DNA break repair by homologous recombination: Mechanism and regulations in S. cerevisiae

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Aurèle Piazza

Titre :

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Title:

DNA break repair by homologous recombination: Mechanism and regulations in *S. cerevisiae*

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Composition du jury :

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Contents

I. *Curriculum vitae*

Birth 05/23/1985; French national
Genome Mechanics Team, Laboratory of Biology and Modeling of the Cell (LBMC) UMR5239
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Research positions

January 2020 – present: Permanent CNRS research and Principal Investigator at the Laboratory of Biology and Modeling of the Cell (LBMC), UMR5239, ENS Lyon.
http://www.ens-lyon.fr/LBMC/equipes/mecanique-du-genome
May 2017 – January 2020: Post-doctoral researcher and permanent CNRS researcher in the Department of Genomes and Genetics at the Institut Pasteur, UMR3525. Supervisor: Romain Koszul.
2013 - 2017: Post-doctoral researcher in the Department of Microbiology and Molecular Genetics at the University of California Davis. Supervisor: Wolf-Dietrich Heyer.
2007 - 2013: Undergraduate and graduate student at the Institut Curie. PhD advisor: Alain Nicolas.
2007: Undergraduate student in the Biophysics department at the National Museum of Natural History. Supervisor: Christophe Escudé.
2006: Volunteer summer internship at the Institut Jacques Monod. Supervisor: Jean Cartaud.

Education

2012: **Ph.D. in Genetics**, « Regulation of G-quadruplex formation and genomic stability in *S. cerevisiae* ». UPMC (Paris VI).
2008: Master in Molecular and Cellular Biology, specialized in Genetics, UPMC (Paris VI).
2006: Bachelor in Life Sciences, UPMC (Paris VI).

Funding and Awards (2012-present)

2020: CNRS Paoletti prize.
2020 - 2022: France-Berkeley Fund award with the Heyer lab (UC Davis). *12 k€.*
2020 - 2024: ERC Starting grant (851006 – 3D-Loop). *1.5 M€.*
2019 - 2021 (interrupted): CNRS Momentum. *180 k€ + 2-years post-doc salary*.
2018: Permanent CNRS researcher position, section 21.
2018 - 2020 (2 years - interrupted): ARC Foundation return post-doctoral fellowship (20171206726).
2018: Marie Skłodowska-Curie actions Seal of Excellence (798062 - PACOM).
2015 - 2018 (3 years): Marie Skłodowska-Curie IOF post-doctoral fellowship (628355 - Multiple Search). *279 k€*.
2016: ASBMB travel award.
2013 - 2015 (2 years): EMBO Long-Term post-doctoral fellowship (ALTF 238-2013). *76 k€.*
2013: Philippe Foundation award.
2013 (6 months): ARC Foundation post-doctoral fellowship.
2012: Alexandre Joël prize of the ARC Foundation.

Publications

As of December, 2021: 18 publications (14 primary research). Citations = 1277. Publication repository

1. **Piazza A*°**, Bordelet H*, Agnès Dumont, Agnès Thierry, Jérôme Savocco, Fabien Girard, Romain Koszul°, "Cohesin regulates homology search during recombinational DNA repair", **Nature Cell Biology**, 2021 Nov;23(11):1176-1186. *co-first and °co-last authors

2. J Savocco, **A Piazza**, "Recombination-mediated genome rearrangements", **Current Opinions in Cell Biology** 2021 Dec 01 (71), 63-71
3. **Piazza A**, Rajput P, Heyer WD, "Physical and genetic assays for the study of DNA joint molecules metabolism and multi-invasion-induced rearrangements in *S. cerevisiae*", **Methods in Molecular Biology** 2021, 2153:535-554
4. Shah SS, Hartono S, **Piazza A**, Som S, Wright W, Chédin F, Heyer WD, "Rdh54/Tid1 inhibits Rad54-mediated D-loop formation and limits D-loop length", **Elife** 2020 Nov 13, 9:e59112
5. Schwartz E, Meyer D, **Piazza A**, Fu B, Heyer WD, "Mus81-Mms4 endonuclease is required for telomere maintenance under replication stress in *Saccharomyces cerevisiae*", **PLoS Genetics** 2020 May 29;16(5):e1008816
6. **Piazza A**, Heyer WD, "Moving forward one step back at a time: reversibility during homologous recombination", **Current Genetics**, 2019 May 23; ahead of print.
7. **Piazza A**, Shah SS, Wright WD, Gore SK, Koszul R, Heyer WD "Dynamic processing of displacement loops during recombinational DNA repair", **Molecular Cell**, 2019 Mar 21;73(6):1255-1266.e4.
8. **Piazza A**, Heyer WD "Homologous recombination and the formation of complex genomic rearrangements", **Trends in Cell Biology**, 2018 Nov 26; (18) 30186-7.
9. Muller H*, Scolari V*, Agier N, **Piazza A**, …, Llorente B, Fischer G, Mozziconacci J, Koszul R, "Characterizing meiotic chromosomes structure and pairing using optimized Hi-C designer chromosome", **Molecular Systems Biology**, 2018 Jul 16;14, e8293.
10. **Piazza A**, Heyer WD, "Multi-invasion-induced rearrangements as a pathway for physiological and pathological recombination", **Bioessays**, 2018 Mar 26:e1700249.
11. **Piazza A**, Koszul R, Heyer WD, "A proximity ligation-based method for quantitative measurement of D-loop extension in *S. cerevisiae*", **Methods in Enzymology**, 2018;601:27-44.
12. **Piazza A**, Wright WD, Heyer WD, "Multi-invasions are recombination byproducts that induce chromosomal rearrangements", **Cell**, 2017 Aug 10; (4):760-773.
13. **Piazza A***, Cui XJ*, Adrian M, Samazan F, Phan AT, Nicolas A, "Non-canonical G-quadruplexes cause the hCEB1 minisatellite instability in *Saccharomyces cerevisiae*", **Elife**, 2017 Jun 29;6. pii: e26884. *co-first authors
14. **Piazza A***, Adrian M*, Samazan F, Heddi B, Hamon F, Serero A, Lopes J, Phan AT, Nicolas A, "Short loop length and high thermal stability determine genomic instability induced by G-quadruplex-forming minisatellites", **The EMBO Journal**, 2015 Jun 12;34(12):1718-34. *co-first authors
15. **Piazza A**, Serero A, Boulé J-B, Legoix-Né P, Lopes J, Nicolas A, "Stimulation of gross chromosomal rearrangements by the human CEB1 and CEB25 minisatellites in *S. cerevisiae* depends on G-quadruplexes or Cdc13", **PLoS Genetics**, 2012; 8(11):e1003033.
16. Lopes J*, **Piazza A***, Bermejo R, …, Foiani M, and Nicolas A, "G-quadruplex-induced instability during leading strand replication", **The EMBO Journal**, 2011 Aug 26;30(19):4033-46. *co-first authors
17. **Piazza A**, Boulé J-B, Lopès J, Mingo K, Largy E, Teulade-Fichou M-P, Nicolas A, "Genetic instability triggered by G-quadruplex interacting Phen-DC compounds in *Saccharomyces cerevisiae*", **Nucleic Acids Research**, 2010 Jul;38(13):4337-48.
18. Ribeyre C, Lopes J, Boulé JB, **Piazza A**, Guédin A, Zakian VA, Mergny JL, Nicolas A, "The yeast Pif1 helicase prevents genomic instability caused by G-quadruplex-forming CEB1 sequences *in vivo*", **PLoS Genetics**, 2009 May;5(5):e1000475.

Scientific Communications (last 5 years)

Invited speaker (October 2021)**:** French SMC meeting, Toulouse, France.
Invited speaker (September 2021)**:** EDRA meetings (online).

Seminar (April 2021): UC Irvine, USA (online).
Seminar (February 2021): Laboratoire de Biologie et Modélisation de la Cellule, ENS, Lyon.
Seminar (December 2020): FMI, Basel, Switzerland (online).
Seminar (November 2019): UC Louvain, Belgium.
Seminar (October 2019): ENS Saclay.
Seminar (September 2019): ENS Lyon.
Seminar (September 2019): FMI, Basel, Switzerland.
Poster (July 2019): FASEB "The genetic recombination and genome rearrangements" conference, Steamboat Springs.
Seminar (July 2019): Institute of Human Genetics, Montpellier.
Poster (May 2019): 13th French 3R meeting, Hyères.
Seminar (October 2018): Laboratoire de Biologie et Modélisation de la Cellule, ENS, Lyon.
Invited Speaker (September 2018): 16th DNA Transactions meeting, Egmond aan Zee, The Netherlands.
Poster (May 2018): Abcam meeting "DNA Recombination", London.
Seminar (March 2018): Institut Pasteur, Paris.
Short Talk (March 2018): 14th Course of Epigenetics. Institut Curie, Paris.
Seminar (February 2018): CEA, Saclay (France).
Seminar (January 2018): Nucleus Club, IBPC, Paris.
Seminar (November 2017): Institut Curie, Paris.
Seminar (June 2017): Institut Pasteur, Paris.
Short Talk (May 2017): 12th French 3R meeting, Hyères.
Short Talk and Poster (April 2017): Keystone Symposia "DNA Replication & Recombination", Santa Fe.
Poster (March 2017): "Chromosome Dynamics" Meeting, Stanford University, Palo Alto.

Mentoring

2020 – present (ENS Lyon)**:** Mentoring of

- **A PhD student**: Yasmina Djeghmoum (Since October 2021).
- **Two Master 2 students:** Vinciane Piveteau and Schayma Ben Marzougui.
- **A Master 1 student**: Nicolas Mendiboure.

2018 - 2020 (Institut Pasteur): Mentoring of a pharmacy student (Orianne Debeaupuis) and of a Master 2 student (Amaury Bignaud).
2014 - 2017 (UC Davis): Mentoring of

- **Two undergraduate students**, Abou Ibrahim-Biangoro (3 years) and Noelle Cabral (2 years). Both received a fellowship or an award for their research.
- **A graduate student**, Shanaya Shah (2 years). 3 co-authors publications.
- **A post-doctoral researcher**: Pallavi Rajput (1 month). 1 co-author publication.
- **Rotation students**.

2010-2012 (Institut Curie): Mentoring of

- **A research engineer**: Frédéric Samazan. 2 co-author publications.
- **A visiting graduate student:** Michael Adrian (NTU, Singapore; 1 month). 2 co-author publications.

Scientific animation and service to the community

2021: Jury for the CNRS Paoletti prize.
2021: Reviewing for the polish academy of sciences.
2017 - 2020: Co-organizer of the Parisian Yeast club (IBPC, Paris). *30 regular participants.*
2018: Examiner of Eliana Moreira-Tavares thesis; Le Cam laboratory (Institut Gustave Roussy, France).

2015 - Present: Reviewing activity for Cell, Molecular Cell, Cell Reports, Genetics, Journal of Molecular Biology, eLife, DNA Repair and Scientific reports.
2013 - 2017: Organizer of the DNA club of the Microbiology department (UC Davis). *10-20 participants*.
2009 - 2010: Organizing committee of the « 13th Young researchers in Life Sciences » meeting, *300 participants*.
2008 - 2010: Secretary of the Association of the PhD Students and Post-docs of the Institut Curie (ADIC).

Outreach

2020-2021: Master ENS Lyon. Course « Cellular dysfunctions leading to Cancer » organized by Bertrand Mollereau.
November 2021: Scientific career forum for middle school students (Collège St-Jean-en-Royans)
September 2018: EMBO Laboratory leadership course (Heidelberg).
June 2018: G&G Fastbreak Scientific Meeting for Graduate Students and Post-docs (Institut Pasteur).
March 2018: Talk at the 14th Graduate course of Epigenetics (Institut Curie).
October 2017: Forum for students in preparatory class for engineering schools.
Summer 2010: Supervision of high school students: reproduction of the seminal senescence experiments of telomerase-deficient *S. cerevisiae* cells.
2005 - 2013: Wikipedia contributor (>20 articles in English and French).

Scientific society memberships

2008 - 2012, **2017 - present**: Société Française de Génétique (SFG).
2015 - 2017: American Society of Biochemistry and Molecular Biology (ASBMB).

II. Introduction

The goal of this introduction is not to provide an extensive overview of the literature on the topics at hand, but rather to introduce ideas and speculations originating from past work and motivating future research directions, to be discussed and debated with jury members. An undeniable influence is "The way things go" by David Weiss and Peter Fischli.

A. DNA: a central, busy, and "active" substrate.

DNA encodes most known living organisms. It is armed with informational, structural, and organizational properties (**Figure 1**). These basic properties are harnessed by proteins in various fundamental biological processes occurring at different scales. They also entail a number of challenges, such as the maintenance of its integrity over millions of nucleotides, or the management of its spatial organization (*i.e.* topology, entanglement, and packing) for inter-generational transmission. Beyond, the universal energetic currency are nucleotides (ATP/GTP) and an across-kingdom family of signaling molecules are made of DNA building blocks or derivatives. Organism's core effort thus lies in DNA metabolism. Comparative genomics initially suggested that 58 of the estimated 256 core essentiality genes (22.7%) were directly devoted to DNA metabolism, packing, segregation and expression in bacteria (Mushegian and Koonin, 1996). Assembly of the minimal *Mycoplasma mycoides* JCV-Syn3.0 genome experimentally confirmed this figure, with 61 of its 324 core genes with known function (19%) belonging to this class (Hutchison et al., 2016). DNA is thus a busy, central biological substrate, teeming with a zoo of relatively static binders (*e.g.* histones, transcription factors, telomere-capping proteins, …), processive molecular motors (*e.g.* SMCs, DNA and RNA polymerases) and their passengers (*e.g.* topoisomerases, histone chaperones) that are often studied in isolation. How are these myriad DNA-dependent activities managed, prioritized, and/or coordinated? Can higher-order biological phenomena emerge from the stochastic actions of independent DNA-dependent processes? How are the informational, structural, and organizational facets of DNA involved?

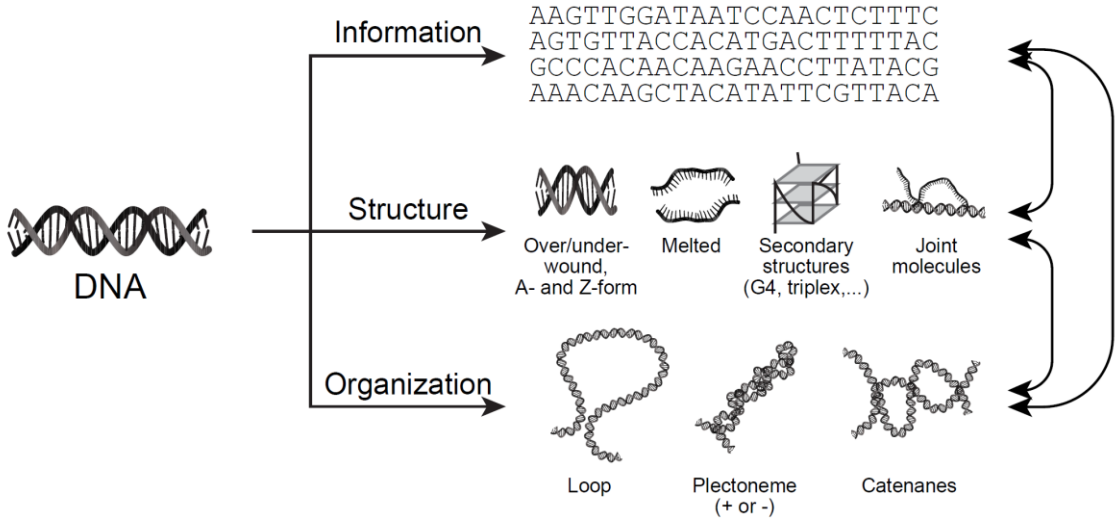

***Figure 1**: **DNA gymnastics**. The informational, structural and organizational facets of DNA, which can impinge on one another.*

We will not discuss here the various spatiotemporal regulations (*e.g.* organelles, genome organization, checkpoints, …) that ensure patterning and avoidance of incompatible DNA-dependent processes (Mazzoccoli et al., 2016). Instead, we will focus on how independent DNA-related processes can influence each other productively, leading to intricated chain reactions that involve the three facets of the DNA polymer. For instance, while transcribing (informational level), RNA polymerases generate torque in the dsDNA molecule (structural level), which can be converted to supercoils (organizational level). These supercoils can either be displaced away by nearby co-directional transcription (informational level), or lead to dsDNA melting or structural transition to other conformations than B-form (structural level) (Kouzine et al., 2004; Selvam et al., 2014; Zhang et al., 2013). These mechanical responses of the DNA substrate can impart built-in feedback and local signaling for protein-dependent interpretation (Bryant et al., 2003), for instance by affecting transcription factors and RNA polymerase loading and/or translocation in *cis* (Gartenberg and Wang, 1992; Joshi et al., 2010; Roca, 2011). They may also be involved in specification and/or firing of replication in metazoans (Brossas et al., 2021). DNA primary sequence (informational level again) can further impinge locally on these mechanical reactions and feedbacks through its influence on duplex stability, secondary structure-forming propensity, DNA bendability (Basu et al., 2021) and plectoneme induction (Kim et al., 2018). These examples illustrate how basic DNA properties of different nature can passively mediate the interplay between DNA-dependent processes.

These interplays can lead to the emergence of higher-order functions. The combination of transcription and chromatin loop folding by cohesin has recently been proposed for mitotic chromosome segregation in *S. cerevisiae* (Paldi et al., 2020): convergent transcription units (informational level) at a typical distance from the centromere organizes cohesin-mediated pericentromeric loop folding (organizational level) that enables centromeres to withstand kinetochore-mediated tensions. This organization participates of chromosome biorientation and accurate segregation during mitosis (Paldi et al., 2020). This spectacular example illustrates how a simple prioritization between minute DNA-dependent processes (transcription by RNA polymerases affecting cohesin localization (Lengronne et al., 2004)) leads to the larger-scale functionalization of a chromosomal region.

Parts of the research projects presented here aims at deciphering these prioritizations in the particular case of DNA break repair by homologous recombination. For instance, how are transcription and recombination prioritized (**Chapter IV.A.4**)? How is homology search tweaked by cohesin blockade in the DSB region (**Chapter IV.A**)? What is the nature of the interplay between chromosome organization and the recombination process that orchestrates the first meiotic division (**Chapter IV.B**)? I suspect that some of these questions can be explained by chain reactions in the framework of simple prioritization rules between DNA-dependent processes, and the built-in properties of DNA that links sequence, structure and organization. I surmise that some of these chain reactions involve an overlooked aspect of nuclear biology: the role of force and mechanical work in regulating protein-DNA transactions (see **C.** below).

B. The multi-scale process of homology search during homologous recombination.

Biological processes often require specific physical interactions between cellular components, which entails their spatial encounter and the association between their cognate interface. How two biological entities such as a protein and a DNA segment bearing a particular sequence or structure can find each other orders of magnitude faster than diffusion-limited reactions, as in the classical example of the Lac repressor binding to the *lac* operator (Riggs et al., 1970), has led to various models of facilitated diffusion (**Figure 2**) (Berg

et al., 1981; Von Hippel and Berg, 1989). In addition to the high-affinity, site-specific binding site that usually involves major-groove or Watson-Crick base-pairing interactions, these models posit the existence of low-affinity, sequence-independent electrostatic interactions between protein and DNA. Facilitated target search has been primarily understood and studied as a matter of variable protein properties with an invariable DNA substrate, whose relevant properties were limited to its polymeric nature (enabling short-range 1D walk) and its concentration (authorizing "hopping" or inter-segmental transfer).

Broadly conserved principles of chromatin organization in the nucleus have emerged more clearly over the last decade (Mirny and Dekker, 2021). They consist mainly in (i) the anchoring of specific loci at the nuclear envelope (*e.g.* telomeres and centromeres in *S. cerevisiae*), (ii) "soft" compartmentalization as membrane-less condensates mediated by multivalent diffusive components such as proteins and RNAs (*e.g.* the nucleolus), and (iii) loop domains of preferential interactions organized by mono- or divalent DNA translocases (*e.g.* condensin and cohesin, respectively). It led to the speculation that these organizing factors may both passively and actively impinge on target search, for instance by regulating intra-domain hopping through differential steady-state DNA condensation or domains of preferential interactions (**Figure 2**). Differently, dynamic juxtaposition of distant *cis* DNA segments at the level of a translocating cohesin may authorize long-range unidimensional search (see **Chapter IV.A.5**) (Dekker and Mirny, 2016). Current work in my laboratory focuses on a particular case of target search: that conducted as part of the templated repair of a DNA break by homologous recombination, which involves the harnessing of the three aforementioned facets of DNA by proteins.

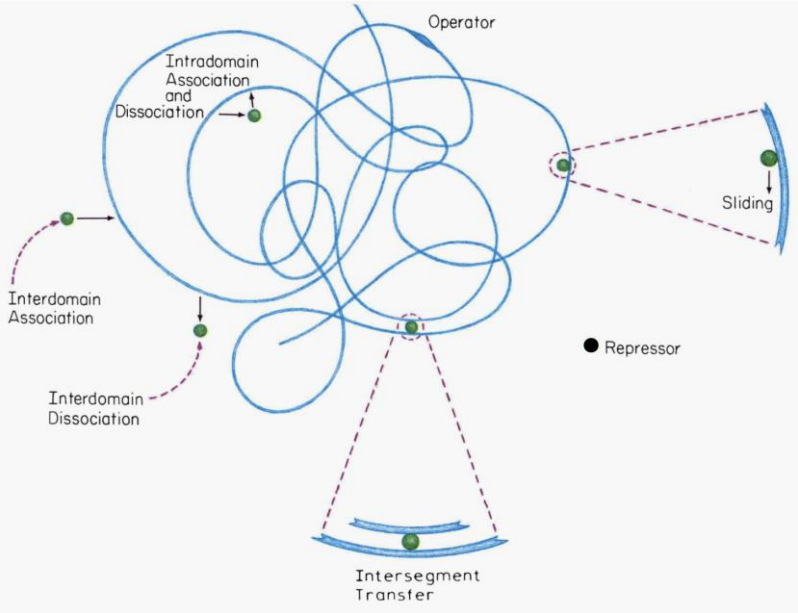

Figure 2: Schematic representation of target search by a diffusive factor within a chromosomal domain*. From (Von Hippel and Berg, 1989).*

Homologous recombination (HR) is a universal pathway for templated DNA double-strand break (DSB) and ssDNA gap repair. At the somatic level, HR promotes cell viability in various genotoxic contexts. At the germinal level, HR guides parental homologous chromosomes recognition in most studied eukaryotes, and operates the reciprocal exchange of parental chromosome fragments that simultaneously ensures homologs attachment and alleles reassortment at the first meiotic division (Hunter, 2015). The central tenet

of DSB repair by HR is the use of an intact homologous dsDNA molecule as a template, which entails its search amidst the vast excess of heterologous DNA of the genome. Like any other target search it has two main requirements: at the molecular scale it requires the ability to identify a target site, in this case by compare the DNA sequence surrounding the break site with that present in other dsDNA molecules; and at the cytological scale opportunities for spatial collisions between the broken molecule and a potential homologous donor, in the form of the sister chromatid, a homologous chromosome, or ectopic repeats. It appears as an atypical case of target search conducted by one or two sub-diffusive entities (*i.e.* the DNA ends) that presumably remain associated; unlike traditional target search conducted by multiple, freely diffusive proteins. Yet donor identification is achieved rapidly in cells: the sister chromatid located at the opposite pole of the cell is found in ~9 minutes in *E. coli* (Wiktor et al., 2021), and ectopic donors are identified within tens of minutes to a few hours in *S. cerevisiae*, depending on their intra- or inter-chromosomal location relative to the DSB site (Hicks et al., 2011; Miné-Hattab and Rothstein, 2012; Piazza et al., 2019, 2021a). What is the mechanism of such an efficient homology search?

Target search in HR is conducted by a conserved nucleoprotein platform composed of RecA (in bacteria) or Rad51/Dmc1 (in eukaryotes) assembled continuously into a helical filament on the hundreds to thousands of nucleotides-long single-strand DNA (ssDNA) generated by resection, which is the informational guide for homology search. The resulting Rad51-ssDNA nucleoprotein filament (hereafter NPF) exhibits striking structural features worth highlighting (**Figure 3**). First, it exhibits two continuous DNA binding sites over the entire NPF length: a high affinity at the axis that contains the guide ssDNA; and a lower-affinity binding site that splays open incoming dsDNA and sequesters one of the two strands, providing the other with opportunities for pairing to the guide ssDNA (Yang et al., 2020) (**Figure 3a**). Successful identification of substantial homology leads to the formation of a DNA joint molecule called a D-loop (structural level; see more on **Chapter II.B.1**). Second, each RecA/Rad51 monomer binds a B-form nucleotide triplet, unstacked from the flanking ones, yielding an uneven 1.5-fold extension compared to the B-form contour length (structural level). The resulting NPF structure is elongated and rigid (organizational level), with persistence length approaching 1 μm *in vitro* (Bell and Kowalczykowski, 2016; Benson et al., 1994; Flory and Radding, 1982; Heuser and Griffith, 1989) (**Figure 3b**).

These features are radically different from that of comparatively small, globular proteins bearing one or a few low-affinity dsDNA binding sites considered for classic target search by proteins (including those guided by nucleic acids such as Cas9 and Ago proteins), and as such may confer atypical target search modes. Single-molecule approaches with bacterial RecA showed that the continuous, multivalent NPF organization authorizes inter-segmental engagement and sampling of multiple, independent dsDNA molecules along the NPF length, thus paralleling the search process (Forget and Kowalczykowski, 2012) (**Figure 3c)**. This search mode is likely conserved in eukaryotes (see **Chapter II.B.2**). The efficiency of inter-segmental contact sampling depends on the conformation of the dsDNA substrate probed (organizational level, see below) (Forget and Kowalczykowski, 2012). Stable synaptic associations are formed past a conserved 8 nt microhomology threshold (informational level) (Danilowicz et al., 2015; Hsieh et al., 1992; Lee et al., 2015; Qi et al., 2015) (**Figure 3d**), much below uniqueness in both bacterial and eukaryotic genomes (see **Chapter IV.A.1**). One NPF thus contains multiple, physically tethered search entities probing for at least 8 nt microhomologies, which solves the "single search entity" problem.

Difficulty in obtaining functional fluorescently-tagged RecA/Rad51 proteins complicated the analysis of NPF structure in cells, which appeared either as thick bundles or as foci (Lesterlin et al., 2014; Lisby et al.,

2004; Waterman et al., 2019). Recently, individual RecA NPF were visualized stretching across the elongated bacterial nucleoid to the opposite cell pole where the sister chromatid resides (Wiktor et al., 2021). By doing so, the NPF would (i) place its search entities in each section of the nucleoid, simplifying a tridimensional search into a bidimensional one, and (ii) overcome the limited diffusion of its originating site. Although appealing and supported by computational modelling, aspects of this model await a formal demonstration: does reducing filament length or its rigidity impact homology search? Furthermore, the applicability of such reduced dimensionality principles in a spherical and much larger eukaryotic nucleus remains unclear. Similarly elongated structures could be identified in eukaryotic cells only recently ((Haas et al., 2018; Horikoshi et al., 2021) and A. Taddei, personal communication).

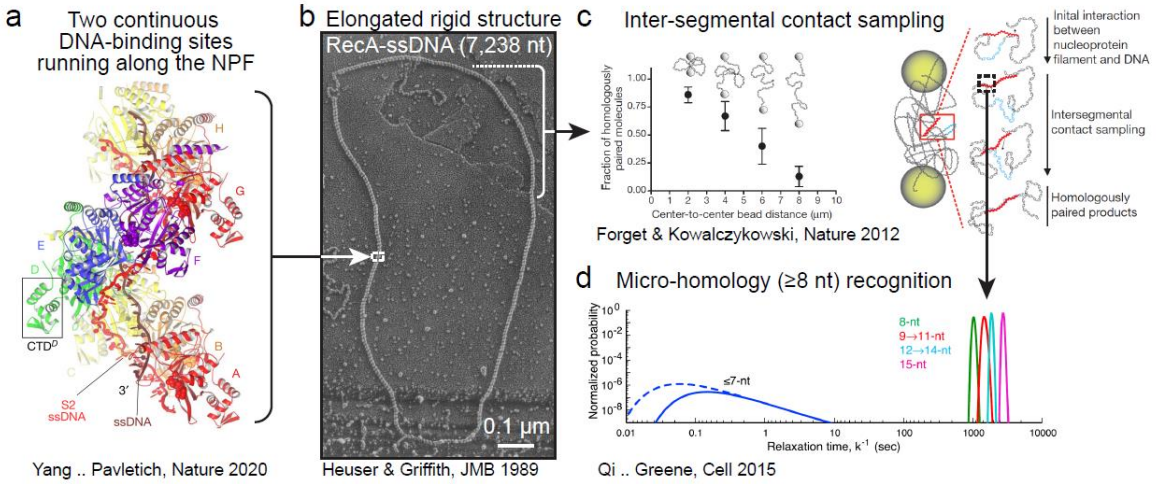

Figure 3: RecA-ssDNA nucleoprotein filament structure and mechanism of dsDNA sampling determined in vitro. ***(a)*** *Structure of fused $(RecA)_9$ protomers (A to I) with ssDNA $(dT)_{27}$ (brown) in the primary DNA binding site, ATPγS and model of a ssDNA (in red) in the secondary DNA binding site. From (Yang et al., 2020)* ***(b)*** *Electron micrograph of a 7 kb-long RecA-ssDNA-ATPγS filament. From (Heuser and Griffith, 1989).* ***(c)*** *Inter-segmental model of homology sampling by RecA-ssDNA NPF, which depends on the coiled nature of the surveyed dsDNA. From (Forget and Kowalczykowski, 2012)* ***(d)*** *Sharp discontinuity in the relaxation time of RecA-ssDNA NPF and dsDNA between 7 and 8 matched nucleotides. From (Qi et al., 2015).*

Finally, HR takes place in the context of a spatially organized and locally constrained genome. The Rabl organization of the *S. cerevisiae* genome, defined by the anchoring of centromeres at the spindle pole body and telomeres at the nuclear envelope, broadly dictates HR efficiency using relatively short (~1 kb) ectopic donors in end-point assays (Agmon et al., 2013; Lee et al., 2016a). Furthermore, we recently showed that cohesin, which organizes chromatin into loops, influences in homology search in various, direct and indirect ways (Piazza et al., 2021a) (see **Chapter III.B.3**).

Work in the lab aims at investigating the role conferred by the informational ((micro-homology sampling, homology length and organization, **Chapter IV.A.1** and **2**), structural (DNA joint molecule metabolism; **Chapter IV.A.3** and **4**) and organizational (spatial chromatin organization and NPF structure; **Chapter IV.A.5** and **6**) aspects of this DNA-centric homology search process, how various proteins impinge on it, and consequences of their defect on the efficiency and accuracy of HR repair.

C. The overlooked role of mechanical work and forces in nuclear biology

Cells and their components are objects that can generate mechanical work and be subjected to it. The study of both work-generating processes, force-sensing processes, resilience to mechanical challenges, and their biological roles is the focus of mechanobiology. Most notoriously, proteins such as Myosin generate work by converting the energy released by the hydrolysis of ATP into translocation along actin filaments. Such mechanochemical coupling is not unique to Myosin or even cytoplasmic proteins: DNA translocases similarly convert ATP hydrolysis into motion along single- or double-stranded DNA. In fact, some dsDNA translocases are amongst the most powerful known molecular motors, such as those involved in phage DNA packaging into its capsid that can generate forces up to 50 pN (Fuller et al., 2007a, 2007b; Smith et al., 2001). Yet, in contrast to research focusing on transmembrane or cytoplasmic processes, and perhaps because of its insulation into an organelle, biological functions of mechanical work and forces in the nucleus remain little explored (Uhler and Shivashankar, 2017).

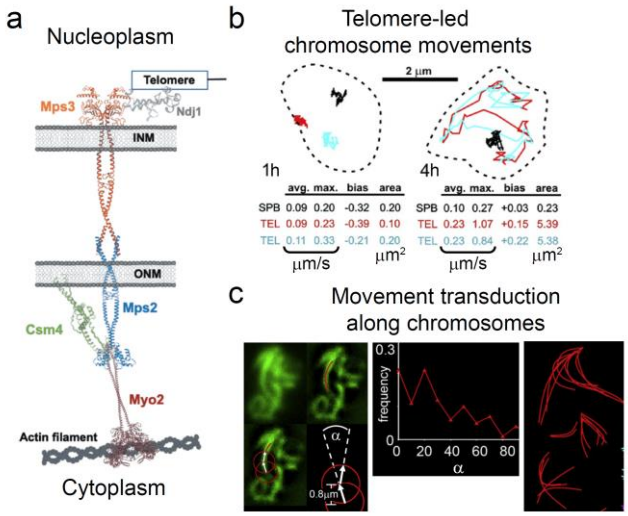

Figure 4: Chromosome movements during meiotic prophase. *(**a**) schematic representation of the cytoplasm-to-nucleoplasm force transduction complex. From (Lee et al., 2020). (**b**) Example of movements of the spindle-pole body (SPB, marked with Tub1-GFP) and the FROS-labeled telomeric region of both chr. IVR (TEL) at 1 and 4h post-meiosis induction (corresponding to the leptotene and zygotene stages, respectively). Average and maximum speed, positional bias, and maximum boxed area containing the total displacement measured over 59 measurements (1 frame per second). From (Conrad et al., 2008) (**c**) Movement and bending of Zip1-GFP labeled chromosomes. Series of chromosome outline over 8-10 seconds are shown on the right. From (Koszul et al., 2008).*

Disparate evidence point at mechanical forces exerted through or within the nuclear envelope as integral components of a variety of nuclear processes: from regulating gene expression (Uhler and Shivashankar, 2017) and nuclear pore architecture and selectivity (Schuller et al., 2021), to meiotic chromosomes pairing and recombination (see below). For instance, the movements induced by cytoplasmic cytoskeleton components can be transduced to chromatin through the nuclear envelope via specialized transmembrane complexes. In *S. cerevisiae* for instance, the microtubule-organizing center called the spindle pole body (SPB) traverses the nuclear envelope, thus connecting the cytoplasmic microtubule network to the centromere-bound kinetochores. This organization transduces the mechanical work generated in the

cytoplasm to the chromosomes, thus allowing for their segregation in the absence of nuclear envelope breakdown (*i.e.* a closed mitosis). In various species, meiotic prophase I features dynamic chromosome movements mediated by the cytoplasmic actinomyosin network, and transduced through the nuclear envelope at the telomere level by the conserved LINC (Linker of Nucleoskeleton and Cytoskeleton) complex. In *S. cerevisiae*, the nuclear envelope is traversed by Mps3-Mps2, connecting on the nucleoplasm side telomeres to Mps3 via Ndj1, and on the cytoplasmic side Mps2 to Myo2 via Csm4 (Lee et al., 2020) (**Figure 4a**). These transducers mediate rapid telomere-led chromosomal movements during the early meiotic prophase I of *S. cerevisiae*, which roughly correspond to the zygotene/pachytene stages, a time at which meiotic recombination occurs (Conrad et al., 2008; Koszul et al., 2008; Wanat et al., 2008). These movements are uncoordinated between chromosomes and can reach up to 1 μm/s (Conrad et al., 2008; Koszul et al., 2008) (**Figure 4b**). They are exerted at the chromosome termini and are transduced interstitially, with decay, along the semi-rigid chromosome axis (Koszul et al., 2008) (**Figure 4c**). They were proposed to promote inter-homolog DSB-donor interactions, break apart non-allelic interactions, and disentangle chromosomes (Koszul and Kleckner, 2009). These intuitions have so far not been translated into a quantitative understanding of this mechanical layer of regulation of the meiotic recombination and chromosome pairing process. I propose to address a model in which mechanical challenges generated between moving chromosomes are the missing link between chromosome structure and recombination, and whose interplay drive the reactions leading to homolog recognition, recombination and segregation at meiosis I (see **Chapter IV.B**)

III. Summary of past research

Past research experiences led me to investigate different aspects of DNA metabolism and genome maintenance. My PhD work focused on the role of exotic DNA structures (G-quadruplexes) formed at certain G-rich DNA in causing chromosomal rearrangements. During my post-doc I investigated the basic mechanisms and regulations of DNA break repair by homologous recombination, focusing on the metabolism of early DNA joint molecules of the pathway. As a permanent CNRS researcher I interrogated the role the spatial chromatin organization and its determinants have on the homology search process of homologous recombination. Most of these works involved interdisciplinary collaborations with chemists, biochemists, biophysicists, structural biologists, and mathematicians.

A. G-quadruplexes are polymorphic DNA secondary structures at risk for genomic stability

Referring to the following publications: (Lopes et al., 2011; Piazza et al., 2010, 2012, 2015, 2017a; Ribeyre et al., 2009)

Although best known in its iconic double-helix form, DNA can adopt a variety of non-canonical structures. G-quadruplexes (G4s) are a family of four-stranded structures formed by certain G-rich nucleic acids, whose folds and biophysical properties were already well-characterized *in vitro* when I started my Ph.D (**Figure 5a**). However, whether they formed in cells and exerted biological functions was largely an open question. When I joined the laboratory of Alain Nicolas in 2007 for my Master 2 internship, observations by Cyril Ribeyre and Judith Lopes in the laboratory were pointing at a specific instability of the chromosomally integrated human G-rich tandem repeats CEB1 in *S. cerevisiae* when the Pif1 helicase was absent or catalytically deficient. This instability would manifest as expansion/contraction of the tandem array, detectable by Southern blot, and was specific to CEB1 (**Figure 5b**). In collaboration with Laurent Lacroix and Jean-Louis Mergny (MNHN, Paris), they hypothesized that it could result from unprocessed G4s within CEB1. This hypothesis was supported by biochemical observations obtained in collaboration with Jean-Baptiste Boulé before he joined the lab, that purified Pif1 could unwind G4s more readily than dsDNA *in vitro*. Using a tandem repeat amplification technique previously devised in the laboratory, I could generate long synthetic tandem repeats, devoid of the canonical G-triplets (CEB1-Gmut) required for G4 formation, integrate them into the yeast genome, and assess their stability in cells. Mutation of the G-triplets abolished CEB1 instability in a *pif1*Δ mutant. This effect was specific to a lack of Pif1, as this mutated array retained instability in the absence of $Rad27^{FEN1}$,a flap endonuclease whose defect destabilizes all tandem repeats (Ribeyre et al., 2009). These results strongly suggested that (i) G4 could form in cells, (ii) Pif1 could unwind them, and (iii) that their pathological presence caused site-specific genomic instability (Ribeyre et al., 2009).

I decided to pursue this promising project during my PhD, and address various open questions: How/when do G4s form in cells? Which DNA metabolic process do they interfere with? How does this perturbation lead to expansion/contraction of the array (we knew it was HR-dependent)? Can G4 cause other types of genome rearrangements than expansion/contraction? Are tandems of G4s required to trigger genomic instability? Why are certain G4-forming sequences destabilized in the absence of Pif1 and not others?

1. Existence of G4 in *S. cerevisiae*

Before I set out to address these questions, I needed to ascertain the dependency of CEB1 instability not only on G-triplets (as demonstrated by mutagenesis), but on the G4 structure. To this end, we initiated a collaboration with the chemists of the Marie-Paule Teulade-Fichou's lab (Institut Curie Orsay), who had developed highly specific G4 binders (hereafter G4-ligands): Phen-DC_3 and Phen-DC_6 (De Cian et al., 2007). Jean-Baptiste Boulé and undergraduate student Katie Mingo showed that these compounds efficiently and specifically inhibited G4 unwinding by Pif1 *in vitro*. Consistently, I could show that both compounds induce CEB1 instability in wild-type cells (**Figure 5a**). This instability depended on the presence of intact G-triplets. These results showed that CEB1 instability was dependent on the formation of G4 structures, and not other features tied to its GC-richness and skewness, thus demonstrating for the first time the existence of G4 in cells (Piazza et al., 2010). They also set a standard to demonstrate the reliance of a phenotype on G4 formation: enhanced upon stabilization with G4-ligands and abolished upon G-triplet mutagenesis, including the enhancement conferred by G4-ligand treatment.

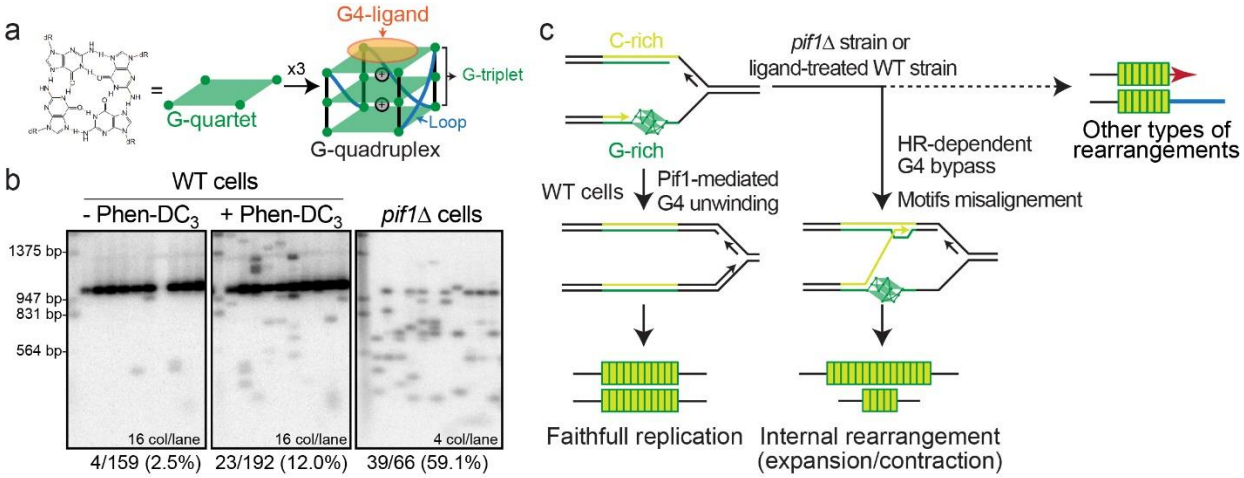

Figure 5: G-quadruplexes interfere with leading strand replication and cause the instability of the underlying hCEB1 tandem repeat. *(**a**) Schematic representation of the structural features of G-quadruplexes, and their recognition and stabilization by G-ligands through external quartet stacking. (**b**) Example of Southern blot showing the destabilization of CEB1 upon wild-type cells treatment with the Phen-DC_3 G4-ligand for 7 generations, or in a pif1Δ mutant for approximately 30 generations. From (Piazza et al., 2015). (**c**) Model for G-quadruplex-induced genome instability during leading strand replication.*

2. G4s induce various types of genome rearrangements

In order to investigate the formation of other types of chromosomal rearrangements than expansion/contraction induced by G-rich sequences, and to screen for the biological efficacy of various types of G4-ligands, I developed a genetic system based on the gross chromosomal rearrangement (GCR) assay of Kolodner and co-workers (Chen and Kolodner, 1999), which scores the rate of loss of a non-essential 30 kb terminal chromosome arm region. This system generalized G4-induced instability to other types of genomic rearrangements than expansion/contraction of CEB1, such as non-reciprocal translocations and frequent telomere additions. It revealed a cooperative behavior between individual G4 motifs in stimulating GCR, up to percent rates (Piazza et al., 2012). It also highlighted the different ability

of other GC-rich tandem arrays to stimulate GCR by telomere addition, although the initiating lesion was G4-independent (see below). We mapped the localization of GC-rich and G4-forming tandem repeats in the human genome and found them strongly enriched near chromosome ends. It raised the possibility that they play a last-resort chromosome healing role, at the cost of short terminal truncations. Alternatively, they could play a benchmarking role for DNA replication and repair machineries, contributing to eliminate deficient cells during ontogenesis with minimal risk for alteration of genes' structure and expression. This last possibility may explain their maintenance in genomes despite the apparent hurdle they represent at the cellular level.

3. G4s are hurdles for leading strand replication

Together with Judith Lopès in the lab, we aimed at determining the mechanism by which G4 cause chromosomal rearrangements. G4 readily form out of ssDNA, while dsDNA efficiently competes with their formation (Phan and Mergny, 2002). Furthermore, *in vitro* work showed that G4s in the template strand interfere with replication progression by various DNA polymerases (Weitzmann et al., 1997). In collaboration with Rodrigo Bermejo and Ariana Colosio in Marco Foiani's lab (IFOM, Milan), we addressed whether the ssDNA generated between Okazaki fragments during replication was conducive to G4 formation and subsequent recombinogenic lesions. To this end, we took advantage of the well-defined directionality of replication in the vicinity of strong replication origins (ARS) in *S. cerevisiae*, by placing CEB1 near *ARS305* in two orientations: one in which the G-rich strand is a lagging strand template, and the other in which it is the leading strand template (**Figure 5c**). To our surprise, CEB1 remained stable in the lagging orientation, while it exhibited strong destabilization in the leading orientation both upon *PIF1* deletion or Phen-DC_3 treatment. Bidimensional gel electrophoresis further revealed the formation of replication-associated, Rad51- and Rad52-dependent DNA joint molecules specifically in the leading orientation. These results indicated that (i) G4s could form on the leading strand template prior to, or during fork passage, (ii) interfere with replication progression, which (iii) required recombination-dependent bypass (Lopes et al., 2011) (**Figure 5c**). It provided a compelling mechanism for G4-induced genomic instability, which contrasted with the main (untested) assumption of the DNA secondary structure field at the time, that such structures formed on, and were detrimental for lagging strand replication. The leading strand was believed to exhibit too little ssDNA between the MCM exit pore and Polε for secondary DNA structure formation. Our results challenged this view, and argued for the presence of substantial amount of ssDNA (>25 nt) on the leading strand to allow for a pioneer G4 to form. This suggestion was later supported by (i) single-molecule work with purified bacterial replisomes, which revealed frequent pausing of the leading strand polymerase and uncoupling from the DnaB helicase (Graham et al., 2017), and (ii) cryo-EM structure of the *S. cerevisiae* and human replisomes, which revealed the presence of a 100-140 Å space between the MCM exit pore and the Polε catalytic site, corresponding to 28-40 nt of unstretched ssDNA (Jones et al., 2021; Yuan et al., 2020).

Our work did not provide information regarding G4 formation between Okazaki fragments, and whether they can form there innocuously remains an open question.

4. G4s ability to induce genomic instability depends on their structural and thermodynamic properties

A puzzle in the laboratory was the inconsistent behavior of another G4-forming human tandem array: CEB25. Unlike for CEB1, *PIF1* deletion and Phen-DC_3 treatment failed to induce expansion/contraction

of CEB25. Furthermore, the high GCR rates induced by CEB25 depended on its high affinity binding sites for the conserved telomere-binding protein Cdc13^{POT1}, but not its G4-forming motifs. I expanded this observation to three additional synthetic G4-forming arrays, two of which remained stable in Pif1-deficient cells and Phen-DC_3-treated wild-type cells. It led me to hypothesize that G4 structural features governed their propensity to induce recombinogenic lesions, which I investigated in collaboration with structural biologists Michael Adrian and Brahim Heddi in Anh Tuan Phan's lab (NTU, Singapore) and Frédéric Samazan in the lab. A main difference between CEB1 and CEB25's G4 motifs was the presence of a long 9-nt loop in CEB25 (**Figure 6a, b**). Replacement of this loop by a single nucleotide was sufficient to trigger CEB25 instability in conditions that destabilize CEB1. Conversely, integration of this 9-nt loop in CEB1's G4 motif abrogated its instability. Systematic loop length analysis using CEB25 revealed that only G4 containing short (1 or 2 nt) loops caused destabilization of the underlying array. Since increasing loop length lowers the G4 structure stability (Guédin et al., 2010), we surmised that long-lived G4s interfered with replication. This hypothesis was verified at equal loop length, by exchanging pyrimidine-containing loops with the least stable purine-containing ones (Guédin et al., 2008), which abrogated the array instability in cells. Bioinformatics analysis and re-analysis of previously published datasets revealed that these "at risk" G4 sequences were depleted from the *C. elegans* and *H. sapiens* genomes and hotspots for genome rearrangements and DNA damage, respectively. Consequently, this work defined a class of highly stable G4 structures at risk for genomic stability in distantly related eukaryotes (Piazza et al., 2015) (**Figure 6c**). More extensive bioinformatics analysis corroborated and generalized this observation to various other eukaryotes (Puig Lombardi et al., 2019).

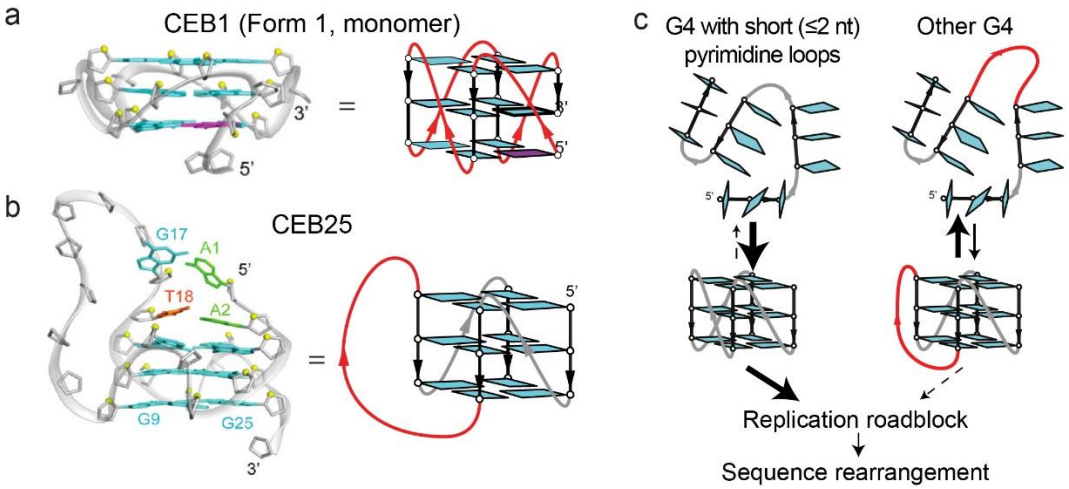

Figure 6: Structure-function relationship identifies the subset of most thermodynamically stable G4 as being at-risk for genomic stability. ***(a, b)*** *Schematic representation of one of the G4 formed by the CEB1 motif* ***(a****, "Form 1") and the monomorphic G4 formed by the CEB25 motif* ***(b)****. From (Adrian et al., 2014; Amrane et al., 2012)* ***(c)*** *Instability rules defined as a function of G4 structural features. From (Piazza et al., 2015).*

5. Evidence for non-canonical G4s in cells

Amusingly, the many G-tracts of CEB1 (on which most of the characterization of G4-induced genomic instability was conducted) did not resemble the "at-risk" G4 motifs defined with CEB25 mutagenesis, thus reversing the initial puzzle. CEB1 motif contained a G-sextet, two G-triplets and a G-doublet separated by

single nucleotide loops, and a last, more distant G-triplet. This G-tract combination formed a mixture of different G4s that could be isolated from one another through motif truncation and mutagenesis. The lab of Anh Tuan Phan had just published a NMR structure of an unusual G4 structure (*Form 1*) for CEB1 bearing single nucleotide loops but containing a terminal G-triad filled by an extra guanine from the G-sextet (Adrian et al., 2014) (**Figure 6a**). This unusual structure did not require the canonical four G-triplets. Would one validate the existence of such G4 structure in cells, the definition of G4-forming motifs in genomes be greatly expanded. In collaboration with AT Phan's lab and Xiaojie Cui in the lab, we determined the NMR structure and the *in vivo* instability of CEB1 variants systematically mutated for their G-tracts, individually or in combination. Four G4 conformations were isolated, all bearing unusual structural features. Most CEB1 instability could be attributed to the aforementioned *Form 1*, and *Form 2* that contained an incomplete G-quartet. This structure-function analysis revealed the existence of G4s with vacant guanine spots (*i.e.* G-triads) in cells, thus expanding the definition of G4 motifs to G-rich sequences lacking four G-triplets (Piazza et al., 2017a).

6. Conclusion

Overall, my Ph.D. work provided the first demonstration of (i) the existence of G4s in cells, (ii) the role of Pif1 in unwinding them, (iii) their pathological interference with DNA replication, and (iv) defined the structural rules governing the level of threat they pose in genomes. These findings were made possible only thanks to prolific inter-disciplinary collaborations.

B. Mechanism of homology search during recombinational DNA repair and consequences for genomic stability

Referring to the following publications (Piazza et al., 2017b, 2018, 2019, 2021a, 2021b; Shah et al., 2020) and reviews (Piazza and Heyer, 2018, 2019a, 2019b; Savocco and Piazza, 2021)

Thanks to the rich scientific environment of the Institut Curie, I was made aware of many outstanding current problems in biology. None sparked more vividly my interest than the puzzle of the homology search process occurring during DNA double-strand break repair by homologous recombination (HR), a formidable "needle-in-the-haystack" feat for which no clear mechanism was in sight (Barzel and Kupiec, 2008). I decided to devote my post-doctoral internship to this problem in the lab of Wolf-Dietrich Heyer (UC Davis), whom I became familiar with during his sabbatical in our lab in 2011. In agreement with him, I devised a research project with two main parts:

- Develop an assay enabling detection of D-loops, *i.e.* the earliest DNA joint molecules formed upon successful homology encounter in cells. Such assay would provide the best available proxy to investigate nucleoprotein factors involved in homology search, and study core regulations of the HR pathway.
- Investigate the existence and potential pathological consequences of a new type of DNA joint molecules the lab had just identified in reconstituted *in vitro* reactions with yeast and human recombination proteins, called "multi-invasions" (MI) (Wright and Heyer, 2014), and which was predicted from the inter-segmental contact sampling homology search model proposed for RecA (Forget and Kowalczykowski, 2012).

Besides enjoying Wolf's full support and expertise to dive into this rich and complex research topic, I benefited from the expert help of William Wright, a post-doc in the lab who trained me to HR biochemistry. I am also grateful to two undergraduate students I had the chance to mentor, Abou Ibrahim-Biangoro and Noelle Cabral, for their help with yeast strains construction and genetics experiments.

This work was pursued upon returning to France in the laboratory of Romain Koszul (Institut Pasteur), to gain access to the spatial genome organization dimension integral to the homology search process. His lab provided a perfect environment to train to high-throughput contact genomic approaches and analysis, and become more familiar with concepts in spatial chromatin organization.

1. Development of proximity-ligation-based assays for the study of D-loop metabolism

HR is a complex pathway that involves various covalent and non-covalent alterations and associations of DNA molecules. Covalent alterations are 5'-3' resection of the broken DNA ends, extension of annealed 3'-OH DNA ends by DNA polymerases, DNA strand exchange junction cleavage, and ligation of DNA extremities. The resulting intermediates can be detected thanks to molecular assays, which greatly advanced our understanding of the molecular mechanisms at play. Non-covalent intermediates and steps are (i) heterologous and homologous NPF-dsDNA associations, (ii) DNA strand invasion resulting in a DNA joint molecule called a D-loop, and other types of DNA joint molecules (some of which can be topologically linked), (iii) D-loop disruption, and (iv) second-end annealing. Physical detection of these intermediates has proven more challenging. Uniquely, the ability to physically detect the DNA joint molecules formed at the pachytene stage of meiosis by bidimensional gel electrophoresis greatly advanced our understanding of their formation and processing by a variety of enzymes (reviewed in (Hunter, 2015)). It contrasted with the mitotic situation, in which DNA joint molecules could only be detected with great difficulty (Bzymek et al., 2010). As a consequence, the mechanisms and regulations of core HR steps (*i.e.* homology search, DNA strand invasion, and D-loop metabolism) in vegetatively growing cells were mainly inferred from physical or genetic end-point assays coupled with protein biochemistry. These works implicated various conserved helicases/topoisomerases such as Srs2^{FBH1}, Mph1^{FANCM}, and the Sgs1-Top3-Rmi1$^{BLM\text{-}TOPO3\alpha\text{-}RMI1/2}$ (STR) complex in protecting against HR-mediated genomic instability and toxicity (reviewed in (Heyer, 2015; Putnam and Kolodner, 2017)). However, their precise substrate(s) and mechanisms of action *in vivo* remained elusive, and their pathway organization difficult to disentangle from end products alone, as they can possibly act at various stages of the pathway. Furthermore, the role of certain proteins in D-loop metabolism, such as the Rad54 paralog Rdh54, or the Rad51 paralogs Rad55-Rad57, could not be straightforwardly addressed *in vitro*, as the substrates, interactors, or the relevant reaction conditions are ill-defined.

In order to better characterize the mechanisms and regulations of the core HR steps, I wished to gain access to the earliest stand-alone DNA joint molecule of the pathway: the D-loop. Informed by the rationale of chromosome conformation capture (3C) methodologies thanks to Elphège Nora in Edith Heard's lab, I realized I could use a similar approach coupled with the high dynamic range of a quantitative PCR output to detect elusive DNA joint molecules formed at an ectopic homologous region. This led to the development of the D-loop Capture (DLC) assay in haploid *S. cerevisiae* cells (**Figure 7a**). The DLC assay involved the dsDNA-specific crosslinker psoralen (previously used for DNA joint molecule detection by bidimensional gel electrophoresis (Bell and Byers, 1983; Schwacha and Kleckner, 1994)) rather than the protein-DNA crosslinker formaldehyde classically used in 3C-type and chromatin immunoprecipitation protocols. The

restriction site lost to resection on the broken molecule was restored by annealing of a long complementary oligonucleotide. This assay proved exquisitely sensitive, enabling detection not only of D-loops, but of even more elusive intermediates such as multi-invasions (see below). Following the same general proximity ligation rationale, but this time without any crosslink, I could detect the initiation of DNA synthesis (**Figure 7b**), as well as formation of BIR, MIR and crossover repair products ((Piazza et al., 2018) and unpublished). These assays filled an important void in our ability to chart HR steps in cells, by enabling kinetics study of both D-loop formation and extension at an inter-chromosomal donor (**Figure 7c**). Nonetheless, the DLC assay suffers from limitations inherent to the crosslink density (estimated at 1/500 bp), as do bidimensional gel electrophoresis-based assays. Indeed, a variation in DLC signal across conditions may be due to a change in average D-loop number or D-loop length in the cell population, which must be considered while interpreting results.

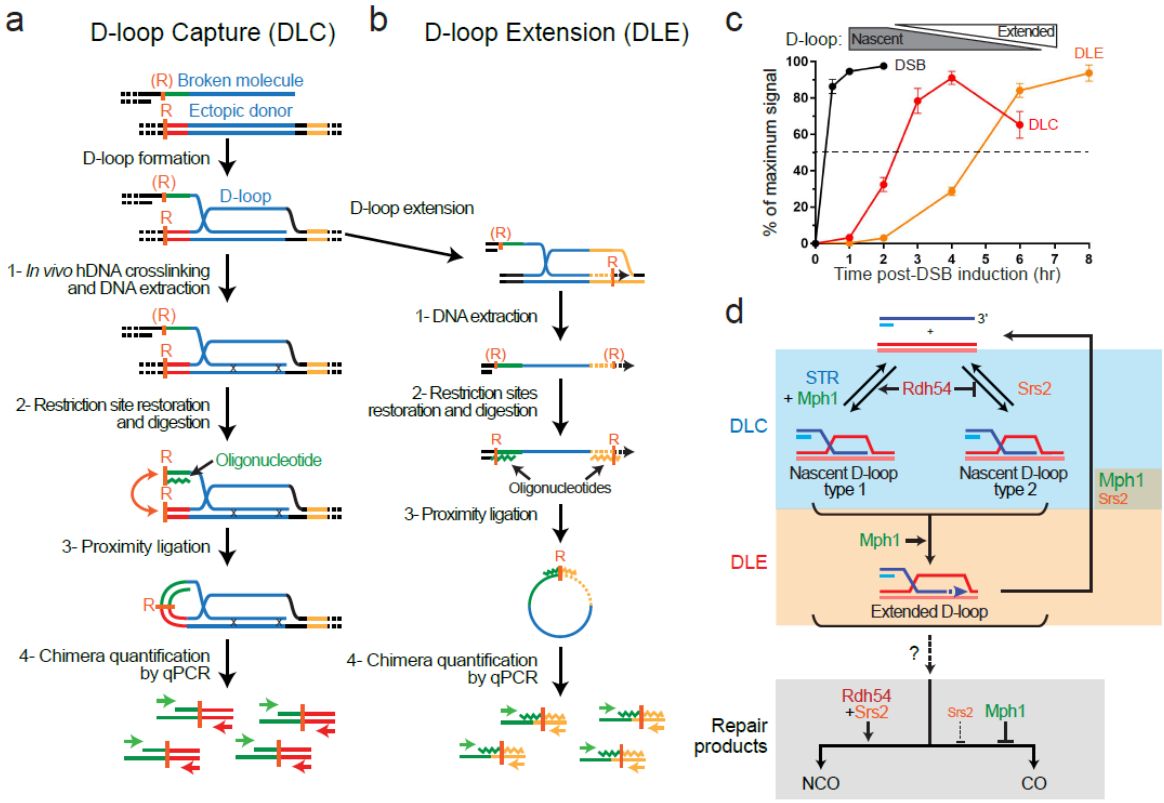

Figure 7: Development of proximity ligation-based assays for the study of D-loop metabolism in S. cerevisiae. *(**a-b**) Rationale of the DLC and DLE assays, which enable (**c**) kinetics study DSB repair by HR following efficient site-specific induction in S. cerevisiae. (**d**) Model of nascent and extended D-loop metabolism by Mph1^{FANCM}, Srs2^{FBH1}, STR and Rdh54 at a perfectly homologous 2 kb-long inter-chromosomal donor in haploid S. cerevisiae cells. From (Piazza et al., 2019).*

First, I investigated the role of various HR regulators in D-loop metabolism, using a 2 kb-long ectopic inter-chromosomal DSB-donor system in haploid cells: Srs2^{FBH1}, Mph1^{FANCM}, the STR complex and, unexpectedly, Rdh54^{RAD54B} defects led to elevated nascent D-loop levels. Determination of their genetic interactions revealed the existence of two D-loop reversal pathways: one supported by the helicase activity of Mph1^{FANCM} together with the topoisomerase activity of the STR complex while the other is supported by

the helicase activity of Srs2^{FBH1}. Rdh54^{RAD54B} acts independently of its catalytic activity, is epistatic with the Mph1-STR disruption pathway, and combined defects to that of Srs2 leads to a synergistic increase in DLC levels. Fine measurements of the kinetics of the initiation of D-loop extension revealed delays upon Mph1-STR defects, but not Srs2 or Rdh54 defects. Altogether, these results suggested a model whereby two types of D-loops are substrate for independent reversal pathways: Mph1-STR on the one hand, and Srs2 on the other hand. Rdh54 delineates these disruption pathways, presumably at the DNA strand invasion step, by providing short D-loops for disruption by Mph1-STR (**Figure 7d**). Defects in Rdh54 results in longer D-loops as shown *in vitro* (Shah et al., 2020), which would now exclusively rely on Srs2 for reversal. Why Srs2 cannot disrupt short D-loops substrate for Mph1-STR, and why its action does not delay the initiation of D-loop extension remains to be determined.

In conclusion, this work identified the complex regulations playing out at the D-loop level, which involved several conserved HR regulators. Perhaps more importantly, they revealed that the majority of D-loops are reversed prior to extension, despite being formed at a 2 kb region of perfect homology. This constitutive D-loop reversal suggests the existence of a kinetic proofreading mechanism at the D-loop level, which improves the donor selection process. Hence, the stringency of the homology search step of HR results from overlaid homology assessment steps: first at the NPF level, and second at the D-loop level (discussed in (Piazza and Heyer, 2019b; Savocco and Piazza, 2021)). Finally, it underscores the value of physically detecting transient non-covalent associations formed during the early steps of HR to determine its mechanisms and the part played by various nucleoprotein actors. These assays continue to be used and improved in the laboratory.

2. Multi-invasion-induced rearrangements: a genome destabilization mechanism originating from homology search byproducts

DNA substrates mimicking physiological resection products, which contained hundreds- to kilobase-long ssDNA and a 5’ dsDNA extremity had been developed by William Wright prior to my arrival in the Heyer lab. Contrary to the classically used 100-mer oligonucleotides, these substrates frequently formed DNA joint molecules involving multiple homologous dsDNA in addition to the classical D-loop in reconstituted recombination reaction *in vitro* (Wright and Heyer, 2014). We demonstrated that these “multi-invasions” (MI) intermediates could form on independent donors, and involved a DNA strand invasion reaction occurring internally to the NPF *in vitro*, in line with the intersegmental contact sampling model defined earlier for RecA-mediated homology search (Forget and Kowalczykowski, 2012). They differed from multi-chromatid joint molecules identified by bidimensional gel electrophoresis during meiosis, which were fully dsDNA, catenated, Holliday junctions-containing intermediates (Oh et al., 2007). These *in vitro* observations raised the possibility that MIs form in cells, tethering intact dsDNA molecules into a multi-branched intermediate possibly at risk for genome stability.

I addressed the existence of these intermediates and their possible pathological consequences for genomic stability upon site-specific DSB induction in diploid *S. cerevisiae* cells (**Figure 8a**). Thanks to the DLC assay I could provide evidence for MI in cells. Importantly, they led to translocation between two intact dsDNA donors with frequencies reaching up to a percent in wild-type cells. This multi-invasion-induced rearrangement (MIR) occurred in the absence of homology between the donors, and inserted the intervening sequence from the NPF between the two invasion sites. It did not require extensive displacement DNA synthesis, as indicated by the modest reliance on Pol32, which is required for long-tract gene conversion and break-induced replication (BIR). Instead, it relied on the redundant action of conserved structure-

selective endonucleases: Mus81-Mms4$^{MUS81-EME1/2}$, Yen1^{GEN1}, and Slx1-Slx4 (Schwartz and Heyer, 2011). Such DNA joint molecule processing caused the transfer of a break on the donor chromosome. Differently, the Rad1-Rad10$^{XPF-ERCC1}$ endonuclease suppressed MIR, as did the D-loop reversal activities of Srs2^{FBH1}, Mph1^{FANCM}, and STR. Substrate variants indicated that Rad1-Rad10$^{XPF-ERCC1}$ prevented MI formation by clipping the 3'-tail produced upon internal invasion. Genetic interactions between other MIR-suppressing activities suggested they target independent parts of MI. Hence, various proteins collaborate in suppressing MIR.

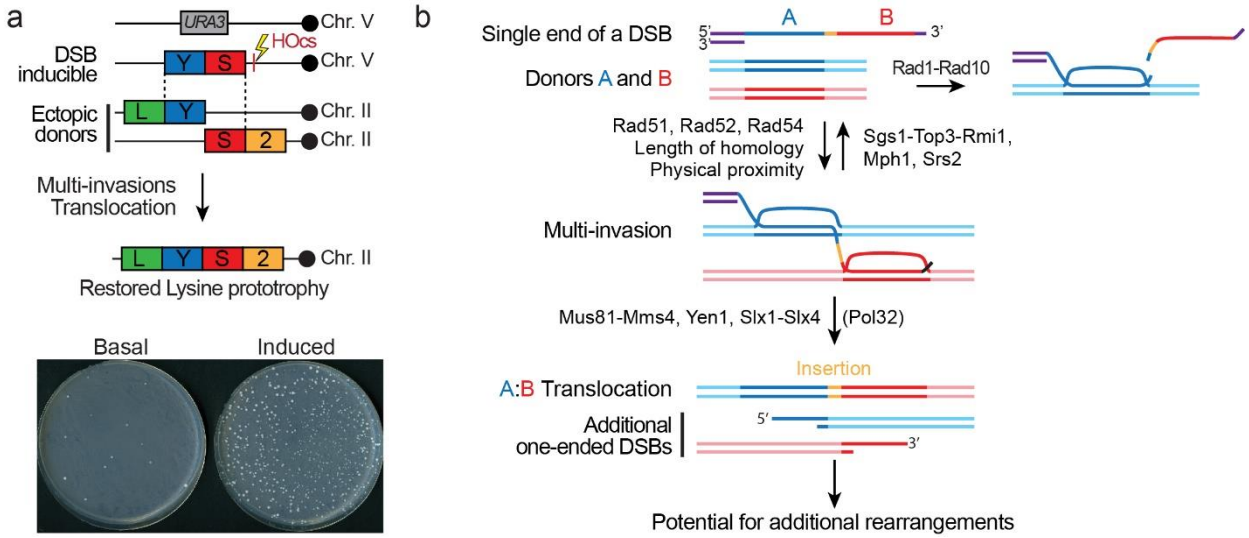

Figure 8: Outline of the Multi-invasion-Induced Rearrangement (MIR) pathway. *(**a**) Top: Experimental system in diploid S. cerevisiae selecting for the translocation of undamaged donors upon break formation at an ectopic site on a third chromosome. Bottom: Example of selective plates before and 2 hours after DSB induction. (**b**) Model for MIR. From (Piazza et al., 2017b).*

Formation of the MIR translocation was occasionally accompanied by additional, unselected rearrangements involving the break and donor sites. The frequency of these secondary rearrangements depended on the sequences flanking the donor sites, from 15% with donors present at an allelic site (*i.e.* sharing flanking homologies) to 75% with ectopic donors. We interpret these results as reflecting the opportunities provided by flanking homologies for accurate repair of secondary breaks generated on the donor during MI processing by structure-selective endonucleases, otherwise leading to additional rearrangements. Finally, examination of the segregation of the MIR translocation with the remaining donors suggested the existence of two specific MIR mechanisms: one fully endonucleolytic applicable in any sequence context and capable of causing additional rearrangements; and the other requiring displacement DNA synthesis resulting in an insertion without unrepaired break at the donor sites. We pursue the fine characterization of these MIR mechanisms and their respective impact on genomic stability (Piazza *et al.* in preparation).

In conclusion, we uncovered and characterized MIR, a genome destabilization mechanism originating in the very process of homology search and DNA strand invasion of HR (Piazza et al., 2017b) (**Figure 8b)**. It bears unique, most detrimental features for genomic stability compared to other low-fidelity HR sub-pathways (discussed in (Piazza and Heyer, 2018, 2019a)). First, MIR not only fails to repair the initial break, but generates two new single-ended breaks that generate secondary rearrangements at high frequency

from a single initiating lesion. This break amplification capacity may participate in the abrupt acquisition of several chromosomal rearrangements known as chromothripsis (Stephens et al., 2011). Second, the extent of the rearrangement does not depend on extensive displacement DNA synthesis as suggested by the minimal involvement of Pol32, unlike BIR (Lydeard et al., 2007). Third, MIR involves DNA strand invasion at regions of homologies distant from the break site. It greatly expands the sequence space at risk for HR-mediated chromosomal rearrangements relative to the canonical DSBR model, which requires the break to fall into a repeated element (Szostak et al., 1983). Fourth, MIR only involves one side of the break. Consequently, single-ended breaks generated upon replication fork cleavage, or long ssDNA accumulating as a result of BIR synthesis, may be a source of MIR. Finally, we discussed the possible involvement of MIR in crossover and non-crossover formation during meiosis (Piazza and Heyer, 2018), as it parsimoniously accounts for the position of conversion and heteroduplex DNA tracts relative to the break site in a large number of cases (Ahuja et al., 2021; Marsolier-Kergoat et al., 2018; Martini et al., 2011), the existence of multi-chromatid joint molecules (Oh et al., 2007), as well as the presence of Dmc1-dependent dsDNA-ssDNA junctions of unexpected polarity in the vicinity of Spo11-mediated hotspots (Mimitou et al., 2017).

3. Spatial reorganization of chromatin following DSB formation in *S. cerevisiae*: determinants and functional consequences for homology search.

Homology identification entails the spatial encounter between the NPF and a homologous dsDNA molecule. It is subordinated to the initial spatial position of the two partners (Agmon et al., 2013; Lee et al., 2016a). Whether, how, and to what extent the spatial chromatin organization is affected in response to a DNA break, and the functional consequences of this reorganization on homology search, had not been investigated. I set out to address these questions by combining the DLC assay with contact genomic approaches (Hi-C) when I joined the lab of Romain Koszul (Institut Pasteur) as a reintegration post-doc. His lab provided a unique environment in France for the study of principles governing spatial genome organization across life kingdoms, which proved instrumental in the realization of this project, both scientifically and technically. This work was pursued as a collaboration after I set up my own laboratory at the ENS Lyon in 2020. I am particularly grateful to Hélène Bordelet, the first post-doc of my lab who performed a 1-year stay in Romain's lab for her enthusiasm, dedication, and sheer experimental and scientific aptitude.

We show that in *S. cerevisiae*, DNA double-strand break repair by HR occurs in a chromatin context spatially reorganized at the global and local levels by cohesins and resection-associated factors, respectively (**Figure 9a-c**). Genome-wide, cohesin folds chromatin as arrays of ~20 kb-long loops into individualized metaphase chromosomes (**Figure 9c**). Loop bases, where cohesin resides, are the regions most insulated from inter-chromosomal interactions, buried at the chromosome axis. This folding is independent of replication and the presence of a sister chromatid. Locally, the dsDNA region across the break and resection tracts are tethered to one another in an MRX-, Exo1-, and Ddc1-Mec3-Rad17 (human 9-1-1) clamp-dependent manner (**Figure 9b**). Downstream HR factors ($Rad52^{BRCA2}$, Rad51 and Rad54), checkpoint activation ($Mec1^{ATR}$), as well as cohesin and the sister chromatid are dispensable. While the Mre11-Rad50-$Xrs2^{NBS1}$ SMC-like complex and Exo1 were previously implicated in end-tethering (Nakai et al., 2011), the 9-1-1 clamp, present at the dsDNA near the resection junction (Bantele et al., 2019; Majka and Burgers, 2003; Majka et al., 2006), was not. Since Rad17 dimerizes *in vitro* (Zhang et al., 2001), end-tethering may involve direct 9-1-1 clamp interactions. Contacts between the resected tracts (RPA-bound ssDNA and NPF)

could not be detected with Hi-C, and whether other tethering interactions take place at that level remains unknown. These local and global layers of organization intersect: the DSB region acts as a cohesin translocation roadblock, causing both (i) their accumulation in the DSB region and (ii) side-specific expansion of chromatin loops on each side of the DSB. Both side-specific loop expansion and cohesin accumulation in the DSB region was lost in the absence of the 9-1-1 clamp. It indicates that, besides mediating end-tethering, the 9-1-1 clamp also blocks cohesin translocation. We suspect they may be two facets of the same coin: end-tethering opposes a barrier to cohesin translocation, akin to that mediated by centromere-kinetochore interaction (**Figure 9b**). Functionally, these local and genome-wide levels of chromatin reorganization constrain homology search locally in two main ways: by inhibiting *trans* contacts upon NPF trapping at the chromosomal axis and upon general chromosome individualization; and by promoting *cis* contacts with the flanking chromatin within the loop expansion span (Piazza et al., 2021a) (**Figure 9d-e**).

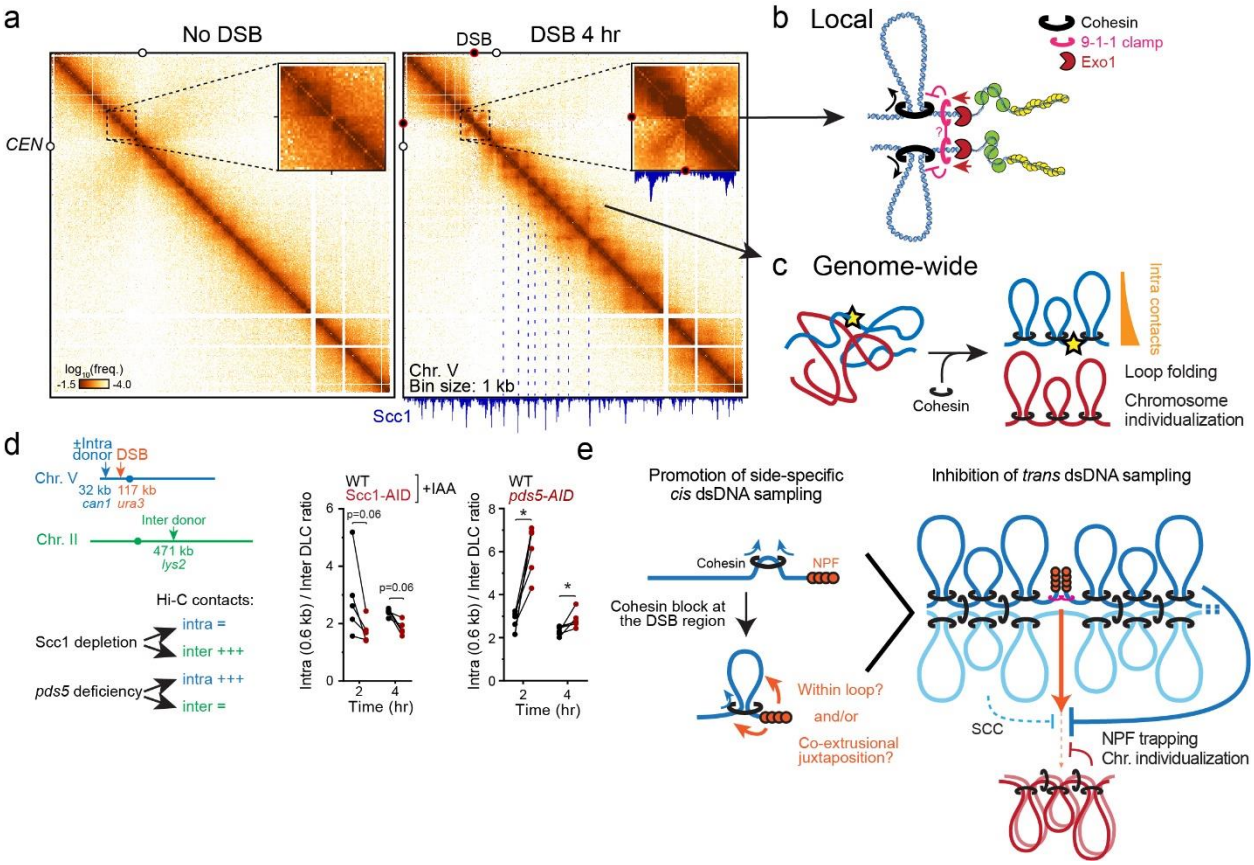

Figure 9: Cohesin regulates homology search in multiple direct and indirect ways*. (**a**) Site-specific DSB induction causes local (**b**) and genome-wide (**c**) reorganization of chromatin. (**b**) Model of the local tethering interaction across the DSB region acting as a cohesin translocation roadblock, and of its determinants. (**c**) Model of the genome-wide reorganization of chromatin as arrays of loops mediated by cohesin, and its consequences for inter-chromosomal contacts. (**d**) Cohesin bias homology search towards intra- over inter-chromosomal donors in two ways: by inhibiting trans DSB-dsDNA interactions (abrogated in Scc1-depleted cells) and by promoting donor identification in the loop extrusion span (extended in a pds5 mutant). Note that both effects are more pronounced at 2 than at 4 hours post-DSB induction (see*

*below). (**e**) Model for the cohesin-mediated regulation of homology search in mitotic cells.* From (Piazza et al., 2021a)

This work thus revealed the existence of two independent layers of spatial chromatin reorganizations elicited by the formation of a DNA break in mitotically dividing cells, delineates at least a subset of their protein determinants, and identifies multiple ways by which cohesin restrain homology search in the local chromatin environment: both by promoting side-specific *cis*, and by inhibiting *trans*, DSB-dsDNA contacts (**Figure 9e**). We noted that opposite order of chromatin loop folding and DSB formation in meiosis may emancipate DSB ends from cohesin entrapment at the chromosome axis, thus relieving a constraint for inter-homolog recombination.

4. Conclusion

My post-doctoral work was dedicated to the study of core HR steps: the homology search process and the metabolism of D-loop joint molecules. It led to (i) the identification of a multi-invasions, a type of DNA joint molecules byproduct of the parallelized process of homology search and DNA strand invasion, (ii) the characterization of a genome rearrangement mechanism originating from their endonucleolytic processing, (iii) a better understanding of D-loop turnover activities and interactions, which broadened the definition of homology search, and (iv) characterized the spatial reorganizations of chromatin following formation of a DNA break, their determinants, and their functional impact on homology search. These projects are currently being expanded upon in my laboratory. This work benefited greatly from the development of dedicated methodologies for HR intermediates and products detection. Although costly and risky, I consider them key investments in the scientific advancement of research goals, and keep dedicating substantial resources to methodological development.

IV. Research projects

Long-term research projects of the laboratory aim at better characterizing the mechanism of homology search in mitotically dividing cells (**part A**), and determine the nature of the regulation enabling homologous chromosomes recognition and recombination in meiosis (**part B**).

A. Mechanism of homology search in somatic cells

Homologous recombination (HR) is a universal pathway for the repair of DNA double-strand breaks (DSBs). HR repair entails the identification of a homologous dsDNA molecule in the genome and nucleus, to be used as a template. It is a particular case of target search in which a single entity (the DSB, itself DNA) searches for a limited set of targets (*i.e.* the sister chromatid, the homolog, or ectopic repeats). Homology search has two requirements: (i) at the molecular level, the ability to interrogate surrounding dsDNA molecules (hereafter "homology sampling"), a process primarily studied *in vitro*; and (ii) at the cytological level, opportunities for spatial encounters between the searching molecule and its target. The first research axis aims at characterizing the basic mechanism of homology search during HR in *S. cerevisiae* cells, and its consequences for genomic (in)stability. It can be broken down into six broad parts:

- Mechanism of homology sampling by the Rad51-ssDNA nucleoprotein filament (NPF).
- Regulation of DNA strand invasion by the Rad54 and Rdh54 dsDNA translocases.
- Role of D-loop reversal activities in homology search.
- Competition between transcription and recombination machineries.
- Role of chromatin spatial organizers in regulating homology search.
- Role of Rad51-ssDNA NPF structure in overcoming initial spatial constraints.

To this end, we will employ high-throughput contact genomic approaches, molecular assays to track HR progression (including transient pairing and joint molecule intermediates), as well as computational modelling. The overall ambition of this research axis is the formulation of a quantitative and predictive framework of homology search in cells.

1. Micro-homology sampling by the NPF: don't get stuck on details.

Thanks to its multivalent nature, the NPF interrogates dsDNA by inter-segmental contacts, thus multiplexing the homology sampling process into discrete, physically tethered units (Forget and Kowalczykowski, 2012; Yang et al., 2020). Stable pairing within the NPF *in vitro* occurs at micro-homologous (microH) match of 8 nt (Danilowicz et al., 2015; Hsieh et al., 1992; Qi et al., 2015). The half-life of such microH pairing is in the range of minutes with bacterial RecA, and yeast and human Rad51 and Dmc1 (Lee et al., 2015, 2016b; Qi et al., 2015). This conserved 8-nt threshold is significantly below uniqueness, theoretically achieved only above 12 nt in the *S. cerevisiae* genome. Hence, for any given position along the NPF, there are ~740 microH matches, against one or a few allelic (macro-)homologous donor (**Figure 10a**). Consequently, whether homology sampling is carried out by 8 nt microH units in cells, and how the massive excess of microHs of the genome affects homology search kinetics and success, remains unknown. We suspect that (i) long-lived binding to the vast excess of microH of the genome titrates the search units of the NPF and thus inhibits homology search, and (ii) that specific NPF components are actively involved in their turnover.

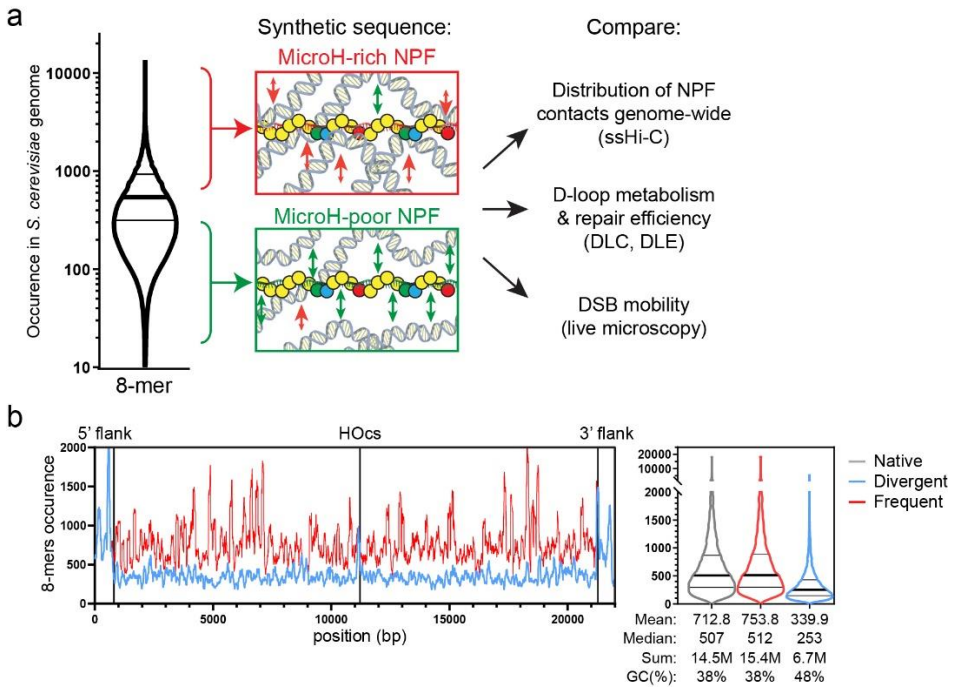

Figure 10: Study the role of microhomology association in homology search upon DSB induction in synthetic chromosomal regions*. (**a**) Distribution of all possible 8-nt microH in the S. cerevisiae genome (left), and rationale for the assembly of microH-rich and -poor synthetic sequences. (**b**) Distribution of 8-nt microH along the 20 kb-long "Frequent" and "Divergent" synthetic regions integrated at the ura3 locus on chr. V.*

To address these possibilities, we generated two 20 kb-long synthetic sequences: one containing as many ("Frequent"), and the other as little ("Divergent") microH match to the *S. cerevisiae* at each position (**Figure 10a-b**). They exhibit an average two-fold difference in the number of microH at every position, with marginal differences in GC content (**Figure 10b**). We assembled and integrated them at two genomic locations in *S. cerevisiae*, and will address their putative impact on homology search efficiency by quantifying (i) the contact profile made by the DSB region by Hi-C and ssDNA-specific Hi-C (in development in the laboratory, see below), (ii) the formation kinetics and yield of D-loops at an ectopically positioned donor using the DLC assay, and (iii) the local DSB mobility reflecting ongoing search by the NPF with cytological approaches (**Figure 10a**). We expect a defect in homology search that should be commensurate with the amount of competing microH to the NPF sequence. Placing a donor on the same chromosome as the NPF should favor their spatial encounter, thus requiring minimal microH sampling compared to an inter-chromosomal donor: the inter-chromosomal situation is expected to exacerbate the defect of the "Frequent" sequence. Finally, we expect a delay in D-loop formation and reduced local DSB mobility in mutants defective for microH turnover to be more pronounced in the "Frequent" than in the "Divergent" class The ATPase activity of Rad54 is a potential candidate, has it promotes the turnover of heterologous NPF-dsDNA association (Tavares et al., 2019), similar to RecA ATPase activity at 8 nt microH (Lee et al., 2016b). Their role in microH turnover will be mechanistically addressed further in reconstituted *in vitro* D-loop reactions challenged by varying amounts of microH in collaboration with the laboratory of WD Heyer.

2. Initiation of DNA strand invasion: a homology search stringency step?

D-loops are produced from Rad51 synaptic intermediates by a DNA strand invasion reaction catalyzed by Rad54 (Petukhova et al., 1998; Tavares et al., 2019; Wright and Heyer, 2014), a dsDNA translocase located both at the extremity and internally to the NPF (Mazin et al., 2000; Sanchez et al., 2013). Rad54 intertwines the homologously paired DNA strands within the NPF while simultaneously removing Rad51 from the resulting heteroduplex DNA (Tavares et al., 2019; Wright and Heyer, 2014) (**Figure 11a**). Rdh54 inhibits this reaction and limits hDNA length (Shah et al., 2020). Despite this detailed biochemical understanding, major questions pertaining to the DNA strand invasion reaction in cells remain.

Homology length and functional NPF segmentation: It is unknown how long the NPF synaptic pairing must be for DNA strand invasion to occur, and if there is a lower threshold preventing D-loop formation at short dispersed repeats. The intra-chromosomal DSB repair efficiency increases linearly with homology length past 250 bp of homology in yeast (Jinks-Robertson et al., 1993), at least an order of magnitude greater than the 8-nt microH recognition unit. Below 250 bp, recombination efficiency drops dramatically. This discontinuity at ~250 bp homology (coined the Minimal Efficient Pairing Segment, or MEPS) is roughly conserved in eukaryotes (Baker et al., 1996; Rubnitz and Subramani, 1984), but much lower (20-50 bp) in bacteria (Shen and Huang, 1986; Watt et al., 1985) and phage (Singer et al., 1982). Since bacterial RecA and phage UvsX catalyze DNA strand invasion autonomously (Shibata et al., 1979; Yonesaki and Minagawa, 1985) but eukaryotic Rad51 requires Rad54 (Petukhova et al., 1998; Sigurdsson et al., 2002; Wright and Heyer, 2014), I hypothesized that the position as well as the homology length threshold of this forward HR reaction is defined by the distribution of Rad54, and that of its antagonist paralog Rdh54 (Shah et al., 2020) in the NPF (Crickard et al., 2020).

To address these possibilities, we will compare the differential efficiency of D-loop formation at donors exhibiting increasingly fragmented homology compared to a continuous donor with the same total length of homology (**Figure 11b**). We expect these constructs to yield the length value at which microH pairing is converted to a nascent D-loop, and identify the factors involved. Preliminary evidence indicates that fragmenting homology from 2 kb to 8x260 bp segments does not affect D-loop formation, while splitting further severely impairs D-loop formation, in agreement with previous estimates of MEPS length. Ablation of Rdh54, which causes a 4-fold increase in the DLC signal with 2 kb-long donors, is reduced to 2-fold with 8x260 bp, and abolished with 16x130 bp split homologies. These results suggest that Rdh54 normally limit the length of D-loop formation by Rad54, and that heterologous block can substitute to this activity. Using fine-tunable expression systems, we will vary the absolute and relative cellular concentrations of Rad54 and Rdh54 to address their role in defining the MEPS. Stochastic *in silico* simulations will help determine the average distributions of Rad54 and Rdh54 in the NPF.

Intriguingly, the MEPS is shorter than the length of retrotransposon solo-long terminal repeat (LTR) elements; the most abundant dispersed repeat in the yeast genome (**Figure 11c**). It suggests that the HR machinery remains permissive to LTR-mediated chromosomal rearrangements, perhaps because it authorizes efficient adaptation to environmental conditions by causing copy number variation at relatively high frequency (Dunham et al., 2002; Gresham et al., 2010; Koszul et al., 2004). This possibility will be addressed using the aforementioned fine-tunable Rad54/Rdh54 expression systems in mutation accumulation experiments or dedicated repeat-mediated genetic systems (Putnam et al., 2009).

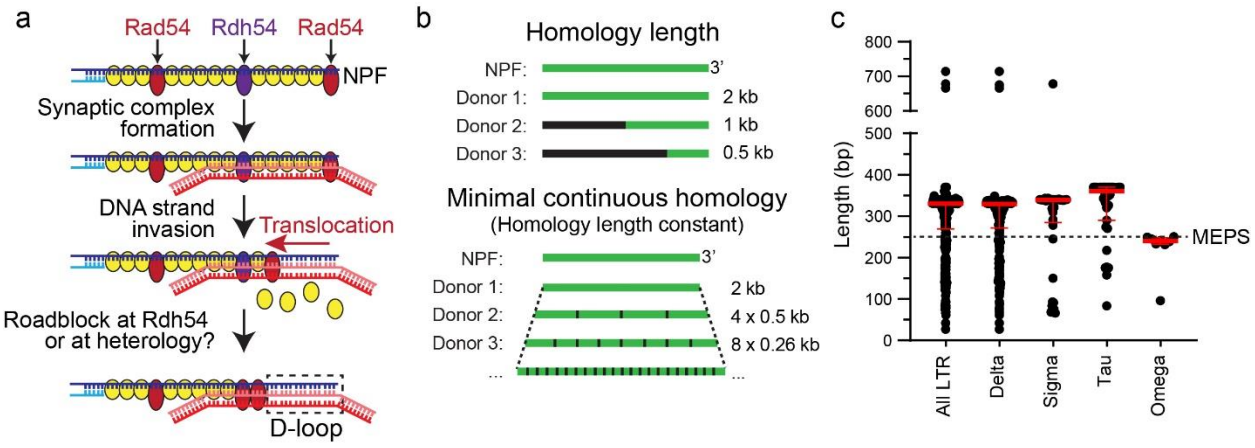

Figure 11: Investigating the determinants of the MEPS in S. cerevisiae*. (**a**) Mechanism of DNA strand invasion by Rad51/Rad54 (Wright and Heyer, 2014), and putative catalytic-independent role of Rdh54 in inhibiting it (Piazza et al., 2019; Shah et al., 2020). (**b**) Substrate variants to define MEPS determinants in S. cerevisiae using the DLC assay. (**c**) Average LTR length is above the MEPS, raising the possibility that HR is tuned to be permissive for LTR-mediated chromosomal rearrangements.*

Internal versus terminal homology usage: Owing to the short, parallelized dsDNA sampling taking place all along the NPF (Forget and Kowalczykowski, 2012; Yang et al., 2020), homology will statistically be encountered internally to the NPF first, not at the 3'-OH extremity. Terminal D-loops are formed only slightly more efficiently than internal D-loops in reconstituted reactions with purified yeast, human and bacterial recombination proteins (Piazza et al., 2017b; Wright and Heyer, 2014). In yeast cells, internal D-loops efficiently form up to 2 kb away from the DSB extremity (Piazza, unpublished). Such internal D-loop can lead to the use of DSB-distal repeats for DSB repair (Inbar and Kupiec, 1999; Jain et al., 2016). Furthermore, this capacity to form D-loops at multiple locations along the NPF also leads to the formation of MI joint molecules at risk for genomic stability (Piazza et al., 2017b). The specific topological requirements for D-loop formation at a distance from a freely rotating DNA end are not known, and will also be investigated thanks to donor substrate variants with homology to sequences at various distance from the DSB end. We will use these constructs further to investigate the role of type 1 (Top1 and Top3) and type 2 (Top2) topoisomerases in internal D-loop formation in single or multiple mutant contexts using inducible protein inactivation/degradation systems (*top3-cd* overexpression and Top2-AID depletion) (Lazar-Stefanita et al., 2017; Oakley and Hickson, 2002). Finally, the role of a set of candidates 3' flapase (Rad1-Rad10 and Mus81-Mms4) in enabling donor usage at internal D-loop will be investigated in the appropriate mutants.

3. Homology sampling is part of a greater loop: role of D-loop dynamics in homology search.

My post-doctoral work revealed that nascent D-loops exist in a dynamic equilibrium governed by multiple activities previously implicated in promoting HR fidelity (*i.e.* accuracy and conservative outcome, see (Putnam and Kolodner, 2017)). How this dynamic is productive in HR remains unknown. We suspect that nascent D-loop dynamics is an integral part of homology search, by embedding the mismatch tolerant sampling process by the NPF (Anand et al., 2017; Danilowicz et al., 2015) within rounds of kinetic proofreading at the D-loop level (discussed in (Piazza and Heyer, 2019b)). In this framework, a key

advantage of long homologies over shorter dispersed repeats is the disproportionate probability to be re-invaded thanks to the physical tethering provided at long homologies by nearby pairing and/or internal D-loops. Hence, kinetic proofreading cycles are expected to funnel the NPF to the longest available homology, provided that the entirety of the genome can be accessed (see below).

To address and achieve a quantitative understanding of this kinetic proofreading scheme, we will determine D-loop formation and extension (i.e. donor usage) in competitive donor situations and fit the results on stochastic simulations (see below). We will engineer an extensive battery of genetic constructs to address the competitor length, number, position (internal or terminal) and spatial localization relative to the NPF. We expect D-loop dynamics enforced by the aforementioned D-loop reversal activities and DSB mobility to increase invasion and usage of the longest available donor, which will be addressed in the appropriate mutants. Preliminary results indicate that, at equal homology length, D-loop reversal activities of STR, Mph1, Rdh54 and Srs2 reinforce usage of the spatially advantaged donor.

4. Transcription-recombination conflicts: *cis*-regulators of HR?

DNA is a template or substrate for various enzymes. How are mutually exclusive DNA metabolic activities coordinated, prioritized, or conflicts between them resolved? In which cellular and genetic contexts? A widely studied case of incompatible DNA-dependent processes are transcription-replication conflicts, in which a single DNA molecule is a template for both DNA and RNA polymerases. This constraint is so primordial that genome's structure mitigating the most detrimental head-on encounters (French, 1992; Srivatsan et al., 2010) were evolutionary selected in bacteria (Brewer, 1988; Zeigler and Dean, 1990). Molecular processes in *cis* and *trans* also evolved to coordinate the two processes: *E. coli* DNA replication is prioritized over transcription (French, 1992) while eukaryotic replication at the ribosomal DNA locus is regulated in *cis* by Fob1 at the replication fork barrier, which blocks replication fork progression unidirectionally (Brewer and Fangman, 1988).

What about recombination-transcription conflicts? Despite the obvious incompatibility between the transcription of the donor site and the formation of a Rad51 synaptic complex, DNA strand invasion and recombinational DNA synthesis, whether and how these processes are coordinated or prioritized remains largely unknown. Most worked focused on upstream steps: DSB repair pathway choice as a function of transcriptional and chromatin status (Aymard et al., 2014; Pfister et al., 2014), or the production of RNA and RNA:DNA hybrids at the break site (Fagagna, 2014; Sharma et al., 2021; Yang et al., 2021). Only recently did inhibition of BIR synthesis by head-on transcription was reported (Liu et al., 2021), akin to canonical DNA replication. Our goal is to investigate potential conflicts at the level of the Rad51 synaptic complex or D-loop intermediate: can transcription prevent the formation, or stimulate the dismantling of D-loops? Or can D-loops interrupt transcription? If transcription inhibit D-loop formation or cause their reversal, could this locally regulate the crossover/non-crossover repair outcome? Could it promote genomic stability by inhibiting half-crossover and MIR?

To this end, we will design ectopic donor sites flanked either by a silent, a strong, or an inducible promoter and compare (i) nascent D-loop levels and (ii) kinetics of the initiation of D-loop extension from these donors. Promoters will be positioned in order to transcribe the template strand or the displaced strand to address the existence of a permissive orientation, as only one end of the DSB needs to invade and be elongated in SDSA. Building upon these constructions, we will address the role of donor transcription in regulating both crossover and non-crossover frequencies, as well as MIR frequencies. Finally, using 5'-

MS2 and 3'-PP7-tagged RNA system, we will investigate whether Rad51 synaptic complex formation and/or a D-loop can stall transcription. Recapitulation of the *in vivo* results in reconstituted *in vitro* experiments will be attempted in order to define the minimal components involved. Preliminary results indicate that donor transcription strongly inhibits D-loop formation and/or stability, as well as their extension (**Figure 12a**). The inhibition can be triggered acutely upon transcription induction right before cell collection and crosslinking, ruling out nuclear relocation or chromatin reorganization of the donor site as the underlying cause. Furthermore, strong transcription of one of the two donor in the MIR system in diploid cells lead to a significant decrease in MIR (**Figure 12b**). These exciting results suggest the existence of a local layer of regulation of DNA recombination responsible for an uneven propensity of the genome to undergo crossovers and genome rearrangements. It may also be of relevance for meiotic recombination in *S. cerevisiae* and other PRDM9-devoid organisms, as DSBs are inflicted in active gene promoters, and thus have invariably at least one end invading in opposite direction relative to transcription.

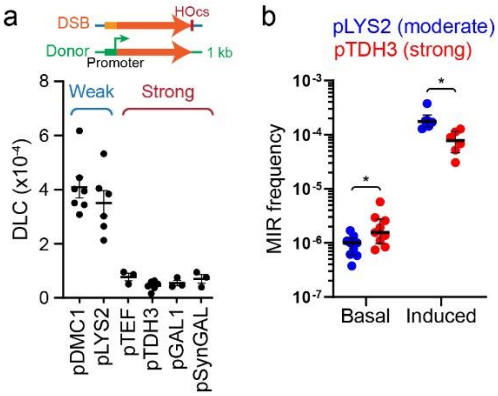

Figure 12: Donor transcription inhibits D-loop formation and MIR. ***(a)*** *D-loop measured by DLC 2 hours post-DSB induction at a 1 kb-long inter-chromosomal donor co-directionally transcribed either weakly or strongly.* ***(b)*** *Influence of the internal donor transcription on MIR.*

5. Role of spatial chromatin organizing activities in homology search and consequences for genome maintenance and evolution

We recently showed, in collaboration with the Koszul lab, that local and global spatial chromatin organization mediated by cohesin and HR-specific factors regulate homology search by regulating DSB-dsDNA contact probability genome-wide in mitotically-dividing *S. cerevisiae* cells (Piazza et al., 2021a). In particular, the localization of cohesin at the resection front tethers the base of the NPF at the chromosome axis. This configuration presumably brings the NPF (i) in close contact with the sister chromatid (which cannot be studied with our experimental setup as both sister chromatids are cut), and (ii) at the chromatin extrusion site. Notably, using a hypomorph mutant of the ancillary cohesin factor Pds5 that increases chromatin loop length ~4-fold (Dauban et al., 2020), we showed that D-loop formation was stimulated at a donor located in the now-expanded loop extrusion span (Piazza et al., 2021a). This coupling between a searching entity (*i.e.* the NPF) and a processive translocase (*i.e.* the cohesin) has the potential to convert a diffusion-limited 3D search into a facilitated directional 1D search. But how to distinguish between this unidimensional search mode and a distributive one within the resulting loop (**Figure 13a**)? To distinguish between these two possibilities, we setup a competitive situation with multiple donors in *cis* located at increasing distances from, yet equivalently contacted by, the DSB site (**Figure 13a-b**). If target search occurs by 3D diffusion within the resulting loops, all donors should be equally identified by the DSB site. If donor identification occurs efficiently via a 1D scanning mechanism, however, proximal donors should be preferred over distal ones (**Figure 13a**). Four donors were positioned in the 30-60 kb range, so as to be

within the loop extrusion span specifically in a *pds5* mutant, with the three most proximal donors 1 kb apart (**Figure 13b**). Their absolute and relative identification and usage was scored over time following DSB induction (**Figure 13c**). Preliminary results reveal that donors were used in decreasing order from their distance to the DSB at early times (2 h) following DSB formation in a *pds5* mutant. Differently, the three closest donors could not be teased apart in a WT strain. Although it does not exclude a diffusive component, these results suggest that homology search partly proceeds via a long-range 1D search over the cohesin-mediated loop expansion span during the early stages of homology search. These preliminary observations will be investigated further with additional donor contexts and in cohesin mutants.

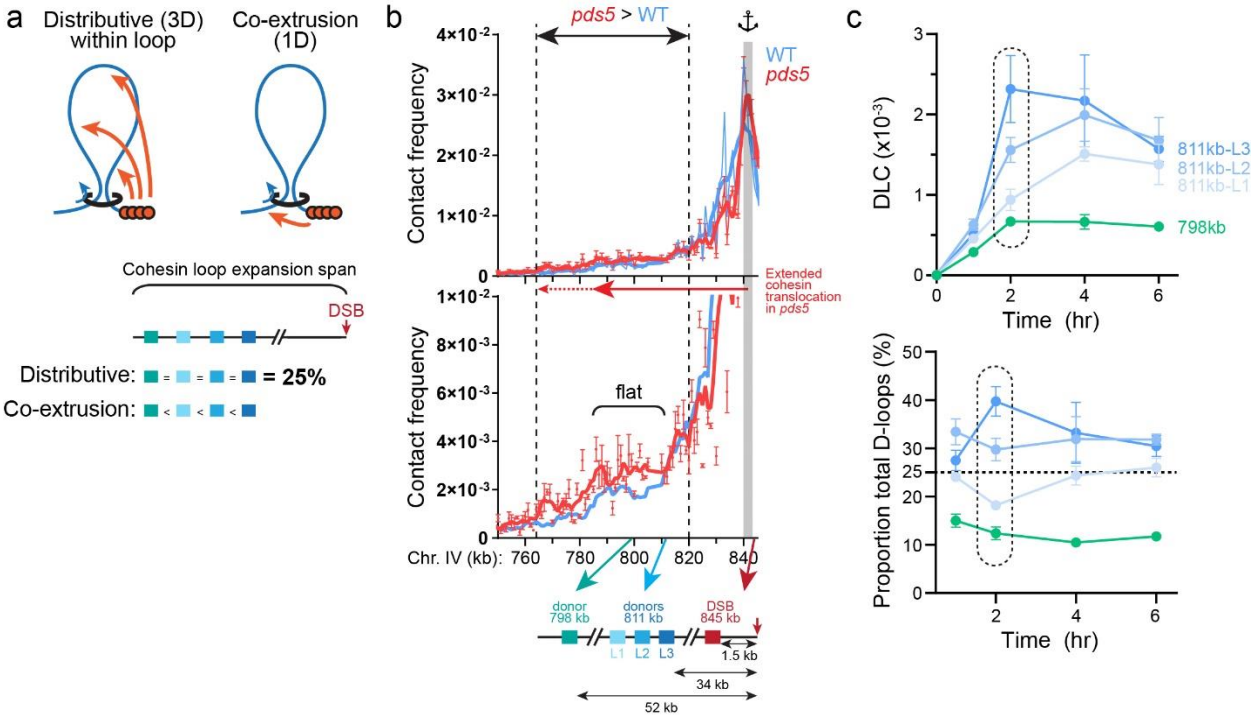

Figure 13: Does NPF piggy-backing on cohesin promotes unidimensional homology search in cis? ***(a)*** *Two possible ways by which homology identification is stimulated within the cohesin-mediated loop expansion span (Piazza et al., 2021a), and rationale of the competitive donor situation to tease them apart.* ***(b)*** *4C-like representation of DSB-flanking contacts obtained by Hi-C 4 hours post-DSB formation on chr. IV in wild-type and pds5 mutant.* ***(c)*** *Time course analysis of D-loop formation at the four cis donors following DSB formation by DLC. Absolute (top) and relative (bottom) donor identification show a monotonous decrease in donor preference following their order relative to the DSB site 2 hours post-DSB induction, but not at other time points.*

Preferential ectopic recombination between *cis* rather than *trans* repeats has long been observed in eukaryotes (Singer, 1988), but whether it resulted solely from preferential 3D contacts, or instead from an active 1D search process is not known. The proposed cohesin-coupled directional homology search is expected to (i) promote ectopic recombination with dispersed repeats located in *cis*, and (ii) be restricted by the processivity of cohesin, itself controlled by conserved *trans* ancillary factors (Wapl, Eco1 and Pds5) and *cis* boundary elements (*e.g.* opposing CTCF in mammals or centromeres in *S. cerevisiae*). Increasing cohesin processivity in corresponding mutants is thus expected to increase both the frequency and the length of repeat-mediated intra-chromosomal rearrangements. Yet, the extensive screens conducted over the last

two decades did not identify cohesin or its regulators as genomic instability suppressor genes (Putnam and Kolodner, 2017; Putnam et al., 2016), possibly because they are essential and not easily amenable to genetic screens. We aim at revisiting the role of cohesin and its regulator in genome maintenance. We performed a preliminary mutation accumulation experiments with bottlenecks in diploid WT and *pds5* mutants either in unchallenged or genotoxic conditions. After a cumulated 400 generations, parallel cultures were sequenced and chromosomal rearrangements determined. Preliminary analysis indicates that, specifically upon camptothecin treatment that creates single-ended DSBs during S-phase, *pds5* defects causes a significant increase both in the number and extent of intra-chromosomal rearrangements involving dispersed repeats. To my knowledge, it is the first time that cohesin ancillary factors are identified as suppressor of genomic instability (Putnam and Kolodner, 2017; Putnam et al., 2016). These preliminary results enjoin us to characterize in-depth this novel layer of homology search regulation implicated in genome maintenance.

6. Homology search adaptation: Role of the NPF structure

RecA/Rad51-ssDNA filament share the conserved property of forming elongated structures with persistence length in the order of a micron (Bell and Kowalczykowski, 2016; Benson et al., 1994; Chen et al., 2008; Flory and Radding, 1982; Sung and Robberson, 1995) (**Figure 3**). What function does this peculiar structure serve?

Kinetics analysis of D-loop formation in various donor contexts suggests a change of regime for homology search over time, transitioning from a predominantly intra-chromosomal *cis* search to a genome-wide *trans* search. Indeed, the preference for a *cis* over a *trans* donor conferred by cohesin wanes over time: it is quantitatively more pronounced at 2 than at 4 hours post-DSB induction (Piazza et al., 2021a) (**Figure 9d**), suggesting that the NPF progressively emancipates from constraints imposed by spatial chromatin organization. This emancipation coincides with the appearance of elongated NPF structures observed cytologically with a new generation of functional fluorescently-tagged Rad51 proteins (A. Taddei, personal communication), consistent with classical filament structures observed for untagged RecA and Rad51 *in vitro* (Benson et al., 1994; Chen et al., 2008; Flory and Radding, 1982; Sung and Robberson, 1995) and recent observation of RecA filaments in *E. coli* (Wiktor et al., 2021). In collaboration with the laboratories of Angela Taddei and Daniel Jost, we will study the structure, dynamics, associated contact profiles, impact on *cis*/*trans* homology identification and their nucleoprotein determinants using a combination of live imaging, D-loop quantification, a single-strand DNA-specific Hi-C protocol we are currently developing in the laboratory, and polymer modelling. This structure-function analysis has the potential to define the biological significance of the elongated NPF structure. It may also reveal the mechanistic underpinning for the apparent non-constant way homology search operates in cells, in which long-range resection is likely to be the main rheostat (Cejka and Symington, 2021).

7. Conclusion: towards a quantitative understanding of the homology search process.

The long-term goal of this first research axis is the development of a quantitative and predictive computational framework of homology search in the cell. In collaboration with the laboratory of Javier Arsuaga at UC Davis, we developed a stochastic simulation of inter-segmental microhomology association/dissociation, DNA strand invasion, 3’ flap cleavage, D-loop reversal, and D-loop extension, which marks the end of the simulation. It ponders microhomology association probability by the genome-wide DSB-dsDNA contact frequencies determined experimentally with Hi-C. DSB and donor sequences can be varied at will. It allows determining individual first passage time, heteroduplex DNA tracts length and position, population-averaged steady-state D-loop levels, and donor preference, to be compared with

experimental data. We expect this cycle between simulations and experiments to achieve quantitative determination of the contributions of NPF components and extrinsic HR regulators to homology search efficiency and accuracy, and thus HR fidelity.

B. Interplay between chromosome structure, dynamics and recombination during meiosis

HR performs the genetic and mechanical work underlying recognition and physical attachment of homologous chromosomes for their proper segregation at the first meiotic division in various organisms, including mammals and *S. cerevisiae* (Hunter, 2015) (**Figure 14a**). It is initiated at the level of self-inflicted DSBs (~150 in *S. cerevisiae* (Lam and Keeney, 2015)), at which a ~1 kb long NPF containing various meiosis-specific proteins assemble (Brown and Bishop, 2015; Mimitou et al., 2017; Zakharyevich et al., 2010). These NPFs perform homology search, leading to the recognition of the homologous chromosome and their full synapsis (Storlazzi et al., 2010). Only a subset of DNA joint molecules (JMs), detectable by 2D-gel electrophoresis, will yield a crossover (CO), such as Single-End Invasion (SEI; presumably an extended D-loop) and the topologically-linked double Holliday Junctions (dHJ) intermediates (Hunter and Kleckner, 2001; Schwacha and Kleckner, 1994, 1995). Activation of the Ndt80/Cdc5 checkpoint triggers the processing of CO-designated intermediates by Sgs1-MutLγ-Exo1 or structure-selective endonucleases (SSEs, *i.e.* Mus81-Mms4, Yen1, Slx1-Slx4) result in class I and class II CO products (Sanchez et al., 2021; Zakharyevich et al., 2010, 2012). The COs provide, together with cohesins, the physical attachment required for proper homolog segregation at meiosis I.

How the meiotic HR process is adapted to result in this spatial reorganization of the genome remains elusive. To achieve homolog attachment, the meiotic HR process has departed from somatic HR repair so as to (i) disproportionately use the homologous chromosome instead of the proximal sister chromatid as a template (*homolog bias*) (Schwacha and Kleckner, 1994), (ii) to achieve the designation of at least one *obligatory CO* per pair of homologs (Jones, 1967; Martini et al., 2006), and (iii) to generate a *CO interference* signal along homologs preventing nearby CO formation (Sturtevant, 1915), thus ensuring formation of one or a few COs per pair of homologs. These reactions involve meiosis-specific NPF proteins and/or structural components of chromosomes, take place in the context of a structured and semi-rigid chromosome organized as arrays of loops anchored to a protein axis agitated by robust telomere-led movements (reviewed in (Hunter, 2007; Zickler and Kleckner, 2015)) (**Figure 14b**). Despite extensive knowledge of the meiosis-specific HR proteins and axis components involved, the mechanistic underpinnings of homolog bias, obligatory CO designation and CO interference as well as the general logic of their relationships remain largely unknown (Wang et al., 2015). They are concomitantly enacted at the level of an intermediate that precedes SEI joint molecules that is not captured with current molecular techniques, possibly at the level of a synaptic intermediate or a short, nascent D-loop (Hunter and Kleckner, 2001) (**Figure 14a**). The long-term ambition of this emerging research axis in my laboratory is to tackle the meiosis-specific HR regulations resulting in these three enigmatic phenomena.

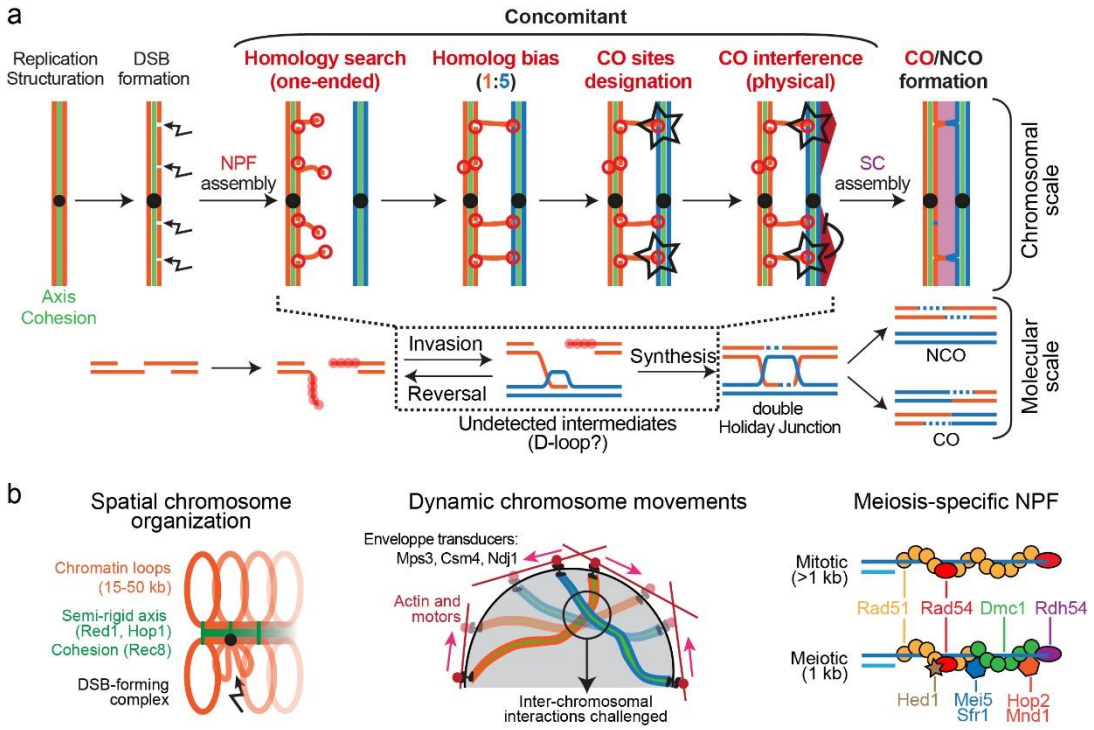

Figure 14: Molecular- and chromosomal-scale events and specificities leading to homologous chromosomes recognition and attachment at the first meiotic division. *(**a**) Overview of the canonical meiosis I progression highlighting concomitant, unexplained phenomena central to the recombination-driven chain of events leading to homolog recognition, pairing, and attachment. (**b**) Specificities of meiosis I relative to somatic HR repair: spatial chromatin organization, telomere-led chromosome movements, and meiosis-specific NPF components.*

1. How do the molecular and chromosomal events of meiosis partner for homologs attachment? The seat-belt hypothesis.

At the chromosomal scale, the meiotic prophase involves conserved:

Chromosome structure. Sister chromatids arrange as cohesin-dependent arrays of 10-50 kb-long chromatin loops anchored via Rec8 (the meiosis-specific kleisin subunit of cohesin) to the semi-rigid chromosome axis composed of Hop1 and Red1 proteins in *S. cerevisiae* (Blat et al., 2002; Grey and de Massy, 2021; Sun et al., 2015) (**Figure 14b**). Direct genomic evidence recently corroborated this organization (Muller et al., 2018; Schalbetter et al., 2019), previously deduced from cytological and ChIP data (Blat et al., 2002; Møens and Pearlman, 1988; Panizza et al., 2011). In addition to be a structural scaffold, the axis recruits factors involved in DSB formation (*e.g.* ANKRD31, RMM and the Spo11 core complex) and HR regulation (*e.g.* the Mek1 kinase) (Boekhout et al., 2019; Kim et al., 2010; Kumar et al., 2018; Murakami et al., 2020; Papanikos et al., 2019). Hence, meiotic chromosomes behave as semi-rigid and cohesive entities functionalized for HR regulation. Although loop and axis location and length varies, this general axis-tethered loop organization and the underlying meiosis-specific protein actors are conserved in eukaryotes sharing the canonical meiotic program in which HR drives homolog pairing (Grey and de Massy, 2021).

Chromosome movements. These relatively stiff chromosomes are agitated by sudden and fast (up to 1 µm/s) cytoskeleton-led movements directly transduced through the nuclear envelope at the level of telomeres by Csm4, Ndj1, Mps2 and Mps3 (Conrad et al., 2008; Koszul and Kleckner, 2009; Lee et al., 2020) (see above,

Figure 4 and **14b**). These movements are a conserved feature of meiotic prophase, and have been proposed to stimulate homology search, disrupt non-allelic pairings, and disentangle chromosomes (Koszul and Kleckner, 2009; Sonntag Brown et al., 2011; Wanat et al., 2008; Wu and Burgess, 2006). The role of nucleoplasmic actin bundles recently observed during meiotic prophase remains to be addressed (Takagi et al., 2021).

At the molecular scale, the meiotic HR involves conserved:

Meiosis-specific NPF proteins (**Figure 14b**). In addition to Rad51/Rad54, the meiotic NPF contains Dmc1/Rdh54 (Nimonkar et al., 2012), an equivalent paralogous couple loaded at the 3' end of the resected molecule with Rad51/Rad54 remaining internal (Brown and Bishop, 2015; Brown et al., 2015). Dmc1/Rdh54 and Rad51/Rad54 exhibit strikingly similar biochemical activities (Brown and Bishop, 2015; Nimonkar et al., 2012), with only subtle differences in mismatch tolerance (Lee et al., 2015; Xu et al., 2021). Yet only the DNA strand invasion activity of Dmc1/Rdh54 is required in meiosis (Cloud et al., 2012; Lao et al., 2013) while that of Rad51/Rad54 is being repressed by Hed1 and phosphorylation of Rad54 by Mek1 (Busygina et al., 2008, 2012; Crickard et al., 2018; Lao et al., 2013).

One-ended homology search. The DSBs are formed at the level of the chromosome axis (Panizza et al., 2011). Unlike somatic HR repair (Lobachev et al., 2004; Nakai et al., 2011; Piazza et al., 2021a), the two DSB ends do not remain spatially associated in meiosis: only one of the two ends exits the axis to undergo homology search (Brown et al., 2015; Storlazzi et al., 2010). This intriguing cytological observation in various species (Zickler and Kleckner, 2015) suggests that a unique and conserved feature of meiotic homology search is its "one-endedness". Further identification of the asymmetric, CO-dedicated and inter-homolog-specific Single-End Invasion (SEI) intermediate (Hunter and Kleckner, 2001) suggests that differential left-right DSB end proficiency for pro-CO inter-homolog repair is non-random.

Requirements for homologs attachment:

Template choice. While the sister chromatid is preferentially used as a template in somatic cells (Kadyk and Hartwell, 1992), meiotic HR disproportionately recombines off the homologous chromosome, 5-fold more than on the sister (Schwacha and Kleckner, 1994). This phenomenon, referred to as *homolog bias*, has two proposed components: a promotion of inter-homolog and an inhibition of inter-sister recombination (Hunter, 2007). They depend on the meiosis-specific NPF proteins Dmc1/Rdh54 and the inhibition of DNA strand invasion by Rad51/Rad54, respectively (Cloud et al., 2012; Hong et al., 2013; Kim et al., 2010; Lao et al., 2013; Schwacha and Kleckner, 1997). Their highly similar biochemical activities (Nimonkar et al., 2012) led to speculate that Dmc1/Rdh54 uniquely integrates a signal specific to the homolog template, the nature of which remains unknown. Homolog bias also depends on axis components, notably sister chromatid cohesion (Hunter and Kleckner, 2001; Kim et al., 2010; Schwacha and Kleckner, 1994). Importantly, depletion of cohesin not only abolishes the normal 1:5 homolog bias, but also the ~10:1 sister bias observed in various NPF mutants, yielding a neutral 1:1 ratio (Hong et al., 2013). This puzzling dominance has not yet been well accounted for.

Repair outcome. While multiple mechanisms inhibit the CO repair outcome in somatic cells, homolog attachment requires at least one CO between homologs. This *obligatory CO* assurance implies a *CO designation* step, a controlled process preceding SEI, whose nature remains elusive (Hunter and Kleckner, 2001; Martini et al., 2006). These designated sites in turn generate a *CO interference* signal, which inhibits similar CO designation in their vicinity, resulting in a tightly controlled and patterned number of CO per pair of homologs. CO interference requires the assembly of a protein scaffold referred to as the synaptonemal complex (SC), which assembles at, and propagates from the CO designated sites to synapse

the paired homologs (Hunter, 2007). Importantly, the SC does not directly convey the interference signal: its nature is physical, whose metrics is the axis length (Wang et al., 2015). CO interference also requires Dmc1/Rdh54 (Shinohara et al., 2003), suggesting that the NPF is capable of integrating this chromosomal-scale physical information.

Despite intense studies and the identification of a plethora of proteins required for their implementation, the mechanistic underpinnings of homolog bias, obligatory CO designation and CO interference and their relationships remain largely unknown. They occur in a concomitant fashion at the level of a recombination precursor or intermediate that escapes detection by current techniques (Hunter and Kleckner, 2001; Owens et al., 2018). Given the key role of meiosis-specific NPF factors, these phenomena are likely a reflection of meiosis-specific regulations of the homology search process, at the homology sampling and/or D-loop metabolism step.

The seat-belt hypothesis

We surmise that the conserved chromosomal events of meiotic prophase (structure and dynamics) generates both a template and an interference signal which is transduced at the molecular level by meiosis-specific NPF components. Specifically, we hypothesize that the homolog bias, obligatory CO designation and CO interference phenomena are the expression of a mechanistic property unique to the meiotic NPF: the ability to sense and, counter-intuitively, to stabilize and/or mature into CO pioneer inter-homolog joints that are challenged by antagonistic forces. The logic of this "seat-belt" hypothesis is depicted in **Figure 15.** The triggering forces originate from the robust telomere-led movements and are propagated along chromosomes by the semi-rigid axis. They are uniquely exerted at pairing sites formed between homologs, whose movements are uncoordinated in the nucleus, while inter-sister pairing interactions remain unchallenged owing to their coordinated movements ensured by cohesins (**Figure 15**). This behavior would account for the homolog bias and obligatory CO designation. The pioneer inter-homolog joint, possibly stabilized by the SC, provides an anchor point that is expected to attenuate antagonistic forces in its vicinity, ensuring CO interference (**Figure 15**). This attenuation should decay with physical axis distance, and be influenced by the elasticity of the axis structure. This view satisfyingly accounts for (i) the dominance of cohesin depletion on any homolog or sister bias observed in wild type cells or NPF mutant, (ii) the physical nature of the interference signal, (iii) the requirement for a meiosis-specific homology search and DNA strand invasion apparatus, and (iv) the requirement for a one-ended homology search, as invasion by both DSB ends could interfere with the force-sensing process.

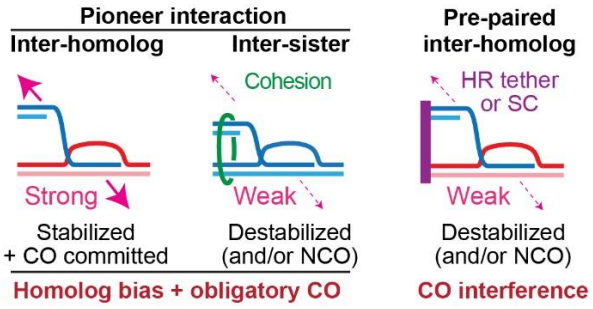

***Figure 15**: **Logic of the seat-belt model: a force-informed template choice, CO designation and CO interference mechanism**. Antagonistic forces (pink) are sensed at the NPF pairing or JM stages (D-loop depicted), which determines the fate of the repair. (SC=synaptonemal complex)*

This proposed seat-belt model shares features of the beam-film model, proposed by the Kleckner lab to account for the CO designation and interference phenomenon (Kleckner et al., 2004; Wang et al., 2015; Zhang et al., 2014a, 2014b; Zickler and Kleckner, 2015, 2016). The beam-film model postulates the accumulation and local release of a CO-promoting mechanical "stress" within meiotic prophase

chromosomes. Differently, we postulate that the CO-designation signal is extrinsic, resulting from mechanical challenges generated at the level of interactions between moving chromosomes. Transduction of this signal from chromosomal extremities to interstitial chromosomal sites is expected to require a semi-rigid axis and be dependent on chromosome axis length, in agreement with published observations (Song et al., 2021; Wang et al., 2017; Zhang et al., 2014b). However, this view is in better agreement with the structure of both mitotic and meiotic chromosomes, who lack a contiguous and rigid scaffold that could build up the postulated inner mechanical stress integral to the beam-film model (Biggs et al., 2020; Poirier and Marko, 2002). Furthermore, the seat-belt model better account for the existence of an obligatory CO, and uniquely accommodates homolog bias if the force-sensing mechanism resides in the HR machinery.

My goal is to address the antagonistic forces-dependent repair commitment hypothesis, define the HR step(s) involved, and characterize the force-sensing mechanism. If verified, this model would represent an important conceptual advance in the HR and meiosis fields, unifying in a single mechanistic model three long-known phenomena whose relationships and general logic had so far remained elusive.

2. SynIV: An experimental system to study chromatin folding, pairing, and recombination progression at high-throughput over a 150 kb-long chromosomal region in *S. cerevisiae*

In order to enable the conjoint study of chromosomal-scale and molecular-scale events of meiosis we, in collaboration with the Koszul lab, designed and assembled two heterozygous, re-designed 150 kb synthetic regions of chromosome IV: SynIV-v1 and v2 (**Figure 16a**). These regions were redesigned for various restriction sites, enabling to distinguish parental homologs using restriction-based techniques, such as Hi-C, DLC, or gel electrophoresis. Care was taken to limit sequence divergence (<1%) in 2-kb hotspot regions. This experimental system should enable to simultaneously track at high temporal resolution (i) the spatial chromatin organization of both parental homologs, (ii) their pairing, as well as (iii) the progression of individual recombination reactions, from early JM formation to product formation, at ~18 contiguous natural hotspots. The koszul lab provided proof of concept of this approach combining SynIV-v1 against the native region successfully identified homolog pairing by Hi-C (Muller et al., 2018).

I also provided evidence that the DLC assay and derivatives could detect inter-homolog JMs and CO at three DSB hotspots, two of which are of low-intensity (**Figure 16b**). This initial design did not allow high resolution study of individual homologs folding nor the study of the remaining recombination hotspots. Consequently, we assembled a second SynIV region with staggered restriction sites, whose assembly has just been completed in SK1 cells. Hi-C analysis pipeline and mid-throughput DLC and CO quantification techniques are being established and evaluated. We anticipate this new generation experimental system to facilitate the conjoined and reciprocal study of chromosomal- and molecular-processes of meiosis. However, we are aware that this approach cannot resolve individual cells. Consequently, analysis will be limited to correlations between population-averaged changes in chromatin organization and recombination reactions following population-level *cis* or *trans* perturbations. They may be complemented with live-microscopy.

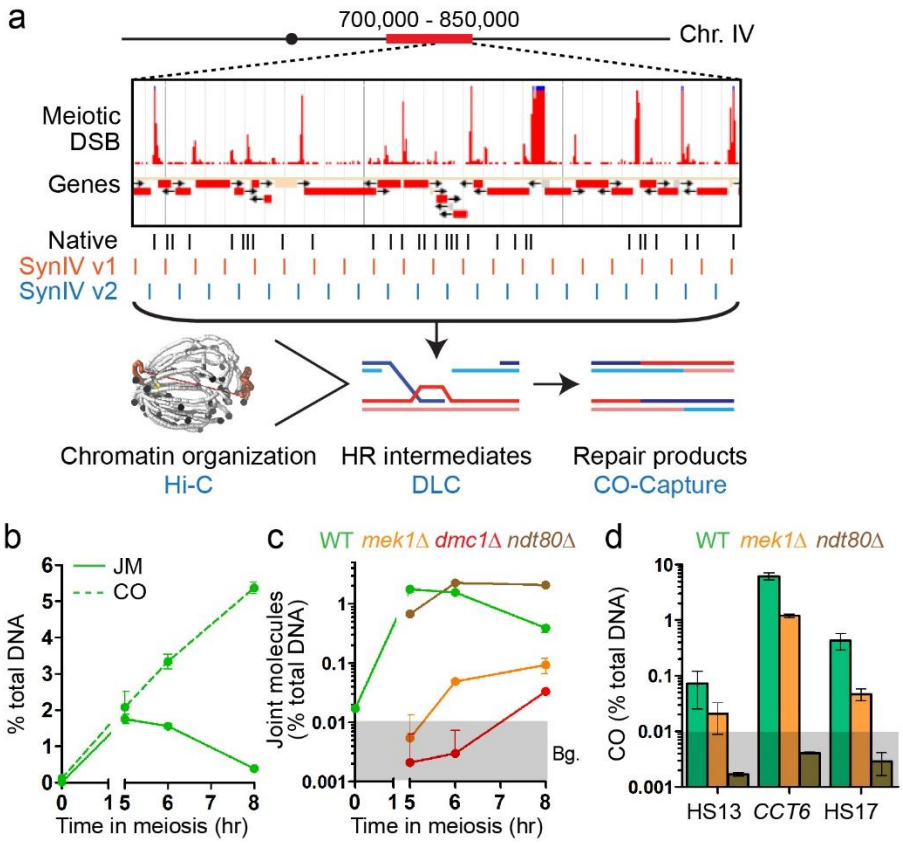

Figure 16: Experimental system to simultaneously track chromatin folding, homolog pairing, and the progression of the HR reaction over a 150 kb chromosomal region. *(a) Redistribution of the restriction sites in the SynIV region allows to determine homolog-resolved chromatin organization and homolog pairing with Hi-C, as well as to quantify inter-homolog DNA joint molecules and CO formed at multiples hotspots within the region. (b) Kinetics of inter-homolog JM and CO formation at the CCT6 hotspot in wild-type cells bearing a native chromosome and SynIV-v1. (**c**) Kinetics of inter-homolog JM formation at the CCT6 hotspot in wild-type cells and in mutants deficient for inter-homolog recombination (dmc1Δ and mek1Δ) or for JM resolution (ndt80Δ).*

3. Prediction 1: dependency of homolog bias, CO designation and CO interference on chromosome dynamics

A key prediction of the seat-belt model is the dependency of both homolog bias, CO designation and CO interference on chromosome dynamics. Consequently, a pair of homologous chromosomes specifically deficient for telomere-led movements should also be deficient for homolog bias and obligatory CO formation. This prediction differentiates the seat-belt model from the beam-film model, which is not expected to depend on chromosome dynamics. We will address it using circularized SynIV-containing chromosome IV, deficient for NE anchorage and movements (Rockmill and Roeder, 1998) (**Figure 17a**). We expect a specific delay and reduction of inter-homolog JMs and CO formation at SynIV, but not at the *HIS4-LEU2* hotspot, located on linear chr. III. This *cis* effect will be verified upon reciprocal chr. III circularization.

Conversely, restoring chromosome dynamics specifically on chr. IV in a strain otherwise deficient for telomere-NE association and transduction of cytoskeleton-mediated movements (*mps3-Δ2-64*, *csm4-AID*)

(Conrad et al., 2008; Kosaka et al., 2008) is expected to restore homolog bias and/or CO formation specifically on that chromosome (**Figure 17a**). Chr. IV-specific anchoring restoration will be attempted upon conditional expression of fusion of Csm4-LacI-DBD and Csm4-TetR-DBD in strains bearing LacO and TetO arrays at each end of chr. IV. In this context, inter-homolog JMs and CO formation should be restored at SynIV, but not at the *HIS4-LEU2* hotspot.

In both contexts, chromosome-specific mobility will be verified by microscopy with FROS-tagged arrays (Lassadi and Bystricky, 2011). To circumvent the anticipated delay in pachytene exit and associated homeostatic up-regulation of DSB formation on chr. IV (Thacker et al., 2014), conditional depletion of a member of the Spo11 core complex will be triggered 4 hr post-meiotic induction.

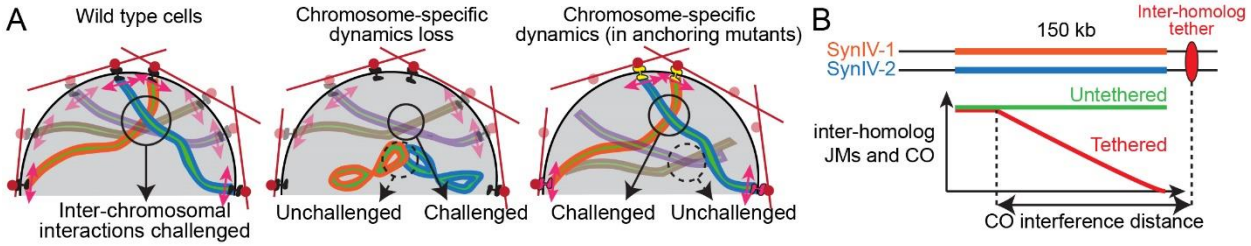

Figure 17: Chromosome tethering strategies to address the force-mediated nature of the homolog bias, CO designation and CO interference phenomena. *(**a**) Homolog-specific abolishment (middle) or restoration (right) of dynamics upon chromosome circularization or artificial anchorage to the cytoskeleton, respectively (**b**) Expected regional effect of an artificial inter-homolog tethering point in the vicinity of SynIV on inter-homolog JM and CO formation.*

4. Prediction 2: Homolog bias and CO designation inhibition in the vicinity of an inter-homolog pre-association point, over the typical interference distance.

The seat-belt model provocatively predicts that an artificial inter-homolog tethering point should abolish homolog bias and CO designation in its vicinity, despite putting the homologs in close spatial proximity. This inhibition should decay from the contact point as CO interference (**Figure 17b**). Such a behavior is not expected from the bean-film model, unless recombination-independent tethering can also relieve mechanical stress stored in chromosomes. We will use a previously developed LacO arrays and tetramerizable LacI system leading to conditional and efficient tethering of allelic loci (Lee et al., 2012). Locus-specific pairing will be verified by microscopy (Lee et al., 2012) and with Hi-C at the level of SynIV. The predicted oriented bias of inter-homolog JM and CO formation will be determined over the natural hotspots spanning the SynIV region. Providing the tether on the other side of SynIV is expected to reverse the bias, which will confirm the *cis*-acting nature of the tether. This regional bias should depend on chromosome dynamics (Csm4), on the semi-rigid axis (Hop1-Red1), and on the force-sensing apparatus presumably lying in the NPF (Dmc1/Rdh54, Hop2-Mnd1), which will be tested in the appropriate mutants.

5. Nature of the force-sensing meiotic apparatus

We intend to identify the force-sensing apparatus and characterize the molecular mechanism underlying its unique ability. It will first require to define the force regime to be sensed. We initiated, in collaboration with Jean-Michel Arbona in the Jost lab, the development of a polymer physics model of meiotic chromosomes and of their dynamic telomere-led movements. We currently fit the model parameters over

published cytological data of chromosome dynamics and rigidity (Conrad et al., 2008; Koszul et al., 2008) to define the physiological range of force applied at pioneer inter-homolog joints, and how pre-existing joints or axis association via synaptonemal complex formation and spreading attenuates mechanical challenges at secondary joints. We will subsequently attempt to characterize the minimal force-sensitive components, which presumably resides in the meiosis-specific HR proteins: Dmc1/Rdh54, Hop2-Mnd1, and Mei5-Sae3. To this end we will explore genetics, biochemistry and structural biology avenues through collaborations.

V. Leading research

The focus put on science at every turn of a young career leaves little brain time available to prepare for the many other aspects integral to leading a research laboratory. One soon realizes the opportunity to instigate a lab culture that can benefit lab members both scientifically and personally, together with its ambition of substantially contributing the scientific enterprise. This is at least what I imagine a "dream lab" to be, and which I hope to achieve. The question is: how?

When randomly browsing for older literature, one cannot help but notice the name of last authors who seem to have dominated their research field for years or decades, as conveyed by streaks of publications in prestigious journals. Yet despite the apparent prominence of their work they quickly faded from scientific memory, their work seldom referenced anymore, the influence exerted at a point in time dissipated. Others have endured. It suggests the existence of an ingredient for an influential and lasting scientific contribution atop the published work itself, and that only becomes apparent in the long run. Since "researchers are at the heart of the research process" (dixit JP Bourguignon), I suspect this ingredient to be the scientific progeny of a laboratory. Only through the number and quality of researchers that go on to establish their own laboratories will the themes and knowledge generated in their parent lab be transmitted and built upon by the next generation. Lab fertility varies greatly despite apparently similar scientific outputs. Reasons for such variations are likely complex, but I suspect two root causes: lab culture and research management. I detail both below, as a nexus for achieving a lasting scientific contribution and a continued place of excitement, growth and joy for its members.

A. Management and freedom

My understanding of research management is that it aims at simultaneously achieving both (i) opportunities for personal, scientific, technical and career progression for team members, and (ii) the lab research goals. Since group leaders are primarily selected based on their scientific curriculum, their managerial success is pretty much down to their own instincts. In 2018 I sought basic management knowledge through an EMBO training, to instigate a collaborative dynamic within the lab and learn how to avoid or defuse potential conflicts. It also provided an opportunity to figure out what management "style" to adopt, which I tie to my own enjoyment of the research experience: freedom. I wish people in my lab, at degrees appropriate to the individual's career stage and abilities, to enjoy freedom in their research. It touches the heart of the scientific and experimental process, to formulate a question and a hypothesis, confront it with reality, and slowly drive the assembly of an understanding of a biological process. I find this point rarely highlighted, that research being at the frontier of human knowledge, it is to some degree emancipated from authority; perhaps one of the few domains of human activity left in which uncompromised expression of one's personality and ideas can be achieved. This exposure is both exhilarating and a source of anxiety: our ego is out for triumph or failure. I know no better fuel and source of elation that this all-in relation with the research topic, which can only exist in enthusiastic individuals if left independent.

Freedom does not mean lack of guidance, especially at the early career stage of graduate studies. To pave the way towards the required independence, I plan a 3-stage build-up for graduate students. The technical independence during the first year, exploring a variety of techniques while advancing projects with (ideally) a solid premise. The theoretical independence acquired within the first two years, over which I will encourage them to independently probe curious and promising side-results. Finally, in the last PhD years,

they may develop a project of their own, to be explored in parallel of the main project. Depending on their experimental and theoretical backgrounds, post-docs will also be encouraged from the get-go to develop their own project alongside a main lab project. This is, at least on paper, the plan to provide a path to scientific independence, while keeping motivation and enjoyment up. In the case of post-docs, it should also prepare a legitimate track for positions of leadership, by granting individuals a promising project to start their own research group. This overall management strategy should provide ground for both a lively lab and lasting scientific contributions.

B. Lab culture

Creating and sustaining a stimulating lab culture is perhaps the most exhilarating and important part of setting up and running a laboratory, as it will imprint the "personality" of its progeny. I wish to build it around three axes: scientific enthusiasm, scientific creativity, and scientific rigor.

Enthusiasm toward science because it is the engine that powers hands and brain. I consider it the key leverage by which to steer the team and stimulate team members toward achieving their research goals, by constantly sharing my own enthusiasm with scientific discoveries and last-night ideas, and responding to theirs. My hope is to reach ignition of the "enthusiasm fusion reaction", which would then power and entertain itself.

Creativity, the recognition and use of the freedom researchers are lucky to enjoy. Creativity is perhaps also our only way to be relevant in a competitive global scientific context in which we are dealt by our institutions a poor financial hand. To do more with less, one must be creative, original, dismiss ideas that come too easily, projects that are thoughtless, or those that require experimental muscles. Operating under such constraints means we must practice scientific aikido, cracking problems with elegance, not brute force. It places scientific creativity at the forefront.

Rigor, finally, which ensures reliable progress and allows others to confidently build onto predecessors' work.

VI. Conclusions

Although being time-consuming, burdensome from an administrative standpoint, costly (I had to pay to become a student again!), and of dubious efficacy in benefiting future PhD students, the exercise of the HDR had an undeniable virtue: that of forcing one to sit, read, and think more deeply than usual, or in different ways, about science and research goals. It also provided time to dive back into the literature and rediscover some formidable contributions by peers, with utmost pleasure. I wish to transmit to students this elating facet of the exercise of science: the simple joy of insightful ideas and experimental elegance.

Strangely enough, writing this manuscript also brought me at the verge of personal and political anguish: why am I in science? And how am I allowed to do this? In hindsight, were the questions I tackled or decided to tackle the most interesting? What can I hope to achieve over a life in science? etc. These questions can also be asked in a collective sense: by which miracle did society agree and organize for some lucky few to essentially fulfil their personal curiosity and even "aesthetic" scientific satisfaction? How beautifully optimistic this sounds! I just hope the erosion of the European Union integrity, the increasing tendency of politicians to pilot research, the intensifying quest for monetizable human productivity, and the slow but steady progression of distrust or defiance of western populations towards science will not endure, so that the next generation of scientists I will contribute to train can keep enjoying the anomaly of the XXst century: free science.

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