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# Régulation de cohésine par le complexe TORC1 chez la levure *Schizosaccharomyces pombe*

Dorian Besson

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Dorian Besson. Régulation de cohésine par le complexe TORC1 chez la levure *Schizosaccharomyces pombe*. *Genetics*. Université de Bordeaux, 2024. English. NNT : 2024BORD0166 . tel-04851251

**HAL Id: tel-04851251**

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THÈSE PRÉSENTÉE  
POUR OBTENIR LE GRADE DE

**DOCTEUR DE**  
**L'UNIVERSITÉ DE BORDEAUX**

ÉCOLE DOCTORALE : SCIENCES DE LA VIE ET DE LA SANTE  
SPÉCIALITÉ : GENETIQUE MOLECULAIRE ET CELLULAIRE

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# Régulation de cohésine par le complexe TORC1 chez la levure *Schizosaccharomyces pombe*

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Soutenue le 20/09/2024

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## Résumés en français et en anglais

Cohésine est un complexe protéique capable de capturer les molécules d'ADN. Cohésine assure la cohésion des chromatides sœurs, essentielle à la ségrégation des chromosomes lors des divisions nucléaires. Elle intervient également en interphase via la formation de boucles d'ADN intra-chromosomiques qui façonnent l'architecture fonctionnelle du génome. L'expression génique est ainsi régulée par l'organisation spatiale des chromosomes, notamment au cours du développement et de la différenciation. Les nombreuses fonctions de cohésine suggèrent une régulation fine dans le temps et l'espace. Le laboratoire adresse cette question via une approche génétique chez l'organisme modèle *Schizosaccharomyces pombe*. La capture de l'ADN par cohésine nécessite l'intervention d'un complexe de chargement, Mis4/Ssl3 (hNIPBL/MAU2). Le mutant *mis4-G1487D* est thermosensible de croissance à 36°C. A température restrictive, il présente un défaut de chargement des cohésines sur les chromosomes et une fréquence élevée de défauts de ségrégation des chromosomes lors de la mitose. Un crible génétique a identifié des mutations extragéniques capables de restaurer la croissance de *mis4-G1487D* à 36°C. Cinq de ces mutations affectent le gène *mip1* et une le gène *tor2*. Mip1 et Tor2 sont des composants du complexe TORC1, l'équivalent de mTOR (Mammalian Target Of Rapamycin) qui est une kinase régulatrice majeure du métabolisme et de la croissance cellulaire. Son activité est stimulée par des signaux tels que la disponibilité en nutriments, niveaux d'énergie, hormones et facteurs de croissance. Chez *S. pombe*, Tor2 est la sous-unité catalytique et Mip1 (hRaptor) participe au choix du substrat. Les gènes *tor2* et *mip1* sont essentiels à la viabilité cellulaire indiquant que les allèles issus du crible sont hypomorphes. Nous avons focalisé nos travaux sur *mip1-R401G* qui ne provoque quasiment pas de défaut de croissance tout en étant un excellent suppresseur. De façon remarquable, *mip1-R401G* restaure l'association de cohésine aux chromosomes et diminue la fréquence de ségrégation anormale des chromosomes du mutant *mis4-G1487D* à 36°C. En fond *mis4<sup>+</sup>*, *mip1-R401G* provoque une augmentation de la quantité de cohésine associée aux chromosomes. Des résultats similaires ont été obtenus en traitant les cellules à la Rapamycine, un inhibiteur de TORC1. Ces données suggèrent que l'activité de TORC1 régule de façon négative le complexe de chargement des cohésines chez *S. pombe*.

Toutes les sous-unités du complexe TORC1 co-purifient avec cohésine et Mis4. La sous unité Psm1 de cohésine et Mis4 sont hypophosphorylées en fond *mip1-R401G*. La combinaison de mutations imitant l'état non-phosphorylé réduit la fréquence des défauts de ségrégation de *mis4-G1487D*. Réciproquement les défauts de ségrégation sont exacerbés par les mutations imitant l'état phosphorylé. Ces données indiquent que TORC1 contrôle l'état de phosphorylation de Psm1 et Mis4. La fonction connue de TORC1 étant d'adapter la cellule aux changements environnementaux, nous avons mis en œuvre une analyse du transcriptome dans diverses situations expérimentales (composition du milieu de culture, température, phase du cycle cellulaire). Toutes expériences confondues, 337 gènes sont différenciellement exprimés en fond *mis4-G1487D* par rapport au contrôle sauvage. De façon remarquable les gènes affectés sont largement différents d'une condition à l'autre, suggérant un défaut de réponse adaptative. D'autre part, la quasi-totalité des gènes dérégulés par *mis4-G1487D* le sont également par *mip1-R401G*. Ces gènes sont préférentiellement localisés aux extrémités des chromosomes et sont impliqués dans la réponse au stress et la différenciation sexuelle.

L'ensemble des données suggère que cohésine est un effecteur de la voie TORC1 pour adapter la cellule aux changements environnementaux. Ce mécanisme ferait intervenir un changement de l'expression génique induit par une modification de l'organisation spatiale du génome.

**Mots clés:** Cohésine; Target Of Rapamycin ; Transcription, structure du chromosome

Cohesin is a protein complex capable of capturing DNA molecules. Cohesin ensures the cohesion of sister chromatids, which is essential for chromosome segregation during nuclear divisions. It is also involved in interphase via the formation of intra-chromosomal DNA loops that shape the functional architecture of the genome. Gene expression is thus regulated by the spatial organisation of chromosomes, particularly during development and differentiation. The many functions of cohesin suggest fine regulation in time and space. The laboratory is addressing this question using a genetic approach in the model organism *Schizosaccharomyces pombe*. DNA capture by cohesin requires the intervention of a loading complex, Mis4/Ssl3 (hNIPBL/MAU2). The *mis4-G1487D* mutant is thermosensitive to growth at 36°C, has a defect in cohesin loading on chromosomes and a high frequency of chromosome segregation defects during mitosis. A genetic screen identified extragenic mutations capable of restoring the growth of *mis4-G1487D* at 36°C. Five of these mutations affect the *mip1* gene and one the *tor2* gene. Mip1 and Tor2 are components of the TORC1 complex, the equivalent of mTOR (Mammalian Target Of Rapamycin), which is a major regulatory kinase for cell metabolism and growth. Its activity is stimulated by signals such as the availability of nutrients, energy levels, hormones and growth factors. In *S. pombe*, Tor2 is the catalytic subunit and Mip1 (hRaptor) is involved in substrate selection. The *tor2* and *mip1* genes are essential for cell viability, indicating that the alleles produced by the screen are hypomorphic. We focused our work on *mip1-R401G*, which causes virtually no growth defects while being an excellent suppressor. Remarkably, *mip1-R401G* restored cohesin association with chromosomes and reduced the frequency of abnormal chromosome segregation in the *mis4-G1487D* mutant at 36°C. In the *mis4<sup>+</sup>* background, *mip1-R401G* increased the amount of cohesin associated with chromosomes. Similar results were obtained by treating the cells with Rapamycin, a TORC1 inhibitor. These data suggest that TORC1 activity negatively regulates the cohesin loading complex in *S. pombe*.

All subunits of the TORC1 complex co-purify with cohesin and Mis4. The Psm1 subunit of cohesin and Mis4 are hypophosphorylated in the *mip1-R401G* background. The combination of mutations mimicking the non-phosphorylated state reduces the frequency of *mis4-G1487D* segregation defects. Conversely, segregation defects are exacerbated by mutations mimicking the phosphorylated state. These data indicate that TORC1 controls the phosphorylation state of Psm1 and Mis4. Given that TORC1's known function is to adapt the cell to environmental changes, we carried out a transcriptome analysis in various experimental situations (culture medium composition, temperature, cell cycle phase). Taking all experiments together, 337 genes were differentially expressed in the *mis4-G1487D* background compared with the wild-type control. Remarkably, the genes affected differed widely from one condition to another, suggesting that *mis4-G1487D* cells have a defective adaptive response. Almost all the genes deregulated by *mis4-G1487D* were also deregulated by *mip1-R401G*. These genes are preferentially located at the ends of chromosomes and are involved in the stress response and sexual differentiation. Taken together, the data suggest that cohesin is an effector of the TORC1 pathway for adapting the cell to environmental changes. Mechanistically, this might involve a change in gene expression induced by a modification in the spatial organization of the genome.

**Keywords: Cohesin; Target Of Rapamycin; Transcription, chromosome structure**

## Remerciements

Je voudrais tout d'abord remercier les membres du jury, Valérie Borde, Sophie Javerzat, Frédéric Beckouët et Pascal Bernard d'avoir accepté d'évaluer mes travaux de thèse.

Ensuite je voudrais remercier l'ensemble du premier étage de l'I.B.G.C. avec les nouveaux arrivants et les retraités. L'entente entre les deux équipes et la bienveillance des personnes donnent à cet endroit un contexte de travail exceptionnel. Je suis reconnaissant de la chance que j'ai eue d'avoir partagé ces quatre années avec vous.

Dans le même registre, je tiens à dire un très grand merci à Jean-Paul, Sabine, Stéphanie et Adrien. Premièrement, Jean-Paul je te remercie de m'avoir recruté en tant que thésard sous ta direction et celle de Karl. Merci pour ta patience et merci pour la formation intellectuelle. Là aussi je me sens chanceux d'avoir eu un directeur de thèse comme toi, qui m'a permis de progresser et de devenir ce que j'espère un scientifique compétent. Ensuite, merci Sabine de m'avoir formé sur le plan expérimental. Les débuts ont été compliqués, tu as dû te demander plusieurs fois « mais sur qui je suis tombé... », il est vrai que monter des western blot à l'envers n'est pas donné à tout le monde. Je suis content de voir qu'il te reste quand même des cheveux sur la tête à la fin de ces quatre années. Je te remercie pour la formation, j'ai beaucoup gagné en rigueur et c'est en très grande partie grâce à toi, qui étais en première ligne, que j'ai pu progresser. Je te remercie aussi d'avoir été à l'écoute et d'être franche, ce qui est pour moi les plus belles qualités chez une personne. Stéphanie, encore une fois, je m'estime chanceux d'avoir partagé avec toi le bureau au début et la fin de ma thèse. Ton sourire, ta joie de vivre, ton énergie sont un moteur au travail. Adrien, tu as rendu ces années de thèse agréables toi aussi par ta bienveillance et ton écoute. J'ai beaucoup apprécié d'avoir pu travailler avec toi, et écouter tes conseils.

Ensuite, j'aimerais dire un grand merci à Karl. Merci de m'avoir accueilli en Suède. Tu as été un super directeur de thèse. J'appréciais beaucoup nos discussions le midi au laboratoire que l'on parle de science ou non. Je tiens aussi à te dire un grand merci pour m'avoir fait découvrir la Suède, Gotland et le Kubb, et de m'avoir appris à faire du patin à glace ! Je n'oublierai jamais ces moments. Enfin, un grand merci pour ta gentillesse.

Je souhaite aussi remercier Adele Marston et son équipe pour m'avoir accueilli dans son laboratoire. Je tiens à remercier particulièrement Hollie de m'avoir formé à la technique de Hi-C.

Je veux aussi remercier mes amis de France et de Suède avec qui j'ai ou j'ai passé des supers moments. Je voudrais quand même remercier plus particulièrement Kaitlin qui a dû me supporter pendant la rédaction de cette thèse. Ça n'a pas été une période facile et tu as su être patiente et aimante malgré tout.

Enfin, je souhaite remercier ma famille, qui malgré la difficulté à comprendre mon travail et mon sujet de thèse, m'a quand même soutenu.







***A mes grands-parents, Ginette et André FOULQUIER...***

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## Introduction générale

Le complexe cohésine est un acteur essentiel de la structure et de la fonction du génome. Son activité est essentielle pour de nombreux processus biologiques, notamment la cohésion des chromatides sœurs, la division nucléaire, la réplication et la réparation de l'ADN et l'expression des gènes. La cohésine est une machine moléculaire alimentée par l'ATP dont la fonction est de capturer les molécules d'ADN. La capture de l'ADN en *cis* (intra-chromosomique) organise le génome interphasique en boucles, qui sont d'une grande importance pour la régulation des gènes, en particulier au cours du développement et des décisions relatives au destin cellulaire. La capture de l'ADN en *trans* assure la cohésion entre les chromatides sœurs, un processus essentiel pour la ségrégation et la réparation des chromosomes.

Le complexe cohésine est constituée de deux protéines SMC (Structural Maintenance of Chromosome), Smc1 et Smc3 (Psm1 et Psm3 dans la levure *Schizosaccharomyces pombe*) dont les têtes ATPases sont reliées par la kleisine (Rad21/Scc1) à laquelle se lie une quatrième sous-unité (hSTAG1-2, spPsc3, scScc3). La capture de l'ADN par la cohésine nécessite le complexe de chargement NIPBL/MAU2 (spMis4/Ssl3, scScc2/Scc4) qui se lie à la cohésine et à l'ADN. L'inactivation de la machinerie de chargement avant la phase S entraîne l'échec de l'établissement de la cohésion entre les chromatides sœurs et une ségrégation aberrante des chromosomes au cours de la mitose suivante. De même, l'extrusion des boucles d'ADN dépend de l'holocomplexe NIPBL-Cohésine. Les boucles de chromatine sont des structures dynamiques qui peuvent se former et disparaître en quelques minutes. Chez l'homme, le facteur de liaison à l'ADN CTCF semble délimiter des domaines de boucles, souvent appelés Topologically Associated Domains (TADs). L'organisation en TAD permettrait de faciliter les interactions entre enhanceurs et promoteurs au sein d'un même domaine tout en limitant les interactions inter-domaines. Les limites des TADs (isolation) semblent régulées car elles sont renforcées lorsque les cellules souches embryonnaires sortent de la pluripotence. De manière cohérente, les mutations des gènes codant pour le CTCF et les composants de cohésine sont liées à des maladies humaines et à des anomalies du développement. Le remodelage de l'architecture du génome semble particulièrement important lors des décisions relatives au destin cellulaire pour établir les programmes transcriptionnels adéquats. Comment la cellule intègre les fonctions cohésine en réponse aux signaux environnementaux est encore largement à découvrir.

Le travail présenté dans cette thèse décrit la découverte de liens biochimiques et fonctionnels entre cohésine et le Target of Rapamycin Complex 1 (TORC1) chez la levure *Schizosaccharomyces pombe*. TORC1 est un régulateur majeur de la croissance cellulaire et du métabolisme, hautement conservé chez les eucaryotes. L'activité kinase de TORC1 est stimulée par divers signaux intra et extracellulaires, notamment les nutriments, les facteurs de croissance, les hormones et les niveaux d'énergie cellulaire. Une fois activée, TORC1 favorise la croissance cellulaire et le métabolisme par la phosphorylation de multiples effecteurs. Chez les mammifères, les facteurs de croissance et l'énergie cellulaire stimulent l'activité de mTORC1 par l'intermédiaire de la GTPase Rheb, et l'inhibition du complexe de la sclérose tubéreuse (TSC), qui fonctionne comme une protéine activatrice de la GTPase pour Rheb. En réponse à la disponibilité en acides aminés, mTORC1 est activé par les GTPases RAG de manière indépendante du TSC. Dans des conditions riches en nutriments, TORC1 favorise les processus anaboliques, tels que la synthèse

des protéines, des nucléotides et des lipides, tout en inhibant les processus cataboliques, tels que l'autophagie.

Chez *S. pombe*, Tor2 porte l'activité catalytique de TORC1, tandis que Mip1, l'équivalent de Raptor, participe à la reconnaissance du substrat. Le complexe contient trois sous-unités supplémentaires, l'orthologue de mLST8 Wat1/Pop3, Toc1 et Tco89. TORC1 joue un rôle crucial dans le passage entre prolifération et différenciation cellulaire en détectant l'abondance de la source d'azote. Lorsqu'elles sont privées d'azote, les cellules s'arrêtent en phase G1 du cycle cellulaire, conjuguent et entrent dans le cycle méiotique. En l'absence d'un partenaire, les cellules entrent dans un état quiescent. Comme son homologue mammifère, la GTPase Rheb Rhb1 est un activateur essentiel de TORC1. Lors de la privation d'azote, l'activité de TORC1 est inhibée par les GTPases Gtr1-Gtr2, le complexe TSC et la kinase Gcn2. Dans des conditions où les nutriments sont abondants, l'activité de TORC1 est élevée mais néanmoins atténuée par l'hétérodimère Gtr1-Gtr2, qui est analogue aux GTPases RAG des mammifères. TORC1 est essentielle à la croissance cellulaire, et la perte de l'activité de TORC1 entraîne l'arrêt du cycle cellulaire en G1. La rapamycine n'inhibe que partiellement l'activité de TORC1 chez *S. pombe* et n'inhibe pas la croissance.

Le lien entre cohésine et TORC1 est né d'un criblage génétique visant à trouver des mutants capables de supprimer le défaut de croissance thermosensible (Ts) de *mis4-G1487D*, qui est déficient pour le chargement de la cohésine, la cohésion des chromatides sœurs et la ségrégation des chromosomes à température restrictive. En principe, le crible avait le potentiel d'identifier des régulateurs de Mis4, avec le raisonnement que la perte d'un régulateur négatif devrait réguler à la hausse l'activité résiduelle de Mis4<sup>G1487D</sup> et restaurer la croissance à la température restrictive. Outre une kinase dépendante des cyclines (CDK) appelée Pef1, qui agit comme régulateur négatif de Mis4, plusieurs mutants de TORC1 ont été identifiés. Le travail présenté montre que la baisse de l'activité TORC1 provoque une augmentation de la quantité de cohésine et de son facteur de chargement Mis4 au niveau de leurs sites réguliers de liaison sur les chromosomes. Dans le contexte où le chargement de cohésine est affecté, la régulation à la baisse de TORC1 restaure la ségrégation des chromosomes, alors que la régulation à la hausse de TORC1 a l'effet inverse. Sur le plan biochimique, TORC1 et la cohésine co-purifient à partir d'extraits protéiques, et le niveau de phosphorylation de résidus spécifiques sur Mis4 et cohésine est réduit dans les mutants TORC1. Les mutants mimant l'état non phosphorylé récapitulent la plupart des effets de la baisse d'activité de TORC1. La cohésine se comporte donc comme un effecteur de la signalisation TOR, ce qui ouvre la possibilité que des signaux environnementaux puissent affecter la robustesse des processus de ségrégation des chromosomes.

Comme TORC1 est connu pour diriger les réponses transcriptionnelles aux signaux environnementaux, nous avons interrogé le rôle potentiel de Mis4 dans cette voie en effectuant une analyse du transcriptome des cellules *mis4-G1487D* dans des situations expérimentales variées sensées mimer des changements qui pourraient se produire dans la nature : composition du milieu de culture, température, temps d'incubation et phase du cycle cellulaire. De façon remarquable les gènes affectés diffèrent largement d'une condition à l'autre, ce qui suggère que le mutant *mis4* a une réponse adaptative défectueuse. Fait remarquable, la plupart des gènes dérégulés par *mis4-G1487D* le sont également par *mip1-R401G*, ce qui indique que la cohésine

et TORC1 agissent dans la même voie. Ces gènes sont préférentiellement situés aux extrémités des chromosomes et sont impliqués dans la réponse au stress et la différenciation sexuelle.

L'ensemble des données indique que TORC1 contrôle l'association de la cohésine et de son facteur de chargement avec les chromosomes, régulant leurs fonctions dans la ségrégation des chromosomes pendant la mitose et dans la réponse transcriptionnelle aux changements environnementaux. Étant donné le rôle de la cohésine et de son complexe de chargement dans le repliement des chromosomes, il est tentant de spéculer que TORC1 pourrait diriger cohésine pour générer une architecture chromosomique compétente pour la réponse transcriptionnelle aux changements environnementaux.

Cohésine est impliquée dans un large éventail de pathologies humaines, y compris le cancer. Initialement, la recherche s'est focalisée sur la relation de causalité entre aneuploïdie, instabilité génétique et cancer. Pour autant, de nombreux cancers avec des altérations de cohésine ne montrent pas d'instabilité chromosomique ou d'aneuploïdie. La découverte que cohésine organise l'architecture des chromosomes en interphase a ouvert la possibilité d'une nouvelle voie causale, un domaine de recherche récent et extrêmement actif.

Plusieurs facteurs agissant en amont (activateurs et répresseurs) et en aval (effecteurs), ainsi que mTOR sont surexprimés ou mutés dans les cancers et mTOR est une cible thérapeutique. Le lien TOR-cohésine présenté ici pourrait être pertinent dans certains cancers. Le contrôle de l'architecture du génome par cohésine et ses dysfonctionnements pathologiques sont des domaines de recherche récents et très actifs. La causalité de cohésine dans certains cancers est établie, via la perturbation de l'équilibre renouvellement versus différenciation. On peut anticiper que ce mécanisme opère également dans d'autres types de cancer.

La rapamycine a attiré beaucoup d'attention pour sa capacité supposée à imiter la restriction calorique, avec pour résultat une amélioration de la santé et de la durée de vie. Notre étude soulève la possibilité que certains effets soient médiés par la cohésine et sa capacité à remodeler les chromosomes en réponse à divers signaux extracellulaires, y compris les nutriments.





# I - INTRODUCTION

## I - Cohesin and the SMC family

### A - General information on the SMC family

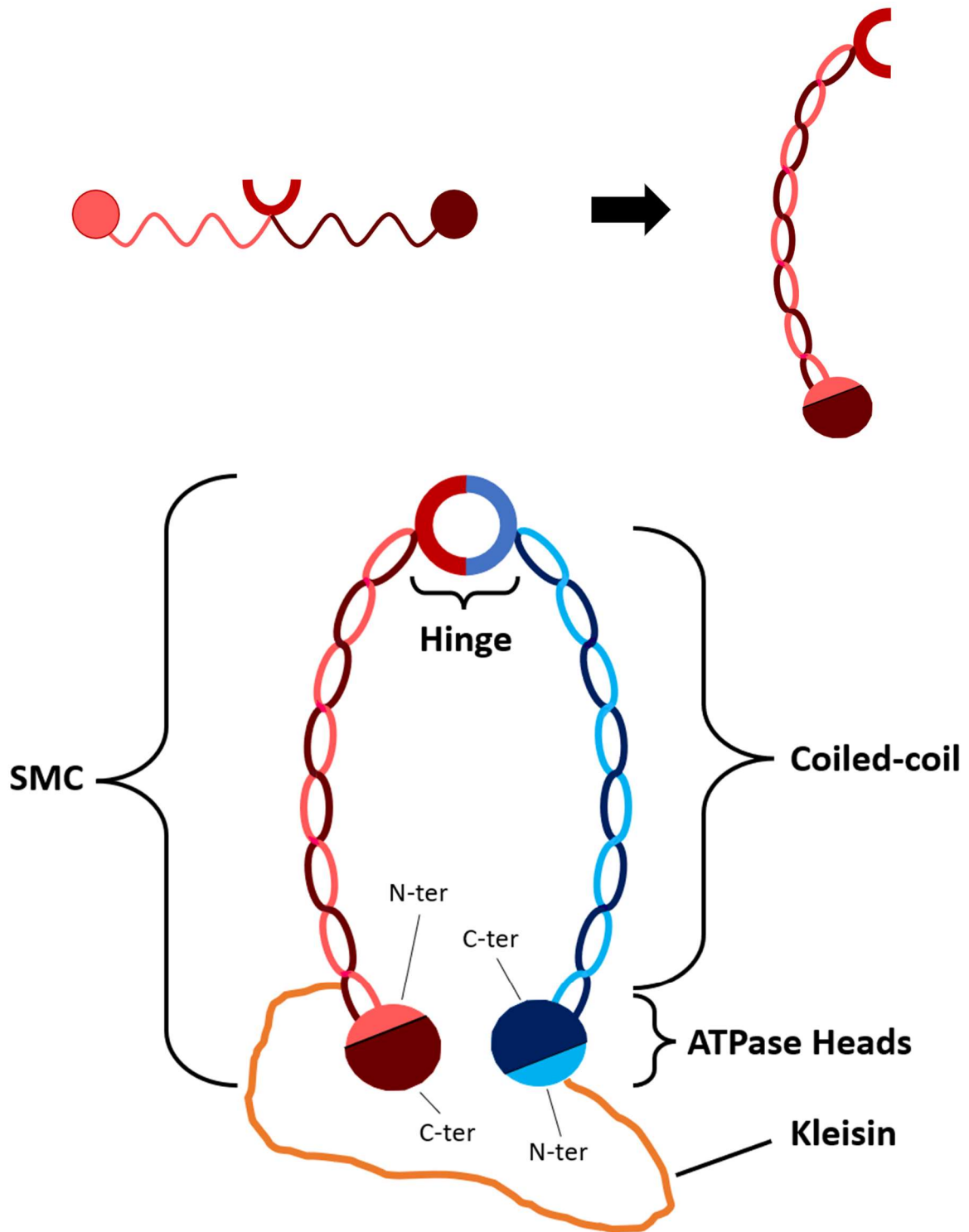
#### *1- SMC History*

SMC (Structural Maintenance of Chromosome) proteins form a family of proteins conserved in all living organisms. These proteins play an essential role in the organisation and maintenance of the genome. The first *smc1* gene was identified in 1985, from a mutant with a defect in the stabilisation of a centromeric minichromosome in *Saccharomyces cerevisiae* (Larionov et al., 1985). Later, in 1991, the *mukB* gene (from the Japanese 'mukaku' meaning 'anucleated') was identified from a heat-sensitive mutant of *E. coli* producing anucleated cells at low temperatures (Hiraga et al., 1989; Niki et al., 1991). Subsequently, in the 1990s, the Smc2 and Smc4 proteins were purified from *xenopus* egg extract and shown to be involved in chromosome condensation. These two proteins form the architecture of the condensin complex (Hirano and Mitchison, 1994). In 1997, a genetic screen for mutants involved in premature sister chromatid separation identified Smc1 and Smc3 as proteins involved in sister chromatid cohesion. The complex was named cohesin (Guacci et al., 1997; Michaelis et al., 1997). In the early 2000s, a third group of SMCs was discovered, Rad18, which was later named Smc6. The Smc5-Smc6 complex is involved in the repair of DNA breaks (Fousteri and Lehmann, 2000; Fujioka et al., 2002; Palecek, 2019).

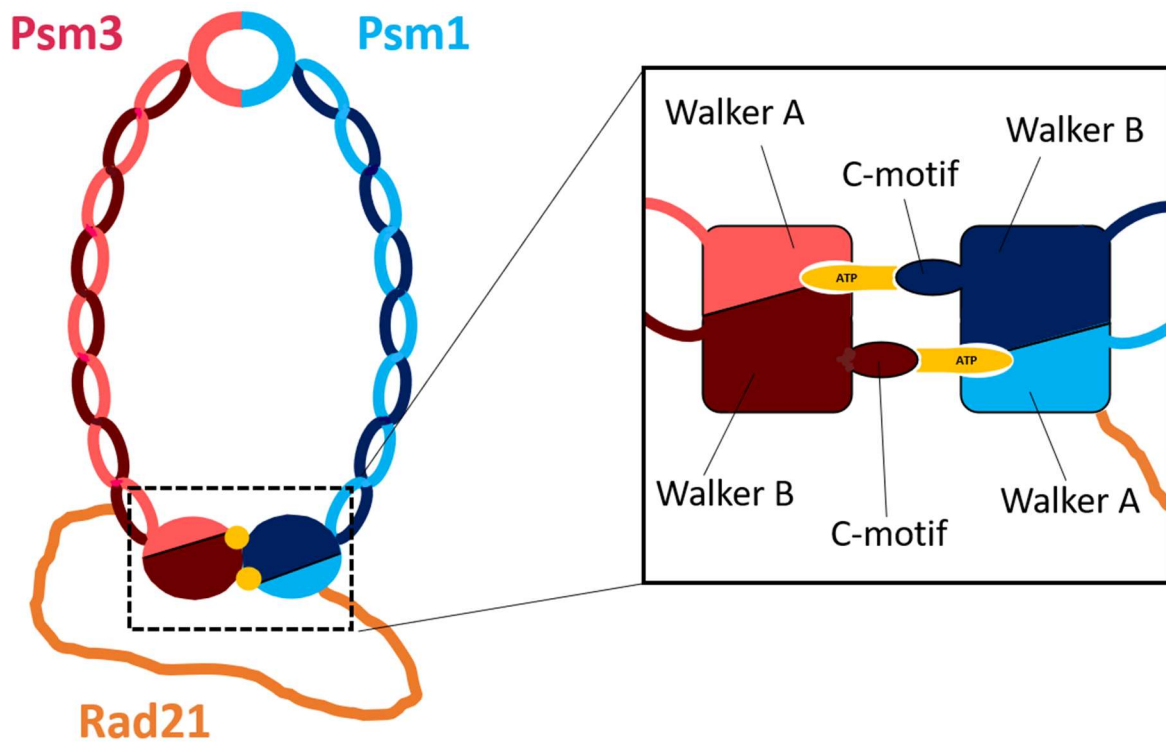


## *2- SMC structure*

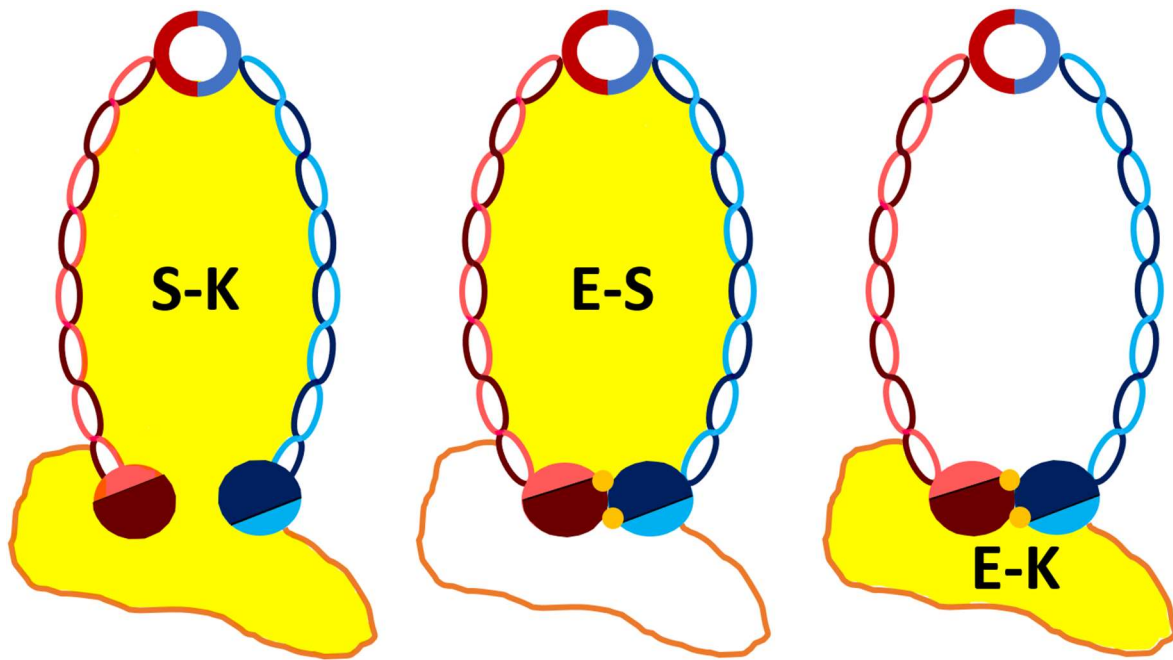
SMC complexes are ATPases with the ability to capture DNA. The structure of SMC complexes is characteristic of this family (Figure 1). An SMC complex is a heterodimer of SMC proteins. SMC proteins comprise two coiled  $\alpha$ -helices folded onto themselves from a hinge domain, forming a superhelix structure. The hinge domain allows dimerization of the two SMC proteins, giving the complex a V-shape. The N- and C-terminal parts join to form a globular domain carrying ATPase activity. The N-terminal part has a Walker A domain and the C-terminal part a Walker B domain (Figure 2). Together they form the globular head. The Walker A domain contains the ATP binding site. This interacts with the N-terminal part of the protein via  $\alpha$  and  $\beta$  phosphates. The Walker B domain has a signature motif that recognises the  $\gamma$  phosphate (Lammens et al., 2004). ATP allows dimerization (or engagement) of the globular heads by binding to the Walker A of one SMC protein and to the signature domain of the other. In this way, each SMC complex binds two ATPs (Figure 2). A protein called kleisin interacts with the heterodimer to form the “circular”, closed structure of the complex. The compartment whose diameter extends from the hinge domain to the kleisin is called S-K. The compartment formed by the SMC proteins and the engaged globular heads is called E-S. And the area between the engaged heads and the kleisin is called E-K (Figure 3) (Chapard et al., 2019; James E Collier et al., 2020; Collier and Nasmyth, 2022).



**Figure 1: SMC structure.** General diagram of a SMC complex showing the different domains that make up its structure. SMC proteins are composed of a central hinge domain from which two alpha helices extend. The two helices wrap around each other to form a coiled-coil. At the end of the coiled-coil, the N and C terminus form a globular head carrying the ATPase activity. SMCs dimerise at their hinge domain. The ATPase heads are linked by Kleisin.

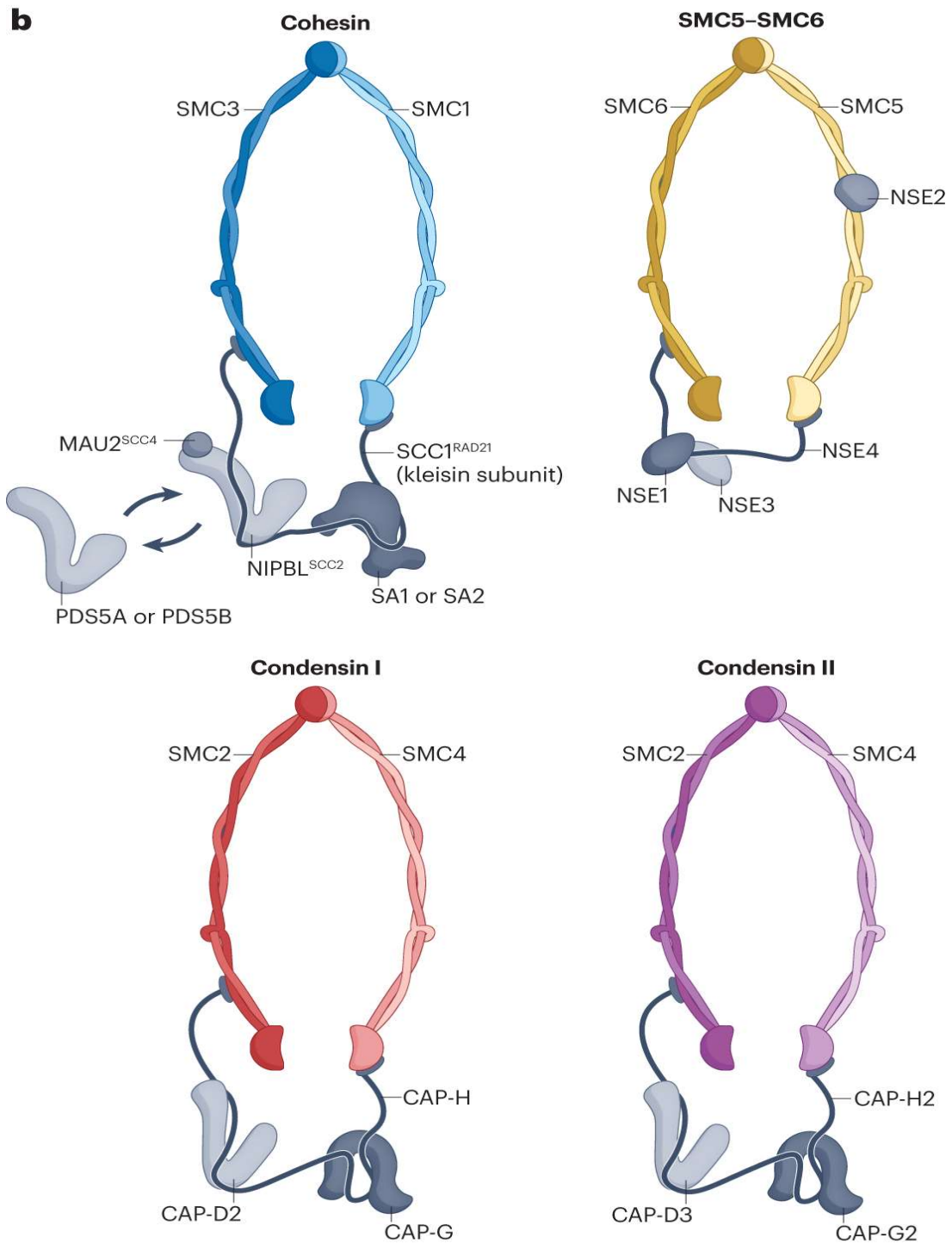


**Figure 2: Cohesin ATPase heads.** Schematic diagram of the structure of cohesin ATPase heads, showing the ATP-binding domains. The N-terminus contains a Walker A domain with an ATP-binding domain. The C-terminal part contains a Walker B domain with an ATP-recognising C motif. This structure makes it possible to bind two ATPs sandwiched between the two heads. ATP binding allows the heads to dimerise, in a position known as the ‘engaged heads’.



**Figure 3: Cohesin compartments.** Cohesin diagram showing the different compartments that make it up. When the heads are disengaged, the compartment is called S-K. When the heads are engaged, cohesin is 'separated' into two compartments. The one formed by the SMCs is called E-S while that one formed by the heads and the kleisin is called E-K.

In *S. pombe*, cohesin is composed of the SMC proteins Psm1 and Psm3 and the kleisin Rad21. Its best known function is the cohesion of sister chromatids, necessary for the attachment and segregation of chromosomes during nuclear divisions (Guacci et al., 1997; Losada et al., 1998; Michaelis et al., 1997). As with other SMC complexes, cohesin also has the capacity to form DNA loops, a process involved in the organisation of the genome in interphase (Rao et al., 2017; Wutz et al., 2017). Cohesin has roles in regulating gene expression, replication, DNA damage repair and V(D)J recombination. Condensin is the SMC complex involved in chromatin condensation during nuclear division. In humans, condensin exists in two isoforms, condensin 1 and 2. Both isoforms are composed of the SMC2 and SMC4 proteins. The difference lies in the kleisin subunit and their regulatory proteins (Ono et al., 2003, 2004). In *S. pombe*, there is only one condensin complex, homologous to condensin I, composed of Cut14, Cut3 and the kleisin Cnd2. Smc5/6 is the most recently discovered eukaryotic SMC complex, and is therefore the least well characterised. Like cohesin, Smc5/6 is thought to be involved in the repair of double-strand breaks by homologous recombination, as well as in replication (De Piccoli et al., 2006; Potts et al., 2006; Palecek, 2019). Recently, it was observed that Smc5/6 is also capable of forming DNA loops *in vitro* (Pradhan et al., 2023). This complex is composed of Smc5, Smc6 and the kleisin Nse4 (Figure 4). The names of the subunits of each complex differ depending on the organism. For the sake of clarity, the organism-dependent equivalent names of the cohesin subunits, the proteins involved in its loading, and the proteins involved in its regulation, have been given in a table (Figure 5).



**Figure 4: Human SMC complexes.** Diagram of the different SMC complexes found in humans. For cohesin, alternative names appear in superscript. Adapted from (Hoencamp and Rowland, 2023).



Role in cohesion	<i>Saccharomyces cerevisiae</i> <sup>a</sup>	<i>Schizosaccharomyces pombe</i>	Vertebrates
Cohesin complex subunits	Scc1/Mcd1	Rad21	Rad21
	Scc3/Irr1	Psc3, <b>Rec11</b>	SA1, SA2, <b>STAG3</b>
	Smc1	Psm1	Smc1 $\alpha$ , <b>Smc1<math>\beta</math></b>
	Smc3	Psm3	Smc3
	<b>Rec8</b>	<b>Rec8</b>	<b>Rec8</b>
Loading	Scc2	Mis4	NIPBL
	Scc4	Ssl3	Scc4/Mau-2
Establishment	Eco1/Ctf7	Eso1	Esco1, Esco2
Maintenance	Pds5	Pds5	Pds5A, Pds5B
	<b>Sgo1</b>	<b>Sgo1</b> , Sgo2	Sgo1, Sgo2
	PP2A	PP2A	PP2A
	Rad61/Wpl1	Wpl1	Wapl
	—	—	Sororin
Dissolution	Esp1	Cut1	Separase
	Pds1	Cut2	Securin
	Cdc5	Plc1	Plk1

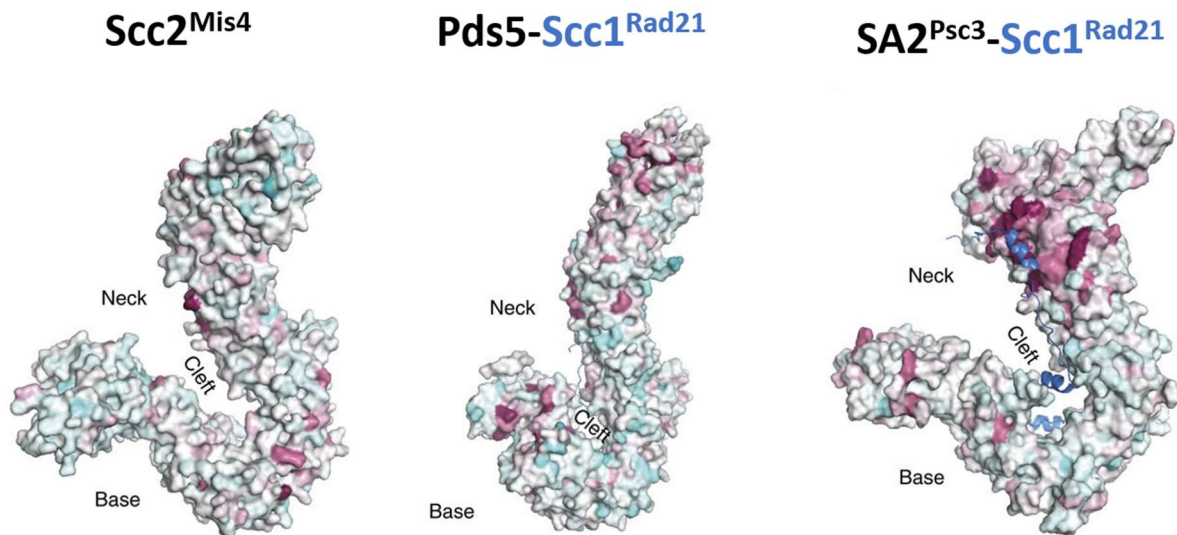
**Figure 5: Proteins from the cohesin pathway, in *S. cerevisiae*, *S. pombe* and vertebrates.** Adapted from (Xiong and Gerton, 2010).



## **B - Models of DNA capture by cohesin**

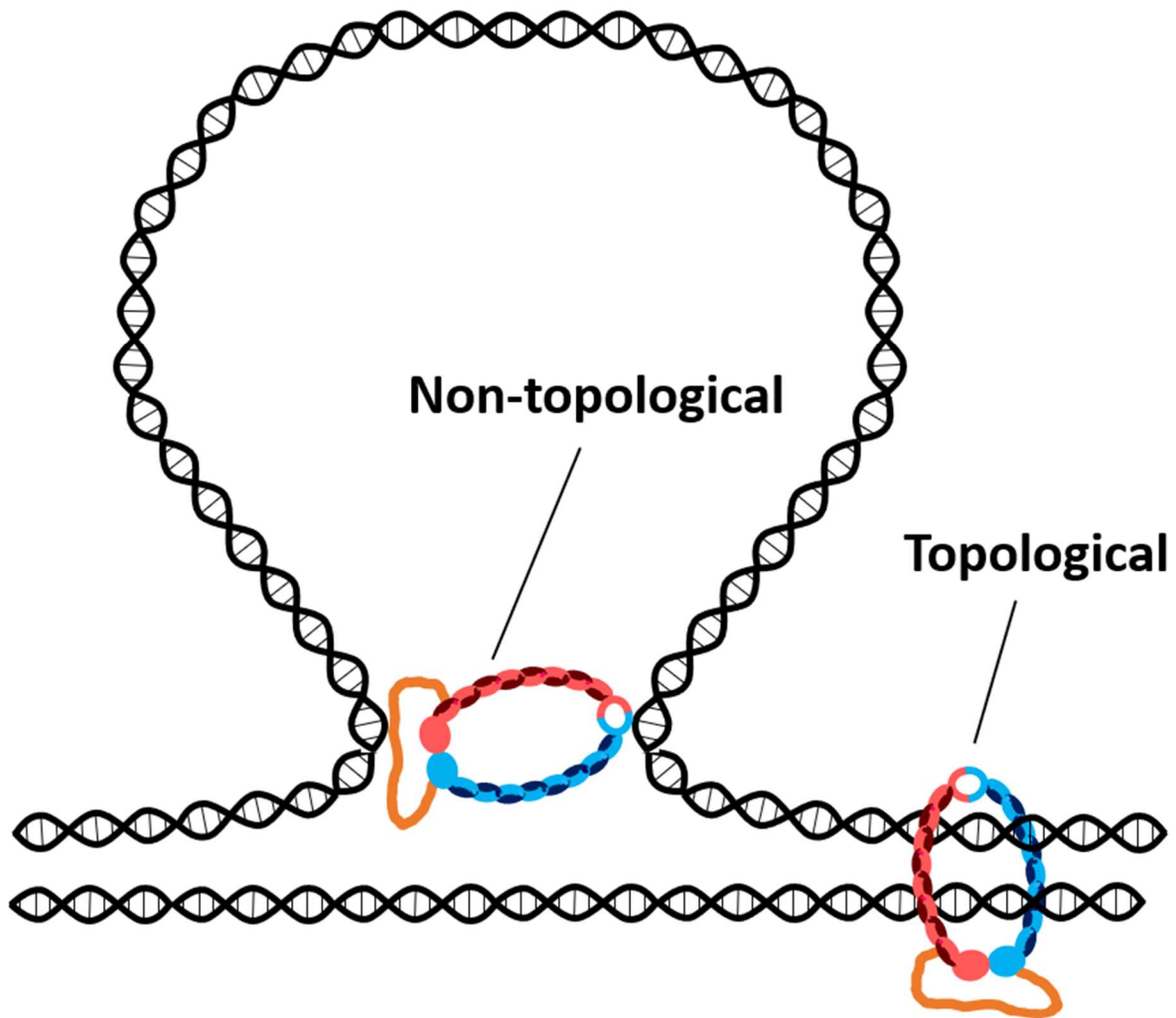
### *1-Topological association*

DNA capture by cohesin requires ATP hydrolysis by the globular heads (Arumugam et al., 2003; Weitzer et al., 2003). However, the intrinsic activity of cohesin ATPase heads is low. The loading of cohesin onto chromosomes requires the intervention of another complex that stimulates ATP hydrolysis. In *S. pombe*, the complex that stimulates cohesin ATPase activity is called Mis4/Ssl3 (Scc2/Scc4 in *S. cerevisiae*, NIPBL/MAU2 in humans) (Kanji Furuya et al., 1998; Rafal Ciosk et al., 2000; Bernard et al., 2006; Petela et al., 2018; Iain F. Davidson et al., 2019). Mis4 is a HAWK (HEAT Repeat Protein Associated With Kleisin) protein (Wells et al., 2017). Like other HAWK proteins, Mis4 is hook-shaped and regulates cohesin through its association with kleisin (Hara et al., 2014; Roig et al., 2014) (Figure 6). Mis4 is capable of binding to naked DNA. It has been identified as the main protein involved in cohesin loading, and has therefore been named 'cohesin loader'. *In vitro* Mis4 alone can load cohesin onto DNA, but *in vivo* loading requires Mis4 to interact with Ssl3 at its N-terminus (Bernard et al., 2006; Murayama and Uhlmann, 2014).



**Figure 6: HAWK proteins.** Hook structure of the HAWK proteins, Scc2<sup>Mis4</sup>, Pds5 and SA2<sup>Psc3</sup>. In blue, part of Scc1<sup>Rad21</sup> associated with Pds5 or SA2<sup>Psc3</sup> during crystallisation. *S. pombe* names are shown in superscript. Adapted from (Chao et al., 2017).

The closed structure of SMCs and the linearity of DNA suggest that DNA capture by cohesin can be achieved topologically and non-topologically (Figure 7). In the remainder of this paragraph, we will explain these two mechanisms in more detail, using cohesin-mediated DNA capture as an example. Topological capture of DNA by cohesin was initially the mechanism with the most support. Indeed, the discovery of separase as an enzyme that cleaves Rad21 at anaphase, freeing sister chromatids, consolidated the hypothesis of topological DNA capture by cohesin (Uhlmann et al., 1999). The association of cohesin with DNA resists concentrated salt washes, suggesting that the DNA is trapped inside the ring. In addition, DNA digestion by nucleases dissociates cohesin from DNA (Rafal Ciosk et al., 2000). Furthermore, cleavage of the Smc3<sup>Psm3</sup> subunit of the cohesin complex, associated with a circular minichromosome, induces dissociation of cohesin from DNA and loss of cohesion (Ivanov and Nasmyth, 2005, 2007). The topological association of cohesin with DNA would therefore be the basis of the sister chromatid cohesion process. For the DNA to be trapped, the cohesin “ring” must open, presumably through the opening of a protein-protein interface. The three cohesin interfaces are between Rad21 and Psm1, Rad21 and Psm3 and the hinge domain between Psm1 and Psm3. Initially, to determine the DNA entry gate(s), the three interfaces were closed. The technique used was to use modified cohesin subunits, either fusion proteins or containing the rapamycin-binding domains FRB and FKBP12, at each interface. The addition of rapamycin to the culture medium resulted in a protein-rapamycin-protein interaction strong enough to assume that the interface could not open. In this way, the laboratory was able to observe that blocking the interface at the hinge domain prevented DNA from entering cohesin (Gruber et al., 2006). This experiment does not formally show that the hinge domain is the gateway for DNA entry, but indicates that the hinge interaction must be intact for cohesin to associate with DNA. Later experiments that reconstructed the loading of cohesin *in vitro*, together with analysis of structural data, suggested that DNA entry occurred via the Psm3-Rad21 interface (Higashi et al., 2020). The debate as to where DNA enters cohesin is still ongoing.



**Figure 7: The two types of cohesin capture.** Cohesin can capture DNA topologically by trapping the DNA in its ring. The other way of capturing DNA is non-topological, in this case the DNA is outside the ring.

Cryo-EM experiments were initially used to solve the 3D structure of  $\text{Scc2}^{\text{Mis4}}$  in *Ashbya gossypii*. These data confirmed the hook-like shape of  $\text{Scc2}^{\text{Mis4}}$ , similar to  $\text{SA2}^{\text{Psc3}}$ .  $\text{SA2}^{\text{Psc3}}$  interacts with  $\text{Scc1}^{\text{Rad21}}$  via its neck, composed of HEAT-repeats, suggesting that  $\text{Scc2}^{\text{Mis4}}$  interacts with these partners with the same surface (Chao et al., 2017). In the same study, the authors also suggested that  $\text{Scc2}^{\text{Mis4}}$  could interact with the hinge and coiled-coil close to the ATPase heads of  $\text{Smc1}^{\text{Psm1}}/\text{Smc3}^{\text{Psm3}}$ . At the same time, another team showed that the C-terminal part of  $\text{Scc2}^{\text{Mis4}}$  in *Chaetomium thermophilum* interacts with the N-terminal region of  $\text{Scc1}^{\text{Rad21}}$  and mutations in the neck region of  $\text{Scc2}^{\text{Mis4}}$  reduce its interaction with  $\text{Scc1}^{\text{Rad21}}$  (Kikuchi et al., 2016). Interestingly, the authors did not observe any binary interaction between  $\text{Scc2}^{\text{Mis4}}$  and  $\text{Scc1}^{\text{Rad21}}$  or between  $\text{Scc2}^{\text{Mis4}}$  and  $\text{Smc1}^{\text{Psm1}}/\text{Smc3}^{\text{Psm3}}$ , suggesting that the interaction of  $\text{Scc2}^{\text{Mis4}}$  with each cohesin subunit requires the complex to be fully formed. Furthermore, it appears that some parts of  $\text{Scc2}^{\text{Mis4}}$  are able to interact with the  $\text{Scc1}^{\text{Rad21}}/\text{Smc3}^{\text{Psm3}}$  interface, suggesting a role for  $\text{Scc2}^{\text{Mis4}}$  in regulating its opening (Gligoris et al., 2014; Huis in 't Veld et al., 2014).

Later, Cryo-EM experiments in humans were used to obtain the structure of  $\text{NIPBL}^{\text{Mis4}}$  bound to cohesin and DNA (Zhubing Shi et al., 2020). These data showed that  $\text{NIPBL}^{\text{Mis4}}$  can interact simultaneously with  $\text{RAD21}$ ,  $\text{SMC1}^{\text{Psm1}}$  and DNA.  $\text{NIPBL}^{\text{Mis4}}$  interacts with  $\text{SMC1}^{\text{Psm1}}$  and  $\text{SMC3}^{\text{Psm3}}$  through three interfaces, one with the globular heads, a second with the coiled-coil domains and finally one with  $\text{SMC3}^{\text{Psm3}}$  at the joint. This last interface also involves  $\text{RAD21}$ . In addition, it appears that the hinge may transiently interact with  $\text{STAG1}^{\text{Psc3}}$  and  $\text{NIPBL}$  simultaneously. Interestingly, the N-terminal part of  $\text{NIPBL}^{\text{Mis4}}$  interacts with  $\text{SMC3}^{\text{Psm3}}$  K105 and K106, involved in regulating the opening of the  $\text{SMC3}^{\text{Psm3}}\text{-RAD21}$  interface (see section II-B- Cohesin dynamic and cohesion establishment, and II- C- Cohesion maintenance). In addition, the DNA interacts with all the subunits by passing through a tunnel in the central position of the complex.

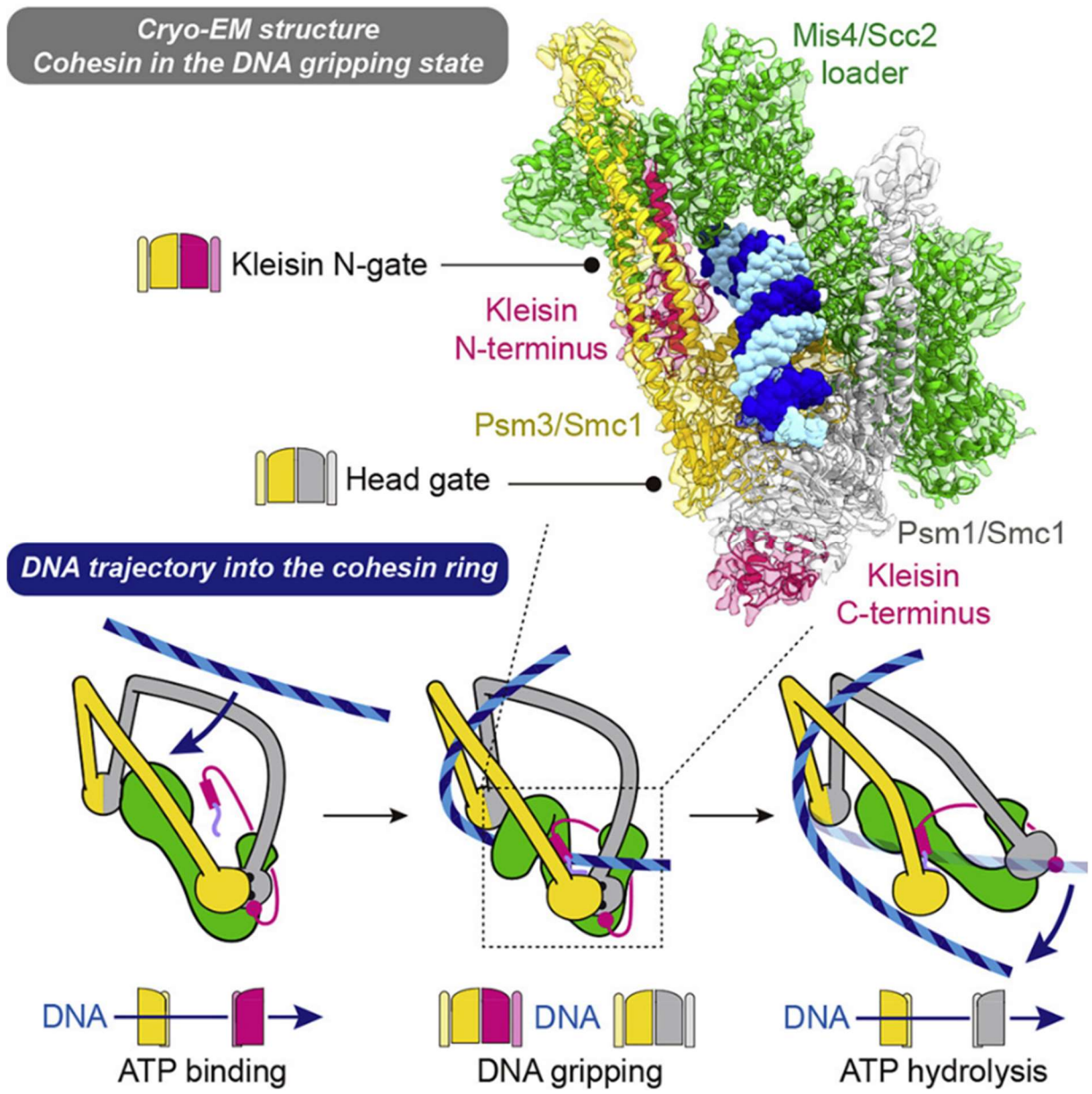




At the same time, a team obtained the structure of cohesin when it is loaded onto DNA in the so called 'gripping state'. From these data, they suggested that initially the DNA slips between the superhelices of a folded cohesin, then the Psm3-Rad21 interface is opened when the ATP binds to the globular heads, which are then engaged. The DNA is then in the E-K compartment. After ATP hydrolysis, the heads are disengaged and the DNA is trapped in cohesin (Higashi et al., 2020) (Figure 8). Kim Nasmyth's team recently observed that simultaneous closure of the  $\text{Smc1}^{\text{Psm1}}\text{-Smc3}^{\text{Psm3}}$  and  $\text{Smc3}^{\text{Psm3}}\text{-Sccl}^{\text{Rad21}}$  interfaces prevents topological capture of DNA by cohesin *in vitro* (Collier and Nasmyth, 2022). In the same study, they suggest that DNA passage through the hinge domain is only possible when DNA is trapped between  $\text{Sccl}^{\text{Mis4}}$  and the engaged globular heads. Interestingly, it appears that DNA entry via  $\text{Smc3}^{\text{Psm3}}\text{-Sccl}^{\text{Rad21}}$  is not dependent on  $\text{Sccl}^{\text{Mis4}}$ . This suggests that there are several mechanisms for topological loading of cohesin. All the data obtained by the two teams suggest a key role for the hinge domain and the Psm3-Rad21 interface in the topological capture of DNA.

## *2- Non-topological association*

Non-topological or pseudo-topological association is defined by the capture of DNA without opening the cohesin ring. Several arguments suggest that this mechanism is involved in the process of DNA loop formation. The hinge domain of cohesin is positively charged. Neutralising these charges induces a cohesion defect without affecting the association of cohesin with DNA (Kurze et al., 2011). In humans, a hinge domain mutant is able to form DNA loops without being able to achieve cohesion (Nagasaka et al., 2023). More importantly, the simultaneous closure of all cohesin interfaces does not prevent its association with DNA or the formation of DNA loops *in vitro* (Srinivasan et al., 2018; Iain F. Davidson et al., 2019). Cohesin is therefore capable of capturing DNA topologically or non-topologically. Topological capture would be involved in the cohesion function, whereas non-topological capture would be involved in the formation of DNA loops.



**Figure 8: DNA entry into cohesin.** Cryo-EM structure of the complex between Mis4, cohesin and DNA in the presence of non-hydrolysable ATP. The schematics below are a model of how ATP hydrolysis would lead to DNA capture by cohesin. Initially ATP binds to cohesin, the heads are engaged, the Psm3-Rad21 interface is opened. Mis4 grips the DNA against the ATPase gate and closed the Psm3-Rad21 interface. Finally, ATP is hydrolysed, the heads disengaged leading to ATPase gate opening, completing DNA entry. Adapted from (Higashi et al., 2020).

## II - Sister chromatid cohesion

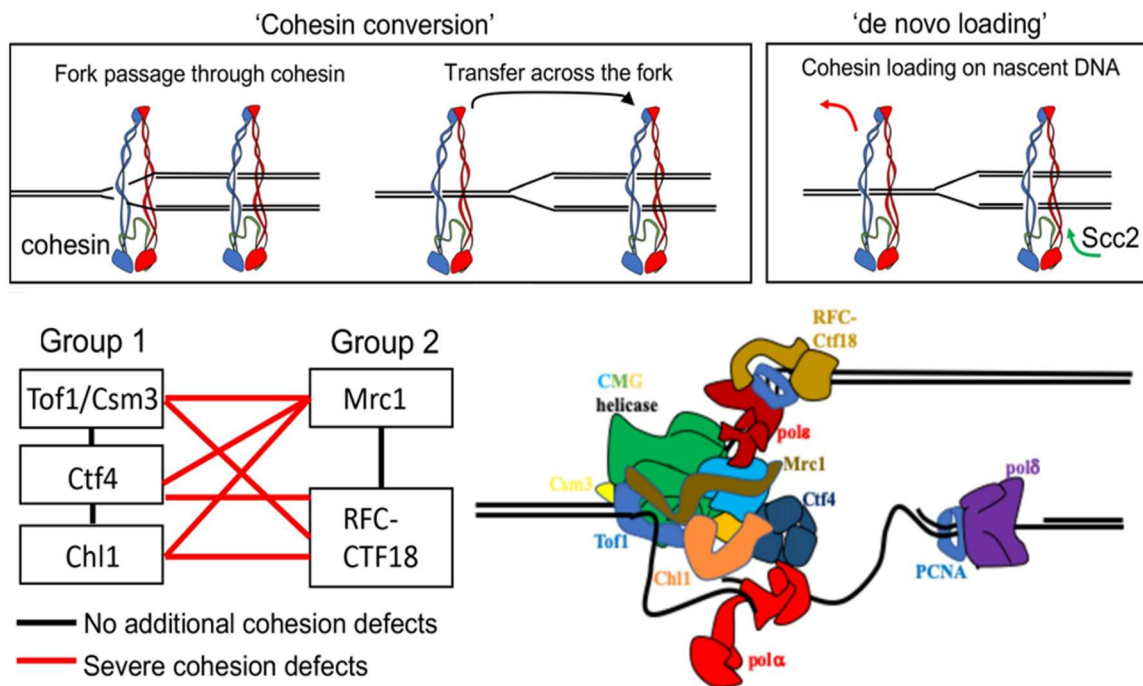
### A - Cohesion establishment and DNA replication

In metazoans, cohesin loading occurs during telophase, whereas in the yeast *S. cerevisiae* loading occurs during S phase and in G1 in *S. pombe* (Guacci et al., 1997; Michaelis et al., 1997; Losada et al., 1998; Rafal Ciosk et al., 2000; Bernard et al., 2006; Gerlich et al., 2006). The establishment of cohesion is restricted to the S phase of the cell cycle, coupled to genome replication.  $\text{Scc2}^{\text{Mis4}}$  exerts its essential function in G1/S and inactivation of  $\text{Scc2}^{\text{Mis4}}$  in G2 does not affect cohesion (Lengronne et al., 2006; Vaur et al., 2012). Cohesin can be loaded after S phase but does not form cohesion (Uhlmann and Nasmyth, 1998). An exception to this rule is the repair of DNA double-strand breaks post-S-phase (see section IV- B- DNA repair). Cohesin become cohesive during DNA replication. How cohesin brings about the cohesion of sister chromatids is not yet fully understood. An early hypothesis suggested that the replisome would pass through cohesin loaded upstream of the replication fork (Lengronne et al., 2006). Using *in vitro* microscopy techniques, it was observed that topologically loaded cohesin cannot be passed through small ( $\approx 19.5$  nm diameter) entities (Stigler et al., 2016). This suggests that cohesin would have to undergo a transient opening for the replication fork to pass through. It is therefore possible that cohesin loses its interaction with DNA during the replisome passage, to regain this interaction afterwards and render the chromatids cohesive.



Genetic analyses in *S. cerevisiae*, studying the involvement of replisome proteins in the establishment of cohesion, revealed two pathways. Initially, it was observed that the replisome proteins Chl1, Ctf4, Tof1/Csm3, Mrc1 and Ctf8, Ctf18, Dcc1 (Ctf18-RFC complex), participate in an important way while being non-essential to cohesion (Hanna et al., 2001; Mayer et al., 2001, 2004; Petronczki et al., 2004; Skibbens, 2004; Xu et al., 2004; Abe et al., 2016; Cortone et al., 2018; Zheng et al., 2018). The genes form two epistatic groups defining two pathways. The deletion of two genes from the same group will have the same effect on cohesion as each of the single mutants, whereas the deletion of two genes from different groups is lethal or sub-lethal and worsens cohesion defects (Xu et al., 2007) (Figure 9). Using circular minichromosomes, K. Nasmyth's group showed that group 1 genes (Chl1, Ctf4, Tof1 and Csm3) are required for the conversion process and group 2 genes (Mrc1 and RFC-CTF18 complex) for the *de novo* pathway. When the conversion pathway is inactivated, cohesin complexes previously associated with DNA do not form cohesion at the end of S phase and are largely dissociated from chromosomes. However, cohesion is established by loaded cohesins, probably at the level of the replisome, in a manner dependent on group 2 genes and the loading factor  $\text{Scc2}^{\text{Mis4}}$  (Srinivasan et al., 2020) (Figure 9). The molecular mechanisms involved in these two processes are still poorly understood.

Recently, it was observed in an *in vitro* system using *Xenopus* egg extracts that 57 to 66% of chromatin-associated cohesin is displaced by the replisome during DNA synthesis (Cameron et al., 2024). The cohesin complexes are moved to the replication termination sites formed by the merging of the forks moving in the opposite orientation. When the replisome is disassembled, these cohesin complexes remain associated with the chromatin and are cohesive. In the same study, the authors showed that a helicase complex dissociation defect during replication termination induces a cohesion defect *in vivo* in *S. cerevisiae*. Taken together, these data suggest that cohesin conversion is localised to replication termination sites (Cameron et al., 2024).

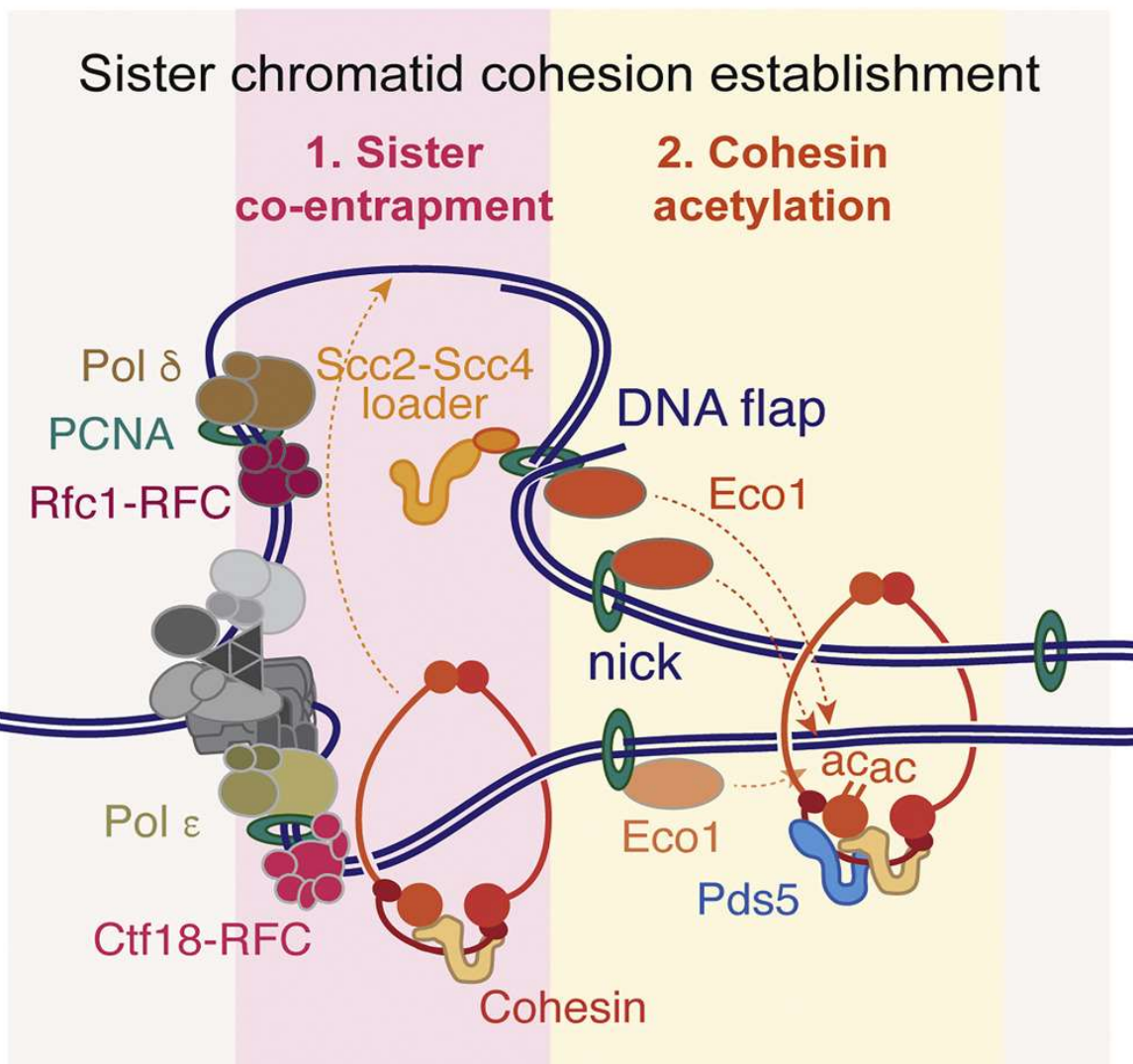


**Figure 9: Cohesin conversion and *de novo* pathway models for the establishment of sister-chromatid cohesion.** The cohesin conversion model involves a transient opening of the cohesin ring, allowing the replisome to pass through. This cohesion process would involve Tof1/Csm3, Ctf4 and Chl1. The other process suggested is the *de novo* loading of cohesin after or during passage of the replication fork. This process would be dependent on Scc2<sup>Mis4</sup> and would involve the Mrc1 and RFC-CTF18 proteins. Adapted from (Srinivasan et al., 2020).

## B - Cohesin dynamic and cohesion establishment

The loading of cohesin onto the DNA is a reversible process. Two cohesin dissociation processes have been discovered that involve the opening of the  $\text{Smc3}^{\text{Psm3}}/\text{Scc1}^{\text{Rad21}}$  interface. The first one, and the best characterised, involves the Wpl1 and Pds5 proteins. Pds5 is a protein with two apparently antagonistic functions. It is involved in the stabilisation of cohesin to DNA, but also in its dissociation (Kueng et al., 2006; Chan et al., 2012; Vaur et al., 2012). Pds5 interacts with cohesin via the kleisin and the  $\text{Smc3}^{\text{Psm3}}$  subunit and allows the recruitment of Wpl1 (Chan et al., 2012; Hara et al., 2014; Lee et al., 2016; Petela et al., 2021). The interaction of Wpl1 with Pds5 stimulates the opening of the  $\text{Scc1}^{\text{Rad21}}\text{-Smc3}^{\text{Psm3}}$  interface, allowing the exit of DNA from cohesin (Chan et al., 2012; Beckouët et al., 2016). Cohesin can be dissociated from the DNA by a second mechanism which does not involve Wpl1. Indeed, it has been shown in budding yeast that depletion of  $\text{Scc2}^{\text{Mis4}}$  at the end of G1 induces unloading of pre-loaded cohesin in a Wpl1-independent manner. This process, induces cohesin dissociation through the opening of the  $\text{Smc3}^{\text{Psm3}}\text{-Scc1}^{\text{Rad21}}$  interface and involves the ATPase head of Smc1. This mechanism which is countered by  $\text{Scc2}^{\text{Mis4}}$  remains largely misunderstood (Srinivasan et al., 2019).

Cohesion establishment during S phase requires an essential acetyltransferase, highly conserved from yeast to humans, called Eso1 in *S. pombe* (Eco1 in *cerevisiae* and Escp1/2 in humans). This acetyltransferase acetylates the cohesin complex on two conserved lysine residues on  $\text{Smc3}^{\text{Psm3}}$  globular head (K105, K106 in *S. pombe* and human, K112, K113 in *S. cerevisiae*). This acetyltransferase is recruited during DNA replication by the replisome via its interaction with PCNA, the clamp protein that stimulates DNA polymerase advancement (Skibbens et al., 1999; Moldovan et al., 2006; Maradeo and Skibbens, 2009). *In vitro* experiments have suggested that these acetylation events would only be possible during the formation of 'nick' and 'flaps' during DNA synthesis (Minamino et al., 2023) (Figure 10). Cohesin acetylation is Pds5-dependent and counteracts the action of the Pds5/Wpl1 proteins. Inhibition of the Pds5/Wpl1-dependent-cohesin release would lead to the stabilization of the  $\text{Scc1}^{\text{Rad21}}\text{-Smc3}^{\text{Psm3}}$  interface thereby allowing the establishment of sister chromatid cohesion. (Zhang et al., 2008; Rowland et al., 2009; Beckouët et al., 2010; Vaur et al., 2012; Chan et al., 2013).



**Figure 10: Cohesion establishment and stabilisation by cohesin acetylation.** Schematic representation of cohesion establishment during DNA replication. Cohesins are stabilised by the action of Eco1, which acetylates cohesin when the PCNA/Eco1 complex encounters a 'nick and flap' structure. Adapted from (Minamino et al., 2023).



## C - Cohesion maintenance

Sister chromatid cohesion must persist throughout G2 and until anaphase. Cohesion maintenance relies on Smc3<sup>Psm3</sup> acetylation and Pds5.

Mechanistically, Pds5 would have more affinity with acetylated cohesin, and would maintain the cohesin in a folded form through its interaction with kleisin and the hinge domain. In this case, the ATPase heads would not be engaged, preventing the cohesin ring from opening. When Smc3<sup>Psm3</sup> is not acetylated, Wpl1 would have more affinity with the hinge domain than Pds5. This would prevent cohesin from folding and therefore cause the ATPase heads to engage and open the ring at the Scc1<sup>Rad21</sup>-Smc3<sup>Psm3</sup> interface (Bauer et al., 2021; Petela et al., 2021; Oldenkamp and Rowland, 2022). In addition, it has been shown that over-expression of the deacetylase Hos1 induces partial deacetylation of Smc3 and a reduction in cohesion. It has been suggested that Pds5 protects Smc3 from deacetylation by Hos1 (Beckouët et al., 2010; Borges et al., 2010; Chan et al., 2013).

In vertebrates, the acetylation of SMC3<sup>Psm3</sup> by ESCO1<sup>Eco1</sup> during replication enables the recruitment of Sororin (Rankin et al., 2005). Sororin opposes Wpl1 by displacing Wpl1 from its binding partner PDS5 and it has been proposed that Sororin maintains cohesion by inhibiting WAPL's ability to dissociate cohesin from DNA (Lafont et al., 2010; Nishiyama et al., 2010; Song et al., 2012).

## D - Release and cleavage of cohesin during mitosis

Mitosis begins with prophase, characterised by the degradation of the nuclear envelope in metazoans, the condensation of chromosomes and the capture of kinetochores by spindle microtubules. In vertebrates, during prophase, cohesin is dissociated from the chromosome arms. This process is known as the Prophase Dissociation Pathway (PDP). During prophase, Sororin is phosphorylated by Cdk1 and Aurora B, preventing its interaction with Pds5 and inducing the release of cohesin by WAPL along chromosome arms (Dreier et al., 2011; Nishiyama et al., 2013). Centromere-associated cohesins are protected from WAPL action by the SGO1-PP2A (Shugoshin - protein phosphatase 2A) complex (Liu et al., 2013). In budding

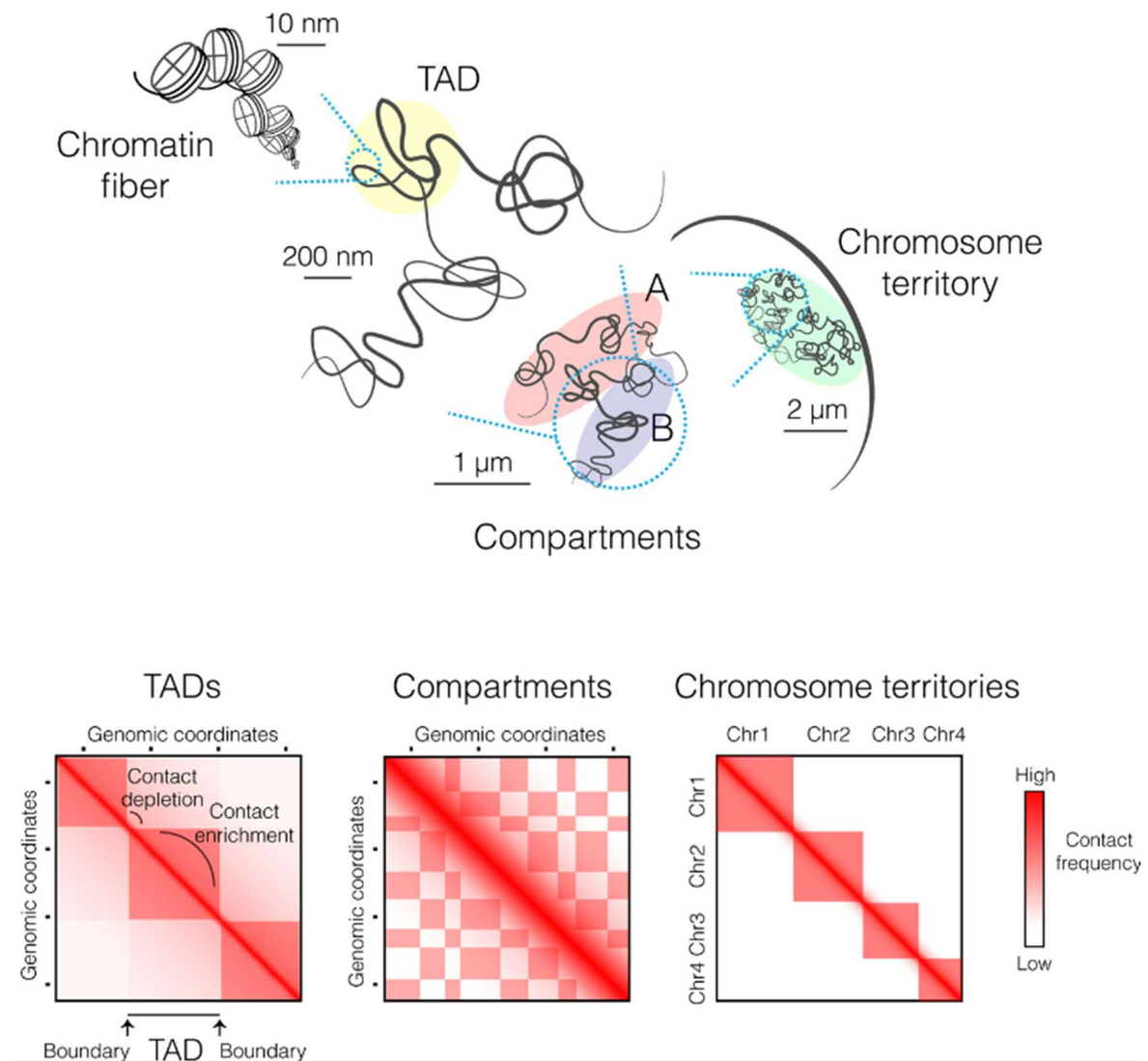
yeast there is probably no PDP, the sister chromatids are linked to each other along their entire length by cohesin, until anaphase. The existence of a PDP was suggested in fission yeast (Christine K. Schmidt et al., 2009). At anaphase, the Rad21 subunit is cleaved by Separase, ending cohesion and allowing segregation of sister chromatids (Uhlmann et al., 1999, 2000) (see section IV- A- Chromosome segregation during nuclear division).

### III- Cohesin and genome architecture

#### A - The genome is organised into compartments and TADs

The advent of the genome-wide chromosome conformation capture (Hi-C) technique has made it possible to discover that the genome is highly structured in interphase by the action of cohesin (Lazar-Stefanita et al., 2017; Rao et al., 2017; Wutz et al., 2017; Iain F. Davidson et al., 2019; Lorenzo Costantino et al., 2020). This technique revealed the presence of compartments, TADs (Topological Associated Domains), initially called globules or TADs like structure in yeast (Lieberman-Aiden et al., 2009; Takeshi Mizuguchi et al., 2014; Rao et al., 2014) (Figure 11). Compartments are structures that can be observed on a large scale. These structures correspond to long-distance *cis*-chromatin interactions and are mutually exclusive (Wang et al., 2016). In mammals, there are two compartments, A and B. Compartment A is characterised as a gene-rich region with active chromatin. Compartment B is enriched in heterochromatin (Lieberman-Aiden et al., 2009; Rao et al., 2014). Within these compartments, chromatin is structured into TADs (Dixon et al., 2012; Nora et al., 2012). A TAD is defined as a genomic region within which numerous *cis* interactions occur. TADs can be observed within a compartment at a scale of 10 to 1000 kpb. The existence of TADs at the level of the single cell remains debated. TADs are only visible by Hi-C, within a population of cells. They cannot be observed in single-cell Hi-C experiments or by microscopy. TADs would therefore be the sum of the interactions within a population of cells (Flyamer et al., 2017; Bintu et al., 2018; Finn et al., 2019; Luppino et al., 2020; Su et al., 2020; Szabo et al., 2020).

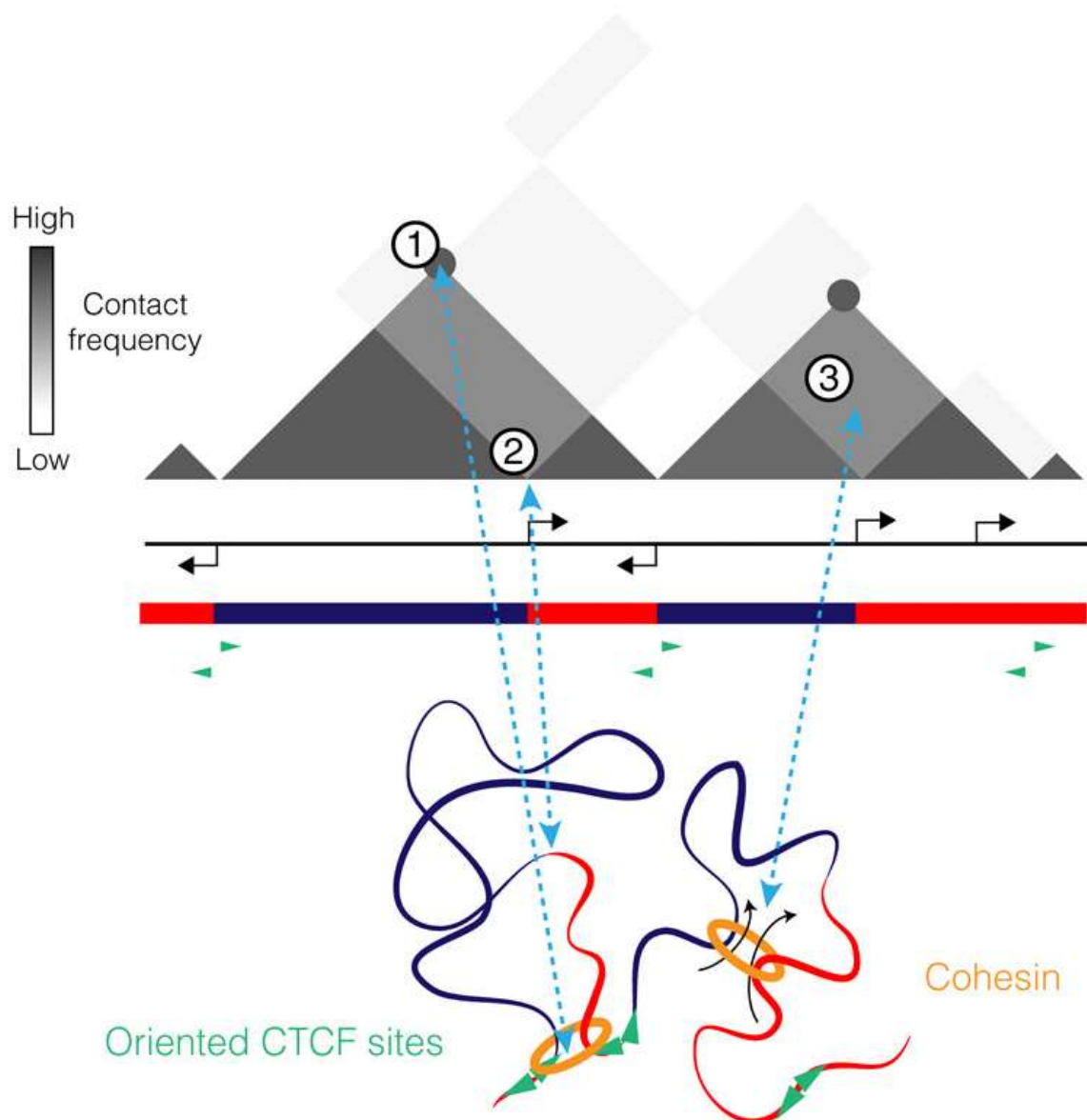
By definition, within a TAD there is strong intra-domain interaction. On the other hand, there is very little inter-domain interaction, i.e. little interaction between TADs. We can therefore observe the presence of borders between TADs (Figure 11) (Dixon et al., 2012; Nora et al., 2012; Sexton et al., 2012). Genes within the same TAD tend to be coregulated during cell differentiation and are under the control of the same enhancer. Alteration of TAD borders can lead to deregulated gene expression (Nora et al., 2012; Symmons et al., 2014; Dixon et al., 2016; Zhan et al., 2017; Ramírez et al., 2018).



**Figure 11: Chromatin compartmentalization.** (A) Diagram showing the different chromosome compartments in the nucleus. These compartments represent areas of strong interaction between different chromosomal regions. The chromosomes occupy preferential locations within the nucleus and compartmentalized into compartments A and B. These two compartments are themselves divided into sub-domains called TADs, defined as domains of high frequency cis-interaction between DNA segments. (B) Diagram of the maps obtained by HI-C, identifying the different levels of interaction between chromatin segments. Adapted from (Szabo et al., 2019)

This deregulation is thought to lead to developmental defects and the formation of cancers (Lupiáñez et al., 2015, 2016; Flavahan et al., 2016; Franke et al., 2016; Hnisz et al., 2016). Despite this, the loss of delineation between TADs has generally little effect on transcription. Moreover, the interaction between two neighbouring TADs is only 2-fold weaker than the interactions within the TAD (Dixon et al., 2012; Dekker and Mirny, 2016; Chang et al., 2020).

The border of a TAD is characterised by the presence of the CCCTC-binding factor (CTCF) protein and cohesin (Phillips-Cremins et al., 2013; Vietri Rudan et al., 2015). CTCF is able to bind to DNA at the CCCTC sequence, otherwise known as the CTCF-binding site. The border of TADs is composed of two CTCF-binding sites in opposite orientation. Interestingly, deletion or change of orientation of this sequence can reduce the definition of the border, such as a border shift (Guo et al., 2015; Lupiáñez et al., 2015; Sanborn et al., 2015; de Wit et al., 2015; Nora et al., 2017). Cohesin or NIPBL<sup>Mis4</sup> depletion induces a defect in TAD formation (Rao et al., 2017; Schwarzer et al., 2017). Conversely, depletion of WAPL induces an increase in loop size and stabilises TADs and their borders (Haarhuis et al., 2017; Wutz et al., 2017). Structural data showed that CTCF binds to cohesin through its N-terminal domain by interacting with RAD21 and STAG2<sup>Psc3</sup>. Interestingly, the binding site of CTCF to cohesin is the same as that of WAPL, suggesting that CTCF is involved in cohesin stabilisation (Li et al., 2020; Nora et al., 2020). Taken together, these data suggest a joint role for cohesin in the formation of TADs and CTCF in their delimitation. One model proposes that cohesin extrudes DNA and forms a loop until it meets two converging CTCF-binding sites or is dissociated from chromatin (Figure 12). However, this model does not explain the formation of all TADs. When CTCF is depleted, 20% of TADs retain a strongly marked border (Nora et al., 2017). There are therefore TADs whose delimitation is not due to CTCF. Furthermore, *in silico*, TADs and DNA loops can be formed by Brownian motion or osmotic pressure alone without cohesin or CTCF activity (Brackley et al., 2018, 2017; Yamamoto and Schiessel, 2017). The formation/stabilisation and the relevance of TADs as functional units of the genome are very active areas of research.



**Figure 12:** Model of loop extrusion by cohesin involved in the formation of TADs. Cohesin extrudes DNA until it is dissociated, meets another cohesin or the CTCF protein. Adapted from (Szabo et al., 2019)

High resolution Hi-C (Micro-C) experiments in *S. cerevisiae* have shown that the bases of DNA loops are located at the CARs (Cohesin Associated Region) (Lorenzo Costantino et al., 2020). As in mammals, depletion of Wpl1 in yeast also induces an increase in loop size. In contrast, Pds5 and Eco1 inhibit the expansion of DNA loops. The acetylation of Smc3 K112 and K113 by Eco1 suppresses loop expansion and acetylated Smc3 requires Pds5 to block loop expansion (Bastié et al., 2022). These results therefore indicate a role for Eco1 in the regulation of DNA loop formation, possibly linked to a regulatory pathway similar to cohesion maintenance. Taken together, these results indicate that cohesin stability plays a role in genome architecture and is finely regulated.



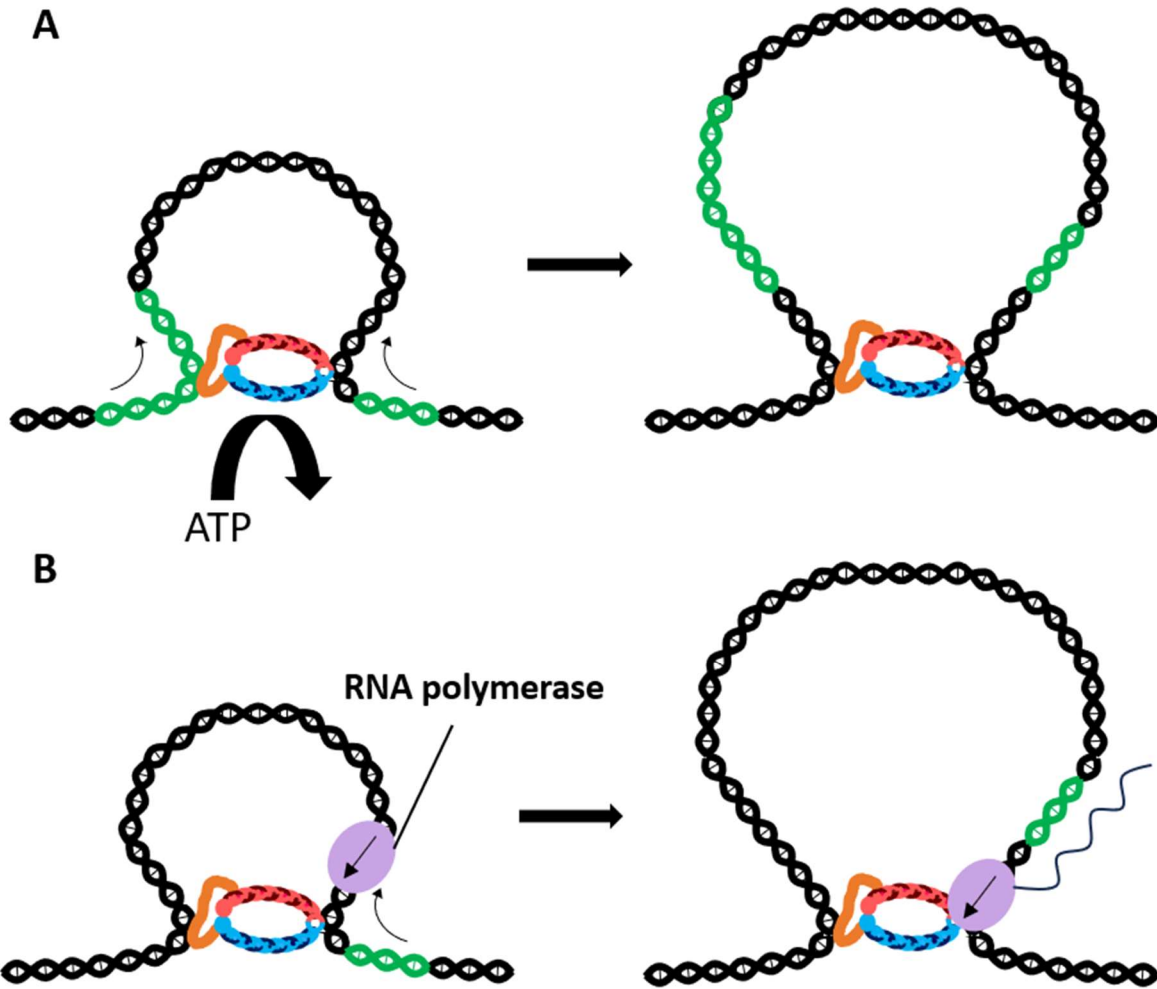


## B - The loop extrusion model

In this model, a cohesin forms a small loop when it is loaded, which grows either by advancing on the DNA or by 'pumping' it (pumping model) (Alipour and Marko, 2012; Fudenberg et al., 2016; Goloborodko et al., 2016) (Figure 13).

Previously we saw that the interaction of cohesin forming DNA loops would take place in a non-topological manner (Iain F. Davidson et al., 2019). In the configuration where the three interfaces are experimentally covalently linked, cohesin can form DNA loops despite the presence of DNA-associated particles larger than the diameter of the largest cohesin compartment (Iain F. Davidson et al., 2019). Thus, the extruded DNA would be outside of cohesin. Cohesin can bind to DNA via its associated proteins such as Wpl1 and Pds5, but also via its hinge domain and kleisin. These are not the only potential DNA binding domains. In fact, several experiments, in particular structure resolution combined with biochemical data, suggest that globular heads and coiled-coils are also potential DNA binding domains (Murayama and Uhlmann, 2014; Y. Li et al., 2018; James E Collier et al., 2020; Higashi et al., 2020; Zhubing Shi et al., 2020). Cohesin would therefore have several domains of interaction enabling it to associate non-topologically with DNA.

The driving force behind DNA extrusion is not yet very well defined. One hypothesis is that DNA extrusion is made possible by the ATPase activity of globular heads. Indeed, it has been observed *in vitro* that loop formation requires the presence of ATP as well as the stimulator of ATP hydrolysis by ATPase heads, i.e. NIPBL<sup>Mis4</sup> (Iain F. Davidson et al., 2019). Moreover, *in vivo*, cohesin translocation onto DNA requires ATP hydrolysis by globular heads (Hu et al., 2011; Petela et al., 2018). ATPase activity would therefore be an intrinsic driver of DNA loop formation (Figure 13.A). Recently, it has been proposed that the motor of DNA extrusion is not or not only intrinsic but also extrinsic. Cohesin ChIP-seq data show cohesin accumulation at the 3' ends of converging genes, suggesting an involvement of transcription (Lengronne et al., 2006; Christine K. Schmidt et al., 2009; Takeshi Mizuguchi et al., 2014). In other words, RNA polymerases would push cohesin along the DNA, inducing the formation of DNA loops (Figure 13.B).



**Figure 13: Cohesin DNA loop extrusion by intrinsic and extrinsic motors. (A) ATP hydrolysis as intrinsic motor for DNA extrusion (B) RNA polymerase activity as extrinsic motor for DNA extrusion.**

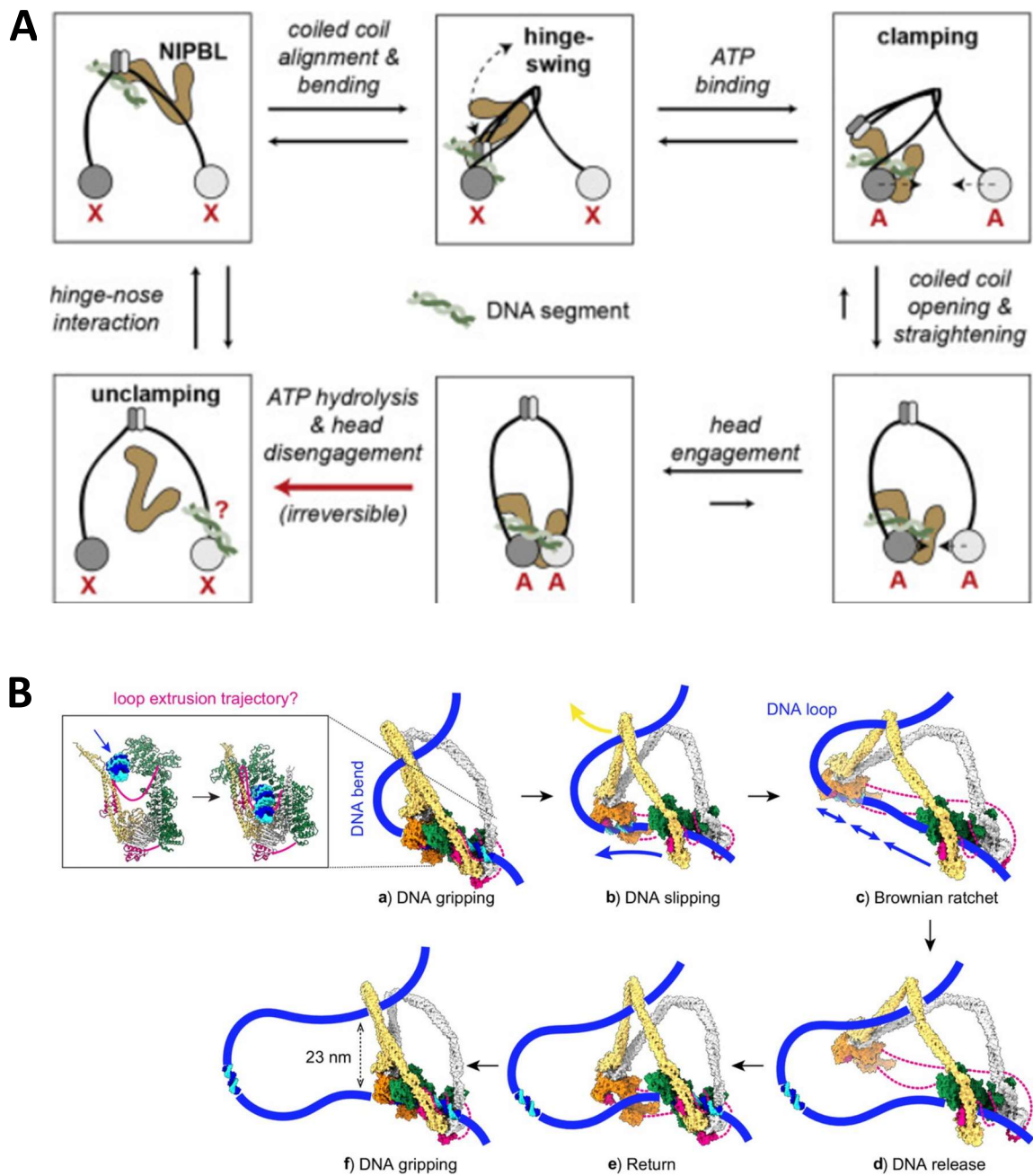
A recent study from Frank Uhlmann's laboratory suggests that DNA loop formation requires transcriptional activity. Depletion of RNA polymerase II induces a total absence of DNA loops (Guérin et al., 2023). According to this study, polymerase activity is the main driver of loop formation. However, in mammalian zygotes, the formation of DNA loops precedes the activation of transcription (Flyamer et al., 2017). Previous studies suggested a concomitant activity of cohesin's intrinsic ATPase activity as well as extrinsic RNA polymerase activity, in the formation of DNA loops. Another recent study in *S. cerevisiae* shows that depletion of cohesin in G1 has no major influence on the long-distance chromatin interactions that result from transcriptional activity (Chapard et al., 2023). What motor(s) is (are) required for DNA extrusion by cohesin is therefore not yet fully understood.



## C - Loop extrusion mechanisms

The mechanism by which DNA loops are extruded is not yet elucidated. As a result, several models have been proposed. The first model, called 'swing and clamp', is based on structural data and FRET microscopy (Bauer et al., 2021). In this model, the authors describe a cycle in which cohesin changes conformation several times and hydrolyses ATP to translocate DNA. Initially cohesin would be in a state where ATP is not bound to the ATPase heads, so the heads are disengaged. NIPBL interacts with the hinge domain, which in turn interacts with the DNA. At this point, cohesin folds, taking the DNA, the hinge domain and NIPBL with it to the globular head of SMC3<sup>Psm3</sup>, the 'swing' stage. ATP then binds to the globular heads, and cohesin is in the 'clamping' conformation. Bringing the ATPase heads together will allow cohesin to straighten and the heads will be engaged. The ATP is then hydrolysed, the heads disengaged and NIPBL is dissociated from cohesin. The cycle can repeat, cohesin interacting with another part of the DNA further away to continue extrusion (Bauer et al., 2021) (Figure 14.A). This model is interesting because at no time will the DNA be trapped inside cohesin. This model is therefore compatible with a non-topological association of cohesin with DNA in the formation of loops.

The second model suggests that the formation of DNA loops is partly due to Brownian motion. This is the 'Brownian-ratchet' model developed by Franck Uhlmann's laboratory (Higashi et al., 2021) on the basis of their data on the structure of the Mis4/cohesin/DNA holocomplex. In this conformation cohesin is folded and Mis4 is bound to Rad21 kleisin at the globular heads and the latter is bound to Psc3 at the hinge domain (Figure 14.B). When ATP binds, the heads are engaged and the DNA is grabbed by Mis4 and Psc3, thus becoming trapped between the heads of the SMCs, Mis4, Psc3 and the hinge domain. What happens next, depending on ATP hydrolysis, remains speculative. ATP hydrolysis would induce separation of the ATPase heads, Mis4 would release the DNA which could be lodged between Mis4 and Rad21. According to their model, unfolding of cohesin would follow and the DNA, trapped between Psc3 and the hinge domain, would be extruded thanks to this Brownian effect (Higashi et al., 2021) (figure 14.B).

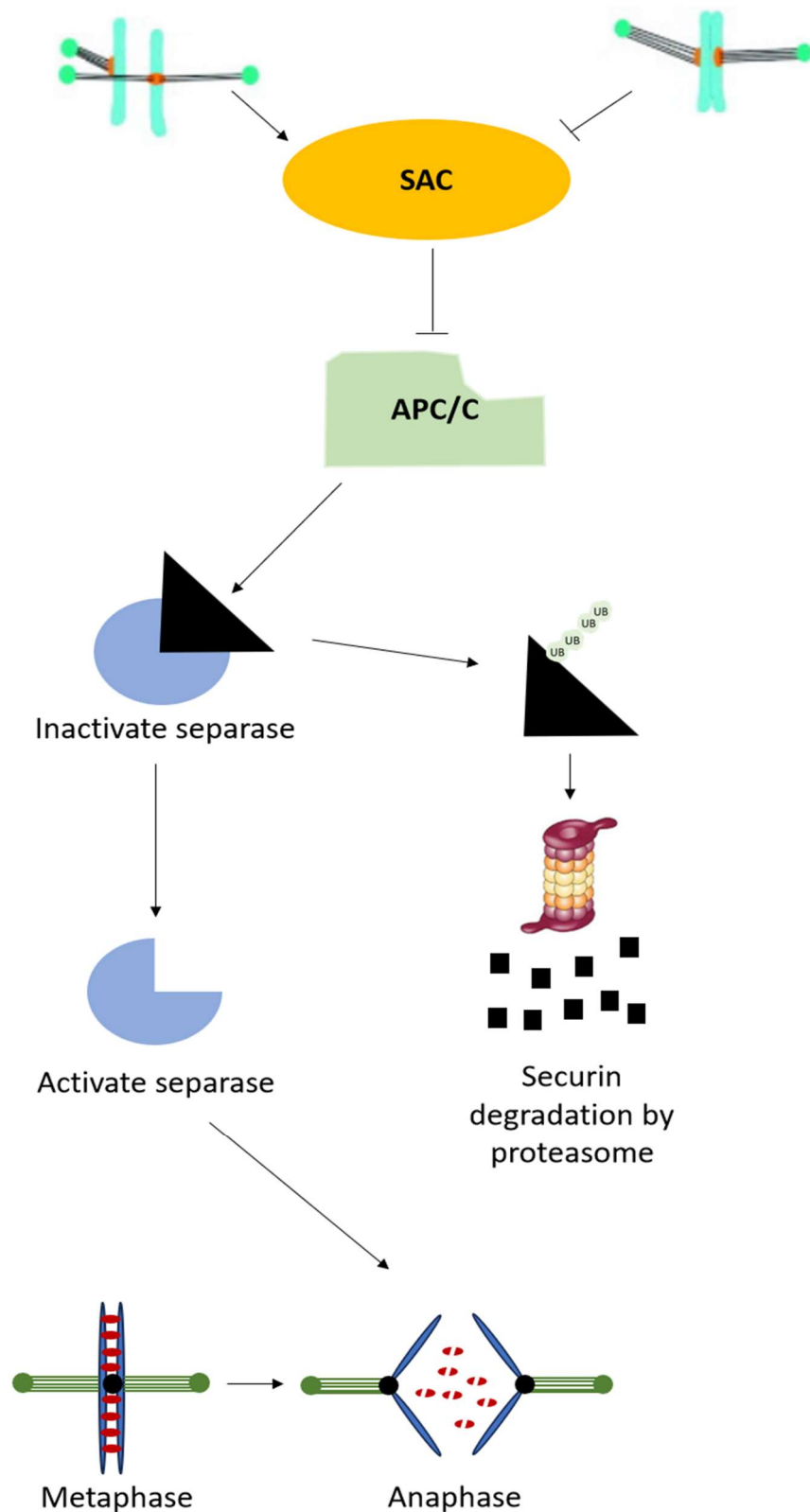


**Figure 14: “Swing and Clamp” and “Brownian-ratchet” models for cohesin DNA loop extrusion.** (A) “Swing and Clamp” model. In this model, NIPBL<sup>Mis4</sup> interacts with the DNA on the cohesin hinge. In follow, cohesin is folded and the hinge/DNA/NIPBL<sup>Mis4</sup> interacts with SMC3<sup>Psm3</sup> head. Then, the heads engage, ATP is hydrolysed and DNA is translocated. (B) “Brownian-ratchet” model. Initially, cohesin and Mis4 grabs DNA (DNA gripping state), following by a DNA loop extrusion powered by Brownian movement. Adapted from (Bauer et al., 2021; Higashi et al., 2021).

## **IV - Cohesin functions**

### **A - Chromosome segregation during nuclear division**

Sister chromatid cohesion is crucial during mitosis. Not only does it allow sister chromatids to be identified by keeping them in pairs, it also allows chromosomes to be attached correctly. During prophase, the kinetochores are progressively captured by the microtubules. Cohesion enables the kinetochores to be placed back to back, which will favour the capture of microtubules coming from the opposite pole of the spindle (Gregan et al., 2007; Juraj Gregan et al., 2011; Courtheoux et al., 2009; Sakuno et al., 2009). Chromosome attachment is controlled by the Spindle Assembly Checkpoint (SAC), a surveillance system that makes the initiation of anaphase conditional on the correct attachment of all chromosomes (for a review see (Musacchio, 2015)). A single unattached kinetochore is sufficient to keep the SAC activated. Correct (bilateral) attachment of a pair of sister chromatids generates tension at the kinetochores, which is taken into account by the SAC. In the absence of tension, Aurora B kinase phosphorylates components of the kinetochore, inducing microtubule detachment and maintaining SAC activation. When bilateral attachment of each pair of kinetochores is achieved, inhibition by the SAC is lifted, APC/C is activated, Securin is ubiquitinated and degraded by the proteasome and anaphase occurs (Figure 15).



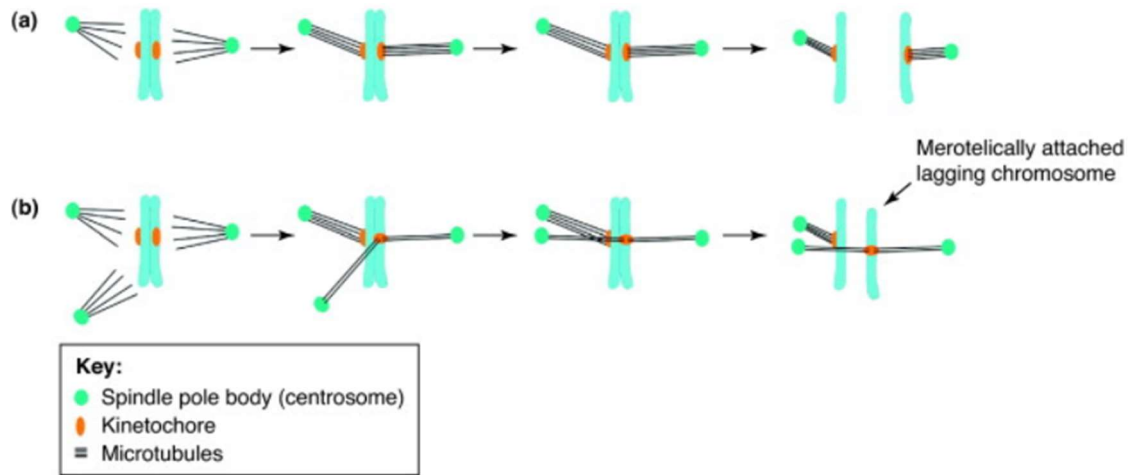
**Figure 15: APC/C activates Separase for cohesin cleavage.** Correct attachment of spindle microtubules to the kinetochores induces the silencing of the SAC. APC/C is activated and ubiquitinates Securin, which is degraded by the proteasome. Separase is activated and cleaves cohesin.



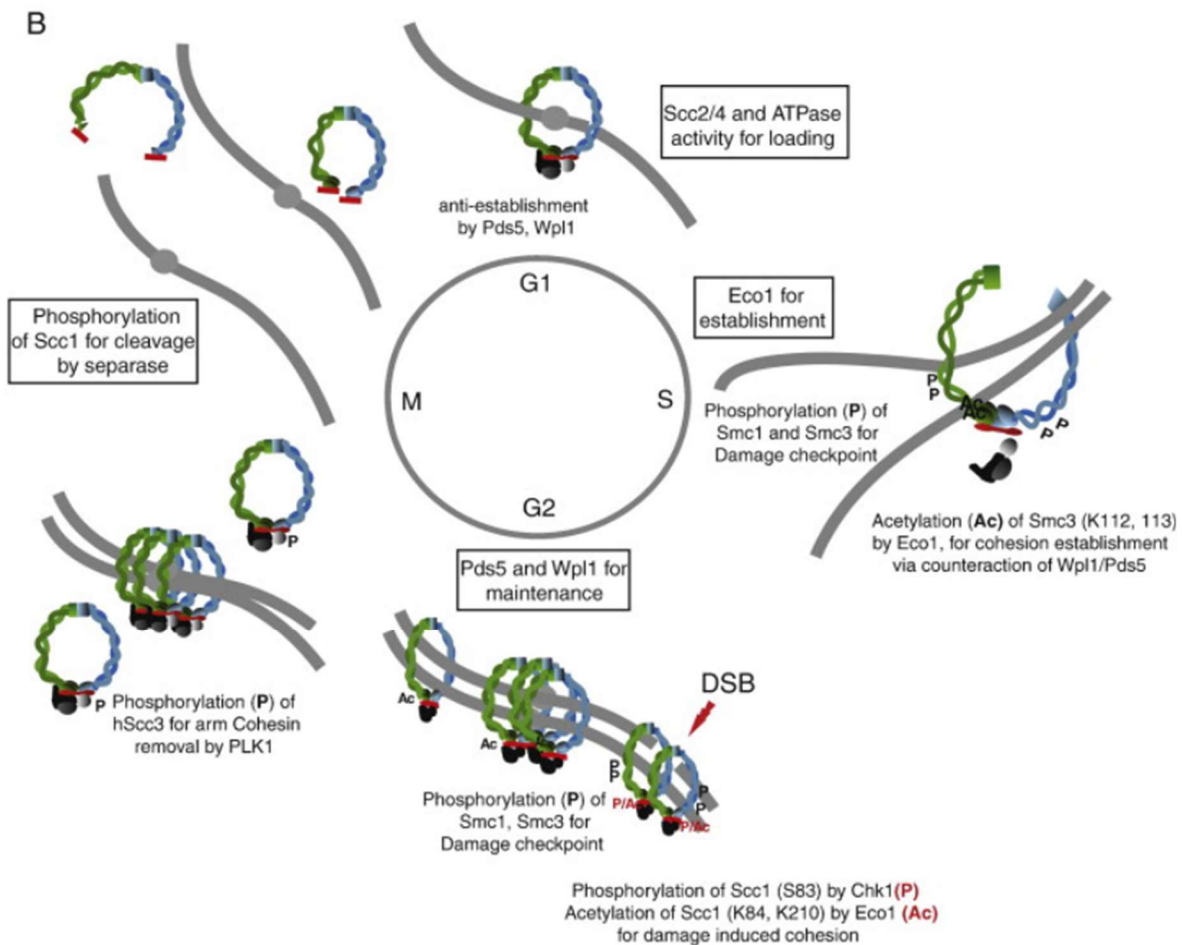
Cohesion defects thus have several consequences during mitosis. A total cohesion defect leads to the loss of sister chromatid information and their random segregation, resulting in the formation of aneuploid daughter cells (Gordon et al., 2012). A total or even partial defect leads to a failure to generate tension at the kinetochores, resulting in a delay in the onset of anaphase. Merotely is a typical defect frequently observed when sister-chromatid cohesion is defective. Merotely is defined as the attachment of the same kinetochore to microtubules from opposite poles of the spindle (Figure 16). The microtubules then exert opposing forces on the kinetochore. The result is that the speed of spindle elongation is reduced during anaphase and the merotelic chromatid 'lags' along the spindle while the correctly attached chromatids have reached the poles.

## **B - DNA repair**

In addition to chromosome segregation in mitosis, cohesion is also necessary for processes involving homologous recombination. This is the case for the repair of DNA double-strand breaks (Sjögren and Nasmyth, 2001). When a DNA break occurs in G2 or S phase, the cell uses homologous recombination (HR) to repair the damaged DNA. HR is made possible by the cohesion of sister chromatids, allowing homologous DNA molecules to be brought together. Surprisingly, it has been observed in *S. cerevisiae* that the presence of a DNA break induces the *de novo* recruitment of cohesin at the site of the break, but also strengthens the cohesion of all the chromosomes. This pathway is known as damage induced-cohesion (DI-C) (Heidinger-Pauli et al., 2008) (Figure 17). After the S phase, a population of cohesin, stably linked to chromatin, coheses sister chromatids. Another, unstable, population associates and disassociates with chromatin. It is this population that is involved in DI-C. Recruitment of these cohesins to breaks is dependent on  $\text{Scc2}^{\text{Mis4}}$  as well as on proteins involved in break recognition and HR. Interestingly, the establishment of DI-C requires Eco1 as well as HR-induced DNA synthesis, suggesting that DI-C is linked to DNA replication (Ström et al., 2007; Ünal et al., 2007).

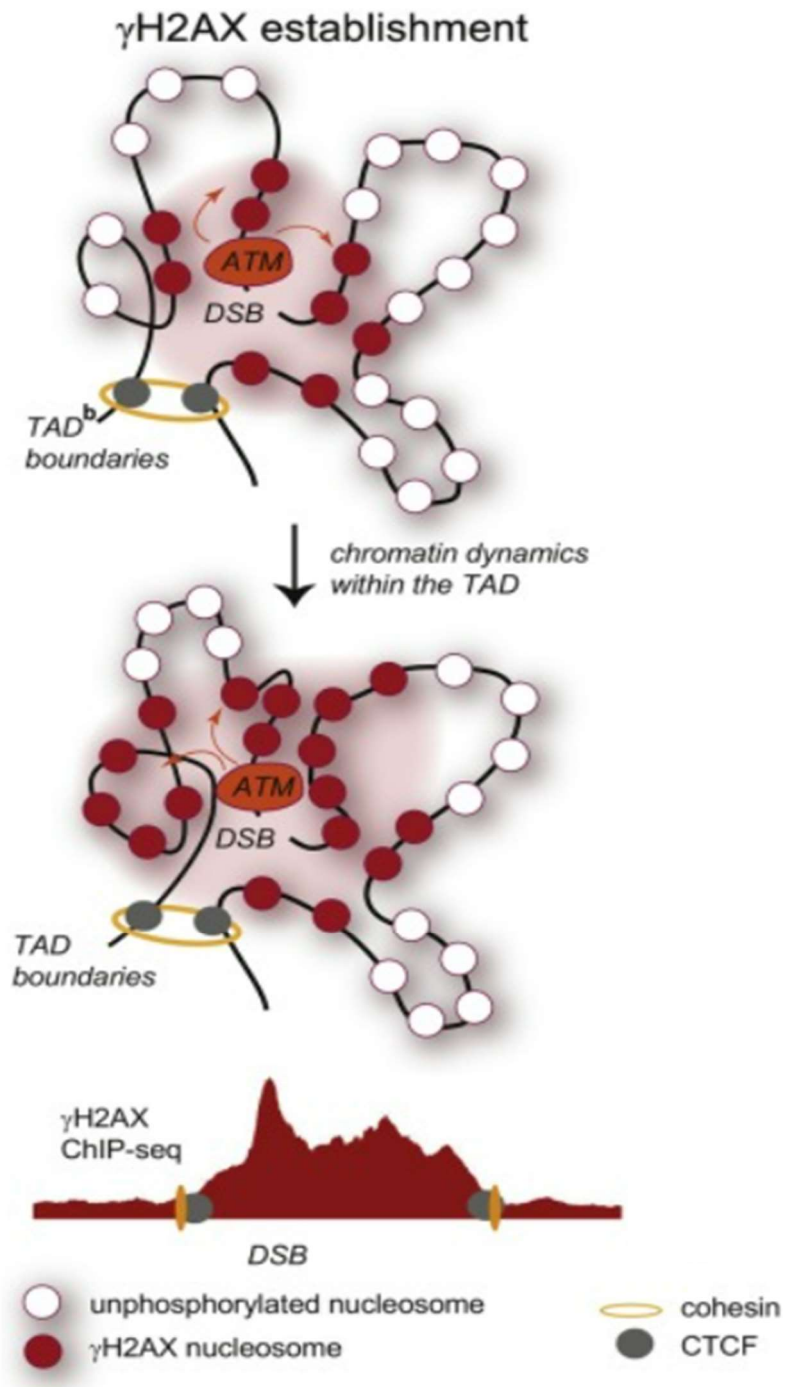


**Figure 16: Merotelic attachment of kinetochores.** Diagram showing correct microtubule attachment (a) and merotelic attachment (b). Merotelic attachment is when microtubules from opposite spindle poles attach to the same kinetochore. Adapted from (Juraj Gregan et al., 2011).



**Figure 17: Damage induced cohesion.** DNA damage in G2 and S phase induce post translational cohesin modification and *de novo* cohesion. Adapted from (Sjögren and Ström, 2010).

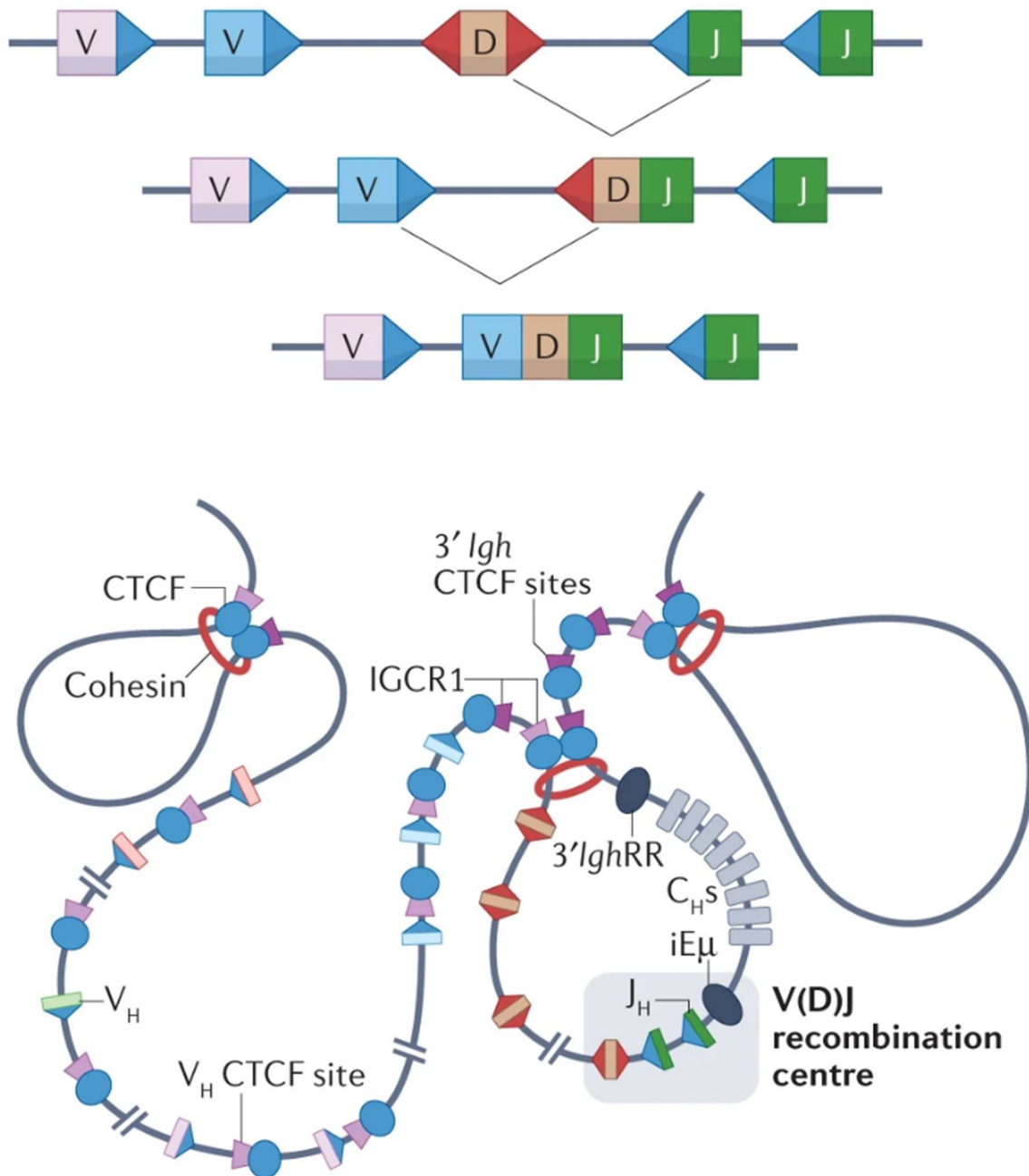
DNA break repair by cohesin involves not only the cohesion process but also the DNA extrusion process. In humans, DNA damage induces local phosphorylation of serine 139 of histone H2AX ( $\gamma$ H2AX) (Rogakou et al., 1998). This modification is propagated around the break and limited by TAD borders (Caron et al., 2012). By microscopy, it was observed that CTCF and  $\gamma$ H2AX are juxtaposed (Natale et al., 2017). These data allowed the construction of the model named 'Intra-TAD'. In this model it is assumed that the propagation of  $\gamma$ H2AX would occur through the modification of histones H2AX spatially close to each other, thanks to the formation of a TAD around the break (Aymard and Legube, 2016; Marnef and Legube, 2017) (Figure 18). Cohesin is therefore able to isolate the break. Furthermore, the ATM kinase involved in H2AX phosphorylation is capable of phosphorylating SMC1<sup>Psm1</sup> S957 and S966 *in vitro* and *in vivo*, suggesting a regulatory pathway for cohesin involved in DNA damage repair (Kastan and Lim, 2000; S.-T. Kim et al., 2002).



**Figure 18: Diagrams illustrating TAD formation following DNA break and H2AX phosphorylation around the break. Adapted from (Arnould and Legube, 2020)**

## **C - V(D)J recombination**

The immune system has to produce a very wide range of antibodies which recognise many different antigens. V(D)J recombination is the key process in creating this diversity. This recombination is possible thanks to the intervention of cohesin and its ability to extrude DNA in the form of a loop (Zhang et al., 2019, 2022). Immunoglobulins are made up of three gene segments: segment V (variable), segment J (junction) and segment D (diversity). There are several loci for each segment in the genome, and segment V has the greatest diversity. The succession of recombination events will first fuse a D segment with a J segment. The D-J set will then be fused to a V domain (Feeney and Verma-Gaur, 2012). Mechanistically, cohesin extrudes the loci that will be recombined. The size of the loop is limited by CTCF and the combined action of this protein and cohesin isolates the loci to be recombined. The RAG endonuclease then scans the loop and recognises specific sequences enabling it to initiate the series of recombination events (Zhang et al., 2019; Ba et al., 2020; Dai et al., 2021; Zhang et al., 2022) (Figure 19).

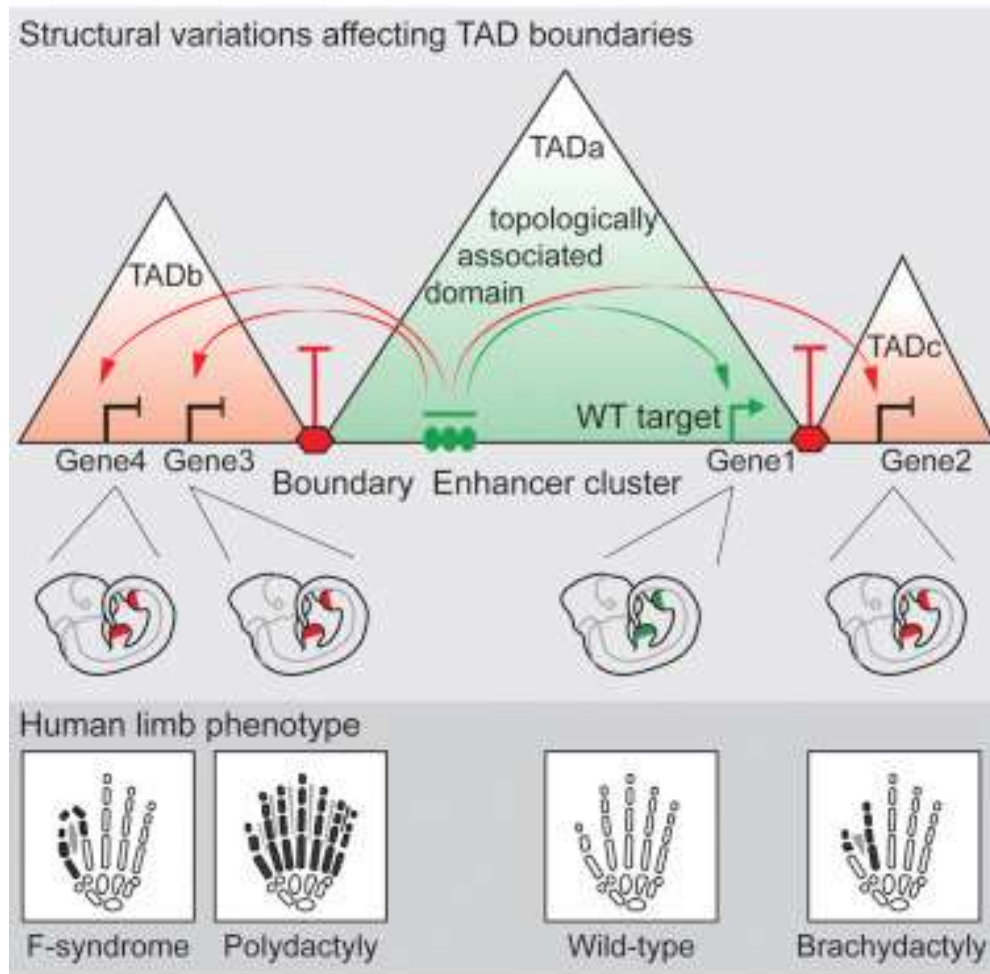


**Figure 19: Diagrams showing the *Igh* locus and the implication of cohesin and CTCF in V(D)J recombination.** (A) V(D)J recombination. First, J domain recombines with D domain, then DJ domain recombines with V domain. (B) Cohesin undergoing DNA loop extrusion insulates V, D and J domain and V(D)J center. Adapted from (Zhang et al., 2022).

## D - Regulation of transcription

The involvement of cohesin in the regulation of transcription is still debated, at least in its mechanism and is a very active area of research. In mammals, depletion of cohesin or NIPBL<sup>Mis4</sup> affects the expression of around 10% of genes in several different cell types (Remeseiro et al., 2012; Seitan et al., 2013; Sofueva et al., 2013; Zuin et al., 2014; Busslinger et al., 2017). The role of cohesin in the regulation of transcription would therefore concern only a few genes. In the remainder of this paragraph, we will detail a few examples of the involvement of cohesin in transcriptional regulation.

Mammalian genes are under the control of enhancer and silencer loci. These loci are capable of controlling a set of genes by associating with their promoter. These promoters can be very far from their enhancer, sometimes by several hundred kilobases. DNA extrusion by cohesin could be the process that brings the distant enhancers and promoters close together. Interestingly, cohesin depletion prevents the expression of genes whose enhancers are more than 400 kpb apart (Kane et al., 2022; Zuin et al., 2022) (Figure 20). Cohesin acts with the Mediator and Polycomb complexes to regulate the expression of genes located far from their promoter (Kagey et al., 2010; Merkenschlager and Nora, 2016). The formation of TADs by cohesin and CTCF insulates genes with their enhancer. A defect in the delimitation of TADs results in their overlap and can induce ectopic expression of genes (Lupiáñez et al., 2015; Rajderkar et al., 2023). Such a defect is causal for developmental syndromes such as polydactyly (Figure 20). In zebrafish, cohesin is involved in activating the embryo genome and in its development by structuring its genome (Meier et al., 2018; Wike et al., 2021).

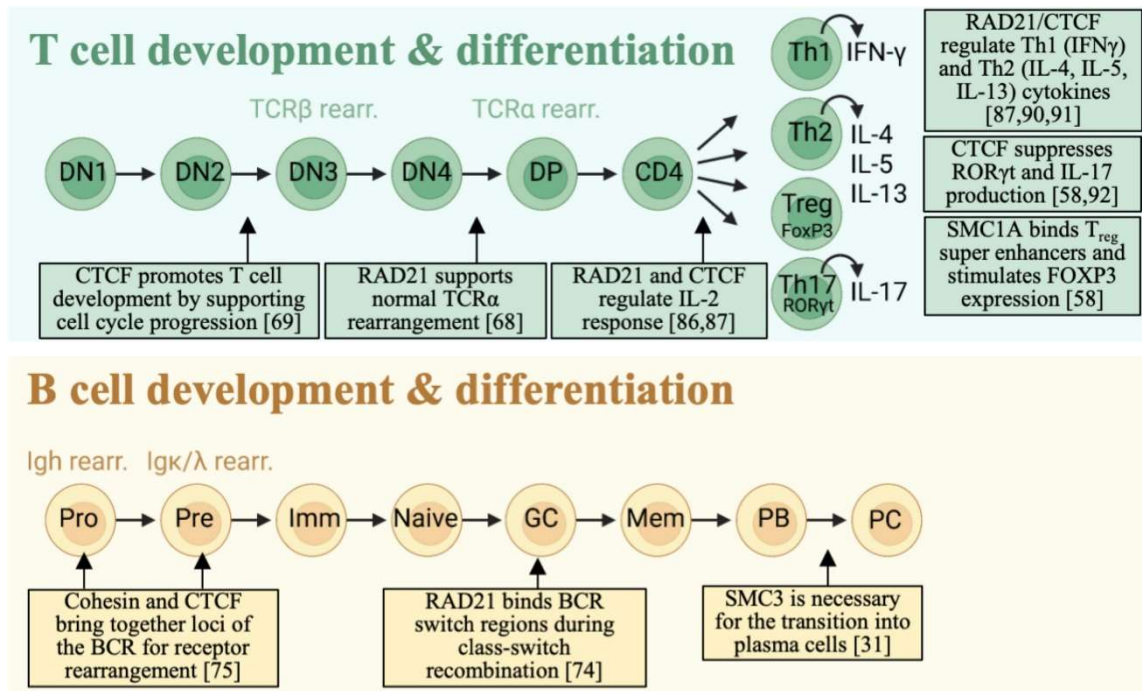


**Figure 20: Diagram showing gene insulation by TAD boundaries.** An enhancer regulates a pool of genes inside a TAD. The TAD boundaries insulate genes from an enhancer located in a different TAD. Variation in TAD boundaries is linked to gene misregulation and developmental defaults like polydactyly. Adapted from (Lupiáñez et al., 2015).



The involvement of cohesin in the regulation of transcription plays an important role in the differentiation of certain cell types, such as the erythropoietic lineage. Immune T cells undergo a series of differentiation steps, the outcome of which depends on cohesin (Figure 21). Depletion of RAD21 causes defects in the recombination of non-self recognition proteins (see section IV- C- V(D)J recombination), as well as the death of T cell progenitors during SP cell division (Heath et al., 2008; Seitan et al., 2011) (Figure 21). This premature death is thought to be due to inhibition of the expression of genes specific to T cell development. During T cell differentiation, the Rag gene must be expressed to induce V(D)J recombination and its expression is dependent on the enhancer-promoter interaction mediated by CTCF-cohesin (Miyazaki et al., 2020). A T cell progenitor with a defect in the rearrangement of its nonself proteins will not be selected, leading to cell cycle arrest (Feng et al., 2011). Differentiation of naive T cells into effective T cells requires the IL-2 protein which plays a role in regulating T cell metabolism (Ross and Cantrell, 2018). Transcription of IL-2-dependent genes requires loading of RAD21 onto chromatin (Iqbal et al., 2021). The use of siRNA to inhibit RAD21 reduces the expression of IL-2 pathway genes (Chisolm et al., 2017) (Figure 21). Furthermore, depletion of SMC3<sup>Psm3</sup> in immune B cells inhibits their differentiation into plasma cells (Rivas et al., 2021) (Figure 21).

In yeasts there is no clear picture on the role of cohesin in regulating gene expression. Cohesin Associated Regions (CARs) are preferentially located between convergent genes and form the borders of TADs (Lengronne et al., 2004; Christine K. Schmidt et al., 2009; Takeshi Mizuguchi et al., 2014). In *S. pombe* this particular location regulates transcription termination, presumably by blocking the advancement of RNA polymerase in the 3' of convergent genes (Gullerova and Proudfoot, 2008; Takeshi Mizuguchi et al., 2014). The cohesin loader Mis4 is required for the regulation of genes in subtelomeric regions. This regulation is thought to be involved in the heterochromatin formation process (Dheur et al., 2011). Interestingly, cohesin alteration in *S. cerevisiae* is also linked to a change in the expression of a number of genes (Skibbens et al., 2010; Bose et al., 2012; Lindgren et al., 2014; Kothival et al., 2021). Moreover, it's interesting to see that ncRNA regulate also cohesin (for review (Kuru-Schors et al., 2021).

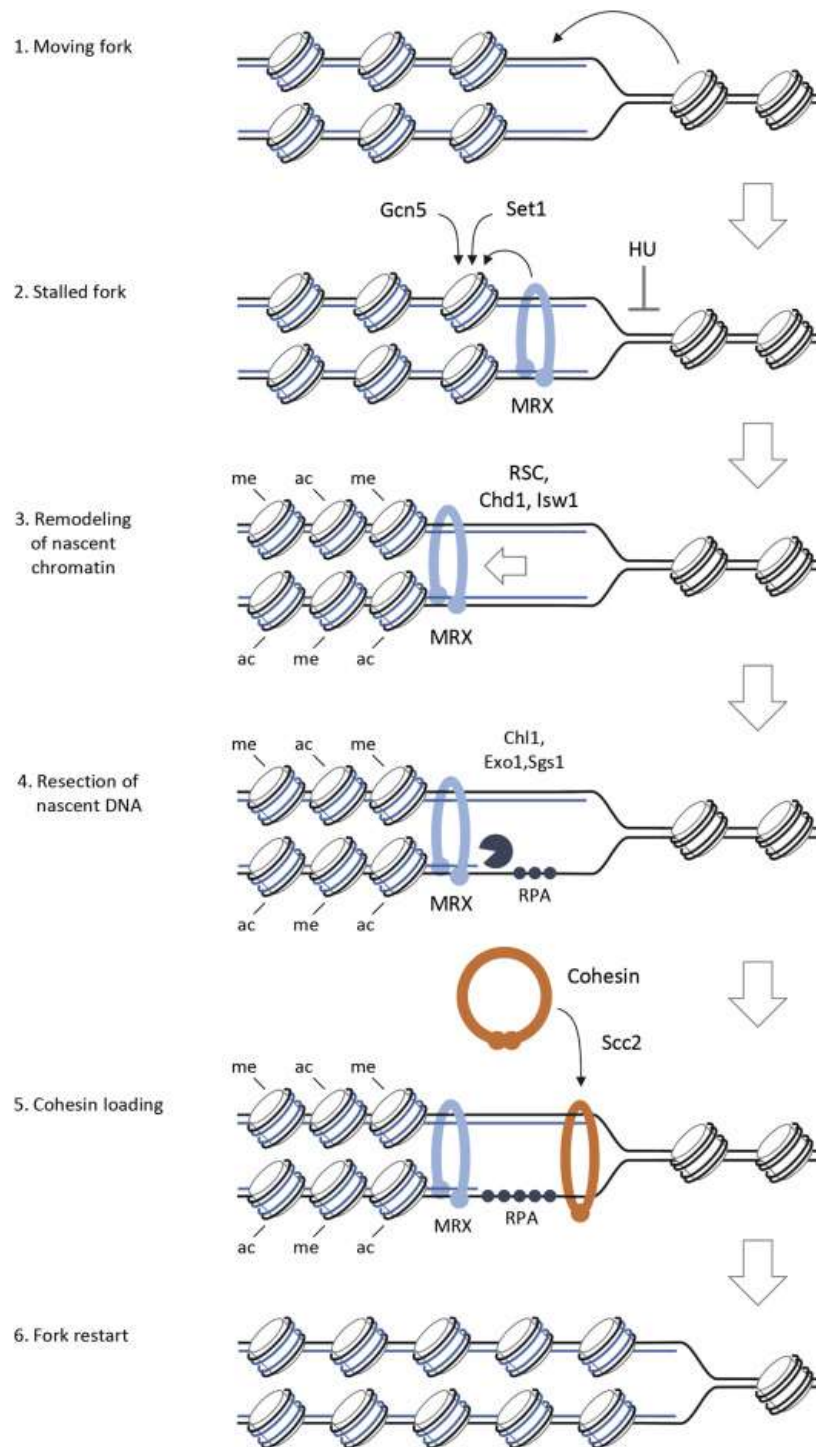


**Figure 21: Cohesin is involved in several steps of T and B cell maturation.** Cohesin and CTCF are implicated in TCR and BCR rearrangements, IL-2 response and T and B cell development, cell cycle progression and differentiation. Adapted from (Chandrasekaran et al., 2022).

## E - DNA replication

We have already seen that the replisome participates in the establishment of cohesion by cohesin. But it could also be that cohesin is involved in the progress of replication. The replication fork is subjected to various stresses, known as replicative stresses, which prevent it from advancing. These stresses can be of different origins, such as DNA damage, a lack of nucleotides or an obstacle to the passage of the helicase (Zeman and Cimprich, 2014). Replication fork arrest induces excessive accumulation of the RPA protein on single-stranded DNA (Branzei and Foiani, 2010). This accumulation induces cohesin loading in a manner dependent on  $\text{Scc2}^{\text{Mis4}}$  and the MRX complex, inducing the restart of the replication fork (Tittel-Elmer et al., 2012; Delamarre et al., 2020) (Figure 22). How *de novo* cohesin loading induces replisome restart is not known. Nevertheless, it is possible that the *de novo* association of cohesin at replication forks may facilitate DNA repair and subsequent replisome restart (Tittel-Elmer et al., 2012; Fumasoni et al., 2015) (Figure 22).

In humans, neighbouring origins of replication can be physically grouped together in a rosette-like structure and activated at the same time. In other words, the origins of replication are synchronised by their interaction through the formation of DNA loops (Jackson and Pombo, 1998; Petronczki et al., 2004). Interestingly, it has been observed that cohesin is enriched at the origins of replication and interacts with pre-replication proteins (Origin recognition complex (ORC) and minichromosome maintenance (MCM) (Guillou et al., 2010). In humans, cohesin could therefore be directly involved in the initiation of replication.



**Figure 22: Cohesin is implicated in fork restart.** Several cell stresses induce pauses in DNA replication followed by chromatin reorganisation. Cohesin loading facilitates sister chromatid exchanges and induces fork restart. Adapted from (Delamarre et al., 2020).

## V - Pathologies linked to cohesin dysfunction

### A - Cohesinopathies

Cohesinopathies are malformations during embryonic development caused by mutations in the cohesin complex or its regulatory proteins. These developmental problems lead to a number of phenotypes, including mental retardation, craniofacial and limb deformities. At present, we do not know which process is defective in patients with these mutations. The hypothesis is that the problem is linked to a transcription defect in genes involved in embryonic development. Cornelia de Lange syndrome (CdLS) is one of the most extensively studied. Patients with CdLS have facial deformity, limb deformity, gastrointestinal abnormalities, cardiac malformations, hirsutism, mental retardation and growth retardation. CdLS is caused by a multitude of mutations, ranging from point mutations to insertions and deletions. These mutations are found in NIPBL<sup>Mis4</sup>, SMC1<sup>Psm1</sup> and SMC3<sup>Psm3</sup> and more rarely in RAD21 and MAU2<sup>Ssl3</sup> (Krantz et al., 2004; Musio et al., 2006; Deardorff et al., 2007; Abarca-Barriga et al., 2023; Peng et al., 2024). Mutations in the NIPBL gene account for half of all cases of CdLS and are located in the coding and non-coding parts of the gene (Gillis et al., 2004). Mutations in SMC1<sup>Psm1</sup> and SMC3<sup>Psm3</sup> are located in the coiled-coils close to the hinge domain and ATPase heads (Deardorff et al., 2007).

Roberts syndrome is another cohesinopathy that is rarer than CdLS. The mutations causing this syndrome are located in the ESCO2<sup>Eco1</sup> gene. Two mutations in this gene have been identified as inhibiting acetyltransferase activity, responsible for maintaining sister chromatid cohesion (Vega et al., 2010). Patient cells show a cohesion defect at the centromere and heterochromatic domains (Schüle et al., 2005; Vega et al., 2005).

Interestingly, the TOR pathway (a chapter will be devoted to this later) is down-regulated in cells with Roberts Syndrome. These cells show a reduction in translation as well as a reduction in rRNA production (Xu et al., 2013). Mimicry of mutations conferring Roberts syndrome in *S. cerevisiae* (mutations in Eco1 and Smc1) induces a significant decrease in rRNA (Bose et al., 2012). The same phenotype is observed in zebrafish affected by Roberts Syndrome. *In vitro* treatment of zebrafish cells and *in vivo* treatment of zebrafish with L-

leucine partially restores the TOR pathway, protein synthesis and increases cell division and fish development (Xu et al., 2013, 2016).

## **B - Cancer**

Cohesin dysfunction caused by mutations induces aneuploidy (Hodges et al., 2005; Zhang et al., 2008; Chiang et al., 2010; Remeseiro et al., 2012; Li et al., 2015). Aneuploidy is thought to be one of the causes of cancer. Mutations in cohesin are present in several cancers. For example, mutations in  $STAG2^{Psc3}$ ,  $RAD21$ ,  $SMC1^{Psm1}$  and  $SMC3^{Psm3}$  are found in myelodysplastic syndrome (Kon et al., 2013). Similarly, several cohesin mutations are found in patients with myeloid leukaemia (Yoshida et al., 2013). Cohesin regulates 3D genome architecture and transcription. CTCF is a tumour suppressor that regulates genome architecture by associating with cohesin (Xiao et al., 2011). CTCF is found mutated in myeloid and lymphoblastic leukaemia and endometrial cancers (Yoshida et al., 2013; Marshall et al., 2017). Its deletion predisposes to cancer (Kemp et al., 2014). Alteration of the 3D structure of the genome by mutations in cohesin could therefore also cause cancer, but this remains to be explored. Inactivation of  $STAG2^{Psc3}$ ,  $RAD21$ ,  $SMC1^{Psm1}$  and  $SMC3^{Psm3}$  in HSPC cells induces an increase in their self-renewal (Viny et al., 2015). Furthermore, deletion of these subunits in mice induces defects in the differentiation of the erythroid and myeloid lineages, leading to early neoplasia (Mazumdar et al., 2015; Mullenders et al., 2015; Viny et al., 2015).

## VI - The Target Of Rapamycin Complex

Cell growth and proliferation are dependent on several extra- and intracellular signals such as nutrient availability, growth factors and energy levels. The cell integrates these different stresses and adapts its response. The integration of these stimuli depends on the TOR (Target Of Rapamycin) protein kinase. In *S. pombe*, the TORC1 complex regulates cell growth and metabolism by influencing autophagy and the synthesis of proteins, nucleotides and lipids. TORC1 is also involved in cell fate (see section VI- D-Functions). In this chapter we will describe the functions of the TORC1 pathway, as well as its regulation, localisation and the structural description of the different complexes, and we will finish by describing the diseases associated with the TOR pathway.

### A - Localisation

#### *1-Lysosome*

To carry out its function, TORC1 must be recruited to the lysosome (vacuoles in yeasts) where it becomes activated. The recruitment is made possible by the RAG protein (see section VI- B-Regulation) and Ragulator. Ragulator is a complex of five proteins called LAMTOR1-5 (Lam1-4 in *S. pombe*). This complex is anchored in the lysosome membrane and serves to recruit and activate RAG proteins (Bar-Peled et al., 2012; Tsun et al., 2013). Ragulator also serves as a platform for other TORC1 regulatory complexes such as GATOR1 (Bar-Peled et al., 2013). The reason for the recruitment of TORC1 to the lysosome could be explained by the presence of its regulatory proteins on the surface of the lysosome, such as Rheb, the main activator of TORC1 kinase activity. Furthermore, it appears that amino acids involved in TORC1 regulation can be detected not only when they are located in the cytosol but also in the lumen of the lysosome (Manifava et al., 2016). Detection of amino acids in the lumen requires V-ATPase and SLC38A9, a transmembrane amino acid transporter protein. These two proteins bind Ragulator and certain V-ATPase mutations prevent TORC1 from rerouting to the lysosome in the presence of amino acids (Zoncu et al., 2011; Jung et al., 2015; Rebsamen et al., 2015; Wang et al., 2015).

## 2-Nucleus

TOR complexes are also found in the nucleus where they regulate chromatin and transcription. Several authors have observed that TORC1 with its RAPTOR subunit is found in the nucleus, as are its regulatory proteins such as Rheb and its effectors such as S6K1 (Kim and Chen, 2000; Vazquez-Martin et al., 2011; Rosner and Hengstschläger, 2008; Rosner et al., 2012; Rosner and Hengstschläger, 2012; Yadav et al., 2013; Alayev et al., 2016; Prakash et al., 2019). CHIP experiments have shown that TORC1/mTORC is localised to promoters in *S. cerevisiae* and mammalian cells (Li et al., 2006; Cunningham et al., 2007; Tsang et al., 2010; Chaveroux et al., 2013; Filer et al., 2017; Audet-Walsh et al., 2017). Interestingly, an NLS and NES domain were found on the *S. cerevisiae* kinase Tor1. Moreover, deletion of the NLS prevents Tor1 binding to rDNA and reduces its transcription (Li et al., 2006).



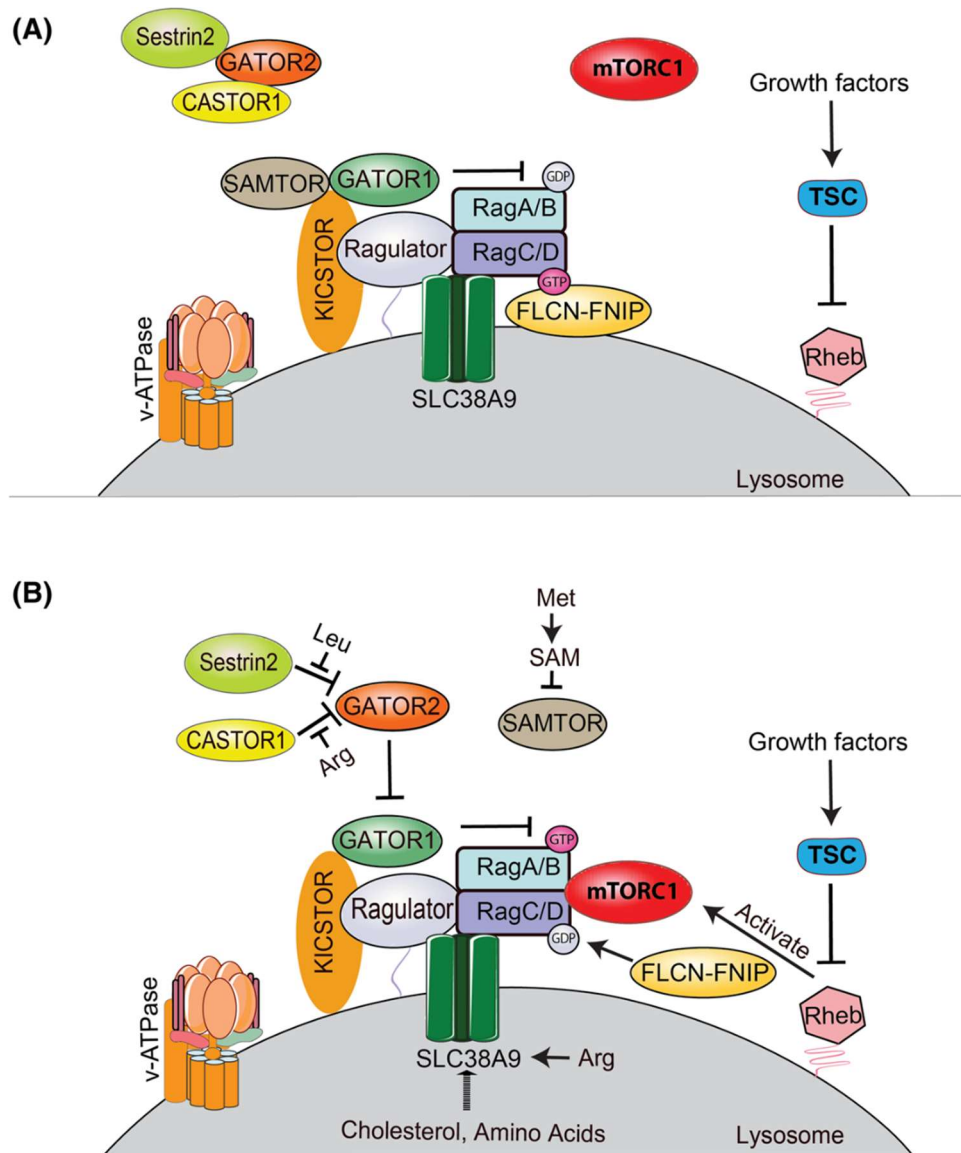
## **B - Regulation**

### *1-TORC1 activation by amino acids*

Regulation of mTORC1 activity in response to external stimuli is mediated by RAG GTPase and Rheb. In mammals there are four RAG proteins, RagA, RagB, RagC and RagD, which can form four heterodimers (Kim et al., 2008; Sancak et al., 2008). The RagA and RagB proteins have 90% homology and RagC has 80% homology with RagD (Sekiguchi et al., 2001). As a result, RagA will be functionally redundant with RagB and RagC with RagD (Sekiguchi et al., 2001; Sancak et al., 2008; Shen et al., 2017). Despite this high homology, it would appear that there are functional differences between RagA and RagB, and between RagC and RagD (Schmitt et al., 1995; Han et al., 2012; Demetriades et al., 2014; Figlia et al., 2022; Gollwitzer et al., 2022). These four GTPases positively regulate TORC1 when amino acids are available, by recruiting it to the lysosome (Kim et al., 2008; Sancak et al., 2008). When GTP binds to RagA/B and GDP to RagC/D, RagA/B forms an active dimer with RagC/D. Conversely, when RagA/B is bound to GDP and RagC/D to GTP, they form an inactive dimer. Nucleotide exchange is mediated by Ragulator and stimulation of GTPase activity by GATOR (Bar-Peled et al., 2012, 2013; Shaw, 2013; Hatakeyama and De Virgilio, 2016) (Figure 23).

GATOR is a complex composed of GATOR1 and GATOR2. In amino acid-rich conditions, GATOR2 inhibits GATOR1. In amino acid-deficient conditions, GATOR1 negatively regulates RAG protein activation by stimulating GTP hydrolysis by RagA/B. Thus, in amino acid-rich conditions, GATOR2 inhibits GATOR1 and the RAG proteins are activated (Figure 23) (Bar-Peled et al., 2013; Panchaud et al., 2013). Under these conditions, TORC1 is recruited to the lysosome by the RAG proteins, and the Rheb protein increases the catalytic activity of TORC1 (Long et al., 2005; Betz and Hall, 2013; Groenewoud and Zwartkruis, 2013) (Figure 23).

FLCN-FNIP is another regulator of RAG proteins, involved in switching RagC/D-bound GTP to GDP. In the absence of amino acid, FLCN-FNIP is bound to RagC/D. In the presence of amino acid, FLCN-FNIP dissociates from RagC/D and promotes the change of GTP by GDP (Tsun et al., 2013; Meng and Ferguson, 2018) (Figure 23).



**Figure 23: mTORC1 regulation by amino acids and growth factors.** (A) In the absence of amino acid, GATOR1 inhibits mTORC1 recruitment and activity by inhibiting RagA/D. (B) Leucine and arginine release GATOR2 from Sestrin2 and CASTOR1. GATOR2 inhibits GATOR1 and mTORC1 is recruited by RAG proteins to the lysosome. In addition, S-adenosylmethionine inhibits SAMTOR activity, reducing GATOR1 activity. Growth factors activate Rheb, which increases the catalytic activity of mTORC1. Adapted from (Lama-Sherpa et al., 2023).

Regulation of mTORC1 is amino acid type-dependent. CASTOR1 is the arginine sensor, Sestrin2 is the leucine sensor and SAMTOR is the S-adenosylmethionine sensor. Arginine and leucine, by binding to CASTOR1 and Sestrin1, lift the inhibition of GATOR1 by GATOR2, inducing the activation of mTORC1 (Wolfson et al., 2017; Kim and Guan, 2019). In the absence of S-adenosylmethionine, SAMTOR is bound to GATOR1 and inhibits mTORC1 activity. SAMTOR is dissociated from GATOR1 when S-adenosylmethionine binds to SAMTOR, increasing mTORC1 activity (Gu et al., 2017; Kim and Guan, 2019; Tang et al., 2022) (Figure 23).

### *2-Regulation by growth factors*

TORC1 is also regulated by growth factors such as insulin. Insulin binds to the receptor tyrosine kinase, inducing activation of PI3K kinase and phosphorylation of Akt by PDK1. In this state, Akt phosphorylates the TSC complex, and more specifically the TSC2 subunit, at multiple residues. TSC2 is a Rheb GTPase inhibitor. Its phosphorylation by Akt induces its inhibition, allowing Rheb to remain in its GTP-bound state. In this condition, Rheb stimulates mTORC1 activity by inducing a conformational change in mTORC1 (see section VI- C-Composition and Structure) (Potter et al., 2002; Inoki et al., 2002, 2003; Garami et al., 2003; Manning and Cantley, 2003; Tee et al., 2003; Dibble et al., 2012; Yang et al., 2017) (Figure 23).



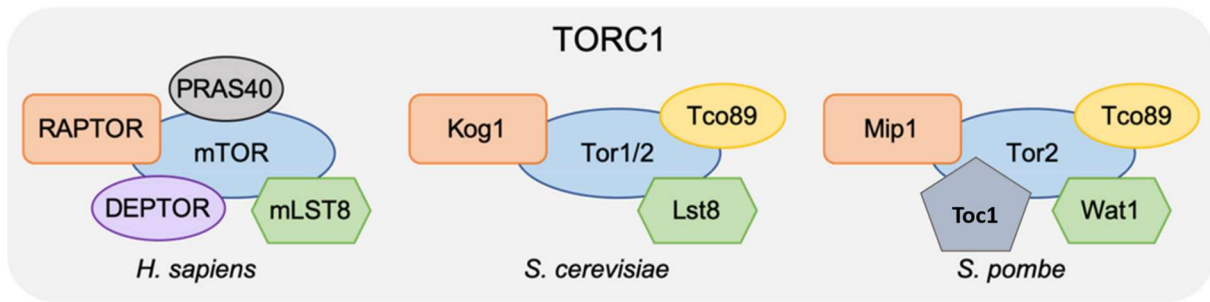
## C - Composition and structure

### 1-TORC1

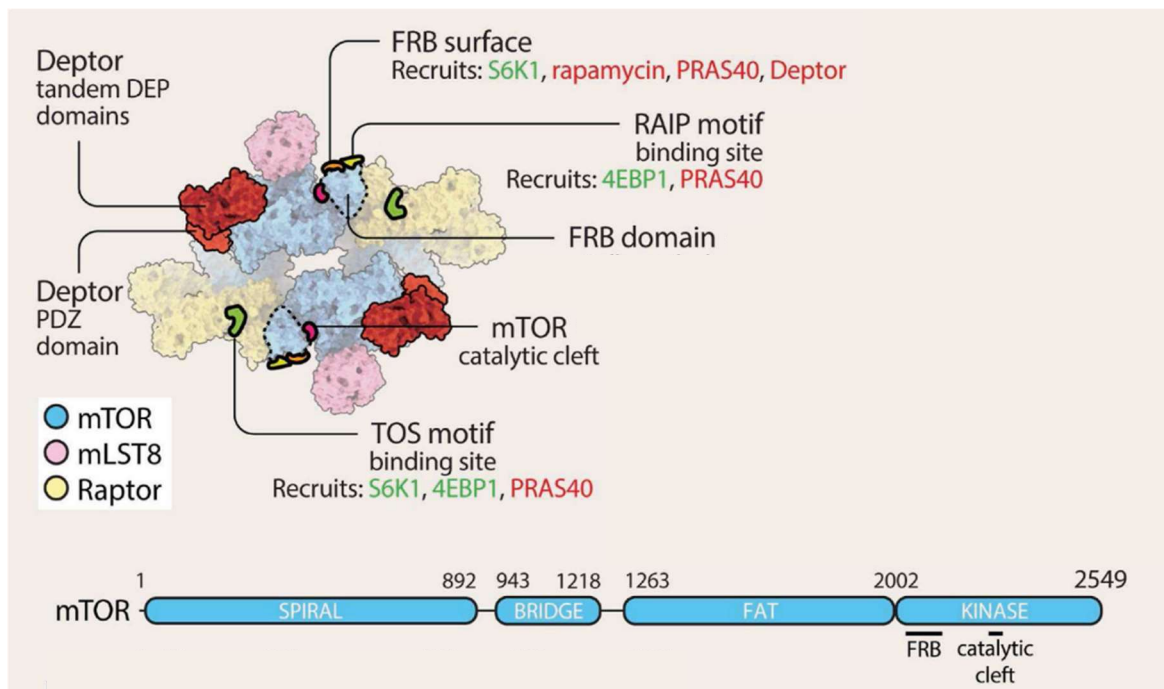
TOR is a member of the phosphoinositide-3 kinase-related protein kinase family, conserved from yeast to human (Brown et al., 1994; Chiu et al., 1994; Sabatini et al., 1994; Sabers et al., 1995). This kinase was discovered in the yeast *S. cerevisiae* thanks to a genetic screen looking for mutants sensitive to rapamycin-induced growth inhibition (Heitman et al., 1991; Cafferkey et al., 1993; Kunz et al., 1993; Helliwell et al., 1994). In humans, mTOR is associated with two structurally and functionally different complexes, mTORC1 and mTORC2. mTORC1 is composed of the RAPTOR protein, PRAS40, DEPTOR, mLTS8 and mTOR, whereas mTORC2 is composed of RICTOR, mSin1, Protor, DEPTOR, mLTS8 and mTOR. In yeast, there are also two TOR complexes, TORC1 and TORC2, and their difference also lies in their structural composition. Unlike mammals, there are two Tor protein kinases in yeast, Tor1 and Tor2. In *S. cerevisiae*, Tor1 and Tor2 are associated with TORC1, whereas TORC2 is associated only with Tor2. In *S. pombe*, Tor2 is specific for TORC1 and Tor1 for TORC2 (Figure 24).

### 2-mTOR/Tor2

The mTOR protein kinase is a large (289 kDa), highly structured protein forming a homodimer (Baretić et al., 2016). It is composed of an FRB domain of around 100 residues, close to the catalytic domain (Figure 25). This domain controls the accession of substrates to the active site and binds regulatory proteins such as PRAS40. The domain also contains the binding site for rapamycin, which by binding to the FRB will reduce accessibility to the active site (Vilella-Bach et al., 1999; McMahon et al., 2002; Yang et al., 2013). mTOR has a FAT domain, composed of 28  $\alpha$ -helices structured as  $\alpha$ -helical repeats. When mTORC1 is stimulated, the FAT domain undergoes a rearrangement to close the catalytic pocket and induce catalysis (Yang et al., 2017). This domain is required for RAPTOR to interact with mTOR (Zoncu et al., 2011; Yang et al., 2013).



**Figure 24: TORC1 subunits in *H. sapiens*, *S. cerevisiae* et *S. pombe*.** Adapted from (Morozumi and Shiozaki, 2021).



**Figure 25: mTORC1 structure.** mTORC1 structure showing principal sub-units (mTOR, RAPTOR and mLST8), substrate binding domain (FRB, TOS and RAIP), catalytic site and structural domain of mTOR. Adapted from (Linde-Garelli and Rogala, 2023).

### *3-RAPTOR/Mip1*

RAPTOR is a long 149 kDa protein composed of three HEATS repeats and seven WD-40 repeats in its C-terminal domain (D.-H. Kim et al., 2002). WD-40 repeats form domains often found in proteins associated with cell cycle progression and signal transduction. They allow the formation of multimers by serving as a scaffold for complex formation (Neer et al., 1994; Smith et al., 1999). RAPTOR is involved in the recruitment and substrate selection of mTORC1 as well as its localisation (Hara et al., 2002; D.-H. Kim et al., 2002; Schalm and Blenis, 2002; Schalm et al., 2003; Yang et al., 2017). RAPTOR homologs are Kog1 in *S. cerevisiae* and Mip1 in *S. pombe* (Wedaman et al., 2003; Álvarez and Moreno, 2006). RAPTOR is also involved in the association of mTORC1 with regulators such as the heterodimeric RAG GTPase proteins (Gtr1/2 in yeast) (see section VI- B-Regulation) (Sancak et al., 2008; Anandapadamanaban et al., 2019).

Recognition of mTORC1 substrates is mediated by RAPTOR's ability to recognise RAIP and TOS (Tor signaling) motifs, present on some, but not all, TORC1 target proteins, such as S6K1 and 4E-BP1 (Yang et al., 2017; Böhm et al., 2021). The TOS motif is composed of five residues FXΦDΦ (Φ hydrophobic, X any residue) (Schalm and Blenis, 2002; Schalm et al., 2003; Nojima et al., 2003; Yang et al., 2017). Phenylalanine appears to be the most important residue in this motif and its mutation inhibits the association of S6K1 and 4E-BP1 with RAPTOR and reduces their phosphorylation by mTORC1 (Choi et al., 2003; Nojima et al., 2003; Schalm et al., 2003). For the RAIP motif, only the IP amino acid appears conserved in vertebrates. It would appear that the RAIP motif is required to phosphorylate certain substrates efficiently and is not necessary for others (Tee and Proud, 2002; Beugnet et al., 2003; Choi et al., 2003; Eguchi et al., 2006). For some substrates, both the TOS and RAIP motifs bind RAPTOR (Böhm et al., 2021).

#### 4-Others sub-units

PRAS40 (proline-rich Akt substrate of 40kDa) is a subunit of TORC1 involved in its negative regulation. PRAS40 binds to the binding domain of mTORC1 substrates, such as TOS or RAIP, preventing their recruitment (see precedent paragraph) (Yang et al., 2017; Anandapadamanaban et al., 2019). PRAS40 is regulated by growth factors such as insulin and the PI3K/Akt pathway (see section VI- B-Regulation) (Sancak et al., 2007). In the presence of growth factor, Akt phosphorylates PRAS40 and induces its dissociation from the TORC1 complex (Kovacina et al., 2003; Nascimento and Ouwens, 2009; Zoncu et al., 2011).

mLST8 (LST8 in *S. cerevisiae*, Wat1 in *S. pombe*) is another subunit common to mTORC2 and mTORC1 whose function is not well known for the latter. Its deletion does not affect the phosphorylation of S6K1, which is a substrate of mTORC1, and instead affects the substrates of mTORC2 (Guertin et al., 2006). The function of mLts8 does not appear to be essential for mTORC1 activity (Kalender et al., 2010). However, in *S. pombe* it has been observed that inactivation of Wat1 prevents phosphorylation of Psk1 and Rps602, which are known targets of TORC1 (see function section VI- D- Functions) (Panigrahi et al., 2023).

Tco89 is a subunit of TORC1 found in yeast but not in mammals. It is a subunit of the TORC1 complex that is not essential (Loewith and Hall, 2011). These functions are not well known, but in *S. cerevisiae*, depletion of Tco89 alters proton flux, decreases K<sup>+</sup> uptake and intracellular pH, but also decreases growth (Mahmoud et al., 2017). In addition, its depletion makes the cell hypersensitive to rapamycin and reduces cellular integrity (Reinke et al., 2004).

Toc1 is another non-essential subunit, specific to *S. pombe*, whose role has not yet been discovered.



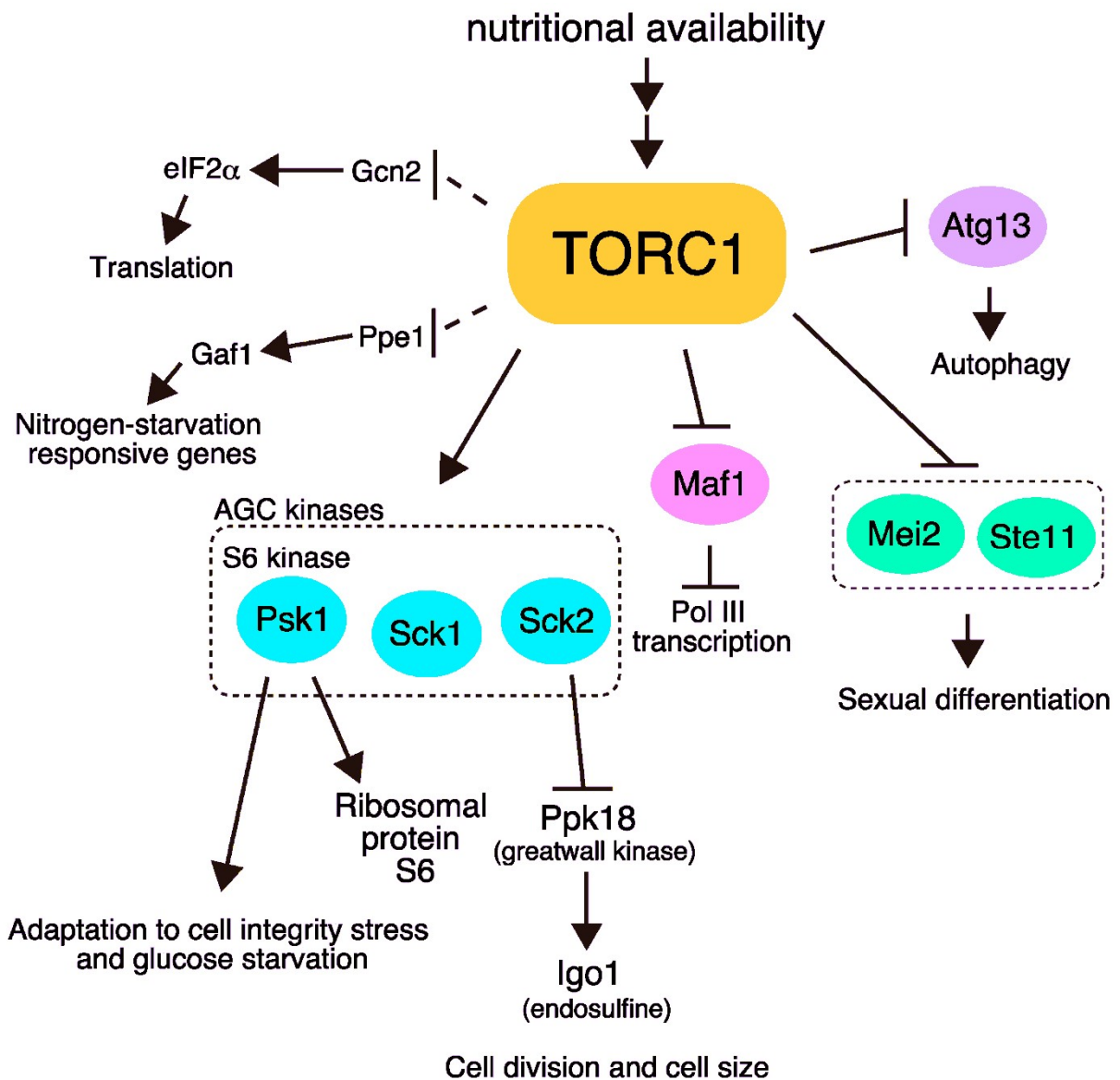
## D - Fonction

The role of the TOR pathway is to adapt the cell to environmental changes (Wullschleger et al., 2006). In *S. pombe*, activation of TORC1 induces anabolic processes such as protein synthesis, ribosome synthesis and transcription and, conversely, inhibits catabolic processes such as autophagy. In this paragraph, we will describe the most well-documented regulatory pathways (Figure 26).

### *1-Regulation of protein synthesis*

The regulation of protein synthesis by TORC1 is the best documented. In humans, TORC1 facilitates protein synthesis by directly phosphorylating the threonine T389 of S6K1 (p70 ribosomal protein S6 kinase 1) and by phosphorylating the 4E-BP1 protein (Pearson et al., 1995; Burnett et al., 1998). S6K1 phosphorylates ribosomal protein S6. The role of 4E-BP1 is to inhibit translation; its phosphorylation by TORC1 induces its dissociation from eIF4E and promotes translation (Brunn et al., 1997; Gingras et al., 1999).

In *S. pombe*, Psk1 is homologous to S6K1 and is a member of the AGC kinase family. This family is involved in cell growth, proliferation and survival. TORC1 directly phosphorylates two sites in Psk1, T415 and T392 (Nakashima et al., 2012). These phosphorylation events are dependent on nutrient availability. Nutrient deficiency conditions (low nitrogen, glucose and glutamine resources) or the addition of rapamycin to the medium induces a reduction in the phosphorylation of these two residues (Nakashima et al., 2010, 2012). Psk1 phosphorylates S235 and S236 of ribosomal proteins Rps601 and Rps602 (ribosomal protein S6), in a TORC1- and nutrient availability-dependent manner (Nakashima et al., 2010). These data suggest that TORC1 regulates protein synthesis via Psk1 and S6 ribosomal proteins in a nutrient availability-dependent manner. However, phosphorylation of Rps601 and Rps602 are not required for cell viability (Nakashima et al., 2010), suggesting the existence of alternative pathways.



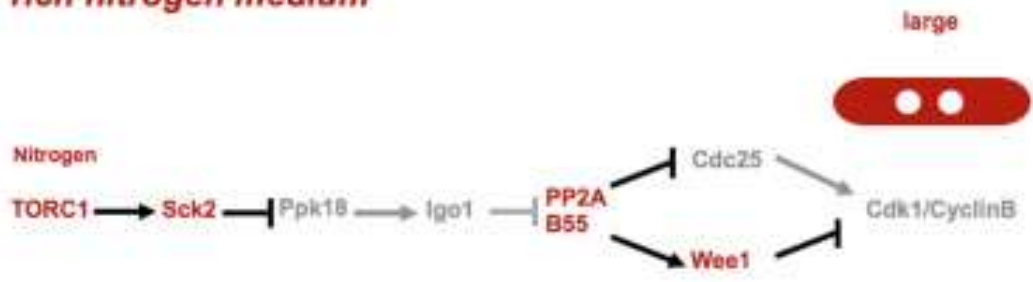
**Figure 26: TORC1 regulated pathways.** In function of the nutritional availability, TORC1 regulates several pathways like autophagy, sexual differentiation, Pol III transcription, cell division and cell size, translation and response to stress like nutrients starvation. Adapted from (Otsubo et al., 2017).

Paul Nurse's laboratory recently studied the effect of TORC1 inhibition on the phosphoproteome of *S. pombe*. The authors observed a marked reduction in protein synthesis. This reduction depended, among other things, on the translation initiation factor Tif471, the homologue of eIF4G. This factor is a target of TOR in *S. pombe*, with 27 TORC1-dependent phospho sites. Mutation of 18 of these sites by alanines induced a 17% reduction in protein synthesis. The authors conclude that Tif471 is a major TOR effector in the control of protein synthesis (Mak et al., 2021).

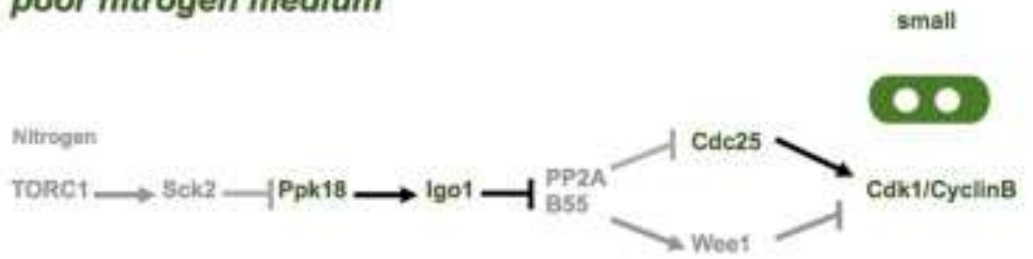
### *2-Regulation of cell growth and cell length at division*

TORC1 is involved in controlling cell size and cell division. TORC1 regulates other AGC kinases than Psk1, such as Sck1 and Sck2. These two kinases are involved in the coupling of cell size with cell division. Initially, it was observed that TORC1 is able to phosphorylate these two kinases directly *in vitro*, and *in vivo* their phosphorylation is dependent on TORC1 activity (Nakashima et al., 2012). Physiologically, it has been observed that TORC1 activates the PP2A-B55 phosphatase by inhibiting the Greatwall-Endosulfine pathway (Ppk18-Igo1 in *S. pombe*). When the cells are moved to a poor medium, TORC1 activity is reduced, the greatwall protein Ppk18 is activated and phosphorylates the endosulphin Igo1 which inhibits PP2A-B55. This event induces activation of Cdc25 and Cdk1/CyclinB, accelerating entry into mitosis and limiting cell growth. In rich media, TORC1 is active, resulting in an increase in cell size (Chica et al., 2016; Martín and Lopez-Aviles, 2018) (Figure 27). Interestingly, overexpression of Sck2 inhibits Ppk18 and also increases cell size (Rallis et al., 2014; Chica et al., 2016; Weston et al., 2017) (Figure 29). Taken together, these data suggest that TORC1 regulates cell size and division in a nutrient-dependent manner. This regulatory pathway is thought to be mediated by the direct phosphorylation of Sck2 by TORC1 (Figure 26)

*rich nitrogen medium*



*poor nitrogen medium*



**Figure 27: Cell size control at division mediated by TORC1.** TORC1 controls mitotic entry in a nutrient dependent manner, by modulating the greatwall protein Ppk18 activity via Sck2. Adapted from (Chica et al., 2016).

### *3-Autophagy regulation*

Autophagy is a process that allows cells in nutrient-poor conditions to recycle their proteins in order to synthesise new ones (Papinski and Kraft, 2016). When nutrients are abundant, TORC1 is active and inhibits autophagy (Kamada et al., 2000). In *S. cerevisiae*, autophagy is regulated by the serine/threonine kinase complex Atg1 (Kabeya et al., 2009). In nutrient-poor conditions, or in the presence of rapamycin, the Atg13 subunit of the Atg1 complex is dephosphorylated. Furthermore, *in vitro*, TORC1 phosphorylates Atg13 at several serine residues. In addition, inhibition of TORC1 induces dephosphorylation of Atg13 and formation of the Atg1 complex, activation of the latter and induction of autophagy (Kamada et al., 2000, 2010). In *S. pombe*, phosphorylation of Atg13 is also dependent on TORC1. The absence of nitrogen in the medium induces inhibition of TORC1 and dephosphorylation of Atg13. In addition, the migration profile of Atg13 in the thermosensitive *tor2-ts6* mutant suggests that Atg13 is hypophosphorylated at restrictive temperature (Kohda et al., 2007). Taken together, these data suggest that TORC1 regulates autophagy by modulating phosphorylation of Atg13 as a function of nutrient availability in the environment (Figure 26).

### *4-Regulation of transcription*

Transcription of the rDNA and tDNA genes is carried out by the Pol III polymerase. Pol III activity is dependent on nutrient availability. Pol III is inhibited by Maf1, which is regulated by phosphorylation (Boguta et al., 1997; Pluta et al., 2001; Johnson et al., 2007; Goodfellow et al., 2008). Phosphorylation of Maf1 induces Pol III-mediated gene transcription (Michels, 2011; Boguta, 2013; Leśniewska and Boguta, 2017). Interestingly, in mammals, TORC1 phosphorylates Maf1 (Kantidakis et al., 2010; Michels et al., 2010; Shor et al., 2010). In yeast, there is no evidence of direct phosphorylation of Maf1 by TORC1, but in *S. pombe*, phosphorylation of Maf1 is dependent on TORC1 (Du et al., 2012). TORC1 therefore regulates rDNA and tDNA via phosphorylation of the Pol III repressor, i.e. Maf1. This regulation is thought to be dependent on nutrient availability. It is possible that when nutrients are available, TORC1 activates rDNA and tRNA transcription by phosphorylating Maf1, thereby facilitating translation. Furthermore, the use of rapamycin or nutrient deprivation reduces ribosomal gene expression (Zaragoza et al., 1998; Powers and Walter, 1999) (Figure 26).

### *5-Regulation of sexual differentiation*

In *S. pombe*, the meiotic pathway is induced by nitrogen deficiency and the presence of partners of the opposite sexual sign. Regulation of meiosis and sexual differentiation is under the control of the RNA-binding protein Mei2 (Watanabe and Yamamoto, 1994; Watanabe et al., 1997; Yamashita et al., 1998). Mei2 physically interacts with TORC1 via the Mip1 subunit and Tor2 kinase (see section VI- C-Composition and Structure) (Shinozaki-Yabana et al., 2000; Álvarez and Moreno, 2006). TORC1 phosphorylates several Mei2 residues, both *in vitro* and *in vivo*. Mechanistically, TORC1 phosphorylates Mei2 to induce its ubiquitination and degradation by the proteasome. Degradation of Mei2 induces inhibition of sexual differentiation (Otsubo et al., 2014). The *mei2* gene is under the control of the Ste11 transcription factor. Ste11 is involved in the differentiation of vegetative cells into meiotic cells. *In vitro* phosphorylation experiments have shown that TORC1 phosphorylates Ste11 (Figure 26).

## **E - Diseases link to TORC1 pathway**

### *1-Type 2 diabete*

The TOR pathway regulates metabolism and is controlled by growth factors such as insulin. Faulty regulation of the TOR pathway can lead to metabolic disorders, insulin resistance and hence type 2 diabete. Patients with type 2 diabete and obesity show hyperactivation of mTORC1 (Um et al., 2004; Khamzina et al., 2005). Excess nutrient availability over-activates TORC1, inducing oxidative stress and inflammation that could be at the origin of insulin-secreting  $\beta$ -cell dysfunction (Ardestani et al., 2018).

### *2-Neurodegenerative diseases*

Defects in regulation of the TOR pathway are linked to the onset of neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's (Shafei et al., 2017; Silva et al., 2019). In patients with Alzheimer's disease, mTOR is over-activated. In mouse models of Alzheimer's disease, treatment with rapamycin and reduction of TOR pathway activity via mutations reduces the deposition of amyloid plaques, reduces toxic phosphorylation of Tau and reduces neurofibrillary tangle. In addition, mice show an increase in cognitive function and a reduction in neuron loss (Sun et al., 2014; Kaeberlein and Galvan, 2019). The effects of TOR pathway deregulation in Alzheimer's disease are thought to be due to autophagy dysfunction, but also to an increase in BCAAs (Branched-Chain Amino Acid) that increase mTORC-dependent phosphorylation of tau (H. Li et al., 2018; Uddin et al., 2019). In addition, defects in the mTOR/Insulin pathway and glucose metabolism induce more oxidative stress and mitochondrial dysfunction, a defect also found in Alzheimer's disease (Neth and Craft, 2017; Sun et al., 2020). The mTOR/Insulin pathway and mitochondria are also deregulated in Parkinson's disease. The use of rapamycin prevents mitophagy defects associated with Parkinson's disease (Siddiqui et al., 2015). Furthermore, A53T  $\alpha$ -sinuclein mutation induces its aggregation and increased mTOR activity. Depletion of mTOR induces autophagy and clearance of A53T  $\alpha$ -sinuclein (Jiang et al., 2013; Gao et al., 2015).

### *3-Cancer*

Cancer cells have the property of having an aberrant and very active metabolism compared with non-cancerous cells (Hanahan and Weinberg, 2011). During the reprogramming of cancer cells, the TOR pathway is upregulated to allow the cells to acquire more nutrients for their growth and proliferation. Several mutations that hyperactivate TOR have been identified in cancer (Sato et al., 2010; Grabiner et al., 2014; Wagle et al., 2014; Yamaguchi et al., 2015; Kong et al., 2016; Murugan et al., 2019). The use of rapamycin-like molecules has therefore been envisaged to combat cancer via inhibition of the TOR pathway (Benjamin et al., 2011; Magaway et al., 2019). The identification of TOR pathway mutations could be useful in the development of specific inhibitor molecules, in a way to propose personal therapy.

### *4-Aging*

Cellular ageing is linked, among other things, to alterations in metabolic pathways. The TOR pathway is implicated in ageing. The use of rapamycin, mutations in the TOR pathway and caloric restriction, inducing a reduction in mTOR activity, increases cell longevity (Harrison et al., 2009; Lamming et al., 2012; Leontieva et al., 2014; Bitto et al., 2016; Swindell, 2017). TOR downregulation is thought to induce a decrease in mitochondrial dysfunction, cell senescence and stem cell exhaustion (López-Otín et al., 2013; Correia-Melo et al., 2016). Reduced mTOR1 activity induces an increase in autophagy. In muscle stem cells, autophagy deficiency induces an accumulation of cellular components, damaged organelles and an increase in ROS compounds involved in senescence. Rapamycin restores autophagy and prevents muscle stem cell senescence (García-Prat et al., 2016).



## VII - Laboratory work underlying the thesis project

The laboratory is interested in cohesin regulation mechanisms. The approach used consists of using the power of genetic tools in the yeast *S. pombe* to identify regulatory pathways.

### **A - The CDK Pef1 and protein phosphatase 4 oppose each other for regulating cohesin binding to fission yeast chromosomes (Biro et al., 2020).**

The Cyclin Dependent Kinase (CDK) Pef1 was identified through a genetic screen for suppressors of *mis4-G1487D*, a conditional, thermosensitive mutant of the cohesin loader Mis4. By constructing an analogue-sensitive allele of *pef1* (*pef1-as*) it was shown that inhibition of Pef1 kinase activity suppressed the thermosensitive growth defect of *mis4-G1487D* and reduced cohesion and chromosome segregation defects. Chromatin Immunoprecipitation analyses revealed an increase in the quantity of cohesin associated with Cohesin Associated Regions (CARs), the regular cohesin binding sites on chromosomes. The effect was particularly marked in the G1 and S phases of the cell cycle.

Remarkably, a similar effect was observed in *mis4<sup>+</sup>* cells. The inhibition or ablation of the CDK in the wild-type background stimulated the association of cohesin and its loader Mis4 at CARs. Ablation of Protein Phosphatase 4 (PP4) had the opposite effect and simultaneous ablation of PP4 and CDK showed that the two activities are in opposition: PP4 stimulates the accumulation of cohesin at their dedicated sites along the chromosomes and Pef1 counteracts this effect.

The Pef1 CDK has three known cyclin partners. Genetic analysis showed that all three cyclins contribute to the regulation of cohesin, suggesting several relevant substrates. One of these is the Rad21 subunit of cohesin. Rad21 is hypo-phosphorylated in the absence of Pef1 and the CDK co-immunoprecipitates cohesin and its loading factor Mis4. The CDK composed of Pef1 and the cyclin Psl1 phosphorylates Rad21-T262 *in vitro* and *in vivo*. The creation and analysis of phosphomutants showed that Rad21-T262 is a relevant substrate. The non-phosphorylatable mutant partially recapitulated the effect of CDK ablation. The form

mimicking the phosphorylated state largely abrogated the effect of CDK ablation, showing that phosphorylation of Rad21-T262 is essential for the functional action of the CDK on cohesin.

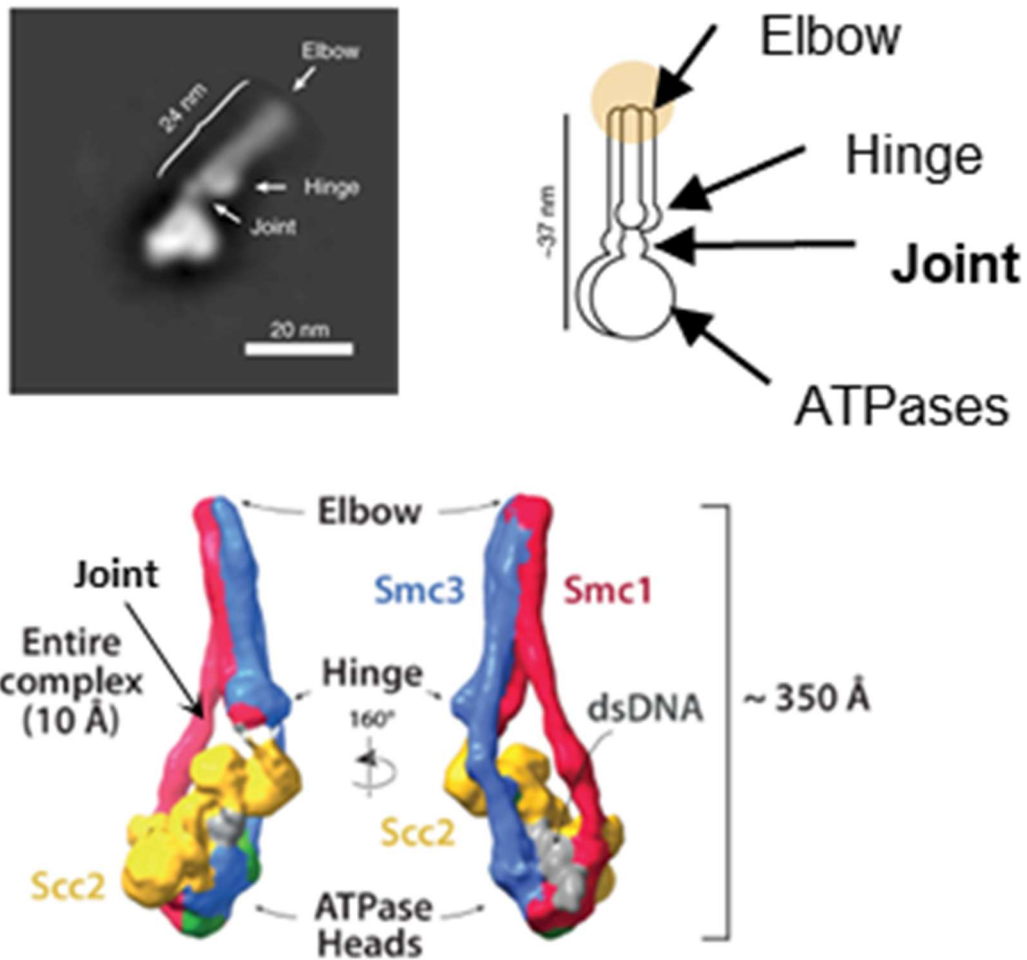
This study opened up exciting perspectives. In addition to the function of sister chromatid cohesion, cohesin controls the architecture of chromosomes in interphase, playing a major role in the control of gene expression, particularly during development, maintenance of the pluripotent state and differentiation. This field of research is currently undergoing considerable expansion. How are the formation, expansion and disappearance of loops controlled in space and time? How is cohesin activity directed towards loop formation versus sister chromatid cohesion? In essence, how cellular signals translate into an appropriate cohesin response? A CDK-Phosphatase mode of regulation has the potential to meet these requirements. It was proposed that CARs correspond to the borders of yeast TAD-like structures (Lorenzo Costantino et al., 2020). Inactivation of Pef1 causes the accumulation of cohesin and its loader at their regular sites on chromosomes. Increased abundance of cohesin at CARs may result from the accumulation of cohesin with loop extrusion activity at TAD boundaries. Co-immunoprecipitation experiments showed that Pef1 ablation increased the amount of cohesin bound to Mis4. Conversely, Protein Phosphatase 4 (PP4) ablation reduced the cohesin-Mis4 interaction and increased Rad21 phosphorylation (Birot et al., 2017, 2020). This suggests that cohesin phosphorylation may regulate the steady state levels of the cohesin/Mis4 interaction. As loop extrusion requires the NIPBL/Cohesin holocomplex (Iain F. Davidson et al., 2019), these considerations suggest that Pef1 and PP4 may regulate loop extrusion through this mechanism.

A collaboration was established with Adele Marston's group in Edinburgh, which has the expertise to generate and analyse Hi-C data. Preliminary data suggested that *pef1* $\Delta$  and the non-phosphorylatable mutant *rad21-T262A* displayed longer DNA loops. Although these experiments have to be repeated, these observations suggest the exciting possibility that a CDK may control genome folding through the phosphorylation of cohesin.

In the meantime, the laboratory in Bordeaux searched for additional Pef1 substrates using phospho-proteomics and found that Psm1-S1022 phosphorylation was *pef1*-dependent. The non-phosphorylatable mutant *psm1-S1022A* suppressed the thermosensitive growth defect of *mis4G1487D*, suggesting that phosphorylation of this residue is likely relevant to cohesin regulation by the CDK.

Studies using electron microscopy revealed that cohesin can adopt a folded configuration (Burmann et al., 2019; James E Collier et al., 2020). The S1022 residue of Psm1 lies within the “joint” region that the “hinge” domain contacts in the folded configuration (Figure 28). Alternatively, cohesin can switch to a “rod” conformation in which the ATPase heads are disengaged. It was suggested that the passage from one configuration to the other is instrumental for DNA capture and/or loop extrusion by cohesin (Zhubing Shi et al., 2020). Recently, imaging of the cohesin holocomplex by high-speed atomic force microscopy revealed that, when ATP is present, complexes alternate between ring-shaped, rod-shaped and bent conformations, leading to the “swing and clamp” model for DNA loop extrusion by cohesin (Bauer et al., 2021).

To see whether the phosphorylation status of Psm1 may be regulating the DNA looping activity of cohesin, Hi-C maps were generated by the group of Adele Marston. Indeed, DNA loops appeared larger in G1-arrested *psm1-S1022A* mutant cells. Altogether, these preliminary data suggest that Pef1 may impinge on chromosome folding through the phosphorylation of cohesin subunits.



**Figure 28:** Top: electron microscopy image of budding yeast cohesin in the folded configuration (Burmann et al., 2019). Bottom: structural model of the yeast cohesin-Scc2-DNA holocomplex based on cryo-EM data . The Psm1-S1022 residue lies near the “joint” domain that the “hinge” contacts when cohesin is in its folded configuration.

## B - The genetic screen for *mis4* suppressors identified components of TORC1

Besides *pef1*, the *mis4* suppressor screen identified three other genes: *tor2*, *mip1*, and *caa1*. The *tor2* and *mip1* genes encode components of the TORC1 complex, both essential for cell survival (Hayashi et al., 2007). Tor2 is the catalytic subunit while Mip1 is the Raptor homolog, which confers some level of substrate specificity (Böhm et al., 2021; Morozumi et al., 2021). The *caa1* gene encodes an aspartate aminotransferase that is required for full activation of the TORC1 complex (Reidman et al., 2019). Interestingly, the literature suggests that Pef1 is an indirect regulator of TORC1. In *S. cerevisiae*, the Pef1 homolog Pho85 is involved in the regulation of autophagy (Yang et al., 2010). Autophagy and cell fate are pathways regulated by TORC1 (see section VI- D-Functions). Recently, the TORC1/Pef1 relationship has been somewhat clarified. TORC1 is thought to be an effector of Pef1, which controls the initiation of cell differentiation, the Ste11-Mei2 pathway and autophagy (Matsuda et al., 2020). In this paper, the authors looked at the genetic interactions between *pef1* and known TORC1 regulators. The TSC complex, which is a negative regulator of TORC1, is the target of several kinases such as AKT in mammals. In addition, Tsc1 appears to be the target of the CDK1-cyclin B complex during the G2/M transition in the HEK293 cell line. These phosphorylation events inhibit TSC1 (Astrinidis et al., 2003). In *S. pombe*, the S862 residue of TSC1 is located within a CDK site (Swaffer et al., 2018). This suggests that TORC1 might be regulated by CDKs such as Pef1. In the Matsuda and colleagues study, a genetic interaction was found between *pef1* and the TSC complex where *pef1* is epistatic on TSC, suggesting that TSC is regulated by Pef1 (Matsuda et al., 2020).

It is remarkable that all *mis4* suppressors link to TORC1 signalling. Pef1 and Caa1 act as positive regulators of TORC1. One attractive hypothesis is that cohesin would be an effector of TORC1 to adapt the cell to environmental changes.

My research work during this thesis was dedicated to characterising the TORC1-cohesin regulatory pathway, determining its mechanisms and physiological relevance. To this end, I started my PhD in Bordeaux with proteomics and phosphoproteomics experiments, looking at interactions between TORC1 and cohesin as well as potential TORC1-dependent phosphorylation sites on cohesin and its loader. I then moved to Karl Ekwall's laboratory at the Karolinska Institutet in Stockholm as part of the IDEX international PhD program from the

University of Bordeaux. I stayed there for a year during which I did experiments asking how *mis4* and *mip1* mutant cells cope with changes in their environment. Back to Bordeaux, I continued experiments aiming at characterizing phosphosites whose phosphorylation appeared modulated by TORC1. During this last period, I spent a month in Adele Marston's laboratory at the Wellcome Centre for Cell Biology, University of Edinburgh. The aim was to repeat Hi-C experiments mentioned earlier. This last part of my work will not be presented in this manuscript. Experiments have been done, the results have to be analysed and compared with previous ones.

The following chapter will present the principal findings of our research in the form of a manuscript that will describe the insights gained into the hitherto unknown control of cohesin by TORC1 signalling. The following chapter will present additional experiments, looking at the potential role of kinases downstream TORC1. A final chapter will discuss the implications of these new data and the perspectives it opens in this area of research.

## II - RESULTS

### I - TORC1-dependent control of fission yeast cohesin

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## ABSTRACT

The Target of Rapamycin Complex 1 (TORC1) integrates cellular cues and adapts cell growth and metabolism through substrate-specific phosphorylation. A genetic screen for suppressors of a conditional mutant of the cohesin loader Mis4 identified several hypomorphic mutants of TORC1. Downregulation of TORC1 enhanced cohesin binding to Cohesin-Associated Regions (CARs). In the context of impaired cohesin loading, TORC1 downregulation rescued chromosome segregation, whereas TORC1 upregulation had the opposite effect, suggesting environmental cues may impinge on the robustness of chromosome segregation. Biochemically, TORC1 and cohesin co-purified from protein extracts, and the phosphorylation level of specific residues on Mis4 and cohesin was reduced in TORC1 mutants. Mutants mimicking the non-phosphorylated state recapitulated most of the effects of TORC1 downregulation. Challenging cells with various conditions revealed that Mis4 and TORC1 regulate a common set of genes involved in the response to environmental changes. These genes are preferentially located far from centromeres and close to telomeres. We propose that cohesin is a downstream effector of TORC1 for adapting cells to environmental changes.

## INTRODUCTION

The cohesin complex is a pivotal player in genome structure and function. Its activity is critical for a variety of biological processes, including sister chromatid cohesion, nuclear division, DNA replication and repair, and gene expression. Cohesin is an ATP-powered molecular machine that is capable of capturing DNA. Intra-chromosomal DNA capture folds the interphase genome into loops, which are of great importance for gene regulation, particularly during development and cell fate decisions. Conversely, DNA capture in trans provides cohesion between sister chromatids, which is essential for chromosome segregation and repair (Davidson and Peters, 2021; Oldenkamp and Rowland, 2022; Zheng and Xie, 2019). The core cohesin complex consists of two Structural Maintenance of Chromosome proteins, Smc1 and Smc3 (Psm1 and Psm3 in the fission yeast *Schizosaccharomyces pombe*) whose ATPase heads are bridged by a kleisin subunit (Rad21/Scc1) to which a fourth subunit (hSTAG1-2, spPsc3, scScc3) binds. DNA capture by cohesin requires the so-called loading complex NIPBL/MAU2 (spMis4/Ssl3, scScc2/Scc4) which binds to cohesin and DNA. Inactivation of the cohesin loading machinery before S phase results in the failure of sister-chromatid cohesion establishment and aberrant chromosome segregation during the ensuing mitosis (Bernard et al., 2006; R. Ciosk et al., 2000; K. Furuya et al., 1998). Similarly, DNA loop extrusion is dependent on the NIPBL-Cohesin holocomplex (I. F. Davidson et al., 2019). Chromatin loops are dynamic structures that form and break on a time-scale of minutes. In human, the DNA binding factor CTCF appears to delineate loop domains, often referred to as topologically associated domains (TADs). Dynamically extruding cohesin complexes are thought to facilitate enhancer-promoter scanning *in cis*. TAD boundaries (insulation) appear regulated as the strengths of CTCF-anchored loop domains are enhanced when mouse embryonic stem cells exit pluripotency. Consistently, mutations in the genes encoding CTCF and cohesin components are linked to human diseases and developmental abnormalities (Davidson and Peters, 2021; Oldenkamp and Rowland, 2022; Zheng and Xie, 2019). The remodelling of



genome architecture appears particularly important for appropriate gene expression during cell fate decisions, suggesting that signalling pathways should convey cellular cues to cohesin. In this context, a recent screen in mammalian cells identified a set of kinases that alter chromosome folding when inactivated (Park et al., 2023).

Here we report a link between fission yeast cohesin, its loader Mis4 and the Target of Rapamycin Complex 1 (TORC1). TORC1 is a master regulator of cell growth and metabolism, highly conserved in eukaryotes. The kinase activity of TORC1 is stimulated by a variety of intra- and extracellular signals, including nutrients, growth factors, hormones and cellular energy levels. Once activated, TORC1 promotes cell growth and metabolism through the phosphorylation of multiple effectors (González and Hall, 2017; Otsubo et al., 2017). In mammalian species, growth factors and cellular energy stimulate the activity of mTORC1 through the Rheb GTPase, and the inhibition of the tuberous sclerosis complex (TSC), which functions as a GTPase-activating protein for Rheb. In response to amino acids availability, mTORC1 is activated via RAG GTPases in a TSC-independent manner. Under nutrient-rich conditions, TORC1 promotes anabolic processes, such as protein, nucleotide, and lipid synthesis, while inhibiting catabolic processes, such as autophagy.

In fission yeast, Tor2 provides the catalytic activity of TORC1, while Mip1, the Raptor equivalent, participates in substrate recognition (Morozumi et al., 2021). The complex contains three additional subunits, the mLST8 orthologue Wat1/Pop3, Toc1 and Tco89 (Hayashi et al., 2007). TORC1 plays a crucial role in switching between cell proliferation and differentiation by sensing nitrogen source. When deprived of nitrogen, fission yeast cells arrest in G1, mate and undergo meiosis. In the absence of a mating partner, G1 cells enter a G0, quiescent state. As its mammalian counterpart, the Rheb GTPase Rbh1 is an essential activator of TORC1. Upon nitrogen deprivation, TORC1 activity is restrained by the Gtr1-Gtr2 GTPases, the TSC complex, and the Gcn2 kinase (van Slegtenhorst et al, 2004; Chia et al, 2017; Fukuda & Shiozaki, 2018; Fukuda et al, 2021). In conditions of abundant nutrients, TORC1 activity is high but nevertheless attenuated by the Gtr1-Gtr2 heterodimer, which is analogous to the mammalian RAG GTPases (Chia et al., 2017; Fukuda and Shiozaki, 2018). TORC1 is essential for cell growth, and loss of the TORC1 activity results in cell cycle arrest in G1. Rapamycin only partially inhibits TORC1 activity in *S. pombe* and does not inhibit growth (Otsubo et al., 2017).

The link between cohesin and TORC1 arose from a genetic screen for mutants able to suppress the thermosensitive growth (Ts) defect of *mis4-G1487D*, which is defective for cohesin loading and chromosome segregation at the restrictive temperature (Birot et al., 2020). In principle the screen had the potential to identify regulators of Mis4, with the rationale that loss of a negative regulator should upregulate residual Mis4<sup>G1487D</sup> activity and restore growth at the restrictive temperature. In addition to a cyclin-dependent kinase (CDK) called Pef1, which acts as a negative regulator of Mis4 (Birot et al., 2020), several mutants of TORC1 were recovered. This study demonstrates that downregulation of TORC1 in fission yeast enhances cohesin binding to Cohesin-Associated Regions (CARs). In the context of impaired cohesin loading, TORC1 downregulation rescued chromosome segregation, whereas TORC1 upregulation had the opposite effect. Biochemically, TORC1 and cohesin co-purified from protein extracts, and the phosphorylation level of specific residues on Mis4 and cohesin was reduced in TORC1

mutants. Mutants mimicking the non-phosphorylated state recapitulated most of the effects of TORC1 downregulation. Hence cohesin behaves as an effector of TOR signalling, opening the intriguing possibility that environmental cues could affect the robustness of chromosome segregation processes.

As TORC1 drives transcriptional responses to environmental cues, we interrogated the role of Mis4 by conducting a transcriptome analysis of *mis4-G1487D* cells in a number of experimental situations, including variations in the composition of the culture medium, temperature, time, and cell cycle phase. Remarkably, the genes affected differed widely from one condition to another, suggesting a defective adaptive response. Remarkably, most of the genes deregulated by *mis4-G1487D* were also deregulated by *mip1-R401G*, consistent with cohesin and TORC1 acting in the same pathway. These genes are preferentially located at the ends of chromosomes and are involved in the stress response and sexual differentiation.

Collectively, the data presented here indicate that TORC1 controls the association of cohesin and its loader with chromosomes, regulating their functions in chromosome segregation during mitosis and in the transcriptional response to environmental changes.

## RESULTS

### A genetic screen for suppressors of *mis4-G1487D* identified components of TORC1

The G1487D substitution in the cohesin loader Mis4 renders the strain thermosensitive for growth (Ts, Figure 1A). At the restrictive temperature the amount of cohesin associated with chromosomes is reduced and cells exhibit defects in chromosome segregation during mitosis (Biot et al., 2020). To identify putative regulators of Mis4, we previously carried out a genetic screen for suppressors of the Ts phenotype of *mis4-G1487D* (Biot et al., 2020). One of the suppressors identified was *Pef1*, a CDK whose activity restrains cohesin binding to Cohesin Associated Regions (CARs). Besides *pef1*, the genetic screen identified three other genes: *tor2* (1 allele), *mip1* (5 alleles) and *caa1* (1 allele). The suppressive effect of the *caa1* mutant was modest (Fig.1, Supplement 1). On the other hand, *tor2* and *mip1* mutants clearly suppressed the Ts phenotype of *mis4-G1487D*, at a level similar to that conferred by the deletion of the *pef1* gene (Fig. 1A).

The *caa1* gene encodes an aspartate aminotransferase that is required for full activation of the TORC1 complex (Reidman et al., 2019). *Tor2* and *Mip1* are components of TORC1 ((Hayashi et al., 2007), Fig.1B). Both genes are essential for cell survival. The *tor2* allele recovered in our genetic screen bears a substitution in the kinase domain which affected colony growth suggesting that TORC1 activity is largely affected. The five *Mip1*/Raptor mutants did not show this phenotype, the growth being similar to that of a wild strain. Still they all show reduced phosphorylation of the S6-kinase *Psk1* (Fig.1C), a well-known TORC1 substrate (Nakashima et al., 2012). Conversely, the *mip1-Y533A* mutant which is deficient for *Psk1* binding and phosphorylation (Morozumi et al., 2021), suppressed the Ts phenotype of *mis4-G1487D* (Fig1C). In a similar manner, the pharmacological downregulation of TORC1 by rapamycin resulted in enhanced growth of *mis4-G1487D* at the restrictive temperature (Fig.1D). Other thermosensitive mutants of the cohesin pathway were not rescued ruling out a general suppressing effect. Hence, the suppression of *mis4-G1487D* may stem from reduced TORC1 kinase activity. Conversely, the genetic upregulation of TORC1 exacerbated the Ts phenotype

of *mis4-G1487D*. TORC1 activity upon nitrogen deprivation is restrained by the Gtr1-Gtr2 GTPases, the TSC complex, and the Gcn2 kinase. Among these, the Gtr1-Gtr2 GTPases dampen TORC1 even in nutrient replete conditions (Fig.1E, (Chia et al., 2017; Fukuda et al., 2021; Fukuda and Shiozaki, 2018; van Slegtenhorst et al., 2004)). The deletion of *gcn2* or *tsc2* was essentially neutral although the suppression by *mip1-R401G* was reduced in a *tsc2*-deleted background. Deletion of *iml1*, *gtr1* or the *gtr1-Q61L* allele (mimicking the GTP-bound inactive state of the Gtr1 GTPase, (Chia et al., 2017)) exacerbated the Ts phenotype of *mis4-G1487D* while mimicking the active GDP-bound form by using the *gtr1-16N* allele, (Chia et al., 2017)) was neutral. The deletion of *tor1*, encoding the catalytic subunit of the related complex TORC2 exacerbated the Ts phenotype of the *mis4* mutant (Fig1, Supplement 1). As TORC1 kinase activity is upregulated when *tor1* is deleted (Ikai et al., 2011), this observation is in line with the notion that *mis4-G1487D* is sensitive to hyperactive TORC1.

The above genetic analyses show that the Ts phenotype of the *mis4* mutant is dampened when TORC1 activity is down-regulated and exacerbated when TORC1 is up-regulated. Because *mip1* alleles merely affected growth on their own and were excellent *mis4* suppressors we focused our analyses on one of them, *mip1-R401G*. We found that *mip1-R401G* was suppressor in another genetic context of crippled cohesin loading. The acetyl-mimicking forms of Psm3 have been demonstrated to impede the loading of cohesin (Biro et al., 2020; Hu et al., 2015; Murayama and Uhlmann, 2015). This phenotype is exacerbated by the deletion of *pph3*, encoding the catalytic subunit of PP4 and the double mutant strain is Ts for growth (Biro et al., 2020). The *mip1-R401G* mutant efficiently suppressed the Ts phenotype (Fig.1F), indicating that the suppression is not restricted to *mis4-G1487D*.

### **The occurrence of chromosome segregation defects in *mis4-G1487D* is modulated by TORC1**

When *mis4-G1487D* cells were shifted at the restrictive temperature cell proliferation was reduced and viability dropped ~25 fold after 24 hours when compared to wild-type. The *mip1-R401G* mutation efficiently suppressed the proliferation and viability defects (Fig.2A). A hallmark of cohesin mutants is the occurrence of abnormal mitoses in which the spindle apparatus attempts to segregate un-cohesed sister chromatids. A single kinetochore is captured by microtubules emanating from opposite spindle poles (merotelic attachment) and moves back and forth on the anaphase spindle as a result of opposite forces (J. Gregan et al., 2011; Pidoux et al., 2000). Live cell analysis confirmed the occurrence of merotelic kinetochores in the *mis4* mutant (Fig.2 supplement 1). In fixed cells, a merotelically-attached chromatid appears as DAPI-stained material lagging on the anaphase spindle. Anaphases with lagging DNA were frequent for *mis4-G1487D* after one doubling of the cell population at the restrictive temperature and their occurrence was significantly reduced by *mip1-R401G* (Fig.2B). Merotelic kinetochores oppose spindle forces and reduce the rate of spindle elongation during anaphase. As the number of merotelic kinetochores increases the spindle elongation rate slows down and may even become negative resulting in spindle shrinkage (Courtheoux et al., 2009). The rate of spindle elongation was strongly affected in *mis4-G1487D* and spindle shrinkage events were frequent, indicative of a high level of merotely. Spindle shrinkage occurred in ~53% of anaphases in *mis4-G1487D* whereas it was reduced to ~21% in the *mip1-R401G* background (Fig.2C and Fig.2 supplement 2). Particularly striking was the observation of spindle shrinkage followed by asymmetric spindle displacement resulting in the

generation of anucleate daughter cells after cytokinesis (Fig.2D). Nearly 80% of cells showed this phenotype in *mis4-G1487D* for the late time points whereas the frequency was down to ~20% in the presence of *mip1-R401G*. Altogether, these analyses indicate that the *mis4* mutant experienced numerous merotelic events during anaphase at the restrictive temperature, leading to asymmetric spindle shrinkage and the production of anucleate cells. The suppression of these phenotypes by *mip1-R401G* is consistent with a lower incidence of merotelic attachments. Although the effect was weaker, rapamycin also reduced the frequency of anaphases with lagging DNA (Fig.2E). This is consistent with the notion that the down-regulation of TORC1 reduced chromosome segregation defects of the *mis4* mutant. Physiologically TORC1 acts as a rheostat, its activity rising with nutrient availability. Consistently, the severity of the Ts phenotype and the frequency of lagging DNA were higher in the rich, complete medium YES than in the synthetic minimal medium EMM2 (Fig.2F). Lagging chromosomes were also less frequent when the nitrogen source was provided by glutamate instead of ammonium chloride (Fig2. Supplement 3). The artificial increase of TORC1 activity by *gtr1-Q61L* resulted in an elevated rate of anaphases with lagging DNA (Fig.1F). Altogether, these analyses show that TORC1 activity modulates the occurrence of chromosome segregation defects of *mis4-G1487D*.

Remarkably, the suppressor effect of the *mip1-R401G* mutation was not restricted to the *mis4* mutant. The frequency of anaphases with lagging DNA was efficiently reduced in the *psm3<sup>K105N-K106N</sup> pph3Δ* background (Fig.2G). As for *mis4-G1487D*, cohesin loading is impaired in this genetic setup (Biro et al., 2020) but Mis4 is wild-type, suggesting the suppression may stem from an upregulation of the cohesin loading machinery.

Although the down-regulation of TORC1 reduced chromosome segregation defects, they were not fully suppressed suggesting that sister-chromatid cohesion was poorly restored. The monitoring of sister-chromatid cohesion at the *ade6* locus (Molnar et al., 2003) revealed a persistent defect and the level of Psm3 acetylation, a marker of sister-chromatid cohesion establishment, remained low. Likewise, rapamycin treatment did not improve Psm3 acetylation (Fig2 supplement 4). We suspect that sister-chromatid cohesion remained largely defective but improved enough to reach the critical threshold allowing cell proliferation.

### **Reduced TORC1 activity increased Mis4 and cohesin binding to Cohesin Associated Regions (CARs)**

The cohesin loading complex performs its essential function during G1/S (Bernard et al., 2006; R. Ciosk et al., 2000; K. Furuya et al., 1998). The down-regulation of TORC1 may rescue *mis4-G1487D* by up-regulating its residual cohesin loading activity. To address this issue, cells were arrested at the end of the G1 phase by the use of the *cdc10-129* Ts mutation and cohesin binding to chromosomes monitored by Rad21 ChIP-qPCR at known CARs (Fig.3A). Cycling *S. pombe* cells are essentially in the G2 phase of the cell cycle (Carlson et al., 1999). When shifted at 36.5°C, *cdc10-129* cells progress through G2, M and arrest in G1. As reported previously, Rad21 binding to CARs was reduced in a *mis4-G1487D* background (Biro et al., 2020; Feytout et al., 2011; Vaur et al., 2012). The *mip1-R401G* mutation efficiently restored cohesin binding to near wild-type levels (Fig. 3B). A similar effect was observed in the *psm3<sup>K105N-K106N</sup> pph3Δ* background (Fig.3 supplement 1) indicating that *mip1-R401G* restores cohesin loading in these

two distinct genetic contexts. Importantly, a similar effect was observed in cells with a wild-type cohesin loading machinery. In an otherwise wild-type background, Mis4 and Rad21 binding to CARs were increased in *mip1-R401G* cells at most chromosomal sites examined (Fig.3C-D). Rapamycin treatment provoked a similar effect although less pronounced (Fig3 supplement 1). We conclude that TORC1 down-regulation increases cohesin abundance at CARs, presumably by stimulating cohesin loading.

### **TORC1 components co-purify with cohesin and its loader**

To see whether TORC1 and cohesin may interact we affinity-purified Mis4 and Rad21 complexes and analysed associated proteins by label-free mass spectrometry (Fig.4A). To avoid cell cycle effects, extracts were prepared from G1 (*cdc10-129*) arrested cells. All TORC1 components co-purified with Mis4 and Rad21 and conversely, additional co-immunoprecipitation experiments showed that cohesin co-purified with TORC1 (Fig.4B-C). This observation suggests that Cohesin and its loader may be TORC1 substrates.

### **Reduced Mis4-S183 and Psm1-S1022 phosphorylation levels in *mip1* mutants**

To see whether *mip1-R401G* may alter cohesin or Mis4 phosphorylation status, Mis4 and Rad21 complexes were purified from *mip1-R401G* and *mip1<sup>+</sup>* cells and triplicate samples analysed by label-free mass spectrometry. As before, protein extracts were prepared from G1-arrested cells to avoid cell cycle induced changes. A serine residue (S183) within the N terminal domain of Mis4 was less phosphorylated in *mip1-R401G*. Likewise, Psm1-S1022 phosphorylation level was decreased (Fig.5A).

Antibodies were raised against the phosphorylated forms (Fig.5 supplement) and confirmed that the levels of Mis4-S183p and Psm1-S1022p were reduced in *mip1-R401G* cells. A similar reduction was also apparent in the unrelated mutant *mip1-Y533A* (Fig. 5B and D).

Mis4-S183 and Psm1-S1022 may be direct TORC1 targets or the substrates for kinases downstream TORC1. Both phosphorylation sites adhere to the CDK consensus (S-T/P) but were not described as direct targets of Cdc2, the main fission yeast CDK (Swaffer et al., 2018). Label-free mass spectrometry indicated that Mis4-S183p was not dependent on the CDK Pef1 either (Fig. 5A supplement). However, Psm1-S1022 phosphorylation was strongly reduced in *pef1* deleted cells (Fig.5C-D supplement 1). The Pef1 CDK was reported as a positive regulator of TORC1 (Matsuda et al., 2020). Indeed, the phosphorylation level of Psk1 was reduced in *pef1* deleted cells and the double mutant *pef1Δ mip1-R401G* grew very poorly (Fig.5F supplement 1). Pef1 may therefore activate TORC1 for the phosphorylation of Psm1. Conversely, Pef1 activity does not seem to depend on TORC1 as Rad21-T262p, which is a Pef1 substrate (Biro et al., 2020), was not affected by *mip1-R401G* (Fig. 5D supplement). The interplay between TORC1, Pef1 and possibly other kinases downstream of TORC1 remains to be elucidated. Whatever the mechanism, the above data show that TORC1 signalling affects the phosphorylation level of Mis4 and Psm1.

### **Mis4-S183 and Psm1-S1022 are relevant targets for the regulation of cohesin by TORC1**

The above data suggest that TORC1 down-regulation may rescue *mis4-G1487D* through reduced phosphorylation of Mis4 and Psm1. To address this question we generated mutants mimicking the non-phosphorylated (S to A) or phosphorylated state (S to E/D). Non-

phosphorylatable *mis4* and *psm1* mutants rescued *mis4-G1487D* growth and chromosome segregation defects while phospho-mimetics had the opposite effect (Figure 6A-C). Cohesin binding to CARs as assayed by ChIP tend to increase for the mutants mimicking the non-phosphorylated state and to decrease with the phospho-mimicking forms (Fig.6D). Likewise, non-phosphorylatable *mis4* and *psm1* mutants efficiently rescued growth and chromosome segregation defects in the *psm3<sup>K105N-K106N</sup> pph3Δ* background (Fig.6 supplement 1). ChIP analyses revealed that cohesin binding to CARs was clearly stimulated, consistent with an upregulation of the cohesin loading machinery when Mis4-S183 and Psm1-S1022 are not phosphorylated. Altogether these data argue that Mis4 and Psm1 are relevant downstream effectors for cohesin regulation by the TOR signalling pathway.

### **Mis4 and TORC1 control a common set of genes involved in the response to environmental changes**

The link between TORC1 and cohesin suggested the possibility of a role for cohesin in the transcriptional response to environmental changes. To address this possibility we looked at the transcriptome by RNA-sequencing of wild-type, *mis4-G1487D* and *mip1-R401G* cells in various experimental conditions (Fig.7). These included cycling cells in the rich complete medium YES at 25°C (V25) and upon a temperature shift to 36.5°C for one doubling (V36.5) or arrested in G1 (*cdc10-129*) at this temperature (G1\_36.5). We also looked upon nitrogen starvation by shifting actively cycling cells from the synthetic medium EMM2 to EMM2 deprived of a nitrogen source (EMM2-N). In this situation, TORC1 activity is restrained, cells arrest in G1 and either mate to enter the reproductive cycle (meiosis) or enter a G0, quiescent state. As no mating partner was available in our experiments, cells homogeneously arrested in G1 after nitrogen deprivation (Fig.7 supplement 1). RNA-sequencing was performed 24 hours after the shift to EMM2-N (T0-N). One half of the remaining culture was then shifted to 36.5°C for 4 days (T4D36.5) while the other half was kept at 25°C for 4 days (T4D25). The results are summarized in Figure 7.

The first striking observation is the low number of genes whose expression was affected in *mis4-G1487D* versus wild-type in actively growing cells in rich medium (Fig.7A, V25, 12 genes), even after G1 arrest (G1\_36.5, 27 genes) or one cell doubling (V36.5, 50 genes) at the restrictive temperature. The number of differentially expressed genes increased upon nitrogen deprivation (T0-N, 118 genes, T4D25, 125 genes) even though 25°C is a permissive temperature for *mis4-G1487D*. A similar number of genes were misregulated in *mis4-G1487D* when nitrogen depleted cells were shifted to 36.5°C for 4 days (T4D36.5, 76 genes). These first observations indicate that the number of genes affected by *mis4-G1487D* is not increased by the shift to the non-permissive temperature. This may suggest that the defect in gene expression of *mis4-G1487D* is not conditional.

A second striking observation emerged when we compared the list of misregulated genes in the different experimental conditions. The diagram in Fig.7B shows that there is little overlap between gene lists. For instance, 70% of the genes misregulated 24 hours after nitrogen depletion (T0-N) were specific to that condition and four days later (T4D25), 77% of the gene list were specific to that stage. Cells deprived of a nitrogen source first arrest in G1 and then progress into the quiescent state. Mis4 appears required for appropriate gene expression

changes as cells enter and progress through quiescence. The other gene lists also showed a low level of overlap, further strengthening the idea that Mis4 is required for the regulation of gene sets involved in the response to specific changes in the environment.

In aggregate, 337 genes were misregulated in *mis4-G1487D* across all experiments. Strikingly, most of these (300/337) were also affected by *mip1-R401G* (Fig.7C). A strong overlap was also apparent with a published set of genes whose expression was upregulated in a *tor2* conditional mutant at the restrictive temperature (Fig. 7D, (Wei et al., 2021)). A significant overlap was also seen with genes up-regulated in G0 (Zahedi et al., 2023), suggesting Mis4 may participate to the proper establishment and/or maintenance of the quiescent state (Fig. 7E). Cell survival remains high in G0 in the *mis4* and *mip1* mutants (Fig.7 supplement 2-3) but upon refeeding a delay in G0 exit was noticeable (Fig.7 supplement 4). This phenotype was quite strong for *mip1-R401G* and much more subtle for the *mis4* mutant. Yet, any delay in G0 exit may reduce the competitive fitness. The analysis of gene lists with AnGeLi (Bitton et al., 2015) indicated that *mis4*-regulated genes showed a strong localization bias towards chromosome ends, as previously reported (Dheur et al., 2011), an enrichment for “stress genes”, the response to nitrogen starvation and differentiation (meiosis). Genes encoding membrane transporters were significantly enriched, suggesting a role for Mis4 in cell homeostasis.

Overall, these data show that Mis4 and TORC1 control a common set of genes involved in the response to environmental changes.

## DISCUSSION

The data presented in this work provide evidence for a regulation of cohesin by the TOR signalling pathway. This is, to the best of our knowledge, the first report to demonstrate such a relationship. Previously, a link had been established between the two pathways, but in the opposite direction. mTORC1 is indeed down-regulated in cells from patients with Roberts syndrome, caused by mutational alteration of the ESCO2 cohesin acetyl-transferase (Xu et al., 2013). The down-regulation of the cohesin loader NIPBL in breast cancer cells induced cell cycle arrest, apoptosis and autophagy through the caspase 3 and mTOR signalling pathways (Zhou et al., 2017). The first hint in the opposite direction came from the observation in fission yeast that the growth of a conditional mutant of *mis4* was enhanced by rapamycin (Sajiki et al., 2018). In mammalian species, a fraction of mTOR controls nuclear processes, notably transcription (Zhao et al., 2024). Likewise, fission yeast TORC1 is involved in the regulation of facultative heterochromatin (Hirai et al., 2023; Wei et al., 2021) and transcriptional processes (Laribee and Weisman, 2020). The present work establishes a close link between cohesin and the TOR pathway, suggesting the exciting possibility that extracellular signals could remodel the functional architecture of chromosomes during differentiation or adaptation processes.

### Mis4 and Psm1 as downstream targets of TORC1

We present evidence showing that cohesin and TORC1 components co-purify from protein extracts and Mis4-S183 and Psm1-S1022 are less phosphorylated in *mip1* mutants. Non-phosphorylatable mutants recapitulated most of the effects of TORC1 down-regulation while

mutants mimicking the phosphorylated state produced phenotypes similar to TORC1 hyper activation. Whether cohesin and Mis4 are substrates of TORC1 or other kinases regulated by TORC1 is currently unknown. Meanwhile, our work provides the first evidence that cohesin is a downstream effector of TORC1. Both phosphorylation events on Mis4 and Psm1 have been reported in the literature but their biological functions are unknown (Halova et al., 2021; Kettenbach et al., 2015; Swaffer et al., 2018; Tay et al., 2019). Mis4-S183 is located within an unstructured, flexible region connecting the Ssl3 interaction domain and the hook domain that provides Mis4 catalytic activity. It was proposed that phosphorylation at CDK sites within the unstructured linker may affect the flexibility of the complex and modulate its activity (Chao et al., 2015). Mis4-S183 and the CDK consensus are conserved in hNIPBL and the S1160 residue was found phosphorylated in a number of phosphoproteomic studies (Beli et al., 2012; Christensen et al., 2010; Dephoure et al., 2008; Mayya et al., 2009; Pan et al., 2009; Rigbolt et al., 2011; Ruse et al., 2008). Cryo-EM structures of cohesin bound to its loader and DNA revealed that the SMC coiled coils are folded around their elbow (J. E. Collier et al., 2020; Z. Shi et al., 2020). The S1022 residue of Psm1 lies close to the “joint” region below the ATPase heads that the “hinge” domain contacts when cohesin in its folded conformation. Whether the phosphorylation of Psm1-S1022 affects the outcome of DNA transactions remain to be investigated.

### **TORC1 activity restrains cohesin binding to CARs**

Reducing TORC1 activity by the *mip1-R401G* mutation or rapamycin treatment enhanced Cohesin binding to CARs. This was observed for two genetic contexts of sensitized cohesin loading and with a wild-type cohesin loading machinery. The most straightforward interpretation is that TORC1 restrains cohesin loading. A distinct possibility would be that TORC1 down-regulation promotes cohesin accumulation at CARs without *de novo* loading. It was proposed that CARs correspond to the borders of yeast TAD-like structures (L. Costantino et al., 2020). Increased abundance of cohesin at CARs may result from the accumulation of cohesin with loop extrusion activity at TAD boundaries. Considering that loop extrusion requires the NIPBL-Cohesin holocomplex (I. F. Davidson et al., 2019), it is interesting to note that Mis4 binding to CARs was similarly enhanced. TORC1 may also impinge on cohesin sliding by transcription-related processes (Lengronne et al., 2004; C. K. Schmidt et al., 2009) and / or influence cohesin accumulation through the phosphorylation of chromatin-bound proteins. Future work will aim at clarifying these issues.

### **TORC1 and chromosome segregation**

Cells with a crippled cohesin loading machinery experience chromosome segregation defects during mitosis. We have shown here that the intensity of the defect is dependent on TORC1 activity. The most prominent defect is the merotelic attachment of kinetochores which, when left uncorrected, lead to aberrant chromosome segregation. Although the down-regulation of TORC1 reduced the frequency of aberrant anaphases, sister-chromatid cohesion was still largely impaired although cohesin binding to CARs in the *mis4* mutant was restored to near wild-type levels in cells arrested at the G1/S boundary. The increase in chromosome bound cohesin may have enhanced cohesion establishment during S-phase. Chromosome segregation defects were indeed reduced but sister-chromatid cohesion remained overall



defective. Consistently, cohesin acetylation was not improved by TORC1 down-regulation. The acetylation of Psm3, mediated by Eso1 in fission yeast, counteracts the cohesin releasing activity of Wapl and is thought to stabilize cohesin binding and cohesion (Biro et al., 2017; Feytout et al., 2011; Kagami et al., 2011; Vaur et al., 2012). However, deletion of the *wpl1* gene does not rescue the *mis4-G1487D* mutant and *S. pombe* cells can survive with non-detectable acetylation or in a context in which Psm3 cannot be acetylated (Biro et al., 2020; Feytout et al., 2011). The primary defect of *mis4-G1487D* may therefore stem from inability to generate sister-chromatid cohesion rather than its maintenance. *In vitro* assays have suggested that sister-chromatid capture by cohesin is a two-step process catalysed by Mis4 (Murayama et al., 2018). The down-regulation of TORC1 may increase the residual loading activity of Mis4 but second DNA capture may remain largely deficient, resulting in poor sister-chromatid cohesion. Increased cohesin binding at peri-centromeres may improve chromosome segregation through other routes. Cohesin organizes the peri-centromere regions in budding yeast (Lawrimore et al., 2018; Paldi et al., 2020). Local depletion of cohesin at centromeres in fission yeast lead to a high incidence of merotelic attachments (Bernard et al., 2001). A recent study revealed that vertebrate kinetochores are bipartite structures and highlighted a role for cohesin in bridging to the two parts into a functional unit (Sacristan et al., 2024). Lastly, enhanced cohesin binding to centromeres may facilitate the correction of merotelic attachments by the Aurora B kinase. The localization of Aurora B relies on histone H3-T3 phosphorylation by Haspin whose localization to centromeres depends largely on the cohesin subunit Pds5 in fission yeast (Goto et al., 2017; Yamagishi et al., 2010).

That chromosome segregation defects increase with TORC1 activity suggests the intriguing possibility that the fidelity of chromosome segregation could be modulated by environmental cues. Chromosome segregation defects in the *mis4* mutant were most intense in the complete rich medium YES than in the synthetic medium EMM2. The use of glutamate as the nitrogen source instead of ammonia further reduced chromosome segregation defects. Microbial species in the wild compete with each other. When nutrients are abundant, it might be preferable to divide rapidly to colonize the niche, even at the expense of individual cells. Conversely, when nutrients become scarce, survival of the species may rely more heavily on individual fitness.

### **Mis4 and TORC1 control the gene response to environmental changes**

In a previous study we reported that *mis4-G1487D* affects the expression of genes located in subtelomeric domains (Dheur et al., 2011). Here we extend the study by challenging cells with various culture conditions. Several conclusions can be drawn. Very few genes were affected when cells were actively cycling in rich media, when TORC1 activity is high. The gene list was much longer upon nitrogen deprivation, when TORC1 activity is repressed, and the list of genes evolved with time spent in G0. In aggregate 337 genes were affected in *mis4-G1487D* when compared with wild-type with a strong bias towards chromosome ends and far from centromeres. Lastly, most genes misregulated in *mis4-G1487D* were misregulated in the *mip1-R401G* mutant suggesting Mis4 and Mip1 act in a same pathway. Paradoxically, the *mis4* and *mip1* mutants remained viable during G0 although a delay was noticeable upon re-entry into the cell cycle at elevated temperature. A defect in quiescence exit was previously reported when the *mis4* gene is affected and the phenotype appears strongly dependent on the allele

considered (Sajiki et al., 2009; Suma et al., 2024). It must be kept in mind that *mis4* mutants were originally screened for a defect during vegetative growth. It would be interesting to screen specifically for *mis4* alleles affecting cell survival in quiescence.

Given the role of cohesin and its loader in chromosome folding, it is tempting to speculate that TORC1 might direct cohesin to generate a chromosome architecture that is competent for transcriptional response to environmental changes. If increased binding of Mis4 and cohesin at CARs does reflect extruding cohesin complexes that have reach TAD boundaries, chromosomes might be more compacted when TORC1 is down regulated. Conversely, high TORC1 would promote the opposite. Even if this hypothesis turned out to be correct, it does not explain why regulated genes show a bias towards chromosome ends. Chromosomes in fission yeast adopt a rabl configuration in interphase (Mizuguchi et al., 2015). Centromeres cluster at the SPBs while telomeres cluster in a few patches at the opposing hemisphere near the nuclear periphery. These constraints generate specific chromosomal regions with distinct molecular environments. Chromosome conformation capture and the mapping of DNA binding sites of inner nuclear membrane proteins have provided evidence of functional sub-nuclear environments that correlate with gene expression activity (Grand et al., 2014; Steglich et al., 2012). The studies suggest more internal locations of actively expressed genes, and nuclear peripheral localization of poorly expressed genes. Chromosome regions containing gene clusters that are up-regulated upon nitrogen starvation re-position from the nuclear periphery to the interior (Alfredsson-Timmins et al., 2009). In this regard, Mis4-regulated genes tend to be lowly expressed and enriched for nitrogen responsive genes. Modifying chromosome compaction may alter the rabl configuration or generate forces that modify the properties of the inner nuclear envelope, leading to misregulated gene expression. Indeed, loss of cohesin causes an increase in inter-chromosomal contacts and a reduction in chromosomal territoriality (T. Mizuguchi et al., 2014).

Cohesin and TORC1 have been implicated with a wide range of human pathologies, including cancer (Waldman, 2020; Yoon, 2020). Rapamycin has attracted much attention for its putative ability to mimic caloric restriction, resulting in improved health and lifespan (Bjedov and Partridge, 2011). Our study raises the intriguing possibility that some effects are mediated by cohesin and its ability to remodel chromosomes in response to various extracellular signals, including nutrients.

## **MATERIALS AND METHODS**

### **Strains, media and genetic techniques**

General fission yeast methods, reagents and media are described in (Moreno et al., 1991). All strains are listed in supplementary Table 1. Experiments were carried out using YES medium unless otherwise stated. EMM-GLU is EMM2 in which NH<sub>4</sub>Cl was replaced with 20mM glutamate. For nitrogen deprivation, cycling cells in EMM2 (~5-10<sup>6</sup>-10<sup>7</sup> cells/ml) were washed three times in EMM2-N and incubated in EMM2-N at the same density. Cell cycle arrest in YES medium was achieved by shifting cycling *cdc10-129* cells (~10<sup>7</sup> cells/ml) at 36°C for 3.5 hours.

Hydroxyurea (HU) was used to arrest cells in early S-phase. HU (12mM) was added to cycling cells in YES (~10<sup>7</sup>/ml) and shifted to 36.5°C for 3.5 hours. All cell cycle arrests were checked by flow cytometry analysis of DNA content. The genetic screen for *mis4* suppressors was described in (Biro et al., 2020). The suppressors fell into four linkage groups. To identify the mutated loci, genomic DNA was extracted from one mutant from each group and from the wild-type *S. pombe* reference strain and co-hybridized to tiling arrays as described (Biro et al., 2020). For the *mip1* group (5 alleles) the mutated site in each strain was identified by PCR followed by DNA sequencing. Phospho-mutants were generated using the SpEDIT method (Torres-Garcia et al., 2020). The introduction of the desired changes was confirmed by PCR followed by DNA sequencing. Gene mapping, sequence and annotations were from Pombase (Rutherford et al., 2024).

### **Cytological techniques**

DNA content was measured by flow cytometry with an Accuri C6 Flow cytometer after Sytox Green staining of ethanol-fixed cells (Knutsen et al., 2011). Data were presented using the FlowJo software. Indirect immunofluorescence was done as described (Biro et al., 2020). Cells were imaged using a Leica DMRXA microscope and a 63X objective. Measurements were made using MetaMorph software.

Live cell analysis was performed in an imaging chamber (CoverWell PCI-2.5, Grace Bio-Labs, Bend, OR) filled with 1 ml of 1% agarose in minimal medium and sealed with a 22 × 22-mm glass coverslip. Time-lapse images of Z stacks (maximum five stacks of 0.5 μm steps, to avoid photobleaching) were taken at 60 sec intervals. Images were acquired with a CCD Retiga R6 camera (QImaging) fitted to a DM6B upright microscope with a x63 objective, using MetaMorph as a software. Intensity adjustments were made using the MetaMorph, Image J, and Adobe Photoshop packages (Adobe Systems France, Paris, France). To determine the percentage of mispositioned spindles, cells were fixed in 3.7% formaldehyde for 6 min at room temperature, washed twice in PBS, and observed in the presence of DAPI/calcofluor.

The position of the SPBs, kinetochores and centromeres were determined by visualization of the Cdc11–GFP, Ndc80–GFP and Mis6–RFP signals. Maximum intensity projections were prepared for each time point, with the images from each channel being combined into a single RGB image. These images were cropped around the cell of interest, and optional contrast enhancement was performed in MetaMorph, Image J or Photoshop where necessary. The cropped images were exported as 8-bit RGB-stacked TIFF files, with each frame corresponding to one image of the time-lapse series. For all channels, custom peak detection was performed. The successive positions of the SPBs and kinetochores/centromeres were determined.

### **Antibodies, protein extracts, immunoprecipitation, western blotting, phosphatase treatment, chromatin immunoprecipitation (ChIP)**

Rabbit polyclonal antibodies against Rad21, Psm1, Psm3, Psm3-K106Ac, Rad21-T262 have been described previously (Biro et al., 2020; Dheur et al., 2011; Feytout et al., 2011). The mouse monoclonal anti-tubulin antibody TAT1 is from (Woods et al., 1989). Anti-Psm1-S1022p and anti-Mis4-S183p antibodies were raised by Biotem (Apprieu, France). Rabbits were immunized with the KLH-coupled peptides. Sera were immune-depleted by affinity with the non-phosphorylated form of the peptide and antibodies were affinity purified against the

phosphorylated peptide. Other antibodies were of commercial source. Rabbit polyclonal anti-GFP A11122 (Molecular Probes), mouse monoclonal anti-GFP (Roche), anti-PK antibodies (monoclonal mouse anti V5 tag, AbD serotec), mouse monoclonal anti-FLAG (Sigma). For the detection of Psk1 (Thr-415) phosphorylation, protein extracts were prepared by the TCA method and western blots probed with the anti-phospho-p70 S6K antibody (cat. no. 9206, Cell Signaling Technology) as described (Morozumi et al, 2021). Protein extracts, immunoprecipitation (IP), cell fractionation and western blotting were as described (Biot et al., 2020). For quantitative western blot analyses, signals were captured with the ChemiDoc MP Imaging System and quantified using the Image Lab software. On beads phosphatase treatment of Mis4-GFP was done as described (Biot et al., 2017). Chromatin Immunoprecipitation (ChIP) was as described (Biot et al., 2020) using anti-FLAG, anti-GFP (A11122) and anti-Rad21 antibodies.

### **RNA sequencing**

Total RNA from biological triplicate samples was subjected to quality control with Agilent TapeStation according to the manufacturer's instructions. To construct libraries suitable for Illumina sequencing, rRNA was depleted using the Ribominus kit (Thermo Fisher) starting with 2000 ng total RNA and then followed by the Illumina stranded ligation sample preparation protocol starting with 100 ng rRNA depleted RNA. The protocol includes fragmentation, denaturation of RNA, cDNA synthesis, ligation of adapters, and amplification of indexed libraries. The yield and quality of the amplified libraries were analyzed using Qubit (Thermo Fisher) and the quality of the libraries was checked by using the Agilent TapeStation. The indexed cDNA libraries were normalized and combined, and the pools were sequenced on the Illumina Nextseq 2000 machine using a P3 100 cycle sequencing run, producing a sequencing length of 58 base paired end reads with dual index.

### **RNA-Seq Data processing and analysis**

Bcl files were converted and demultiplexed to fastq using the bcl2fastq v2.20.0.422 program. STAR 2.7.10a (Dobin et al., 2013) was used to index the *S. pombe* reference genome (ASM294v2) and align the resulting fastq files. Mapped reads were then counted in annotated exons using featureCounts v1.5.1 (Liao et al., 2014). The gene annotations (Schizosaccharomyces\_pombe.ASM294v2.35.gff3) and reference genome were obtained from Ensembl Fungi. The count table from featureCounts was imported into R/Bioconductor and differential gene expression was performed using the EdgeR (Robinson et al., 2010) package and its general linear models pipeline. For the gene expression analysis genes with no or very low expression were filtered out using the filterByExpr function and subsequently normalized using TMM normalization. Genes with an FDR adjusted p value < 0.05 were termed significantly regulated. Venn diagrams were generated using jvenn (Bardou et al., 2014).

### **Mass spectrometry**

Sample preparation and protein digestion. Protein samples were solubilized in Laemmli buffer and proteins were deposited onto SDS-PAGE gel. After colloidal blue staining, each lane was cut out from the gel and was subsequently cut in 1 mm x 1 mm gel pieces. Gel pieces were destained in 25 mM ammonium bicarbonate 50% ACN, rinsed twice in ultrapure water and shrunk in ACN for 10 min. After ACN removal, gel pieces were dried at room temperature,

covered with the trypsin solution (10 ng/ $\mu$ l in 50 mM  $\text{NH}_4\text{HCO}_3$ ), rehydrated at 4°C for 10 min, and finally incubated overnight at 37°C. Spots were then incubated for 15 min in 50 mM  $\text{NH}_4\text{HCO}_3$  at room temperature with rotary shaking. The supernatant was collected, and an  $\text{H}_2\text{O}/\text{ACN}/\text{HCOOH}$  (47.5:47.5:5) extraction solution was added onto gel slices for 15 min. The extraction step was repeated twice. Supernatants were pooled and dried in a vacuum centrifuge. Digests were finally solubilized in 0.1%  $\text{HCOOH}$ .

**nLC-MS/MS analysis and Label-Free Quantitative Data Analysis.** Peptide mixture was analyzed on a Ultimate 3000 nanoLC system (Dionex, Amsterdam, The Netherlands) coupled to a Electrospray Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA). Ten microliters of peptide digests were loaded onto a 300- $\mu$ m-inner diameter x 5-mm C18 PepMap™ trap column (LC Packings) at a flow rate of 10  $\mu$ L/min. The peptides were eluted from the trap column onto an analytical 75-mm id x 50-cm C18 Pep-Map column (LC Packings) with a 5–27.5% linear gradient of solvent B in 105 min (solvent A was 0.1% formic acid and solvent B was 0.1% formic acid in 80% ACN) followed by a 10 min gradient from 27.5% to 40% solvent B. The separation flow rate was set at 300 nL/min. The mass spectrometer operated in positive ion mode at a 2-kV needle voltage. Data were acquired using Xcalibur software in a data-dependent mode. MS scans ( $m/z$  375-1500) were recorded in the Orbitrap at a resolution of  $R = 120\,000$  (@  $m/z$  200) and an AGC target of  $4 \times 10^5$  ions collected within 50 ms. Dynamic exclusion was set to 60 s and top speed fragmentation in HCD mode was performed over a 3 s cycle. MS/MS scans were collected in the Orbitrap with a resolution of 30,000 and maximum fill time of 54 ms. Only +2 to +7 charged ions were selected for fragmentation. Other settings were as follows: no sheath nor auxiliary gas flow, heated capillary temperature, 275 °C; normalized HCD collision energy of 30%, isolation width of 1.6  $m/z$ , AGC target of  $5 \times 10^4$  and normalized AGC target of 100%. Monoisotopic precursor selection (MIPS) was set to Peptide and an intensity threshold was set to  $2.5 \times 10^4$ .

**Database search and results processing.** Data were searched by SEQUEST and BYONIC through Proteome Discoverer 2.5 (Thermo Fisher Scientific Inc.) against an *Schizosaccharomyces pombe* uniprot database (5,098 entries in v2021-01). Spectra from peptides higher than 5000 Da or lower than 350 Da were rejected. Precursor Detector node was included. Search parameters were as follows: mass accuracy of the monoisotopic peptide precursor and peptide fragments was set to 10 ppm and 0.02 Da respectively. Only b- and y-ions were considered for mass calculation. Sequest HT was used as the search algorithm: Oxidation of methionines (+16 Da), methionine loss (-131 Da), methionine loss with acetylation (-89 Da), protein N-terminal acetylation (+42 Da) and phosphorylation of Serine, Threonine and Tyrosine (+80 Da) were considered as variable modifications while carbamidomethylation of cysteines (+57 Da) was considered as fixed modification. Two missed trypsin cleavages were allowed. Peptide validation was performed using Percolator algorithm (Kall et al., 2007) and only “high confidence” peptides were retained corresponding to a 1% False Positive Rate at peptide level. Peaks were detected and integrated using the Minora algorithm embedded in Proteome Discoverer. Normalization was performed based on total peptide amount. Protein ratios were calculated as the median of all possible pairwise peptide ratios. A t-test was calculated based on background population of peptides or proteins. Quantitative data were considered at peptide level.

## ACKNOWLEDGMENTS

We would like to thank Tomoyuki Fukuda, Yuichi Morozumi, Janni Petersen, Kazuhiro Shiozaki, Ronit Weisman, Mitsuhiro Yanagida and the National BioResource Project for providing *S. pombe* strains and advices. Keith Gull for the gift of anti-tubulin antibodies. This work was supported by the Centre National de la Recherche Scientifique, l'Université de Bordeaux, la Région Aquitaine, la Fondation ARC pour la recherche contre le cancer (ARCPJA2021060003810) and La Ligue contre le Cancer. Dorian Besson was supported by the International Doctorate Program – IdEx University of Bordeaux and a fellowship from the Fondation ARC. Work in the K.E laboratory was financed by the Swedish research council (VR-MH) and the Swedish Cancer Society (CF). We also would like to thank BEA, The core facility for Bioinformatics and Expression Analysis, which is supported by the board of research at the Karolinska Institutet and the research committee at the Karolinska hospital.

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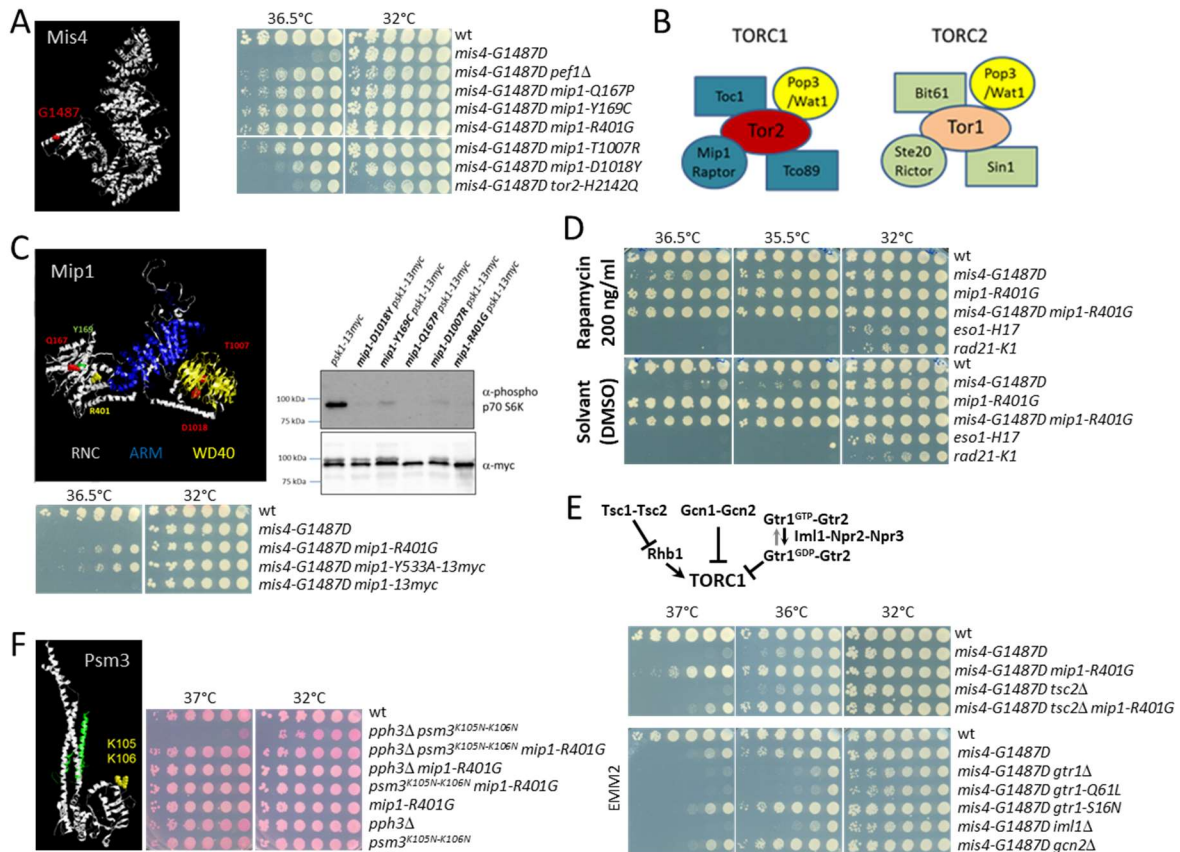
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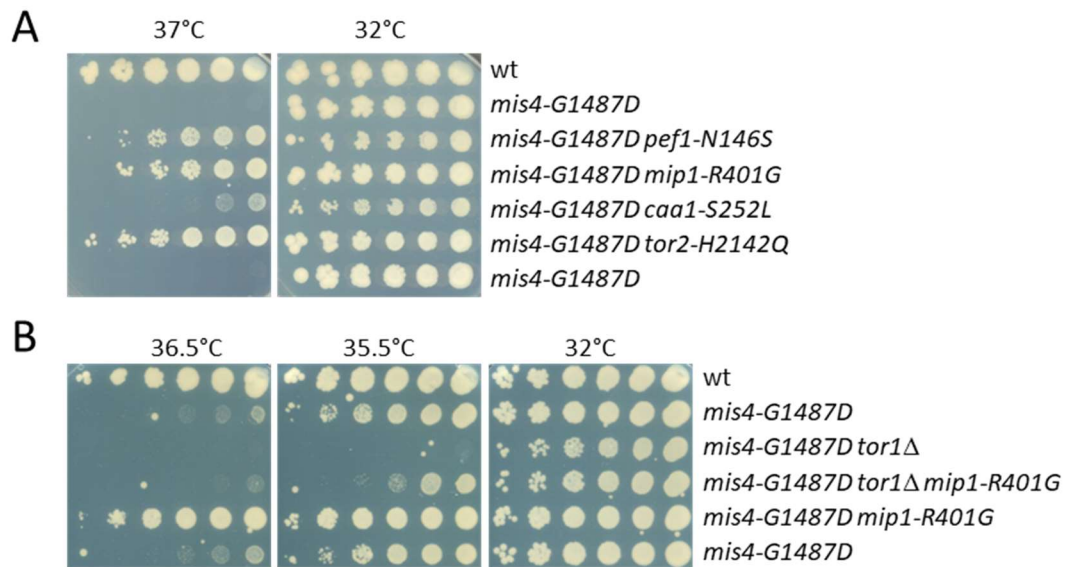
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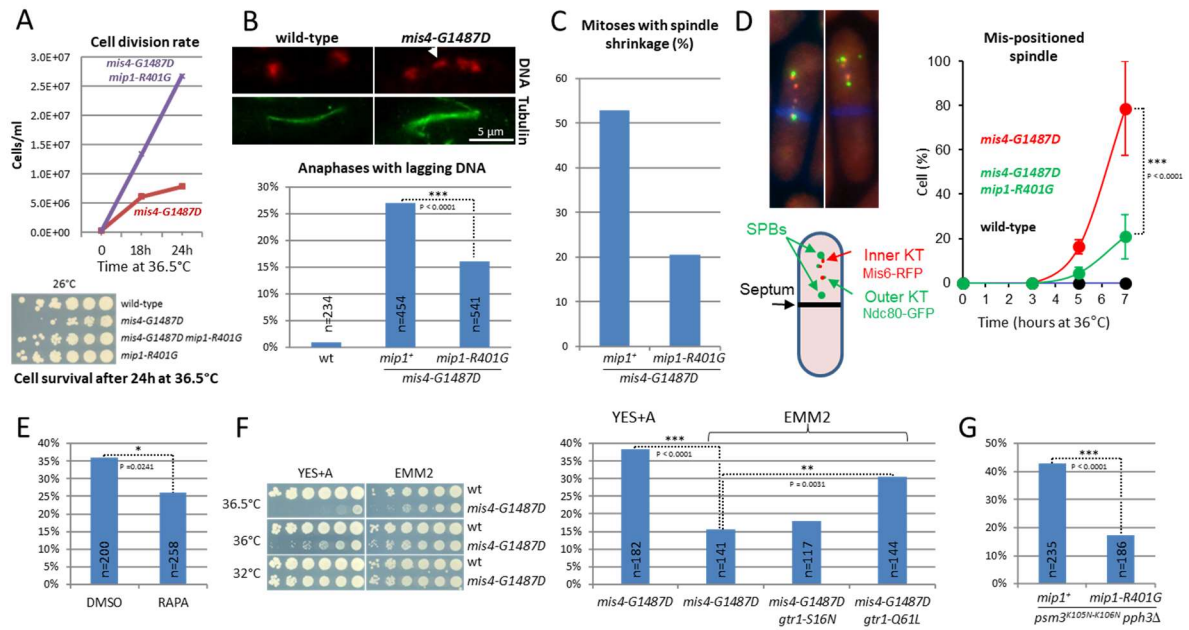
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**Figure 1. Genetic evidence linking TORC1 to cohesin.** (A) Model structure of Mis4. The G1487D substitution confers a thermosensitive growth (Ts) phenotype. The cell growth assay shows the suppression of the Ts phenotype by the indicated mutations. (B) Composition of fission yeast TORC complexes (adapted from (Hayashi et al, 2007)). (C-D) Down-regulation of TORC1 suppresses the Ts growth defect of *mis4-G1487D*. (C) Model structure of Mip1 highlighting amino-acid substitutions in the suppressor mutants (RNC: Raptor N-terminal CASPase-like domain, ARM: Armadillo-type fold, WD40: WD-40 repeats). All *mip1* mutant alleles from the genetic screen showed reduced phosphorylation of the S6-kinase Psk1, a known TORC1 substrate. Conversely, *mip1-Y533A* which is deficient for Psk1 binding and phosphorylation, suppressed the Ts phenotype of *mis4-G1487D*. (D) The TORC1 inhibitor rapamycin rescued *mis4-G1487D* but not *eso1* and *rad21* mutants. (E) Genetic upregulation of TORC1 exacerbates the Ts phenotype of *mis4-G1487D*. The deletion of *gcn2* or *tsc2* was essentially neutral, although the suppression by *mip1-R401G* was reduced in a *tsc2* deleted background. Deletion of *gtr1*, *iml1* or *gtr1-Q61L* mimicking the GTP-bound, inactive state of GATOR exacerbated the Ts phenotype of *mis4-G1487D* while mimicking the active GDP-bound form (*gtr1-16N*) was neutral. (F) *Psm3<sup>K105N-K106N</sup>* confers a Ts phenotype when combined with the deletion of *pph3*, encoding the catalytic subunit of PP4, a phenotype efficiently rescued by *mip1-R401G*.

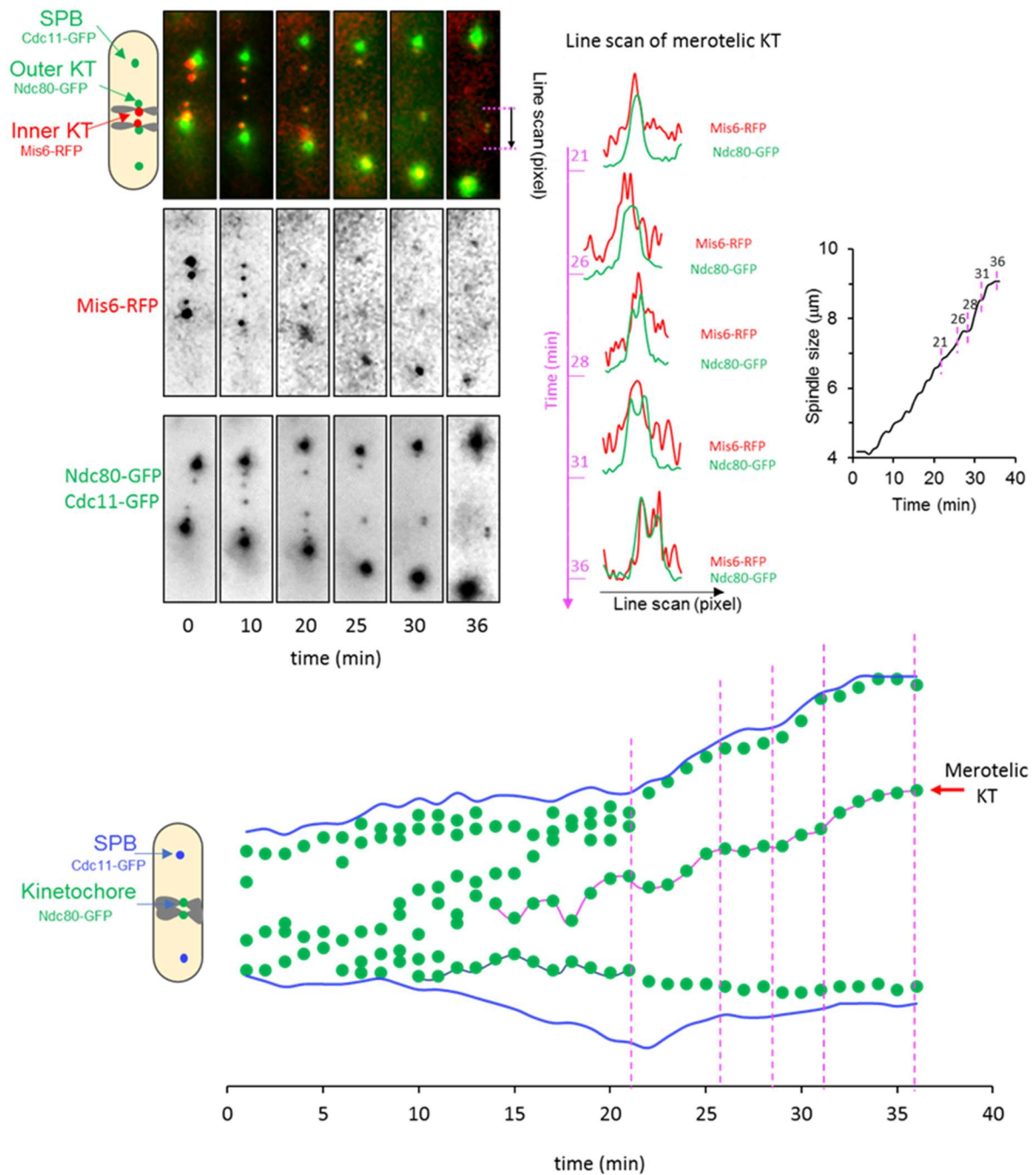


**Fig.1 supplement 1. (A)** Cell growth assay showing the suppression of the Ts phenotype of *mis4-G1487D* by the indicated mutations. Plates were incubated for 6 days at the indicated temperatures. The *caa1* mutant is slow growing and 6 days were required to see a weak suppressor effect. **(B)** The deletion of *tor1*, encoding the catalytic subunit of TORC2, shows a negative genetic interaction with *mis4-G1487D*.

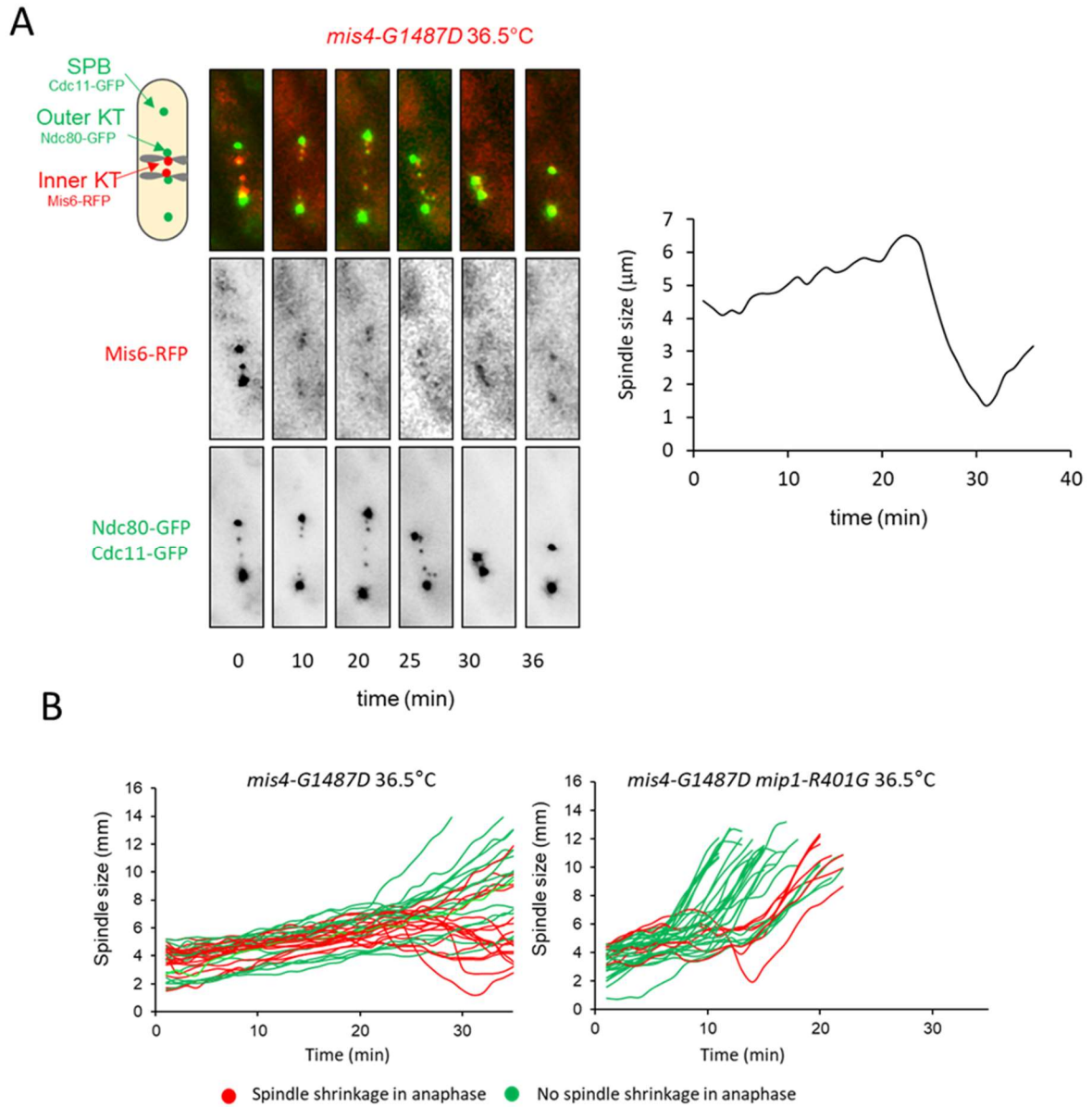


**Figure 2. The incidence of chromosome segregation defects in *mis4-G1487D* is modulated by TORC1.**

(A) Exponentially growing cells at 25°C were shifted at 36.5°C for 24 hours. The growth curve shows that *mip1-R401G* suppressed the *mis4-G1487D* temperature growth defect. Cell survival after the 24 hours temperature shift was addressed by plating serial 5-fold cell dilutions at permissive temperature for *mis4-G1487D* ( $4 \times 10^4$  cells in the first row). Cell survival was reduced ~25 fold in *mis4-G1487D* and restored to wild-type levels when combined with *mip1-R401G*. (B) Exponentially growing cells at 25°C were shifted at 36°C for one doubling. DNA was stained with DAPI (red, pseudo-colour) and tubulin detected by indirect immunofluorescence (green). Lagging DNA (white arrow) was defined as DAPI-stained material on the anaphase spindle (length>5µm). (C) Spindle shrinkage events (shrinkage of more than 0.5µm) were detected by live analysis of cells undergoing anaphase at 36.5°C (*mis4-G1487D*, n=34; *mis4-G1487D mip1-R401G*, n= 35). (D) Anaphase cells with displaced spindle. Cells were cultured at 36.5°C, fixed and stained with Calcofluor to visualize septa. Shown are averages and SD obtained from three independent experiments (n>70 cells for each condition). (E) Rapamycin reduces the incidence of anaphases with lagging DNA. Rapamycin (RAPA, 200ng/ml) or solvent alone (DMSO) was added to cycling cells at 25°C and the cultures shifted to 36°C for one doubling of the cell population. Samples were treated as in B. (F) Left, cell growth assay showing that the Ts growth defect of *mis4-G1487D* is more severe in YES+A medium than in EMM2. Right, the frequency of anaphases with lagging DNA is higher in YES+A than in EMM2. The inactive form of the TORC1 inhibitor Gtr1 (Gtr1-Q61L) increased the frequency of abnormal anaphases while the active form was neutral (Gtr1-S16N). Cells were treated as in B. (G) The *mip1* mutant reduced the frequency of anaphases with lagging DNA in the *psm3<sup>K105-K106N</sup> pph3Δ* background. Cells were treated as in B. (\*) (\*\*) (\*\*\*), P-values from two-sided Fisher's exact test.

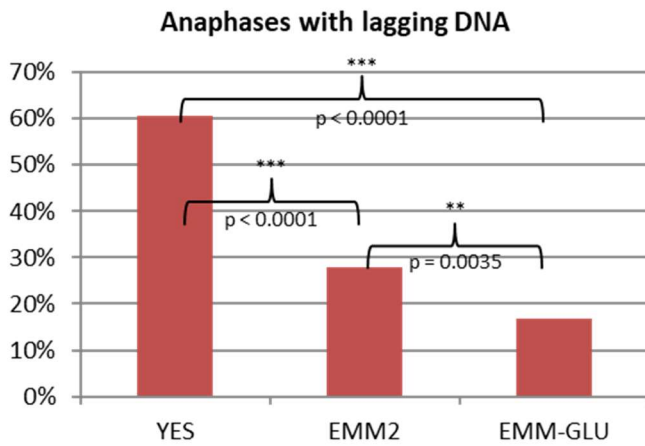


**Fig.2 supplement 1. Live analysis of *mis4-G1487D* undergoing anaphase at 36.5°C. A merotelic kinetochore is evidenced by the stretching of the outer kinetochore protein Ncd80 (line scan time 28 to 36 min).**

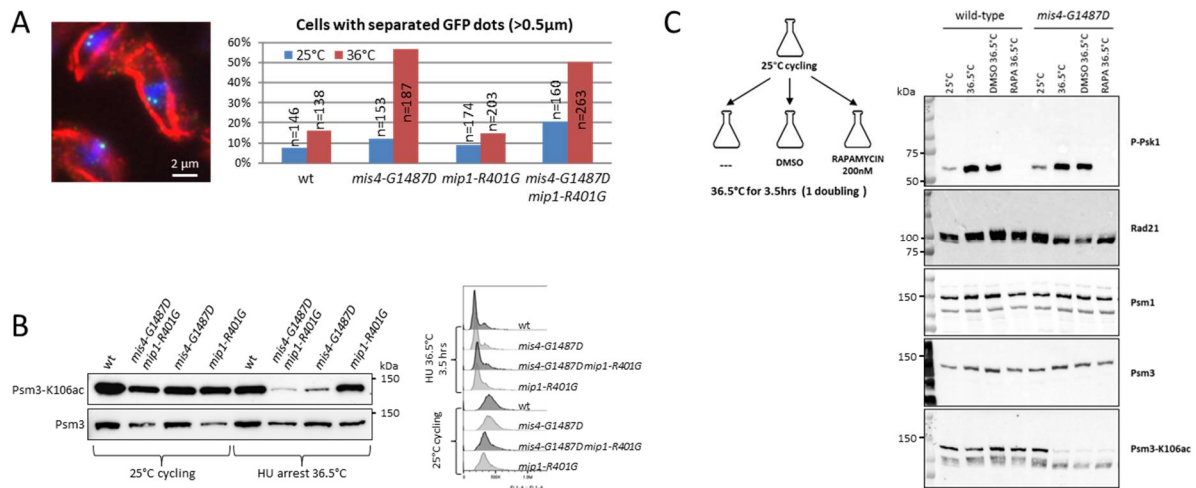


**Fig.2 supplement 2. Live analysis of anaphase cell revealed spindle shrinkage events. (A)** Example of an anaphase cell showing spindle shrinkage. **(B)** As shown in Figure 2C the proportion of mitotic cells with the occurrence of spindle shrinkage ( $>0.5\mu\text{m}$ ; red curves) is reduced in the *mip1-R401G* background (*mis4-G1487D*,  $n=34$ ; *mis4-G1487D mip1-R401G*,  $n=35$ ).

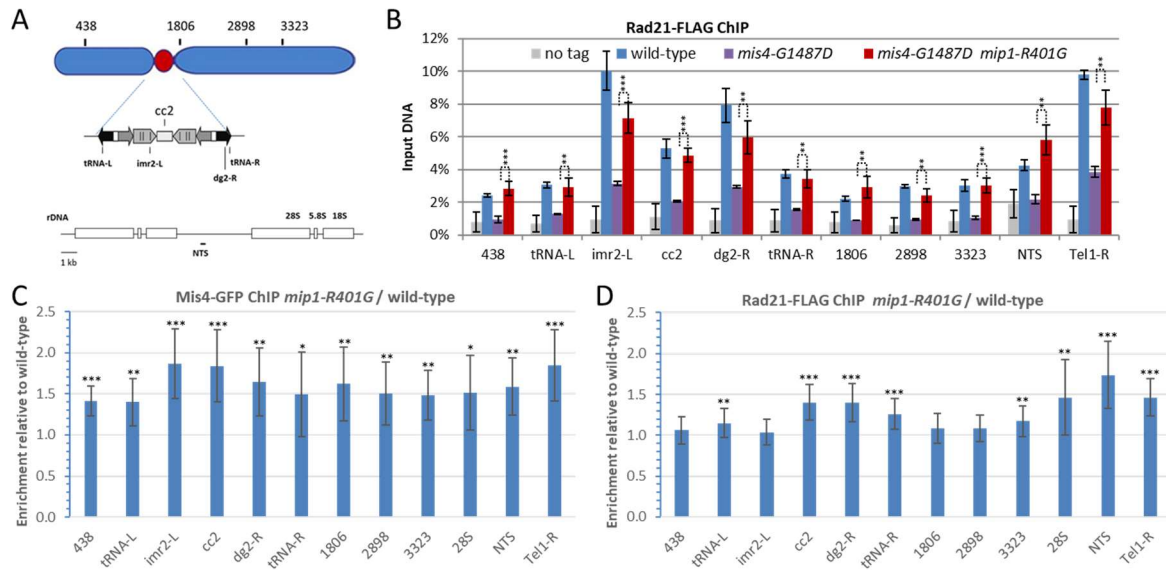




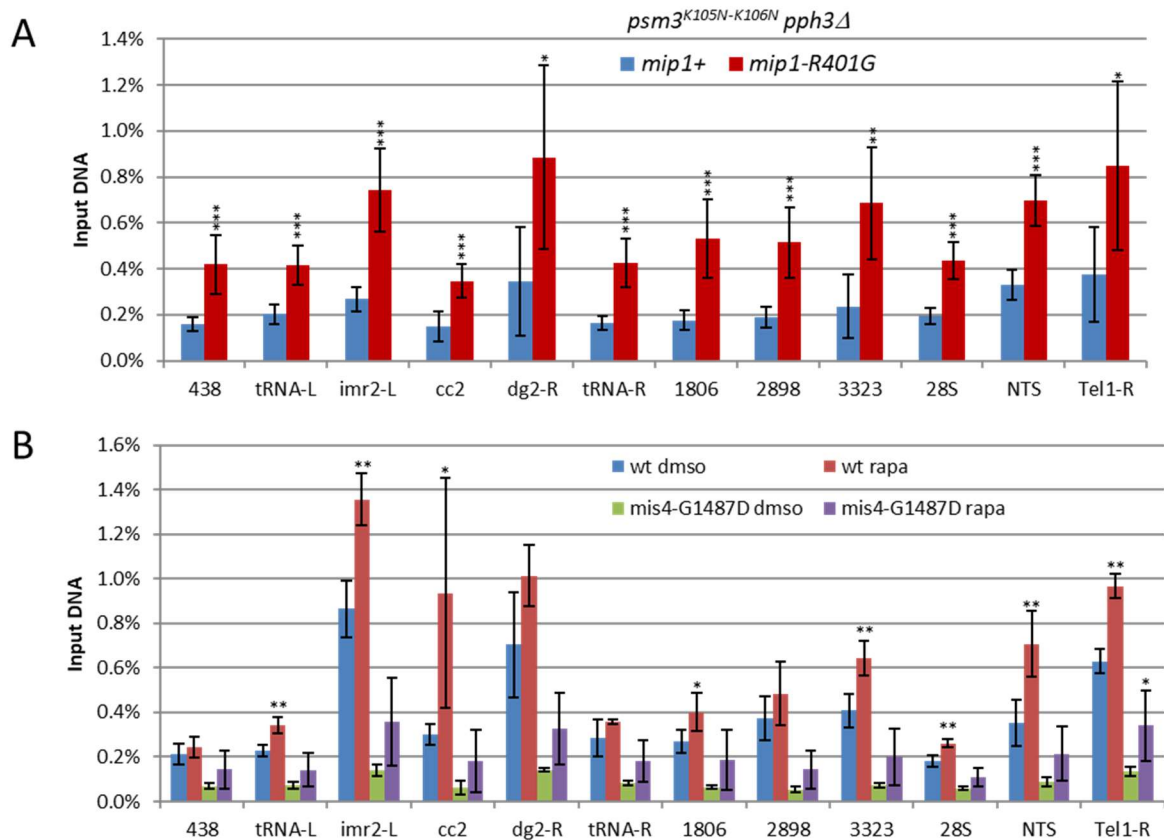
**Figure 2 supplement 3. The frequency of anaphases with lagging DNA is dependent on the culture medium.** Exponentially growing *mis4-G1487D* cells at 25°C in the indicated media were shifted at 36.5°C for one doubling of the cell population. DNA was stained with DAPI and tubulin detected by indirect immunofluorescence. Lagging DNA was defined as DAPI-stained material on the anaphase spindle (length > 5µm). EMM-GLU is EMM2 in which NH<sub>4</sub>Cl was replaced by 20 mM glutamate. (\*) P-values from two-sided Fisher's exact test.



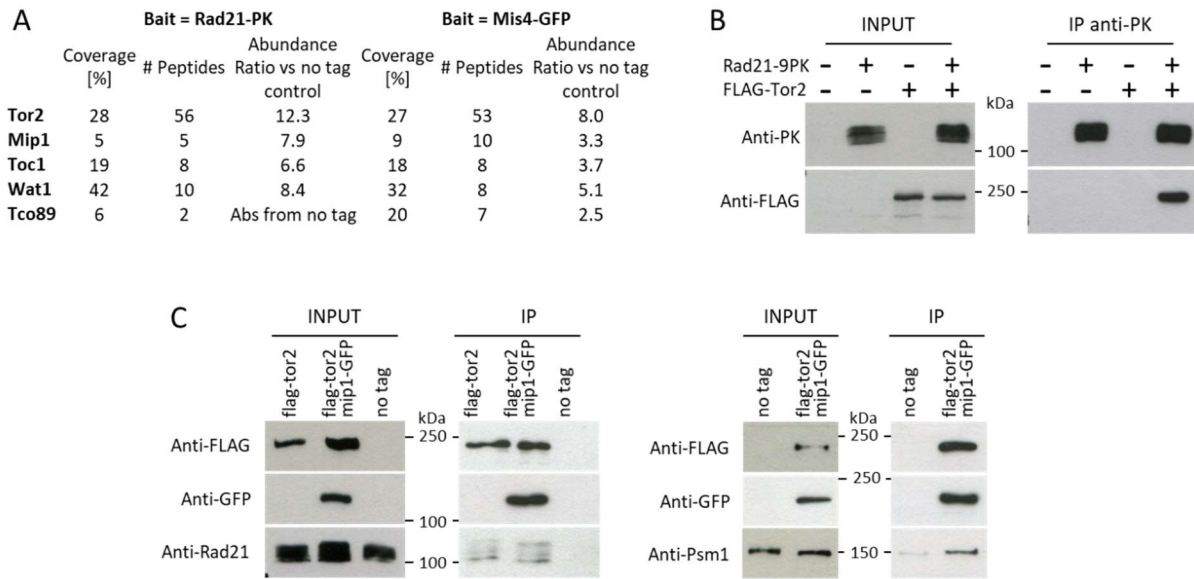
**Figure 2 supplement 4. (A) Sister-chromatid cohesion assay at the *ade6* locus.** Exponentially growing cells at 25°C were shifted at 36°C for one doubling. Fixed cells were treated for detection of GFP dots and tubulin by indirect immunofluorescence. DNA appears in blue, tubulin in red and the GFP-marked *ade6* locus in green. Separated GFP dots (>0.5μm) were scored in G2 cells as judged by the presence of the interphase array of microtubules and a single nucleus. **(B-C) Psm3-K106 acetylation levels.** **(B)** Exponentially growing cells at 25°C were shifted at 36.5°C. HU (12mM) was added at the time of the shift and cells incubated for 3.5 hours. The level of Psm3-K106 acetylation was determined by western blotting. Cell cycle arrest in early S-phase was checked by DNA-content analysis (Right). **(C)** Rapamycin (200nM), solvent alone (DMSO) or nothing (---) was added to exponentially growing cells at 25°C and the cultures shifted to 36.5°C for one doubling of the cell population. Protein extracts were analyzed by western blotting using antibodies detecting phosphorylated Psk1 and the cohesin core subunits Rad21, Psm1 and Psm3. The acetylated form of Psm3 was detected with anti Psm3-K106ac antibodies.



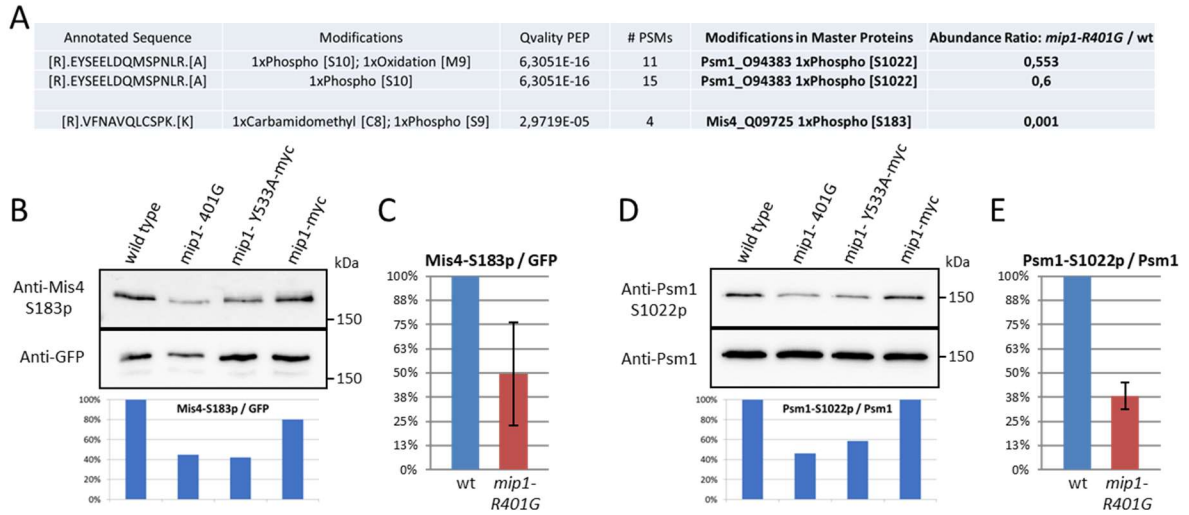
**Figure 3. Increased cohesin binding to Cohesin Associated Regions in the raptor mutant *mip1-R401G*.** (A) Chromosomal sites examined were Cohesin Associated Regions along the arms (438, 1806, 2898 and 3323) and centromere of chromosome 2, the non-transcribed spacer region (NTS) of the rDNA gene cluster on chromosome 3 and the chromosome 1 right telomere. Within the centromere, the central core (cc2) which is the site of kinetochore assembly, the imr and dg repeats that flank the central core on either side and at tRNA rich domains that delineate the centromere. (B) The *mip1-R401G* mutation restores cohesin binding in *mis4-G1487D* cells. Cohesin binding to chromatin was monitored by Rad21-FLAG ChIP in G1 cells (*cdc10-129* arrest) at 36.5°C. Error bars = SD from 4 ChIPs, except for *mis4-G1487D* (3 ChIPs). \*\*\* $P \leq 0.001$ , \*\* $P \leq 0.01$ , \* $P \leq 0.05$  by two-tailed, unpaired t-test with 95% confidence interval. (C-D) The *mip1-R401G* mutation increased Mis4 and Rad21 binding in *mis4<sup>+</sup>* cells. ChIP assays were made with *cdc10-129* arrested cells. (C) Mean and SD were calculated from 2 independent experiments and 4 technical replicates per experiment. (D) Mean and SD were calculated from 4 independent experiments and 4 technical replicates per experiment. \*\*\* $P \leq 0.001$ , \*\* $P \leq 0.01$ , \* $P \leq 0.05$ , by two-tailed, one sample t-test with 95% confidence interval.



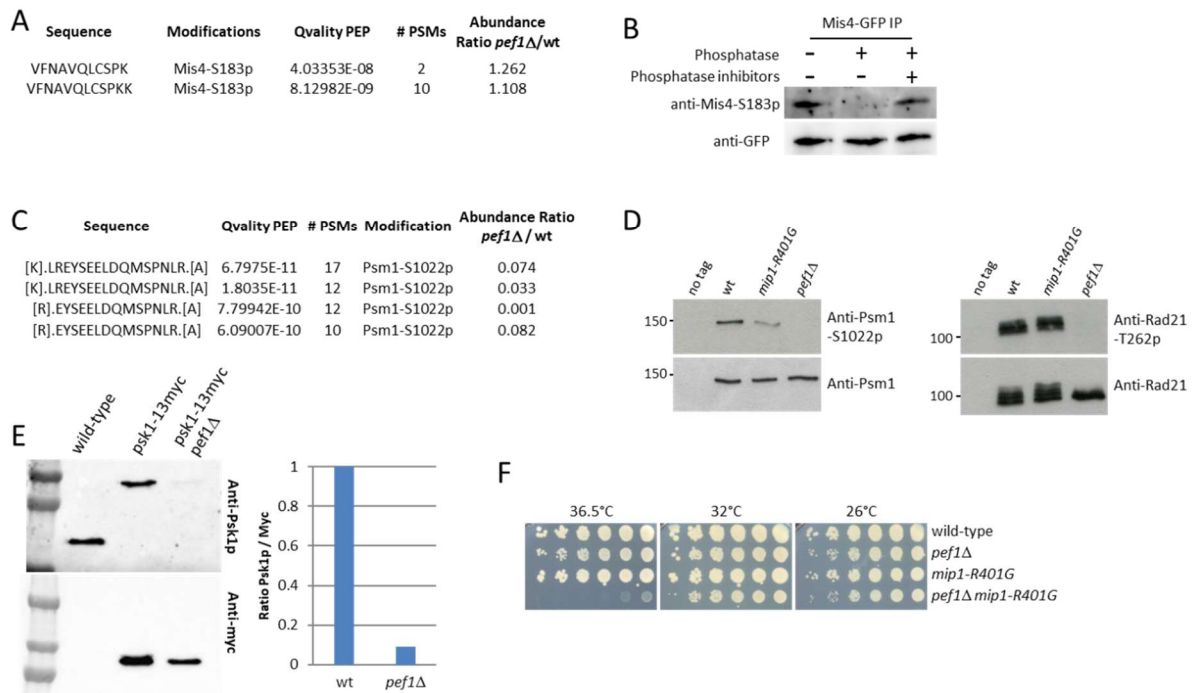
**Fig.3 supplement 1. (A) The *mip1-R401G* mutation increases cohesin binding to Cohesin Associated Regions in *pph3Δ psm3<sup>K105N-K106N</sup>* cells.** Exponentially growing cells at 25°C were shifted to 36°C for one doubling of the cell population. Cohesin binding to chromatin was monitored by ChIP using anti-Rad21 antibodies. Mean and SD from 6 ChIPs. **(B) Effect of Rapamycin treatment on cohesin binding.** Exponentially growing cells at 25°C were shifted to 36°C for one doubling of the cell population. Rapamycin (200ng/ml) or solvent alone (dmsso) was added at the time of the temperature shift. Cohesin binding to chromatin was monitored by ChIP using anti-Rad21 antibodies. Mean and SD from 4 ChIPs. \*\*\* $P \leq 0.001$ , \*\* $P \leq 0.01$ , \* $P \leq 0.05$ , by two-tailed, one sample t-test with 95% confidence interval.



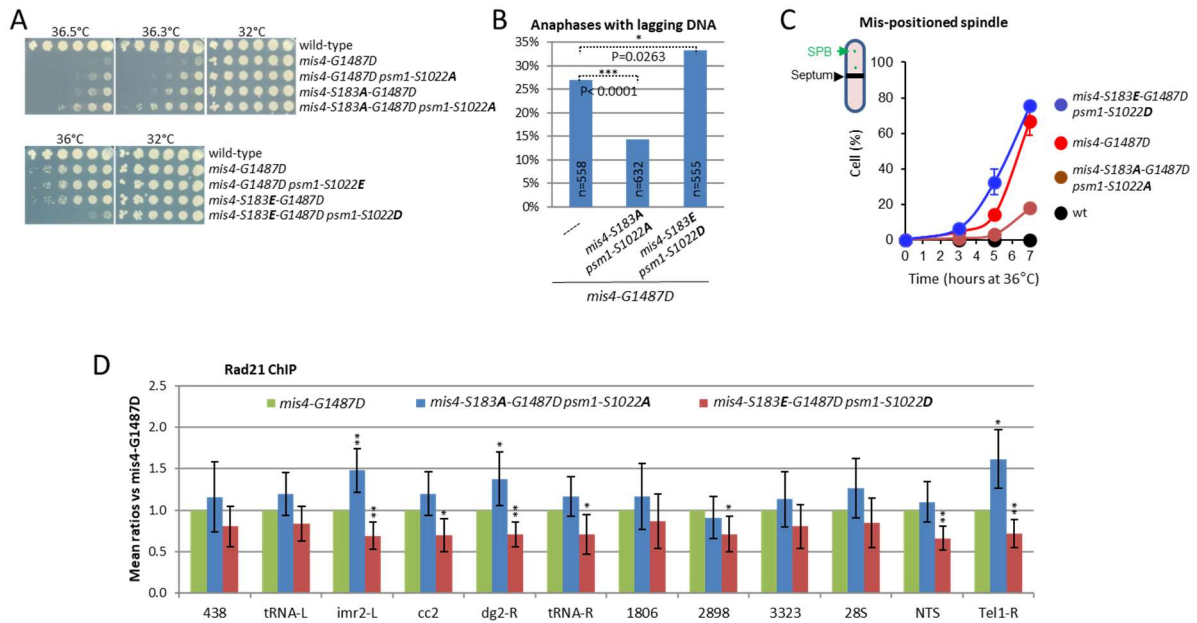
**Figure 4. TORC1 components co-purify with Rad21 and Mis4. (A)** Affinity purifications (triplicate samples) were made from G1-arrested cells (*cdc10-129* arrest) and proteins analysed by label-free mass spectrometry. **(B-C)** Reciprocal co-immunoprecipitation experiments. Western blots were probed with the indicated antibodies. **(B)** Rad21-PK IP (*cdc10-129* arrested cells). **(C)** Tor2-FLAG IP (cycling cells).



**Figure 5. Reduced phosphorylation of Mis4-S183 and Psm1-S1022 in the *mip1* mutants.** (A) Mis4-GFP and Cohesin (Rad21-PK) were affinity-purified from G1-arrested cells (*cdc10-129* arrest). Triplicate samples were analyzed by label-free mass spectrometry. Mis4-S183 and Psm1-S1022 phosphorylation levels were reduced in the *mip1-R401G* background. (B) Mis4-GFP immunoprecipitations made from cycling cells. Western-blot was probed with anti-Mis4-S183P and anti-GFP antibodies. (C) Mean ratio +/- SD from three Mis4-GFP immunoprecipitations (*cdc10* arrested cells). (D) Rad21-FLAG immunoprecipitations made from G1 cells (*cdc10-129* arrest). Western blot was probed with anti-Psm1-S1022P and anti-Psm1 antibodies. (E) Mean ratio +/- SD from three experiments (*cdc10-129* arrested cells).

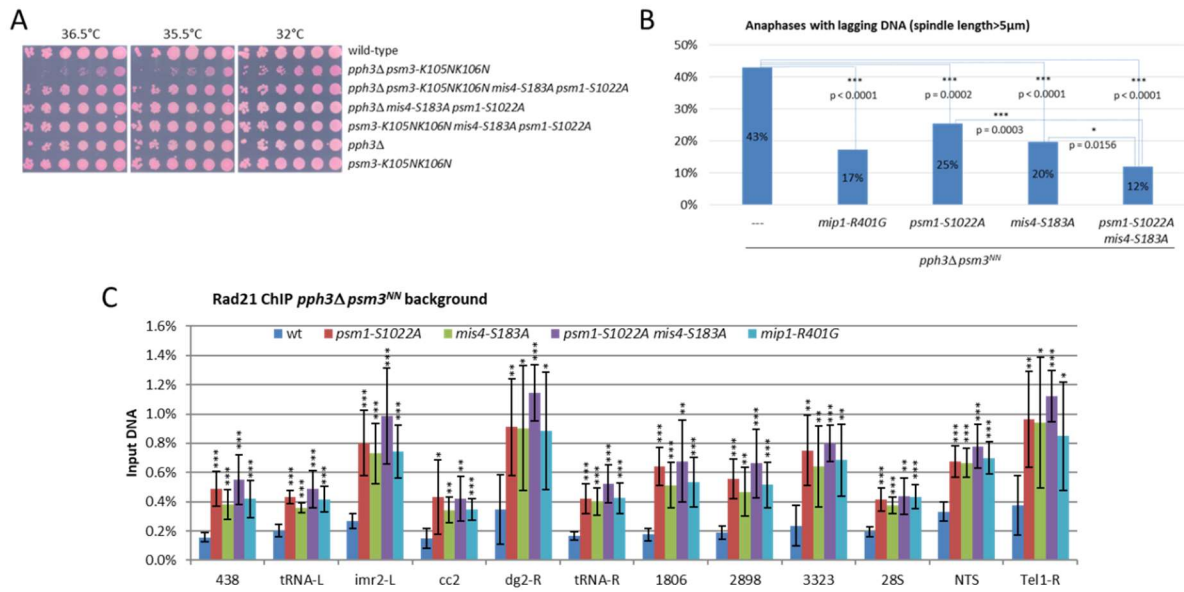


**Fig.5 supplement 1. (A) The phosphorylation of Mis4-S183 is not dependent on Pef1.** Mis4-GFP was affinity-purified from *pef1<sup>+</sup>* and *pef1Δ* cells (*cdc10-129* arrest) and triplicate samples analyzed by label-free mass spectrometry. **(B) Phosphatase assay showing the specificity of anti-Mis4-S183p antibodies.** Mis4-GFP was affinity-purified from *cdc10*-arrested cells. Phosphatase treatment was performed in the presence or absence of phosphatase inhibitors and proteins analyzed by western blotting with the indicated antibodies. **(C-D) The phosphorylation of Psm1-S1022 is dependent on Pef1.** **(C)** Rad21-PK was affinity-purified (triplicate samples) from *pef1<sup>+</sup>* and *pef1Δ* cells (*cdc10-129* arrest) and proteins were analyzed by label-free mass spectrometry **(D)** Rad21-FLAG was affinity-purified from *pef1<sup>+</sup>* and *pef1Δ* cells (*cdc10-129* arrest) and proteins analyzed by western blotting using the indicated antibodies. **(E) Psk1 phosphorylation is reduced in *pef1Δ*.** TCA extracts from cycling cells were probed by western blotting with the indicated antibodies. **(F) Cell growth assay showing the negative genetic interaction between *pef1Δ* and *mip1-R401G*.**

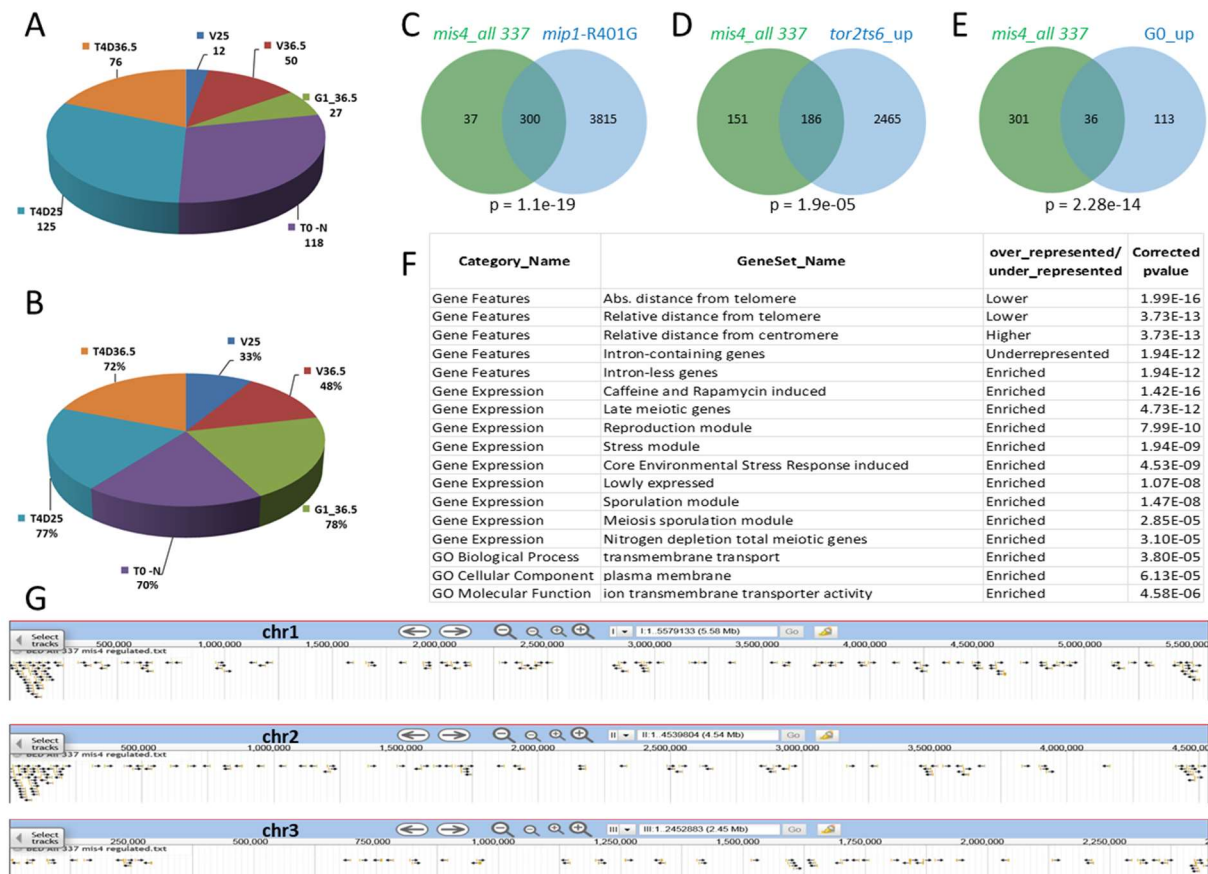


**Figure 6. Mimicking the non phosphorylated state of Psm1-S1022 and Mis4-S183 alleviates *mis4-G1487D* phenotypes while phospho-mimetics have the opposite effect. (A)** Cell growth assays. **(B)** Exponentially growing cells at 25°C were shifted at 36°C for 3.5hrs (one doubling). DNA was stained with DAPI and tubulin detected by indirect immunofluorescence. Lagging DNA was defined as DAPI-stained material on the anaphase spindle (length>5µm). (\*) Two-sided Fisher's exact test. **(C)** Anaphase cells with displaced spindle. Cells were cultured at 36°C, fixed and stained with Calcofluor to visualize septa. **(D)** Rad21 ChIP. Exponentially growing cells at 25°C were shifted at 36°C for 3.5hrs (one doubling). Mean and SD from 6 ratios and 2 independent experiments. \*\* $P \leq 0.01$ , \* $P \leq 0.05$ , by two-tailed, one sample t-test with 95% confidence interval.

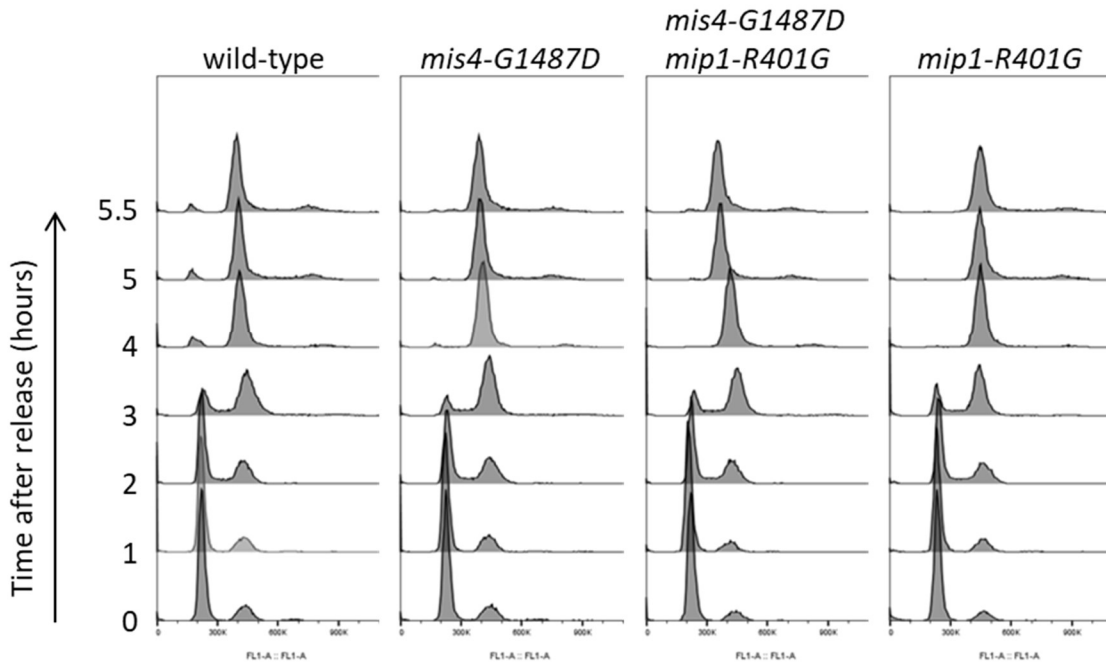




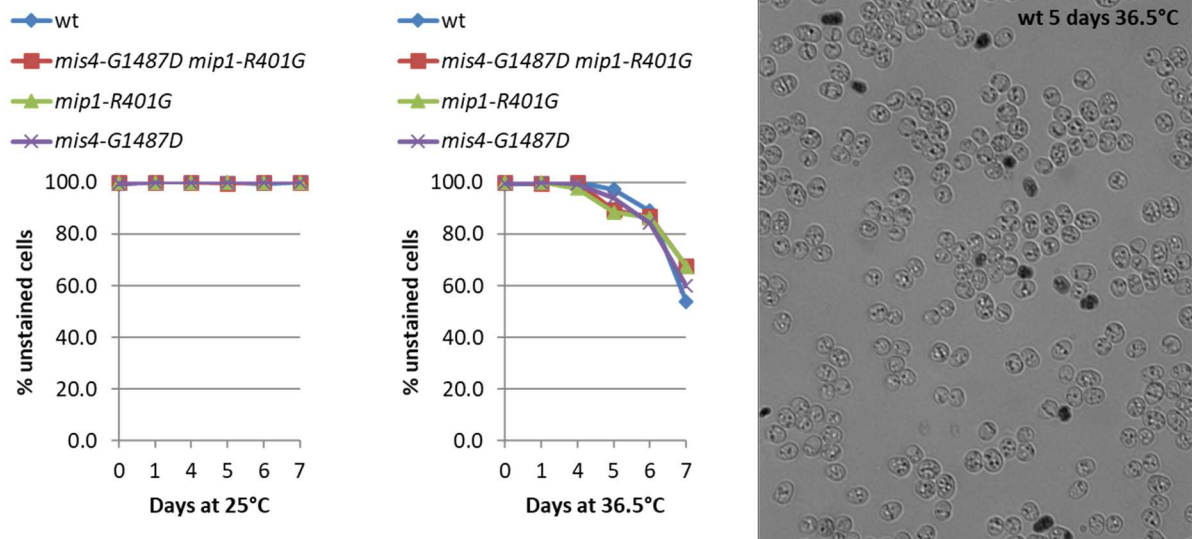
**Fig.6 supplement 1. Mimicking the non phosphorylated state of Psm1-S1022 and Mis4-S183 suppressed *pph3Δ psm3-K105K106N* phenotypes.** (A) Cell growth assay showing the suppression of the Ts phenotype. (B) Exponentially growing cells at 25°C were shifted at 36°C for one doubling. DNA was stained with DAPI and tubulin detected by indirect immunofluorescence. Lagging DNA was defined as DAPI-stained material on the anaphase spindle (length > 5μm). P-values from two-sided Fisher's exact tests. (C) Exponentially growing cells at 25°C were shifted to 36°C for one doubling of the cell population. Cohesin binding to chromatin was monitored by ChIP using anti-Rad21 antibodies. Mean and SD from 6 ChIPs. \*\*\*P ≤ 0.001, \*\*P ≤ 0.01, \*P ≤ 0.05, by two-tailed, one sample t-test with 95% confidence interval.



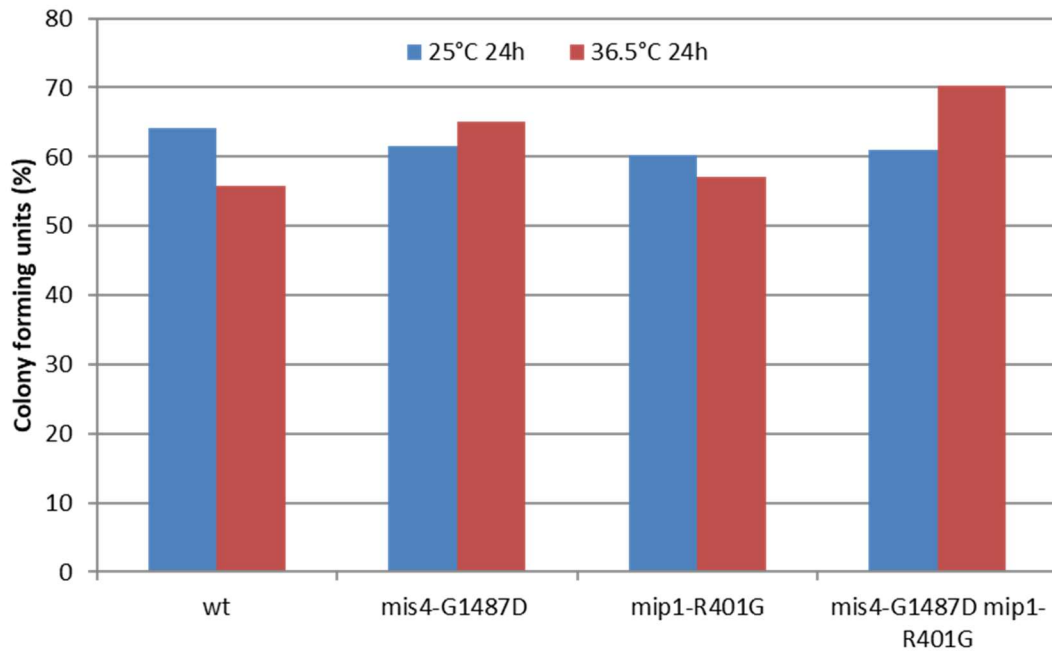
**Figure 7. *mis4-G1487D* affects gene response to environmental changes.** RNA-sequencing (biological triplicates) was performed on cells growing in rich medium at 25°C (V25), after one cell doubling at 36.5°C (V36.5) and on G1-arrested cells (*cdc10-129*) at 36.5°C (G1\_36.5). For nitrogen starvation, cells grown in EMM2 were deprived from nitrogen for 24H at 25°C (TO-N). One half of the culture was shifted to 36.5°C for 4 days (T4D36.5) while the other half was left at 25°C for 4 days (T4D25). **(A)** Number of genes misregulated in *mis4-G1487D* versus wild-type for each condition. **(B)** Proportion of misregulated genes that are specific to each experimental condition. **(C)** Overlap between genes misregulated in *mis4-G1487D* and *mip1-R401G* versus wild-type in all experimental conditions. **(D-E)** Overlap with genes upregulated in *tor2ts6* (Wei et al., 2021) and genes upregulated during G0 (Zahedi et al., 2023) **(F)** Gene features and GO-term analysis for the 337 *mis4* regulated genes. **(G)** Browser view of *mis4*-regulated genes showing the distribution bias towards the ends of chromosomes 1 and 2.



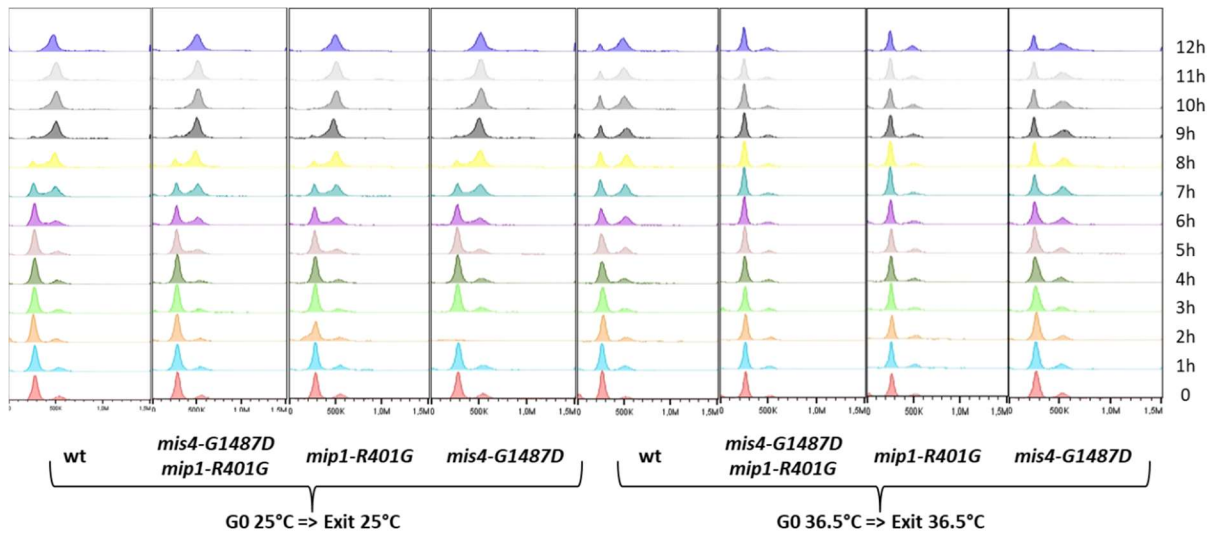
**Fig.7 supplement 1. Response to nitrogen starvation and refeeding.** Exponentially growing cells in EMM2 medium at 25°C were washed three times in EMM2-N and incubated in EMM2-N for 16 hours at 25°C (time 0). DNA content analysis showed that all strains arrested with a 1C DNA content. G1-arrested cells were then released into the cell cycle in rich medium at 36.5°C. DNA content analysis showed that all strains progressed through S phase with similar kinetics.



**Fig.7 supplement 2. Cell survival during quiescence.** Exponentially growing cells in EMM2 medium at 25°C were washed three times in EMM2-N and incubated in EMM2-N for 24 hours at 25°C (time 0). Half of the culture was transferred to 36.5°C while the other half remained at 25°C. Cell viability was monitored over time by methylene blue staining (sample image on the right).



**Fig.7 supplement 3. Quiescence exit at 25°C.** Exponentially growing cells in EMM2 medium at 25°C were washed three times in EMM2-N and incubated in EMM2-N for 24 hours at 25°C. Half of the culture was transferred to 36.5°C while the other half remained at 25°C. After 24 hours, cells were plated on YES medium (25°C) to assess cell's ability to exit G0 and form colonies.



**Fig.7 supplement 4. Quiescence exit at 36.5°C.** Exponentially growing cells in EMM2 medium at 25°C were washed three times in EMM2-N and incubated in EMM2-N for 24 hours at 25°C. Half of the culture was transferred to 36.5°C while the other half remained at 25°C. After 3 days, nitrogen (NH<sub>4</sub>Cl 5g/l) was added (time 0). Exit from G<sub>0</sub> was monitored by flow cytometry analysis of DNA content. At 25°C (G<sub>0</sub> 25°C => exit 25°C) the onset of DNA replication occurred ~7hrs after nitrogen addition for all strains. At 36.5°C (G<sub>0</sub> 36.5°C => exit 36.5°C) DNA replication was slow or asynchronous even for the wild-type. The *mis4* mutant behaved mostly as wild-type even though a larger fraction of cells did not replicate 12 hours after nitrogen addition. The *mip1* mutants did not show any sign of DNA replication during the duration of the experiment.

**Supplementary Table 1. Strains used in this study**

strain #	genotype	Figure
<b>2</b>	<i>h-</i>	1A
<b>2729</b>	<i>h- mis4-G1487D</i>	1A
<b>6849</b>	<i>h- mis4-G1487D pef1Δ::natR</i>	1A
<b>12208</b>	<i>h- mis4-G1487D mip1-Q167P</i>	1A
<b>11562</b>	<i>h- mis4-G1487D mip1-Y169C</i>	1A
<b>10874</b>	<i>h- mis4-G1487D mip1-R401G</i>	1A
<b>12226</b>	<i>h- mis4-G1487D mip1-T1007R</i>	1A
<b>11558</b>	<i>h- mis4-G1487D mip1-D1018Y</i>	1A
<b>11200</b>	<i>h- mis4-G1487D tor2-H2142Q</i>	1A
<b>11478</b>	<i>h- psk1-13myc-hygR</i>	1C
<b>12191</b>	<i>h- mip1-D1018Y psk1-13myc-kanR</i>	1C
<b>12183</b>	<i>h- mip1-Y169C psk1-13myc-HygR</i>	1C
<b>12252</b>	<i>h- mip1-Q167P psk1-13myc-HygR</i>	1C
<b>12248</b>	<i>h- mip1-T1007R psk1-13myc-hygR</i>	1C
<b>11448</b>	<i>h- mip1R401G psk1-13myc-hygR</i>	1C
<b>2</b>	<i>h-</i>	1C
<b>2729</b>	<i>h- mis4-G1487D</i>	1C
<b>10874</b>	<i>h- mis4-G1487D mip1-R401G</i>	1C
<b>11584</b>	<i>h- mis4-G1487D mip1-Y533A-13myc-kanR</i>	1C
<b>11636</b>	<i>h- mis4-G1487D mip1-13myc-kanR</i>	1C
<b>2</b>	<i>h-</i>	1D
<b>2729</b>	<i>h- mis4-G1487D</i>	1D
<b>11002</b>	<i>h- mip1-R401G</i>	1D
<b>10874</b>	<i>h- mis4-G1487D mip1-R401G</i>	1D
<b>5913</b>	<i>h- eso1-H17</i>	1D
<b>12318</b>	<i>h- rad21-K1</i>	1D
<b>2</b>	<i>h-</i>	1E
<b>2729</b>	<i>h- mis4-G1487D</i>	1E
<b>10874</b>	<i>h- mis4-G1487D mip1-R401G</i>	1E
<b>11420</b>	<i>h- mis4-G1487D tsc2Δ::kanR</i>	1E
<b>12115</b>	<i>h- mis4-G1487D tsc2Δ::kanR mip1-R401G</i>	1E
<b>2</b>	<i>h-</i>	1E
<b>2729</b>	<i>h- mis4-G1487D</i>	1E
<b>12175</b>	<i>h- mis4-G1487D grt1Δ::kanR</i>	1E
<b>12179</b>	<i>h- mis4-G1487D grt1-Q61L-kanR</i>	1E
<b>12167</b>	<i>h- mis4-G1487D grt1-S16N-kanR</i>	1E
<b>12177</b>	<i>h- mis4-G1487D iml1Δ::kanR</i>	1E
<b>12171</b>	<i>h- mis4-G1487D gcn2Δ::hygR</i>	1E

<b>2</b>	<i>h-</i>	1-sup1A
<b>2796</b>	<i>h-leu1-32 mis4-G1487D rec8-GFP-kanR</i>	1-sup1A
<b>2899</b>	<i>h-leu1-32 mis4-G1487D rec8-GFP-kanR pef1-N146S</i>	1-sup1A
<b>2912</b>	<i>h-leu1-32 mis4-G1487D rec8-GFP-kanR mip1-R401G</i>	1-sup1A
<b>2852</b>	<i>h-leu1-32 mis4-G1487D rec8-GFP-kanR caa1-S252L</i>	1-sup1A
<b>2923</b>	<i>h-leu1-32 mis4-G1487D rec8-GFP-kanR tor2-H2142Q</i>	1-sup1A
<hr/>		
<b>2</b>	<i>h-</i>	1-sup1B
<b>2729</b>	<i>h-mis4-G1487D</i>	1-sup1B
<b>12650</b>	<i>h-ura4-D18 mis4-G1487D tor1Δ::ura4+</i>	1-sup1B
<b>12667</b>	<i>h-ura4-D18 tor1Δ::ura4+ mip1-R401G</i>	1-sup1B
<b>10874</b>	<i>h-mis4-G1487D mip1-R401G</i>	1-sup1B
<b>2729</b>	<i>h-mis4-G1487D</i>	1-sup1B
<hr/>		
<b>2</b>	<i>h-</i>	2A-B
<b>2729</b>	<i>h-mis4-G1487D</i>	2A-B
<b>10874</b>	<i>h-mis4-G1487D mip1-R401G</i>	2A-B
<hr/>		
<b>12365</b>	<i>h-cdc11-GFP-kanR ndc80-GFP-kanR mis6-RFP-hygR</i>	2C-D
<b>12363</b>	<i>h-cdc11-GFP-kanR ndc80-GFP-kanR mis6-RFP-hygR mis4-G1487D</i>	2C-D
<b>12393</b>	<i>h-cdc11-GFP-kanR ndc80-GFP-kanR mis6-RFP-hygR mis4-G1487D mip1-R401G</i>	2C-D
<hr/>		
<b>2729</b>	<i>h-mis4-G1487D</i>	2E
<hr/>		
<b>2</b>	<i>h-</i>	2F
<b>2729</b>	<i>h-mis4-G1487D</i>	2F
<hr/>		
<b>2729</b>	<i>h-mis4-G1487D</i>	2F
<b>12167</b>	<i>h-mis4-G1487D gtr1-S16N-kanR</i>	2F
<b>12179</b>	<i>h-mis4-G1487D gtr1-Q61L-kanR</i>	2F
<hr/>		
<b>11326</b>	<i>h-pph3Δ::hygR psm3K105NK106N</i>	2G
<b>11395</b>	<i>h-pph3Δ::kanR psm3K105NK106N mip1-R401G</i>	2G
<hr/>		
<b>12363</b>	<i>h-cdc11-GFP-kanR ndc80-GFP-kanR mis6-RFP-hygR mis4-G1487D</i>	2-sup1
<b>12393</b>	<i>h-cdc11-GFP-kanR ndc80-GFP-kanR mis6-RFP-hygR mis4-G1487D mip1-R401G</i>	2-sup1
<hr/>		
<b>12363</b>	<i>h-cdc11-GFP-kanR ndc80-GFP-kanR mis6-RFP-hygR mis4-G1487D</i>	2-sup2
<b>12393</b>	<i>h-cdc11-GFP-kanR ndc80-GFP-kanR mis6-RFP-hygR mis4-G1487D mip1-R401G</i>	2-sup2
<hr/>		
<b>2729</b>	<i>h-mis4-G1487D</i>	2-sup3
<hr/>		
<b>12634</b>	<i>h90 ade6Δ::LacO his7+-Lacl</i>	2-sup4A
<b>12635</b>	<i>h90 ade6Δ::LacO his7+-Lacl mis4-G1487D</i>	2-sup4A



12648	<i>h90 ade6Δ::LacO his7+-Lacl mip1-R401G</i>	2-sup4A
12653	<i>h90 ade6Δ::LacO his7+-Lacl mis4-G1487D mip1-R401G</i>	2-sup4A
<hr/>		
2	<i>h-</i>	2-sup4B
2729	<i>h- mis4-G1487D</i>	2-sup4B
10874	<i>h- mis4-G1487D mip1-R401G</i>	2-sup4B
11002	<i>h- mip1-R401G</i>	2-sup4B
<hr/>		
2	<i>h-</i>	2-sup4C
2729	<i>h- mis4-G1487D</i>	2-sup4C
<hr/>		
3985	<i>h- cdc10-129</i>	3B
6392	<i>h+ cdc10-129 rad21-FLAG3-KanR</i>	3B
8148	<i>h+ cdc10-129 rad21-FLAG3 mis4-G1487D</i>	3B
11287	<i>h+ cdc10-129 rad21-FLAG3-KanR mis4-G1487D mip1-R401G</i>	3B
<hr/>		
3985	<i>h- cdc10-129</i>	3C
9748	<i>h- cdc10-129 leu1-32 mis4-EGFP-LEU2</i>	3C
11357	<i>h- cdc10-129 leu1-32 mis4-EGFP-LEU2 mip1-R401G</i>	3C
<hr/>		
3985	<i>h- cdc10-129</i>	3D
6392	<i>h+ cdc10-129 rad21-FLAG3-KanR</i>	3D
11283	<i>h+ cdc10-129 rad21-FLAG3-KanR mip1-R401G</i>	3D
<hr/>		
11326	<i>h- psm3-K105NK106N pph3Δ::hygR</i>	3-sup1A
11395	<i>h- psm3-K105NK106N pph3Δ::hygR mip1-R401G</i>	3-sup1A
<hr/>		
2	<i>h-</i>	3-sup1B
2729	<i>h- mis4-G1487D</i>	3-sup1B
<hr/>		
3985	<i>h- cdc10-129</i>	4A
9748	<i>h- cdc10-129 leu1-32 mis4-EGFP-LEU2</i>	4A
3984	<i>h- cdc10-129 rad21-9PK-kanR</i>	4A
<hr/>		
3985	<i>h- cdc10-129</i>	4B
3984	<i>h- cdc10-129 rad21-9PK-kanR</i>	4B
10976	<i>h- cdc10-129 FLAG-tor2-kanR</i>	4B
10984	<i>h- cdc10-129 FLAG-tor2-kanR rad21-9PK-kanR</i>	4B
<hr/>		
2	<i>h-</i>	4C
10918	<i>h- FLAG-tor2-kanR mip1-GFP-hygR</i>	4C
10940	<i>h- FLAG-tor2-kanR</i>	4C
<hr/>		
3985	<i>h- cdc10-129</i>	5A
3984	<i>h- cdc10-129 rad21-9PK-kanR</i>	5A
11029	<i>h- cdc10-129 rad21-9PK-kanR mip1-R401G</i>	5A

3985	<i>h- cdc10-129</i>	5A
9748	<i>h- cdc10-129 leu1-32 mis4-EGFP-LEU2</i>	5A
11357	<i>h- cdc10-129 leu1-32 mis4-EGFP-LEU2 mip1-R401G</i>	5A
12287	<i>h- mis4-GFP-hygR</i>	5B
12289	<i>h- mis4-GFP-hygR mip1-R401G</i>	5B
12220	<i>h- mis4-GFP-hygR mip1-Y533A-13myc</i>	5B
12222	<i>h- mis4-GFP-hygR mip1-13myc</i>	5B
9748	<i>h- cdc10-129 leu1-32 mis4-EGFP-LEU2</i>	5C
11357	<i>h- cdc10-129 leu1-32 mis4-EGFP-LEU2 mip1-R401G</i>	5C
3985	<i>h- cdc10-129</i>	5D-E
6393	<i>h-cdc10-129 rad21FLAG3-kanR</i>	5D-E
11284	<i>h- cdc10-129 rad21FLAG3-kanR mip1-R401G</i>	5D-E
3985	<i>h- cdc10-129</i>	5-sup1A
9748	<i>h- cdc10-129 leu1-32 mis4-EGFP-LEU2</i>	5-sup1A
10289	<i>h- cdc10-129 leu1-32 mis4-EGFP-LEU2 pef1Δ::hygR</i>	5-sup1A
9748	<i>h- cdc10-129 leu1-32 mis4-EGFP-LEU2</i>	5-sup1B
11357	<i>h- cdc10-129 leu1-32 mis4-EGFP-LEU2 mip1-R401G</i>	5-sup1B
3985	<i>h- cdc10-129</i>	5-sup1C
3984	<i>h- cdc10-129 rad21-9PK-kanR</i>	5-sup1C
6228	<i>h- cdc10-129 ura4-D18 pef1Δ::ura4+ rad21-9PK-kanR</i>	5-sup1C
3985	<i>h- cdc10-129</i>	5-sup1D
6393	<i>h-cdc10-129 rad21FLAG3-kanR</i>	5-sup1D
11284	<i>h- cdc10-129 rad21FLAG3-kanR mip1-R401G</i>	5-sup1D
6360	<i>h- cdc10-129 ura4-D18 rad21FLAG3-kanR pef1Δ::ura4+</i>	5-sup1D
2	<i>h-</i>	5-sup1E
11478	<i>h- psk1-13myc-hygR</i>	5-sup1E
11744	<i>h+ pef1Δ::natR psk1-13myc-hygR</i>	5-sup1E
2	<i>h-</i>	5-sup1F
10369	<i>h- pef1Δ::natR</i>	5-sup1F
11002	<i>h- mip1-R401G</i>	5-sup1F
11456	<i>h- pef1Δ::natR mip1-R401G</i>	5-sup1F
2	<i>h-</i>	6A
2729	<i>h- mis4-G1487D</i>	6A
11164	<i>h- mis4-G1487D psm1-S1022A</i>	6A
11897	<i>h- mis4-G1487D-S183A</i>	6A

<b>11985</b>	<i>h- mis4-G1487D-S183A psm1-S1022A</i>	6A
<b>2</b>	<i>h-</i>	6A
<b>2729</b>	<i>h- mis4-367</i>	6A
<b>11076</b>	<i>h- mis4-367 psm1-S1022E</i>	6A
<b>11857</b>	<i>h- mis4-367-S183E</i>	6A
<b>11987</b>	<i>h- mis4-367-S183E psm1-S1022D</i>	6A
<b>2729</b>	<i>h- mis4-367</i>	6B
<b>11985</b>	<i>h- mis4-367-S183A psm1-S1022A</i>	6B
<b>11987</b>	<i>h- mis4-367-S183E psm1-S1022D</i>	6B
<b>12363</b>	<i>h- cdc11-GFP-kanR ndc80-GFP-kanR mis6-RFP-hygR mis4-367</i>	6C
<b>12365</b>	<i>h- cdc11-GFP-kanR ndc80-GFP-kanR mis6-RFP-hygR</i>	6C
<b>12626</b>	<i>h- cdc11-GFP-kanR ndc80-GFP-kanR mis6-RFP-hygR mis4-367-S183A psm1-S1022A</i>	6C
<b>12644</b>	<i>h- cdc11-GFP-kanR ndc80-GFP-kanR mis6-RFP-hygR mis4-367-S183E psm1-S1022D</i>	6C
<b>2729</b>	<i>h- mis4-367</i>	6D
<b>11985</b>	<i>h- mis4-367-S183A psm1-S1022A</i>	6D
<b>11987</b>	<i>h- mis4-367-S183E psm1-S1022D</i>	6D
<b>2</b>	<i>h-</i>	6-sup1A
<b>11326</b>	<i>h- pph3::hygR psm3-K105NK106N</i>	6-sup1A
<b>12621</b>	<i>h- pph3::hygR psm3-K105NK106N mis4-S183A psm1-S1022A</i>	6-sup1A
<b>12617</b>	<i>h- pph3::hygR mis4-S183A psm1-S1022A</i>	6-sup1A
<b>12613</b>	<i>h- psm3-K105NK106N mis4-S183A psm1-S1022A</i>	6-sup1A
<b>6095</b>	<i>h+ pph3::hygR</i>	6-sup1A
<b>4573</b>	<i>h- psm3-K105NK106N</i>	6-sup1A
<b>11326</b>	<i>h- pph3::hygR psm3-K105NK106N</i>	6-sup1A
<b>12621</b>	<i>h- pph3::hygR psm3-K105NK106N mis4-S183A psm1-S1022A</i>	6-sup1A
<b>12617</b>	<i>h- pph3::hygR mis4-S183A psm1-S1022A</i>	6-sup1A
<b>12613</b>	<i>h- psm3-K105NK106N mis4-S183A psm1-S1022A</i>	6-sup1A
<b>11326</b>	<i>h- pph3::hygR psm3-K105NK106N</i>	6-sup1BC
<b>11395</b>	<i>h- pph3::kanR psm3K105NK106N mip1-R401G</i>	6-sup1BC
<b>11519</b>	<i>h- pph3::hygR psm3-K105NK106N psm1-S1022A</i>	6-sup1BC
<b>11918</b>	<i>h- pph3::kanR psm3K105NK106N mis4-S183A</i>	6-sup1BC
<b>12621</b>	<i>h- pph3::hygR psm3-K105NK106N mis4-S183A psm1-S1022A</i>	6-sup1BC
<b>11589</b>	<i>mat1-M-smt0</i>	7

<b>11654</b>	<i>mat1-M-smt0 mis4-367</i>	7
<b>11628</b>	<i>mat1-M-smt0 mip1-R401G</i>	7
<b>11589</b>	<i>mat1-M-smt0</i>	7-sup1-4
<b>11654</b>	<i>mat1-M-smt0 mis4-367</i>	7-sup1-4
<b>11628</b>	<i>mat1-M-smt0 mip1-R401G</i>	7-sup1-4
<b>11626</b>	<i>mat1-M-smt0 mis4-367 mip1-R401G</i>	7-sup1-4



## II - Additional results

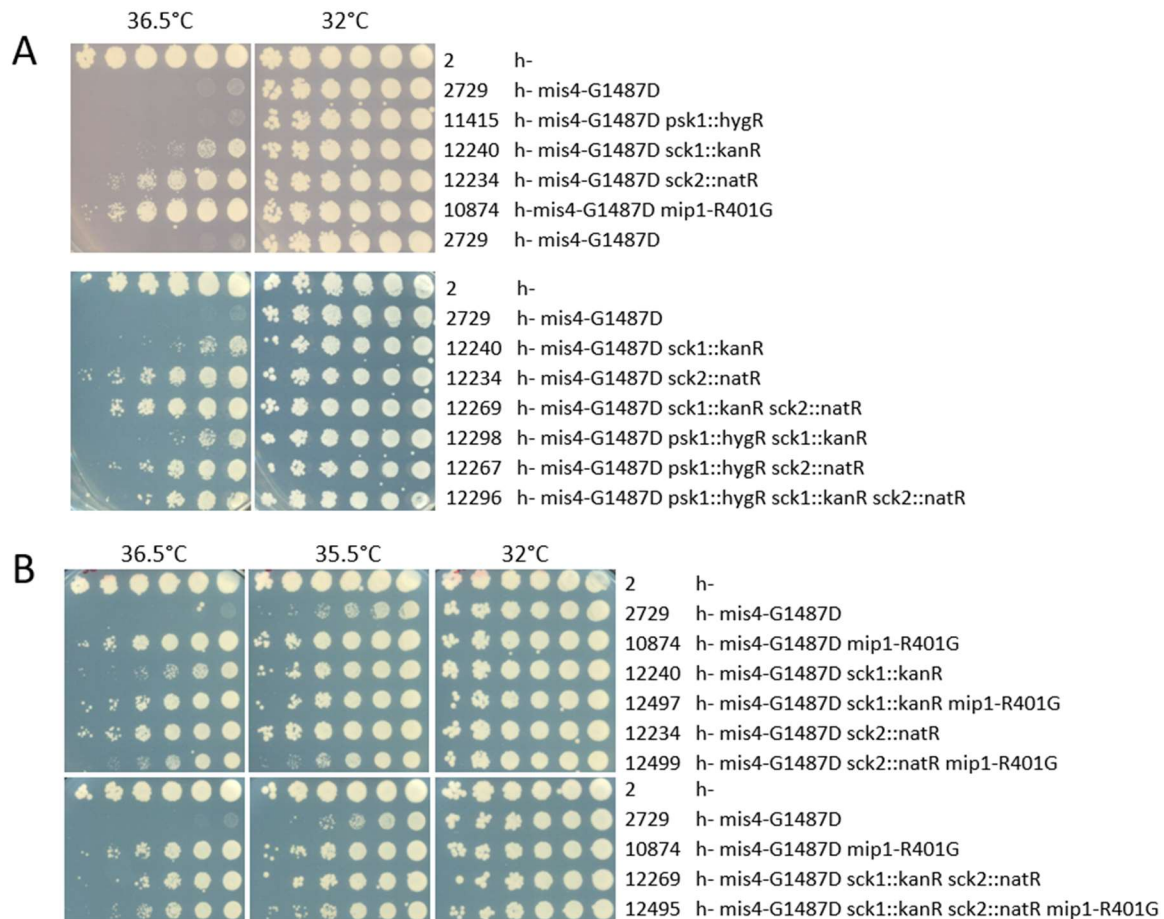
The data presented in the previous chapter argue that TORC1 activity impinges on cohesin through the phosphorylation of Psm1-S1022 and Mis4-S183. TORC1 could phosphorylate these residues directly but it is also possible that TORC1 regulates cohesin indirectly, *via* the activation of downstream kinases. In fission yeast there are three known AGC kinases phosphorylated and thereby presumably activated by TORC1: Psk1, Sck1 and Sck2 (Chica et al., 2016; Nakashima et al., 2012). The ribosomal S6 kinase Psk1 was introduced in the previous chapter. The targets of Sck1 and Sck2 are largely unknown in fission yeast. One characterized substrate is the heterotrimeric G protein alpha-2 subunit Gpa2. Phosphorylation by Sck1 inhibits its activity and negatively regulates glucose signalling (Mudge et al., 2014). Sck2 phosphorylates and inhibits the greatwall kinase Ppk18, involved in the negative regulation of G2/M transition of mitotic cell cycle (Chica et al., 2016). The closest homologs of Sck1-2 are SGK1-3 (Serum/glucocorticoid regulated kinase 1, 2 and 3) in human and SCH9 in budding yeast.

AGC kinase genes are dispensable for growth in fission yeast, either alone or in combination, which rendered possible a straightforward analysis. We looked at the suppression of *mis4-G1487D* phenotypes, as well as at cohesin binding at CARs and the phosphorylation levels of Psm1-S1022 and Mis4-S183. These data are still preliminary, and more experiments are required to draw any conclusions.

## **A - Deletion of *sck1* and *sck2* suppressed the temperature growth defect of *mis4-G1487D***

Deletion of the *psk1* gene was essentially neutral for *mis4-G1487D* (Figure 29.A) The deletion of *sck1* had a weak suppressor effect whereas *sck2* deletion suppressed the growth defect to a level similar to *mip1-R401G*. The *psk1* $\Delta$  *sck1* $\Delta$  and *psk1* $\Delta$  *sck2* $\Delta$  double mutants had a similar effect that the single *sck1* and *sck2* mutants on *mis4-G1487D* growth. The same was true for the *psk1* $\Delta$  *sck1* $\Delta$  *sck2* $\Delta$  triple mutant, having the phenotype of *sck2* $\Delta$ . The *psk1* deletion is therefore neutral towards the growth of *mis4-G1487D*. The double deletion of *sck1* and *sck2* had the same effect as the single deletion of *sck2*. This suggests that *sck2* is epistatic on *sck1*. However, since *sck2* $\Delta$  alone gave quite a high level of suppression it can be argued that an additive effect with *sck1* $\Delta$  may be not observable.

Next, we addressed the question of *mip1* epistasis on *sck1* and *sck2* (Figure 29.B) The *mip1-R401G* *sck1* $\Delta$  double mutant showed the same phenotype as *mip1-R401G*, suggesting that *mip1* is epistatic on *sck1*. Again, this should be taken with caution as *mip1-R401G* is an excellent suppressor of the thermosensitive growth defect of *mis4-G1487D*. The *mip1-R401G* mutation and *sck2* deletion had a negative genetic interaction with respect to *mis4-G1487D* suppression. TORC1 being essential, mutation of two genes in the pathway may have reduced its activity too drastically.

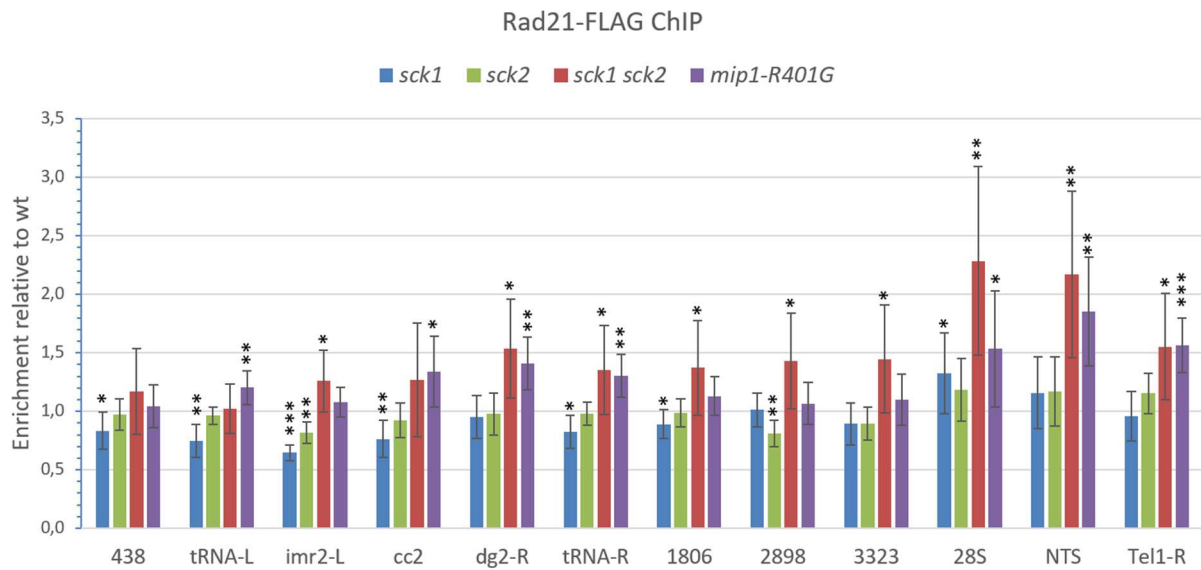


**Figure 29: The deletion of *sck1* or *sck2* genes rescued the temperature growth defect of *mis4-G1487D*.** (A) The deletion of *psk1* was neutral for *mis4-G1487D*. The deletion of *sck1* rescued weakly the growth at 36.5°C and deletion of *sck2* rescued to a similar level as *mip1-R401G*. The double mutant *sck1Δ sck2Δ*, rescued at the same level than *sck2Δ* and *psk1Δ* was neutral in combination with *sck1Δ sck2Δ*. (B) *mip1-R401* is epistastic on *sck1Δ*. The combination of *mip1-R401* with *sck2Δ* showed a reduced suppressor effect when compared with the effect of each single mutant.



## **B - Increased cohesin binding to CARs in the *sck1Δ sck2Δ* double mutant**

Using Rad21-FLAG ChIP-qPCR on G1-arrested cells (*cdc10-129*), we determined the relative enrichment of cohesin localised at CARs in the mutants strains versus wild-type, in a *mis4<sup>+</sup>* background (Figure 30). A small decrease was observed for *sck1Δ* at several CARs, such as 438, *tRNA-L*, *cc2*, *imr2-L* and 1806. The *sck2Δ* single mutant behaved quite similar to wild-type. A small reduction was observed at *imr2-L* and 2898. In contrast, the *sck1Δ sck2Δ* double mutant showed an increase relative to wild-type at almost all loci and at a level similar to *mip1-R401G*. This suggests a synthetic effect of the simultaneous deletion of *sck1* and *sck2* on cohesin association at CARs in *cdc10-129* arrested cells.



**Figure 30 : Increased cohesin binding to CARs in the *sck1Δ sck2Δ* double mutant.** Cohesin binding to chromatin was monitored by Rad21-FLAG ChIP in G1 cells (*cdc10-129* arrest). Mean ratios and SD were calculated from 2 independent experiments and 4 technical replicates per experiment. \*\*\* $P \leq 0.001$ , \*\* $P \leq 0.01$ , \* $P \leq 0.05$ , by two-tailed, one sample t-test with 95% confidence interval.

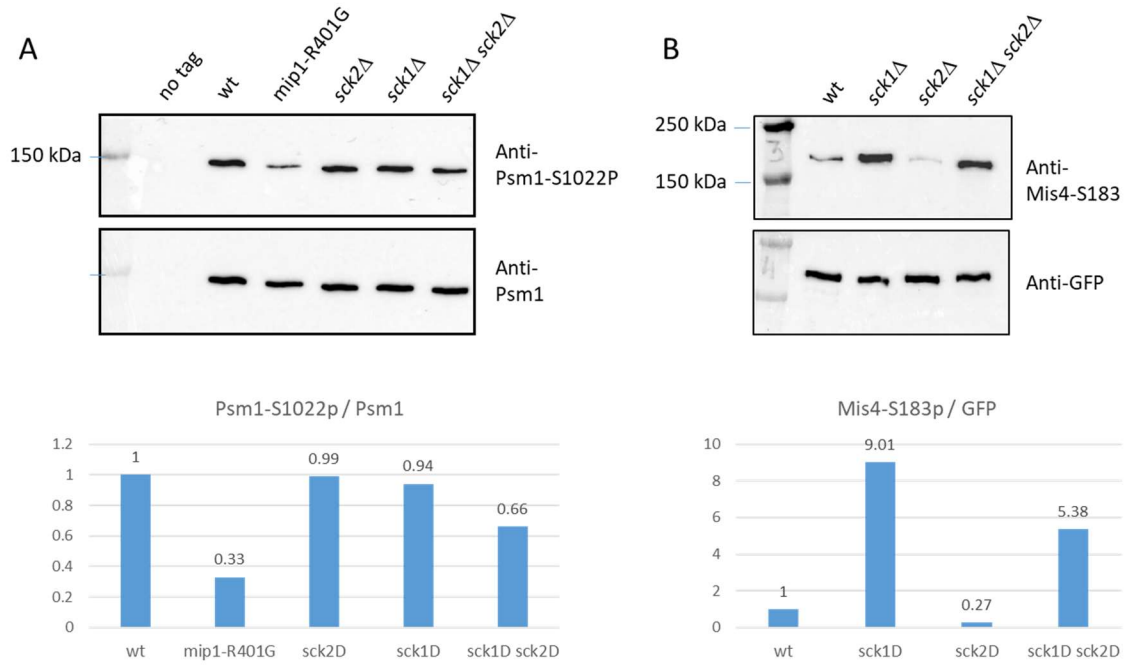
### **C - The phosphorylation level of Psm1-S1022 is decreased in the *sck1Δ sck2Δ* background**

In the previous chapter we presented evidence that the *mip1-R401G* mutation reduced the phosphorylation levels of Psm1-S1022 and Mis4-S183. Using the same assay, the phosphorylation level of Psm1-S1022 was similar to wild-type in the *sck1Δ* and *sck2Δ* single mutants (Figure 31). The *sck1Δ sck2Δ* double mutant showed a 33% decrease in phosphorylation. In this experiment, the *mip1-R401G* mutant showed a 66% decrease.

These results suggest that *sck1* and *sck2* would be redundant for the regulation of Psm1-S1022 phosphorylation. This experiment will have to be repeated several times to be able to draw any firm conclusions. As Psm1-S1022 phosphorylation was never abolished, these kinases may not directly phosphorylate Psm1. Even this suggestion should be taken with caution as several kinases may be able to phosphorylate Psm1.

### **D - The phosphorylation level of Mis4-S183 is increased in *sck1Δ* and decreased in *sck2Δ***

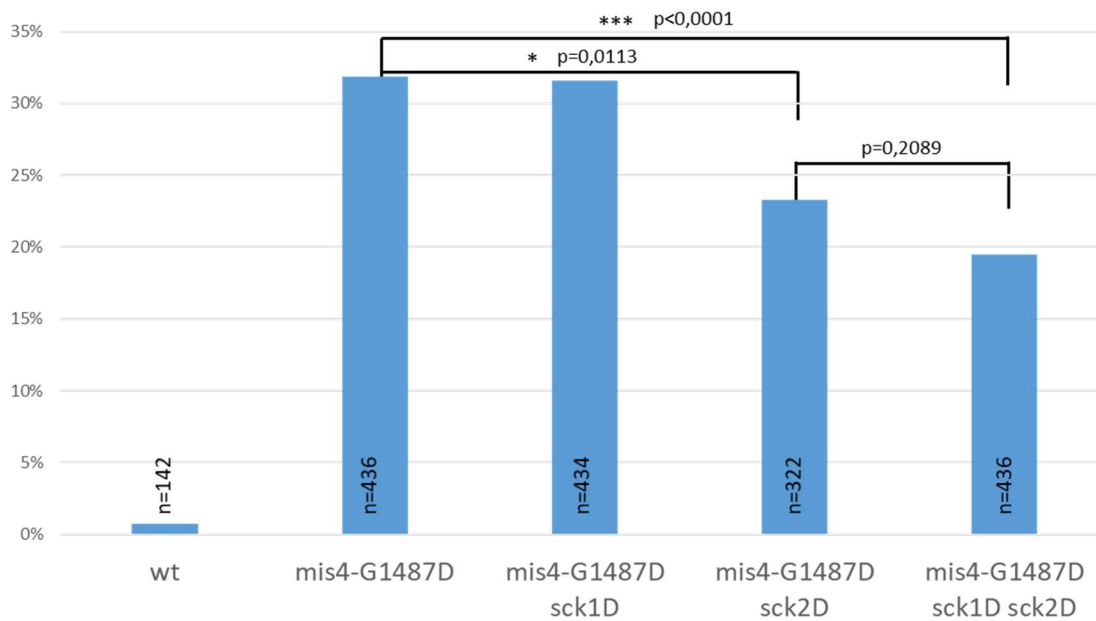
The phosphorylation level of Mis4-S183 was assayed as in the previous chapter. Mis4-S183p was up to 9-fold in *sck1Δ* compared to wild-type. Conversely, Mis4-S183p was reduced in the *sck2Δ* strain relative to wild-type and the double mutant showed a 5-fold higher signal than the wild-type. As Mis4-S183 is phosphorylated in the double mutant this indicates that Mis4-S183 is phosphorylated by another, unknown kinase, at least in this genetic context. In this scenario, the unknown kinase would be positively regulated by Sck2 and negatively by Sck1.



**Figure 31: Psm1-S1022 and Mis4-S183 phosphorylation levels in AGC kinase mutants in *cdc10-129*-arrested cells.** Rad21-FLAG (A) and Mis4-GFP (B) IPs were analysed by western blotting using the indicated antibodies and the ratios of signal intensities were calculated

## **E - The *sck1Δ sck2Δ* double mutant reduced *mis4-G1487D* chromosome segregation defects**

The presence of lagging chromosomes during anaphase is a typical phenotype of the *mis4-G1487D* mutant at the restrictive temperature. Previously we have seen that *mip1-R401G* reduced the occurrence of lagging chromosomes. Here, we questioned whether deletion of *sck1* and *sck2* would behave similarly (figure 32). The deletion of *sck1* had no effect, approximately 32% of anaphase cells displayed lagging DNA, as in the *mis4-G1487D* single mutant. The deletion of *sck2* reduced the occurrence of lagging DNA and the effect was enhanced by the additional deletion of *sck1*. The suppression with *sck1Δ sck2Δ* is in the range of previously observed with *mip1-R401G*. A direct comparison within the same experiment is needed to confirm this point.



**Figure 32: Frequency of anaphases with lagging DNA.** Exponentially growing cells at 25°C were shifted at 36.5°C for one doubling of the cell population and fixed. DNA was stained with DAPI and tubulin detected by indirect immunofluorescence. Lagging DNA was defined as DAPI-stained material lagging on the anaphase spindle (length>5µm). P-values from two-sided Fisher's exact tests.

### III - Conclusions and outlook

Taken together, these additional results suggest that the AGC kinases Sck1 and Sck2 are relevant to cohesin regulation by TORC1. It is clear however that the deletion of *sck2* or the additional deletion of *sck1* does not equal the effect of *mip1-R401G*. The relationship between components of the TORC1 pathway down to cohesin appears complex, at least with the data we have in hand. The aim of this preliminary analysis was to evaluate whether AGC kinases downstream TORC1 might play a role. The answer is yes. The suppression of the Ts phenotype and chromosome segregation defects of the *mis4* mutant by the double deletion of *sck1* and *sck2* mimics largely the effect of *mip1-R401G*. Likewise, cohesin abundance at CARs is similar. The discrepancy is observed for the phosphorylation levels of Psm1-S1022 and Mis4-S183. Interestingly, the two sites respond differentially to *sck1* and *sck2* deletion. For Psm1, both kinases seem to contribute whereas Sck1 appears to oppose Sck2 for Mis4-S183. The *mip1-R401G* mutation may affect differently Sck1 and Sck2 activities. The negative genetic interaction observed between *mip1-R401G* and *sck2* $\Delta$  is consistent with the notion that Sck2 activity is not abolished in *mip1-R401G*.

Whether Sck1, Sck2 or TORC1 directly phosphorylates cohesin components are the next questions to address, using *in vitro* assays. The fact that the phosphorylation of Psm1-S1022 and Mis4-S183 were never abolished in the *sck1-2* mutants may suggest they are indirectly involved. This should be taken with caution because we have been using deletion mutants. In this situation, cells adapt and other kinases may substitute, at least partially (Plank et al., 2020). Asking whether cohesin components display consensus sites for AGC kinases is a complementary approach. The consensus site for Sck1 and Sck2 is unknown. If we assume some conservation with budding yeast SCH9, the consensus sequence would be RRxS/T. Psm1-S1022 (DQMSP) and Mis4-S183 (QLCSP) do not fit the consensus. However, this kind of information should be taken with care as many SCH9-dependent phosphorylation sites did not fall within the consensus in a study using analogue-sensitive AGC kinases and phosphoproteomics (Plank et al., 2020). Alternatively, Sck1-2 may regulate other kinases. Indeed Psm1-S1022 and Mis4-183 lie within CDK consensus sites and Psm1-S1022 phosphorylation is Pef1-dependent. It will be worth looking at other kinases, for instance those that co-purified with cohesin, Mis4 and Pef1 in our proteomic analyses. Lastly, phosphatases have been largely ignored although their activities can be regulated by the

TORC1 pathway. For instance Sck2 is known to downregulate PP2A-B55 through the greatwall pathway (Chica et al., 2016).

Taken together, the data generated during the course of this thesis suggest that cohesin is an effector of TORC1. We suggest that such a link may be relevant to the adaptation of cells to changes in their environment, possibly via a change in genome folding. Rapamycin has attracted much attention for its supposed ability to mimic caloric restriction, resulting in improved health and lifespan. Our study raises the possibility that some of these effects are mediated by cohesin and its ability to remodel chromosomes in response to various extracellular signals, including nutrients.

Whether nutriment availability directly impinges on genome folding is however poorly documented. A recent study revealed that fasting induces a tissue-specific reorganization of the whole genome in *C. elegans* (Al-Refaie et al., 2023). The mechanism is dependent on RNA Pol I transcription, itself regulated by mTOR. In mouse, no obvious changes in TADs were observed in hepatocytes under a high-fat or high-carbohydrate diet. However, promoter capture Hi-C revealed the rewiring of promoter-enhancer interactions, extensive changes in transcription factors binding and reprogramming of the liver transcriptional network (Qin et al., 2020). Whether changes are initiated first by cohesin or by transcription factors is unknown. Our study suggests that cohesin may be targeted by TORC1 signalling for genome remodelling in response to environmental changes. Some key experiments should tell whether this hypothesis might be correct. Hi-C experiments in TORC1 mutants and rapamycin-treated cells should provide the answer. Rapamycin is particularly attractive as it would address the kinetics of changes after TORC1 inhibition. Crucially, Hi-C experiments in the mutant mimicking the non-phosphorylated state of Psm1-S1022 Mis4-S183 will ask whether the phosphorylation status of Psm1 and Mis4 is indeed causal. Likewise, RNA-seq experiments in this genetic context should tell whether gene expression is affected and whether the changes are specific to the set of genes identified in this study.

Cohesin is implicated in a wide range of human pathologies, including cancer. Initially, research focused on the causal relationship between aneuploidy, genetic instability and cancer. However, many cancers with alterations in cohesin do not show chromosomal instability or aneuploidy. The discovery that cohesin organises the architecture of



chromosomes in interphase has opened up the possibility of a new causal pathway, a recent and extremely active field of research.

Several factors acting upstream (activators and repressors) and downstream (effectors), as well as mTOR, are overexpressed or mutated in cancers and mTOR is a therapeutic target. The TOR-cohesin link presented here could be relevant in certain cancers. The control of genome architecture by cohesin and its pathological dysfunction are recent and very active areas of research. The causality of cohesin in certain cancers has been established, via disruption of the balance between renewal and differentiation. We can anticipate that this mechanism also operates in other types of cancer.



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