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Actin-Related Protein 6 (Arp6) Influences Double-Strand Break Repair in Yeast

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Abstract: DNA double-strand breaks (DSBs) are the most deleterious form of DNA damage and are repaired through non-homologous end-joining (NHEJ) or homologous recombination (HR). Repair initiation, regulation and communication with signaling pathways require several histone-modifying and chromatin-remodeling complexes. In budding yeast, this involves three primary complexes: INO80-C, which is primarily associated with HR, SWR1-C, which promotes NHEJ, and RSC-C, which is involved in both pathways as well as the general DNA damage response. Here we identify *ARP6* as a factor involved in DSB repair through an RSC-C-related pathway. The loss of *ARP6* significantly reduces the NHEJ repair efficiency of linearized plasmids with cohesive ends, impairs the repair of chromosomal breaks, and sensitizes cells to DNA-damaging agents. Genetic interaction analysis indicates that *ARP6*, *MRE11* and RSC-C function within the same pathway, and the overexpression of *ARP6* rescues rsc2Δ and *mre11*Δ sensitivity to DNA-damaging agents. Double mutants of *ARP6*, and members of the INO80 and SWR1 complexes, cause a significant reduction in repair efficiency, suggesting that *ARP6* functions independently of SWR1-C and INO80-C. These findings support a novel role for *ARP6* in DSB repair that is independent of the SWR1 chromatin remodeling complex, through an apparent RSC-C and *MRE11*-associated DNA repair pathway.

Keywords: DNA breaks; DNA end-joining repair; chromatin assembly and disassembly; *Saccharomyces cerevisiae* proteins/genetics

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1. Introduction

DNA double-strand breaks (DSBs) occur when both strands of the phosphodiester backbone are severed, and the integrity of the DNA molecule is compromised. Such breaks are regarded as the most genotoxic form of DNA damage, and can lead to genomic

instability, chromosomal rearrangement, carcinogenicity or cell lethality if ineffectively repaired [1]. Much research has focused on achieving a comprehensive understanding of DSB repair pathways because of their complex nature, biological importance and links to human diseases such as cancer [2,3]. Eukaryotic DSB repair proceeds primarily through one of two distinct pathways: homologous recombination (HR), which uses a homologous template and is considered error-free; or non-homologous end-joining (NHEJ), which directly re-joins the broken ends through an error-prone process.

Budding yeast, *Saccharomyces cerevisiae*, has long served as a model for studying the molecular mechanisms underlying human DSB repair, owing to the highly conserved nature of the core eukaryotic repair machinery [4,5]. Three protein complexes comprise the core yeast NHEJ repair machinery required for initiation, end-bridging/processing, and ligation steps. The YKU complex (Yku70/Yku80) initiates NHEJ by forming a ring over each broken strand, which stabilizes the structure and protects the DNA from degradation. Next, the MRX complex (Mre11/Rad50/Xrs2) brings the DNA ends in close proximity and processes the strands in preparation for rejoining. Finally, the DNA ligase IV complex (Dnl4/Lif1/Nej1) performs ligation by filling in missing nucleotides and joining the broken ends [6]. Before any of these processes can occur, the DSB must be detected, repair factors must be recruited, and adjacent chromatin must be significantly modified to provide access to the break site, while maintaining communication with the DNA damage response.

There is considerable cross-talk that occurs between DNA damage response proteins, cell cycle regulators, chromatin remodeling complexes and histone-related proteins throughout the repair process [7]. DSBs are first detected by sensors, including the RSC (remodels the structure of chromatin) complex and Mre11 [8]. Following detection, the key DNA checkpoint proteins Mec1 and Tel1, orthologs of human ATR and ATM, are recruited to the break site subsequently phosphorylating core histone protein H2A to form γ-H2A [9]. This phosphorylated H2A acts as a docking site for histone modifiers, such as Arp4 and Esa1, and recruits various chromatin remodeling complexes involved in DSB repair. Three complexes are primarily responsible for chromatin remodeling following a DSB event: INO80-C, SWR1-C and RSC-C [9–11].

Following a DSB, INO80-C and SWR1-C are recruited to the site of damage in an Arp4-dependent manner [10]. Each complex promotes unique repair dynamics through its various subunits. INO80-C plays a predominant role in the repair of DSBs at replication forks, and promotes end-resection to induce HR-dependent repair [9]. SWR1-C facilitates the recruitment and binding of the YKU heterodimer to the break site in preparation for NHEJ repair [12]. Both INO80-C and SWR1-C facilitate the damage-induced exchange of H2A with its variant H2A.Z, which relaxes the chromatin structure to provide access for repair proteins [11,13]. In addition to these two key chromatin modifiers, the ~17-member RSC complex plays various roles in DSB repair, including detecting breaks, signaling damage checkpoints, and facilitating nucleosome restructuring [8,14].

As a chromatin remodeling complex, RSC-C mobilizes nucleosomes in response to DSBs, and its activity is correlated with the level of H2A phosphorylation in a dose-dependent manner [15]. RSC-C binds directly to nucleosomes and translocates along DNA ends, relaxing and releasing histones up- and downstream of the break site [15,16]. Members of the RSC complex include Rsc1 and Rsc2, which interact with Yku80 and Mre11 and function in the process of NHEJ repair. The complex also contains two members of the highly conserved actin-related protein (ARP) family, Arp7 and Arp9. Members of the ARP family are common to all three chromatin remodeling complexes. INO80-C contains Arp8, Arp5 and Arp4, SWR1-C contains Arp4 and Arp6, and RSC-C is reported to contain Arp7 and Arp9 [14,17,18].

Arp6 is a lesser-studied member of the nuclear ARP family that has been reported to function both within, and independently of, the SWR1 complex. It has been shown to regulate gene expression, often of ribosomal proteins, by the exchanging of H2A for H2A.Z [19]. Additionally, it has been indirectly implicated as a potential participant in NHEJ because of reported interactions with Swc2, Swc6 and Swc3, evidence that it may

form a sub-complex within SWR1, as well as indications from large-scale analyses of DNA repair proteins; a direct role in DSB repair, however, has not been reported [20,21]. In this report, we investigated Arp6 as a potential participant in DSB repair. Once preliminary testing confirmed an important role for *ARP6* in this pathway, we aimed to develop a general understanding of the role it may play in this process. Here we report a novel function for Arp6 in DSB repair through NHEJ that appears to be independent of its activities within the SWR1-C.

Our findings suggest that Arp6 plays an important role in promoting NHEJ by functioning with RSC-C and Mre11.

2. Materials and Methods

2.1. Yeast Strains and Meterial

The mutant strains used are in a BY4741 background (*MATa orf*Δ*::KanMX4 his*3Δ *leu2*Δ *met15*Δ *ura3*Δ) [22]. *ARP6* was deleted in a Y7092 background (*MATα can1*Δ::STE2pr-*HIS3 lyp11*Δ *ura31*Δ *leu21*Δ *his31*Δ *met151*Δ) for synthetic genetic array (SGA) analysis, and a JKM139 background (*MATa hmr*Δ*::ADE1 hml*Δ*::ADE1 ade1-100 leu2-3,112 lys5 trp1::hisG ura3-52 ade3*::*GAL*-HO) for chromosomal repair and repair fidelity analysis, through a PCR-based transformation in which *ARP6* was replaced by Nat^R [23]. Plasmid repair assays were carried out using p416 containing a *URA3* marker using XbaI digestion. A blunt end repair assay was carried out using YCplac111, with *LEU2* marker, using SmaI digestion. Plasmid pMV1328 carrying a *LEU2* marker and Amp^R was used for the repair fidelity assay [24]. Derivatives of pGEH expression plasmids were used in suppression analysis via galactose induction [25]. Plasmid pGV-255/LIVE and its derivative pGV-256/DEAD were used for the homologous recombination assay [26]. All restriction enzymes were purchased from New England Biolabs Canada. All antibiotics were acquired from Sigma Aldrich Canada.

2.2. Plasmid Repair Assay

Ycplac111 and p416 were single-digested at SmaI and XbaI sites, respectively. These sites lack homology to yeast chromosomal DNA and require NHEJ repair for proliferation. Parallel transformation of digested and intact plasmid was carried out in WT and mutant strains. After three days of growth on synthetic minimal media lacking uracil or leucine, colonies were counted. The ratio of colonies formed by digested transformants to circular transformants in mutant strains, normalized to WT, was used to assess the repair efficiency [27,28].

2.3. Repair Fidelity Assay

Plasmid pMV1328 was digested by PstI within its KanMX6 marker. Digested and circular plasmids were transformed into mutant and WT strains in JKM139 background. Cells were grown on synthetic media lacking leucine for 2–3 days. To test the accuracy of repair, 50–100 transformants (colonies) were grown on YPD media containing G418 (200 µg/mL). Growth on G418 represented accurate repair [29]. Repair fidelity is represented as the ratio of viable cells over total transformants transferred and normalized to the WT ratio.

2.4. DNA Damaging Drug Sensitivity Analysis

Mutant and WT strains from a BY4741 background were grown to saturation in 5 mL of YPD media for 2 days. Saturated cultures were serially diluted at 10^{-1}–10^{-4}, and 15 µL of each dilution was spotted on YPD media containing 3 µg/mL phleomycin (PHLM) or 70 mM hydroxyurea (HU). The viability of each strain was visually examined after 2–3 days of growth at 30 °C.

2.5. Synthetic Genetic Array Analysis of Genetic Interactions

SGA analysis was performed as in [23], and phenotypic suppression analysis (PSA) was performed as in [25]. Strains carrying deletion or overexpression of *ARP6* in Y7092 background were crossed to a DNA damage array containing deletion strains of 384 genes associated with DNA repair, DNA damage response, cell cycle, and chromatin remodeling. The resultant strains were then grown on media containing 30 mM HU and 1.6 µg/mL PHLM, to test for conditional genetic interactions. Results were visually scored. Each experiment was repeated three times and the average results are presented below.

2.6. Chromosomal Repair Assay

A JKM139 background was used to examine the efficiency of repair of chromosomally induced breaks. In this strain, a HO site-specific endonuclease under a *GAL* promoter induces DSBs at the *MAT* locus. *HML* and *HMR*, homologous regions of *MAT*, were deleted to prevent the possibility of HR repair, which allowed the evaluation of NHEJ repair efficiency of chromosomal breaks [28]. WT, mutants, and mutants carrying overexpression plasmids were grown in YPD or selective media to saturation and were then serially diluted to concentrations of 10^{-1} to 10^{-4}. Then, 15 µL of each dilution was spotted on YP-Galactose and YPD. Fitness was visually assessed after 3 days of incubation at 30 °C.

2.7. Homologous Recombination Assay

In this assay, a pGV-256/DEAD plasmid was digested at its non-functional *lacZ* gene with BglII [26]. Separately, a PCR product containing functional *lacZ* was amplified using the pGV-256/LIVE plasmid, to provide a potential template for HR repair. Then, 10 ng of "dead" plasmid and 200 ng of PCR product containing the functional *lacZ* cassette were co-transformed into mutant and WT strains in BY4741 and grown for 2 to 3 days on selective media. A minimum of 50 colonies was transferred to a new plate and grown for 1 day. To measure the frequency of HR repair, a colony-lift assay was performed by transferring colonies onto a nitrocellulose membrane and lysing with liquid nitrogen, prior to incubation in Z-buffer containing X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) at 30 °C for ~4 h [28]. The frequency of blue colonies was normalized to the WT ratio to assess HR event frequency.

3. Results

3.1. Deletion of ARP6 Impairs NHEJ Repair of Linearized Plasmids and Chromosomal Breaks

Because Arp6 has been loosely linked to NHEJ, we first investigated if *ARP6* influences the NHEJ pathway, using a series of DNA repair assays. A classic assay for investigating the role of a gene in NHEJ repair is to investigate the effect of gene deletion on the efficiency of rejoining linearized plasmids [29]. A deficiency in the ability to ligate a digested plasmid that lacks homology to intracellular DNA by a gene mutant is an indication of involvement of the deleted gene in NHEJ repair. This plasmid repair assay can also assess the repair of multiple break structures, including blunt and cohesive ends, depending on the restriction enzyme used [24]. When *ARP6* mutants were presented with breaks containing 5′ cohesive overhangs (XbaI digestion), repair efficiency was reduced to 41% compared to WT (Figure 1A), supporting previous findings, and indicating that *ARP6* may contribute to efficient NHEJ repair.

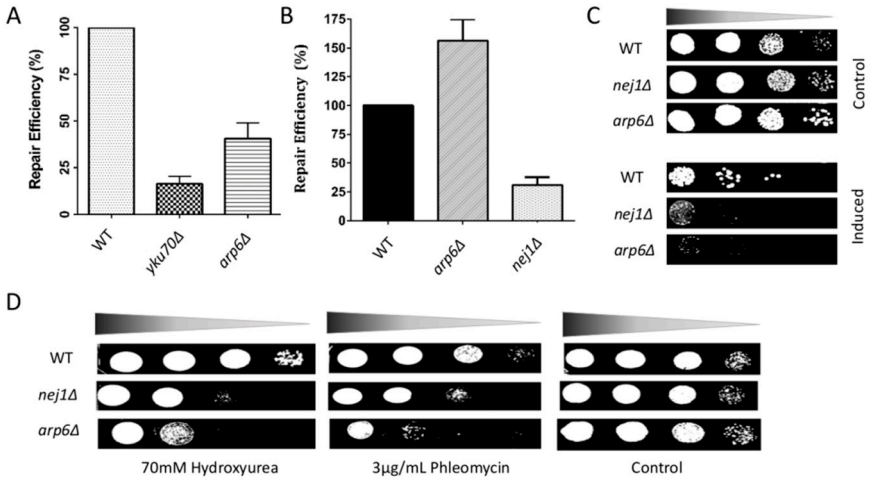

Figure 1. Plasmid repair, chromosomal repair, and drug sensitivity assays support a role for *ARP6* in NHEJ repair. (**A**) Efficiency of repair of a linearized (XbaI digestion) p416 plasmid with cohesive ends via NHEJ is reduced to 40% when *ARP6* is deleted. *yku70*Δ is used as a control. (**B**) Contrarily, when YcPlac111 is digested to produce blunt ends (SmaI digestion), deletion of *ARP6* increases repair efficiency (>60% more efficient than WT). Plasmid repair assays were repeated 5 times, and error bars indicate standard deviation among trials. (**C**) Chromosomal repair assay indicates that the deletion of *ARP6* sensitizes cells to repeated chromosomal breaks. (**D**) Drug sensitivity analysis. Deletion mutants for *ARP6* are sensitive to the DNA-damaging agents phleomycin (3 µg/mL) and hydroxyurea (70 mM). Cells were grown to saturation and serially diluted before spotting on media with and without the drug. *NEJ1* mutants are used for (**B**) as the YKU complex is not believed to be involved in the repair of blunt-end breaks.

We also examined the ability of *arp6*Δ to repair blunt-end breaks. The deletion of *ARP6* enhanced the repair of blunt-end breaks compared to WT, resulting in an efficiency of 155% (Figure 1B). In yeast, blunt-end breaks are repaired through an alternative YKU-independent mechanism [24,30]. This observation suggests a role for *ARP6* in the classic NHEJ repair of breaks with cohesive ends, and not those with blunt ends. The fact that the deletion of *ARP6* increased the efficiency of blunt-end repair merits further investigation of a potential suppressing function for *ARP6* that may regulate the repair of breaks with no overhangs. If *ARP6* functions to promote NHEJ, this activity is likely independent of YKU binding. It is also noteworthy that the deletion of *YKU70* enhances blunt-end repair efficiency, a trend that is not seen with other key NHEJ genes [24,30].

Plasmid DNA and chromosomal DNA molecules differ in their topology, which may lead to variances in repair dynamics [31]. To assess if *ARP6* also influences the repair of severed chromosomes, a chromosomal repair assay was performed. This assay utilized a JKM139 background that possesses a single-loci-specific HO-endonuclease regulated by a *GAL* promoter, which continuously induces DSBs at the *MAT* locus when in the presence of galactose [32]. These breaks must be repaired via NHEJ, as the target sequence is not homologous to other chromosomal regions. A severe reduction in the survivability of *arp6*Δ cells was observed following consistent chromosomal DSB induction (Figure 1C), suggesting that *ARP6* is important in the repair of chromosomal breaks, and further supporting its involvement in NHEJ.

3.2. ARP6 Deletion Is Sensitive to DNA-Damaging Agents

To further analyze if *ARP6* is an important participant in DNA repair activities, we subjected it to drug sensitivity analysis using DNA-damaging agents. The sensitivity of gene mutants can be used as an indicator for involvement in DNA repair [33]. The loss of important DNA repair genes generally, but not necessarily, increases the vulnerability of cells to DNA-damaging drugs [34]. Most drug sensitivity assays involve the induction of

persistent DNA damage stress that invokes a comprehensive damage response involving a range of repair pathways. The damage induced by the agents employed in this study does not exclusively result in DSBs but rather induces stress events that eventually lead to DSBs, in addition to other forms of DNA lesions. Hydroxyurea (HU) depletes the dNTP pool, which eventually results in the collapse of replication machinery and the induction of double-strand breaks at replication forks [35]. Phleomycin (PHLM) is believed to intercalate DNA directly and promote the production of free radicals that react to form a variety of DNA lesions, including DSBs [36]. As is consistent with the involvement of *ARP6* in DNA repair, cells with deletions of *ARP6* were sensitive to PHLM (3 μg/mL) and HU (70 mM) (Figure 1D).

3.3. The Loss of ARP6 Increases NHEJ Accuracy

NHEJ is considered to be an error-prone pathway that can introduce mutations within the repaired region of the DNA [37]. However, the genetic composition of repair factors can significantly influence the error rate of NHEJ repair [38]. We examined the role of *ARP6* in facilitating accurate NHEJ repair by subjecting *arp6*Δ to a repair fidelity assay.

In this dual-selection assay, a plasmid digested within its KanMX6 cassette is used to indicate the rate of accurate NHEJ repair, based on the ability to grow on G418. Accurate repair, indicated by functional KanMX6, was observed 172% more often in *arp6*Δ mutants compared to WT (Figure 2). The efficiency of repair of the same plasmid was reduced by 82% in *arp6*Δ cells, which is consistent with our previous finding that *ARP6* impairs the repair efficiency of cohesive ends. This suggests that even though the deletion of *ARP6* causes defects in the efficiency of the repair process, NHEJ proceeds through an alternative mechanism with very high accuracy. Furthermore, these results indicate that *ARP6* may affect the balance between the efficiency and accuracy of repair, suggesting its function may be in a regulatory capacity independent of the core repair machinery. Several proteins can influence NHEJ fidelity, but the most important factor in fidelity appears to be the MRX complex. MRX influences multiple pathways that promote the timely dissociation of YKU, an event shown to lead to accurate repair [39].

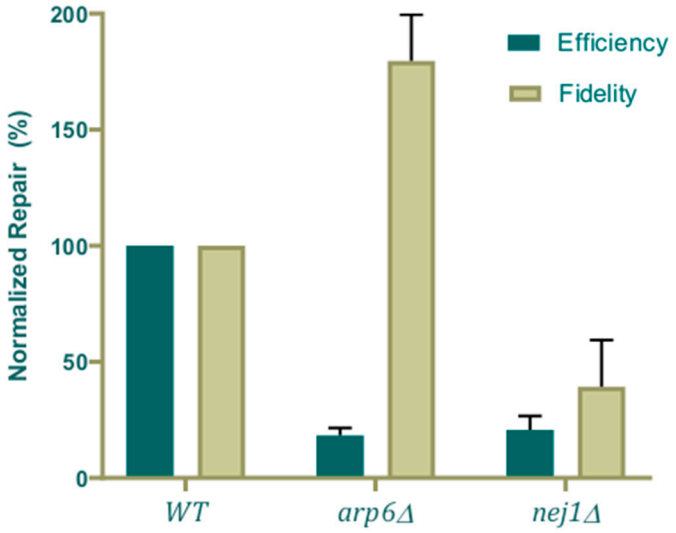

Figure 2. Loss of *ARP6* results in less efficient but more accurate repair. *Arp6*Δ decreases the efficiency of the NHEJ repair of linearized plasmid pMV1328 (18%) and drastically increases the accuracy of repair by 172%. A dual-selection plasmid repair assay was repeated five times and error bars indicate standard deviation among trials.

This finding of increased repair fidelity may help explain the severe sensitivity of *arp6*Δ found in our chromosomal repair analysis, where cells were unable to recover from the repeated induction of chromosomal DSBs (Figure 1C). Fidelity of repair plays an important

role in this assay, as mutations in the recognition site cause the cells to become resistant to further assault by HO-endonucleases. Consequently, the extreme phenotype observed for *ARP6* deletion mutants could be explained by a decrease in repair efficiency combined with an increase in repair fidelity, which would leave break sites prone to increased rounds of continual assault.

3.4. Interaction Analysis Suggests a Role with DNA Repair and Chromatin Remodeling

To better understand how *ARP6* functions within DNA repair, we examined how it interacts with other known DNA repair genes using a variety of genetic interaction (GI) and conditional GI analyses. GI network analysis is used in functional genomics to investigate novel gene functions and/or to elucidate the role of genes in various cellular pathways [40]. Furthermore, conditional GI analysis can also be employed to identify interactions that only occur under controlled stress conditions, such as interactions that only occur under DNA damage stress [41]. Specifically, negative interactions resulting from the deletion of two genes can be identified as a synthetic sick or lethal phenotype and may suggest that the target genes function in compensating, and often parallel, pathways that perform similar functions [25,40].

We performed synthetic genetic array (SGA) analysis and phenotypic suppression analysis (PSA) on *ARP6* using a DNA damage array containing 384 genes implicated in DNA repair, DNA damage response and other related pathways. SGA analysis involved the systematic crossing of an *arp6*Δ single deletion strain with an array of single mutants, to produce double mutants [23]. Our analysis showed that *ARP6* has negative genetic interactions with several NHEJ genes, namely *RAD50*, *RTT109* and *RAD27* (Figure 3). In addition to these NHEJ genes, *ARP6* also interacts with key genes involved in the DNA damage response, *ARP8*, *RAD53* and *DDC1*, mismatch repair, *MLH1* and *MLH2*, and post-replication repair, *RAD18*. Interestingly, under DNA-damaging conditions, negative genetic interactions were observed between *ARP6* and the key HR genes *RAD55* and *RAD52*, suggesting a DNA damage-induced functional relationship with HR genes.

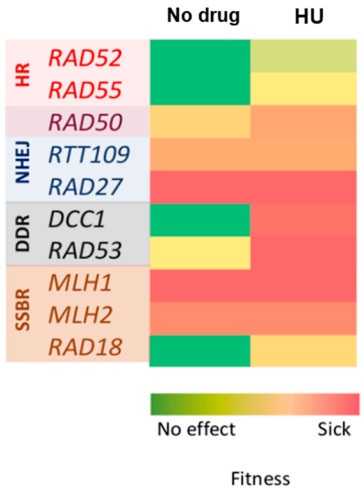

Figure 3. Genetic interaction profile for *ARP6*. Genetic interactions of *ARP6* include genes from various DNA repair pathways. SGA analysis of double mutants of *ARP6* and various genes involved in the DNA damage response, DNA repair pathways, and chromatin remodeling were analyzed under drug-free conditions and under 30 mM hydroxyurea.

The GI network of *ARP6*, which included genes involved in HR, NHEJ, and the DNA damage response, hints at a potential role for *ARP6*, upstream of NHEJ repair machinery.

SGA analysis suggested a role for *ARP6* in various DNA repair pathways, a result that is not unexpected if Arp6 is involved in repair-related chromatin remodeling of DNA damage signaling. Additionally, the analysis of known Arp6 protein–protein interactions indicated significant enrichment of the proteins associated with both SWR1-C and INO80

(Figure S1) and (Table S1). To further hone in on the specific function of *ARP6*, PSA was performed. PSA involves overexpressing one gene in a gene deletion background under stress conditions, to examine if the overexpressed gene can "rescue" the sensitive phenotype of the deletion mutant [42]. Phenotypic rescue is often observed in genes that perform similar functions within a pathway [43]. For example, the overexpression of *PPH3* or *PSY2* was previously shown to compensate the sensitivity of *CHK1* deletion to DNA-damaging conditions [27]. To this end, we introduced conditionally expressed *ARP6* overexpression plasmids into the 384 single-gene deletion mutants in the DNA damage array. Under DNA damage induction using PHLM and HU, *ARP6* overexpression compensated for sick phenotypes of four interesting gene deletion strains, which helped suggest a possible function of NHEJ.

The overexpression of *ARP6* rescued four deletion mutants that were sensitive to DNA-damaging drugs (Table S2). These genes included *MMS22*, a stabilizer of replication forks, *MRE11*, a key NHEJ and DNA damage-response gene, *RSC2*, a key component of the RSC-C chromatin remodeling complex, and *NUP84*, a member of the nuclear pore complex involved in translocating chromosomes to the nuclear periphery for DSB repair. Both *NUP84* and *MMS22* are associated with key DSB repair-related chromatin remodeling proteins. For example, *NUP84* is known to genetically interact with both *ARP6* and *ARP8* [44], while *MMS22* has known physical interactions with three members of the RSC complex, Rsc8, Rsc9 and Arp9 [45]. These results further support the implication of *ARP6* in the repair pathway upstream of DSB repair and suggest a possible connection between *ARP6*, *MRE11* and the RSC-C chromatin complex.

3.5. Studying ARP6 Function in Relation to SWR1-C, INO80-C and RSC-C

Following a DSB, regulation of repair is largely controlled by H2A.Z (coded by HTZ1), a variant of H2A which is incorporated at the break site [12]. The recruitment of various chromatin remodeling complexes following these events can direct repair dynamics. SWR1-C promotes NHEJ and is required for response to DNA damage and the efficient binding of YKU. Additionally, INO80-C, which promotes HR, and RSC-C, which is involved in both HR and NHEJ, are also involved in chromatin remodeling following a DSB. To study the role of *ARP6* in NHEJ, we examined its functional relationship with members of SWR1-C, INO80-C and RSC-C chromatin remodeling complexes, in addition to Htz1. This was achieved by analyzing the double deletion mutants of *ARP6* and key members of each of the three relevant complexes.

Since *ARP6* has been reported to be a part of SWR1-C, we first investigated the functional relation between *ARP6* and two members of SWR1-C, *SWC2* and *SWR1* in the re-joining of linearized plasmids (Figure 4B). The double deletion mutants *arp6*Δ/swr1Δ and *arp6*Δ/swc2Δ were subjected to the plasmid repair assay, and compared to single mutants of the relevant strains, to determine if genetic interactions were occurring. The premise of this experiment is that if two genes participate in the same pathway, their double deletion should not produce a phenotype significantly different from the two single gene deletions. However, if the genes affect the same process via different pathways, a combinatory effect should be observed, where the double deletion causes a more severe effect than the individual gene deletions.

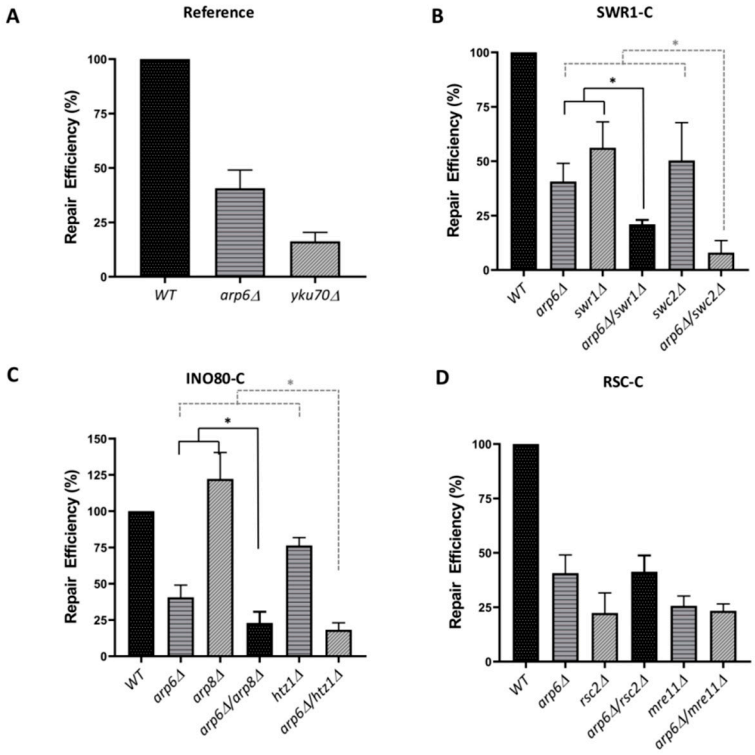

Figure 4. Genetic interaction analysis suggests *ARP6* is functionally related to *RSC2* and *MRE11* in the context of NHEJ repair. (**A**–**D**) Plasmid repair analysis of linearized p416 with overhangs. Single and double mutant repair efficiencies are used to analyze functional genetic relationships. Comparison of repair efficiencies of *ARP6* single mutants to the repair efficiencies of double mutants of genes involved in the *SWR1* (**B**), *INO80* (**C**), and *RSC* (**D**) complexes was performed using one-way ANOVA analysis (Dunnett's multiple comparison test) post hoc. There is no significant reduction in double mutants of genes in the RSC-C complex (*MRE11* and *RSC2*), indicating that *ARP6* function in NHEJ is related to the role of RSC-C (**D**). * represents statistically significant differences, p value < 0.05.

The deletion of SWC2 reduced the repair efficiency to 50%, roughly comparable to the 41% efficiency observed in the *arp6*Δ background. However, the double deletion of *ARP6* and SWC2 (*arp6*Δ/swc2Δ) significantly impairs the repair process and produced a synergistic effect that resulted in only 8% repair efficiency compared to WT, suggesting their involvement in additive pathways (Figure 4B). In the case of SWR1 single deletion, repair efficiency was calculated at 56%, while double deletion of *ARP6* and *SWR1* (*arp6*Δ/swr1Δ) further reduced the repair efficiency to 20%. These data showed the additive influence of *ARP6* with *SWR1* and *SWC2*, suggesting that during NHEJ repair, *ARP6* likely functions in a parallel pathway to SWR1-C. This functional relationship suggests that *ARP6*'s cumulative influence on NHEJ appears independent of the SWR1 complex.

Next, we examined whether the role that *ARP6* plays in NHEJ is related to the functioning of the INO80 complex or generalized chromatin remodeling following DNA damage via HTZ1 (Figure 4C). *ARP8* is an important component of INO80-C, and arp8Δ mutants have been used to represent *INO80* disruption for phenotypic analysis [9]. We investigated the repair efficiency of double mutants of *ARP6* with *ARP8* and *HTZ1*. Our results show that, although the deletion of *ARP8* did not reduce the efficiency of NHEJ repair compared to WT, the double deletion of *ARP8* and *ARP6* lowered the repair efficiency to 23%, whereas the *ARP6* single deletion displayed 41% efficiency (Figure 4C). Furthermore, HTZ1 single mutants show a moderate reduction in repair (76%), while the double deletion of *ARP6* and *HTZ1* significantly lowered the repair efficiency to 18%. Together, these observations suggest that *ARP6*'s role in NHEJ is independent of the INO80-C and *HTZ1* pathways.

We then investigated the role of *ARP6* in association with RSC-C. In addition to its traditional role in transcription regulation, RSC-C functions as an early sensor of DSBs, an activator of the DNA damage response, and an ATP-dependent chromatin remodeler in both NHEJ and HR [8]. RSC-C responds rapidly to DSBs, and recruits key DNA damage response proteins Tel1, Mec1 and Rad9. It also interacts physically with Mre11, the earliest sensor of DSBs. RSC-C's function in DSB appears to primarily be involved with the earliest stages of repair.

Normalized repair efficiency of rsc2Δ, a key component of the RSC-C complex was 22%, which is not significantly different from the repair efficiencies of *arp6*Δ and *arp6*Δ/rsc2Δ which are both 41% (Figure 4D). This type of non-additive phenotype is often indicative of genes that encode members of the same non-essential pathways [40]. The genetic relationship between *ARP6* and *RSC2*/*MRE11* suggests that the role of *ARP6* in NHEJ may be related to RSC-C activities.

Furthermore, *mre11*Δ and *arp6*Δ/*mre11*Δ showed comparable repair efficiencies at 26% and 23%, respectively (Figure 4D), suggesting that *ARP6* and *MRE11* function within the same pathway [40]. The deletion of *ARP6* did not seem to have an additive effect on the reduced efficiency of NHEJ, caused by the deletion of *RSC2* or *MRE11*. The NHEJ repair activity of *ARP6* appears to be functionally associated with *MRE11* and *RSC2*. Together, these results suggest possible functions at the early stages of damage sensing, repair factor recruitment or early chromatin remodeling.

3.6. Further Conditional Genetic Evidence for the Association of ARP6 with RSC-C2 and MRE11

To further study the functional association between *ARP6* and the RSC-C pathway, we investigated the drug sensitivity profiles of single and double mutants. The deletion of either *RSC2* or *MRE11* causes severe growth defects when exposed to HU or PHLM, but *arp6*Δ mutants are only moderately sensitive to both drugs (Figure S2). The double mutants *arp6*Δ/rsc2Δ and *arp6*Δ/mre11Δ rescue this sensitivity and increase fitness through an apparent phenotypic suppression that results in cells that phenocopy the WT. In this case, the deletion of a second gene (*RSC2* or *MRE11*) relieves the cell requirement for the presence of another compensatory gene, resulting in a positive genetic interaction (suppression) [46]. This asymmetric positive interaction provides further evidence that *ARP6* and *MRE11*/*RSC2* perform related functions in response to severe DNA damage that are not limited to NHEJ [46].

3.7. ARP6 Severely Reduces the Efficiency of Homologous Recombination

RSC-C and *MRE11* participate in the earliest stages of DSB repair, including break detection and damage signaling that can induce either NHEJ or HR repair. Because both RSC-C and *MRE11* participate in both pathways, it is important to understand how *ARP6* affects HR efficiency. To assess this, *ARP6* mutants were subjected to a homologous recombination assay that is a variation of the previously described plasmid repair assay, but the vector used is digested within a non-functional *lacZ* gene. Linearized plasmids are co-transformed with an excess amount of a PCR-amplified linear cassette encoding a functional *lacZ* into both WT and mutant strains [26]. Cells that have integrated the cassette via HR will produce a blue color in the presence of x-gal solution. Repair through NHEJ is indicated by a colony remaining white in color [28]. The relative frequency of HR or NHEJ is reflected by the ratio of blue or white colonies/total colonies, normalized to the WT frequency.

The deletion of *ARP6* severely depressed the recombination repair rate, with only 12% of repair events occurring via HR, compared to the WT ratio (Figure 5). The efficiency of HR in *arp6*Δ is even lower than positive control *rad52*Δ, in which HR accounts for 24% of repair events. Here, we show that the deletion of *ARP6* decreases the efficiency with which DSBs are repaired, whether that repair be through NHEJ or HR. If *ARP6* function in repair is strictly related to its role within SWR1-C, one might expect that the loss of *ARP6* would lead to increased rates of HR. SWR1-C promotes the binding of YKU, thereby facilitating

NHEJ, so, if *arp6*Δ causes no other effects other than decreased SWR1-C functionality, NHEJ should be impaired [9]. However, the results in Figure 5 indicate that *arp6*Δ mutants promote NHEJ and/or are severely deficient in HR repair, which again supports our claim that *ARP6* functions in DSB repair independently of SWR1-C, or in addition to its role within that complex.

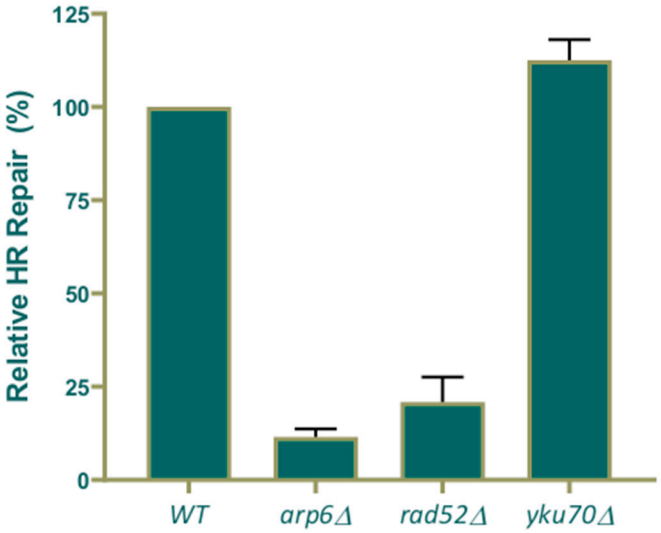

Figure 5. The deletion of *ARP6* pushes DSB repair toward the NHEJ pathway. A plasmid-based assay evaluates repair pathway choice for single mutants compared to WT. HR efficiency is presented as the ratio of blue/total colonies, normalized to the blue/total ratio of the WT +/− SD.

4. Discussion

The evidence presented herein indicates a novel function for *ARP6* in promoting accurate NHEJ repair through the RSC-C complex and *MRE11*. Members of the ARP family have general sequence homology but fulfill vastly different functions within the cell. In yeast, the ARP family is comprised of 10 members, including cytoplasmic proteins Arp1, Arp2, Arp3, and Arp10, and nuclear proteins Arp4, Arp5, Arp6, Arp7, Arp8, and Arp9 [47]. Some ARP proteins, such as Arp8 and Arp5, are involved in the DNA repair process [48]. Arp4 is an essential protein required for chromatin remodeling at DSB sites [49]. Arp6 is a lesser-studied member of the ARP family, and a specific role for Arp6 in DNA repair has not been reported. However, certain evidence has indirectly implicated it as a potential participant in DSB repair. such as sensitivity to methyl methanesulfonate (MMS), and a reported increase in recombination frequency when *ARP6* is deleted [47].

Kawishma et al., 2007 reported that the deletion of *ARP6* inhibits the function of the SWR1 complex and has no effect on HR efficiency, but causes minor increases in the frequency of spontaneous and MMS-induced unequal sister chromatid recombination events. This increase in recombination frequency was attributed to a dysregulation of the H2A↔H2A.Z exchange that was proposed to lead to an increased frequency of exchange events. However, an alternative explanation could be that the loss of *ARP6* promotes NHEJ that may be mutagenic, and lead to an increased rate of secondary HR events, and our study provides some preliminary evidence to support this hypothesis.

Here we report a novel role for *ARP6* in NHEJ linked to RSC-C activities through its genetic association with *RSC2*. This result is not surprising, since *ARP6* has been reported to function independently of SWR1-C in other processes, such as transcription regulation [19]. Arp6 may function alone, with SWR1-C, or, as reported here, in a role related to RSC-C function.

We also report that *ARP6*'*s* role in NHEJ may be independent of INO80-C, despite physical interconnectivity with the complex (Figure S1), whose role in DSB repair is primarily in HR through the recruitment of RPA and Rad51 in an MRX-dependent manner [50].

Instead, we observe *ARP6* functions related to the RSC-C complex and *MRE11*, important DSB first-responders required for progression of the DNA damage response. Rsc2 is important for the recruitment of Tel1 and Mec1 to the site of damage, and activation of Rad53 in the DNA damage checkpoint pathway [8]. The observed increase in repair fidelity (Figure 3) in *ARP6* mutants may be related to the activity of MRX, potentially via MRX endonuclease activity, and the activation of Tel1, which are important for facilitating accurate repair [39]. The deletion of *RSC2* reduces the efficiency of NHEJ repair, possibly due to its physical interaction with Mre11 and Yku70 [8,15,51], and this activity may also be influenced by the presence/absence of Arp6. Additionally, the loss of *ARP6* leads to impaired HR (Figure 5), again suggesting that *ARP6*′*s* role in resolving breaks is likely not involved in the core NHEJ repair machinery but instead is functioning in the early stages of DSB repair.

In conclusion, NHEJ is an expansive and multifaceted process, and it is important to identify all genes involved, from damage sensing to repair and return to the cell cycle. This was the premise for our project, which aimed to identify novel NHEJ repair genes. Molecular mechanisms underlying NHEJ in yeast continue to be used to understand how the pathway functions in human cells [15,52]. We report that *ARP6* is in fact a participant in NHEJ through *MRE11* and RSC-C complex activity. Additionally, this novel repair function is independent of SWR1-C activity in response to DNA damage. Additional biochemical investigations are needed to fully elucidate the association of Arp6 and Rsc2/Mre11 in the context of DNA repair. It is also interesting to examine whether the role of Arp6 in DNA repair is conserved in other organisms, such as humans.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/applmicrobiol1020017/s1, Figure S1: Protein-protein interaction network of Arp6, Table S1: Functional gene ontology (GO) analysis of proteins within the Arp6 physical interaction network, Table S2: *ARP6* phenotypic suppression analysis, Figure S2: Conditional chemical-genetic analysis indicates a functional relationship between *ARP6* and *MRE11*/*RSC2*.

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