Title

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Authors

Piazza, Aurèle
Rajput, Pallavi
Heyer, Wolf-Dietrich

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Physical and genetic assays for the study of DNA joint molecules metabolism and multi-invasion-induced rearrangements in *S. cerevisiae*

Aurèle Piazza[1,2], **Pallavi Rajput**[2], **Wolf-Dietrich Heyer**[2,3,*]

[1]Spatial Regulation of Genomes, Institut Pasteur, UMR3525 CNRS, 25-28 Rue du Docteur Roux, 75015 Paris, France.

[2]Department of Microbiology and Molecular Genetics, University of California, Davis, CA 95616, USA

[3]Department of Molecular and Cellular Biology, One Shields Ave, University of California, Davis, CA 95616, USA

Abstract

DNA double-strand breaks (DSBs) are genotoxic lesions that can be repaired in a templated fashion by homologous recombination (HR). HR is a complex pathway that involves the formation of DNA joint molecules (JMs) containing heteroduplex DNA. Various types of JMs are formed throughout the pathway, including Displacement loops (D-loops), multi-invasions (MI), and double Holliday Junction intermediates. Dysregulation of JM metabolism in various mutant contexts revealed the propensity of HR to generate repeat-mediated chromosomal rearrangements. Specifically, we recently identified MI-Induced Rearrangements (MIR), a tripartite recombination mechanism initiated by one end of a DSB that exploits repeated regions to generate rearrangements between intact chromosomes. MIR occurs upon JM processing by endonucleases and is suppressed by JM disruption activities. Here, we detail two assays: a physical assay for DNA joint molecule detection in *S. cerevisiae* cells and a genetic assay to determine the frequency of MIR. These assays enable studying the regulation of the HR pathway and the consequences for genomic instability by MIR.

Keywords

Homologous recombination; D-Loop; multi-invasion-induced rearrangement; MIR; DSB repair; genomic instability

1. INTRODUCTION

Homologous recombination (HR) repairs DNA double-strand breaks by using the sequence information present on an independent homologous dsDNA molecule. This mechanism entails the formation of DNA joint molecules (JMs) that contains a heteroduplex DNA

*Corresponding author. wdheyer@ucdavis.edu Phone: +1 (530) 752-3001.

(hDNA) region between the broken molecule and its complement in the "donor" duplex DNA, such as the in the Displacement-loop (D-loop) intermediate (Fig. 1A). Regulation of the formation and stability of these JMs is paramount for the progression and outcome of the HR pathway, and limits its propensity to generate chromosomal rearrangements between repeated sequences (Piazza and Heyer, 2019). In particular, the homology search process is at risk of encountering homology and forming hDNA on independent molecules. We recently provided physical evidence for such Multi-invasions (MI) JMs *in vitro* and in *S. cerevisiae* cells (Fig. 1B) (Piazza et al., 2017; Wright and Heyer, 2014). These MI-JMs are at the basis of a tripartite chromosomal rearrangement mechanism called MI-induced rearrangement (MIR) in which the two donors are translocated when bridged by an invading ssDNA (Fig. 1B) (Piazza and Heyer, 2018; Piazza et al., 2017). We detail here assays allowing to study JM metabolism and its consequences for genomic instability by MIR in *S. cerevisiae*.

Rationale of the genetic MIR assay.

The genetic MIR assay quantifies the formation of a translocation between two undamaged genomic regions upon induction of a single site-specific DSB in a third, ectopic genomic region (Fig. 2A). The translocation restores a functional *LYS2* gene, providing a mean for selection and determination of its frequency in a cell population. The DSB-inducible construct must have homology to both donors on one side of the DSB (*e.g.* YS; Fig. 2A). The two donors do not share homology to one another (LY and S2; Fig. 2A). The various genetic constructs are described in section 2.1, as illustrated in Fig. 2B, and the precise annotated sequences are available in Dataset S1. The expression of the *HO* gene under the *GAL1/10* promoter is induced upon galactose addition in exponentially growing cells in a media lacking glucose (YEP-Lactate) (Fig. 3A). This induction results in rapid DSB formation in the cell population (~99% in 1 hr, Fig. 3B). Cells are thus plated prior to, and 2 hr post-DSB induction (Fig. 3A), enabling determination of the basal and the induced Lys^+ (*i.e.* MIR translocation) frequencies, respectively (Fig. 3C). The 2 hr time point was selected because it corresponds to a time at which DSB repair using the undamaged chromosome V (chrV) has not yet happened (Fig. 3B). This repair presumably accounts for the gradual decline of the Lys^+ frequency over time (Fig. 3D), as cells repairing the DSB without undergoing MIR will resume growth more quickly, leading to a relative dilution of those undergoing MIR. This early plating facilitates comparison of MIR frequencies in various mutants, as they will not be affected by additional changes in the DSB repair kinetics.

Rationale of the physical JM-Capture assay.

The Joint Molecule-Capture (JM-Capture) assay is a proximity ligation-based methodology (Dekker et al., 2002) for physical JM detection (Fig. 4A) (Piazza et al., 2019). It consists (i) in the stabilization of JMs upon UV-induced inter-strand DNA crosslink with psoralen (Oh et al., 2009; Schwacha and Kleckner, 1994), (ii) restriction digestion and (iii) proximity ligation of the digested DNA extremities covalently held together in space by the crosslinked hDNA. The resulting chimera is a readout of the amount of this JM in the cell population, determined by quantitative PCR. The sites used are located upstream of the region of homology in both the DSB-inducible and donor molecules, so as to avoid scoring permanent acquisition of the donor restriction site by the broken molecule upon initiation of DNA

synthesis. An independent assay exploits this feature as the basis to determine the initiation of recombination-associated DNA synthesis (Piazza et al., 2018). The approach requires restoration of the restriction site ablated by resection of the broken molecule through annealing of a long complementary oligonucleotide to the resected end. Detection of MI JMs follows the same principle, except that it scores the physical tethering of the two donors by the broken molecule and does not require restriction site restoration on the broken molecule (see Fig. 4B) (Piazza et al., 2017). Limitations of the assay have been extensively discussed (Piazza et al., 2019), and the major limit is the crosslinking density of 1/~500 bp).

2. MATERIALS

2.1 *Saccharomyces cerevisiae* strains

We detail here the content of the *Saccharomyces cerevisiae* strains we developed for the genetic study of MIR and the detection of DNA joint molecules. The relevant genetic components are depicted in Fig. 2, listed in Table 1, and the precise annotated constructs are provided in Dataset S1.

Strains for the MIR assay.—The *HO* gene (which encodes the site-specific HO endonuclease) under the control of the glucose-repressed and galactose-inducible *GAL1* promoter has been inserted at the *TRP1* locus alongside the *hphMX* marker to allow for controlled HO endonuclease expression upon galactose addition in the culture media. The HO cut-sites (HOcs) at *MAT* on chrIII were mutated (*MAT*-**a-inc** or *MAT*-**α-inc**) to prevent their cleavage by HO. The DSB-inducible construct replaces the *URA3* coding sequence on chrV in all cases (Figs. 2A and B). The construct contains the 117 bp HO cut-site (Kostriken et al., 1983) flanked on its left by the 2 kb-long middle part of the *LYS2* gene (YS), and 327 bp of the PhiX174 genome flanked on each side by multiple restriction sites. This last region is used in the JM-Capture assay (see below). The donor loci consist of the first (LY) and the second half (S2) of the *LYS2* gene, respectively. In the main construct used to study MIR so far, referred to as “inter-chromosomal”, the donors are present at an allelic position, at the native *LYS2* locus on chrII (Figs. 2A, B). The *URA3* and *LYS2* loci have been extensively used by others to study DSB repair by HR (Inbar and Kupiec, 1999; Mine-Hattab and Rothstein, 2012). They are located in the middle of chromosome arms and represent unconstrained and untethered chromosomal regions in the nuclear space (Agmon et al., 2013; Berger et al., 2008).

In a variant donor construct referred to as intra-chromosomal, the LY and S2 donors at *LYS2* are separated by a ~1 kb spacer containing the *HIS3* gene for selection purposes (Fig. 2B). The other copy of LYS2 is replaced with *URA3*. The remaining endogenous *URA3* gene on chrV is deleted. In variant donor constructs, referred to as “ectopic”, the S2 donor together with the *HIS3* marker is inserted at the *CAN1* locus on the left arm of chrV, 83 kb away from the *URA3* locus. It is inserted either on the same chromosome as the DSB-inducible construct (ectopic-*cis*) or on the homologous chromosome (ectopic-*trans*) (Fig. 2B). The other *CAN1* gene is the *can1–100* allele of the W303 background.

Strains for the JM-Capture assay—The haploid strain used for physical detection of D-loops bears a DSB-inducible construct that replaces *URA3* on chrV. It is similar to the

DSB-inducible construct, except that the region of homology is the first 2 kb of the *LYS2* gene (LY) (Fig. 2C). The donor locus is of identical sequence and located at *LYS2* on chrII.

For the study of internal D-loops, terminal D-loops, and MI, a variant of the intra-chromosomal donors configuration is used, in which an additional 193 bp pad (PhiX genome) together with an *Eco*RI site was placed immediately 5' of the S2 donor for the physical detection of D-loops formed at S2 and of MI formed between the LY and S2 donor (Fig. 2D).

2.2 Yeast culture media

1. YPD medium: 1 % (w/v) of Bacto yeast extract (BD Biosciences), 2 % (w/v) Bacto peptone (BD Biosciences), 2 % (w/v) D-glucose. Autoclave or filter-sterilize and store at room temperature (RT).
2. YEP-Lactate medium: 1 % (w/v) of Bacto yeast extract (BD Biosciences), 2 % (w/v) Bacto peptone (BD Biosciences), 2 % (v/v) Sodium DL-lactate (60 %, Sigma-Aldrich). Autoclave or filter-sterilize and store at RT.
3. Optional: SD-Histidine medium: 0.67% (w/v) nitrogen base w/o amino acids (BD Biosciences), 0.087% (w/v) histidine drop out mix, 2 % (w/v) D-glucose. Autoclave or filter-sterilize and store at RT.
4. SD-Lysine agarose plates: 0.67% (w/v) nitrogen base w/o amino acids (BD Biosciences), 0.087% (w/v) lysine drop out mix, 2 % (w/v) D-glucose, 2% agar. Autoclave and store at 4 °C.
5. YPD agarose plates: 1 % (w/v) of Bacto yeast extract (BD Biosciences), 2 % (w/v) Bacto peptone (BD Biosciences), 2 % (w/v) D-glucose, 2% agar. Autoclave and store at 4 °C.
6. galactose 20 % (w/v). Dissolve with moderate heating. Filter-sterilize and store at RT.
7. Optional: SD-Histidine agarose plates: 0.67% (w/v) nitrogen base w/o amino acids (BD Biosciences), 0.087% (w/v) histidine drop out mix, 2 % (w/v) D-glucose, 2% agar. Autoclave and store at 4 °C.

2.3 Equipment and reagents

Both assays

1. Standard yeast culture equipment (orbital shaker, rotator, and incubator at 30 °C, 100 ml and 250 mL culture flasks, 15 and 50 mL tubes).
2. Spectrometer (or hemocytometer).
3. Benchtop centrifuge with 50 mL tubes adaptors.
4. Microcentrifuge.

JM-Capture assay

5. Dry bath at 30 °C, 37 °C, 55 °C and 65 °C.

6. Water bath at 16 °C.
7. quantitative PCR instrument (we used Roche LightCycler 96 and 480).
8. UV irradiator (we used Spectrolinker XL-1500 and Bio-Link BLX) with 365 nm UV bulbs.
9. Precision balance.
10. Orbital shaker.
11. Jack and thick glass or plexiglass plate.
12. Petri dish (⌀ 60 mm).
13. Spheroplasting buffer: 0.4 M sorbitol, 0.4 M KCl, 40 mM Sodium Phosphate Buffer pH 7.2, 0.5 mM $MgCl_2$. Filter sterilize and store at 4 °C.
14. Zymolyase 100T solution: 2 % D-glucose, 50 mM Tris HCl pH 7.5, 5 mg/mL Zymolyase 100T (US Biological). Mix well and store up to 3 months at 4 °C.
15. Cutsmart buffer 10X (NEB): 500 mM Potassium Acetate, 200 mM Tris Acetate pH 8.0, 100 mM Magnesium Acetate, 1 mg/mL BSA. Store at 4 or −20 °C.
16. *Eco*RI-HF 20,000 U/mL (NEB, cat. R3101L).
17. Ligation buffer 10X: 500 mM Tris HCl pH 8.0, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP pH 8.0, 25 µg/mL BSA. Store at −20 °C.
18. Ligation mix (660 µL per sample): Ligation buffer 1.2 X, 82.5 µg/mL BSA, 7 µg/mL (Bayou Biolabs) or 240 U/mL (NEB M0202L) DNA T4 ligase. Make fresh right before use.
19. 10 % Triton X100.
20. 1 % and 10 % SDS solution.
21. 25:24:1 Phenol:Chloroform:Isoamyl Alcohol.
22. 3 M Sodium Acetate pH 5.2.
23. 100 % isopropanol.
24. 100 % ethanol.
25. 70 % ethanol.
26. 1X TE pH 8.0 buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)
27. Tri-Methyl-psoralen (TMP or Trioxsalen) ≥98% (Sigma-Aldrich, cat. T6137)
28. TE1 buffer : 50 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0)
29. 10 mg/mL Proteinase K.
30. Faststart Essential DNA Green Master (Roche, cat. 06402712001).
31. LightCycler Multiwell plate 96 white (Roche, cat. 04729692001) and adhesive plate sealer (Sigma, cat. Z722553).

32. Hybridization oligonucleotide (olWDH1770, Table 2, and see Note 1).

33. Quantitative PCR primers (Table 2).

3. METHODS

3.1 Genetic assay to determine MIR frequency.

Day 1: Pre-culture

1. Inoculate 5 mL of YPD with a fresh colony taken from a YPD plate and grow to saturation overnight at 30 °C with shaking (Note 2).

Day 2: DSB induction and cells spreading

2. In the morning, measure the cell concentration of the overnight culture.

3. Dilute the culture at ~2.10^6 cells/mL in 30–40 mL of YEP-lactate and grow for 5–6 hr at 30 °C with shaking until the cells have reach the exponential phase (5.10^6 to 10^7 cells/mL).

4. Meanwhile, label the plates and tubes required for spreading cells (Note 3).

5. Measure the cells concentration and take the “uninduced” time point (Note 4). Harvest the cells by centrifugation at 1,000 g for 5 min at RT. Remove the supernatant and spread the pellet (~100 µL) on a SD-LYS plate. Perform a control plating of 100 cells on rich (YPD) media, after two serial dilutions of the culture in sterile water. (Note 5).

6. Induce DSB formation by adding galactose at a final concentration of 2 %. Incubate in the 30 °C shaker for 2 hr.

7. Measure the cells concentration and spread cells on SD-LYS. Perform a control plating of 100 cells on rich (YPD) media after two serial dilutions of the culture in sterile water.

8. Plates are grown in the incubator at 30 °C for 3 days (Note 6).

9. Count colonies on both SD-LYS and YPD plates. Calculate the basal and induced Lys^+ frequencies by counting colonies formed on SD-LYS, normalized for the plating efficiency determined with colonies formed on YPD. Also, calculate cells viability following DSB formation by comparing plating efficiencies before and after DSB formation.

3.2 Physical assay for the detection of DNA joint molecules in *S. cerevisiae.*

Pre-culture, site-specific DSB induction, and psoralen crosslinking

Day 1: Pre-culture

1. Inoculate 5 mL of YPD with a fresh colony and grow to saturation overnight at 30 °C.

Day 2: Culture

2. In a 50 mL falcon tube wrapped in an aluminum foil, prepare the 5X psoralen stock solution in a maximum of 7 mL of absolute ethanol and seal with parafilm. Place the tube in orbital agitation at a ~30° angle at ~150 rpm overnight at RT (Note 7).

3. In the evening, dilute the saturated culture at ~2.10^5 cells/mL in 80 mL YEP-lactate in a 500 mL flask, so that the cells are in exponential phase the next morning (5.10^6 to 10^7 cells/mL) (Note 8).

Day 3: Time course

4. Harvest a “no DSB’ time point prior to galactose addition. Add galactose at a final concentration of 2 %.

5. At each time point, collect 1.5×10^8 cells and place in ice.

6. Harvest the cells by centrifugation at 1,500 g at 4 °C for 5 min. Meanwhile, prepare the 1X psoralen solution (50 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 0.1 mg/mL Psoralen, 20% ethanol; Note 9) in a 50 mL Falcon tube wrapped in aluminum foil and label the 60 mm petri dishes. Discard the supernatant and drain well. Keep cells in ice.

7. Resuspend the pellet with pipet in 2.5 mL of 1X psoralen solution and transfer to a 60 mm petri dish.

8. Align the petri dishes on a pre-chilled Plexiglas plate.

9. Place the plates in the UV irradiator and jack it up so that they are well aligned and ~ 1 cm under the UV bulb (Fig. 4C; Note 7).

10. Irradiate at max. setting for 10 min, under orbital agitation of the whole apparatus at 70 rpm. Meanwhile, prepare 2.5 mL of cold 1X TE solution, and label a new 5 mL tube per sample.

11. Transfer the cells in a pre-chilled tube in ice. Rinse the petri dish with 2.5 mL of cold 1X TE.

12. Harvest the cells by centrifugation at 5,000 g at 4 °C for 5 min. Discard the supernatant and drain well.

13. Store the pellet at −20 °C. Pellets can be stored for at least a month.

Day 4: Cells lysis (1.5 hours)

14. Thaw pellet in ice, resuspend it in cold Spheroplasting buffer and transfer in a 1.5 mL microfuge tube in ice.

15. Add 3.5 µL of 1 mg/mL Zymolyase solution and mix by inversion. Keep in ice until Zymolyase has been added to all the tubes.

16. Incubate at 30 °C for 15 min. Mix by inversion once or twice during this time (Note 10).

17. Harvest spheroplasts by centrifugation at 2,500 g for 3 min at 4 °C.
18. Discard the supernatant. Keep cells in ice at all time.
19. Wash the cells with 1 mL of cold spheroplasting buffer at 2,500 g for 3 min at 4 °C. Resuspend the pellet carefully by pipetting up and down (Note 11).
20. Repeat wash twice. Meanwhile, prepare the 1X Cutsmart buffer (3 mL per sample) and 1.4X Cutsmart buffer without 6 pM hybridization oligonucleotide (olWDH1770; 360 µL per sample).
21. Lyse and wash the cells debris with 1 mL of cold 1X Cutsmart buffer at 16,000 g for 3 min at 4 °C.
22. Repeat wash twice (Note 12).
23. Resuspend the pellet in 360 µL of 1.4X Cutsmart + hybridization oligonucleotide.
24. Immediately freeze in dry ice and store at −80 °C (Note 13).

Day 5: Joint Molecule Capture (8 hours)

25. Thaw the pellet in ice or at RT. Prepare two 1.5 mL and one 2 mL microfuge tubes per sample.
26. Pipet 36 µL of the cells extract (~ 3×10^7 genomes) into a 1.5 mL microfuge tube at RT.

Chromatin solubilization, restriction site restoration, and restriction digestion

27. Add 4 µL of 1% SDS to the extract (0.1% final) and mix by flicking the tube 4–5 times.
28. Incubate at 65 °C for 10–15 min. Mix by flicking the tube once or twice during this time.
29. Place the cells back in ice. Add 4.5 µL of 10% Triton X100 (1% final) and mix by swirling and pipetting up and down.
30. Add 1 µL of *Eco*RI-HF (20 U/µL). Mix by flicking the tube.
31. Incubate at 37 °C for 1 hr. Mix by flicking the tube once or twice during this time. Meanwhile, set a water bath at 16 °C.

Proximity ligation

32. Add 8.6 µL of 10% SDS (2% final) and mix by flicking the tube 4–5 times.
33. Incubate at 55 °C for 10 min. Mix by flicking the tube once or twice during this time. Meanwhile, prepare the 1X Ligation solution in ice.
34. Place the tubes in ice. Add 80 µL of 10% Triton X100 (5% final). Mix slowly by pipetting up and down.

35. Add 660 μL of 1X Ligation solution and mix by inversion. Incubate at 16 °C for 1.5 hr. Mix by inversion once during this time.

DNA purification

36. Add 1 μL of 20 mg/mL proteinase K, vortex, and incubate at 65 °C for 30 min.

37. Transfer in a 2 mL microfuge tube and perform phenol:chloroform:isoamyl alcohol extraction, followed by a regular DNA precipitation with isopropanol.

38. Resuspend the pellet in 50 μL 1X TE pH 8.0 buffer (Note 14). Meanwhile, prepare the quantitative PCR reactions. The remaining DNA can be stored at −20 °C.

Quantitative PCR, analysis, and normalization

39. Setup the 20 μL qPCR reactions according to the Power SYBR Green PCR Master Mix manufacturer instructions. Use 2 μl of DNA (~10^5 genomes) per reaction. Each reaction is run in technical duplicate. Several reactions are performed: their function and primers are indicated in Fig. 4C. Primer sequences are available in Table 2. For primer design, see Note 15.

40. The qPCR cycles are as follow: one initial denaturation step (10 min at 95 °C), 50 amplification steps (15 seconds at 95 °C, 12 seconds at 61 °C and 15 seconds at 72°C followed by a fluorescence recording), and a final melting curve (65 °C to 97 °C with 0.11 °C/second increment and continuous fluorescence recording) to determine the melting temperature of the amplified product (Note 15).

41. Quantitative PCR results are analyzed on the LightCycler 96 Software 1.1 (Roche). All amplification results are normalized to the reference *ARG4* locus on chrVIII.:

    - The efficiency of DSB formation at the HOcs is normalized to the uncut level (*i.e.* prior to galactose addition). A decrease in signal indicates cutting.
    - The digestion and intramolecular ligation efficiency of a 1,904 bp linear dsDNA fragment from chrVIII (*YHR028C*) allow determining the restriction digestion and ligation efficiency (Note 16).
    - The crosslinking efficiency can be measured by amplification of the unique PhiX sequence on the 5’ side of the homology region on the broken molecule (Note 17).
    - The internal and terminal JM-Capture signals (Note 18).
    - The MI Capture signal (Note 18).

CONCLUSION

These assays allow determining changes in both MIR frequency and amount of JMs in various sequence or mutant contexts. We provide here an example of diploid cells defective

for the Mph1 helicase (human FANCM; a protein involved in D-loop disruption (Mazon and Symington, 2013; Piazza et al., 2019; Prakash et al., 2009)) and the Rad1-Rad10 endonuclease (human XPF-ERCC1) involved in 3' flap cleavage during HR (Fishman Lobell and Haber, 1992; Ivanov et al., 1996; Pâques and Haber, 1997; Sugawara et al., 1995)). Both deletions cause a significant 2- to 3-fold increase in MIR frequency (Fig. 5A). Combining both mutations yields a synergistic 10-fold increase (Fig. 5A).

The metabolism of internal and terminal D-loops, as well as MI was determined in diploid cells with the JM-Capture assays (Fig. 2D). As reported previously (Piazza et al., 2019; Piazza et al., 2017), JM-Capture depends on DSB formation and Rad51 (Fig. 5B). Furthermore, D-loop Capture depends on the restoration of the *Eco*RI restriction site on the broken molecule with a complementary oligonucleotide (olWDH1770) while MI does not (Fig. 5B). None of the mutation or their combination significantly affect the amount of internal D-loops (Figs. 5B and C). However, deletion of both genes leads to a significant 2.5-fold increase of terminal D-loops, as well as MI while individual mutations have modest effects (Figs. 5B and C) (Piazza et al., 2017). Hence, the increased MIR frequency observed in the *mph1Δ rad1Δ* mutant is associated with an increased amount of MIs precursors in the cell population. The associated increase in terminal D-loops (but not internal D-loops) suggest that Rad1-Rad10 and Mph1 limit MI formation and/or stability by specifically preventing formation or destabilizing terminal D-loops. The results illustrate the benefit of combining these physical and genetic assays to decipher the regulation of the metabolism of different types of JMs and the consequences of defects on genomic instability by MIR.

NOTES

1. The "hybridization oligonucleotide" is used to restore the restriction sites on the ssDNA produced by resection and DNA synthesis (in the 5' and 3' unique sequences flanking the region of homology "A", respectively). It has been designed to be properly oriented and encompasses the restriction site with at least ~80 nt internal to the restriction fragment, in order to remain bound during the restriction enzyme inactivation steps (55 °C) and up to the ligation step of the procedure. To avoid artifactual amplification during the qPCR, the five nucleotides at the 3' end are heterologous.
2. The strains bearing the donors in the intra-chromosomal configuration (Fig. 2B) have a higher basal Lys^+ frequency than other strains. To limit the basal Lys^+ frequency, cells can be grown on SD-HIS plates and media prior to dilution in YEP-Lactate.
3. For each time point, label one SD–LYS, one YPD plate, one 15 mL Falcon tube (for collecting 5.10^7 cells at the t0 time-point), two 1.5 mL microfuge tubes with 1 mL of sterile water for the dilution required for plating 100 cells on YPD.
4. For wild type cells with the inter-chromosomal and ectopic donors, this is usually 5.10^7 cells (basal) and 10^6 cells (induced). Collect cells and harvest them by centrifugation at 2,500 g for 5 min. Resuspend in 100 µl autoclaved water and spread on a plate.

5. The basal spreading enables determining the proportion of translocants in the culture assayed prior to DSB induction. In rare instances, an early clonal translocation event will yield a high basal Lys^+ frequency (Lea and Coulson, 1949; Luria and Delbrück, 1943), preventing determination of the induced Lys^+ frequency in this culture. Consequently, such a sample should be removed from the analysis.

6. This can be longer for slow-growing mutants.

7. Multiple factors influence the efficiency of the psoralen crosslink, and thus the inter-experiment reproducibility of the JM-Capture assay. Notably, the quality of dissolving the psoralen in the 5X stock solution, the time between dilution of the working psoralen 1X solution and time of use, the optical density and volume of the solution at the time of crosslink, and the distance between the lamp and the petri dish. These parameters should be kept as constant as possible. A number of inter-experimental variations can be offset by the use of a reference (*e.g.* wild type) strain included for comparison at each time point, onto which the mutant results are normalized. Additionally, the JM-C values can be normalized over the crosslinking efficiency values determined by qPCR.

8. Count ~ 3h of lag phase and ~ 2h per doubling time. This corresponds to inoculating the culture at approximately 2.10^5 cells/mL at 6 pm to result in the expected cell density the next morning.

9. Make the psoralen 1X solution fresh at every time point. Place the 5X psoralen stock solution back in agitation between each time point. Psoralen must be handled under the hood.

10. Check spheroplasting under the microscope. Spheroplasting must not be carried out for too long. Spheroplasting time may need to be redefined for each Zymolyase stock solution.

11. The pellet can be moderately slushy. Make sure it does not remain sticking in the pipet tip.

12. Extensive washing is critical for eliminating enzymatic activities presumably present in the Zymolyase preparation that inhibit ligation efficiency.

13. We noted that not doing so resulted in a progressive degradation of the ssDNA, and thus of the JM-C signal.

14. Let the pellet dissolve at RT for 30 min, vortex briefly, and incubate at 37 °C for 30 min. We usually perform quantitative PCR immediately. A 2 μL volume is used per qPCR reaction, *i.e.* 12 duplicate qPCR reactions per sample can be performed.

15. The qPCR primers must have similar Tm and be positioned so as to generate amplicons of similar size (100–180 bp). Designing new primers require careful determination of the optimal annealing temperature, to minimize non-specific background amplification. Determination of the PCR product melting temperature can allow identifying such spurious amplification.

16. In a typical experiment, 10–40% of the molecules have been circularized.

17. Resection prevents stacking and crosslinking of psoralen in the region flanking the DSB site. Hence, all ssDNA molecules amplify, while the majority of the dsDNA (crosslinked) molecule do not. In a typical experiment, the ssDNA amplification is 10- to 50-fold greater than the reference *ARG4* dsDNA control.

18. Optional: in case of important variation in the efficiency of crosslinking, or of digestion-ligation efficiency, the capture signal can be normalized onto the intra-molecular ligation signal. We noticed that better reproducibility is achieved by normalizing the JM-Capture signal onto the intra-molecular ligation efficiency, and used it routinely (Piazza et al., 2019).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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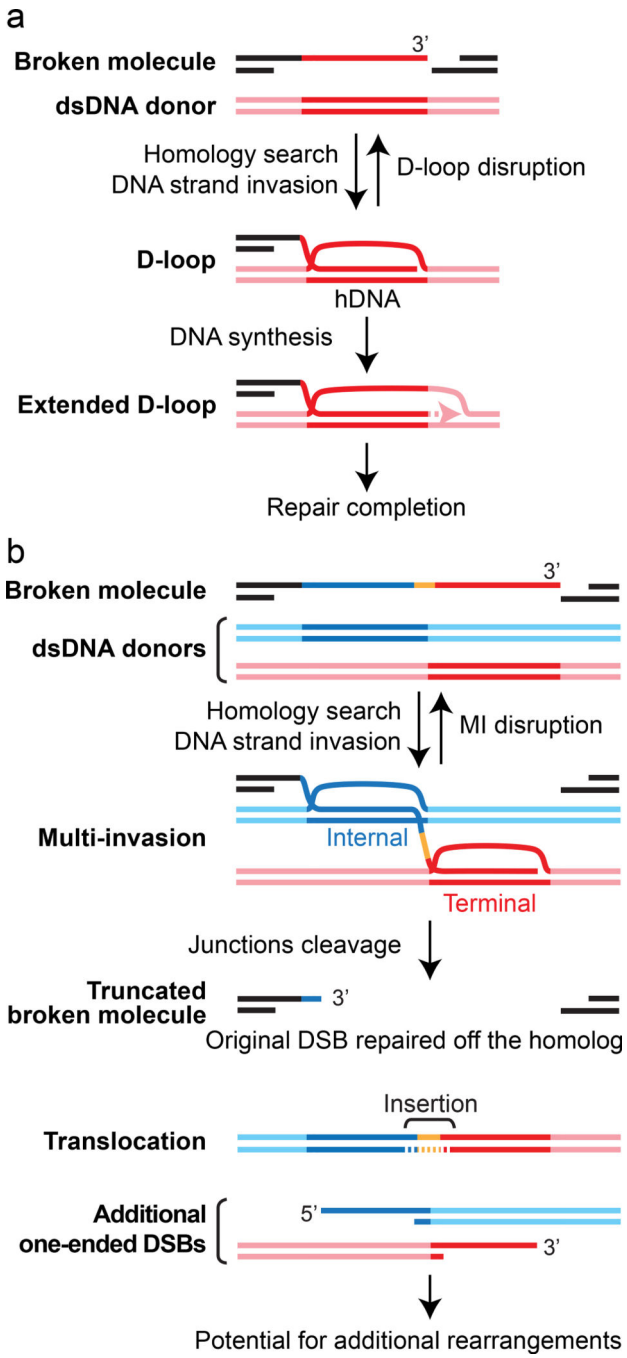

Figure 1: Overview of D-loops and MIs metabolism, as well as the formation of chromosomal rearrangements by MIR.

(**A**) D-loops are metastable reversible JM intermediates formed at a homologous region. (**B**) Overview of the MIR pathway. The MI JM contains two D-loops: one terminal, and one internal relative to the end of the broken molecule.

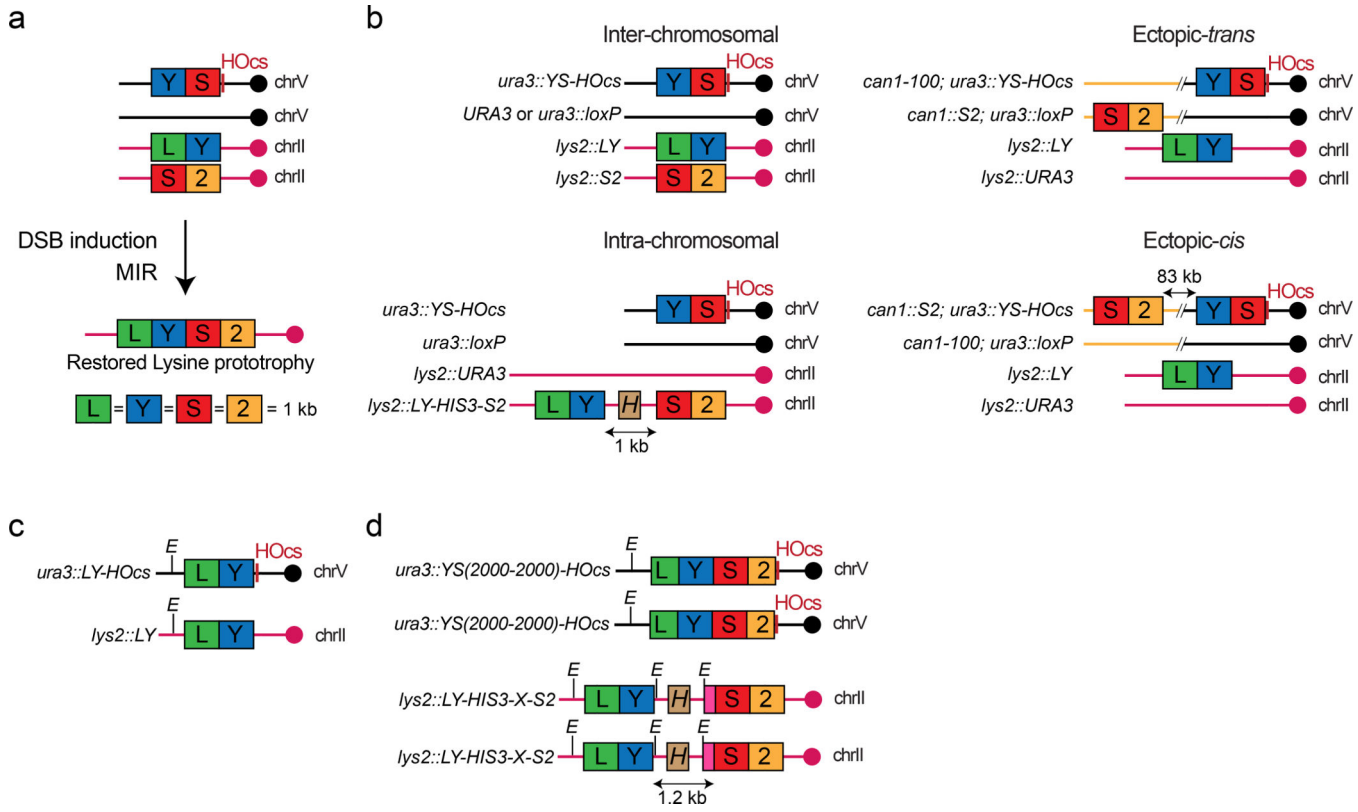

Figure 2: Genetic constructs for studying JM metabolism and MIR in *S. cerevisiae* cells.

(**A**) Rationale of the MIR assay. The translocation of the two undamaged LY and S2 donors on chrII upon DSB formation on chrV at one side of an overlapping homology region YS leads to the restoration of the *LYS2* gene. This event confers Lysine prototrophy to the cell, which can be selected on media lacking Lysine (SD-LYS). Each "letter" is ~ 1 kb-long. The DSB is repaired by gene conversion using the homologous chrV, which results in the conversion of the DSB-inducible construct to *URA3* (Piazza et al., 2017). (**B**) Genetic constructs with varying donor configurations used to study MIR in diploid cells: inter-chromosomal (WDHY5144), intra-chromosomal (WDHY4917), ectopic-*cis* (WDHY5315) and ectopic-*trans* (WDHY5316) (Piazza et al., 2017). (**C**) Genetic construct used to study nascent and extended D-loops metabolism with the JM-Capture assay in haploid cells (WDHY5511) (Piazza et al., 2019). Because it lacks homology on the other side of the DSB, this design precludes the formation of JM intermediates downstream of extended D-loops (such as double Holliday Junctions). Repair completion by break-induced replication is lethal. This strategy prevents changes in the amount of the JM intermediates due to the dilution of cells undergoing repair by those having resumed growth. (**D**) Genetic construct used to study MI as well as internal and terminal D-loop metabolism in diploid cells (WDHY5325). As in (**C**), repairing the break is impossible because all the chromosome V chromatids are broken. A 193 bp phage PhiX sequence together with an *Eco*RI site (pink) has been inserted immediately 5' of the S2 donor relative to the intra-chromosomal construct used for MIR (**B**). This construct provides the restriction site and unique sequence required for JM-Capture of terminal D-loops and MI.

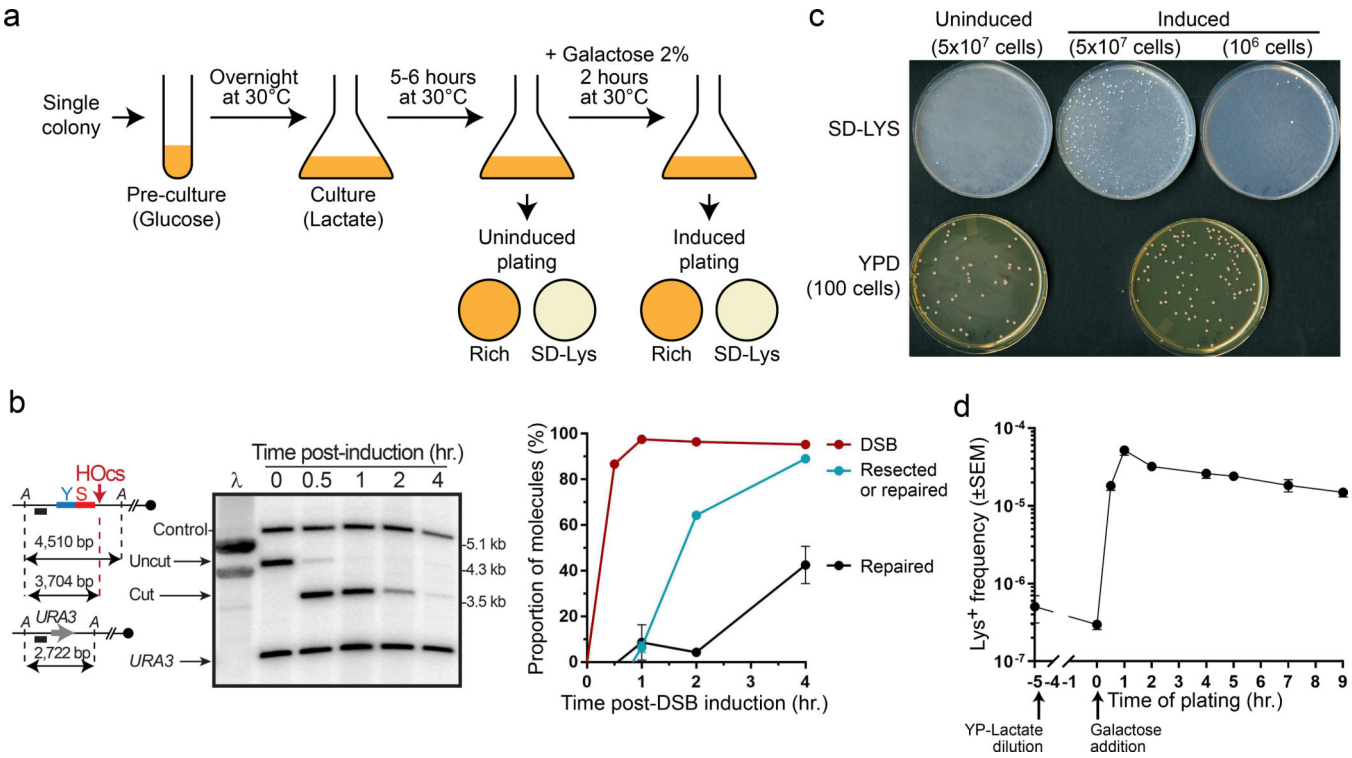

Figure 3: Genetic assay for determining MIR frequency.

(**A**) MIR assay experiment. (**B**) Kinetics of DSB formation, resection, and repair determined by Southern blot analysis in WDHY5144 (described in (Piazza et al., 2017)). *A* represents *Avr*II sites. (**C**) Example of plates obtained in a typical experiment. (**D**) Translocation (Lys^+) frequency before and after DSB induction in wild type cells with donors in the inter-chromosomal configuration (WDHY5144) plated at different time before and after galactose addition.

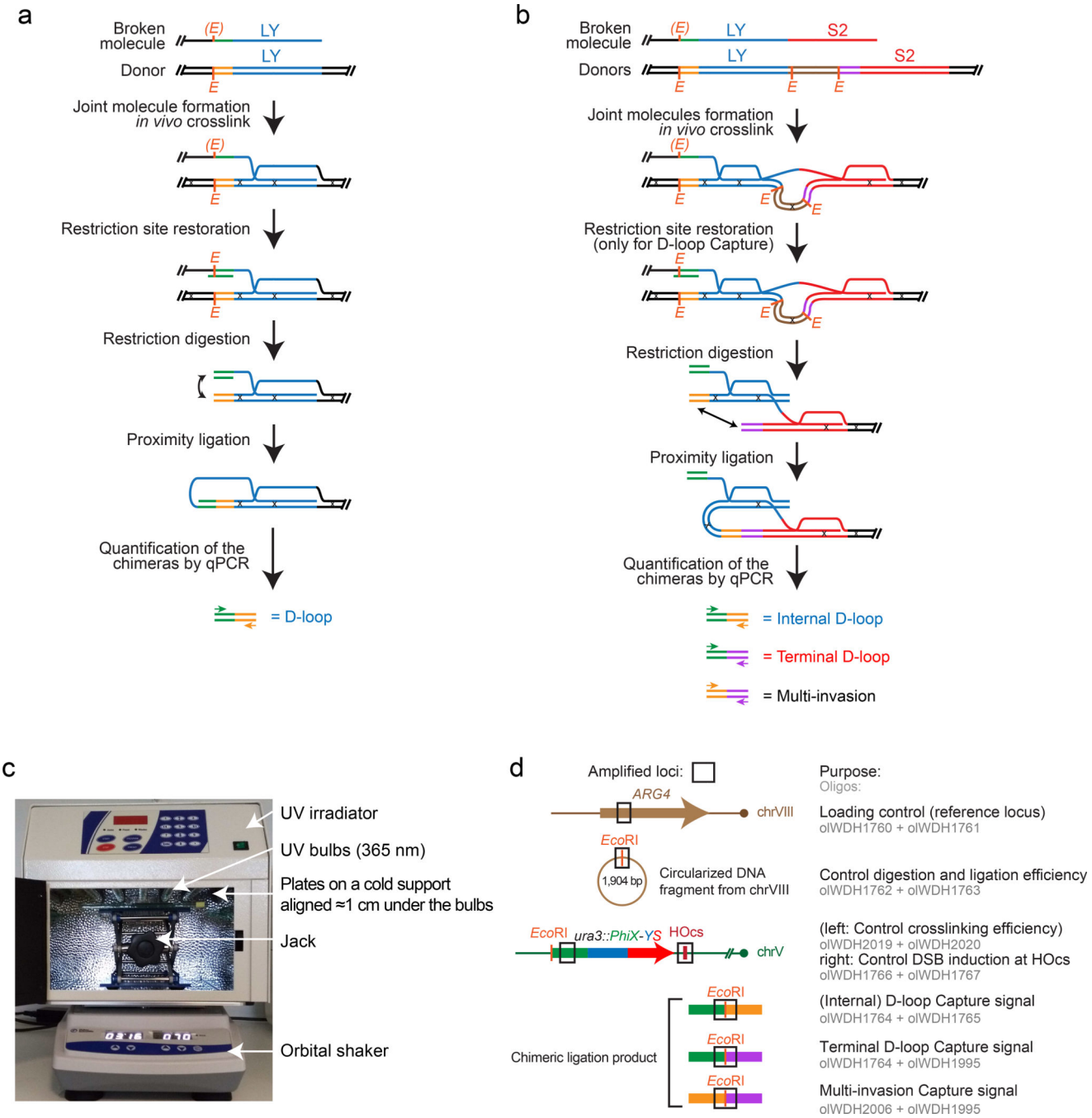

Figure 4: JM-Capture assay.

(**A**) Outline of the D-loop Capture assay. Restriction sites for *Eco*RI are denoted by an *E* and in parenthesis in their ssDNA form. (**B**) Outline of the MI-Capture assay. (**C**) Apparatus used for UV-induced DNA crosslink with psoralen. (**D**) Quantitative PCR reactions performed and their purposes. See Table 2 for the primers used.

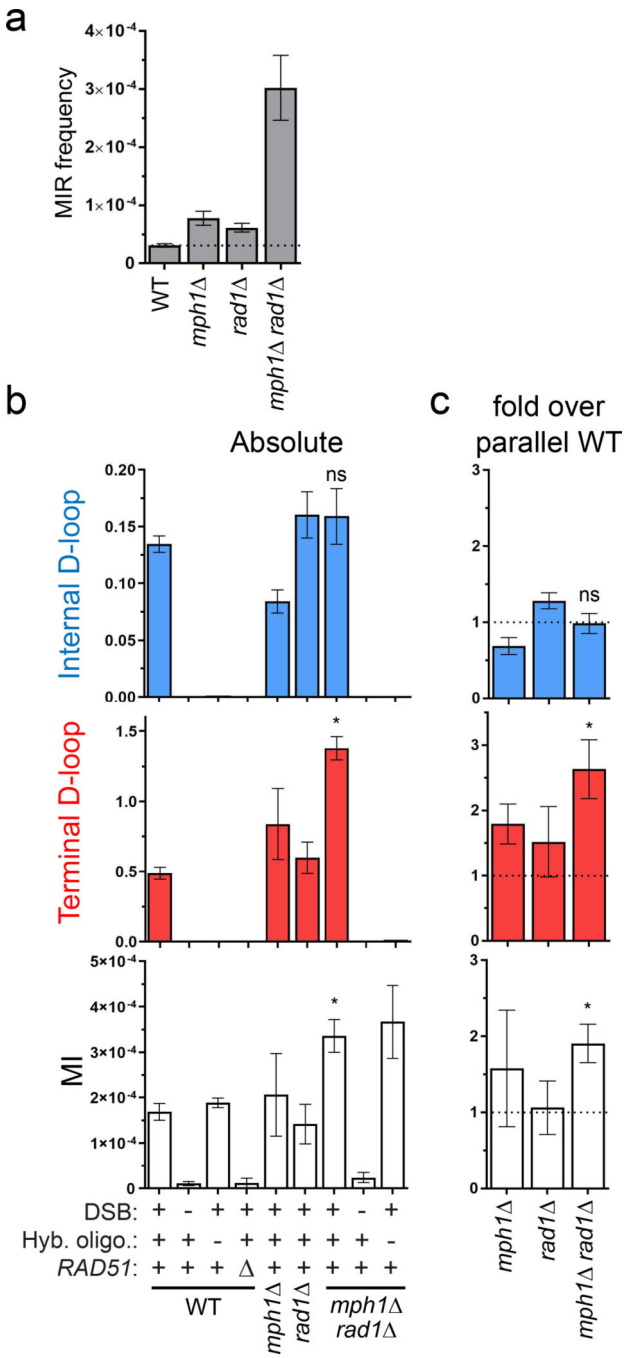

Figure 5: Requirements and example of the regulation of JMs and MIR by Mph1 and Rad1-Rad10.

(**A**) MIR frequencies in *mph1Δ* (WDHY4431) *rad1Δ* (WDHY4273) and *mph1Δ rad1Δ* (WDH4490) strains. (**B** and **C**) Detection of internal D-loops, terminal D-loops and MI JMs 3 hr post DSB induction in diploid wild type (WDHY5325), *rad51Δ* (WDHY5147), *mph1Δ* (WDHY5367), *rad1Δ* (WDHY5326) and *mph1Δ rad1Δ* (WDHY5368) strains. See Table 1 for full genotypes.

Table 1:

Genotypes of *Saccharomyces cerevisiae* strains used in this study.

Strain	Purpose	Relevant genotype	Source
WDHY5144	MIR (Inter-chromosomal donors)	**a**-*inc*/**α**-*inc, ura3::YS(1000–1000)-HOcs/URA3, lys2::LY/lys2::S2, trp1::GAL-HO-KanMX/TRP1, his3 Δ 200/his3 Δ 200, can1–100/can1–100, ade2–1/ade2–1, leu2–3,112/leu2–3,112, RAD5/RAD5*	Piazza et al. Cell 2017
WDHY4917	MIR (Intra-chromosomal donors)	*ura3::YS(1000–1000)-HOcs/ura3::loxP, lys2::LY-HIS3-S2/lys2::URA3*	Piazza et al. Cell 2017
WDHY5316	MIR (Ectopic-*trans* donors)	*can1::HIS3-S2(trans)/can1–100, ura3::YS(1000–1000)-HOcs/ura3::loxP, lys2::LY/lys2::URA3*	Piazza et al. Cell 2017
WDHY5315	MIR (Ectopic-*cis* donors)	*can1::HIS3-S2(cis)/can1–100, ura3::YS(1000–1000)-HOcs/ura3::loxP, lys2::LY/lys2::URA3*	Piazza et al. Cell 2017
WDHY4331	MIR (*mph1*Δ)	*WDHY5144 mph1::KanMX/mph1::KanMX*	Piazza et al. Cell 2017
WDHY4273	MIR (*rad1*Δ)	*WDHY5144 rad1::LEU2/rad1::LEU2*	Piazza et al. Cell 2017
WDHY4490	MIR (*mph1*Δ *rad1*Δ)	*WDHY5144 mph1::KanMX/mph1::KanMX, rad1::LEU2/rad1::LEU2*	Piazza et al. Cell 2017
WDHY5325	MI-Capture (WT)	*ura3::YS2000–2000-HOcs/ura3::YS2000–2000-HOcs, lys2::LY-HIS3-X-S2/lys2::LY-HIS3-X-S2, trp1::GAL-HOhphMX/trp1::GAL-HO-hphMX*	Piazza et al. Cell 2017
WDHY5147	MI-Capture (*rad51*Δ)	*ura3::YS2000–2000-HOcs/ura3::YS2000–2000-HOcs, lys2::LY-HIS3-X-S2/lys2::LY-HIS3-X-S2, trp1::GAL-HOhphMX/trp1::GAL-HO-hphMX, rad51::natMX/rad51::natMX*	Piazza et al. Cell 2017
WDHY5367	MI-Capture (*mph1*Δ)	*ura3::YS2000–2000-HOcs/ura3::YS2000–2000-HOcs, lys2::LY-HIS3-X-S2/lys2::LY-HIS3-X-S2, trp1::GAL-HOhphMX/trp1::GAL-HO-hphMX, mph1::KanMX/mph1::KanMX*	Piazza et al. Cell 2017
WDHY5326	MI-Capture (*rad1*Δ)	*ura3::YS2000–2000-HOcs/ura3::YS2000–2000-HOcs, lys2::LY-HIS3-X-S2/lys2::LY-HIS3-X-S2, trp1::GAL-HOhphMX/trp1::GAL-HO-hphMX, rad1::LEU2/rad1::LEU2*	Piazza et al. Cell 2017
WDHY5368	MI-Capture (*mph1*Δ *rad1*Δ)	*ura3::YS2000–2000-HOcs/ura3::YS2000–2000-HOcs, lys2::LY-HIS3-X-S2/lys2::LY-HIS3-X-S2, trp1::GAL-HOhphMX/trp1::GAL-HO-hphMX, mph1::KanMX/mph1::KanMX, rad1::LEU2/rad1::LEU2*	Piazza et al. Cell 2017
WDHY5511	D-loop-Capture (WT)	*ura3::LY-HOcs, lys2::LY, trp1::GAL-HO-hphM, ade2–1, leu2–3,112, his3–11,15, can1–100, RAD5*	Piazza et al. Mol. Cell 2019

Table 2:

Primers used in this study

Name	Sequence (5'-3')	Purpose	Annealing temp. (°C)
olWDH1760 olWDH1761	AGACAGAATTGGCAAAGATCC GGCCAATTAGTTCACCAAGACG	Reference locus (chrVIII); used for copy number reference and dsDNA loading control in quantitative PCR experiments.	61 and 66
olWDH1762 olWDH1763	ACTTCGAATTTCGGCACTTC CGATGAAACGTTAAGTGACCAC	Quantify the intra-molecular ligation efficiency of a 1904 bp *Eco* RI fragment at the *DAP2* locus.	61
olWDH1766 olWDH1767	GTTTCAGCTTTCCGCAACAG GGCGAGGTATTGGATAGTTCC	Measure dsDNA integrity at the HOcs. DSB induction causes a decrease of signal.	61
olWDH1764 olWDH1765	AGAGCGGTCAGTAGCAATCC CACACGCGAAAAACCGCC	Quantify the chimera formed upon internal D-loop formation	61
olWDH1764 olWDH1995	AGAGCGGTCAGTAGCAATCC AGCGTATGCCGCATGACC	Quantify the chimera formed upon terminal D-loop formation	61
olWDH2006 olWDH1995	CACGCGAAAAACCGCCGG AGCGTATGCCGCATGACC	Quantify the chimera formed between the donors upon MI formation	66
olWDH2019 olWDH2020	CTTTAACCGGACGCTCGA TTGAGTTTATTGCTGCCGTC	Estimate the quality of psoralen crosslinking only in strains that have resected the broken molecule (chrV) past the PhiX region.	61
Hybridization oligonucleotide			
Name	**Sequence (5'-3')**	**Purpose**	
olWDH1770	CGAAATCATCTTCGGTTAAATCCAA AACGGCAGAAGCCTGAATGAAACA TATGAACCAATTGGAGGACGTCAAT GAATTCTGGGGATCCATTGCATTTT T	Hybridize at the *Eco* RI site upstream of the PhiX genome fragment on the resected broken molecule (chrV).	