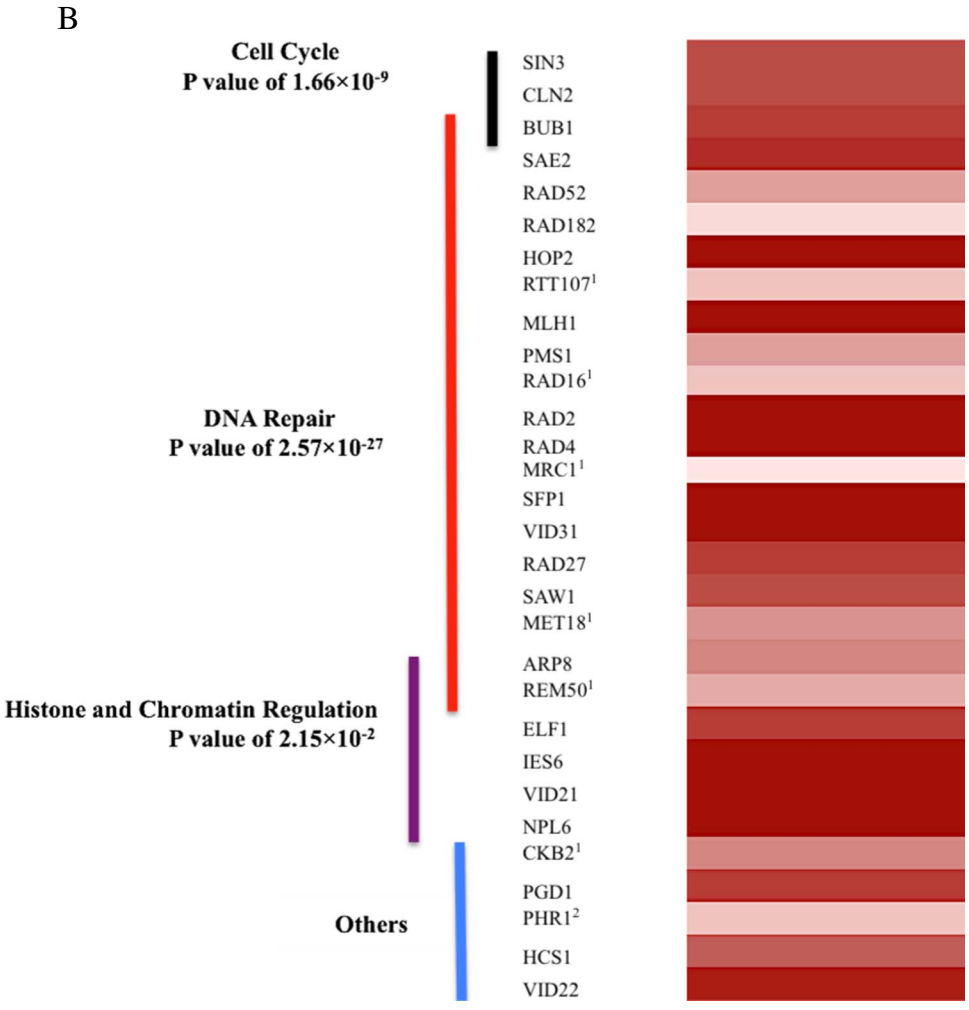


Fig. 1. Protein-protein interaction prediction and genetic interactions analysis for *HUR1*. A) Hur1p was computationally predicted to interact with Tpk1p, Tpk2p and Tpk3p. These interactions were predicted on the basis of the co-occurrence of short polypeptide regions that can mediate interactions. Numbers represent the predicted sites of interactions on the primary sequence of the proteins. B) *HUR1-A* formed negative genetic interactions with a number of genes involved in DNA damage repair pathways. These interactions represent a higher level of functional connection, parallel and compensating, between *HUR1-A* and the selected genes. Conditional negative interactions representing conditional dependency were selected in the presence of 50 mM HU ([1]) and 4 μg/ml bleomycin ([2]). C) Overexpression of *HUR1* compensated for key NHEJ genes in the presence of 50 mM HU (Solid lines) and 4 μg/ml bleomycin (Dash lines). Sensitivity of the identified gene deletion mutant strains were reversed when *HUR1* was overexpressed suggesting a functional correlation between *HUR1* and the selected genes.

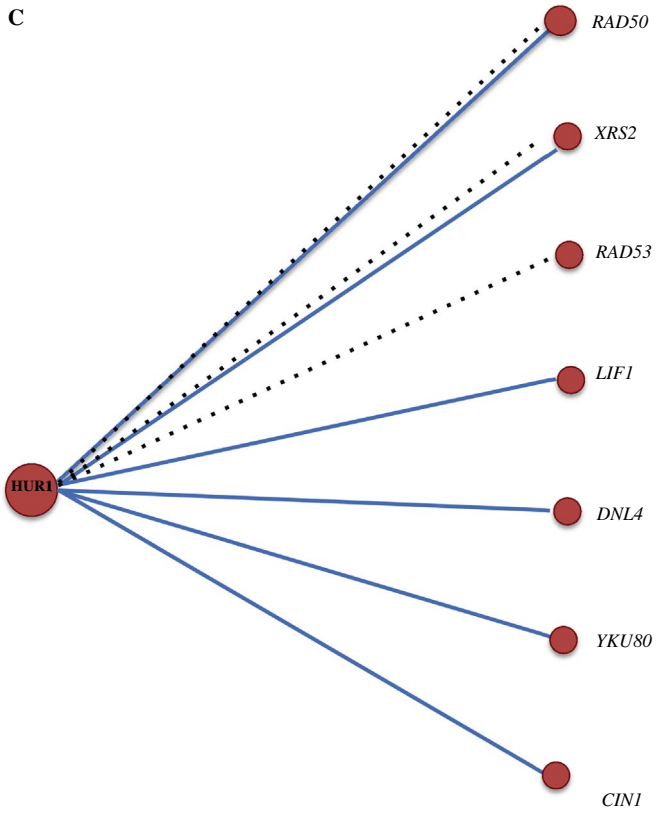


Fig. 1. (*continued*)

plasmid repair assay was utilized (Boulton and Jackson, 1998). This assay has been used to identify novel genes involve in NHEJ pathway (Shim et al., 2005, Jessulat et al., 2008,Yu and Volkert, 2013). Equal amounts of intact and linearized plasmids with overhangs were used to separately transform the wild-type, Δ*hur1-A*, Δ*pmr1-A* and Δ*yku70* strains. Δ*hur1-A* and Δ*pmr1-A* represent partial gene deletions for *HUR1* and *PMR1*, respectively, without compromising the overlap region between the two genes. In this way the influence of each gene on NHEJ can be evaluated independent of the other. In this assay, only the cells that contain circular (repaired) plasmids would form a colony. DNA repair is limited to NHEJ due to the lack of homology between the break site on the plasmid and *S. cerevisiae* genome. The number of colonies formed from transformation with linearized plasmids is related to colonies formed from circular plasmids, and the ratio represents the efficiency of plasmid repair that have occurred. It was observed that Δ*hur1-A* showed approximately 86% reduction in plasmid repair efficiency. In contrast the efficiency of NHEJ was reduced by approximately 18% for Δ*pmr1-A*. The efficiency of NHEJ in Δ*hur1* was similar to Δ*hur1-A* (Fig. 3). This observation suggests that *HUR1* influences the efficiency of NHEJ of plasmid DNA and that this activity seems independent of its overlap with *PMR1* gene.

Next we investigated the repair efficiency of Δ*hur1-A* for DSBs with blunt ends. For this, we tested the transformation efficiency using both YCplac111 and pMV1328 plasmids (Bahmed et al., 2010) cut with Sma1 and *Nru*I, respectively (leaving blunt ends). As before, equal amounts of intact and linearized plasmids with blunt ends were used to transform wild-type, Δ*hur1-A*, and Δ*yku70*. The Δ*hur1-A* strain showed 84% and 69.75% reduction in plasmid repair efficiency for blunt end repair cut with Sma1 and *Nru*I, respectively, suggesting a role for *HUR1* in blunt end DNA repair (Fig. 4A, D). In agreement with previous observations (Boulton and Jackson, 1996) deletion of *YKU70* did not show a reduction in blunt end repair; the reason for this observation is still unclear. Repair of the blunt end DSBs is generally accepted to be independent of YKU activity (Yu and Volkert, 2013). In this way it appears that the influence of *HUR1* on NHEJ might be different from *YKU70*'s activity.

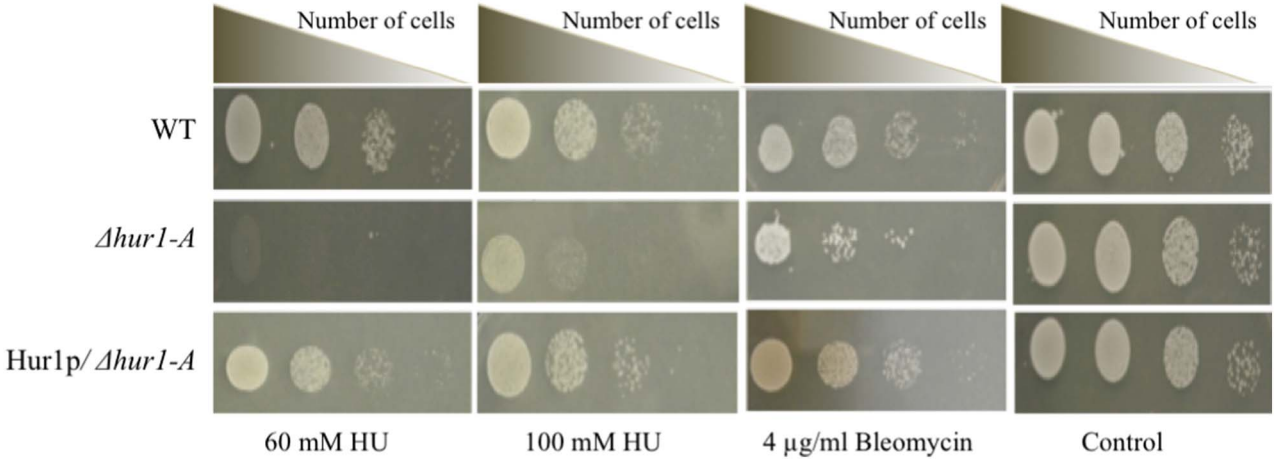


Fig. 2. Drug sensitivity analysis to bleomycin and HU. Single deletion mutant for *HUR1-A* showed increased sensitivity to bleomycin, 4 μg/ml, and HU, 60 mM. The same strain showed less sensitivity to 100 mM HU (compare to 60 mM HU). This might be explained by the activation of an alternative compensating pathway in the presence of 100 mM. *Re*-introduction of *HUR1* into Δ*hur1-A* (Hur1p/Δ*hur1-A*) reversed the sensitive phenotype of the mutant cells.

region used in our Δ*hur1-A* mutant strain. It is possible that the mutated *HUR1* ORF in Jordan et al. might have retained some of its activity. This may explain the observed sensitivity difference between the two studies. To investigate if reintroduction of *HUR1* into its deletion mutant strain would reverse the observed sensitivity, a *HUR1* overexpression plasmid was used. Introduction of plasmid-born Hur1p into the Δ*hur1-A* deletion mutant reversed the drug sensitivities, suggesting that the observed phenotypes was as a result of deletion of *HUR1-A*.

3.3. HUR1 deletion reduced the efficiency of NHEJ in a plasmid based repair assay independent of its overlap with PMR1

To investigate the activity of Hur1p on the efficiency of NHEJ, a

3.4. Plasmid repair analysis of double mutant strains suggest that HUR1-A, TPK1 and NEJ1 function in the same pathway and parallel to YKU70

Single gene deletion mutant strains for *HUR1-A*, *YKU70*, *NEJ1*, and *TPK1* showed significant reduction in NHEJ compare to the WT. Deletion of *HUR1-A* showed NHEJ efficiency of 14.25%, 14.6% and

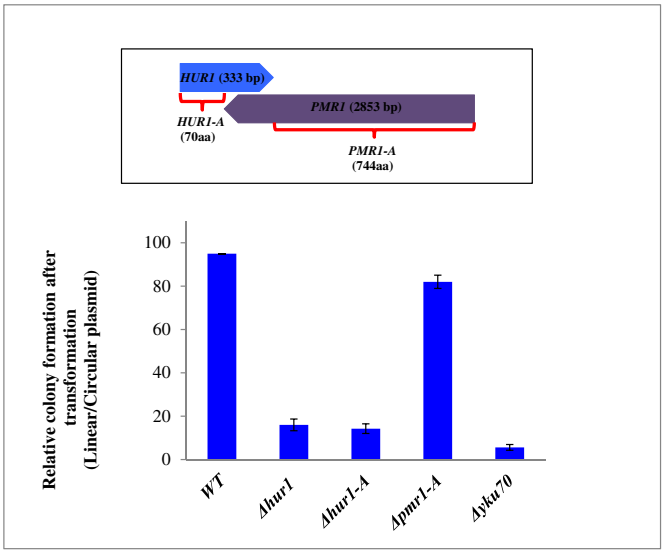


Fig. 3. Plasmid repair assay for *HUR1* and *PMR1* deletion strains. Deletion of *HUR1* reduced the efficiency of repair for cut plasmids that contain overhangs. *Δhur1-A* (with intact *PMR1*) reduced the efficiency of NHEJ similar to *Δhur1* but *Δpmr1-A* (with intact *HUR1*) did not affect the efficiency of NHEJ. These observations suggest that reduced efficiency of NHEJ is associated with *HUR1*. Error bars represent standard deviation. Wild-type values are normalized to 100% and other values are related to this value. *Δyku70* was used as a positive control. Each experiment was repeated at least five times. Inset: Schematic representation of the 181 bp (60 amino acids) overlap between *HUR1* and *PMR1* ORF on Chromosome VII. The arrows represent the direction of transcription for each ORF. The first 70 amino acids of *HUR1* were deleted (*Δhur1-A*) without affecting its overlap with *PMR1*. In *Δpmr1-A* the first 744 amino acids of *PMR1* were deleted without affecting its overlap with *HUR1*. The diagram is not in scale.

19.4% for overhang ends produced by enzymatic digestions with *Xba*I, Pst1 and *Hin*dIII, respectively (Fig. 4). To further investigate the functional relationship between these genes, a plasmid repair assay for double gene deletion strains was carried out. Deletion of two genes in the same pathway within a cellular process generally causes phenotypes similar to deletion of a single gene alone. However, an aggravating change in the phenotype is expected if the two deleted genes work in parallel pathways that can compensate one another. It was observed that strains carrying gene deletions for *HUR1-A* along with *TPK1* (*Δhur1-A/Δtpk1*) or *NEJ1* (*Δhur1-A/Δnej1*) have the same reduced repair efficiency as their corresponding single gene deletion mutants for *HUR1*, *TPK1* and *NEJ1*. Double mutant strains *Δhur1-A/Δtpk1*, and *Δhur1-A/Δnej1* showed NHEJ efficiency of 12.5% and 13.75%, respectively for *Xba*I overhang ends repair (Fig. 4A) and 11.75% and 12%, respectively for Pst1 overhang ends repair (Fig. 4B) suggesting that *HUR1* functions in the same pathway as *TPK1* and *NEJ1*. In contrast, the double mutant strain for *HUR1-A* and *YKU70* (*Δhur1-A/Δyku70*), showed an increase in reduction of repair efficiency compared to single mutants (p-value ≤ 0.5). *Δhur1-A/Δyku70* showed NHEJ efficiency of approximately 2.75% and 3% for XbaI and Pst1 overhang ends, respectively, which are lower than NHEJ efficiency of either *HUR1-A* or *YKU70* single mutants alone (Fig. 4A, B). As indicated, unlike *TPK1* and *NEJ1* (Hooshyar et al., 2017) DSBs with blunt ends are repaired independently of the YKU complex (Boulton and Jackson, 1996; Yu and Volkert, 2013). We observed that *Δyku70* showed < 5% reduction in NHEJ efficiency of blunt ends (Fig. 4C, D). However, similar to above, *Δhur1-A/Δyku70* had a decrease in the repair efficiency by approximately 81%, equivalent to that for *Δhur1*-A (Fig. 4C). Consequently it appears that *HUR1-A* influences blunt end repair, and that this activity, like that for *TPK1* and *NEJ1* (Hooshyar et al., 2017) seems to be independent of YKU complex.

3.5. The effect of Hur1p half on efficient NHEJ is relevant in a chromosomal context

We used the JKM139 strain to further investigate and confirm the effect of *HUR1-A* on the efficiency of NHEJ to repair chromosomal DSBs (Moore and Haber, 1996). In this assay, the target gene is deleted in a JKM139 strain background and the viability of target gene deletion mutant is evaluated after DSB induction by exposure to galactose. JKM139 strain carries a GAL promoter in front of an endonuclease specific to the HO site. The presence of galactose induces the production of this endonuclease and consequently results in chromosomal breakage at the HO sites. There is no homologous region to HO site in this strain limiting the repair to NHEJ. Equal amount of cells for WT, and *Δhur1-A* (JKM139 background) were serially diluted and plated on both galactose and glucose (control) medium to form the colonies. The ratio of comparing the number of colonies in glucose and galactose were used as a measure of survival and were related to the ability of the cell to repair induced DSB (Fig. 5). As expected, *Δhur1-A* had a reduced ability to survive when DSB was induced. This observation confirms the results of the plasmid repair assay in a chromosomal context for *Δhur1-A* gene. To further investigate if the observed inability of *Δhur1-A* is in fact a result of the deletion of *HUR1-A* and not a secondary mutation within the genome, the *HUR1* expression plasmid was reintroduced into *Δhur1-A*. The introduction of this plasmid compensated for the deletion of *HUR1-A* in chromosomal break assay confirming that the observed phenotype was a consequence of *HUR1-A* deletion and not a secondary unwanted mutation. Overexpression of Hur1p alone did not affect the phenotype of a wild-type JKM139 strain.

3.6. HUR1 is involved in homologous recombination repair

To investigate a potential role of *HUR1* in HR, a plasmid-based assay for HR was used (Erdimir et al., 2002). In this assay, plasmid pGV-255-dead is digested with restriction enzyme *Bgl*II within a non-functional *LacZ* gene. Linearized plasmids are co-transformed with PCR products containing functional *LacZ* gene into both wild-type and mutant strains. The ratio of colonies expressing β-galactosidase indicates plasmids acquiring functional *LacZ* through HR, while cells carrying plasmids with non-functional *LacZ* repair plasmids through a re-joining pathway (Jessulat et al., 2008). The deletion mutant for *HUR1* repressed recombination repair, to approximately 45% of wild-type (Fig. 6). HR efficiency was observed at 7.3% for *Δrad52*, used as a positive control. These observations suggest that *HUR1* might also influence the efficiency of HR and that its role in DNA repair may not be limited to NHEJ. In fact, this is in agreement with data from our PPI and GI analysis above that do not exclude the possible involvement of *HUR1* in other DNA repair pathways.

4. Concluding remark

In this study, we use genetic evidence to report a role for functionally uncharacterized ORF *HUR1* in the process of NHEJ in *S. cerevisiae*. We provide evidence linking *HUR1* to NHEJ through its interaction with *TPK1* and *NEJ1* (Fig. 7). In this way, *HUR1* may influence both classic and alternative NHEJ (Fig. 7). Our genetic interaction analyses revealed negative interaction between *HUR1-A* and genes involve in DNA damage repair pathways. Deletion of the region of *HUR1* that has no overlap with *PMR1* (*HUR1-A*) reduced NHEJ efficiency in both chromosomal and plasmid repair assays suggesting that the NHEJ activity observed is related to *HUR1* and seems independent of *PMR1*. Similar to *TPK1* and *NEJ1*, and unlike YKU complex, Hur1p appears to be involved in blunt end DSB repair as well as repair of overhangs (Fig. 7). In agreement with this, double gene deletion mutant strains *Δhur1/Δtpk1*, and *Δhur1/Δnej1* show similar reduction compare to single gene deletion mutant strains. Deletion of *HUR1-A* also reduced the efficiency of HR suggesting the involvement of *HUR1* in other repair

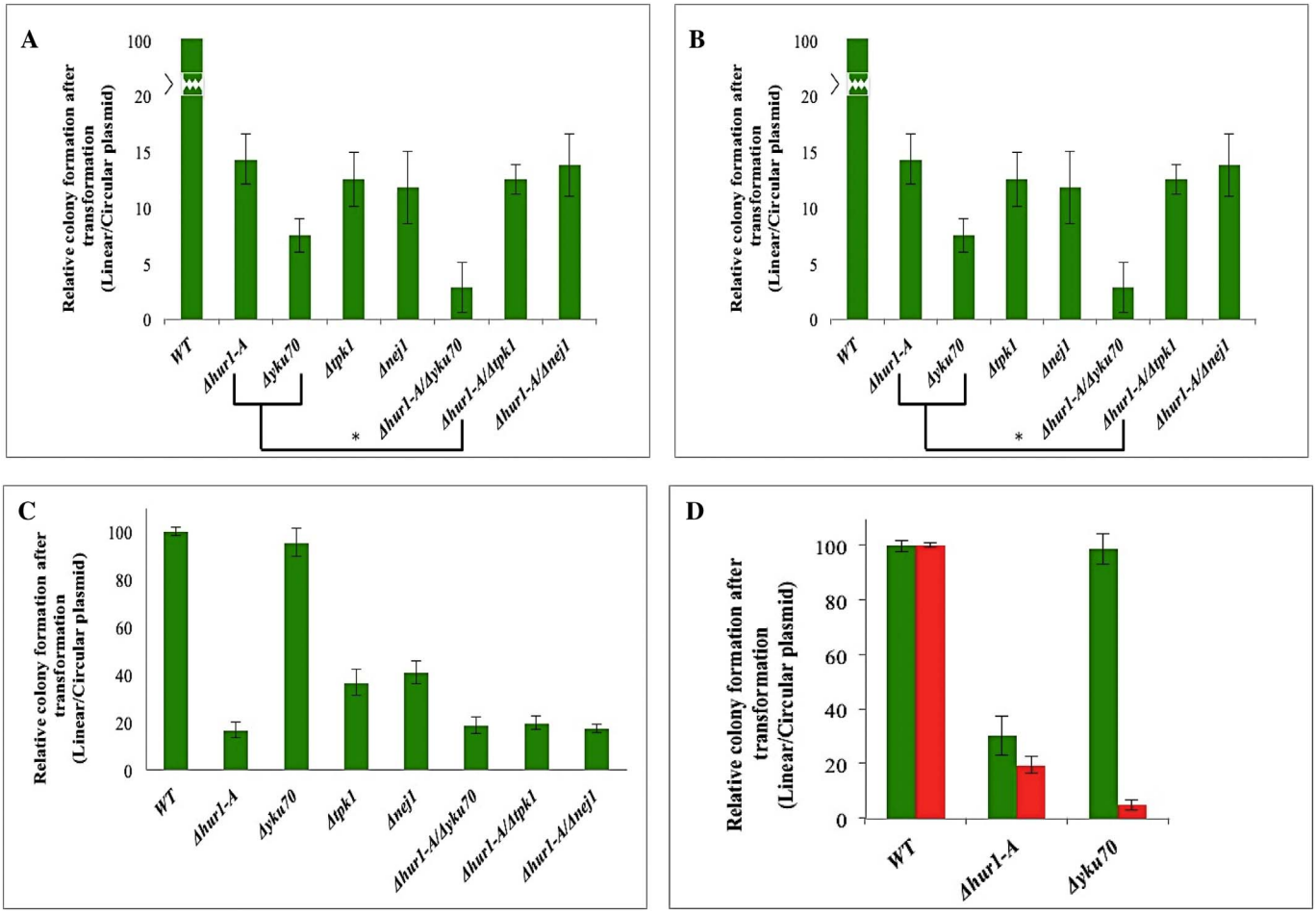


Fig. 4. Plasmid repair efficiency for different yeast strains. *Δhur1-A* showed reduction in NHEJ efficiency for plasmid repair assays where plasmids were digested by (A) *Xba*I that produces 5′ overhangs, (B) Pst1 that produces 3′ overhangs, (C) *Sma*I that produces blunt ends, (D, green) *Nru*I that produces blunt ends, and (D, red) *Hin*dIII that produces 5′ overhangs. *Δyku70* has reduced efficiency when the digested plasmids had overhangs only and not blunt ends. Double gene deletion mutant *Δhur1-A/Δyku70* showed additional reduction compared to single gene deletion mutants *Δhur1-A*and *Δyku70* alone when the digested plasmids carried overhangs (A) and (B) but not blunt ends (C). Double gene deletion mutants *Δhur1-A/Δtpk1* and *Δhur1-A/Δnej1* showed similar reduction compared to corresponding single gene deletion mutants. Altogether these data support that *HUR1* seem to function in the same pathway as *NEJ1* and *TPK1* and in parallel to *YKU70*. Error bars represent standard deviation. Wild-type values are normalized to 100% and other values are related to this value. Each experiment was repeated at least five times. * Statistically significant at p-value ≤ 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

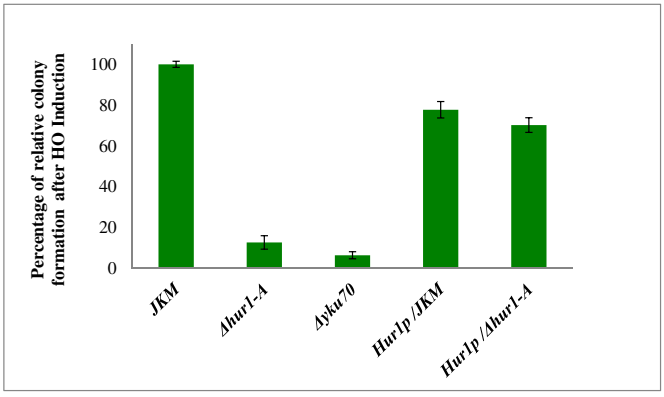


Fig. 5. Repair efficiency of chromosomally induced breaks. Comparing colony formation before and after DSB induction (in the presence of galactose) is used to study the efficiency of DSB in a chromosomal context using JKM139-based strains. In this assay the presence of galactose induces the production of an endonuclease that causes chromosomal breakage at the HO sites in JKM-based yeast strains. Deletion mutant for *HUR1* had a reduced relative colony survival (87.25% reduction). Wild-type values are normalized to 100% and other values are related to this value. Hur1p/JKM was used as a control to investigate possible consequences of Hur1 overexpression. Error bars represent standard deviation. Each experiment was repeated at least five times.

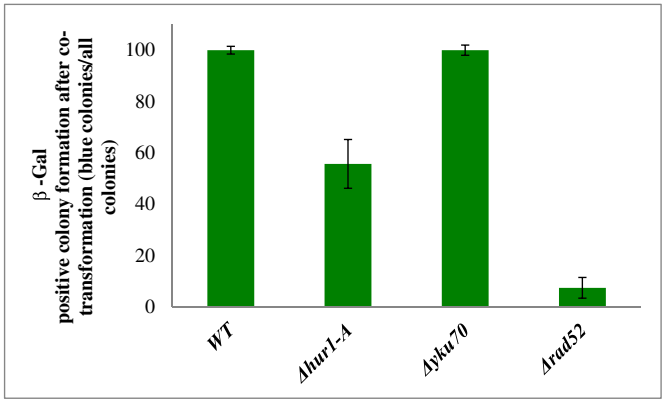


Fig. 6. Plasmid repair efficiency of different yeast strains through homologous recombination. In this assay the plasmids that are repaired through HR contain a functional *LacZ* gene, which is co-transformed into the cells along with digested plasmids that carry non-functional *LacZ* genes. Deletion of *HUR1-A* reduced plasmid repair through HR. *Δhur1-A* shows 45% reduction in HR repair efficiency. Error bars represent standard deviation. Wild-type values are normalized to 100% and other values are related to this value. *Δrad52* was used as a positive control and *Δyku70* was used as a negative control.

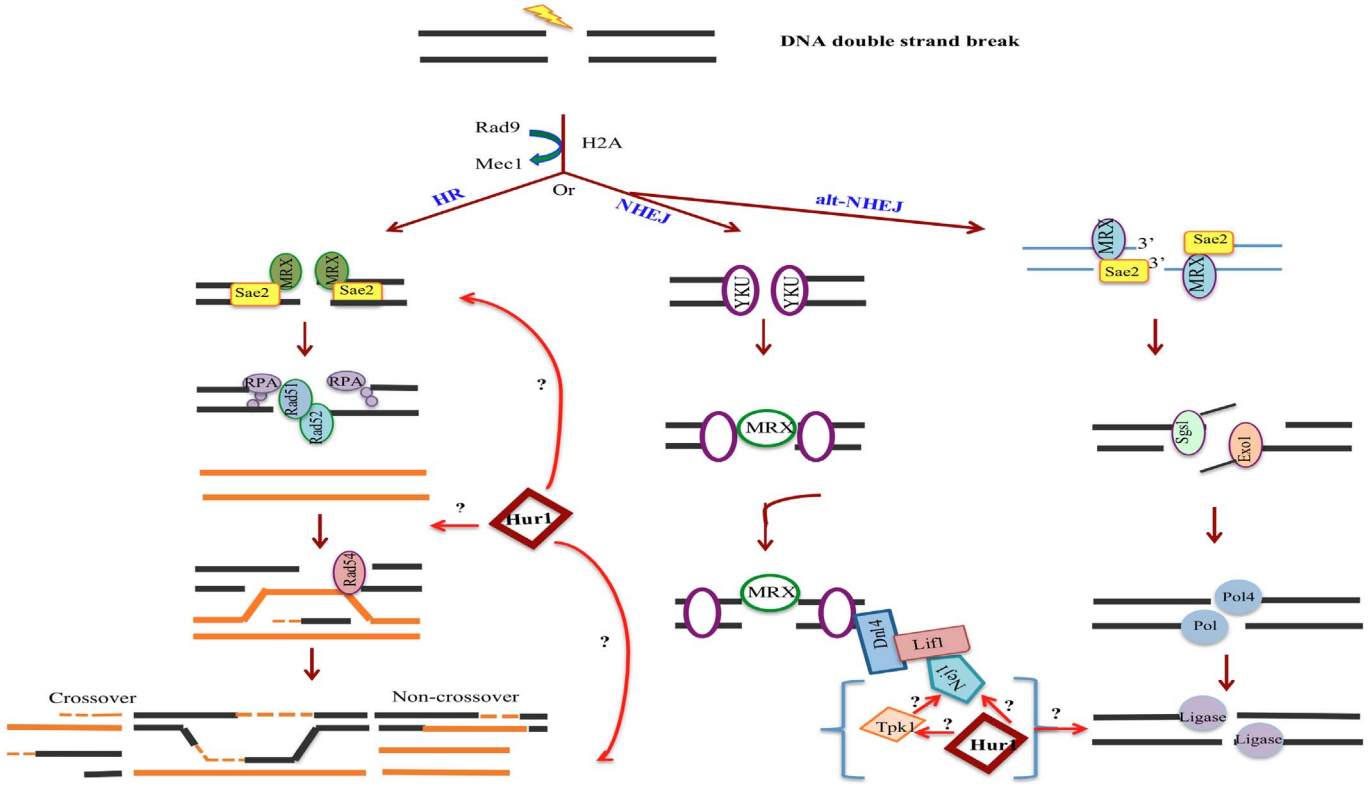


Fig. 7. Double Strand DNA Break (DSB) repair pathways in *S. cerevisiae*. There are three main pathways to repair DSBs. Homologous recombination (HR), Non-homologous end joining (NHEJ), and alternative Non-Homologous end joining (alt-NHEJ). Here, we have suggested the involvement of *HUR1* in NHEJ through its interaction with *TPK1* and *NEJ1*. Its role does not seem to be limited to NHEJ, and *HUR1* might be involved in HR and alt-NHEJ pathways.

pathways including HR (Fig. 7). Additional investigations of *HUR1* in the context of DNA repair would help us further understand the activity of this protein in yeast.

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