

Tesis doctoral

*Cohesin ubiquitylation and  
mobilisation by an Rsp5<sup>Bul2</sup>-Cdc48 axis  
facilitate stalled fork dynamics and  
integrity*

Sara VILLA HERNÁNDEZ

Director: Rodrigo Bermejo Moreno  
Tutora: Mónica Segurado Carrascal

SALAMANCA, 2016

**Dr. Rodrigo Bermejo Moreno**, científico titular del Consejo Superior de Investigaciones Científicas (CSIC)

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*Rodrigo Bermejo Moreno*

**Director de la Tesis Doctoral**



***Dra. Mónica Segurado Carrascal***, profesora del departamento de Microbiología y Genética de la Universidad de Salamanca.

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Que la licenciada ***Sara Villa Hernández*** ha realizado el trabajo titulado ***“Cohesin ubiquitylation and mobilisation by an Rsp5<sup>Bul2</sup>-Cdc48 axis facilitate stalled fork dynamics and integrity”***, bajo mi tutoría en el programa de Doctorado en Biología Funcional y Genómica.

Y para autorizar su presentación y evaluación por el tribunal correspondiente, expide el presente certificado en Salamanca, a 15 de Diciembre de 2016

*Mónica Segurado Carrascal*

***Tutora de la Tesis Doctoral***



**Dr. Francisco del Rey Iglesias**, Catedrático del departamento de Microbiología y Genética de la Universidad de Salamanca.

**CERTIFICA:**

Que el trabajo titulado **“Cohesin ubiquitylation and mobilisation by an Rsp5<sup>Bul2</sup>-Cdc48 axis facilitate stalled fork dynamics and integrity”**, presentado por la licenciada **Sara Villa Hernández** para optar al grado de Doctor en Biología Funcional y Genómica por la Universidad de Salamanca, ha sido realizado bajo la dirección del Dr. Rodrigo Bermejo Moreno y la tutela de la Dra. Mónica Segurado Carrascal, en el Instituto de Biología Funcional y Genómica (IBFG), centro mixto de la Universidad de Salamanca (Departamento de Microbiología y Genética) y del Consejo superior de Investigaciones Científicas (CSIC).

Y para autorizar su presentación y evaluación por el tribunal correspondiente, expide el presente certificado en Salamanca, a 16 de Diciembre de 2016

*Dr. Francisco del Rey Iglesia*



# RESUMEN

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En la tesis titulada ‘La ubiquitinación y movilización de cohesina por Rsp5<sup>Bul2</sup> y Cdc48 facilita la dinámica e integridad de las horquillas de replicación detenidas’ se ha caracterizado el papel de los complejos señalados en respuesta a estrés replicativo. Para ello, se han utilizado herramientas genéticas y de biología molecular en la levadura de gemación *Saccharomyces cerevisiae*.

La replicación del ADN es necesaria para que en la división celular o mitosis, cada copia del genoma original se segregue a cada célula ‘hija’. Este proceso debe ser llevado a cabo de manera fidedigna para poder conservar la estabilidad del genoma. La replicación de ADN requiere el ensamblaje de complejos especializados, llamados replisomas, los cuales están especializados en la duplicación del genoma de manera eficiente y acoplada al resto de procesos que ocurren en el mismo marco espacio-temporal, como son la cohesión de cromátidas hermanas o la herencia de nucleosomas.

La cohesión de cromátidas hermanas es esencial para la correcta segregación de los cromosomas y la viabilidad celular. El proceso de cohesión está mediado por el complejo cohesina, el cual está compuesto por dos proteínas de tipo SMC, Smc1 y Smc3 que conforman la estructura fundamental del complejo con forma de anillo, una proteína kleisina Scc1 que cierra dicho anillo y otra proteína esencial, Scc3, con características regulatorias. El anillo de cohesina es capaz de ‘abrazar’ las moléculas de ADN, siendo ésta su principal característica funcional. El complejo de cohesina de carga en la cromatina en la fase G1 del ciclo celular, y una vez el ADN se ha duplicado, el complejo de cohesina es acetilado por la acetiltransferasa Eco1. Esta modificación determina el cierre estable del anillo, que mantiene la asociación de las dos cromátidas hermanas hasta que la célula llega a anafase, donde tienen que segregarse cada una a una célula ‘hija’.

El complejo de cohesina desempeña además funciones importantes en la regulación de la transcripción génica y la reparación del ADN. Recientemente se ha descrito la

acumulación de cohesina en horquillas detenidas a consecuencia de daño en el ADN molde o a estrés replicativo. La asociación de cohesina se cree necesaria para el reinicio de la progresión de la horquilla detenida, si bien los mecanismos implicados aún no han sido elucidados.

En este trabajo de tesis se ha caracterizado el papel de la ubiquitinación del complejo de cohesina, que ocurre de manera dependiente del complejo ligasa Rsp5<sup>Bul2</sup>, en el control de su asociación a cromatina durante la replicación del ADN. La ubiquitinación de cohesina durante estrés replicativo se ve determinada además, por la respuesta de checkpoint de daño en el ADN, una vía de señalización que responde a problemas durante el proceso de replicación.

Los datos obtenidos apuntan a que la ubiquitinación de cohesina estimula su función en la respuesta a estrés replicativo, necesaria para la correcta progresión de horquillas detenidas. El complejo de cohesina ubiquitinado sería reconocido por la segregasa Cdc48 lo que conllevaría su movilización y asociación a cromátidas neosintetizadas durante estrés replicativo.

Mediante experimentos de sobreexpresión de Wpl1, uno de los factores que regula la extracción de cohesina de cromatina, se demuestra que la segregasa Cdc48 trabaja conjuntamente con este factor en la movilización del complejo de cohesina y que la función de ambos es esencial en las horquillas de replicación en condiciones de estrés replicativo. La acetiltransferasa encargada del cierre estable del anillo también está implicada en esta vía.

Los resultados obtenidos en esta tesis permiten proponer un mecanismo de estabilización de horquillas en estrés replicativo mediado por el complejo de cohesina. Según el modelo propuesto, cohesina sería ubiquitinada de manera dependiente de la respuesta de checkpoint cuando la horquilla se detiene, por ejemplo, por la depleción del acervo de dNTPs. La ubiquitinación de cohesina promovería a su vez la acción conjunta de la segregasa Cdc48 y el factor Wpl1 en la extracción de cohesina desde el ADN no replicado a las cromátidas recién sintetizadas detrás del punto de ramificación de la horquilla. Una vez asociada a las cromátidas hermanas, cohesina sería acetilada por Eco1, favoreciendo el cierre estable del anillo, que a su vez

garantizaría la estabilidad estructural del conjunto replisoma-ADN, hasta que las condiciones se restablezcan para proseguir con la correcta síntesis del ADN.



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# ABSTRACT

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Replication fork stability is challenged in conditions of replication stress and protected by the Mec1/ATR checkpoint to preserve genome integrity. An enigmatic role in fork protection is played by cohesin, which mediates key chromosome transactions by topologically entrapping DNA. Searching for factors interfacing with the checkpoint response, we found that the Rsp5<sup>Bul2</sup> ubiquitin ligase promotes stalled fork progression. Rsp5<sup>Bul2</sup> physically interacts with cohesin and the Mec1 kinase, thus mediating checkpoint-dependent cohesin ubiquitylation and stimulating cohesin function in fork protection. The Cdc48/p97 ubiquitin selective segregase, together with Rsp5<sup>Bul2</sup>, promotes cohesin dissociation from replicating chromatin. Mobilization by Cdc48 involves Wpl1 function and is required for cohesin relocation to newly synthesized chromatids and replication stress survival. Cohesin-mediated fork protection also relies on Eco1, which secures sister chromatid entrapment. The results here presented indicate that ubiquitylation facilitates cohesin interfacing with stalled forks to protect fork-replisome dynamic architecture and sustain replication progression.



# INTRODUCTION

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## 1. EUKARYOTIC DNA REPLICATION.

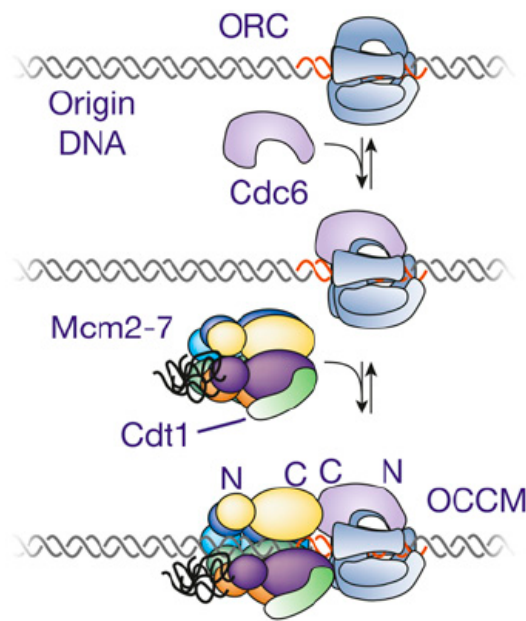
### 1.1 Chromosome duplication.

Faithful DNA replication is essential for cell proliferation. DNA must be correctly duplicated in order to segregate two copies of the genetic complement to each daughter cell without the inheritance of deleterious mutations. The mechanism of DNA replication is very well conserved in eukaryotes. Thus, the yeast *Saccharomyces cerevisiae* experimental model allows answering open questions about the mechanisms of chromosome replication, as well as its regulation and interplay with other cellular processes. Insight obtained in simpler experimental models facilitates the research of more complex systems like mammalian cells (Siddiqui et al. 2013; Doubl   and Zahn 2014).

DNA replication involves the assembly of multi-enzyme complexes, called replisomes. The replisome is responsible for copying chromosomal DNA with high fidelity. After duplication, each of the new copies of the DNA contains one strand from the parental duplex DNA and one nascent antiparallel strand. DNA replication is thus semiconservative. The process of semiconservative replication imparts a geometry to the sites of DNA replication, a fork-like structure where the DNA helix is unwound and the unpaired DNA strands are used as template for the incorporation of free nucleotides into double-stranded DNA molecules (Leman and Noguchi 2013). The complexity of the eukaryotic replisome is not yet fully understood and replisome components have emerged as main regulators not only of DNA synthesis, but also replication-coupled processes such as chromatin dynamics, epigenetic marks inheritance and establishment of sister chromatid cohesion (Bell and Labib 2016).

DNA replication is initiated at genomic sites called origins of replication defined in *S. cerevisiae* by the presence of Autonomously Replicating Sequences (ARS) elements, where the Origin Recognition Complex (ORC) binds (Bell and Stillman 1992). ORC

mediates the recruitment of the MCM2-7 complex to origin DNA, through the association of the Cdc6 and Cdt1 proteins, in a process termed origin licensing (Diffley et al. 1994). These sequentially loaded proteins conform the pre-replicative complex (pre-RC), which marks sites of potential replication initiation. Origin licensing is restricted to G1 phase, as Cdc6 and Cdt1 function is counteracted by Cyclin-dependent kinase (CDK) activities rising upon entry in S-phase, thus ensuring that just one round of replication occurs per cell cycle (Siddiqui et al. 2013) (**Figure 1**).



**Figure 1: Origin licensing.** The ORC complex attracts Cdc6 to origin DNA to recruit MCM2-7/Cdt1 and form the ORC-Cdc6-Cdt1-MCM (ORCM or pre-RC) complex, which marks the sites of potential replication initiation (From Bell and Labib 2016).

In G1-S phase transition, the pre-initiation complex (pre-IC) is formed upon upregulation of CDK and DDK (Dbf4-dependent kinase) activities, allowing helicase and origin firing. DDK and CDK phosphorylate several essential replication factors to promote their loading onto origin DNA (Francis et al. 2009; Randell et al. 2010). Among these we can find Cdc45 and the GINS (Go-Ichi-Ni-San) complex, which together with MCM2-7 conform the functional CMG (Cdc45-MCM-GINS) helicase (Ilves et al. 2010), as well as Mcm10, Dpb11 and DNA polymerase Pol  $\epsilon$  (Muramatsu et al. 2010). Once MCM2-7 complex is phosphorylated and activated, the double hexamer divides into two hexamers that start unwinding DNA (Quan et al. 2015). Helicase

activation generates two replication forks to which additional factors are recruited to build replisomes capable of efficient DNA synthesis (Bell and Labib 2016).

The unwound DNA is then duplicated by three replicative polymerases, each one essential and with a distinct role at replication forks (Doubl   and Zahn 2014; Lujan et al. 2016). Due to the antiparallel nature of the DNA helix, DNA synthesis must progress in opposite directions in the two strands of the replication fork. However, all DNA polymerases synthesize DNA in the 5' to 3' direction. Pol  $\alpha$ /primase creates and extends RNA primers that are prolonged by the other two polymerases (Figure 2). DNA polymerase  $\epsilon$  synthesizes DNA in a continuous manner, in the same direction of DNA unwinding, constituting the 'leading strand'. DNA polymerase  $\delta$ , instead, extends these short RNA/DNA molecules on the opposite template strand in a discontinuous fashion to synthesize the 'lagging strand' (Clausen et al. 2015; Williams et al. 2016). The discrete DNA stretches occurring in the replication of the lagging strand are known as Okazaki fragments, and are about 100 to 200 bp long. Single stranded DNA (ssDNA) is formed upon helix unwinding and is more prominently exposed in the lagging strand. ssDNA is stabilized by the single-stranded DNA binding protein complex Replication Protein A (RPA), which protects it and prevents the formation of secondary structures (Alani et al. 1992).

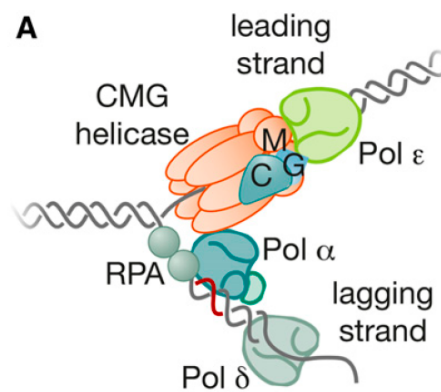
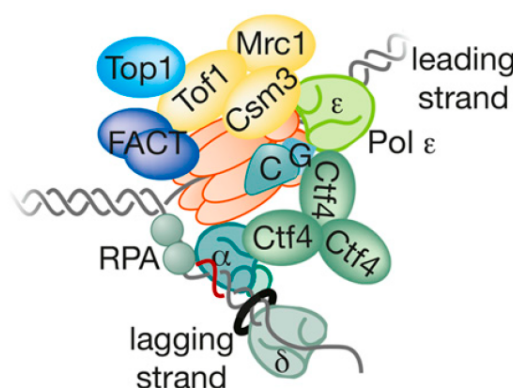


Figure 2: The division of labour among DNA polymerases at the yeast replisome. DNA polymerase  $\epsilon$  primarily synthesizes the leading strand, while DNA polymerase  $\delta$  synthesizes the Okazaki fragments at the lagging strand extending the short RNA/DNA molecules created by DNA polymerase  $\alpha$  (From Bell and Labib 2016).

Pol  $\delta$  recruitment to the replisome is regulated by the loading of the Proliferating Cell Nuclear Antigen (PCNA), which promotes its processivity (Georgescu et al. 2014).

Although PCNA is loaded at both strands, its role in leading strand synthesis remains unclear, since pol  $\epsilon$  is inherently a processive polymerase. Pol  $\delta$  extends Okazaki fragments at the lagging strand, and when it reaches the 5' extreme of the preceding fragment, it can continue synthesizing thus forming a 5' flap (Garg et al. 2004) that is processed leading to the ligation of all lagging strand fragments by the DNA ligase I. Among the nucleases involved in the flap processing, Fen1 and Dna2 play most prominent roles.

Biochemical analysis of replisome-associated proteins revealed the replisome progression complex (RPC) (**Figure 3**). In addition to the aforementioned factors, the RPC is composed by Ctf4, DNA topoisomerase I (Top1) (required to remove the supercoiling from in front of replication forks), the histone chaperone complex FACT and three factors conforming the “fork protection complex” (Csm3, Tof1 and Mrc1) which support replication progression (Gambus et al. 2006). Ctf4 has recently emerged as a hub that links different proteins with replication-associated activities to the replisome through the CMG helicase (Villa et al. 2016). It forms a trimer that exposes three identical binding sites, which are recognition sites for a short peptide motif present, among others, in GINS and Pol  $\alpha$  (Simon et al. 2014). The current view is that Ctf4 uses its binding sites to associate to many other proteins, such as Dna2 and Tof1, supporting its function as a hub allowing the dynamic association of different factors to the replisome (Villa et al. 2016).



**Figure 3: The Replisome Progression Complex.** Replication requires the regulated assembly of multi-enzyme complexes (replisomes) that synthesize carry out chromosome replication (From Bell and Labib 2016). See text for details.

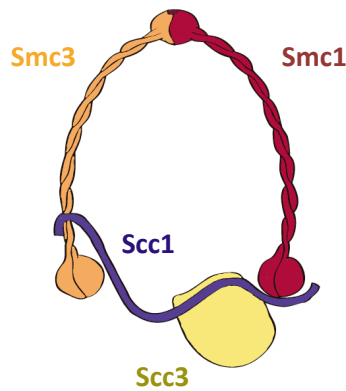
The final steps in chromosome duplication are less well understood. Whereas origin sites are placed at specific loci in the budding yeast genome, sites of replication

termination are more stochastically determined. Replication termination occurs at sites where two converging forks happen to encounter each other, or when a fork meets the end of a chromosome. DNA topoisomerase I and II activities are required during DNA replication to remove the positive supercoils from in front of replication forks (Bermejo et al. 2007). Furthermore, topoisomerases play a crucial role in termination of DNA replication, when two forks converge. In this context topological constraints are thought to be transmitted backwards by fork rotation, where Top2 can resolve the entanglement of the replicated duplexes (Baxter and Diffley 2008). Rrm3 helicase also plays a role in replication termination (Fachinetti et al. 2010), most prominently at rDNA repeats, where it would promote efficient fork pausing (Ivessa et al. 2000). It was recently described that replication termination involves the disassembly of the CMG helicase. This process appears to be restricted to converging forks through mechanisms that are not yet fully understood. Termination of replication is tightly regulated by ubiquitylation: both in budding yeast and *Xenopus* extracts K48 ubiquitin chains are linked to Mcm7 by the SCF<sup>Dia2</sup> ubiquitin ligase during the last steps of replication. Mcm7 ubiquitylation leads to the disassembly of the CMG helicase from chromatin by action of the Cdc48/p97 ubiquitin selective segregase (Priego Moreno et al. 2014; Maric et al. 2014; Maculins et al. 2015).

### 1.2. Establishment of Sister Chromatid Cohesion during replication.

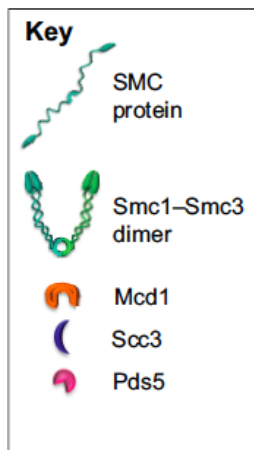
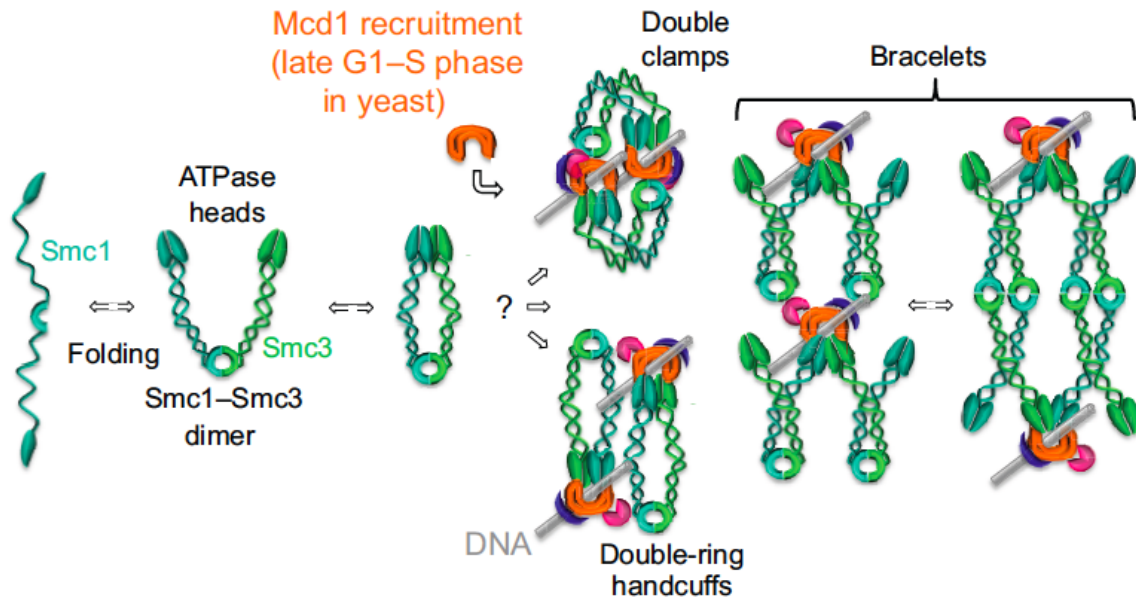
The process of sister chromatid pairing, or cohesion establishment, is coupled to DNA replication and is essential for accurate chromosome segregation and cell viability. Cohesion is mediated by cohesin complexes that are thought to embrace sister chromatids as large rings (Carretero et al. 2010). The cohesin complex is formed of long coiled-coil domains of two SMC subunits, which fold over themselves, and shape the core of a ring structure. Smc1 and Smc3 are connected by a stable dimerization interface known as the 'hinge' (Figure 4). At the other end of Smc1 and Smc3 globular heads with ATP-binding cassettes (ABC) can be found (Gligoris and Löwe 2016). These ATPase domains dimerize in the presence of ATP, which becomes sandwiched between the two heads. These two heads are then connected by the  $\alpha$ -kleisin subunit Scc1: the Scc1 N-terminus binds to Smc3 and the C-terminus associates to Smc1

(Haering et al. 2002). A fourth cohesin subunit, Scc3, binds Scc1 to complete the core of the cohesin ring (Michaelis et al. 1997).



**Figure 4: The core cohesin complex.** Cohesin is composed of Smc1 and Smc3, the kleisin subunit Scc1 and Scc3/SA. SMC proteins fold back on themselves by anti-parallel coiled-coil interactions resulting in a molecule with a 'hinge' domain at one end and a globular head with an ATPase domain at the other. The ATPase heads are connected by the Scc1 subunit, which also associates with a fourth subunit, Scc3.

Cohesin function relies on the topological embracing of the DNA, which is widely accepted to occur within a single cohesin ring (Nasmyth 2011). However, other plausible models have been proposed that are worth considering in the light of the existing evidence (Skibbens 2016) (**Figure 5**). On the one hand, protein engineering used to seal cohesin subunit interfaces *in vivo* has allowed to identify the paths through which DNA enters or exits the cohesin ring, and studies with minichromosomes shown that the ring embraces circular bacterial chromosomes. Structural studies also suggest that the one ring model is feasible. However, the approaches through which cohesin is assembled or enriched, for example, may impact the resulting structure in structural studies. Depending on the subunits studied, for example, enriched rod-like forms of cohesin are predominant. If this type of structures occur *in vivo*, other ways of DNA entrapment by cohesin can be envisaged.

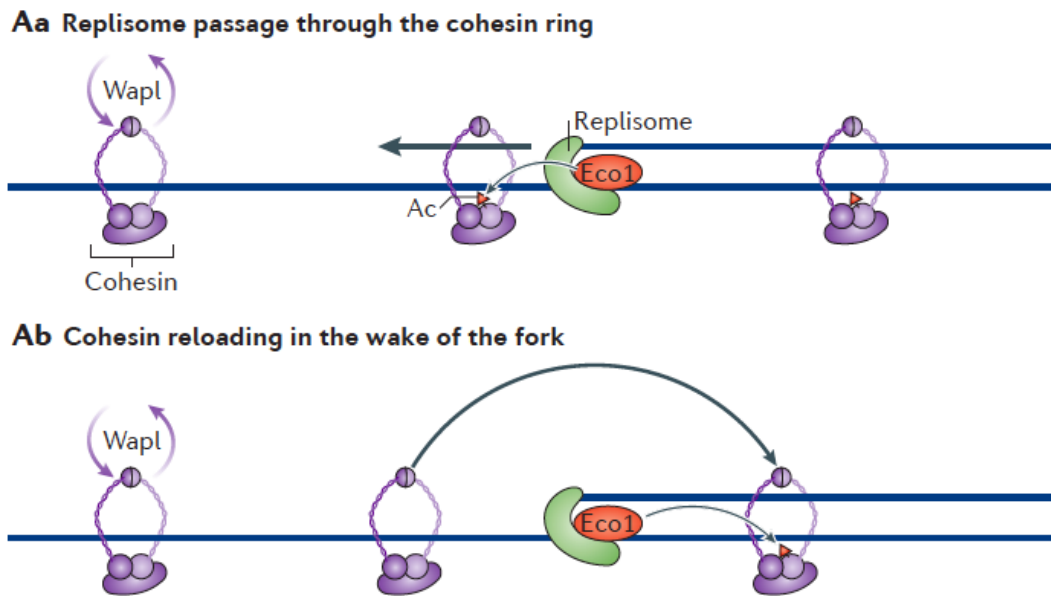


**Figure 5: Alternative models for DNA entrapment by the cohesin complex.** Once the SMC1-SMC3 dimer is formed and loaded on the chromatin, Scc1 (Mcd1) might close the ring in different ways leading to different types of entrapment. Two rings can co-entrap two different DNA strands, or form a handcuff-like structure with the DNA. Cohesin may also form bracelet-like structures to tether the DNA molecules (From Rudra and Skibbens 2013).

Cohesin is loaded onto DNA in G1/S phase transition in yeast, in telophase in mammalian cells, by the cohesin loading complex Scc2/Scc4 (Lengronne et al. 2004; Woodman et al. 2014). The SMC3-SMC1 hinge interface seems to be important for DNA entry into the ring, while the SMC3-Scc1 interface appears to be important for DNA exit.

Cohesin complexes are in dynamic contact with chromosomes (Rankin 2006): once DNA has been duplicated, cohesin holds sister chromatids in a stable manner until cells reach anaphase where sister chromatids must be segregated (Mehta et al. 2012). The way cohesin ring interfaces with incoming forks to be placed behind and associated to nascent sister chromatids still remains unclear. Currently, two non-mutually exclusive ways to entrap the sister chromatids upon replisome passage are contemplated (Uhlmann 2016). One option is that the replication machinery passes through cohesin rings. This option would allow to easily couple DNA replication with sisters entrapment, and would be supported by the notion that Scc2-Scc4 loader is no longer required during DNA replication (Lengronne et al. 2006). Alternatively, the

cohesin ring could be displaced upon engagement by forks and be reloaded behind the replication machinery (Figure 6). Cohesin is prone to establish links between nearby DNA molecules, and co-entrapment of the sister chromatids once they are synthesised seems plausible in this context.



**Figure 6: Different models for cohesin- replisome interfacing.** (Aa) The replisome passes through the cohesin ring and cohesin becomes acetylated by Eco1 once it tethers the sister chromatids. (Ab) Cohesin is dislodged from chromatin upon encountering the replisome and is translocated behind the replication machinery where it entraps sister chromatids. (From Uhlmann 2016).

There is abundant evidence pointing that once cohesin tethers the replicated chromatids, it becomes acetylated by Eco1 in order to lock the exit interphase and thus establish cohesion (Ivanov et al. 2002; Rowland et al. 2009). Eco1 is an acetyltransferase that travels with the replisome, therefore permitting acetylation of cohesion in coordination with DNA replication. Eco1 acetylates Smc3 K112 and K113 to prevent the anti-establishment action of another important factor, Wpl1, which otherwise can dislodge the Smc3-Scc1 opening through a not very well understood mechanism permitting DNA exit from the cohesion ring (Nasmyth 2011; Lopez-Serra et al. 2013). Because of this disengaging activity, Wpl1 is best known as a cohesion anti-establishment factor. Whether Wpl1 is needed for cohesion establishment remains controversial, but importantly, Wpl1 is required to remove cohesin complexes from chromatin (Kueng et al. 2006). In order to do so, it has been proposed that Wapl acts in concert with another cohesin-associated protein, Pds5, which is important for

both establishment and anti-establishment activities (Sutani et al. 2009; Chan et al. 2013), although its mechanism of action still remains poorly understood. Cohesin tethers sister chromatids together until cells reach prophase: a Plk1 and Wapl-mediated mechanism removes cohesin loaded on chromosome arms. In anaphase, APC/Cdh1-dependent degradation of Securin, an inhibitor of the Separase protease responsible of cleaving Scc1, triggers removal of centromere-associated cohesin (Alexandru et al. 2001; Nishiyama et al. 2013) permitting sister chromatid separation.

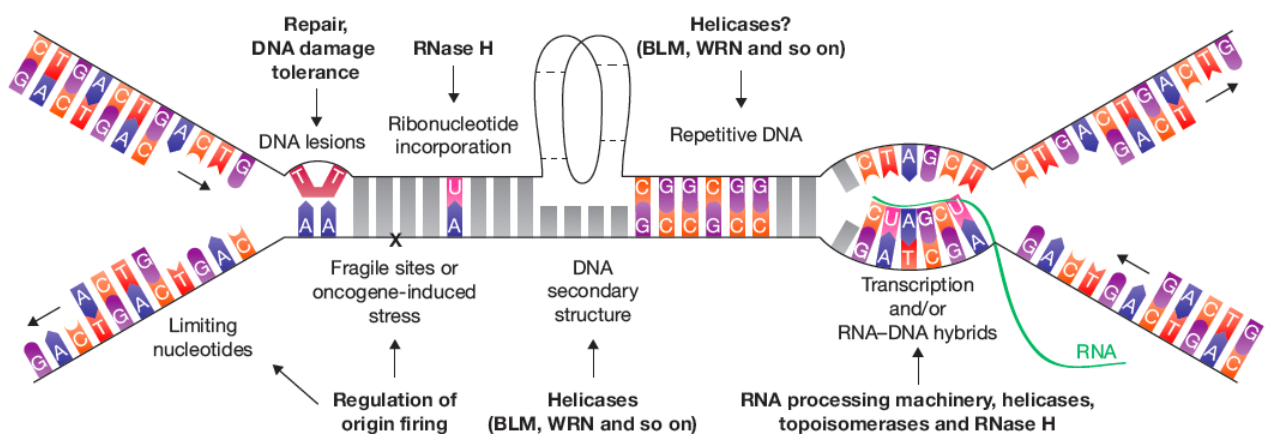
Apart from its role in sister chromatid cohesion, cohesin is involved in gene expression and is important for organism development (Dorsett 2011). Its ability to organize DNA higher order structures makes cohesin one of the mayor factors modulating chromatin architecture. Cohesin colocalizes in the genome with other factors, such as CTCF and Mediator, which function together in the regulation of gene activation in mechanisms that may involve DNA loop formation (Kagey et al. 2010). Mutations cohesin coding genes and its regulators causes developmental syndromes, known as cohesinopathies, which include Roberts or Cornelia di Lange syndromes. Cohesinopathies are characterized by growth and mental retardation, limb deformities and craniofacial abnormalities, indicating that the subjacent cause would be deregulation of gene expression during embryogenesis (Bose and Gerton 2010; Zakari et al. 2015). Cohesin somatic mutations can also be found with high frequency in a select subset of human tumours like glioblastoma, Ewing sarcoma, urothelial carcinoma and leukemia. There are many potential functional effects of cohesin mutations in this context, including the initiation of genomic instability and aneuploidy, alterations in gene expression, replication stress response defect and enhanced susceptibility to DNA damage. However, the relevance of cohesin mutations for cellular transformation and oncogenesis remains unclear (Solomon et al. 2014; Hill et al. 2016, de Koninck and Losada, 2016). A better understanding of cohesin functions and its contribution to tumorigenesis will improve current diagnosis and treatments of cohesin-related diseases.

## 2. DNA REPLICATION DURING PERTURBED CONDITIONS.

DNA replication can be challenged in conditions that impede replication fork progression, broadly termed as replication stress. Replication stress can alter fork

structure and functionality and ultimately lead to DNA breakage, rearrangement and the missegregation of chromosomes (Branzei and Foiani 2010; Gaillard et al. 2015). It is widely accepted that replication stress can be an important source of spontaneous genomic instability leading to malignant transformation of pre-cancerous cells (Bartek and Lukas 2007). Oncogene expression can cause replication stress as well, supporting the direct links between replication stress and tumorigenesis (Bartkova et al. 2005).

Replication fork stalling can be due to various causes (**Figure 7**). Intrinsically, topological constraints, higher order DNA structures and tightly DNA bound proteins can counteract helicase DNA unwinding (Azvolinsky et al. 2006; Bermejo et al. 2007). Interference with other DNA metabolism machineries is also a major potential cause of replication fork stalling and collapse, like the encounter with the transcription machinery, where DNA and RNA polymerases compete for the same template during S phase (Hamperl and Cimprich 2016). The collision between both machineries is thought generate topological distortions and promote the formation of toxic DNA:RNA hybrids (Aguilera and García-Muse 2012). On the other hand, exogenous factors can hinder replication fork progression, either directly inhibiting DNA synthesis (e.g. during hydroxyurea treatment which leads to dNTP pool depletion) or blocking DNA helicases by the generation of DNA-protein crosslinks (DPCs), intra/inter-strand crosslinks or bulky DNA adducts (Jossen and Bermejo 2013).

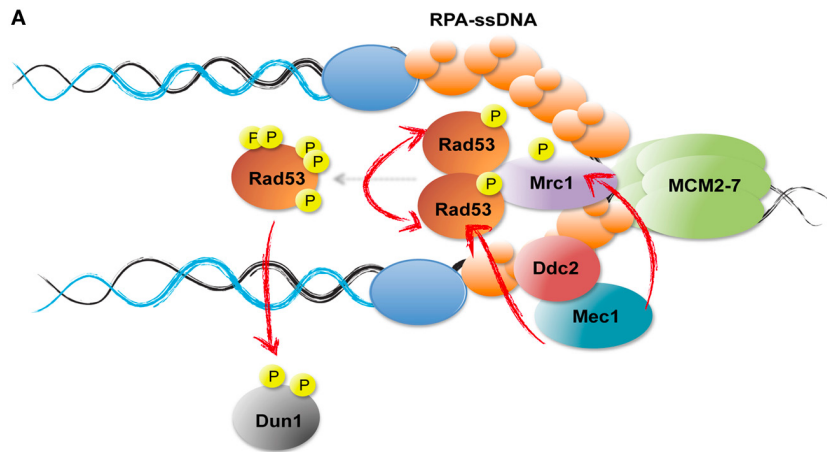


**Figure 7: Sources of DNA replication stress.** (From Zeman and Cimprich 2013).

Several mechanisms have been described that contribute to stabilize replication forks and prevent their collapse. A prominent role in preserving genome stability is played

by the replication checkpoint, which interplays with factors dedicated to preserve replisome structure and functionality, and with DNA repair and DNA damage tolerance pathways.

Replication fork stalling can generate an excessive accumulation of ssDNA, which is coated by the single-strand DNA-binding protein RPA complex (composed in budding yeast by the Rfa1, Rfa2 and Rfa3 subunits) (Zou and Elledge 2003). This is thought to be the main signal triggering checkpoint activation in response to replication stress. RPA complex recruits the checkpoint apical kinase Mec1 to stalled forks through its associated factor Ddc2 (**Figure 8**). Upon recruitment to replication forks, Mec1 phosphorylates several targets, including the Mrc1 protein (Alcasabas et al. 2001; Tanaka and Russell 2001). Mrc1 is a structural replisome component necessary for fork progression in the absence of replication stress (Tourrière et al. 2005). Upon replication fork stalling, Mrc1 acts as a mediator facilitating Rad53 *in trans* autophosphorylation leading to its full kinase activation, in analogy to the Rad9 checkpoint transducer, thought to act as a scaffold for the Rad53 activation events. Moreover, Mrc1 is also proposed to somewhat tether DNA polymerases with helicases, thus preventing uncoupling between them (Lou et al. 2008). Checkpoint activation determines various phosphorylation events carried out by Mec1, Rad53 and Dun1 kinases, which modulate the cellular response to replication stress. This checkpoint-mediated response involves the transcriptional induction of damage inducible genes, dNTP pool upregulation, inhibition of origin firing, stabilization of replication forks and modulation of DNA repair.



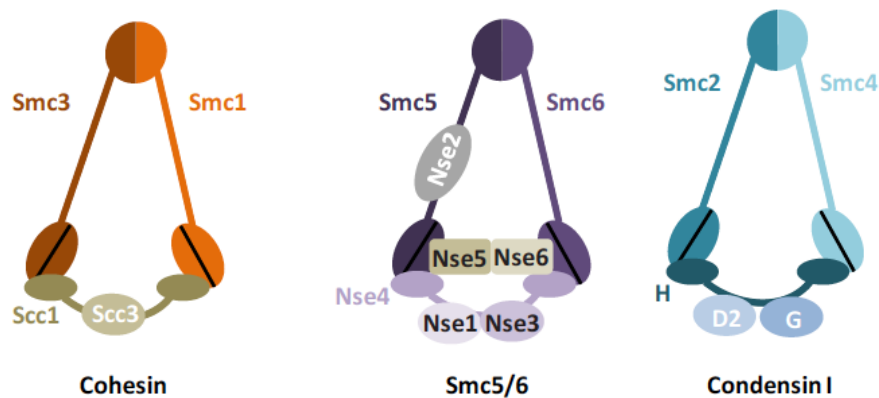
**Figure 8: DNA replication checkpoint activation.** Accumulation of RPA-bound single stranded DNA (ssDNA) triggers Mec1/ATR recruitment via its partner Ddc2/ATRIP. Mec1 phosphorylates Mrc1/CLASPIN, which serves as a scaffold for Rad53 autophosphorylation and full activation. Mec1, Rad53/CHK2 and the downstream kinase Dun1 mediate phosphorylation events that regulate the response to replication stress. (From Jossen and Bermejo 2013).

Protection of fork stability is considered the essential function exerted by checkpoint kinases to promote cell viability in response to replication stress (Tercero and Diffley 2001; Tercero et al. 2003; Segurado and Diffley 2008). Checkpoint mutants are not able to resume DNA synthesis upon recovery from replication stress and accumulate DNA breaks (Branzei and Foiani 2006). Non-functional forks (collapsed forks) exhibit structural alterations of replication intermediates and are thought to be a major source of gross chromosomal rearrangements in checkpoint-deficient cells. Suppression of the formation of abnormal replication intermediates and unscheduled nucleolytic fork processing are two main functions of the checkpoint response promoting fork protection (Jossen and Bermejo 2013).

Checkpoint kinases also interplay with other factors in order to achieve fork protection, including factors safeguarding the dynamic integrity of stalled forks, such as Mrc1 and Tof1. Mrc1 and Tof1 act to promote a stable replication-pausing complex (Katou et al. 2003; Calzada et al. 2005; Bando et al. 2009) and mediate checkpoint-independent replication stress-recovery (Tourrière et al. 2005). In addition, specialized helicases assist replication forks to overcome obstacles hindering their progression. Senataxin and Rrm3 associate with replication forks and are essential when intrinsic replication stress arises from transcription-replication collisions or at replication of specific difficult-to-replicate sites, respectively (Azvolinsky et al. 2006; Alzu et al. 2012). Helicases can act on stalled replication forks exerting functions affecting fork

stability. For instance, Sgs1 helicase function is required to promote, together with Mrc1, DNA polymerase  $\epsilon$  stabilization at stalled replication forks (Bjergbaek et al. 2005), while the checkpoint response inhibits Rrm3 and Pif1 helicases in order to prevent fork reversal and collapse (Rossi et al. 2015).

In addition, chromatin organizing SMC (Structural Maintenance of Chromosomes) complexes play intriguing roles in promoting stalled fork integrity. All SMC complexes are characterized by their ring shape and their ability to entrap DNA molecules. Three different complexes have been described in eukaryotic cells: the cohesin complex, condensin, and the Smc5-6 complex. All are composed by two SMC subunits, one kleisin subunit that closes the ring and HEAT subunits, which function is essential and mainly regulatory (Figure 9).



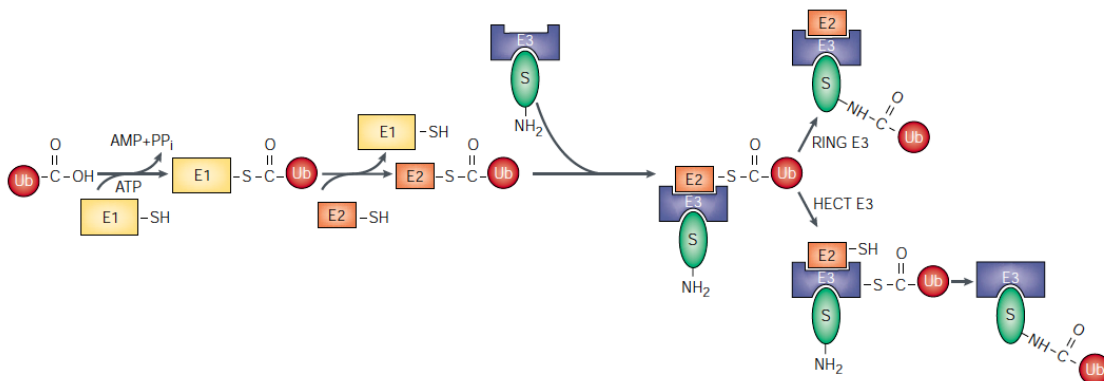
**Figure 9: Architecture of SMC complexes.** The core of SMC complexes are formed by two SMC subunits, which contain an ATPase head domain, a hinge domain and an antiparallel coiled coil domain that folds into itself. Each complex contains additional non-SMC subunits, which modulate its function (From Wu and Yu 2012).

All three SMC complexes accumulate at stalled or collapsed replication forks in yeast (D'Ambrosio et al. 2008; Kegel et al. 2011) and respond to DNA lesions (Ström et al. 2004; Unal et al. 2004; D'Ambrosio et al. 2008). It has been proposed that SMC complexes are necessary in order to support sister chromatid cohesion around DNA break sites thus facilitating repair through homologous recombination upon DNA damage induction. Cohesion establishment after DNA damage also depends on Eco1, which in these conditions acetylates Scc1 subunit instead of Smc3. This modification depends on checkpoint-dependent phosphorylation of Scc1, indicating that Scc1 is the key target of the DNA damage response. It has been also shown that cohesin is required in order to maintain the proximity between broken DNA ends (Gelot et al. 2016). Another SMC-related complex, MRX/N can tether duplexed DNA molecules

(Hopfner et al. 2002) and has key functions in response to DSB. The MRX/N complex (composed by Mre11-Rad50-Xrs2/Nbs1) acts as a sensor of the breaks, regulating DNA repair pathways and signalling to the checkpoint (Lee and Dunphy 2013). Recently it has been described that MRX complex, dependently on its interaction with RPA, keeps sister chromatids together in order to promote DSB repair (Seeber et al. 2016). Upon replication stress induction, cohesin is recruited to stalled forks dependently on MRX complex in order to promote fork restart (Tittel-Elmer et al. 2009), probably by a mechanism distinct from that exerted by the complex during DSB repair. MRX is also recruited to stalled forks where it stabilizes the association of replisome components (Tittel-Elmer et al. 2009). It has been suggested that SMC complexes functions during replication stress may rely on their ability to embrace and stabilize altered structures, such as stalled replication forks (Uhlmann 2016). Nevertheless, the mechanism of action of MRX/N in this context remains to be clarified.

### 3. UBIQUITYLATION DURING DNA REPLICATION.

Ubiquitylation is a highly regulated three-enzyme process consisting in the covalent attachment of an ubiquitin moiety to a determined substrate. First, an E1 ubiquitin-activating enzyme forms a high-energy thioester bond with the carboxyl group of the terminal glycine residue of ubiquitin. This activated ubiquitin is then transferred to an E2 ubiquitin-conjugating enzyme by transesterification. A E3 ubiquitin ligase then catalyses the formation of an isopeptide bond between a lysine in the substrate and the activated carboxyl group of ubiquitin (Komander and Rape 2012). Multiple rounds of this process, using lysines on ubiquitin as a substrate, lead to the formation of different types of polyubiquitin chains. Any of the seven lysines present on ubiquitin (K6, K11, K27, K29, K33, K48 and K63), as well as the amino-terminal methionine (Met1) of the ubiquitin monomer, can serve as isopeptide bond acceptors (Komander and Rape 2012; Kulathu and Komander 2012). Moreover, not only homotypic ubiquitin chains can be formed, but also atypical chains, such as mixed chains (in which different lysines are successively used to link ubiquitin moieties) or branched chains (in which different lysines are poly-ubiquitylated in a given ubiquitin molecule).



**Figure 10: Mechanism of ubiquitin conjugation.** Ubiquitin conjugation requires the activities of three factors: an E1 activating enzyme, an E2 conjugating enzyme and an E3 ubiquitin ligase. Depending of its mechanism of action, the E3 ligases can be RING ligases (that transfer directly the ubiquitin to the substrate) or HECT ligases, which first accept the ubiquitin and then catalize its binding to the substrate. (From Weissman 2001).

Ubiquitin chain variants are readily detected in cells and determine different outcomes of the modified substrates. While K11 and K48 chains more frequently signal proteins for degradation, monoubiquitylation and K63 chains usually modulate protein-protein interactions. However, these regulatory functions are not strict and proteolytic outcomes of K63 chains and non-proteolytic ones for K48 have been described (Shibata et al. 2012; Maric et al. 2014). K6, K27, K29 and K33-ubiquitin chains have been reported only for a small number of substrates and their function is still poorly understood (Kulathu and Komander 2012; Pinder et al. 2013; Yau and Rape 2016).

As in the case of other post-translational modifications, ubiquitylation can be reversed by specific ubiquitin proteases or DUBs. DUBs are cysteine proteases (with the exception of Rpn11 in yeast that is a zinc metalloprotease), which catalyse the hydrolysis of the isopeptide bonds connecting ubiquitin with its substrate and have been classified based on their molecular structure (Nijman et al. 2005; Sahtoe and Sixma 2015). Ubiquitylation modulates a great variety of cellular processes and is regulated in a more sophisticated way than initially anticipated by factors that promote either substrate ubiquitylation or deubiquitylation. Noteworthy, pairs of E3 ligases and DUBs acting in coordination have been identified, in which the two enzymes act on the same substrates to fine-tune ubiquitylation levels (Kee et al. 2005; Sowa et al. 2009). Another important layer of regulation comes from ubiquitin chain

editing, which requires the concerted action of additional ubiquitin ligases and/or DUBs that change the topology of the ubiquitin chains and potentially alter substrate fate (Newton et al. 2008).

DNA replication is a highly regulated process and several mechanisms control replication timing and fidelity both during unperturbed cell cycles and in response to replication stress. Ubiquitylation plays an important role mediating G1-S phase transition, promoting mitotic cyclins and CDK-activating phosphatase Cdc25 degradation by the anaphase promoting complex (APC) ubiquitin ligase through its adaptor Cdh1 (Donzelli et al. 2002). Ubiquitylation and proteolysis of Cdc6 and Cdt1 factor is required in order to prevent re-replication of the DNA (Sánchez et al. 1999; Li et al. 2003; Walter et al. 2016). Termination of replication is also regulated by the ubiquitylation on the CMG helicase, leading to its disassembly from chromatin by the action of Cdc48/p97 ubiquitin selective segregase (Priego Moreno et al. 2014; Maric et al. 2014).

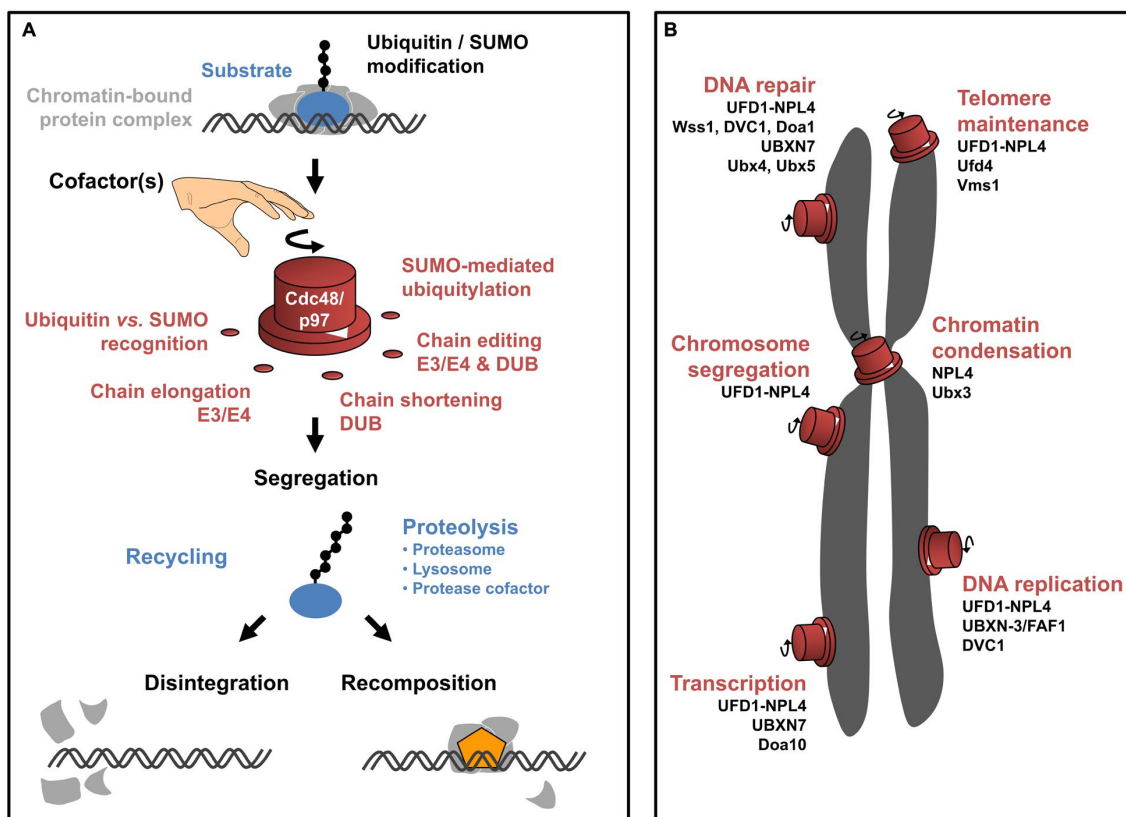
Replication in altered conditions, such as in the presence of DNA damage or replication stress, requires rapid and flexible coordination among the different responses triggered in these conditions. This fine level of regulation can be achieved by posttranslational protein modifications, being ubiquitylation one of the major regulators involved (García-Rodríguez et al. 2016). Ubiquitylation has shown to be crucial in response to DSB and replication stress by regulating HR-mediated repair (Ulrich and Walden 2010; Jackson and Durocher 2013) and in the DNA damage tolerance pathway, where the key regulatory event is the ubiquitylation of the PCNA factor (Friedberg 2005), for example. Ubiquitylation of replicative polymerases regulates their interaction and exchange within the replisome (Mosbech et al. 2012; Roseaulin et al. 2013; Daraba et al. 2014) and other key components of the replisome, like the RPA complex, are ubiquitylated upon replication stress (Elia et al. 2015).

The importance of ubiquitylation seems evident as deregulation of this process is directly involved in carcinogenesis (Zhou et al. 2014). Different type of ubiquitin ligases and ubiquitin readers are usually found mutated in different cancer types and degenerative diseases (Bernassola et al. 2008; Vekaria et al. 2016). Among them, the HECT family of E3 ubiquitin ligases is known to actively contribute to tumorigenesis

processes (Bernassola et al. 2008). HECT ligases are unique among the E3s because they possess intrinsic catalytic activity (unlike the other major group of E3 ligases, the RING-finger E3s). Interestingly, the enzymatic activity of the HECT E3 ligases can be modulated by their interaction with adaptor proteins. The Rsp5/NEDD4 E3 ligase is one of the best examples of this ability. Rsp5 is the only yeast HECT E3 essential for viability and it mediates the ubiquitylation of a large number of substrates, regulating pathways like endocytosis, sorting of transmembrane proteins, mitochondrial inheritance and transcription (Belgareh-Touzé et al. 2008; Shcherbik and Pestov 2011; Sommer et al. 2014). Rsp5 can achieve all these functions thanks to the interaction with several different adaptor proteins. Ubiquitylation by Rsp5 can lead to different outcomes. For example, Rsp5 regulates the sorting of the Gap1 permease through its monoubiquitylation, that leads to Gap1 transport to the plasma membrane in the presence of a poor nitrogen source. When cells are grown in a rich nitrogen source, a Gap1 function is no longer needed, Rsp5 polyubiquitylates this factor with the aid of the Bul1 and Bul2 adaptor proteins, promoting its relocation to the vacuole where it is degraded (Helliwell et al. 2001). Rsp5 also works together with an ubiquitin protease, Ubp2, that functions hydrolysing Rsp5-mediated ubiquitylation (Kee et al. 2005; Kee et al. 2006; Lam and Emili 2013). Therefore, Rsp5 can ubiquitylate a substrate by itself, or with the aid of specific adaptors. The role of most adaptors is thought to be providing substrate specificity, although further work is needed to provide a comprehensive picture of the functions of Rsp5 adaptors, as other roles such as switch from mono to polyubiquitylation have been proposed.

Cdc48/p97, another important ubiquitin-related factor, has also been involved in modulating protein association to chromatin, and its mutation is directly linked to oncogenesis, neurodegenerative disorders and premature aging (Ramadan et al. 2016; Vekaria et al. 2016). Cdc48/p97 is an AAA+ ATPase that functions as a segregase facilitating the extraction of protein complexes marked with ubiquitin or SUMO from different cellular compartments (Stolz et al. 2011; Dantuma and Hoppe 2012). Cdc48 mediated-extraction of the substrate from its context usually leads to its degradation by the proteasome, although this is not always the case. Cdc48 works as an ATP-dependent segregase, but its function requires its binding to an arsenal of regulatory cofactors. These cofactors are required for recognition and processing of the substrate, therefore they have an important role in its final destination.

Cdc48 best understood function is the endoplasmic reticulum-associated degradation (ERAD), where it is essential for the protein quality control. Cdc48 is involved in other cellular processes such as mitochondrial-associated degradation (MAD), ribosome-associated quality control and lysosomal degradation (Wolf and Stolz 2012; Dargemont and Ossareh-Nazari 2012; Vaz et al. 2013). It has been recently described the function of Cdc48 in the direct regulation of chromatin association of factors involved in several DNA metabolic processes (Vaz et al. 2013; Dantuma et al. 2014; Franz et al. 2016), such as DNA repair, transcription and DNA replication.



**Figure 11: Cdc48/p97 function in chromatin associated processes.** (A) Cdc48 recognizes ubiquitylated or SUMOylated substrates. Depending on the modification of the substrate, the different Cdc48-associated cofactors can trigger the processing of the substrate by extending, removing or editing its modification. Finally, Cdc48 segregates the substrate from higher order protein complexes and promotes its recycling or proteolysis. (B) Cdc48 can be assisted by different co-factors and function in pathways involved in chromosome metabolism (From Franz et al. 2016).

Rsp5 and Cdc48 have been described to have both an important role in de degradation of the Rpb1 subunit of the RNA polymerase II upon UV irradiation (Harreman et al. 2009; Verma et al. 2011). Rsp5 is necessary for the ubiquitylation of Rpb1 upon UV irradiation, which in turn is promotes recognition by Cdc48 mediating its extraction at

sites of stalled transcription. Both factors were also described to control the ubiquitin-mediated degradation of Sec23, a COPII complex component involved in the transport between the endoplasmatic reticulum and Golgi apparatus (Ossareh-nazari et al. 2010). Whether these two factors cooperate in the regulation of other processes, such as DNA replication, remains to be known.



# OBJECTIVES

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1. IDENTIFYING RELEVANT TARGETS OF  $\text{Rsp5}^{\text{BUL2}}$  IN THE CELLULAR RESPONSE TO REPLICATION STRESS.
2. ANALYSING THE INTERPLAY BETWEEN  $\text{Rsp5}^{\text{BUL2}}$  AND COHESIN COMPLEXES IN STALLED FORK PROTECTION.
3. CHARACTERIZING THE ROLE OF COHESIN UBIQUITYLATION IN STALLED FORK DYNAMICS.







# MATERIALS AND METHODS

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## 1. MEDIA AND BUFFERS.

All W303 strains used in this study are isogenic derivatives of **W303-1a** RAD5 background (Thomas & Rothstein, 1989). The genotype is listed in the **Table 1**. Deletion strains were constructed using PCR-based gene disruption strategy (Brachmann et al., 1998; Longtine et al., 1998). Protein tagging was performed by introducing the in-frame sequence of the corresponding epitope (FLAG, HA, MYC, PK) at the C-terminal end of the gene of interest.

### 1.1 Solid media.

- Complete media YPDA:

Yeast extract	4 g
Peptone	8 g
D-glucose 40%	20 ml
Agar	8 g
H <sub>2</sub> O (milli Rho)	380 mL

Insoluble Adenine was added at a final concentration of 25 µg/mL.

- Minimum media:

YNB (w/o aa)	2.8 g
Agar	8 g
D-glucose 40%	20 mL
H <sub>2</sub> O (milli Rho)	380 mL
<b>Drop-out*</b>	16 mL

Aminoacids used for selection (HIS, TRP, LEU, URA) were added at the final concentration (Cf) of 25 µg/mL

**\*DROP-OUT (final volume 2 L):**

Thr	1.2 g
Phe	1.2 g
Ile	1.2 g
Lys	1.2 g
Arg	1.2 g
Tyr	1.2 g
Ino	1.74 g
Ade	1.2 g
Etoh 100%	120 mL
ddH <sub>2</sub> O	1800 mL

- YNB (Yeast Nitrogen Base):

YNB (w/o aa)	2.8 g
Agar	8 g
D-glucose 40%	20 mL
H <sub>2</sub> O (milli Rho)	380 mL

- Sporulation medium (VB):

Anhydro CH <sub>3</sub> CO <sub>2</sub> Na	3.28 g
KCl	0.76 g
NaCl	0.48 g
MgSO <sub>4</sub>	0.14 g
Agar	6 g
H <sub>2</sub> O (milli Rho)	400mL

## 1.2 Liquid media.

- Complete medium YPD:

Yeast extract	4 g
Peptone	8 g
D-glucose 40%	20 mL
H <sub>2</sub> O (milli Rho)	380 mL

- Complete medium YPDA:

Yeast extract	4 g
Peptone	8 g
D-glucose 40%	20 mL
H <sub>2</sub> O (milli Rho)	380 mL

Insoluble Adenine was added at a final concentration of 25 µg/mL

- -URA medium:

YNB (w/o aa)	2.8 g
Agar	8 g
D-glucose 40%	20 mL
H <sub>2</sub> O (milli Rho)	380 mL
<b>Drop-out *</b>	16 mL

HIS, TRP, LEU, Cf 25 µg/mL

### 1.3 Media with drugs.

To minimal or complete (YPDA) liquid/solid the corresponding amounts of hydroxyurea (HU) were added depending on the final concentration desired.

### 1.4 List of buffers.

- **Buffer A (pH 8.0):** 8M urea, 10mM Tris-HCl pH 7.5, 0.05% Tween-20, 6.9mM NaH<sub>2</sub>PO<sub>4</sub> + 94mM Na<sub>2</sub>HPO<sub>4</sub> to reach a pH around 8.0
- **Buffer C (pH 6.3):** 8M urea, 10mM Tris-HCl pH 7.5, 0.05% Tween-20, 88mM NaH<sub>2</sub>PO<sub>4</sub> + 12mM Na<sub>2</sub>HPO<sub>4</sub> to reach a pH around 6.3
- **Laemmli Buffer 1X:** 2% SDS, 10% Glycerol, 5% β-mercaptoethanol, 0.002% Bromophenol blue, 0.125 M Tris-HCl pH 6.8
- **PBS:** 137mM NaCl, 10mM PO<sub>4</sub>, 2.7mM KCl
- **Ponceau S:** 0.1% Ponceau S, 1% acetic acid, H<sub>2</sub>O
- **Red Mix buffer:** contains all the necessary reagents required for PCR (Taq HS polymerase and dNTPs), it only requires the addition of template, primers and water

- **Running buffer 1X:** 25mM Tris-base, 192mM Glycine, 0.1% SDS
- **SSR 2X:** 0.25M Tris-HCl pH 6.8, 4% SDS, 10% sucrose, 0.025% Bromophenol blue, 1%  $\beta$ -mercaptoethanol
- **TAE:** 0.04M Tris-Acetate, 0.001M EDTA
- **TBS:** 20mM Tris-HCl pH 7.5, 150mM NaCl
- **TE:** 10mM Tris-HCl pH 7.4, 1mM EDTA
- **Transfer buffer:** Glycine 1%, Tris-base 0.02M, Methanol 20%

## 2. POLYMERASE CHAIN REACTION (PCR).

The Polymerase Chain Reaction is used for the in vitro amplification of specific DNA sequences to transform yeast cells and produce yeast mutants. A PCR reaction requires two oligonucleotide sequences (17-30 base pairs) flanking the DNA region to amplify (primers). PCR reaction is divided into three steps, each of them with a specific temperature. The cycle of denaturing-annealing-extension is repeated 20-30 times to have a satisfactory amplification of the desired sequence.

- **Temperature denaturing:** the double helix is separated into the two single helices by heating ( $T = 94^{\circ}\text{C}$ ).
- **Temperature annealing:** at a lower temperature each primer recognizes and binds to its complementary sequence in one of the two separated helices ( $T = 45-60^{\circ}\text{C}$ ). The primers have a free 3'-end in order to make possible the synthesis on both DNA strands.
- **Temperature extension:** starting from the primers the DNA polymerase synthesizes new DNA helix in a 5'-3' direction using the four dNTPs added to the reaction ( $T = 72^{\circ}\text{C}$ ).

Different PCR reaction mixtures and programmes were used according to the specific DNA sequence (cassette) to amplify:

- **DELETION (HIS, TRP, URA) or MYC-TAG cassette**

Buffer 10X (Biotools 10.002)	100 µL	94°C	3'	
dNTPs (2mM)	100 µL	94°C	30"	} 8 cycles
Primer forward (250 ng/µL)	20 µL	42°C	30"	
Primer reverse (250 ng/µL)	20 µL	72°C	1'30"	
Specific DNA template (20 ng/µL)	10 µL	94°C	30"	
Dynazyme polymerase	20 µL	58°C	30"	} 30 cycles
ddH <sub>2</sub> O sterile	730 µL	72°C	1'30"	
-----		72°C	7'	
Final volume	1000 µL			

- **PK-TAG cassette**

Buffer 10X (Biotools 10.002)	100 µL	94°C	5'	
dNTPs (2mM)	100 µL	94°C	15"	} 32 cycles
Primer forward (250 ng/µL)	10 µL	45°C	15"	
Primer reverse (250 ng/µL)	10 µL	72°C	2'	
BB6 (10 ng/µL)	20 µL	72°C	7'	
Dynazyme polymerase	20 µL			
ddH <sub>2</sub> O sterile	730 µL			
-----				
Final volume	1000 µL			

**CLONAT (NAT) and HYGROMYCIN (HPH) DELETION cassette**

iProof GC Buffer 5X (BIORAD)	200 µL	98°C	2'	
dNTPs (2mM)	100 µL	98°C	15"	} 5 cycles
Primer forward (250 ng/µL)	20 µL	49°C	20"	
Primer reverse (250 ng/µL)	20 µL	72°C	40"	
BB19/BB70 (10 ng/µL)	10 µL	98°C	15"	
Phusion polymerase	10 µL	65°C	20"	} 35 cycles
ddH <sub>2</sub> O sterile	640 µL	72°C	40"	
-----		72°C	5'	
Final volume	1000 µL			

PCR products were analysed on a 0,8% agarose/TAE 1X gel and precipitated by the addition of 1/10 volume of 3M Sodium Acetate ( $\text{CH}_3\text{CO}_2\text{Na}$ ) and 2.5 volume of cold 100% EtOH and a 10 minutes centrifugation at maximum speed at 4°C. Pellets were washed with 1 ml of cold 70% EtOH, centrifuged (2 minutes, maximum speed, 4°C), dried and re-suspended in sterile TE 1X to reach a final concentration of 1  $\mu\text{g}/\mu\text{l}$  DNA. Different DNA quantities from this stock solution were then used for transformation.

### 3. HIGH EFFICIENCY LiAC TRANSFORMATION.

To generate knock out mutants or strains that express a tag version of the protein of interest we used a high efficiency transformation protocol. Strains to be transformed were grown in a pre-culture of 5 mL of YPDA in a 50 mL falcon tube. Cells were then counted at the microscope, diluted in 50 mL of YPD and let grow over night to reach the day after the final concentration of  $5 \times 10^6$  cell/mL. The following day, the culture were centrifuged for 3 minutes at 4000 rpm and the pellet was rinsed with 25 mL of sterile water to wash away completely the medium. The pellet obtained after a second centrifugation was resuspended in 1 mL di 0.1M LiAc/TE 1X and transferred in a 1,5 mL Eppendorf tube. Cells were centrifuged at maximum speed for 15 seconds and resuspended in a final volume of 500  $\mu\text{L}$  di 0.1M LiAc/TE 1X. The cell suspension is vortexed and split into individual 50  $\mu\text{L}$  aliquots for each transformation. Meanwhile salmon sperm DNA (ssDNA) was boiled 5 minutes at 95°C in order to use it as DNA carrier.

The 50  $\mu\text{L}$  cell suspension was centrifuged at maximum speed for 15 seconds and the transformation mix was added to the pellet in the following order:

PEG (50% W/v)	240 $\mu\text{L}$
1M LiAc	36 $\mu\text{L}$
ssDNA (9.5 mg/mL)	10.5 $\mu\text{L}$
DNA (plasmid or PCR product)	1-5 $\mu\text{g}$ (x $\mu\text{L}$ )
Sterile ddH <sub>2</sub> O	73.5 - x $\mu\text{L}$
	-----
Final volumen	360 $\mu\text{L}$

For cell transformation different amounts (1-5 µg) of DNA were used and the corresponding µL of ddH<sub>2</sub>O were added to reach the final volume of 360 µL. The transformation mix was vortexed vigorously for at least 1 minute to obtain a homogenous mixture that was incubated 40 minutes at 42°C: in this step, called “heat shock”, cells incorporate the DNA contained in the mix. After the heat shock, cells were centrifuged for 15 seconds at 7000 rpm, the transformation mix was removed with the vacuum pump and the pellet was resuspended in a small volume of sterile water to be easily plated in the corresponding selective medium. If the cassette used to transform carried an antibiotic resistance marker, for example naturomycin (NAT), kanamycin (KAN) or hygromycin (HPH), cells were let grow for at least 3 hours in 3 mL YPDA before plating to allow them express the resistance gene. Deletion transformants were then selected and analysed by Colony PCR; protein extraction with subsequent SDS page electrophoresis and WB analysis was performed to check protein tags.

#### 4. COLONY PCR.

This technique was used to verify gene deletions. The Polymerase Chain Reaction amplifies the specific nucleotide sequence after cell breakage. A little amount of cells from the colonies of interest was resuspended in 3 µL of 20mM NaOH in PCR tubes and boiled at 99° C for 10 minutes.

For the PCR reaction, the following mix was added to 1.5 µL of boiled solution:

Red Mix 10X (MyTaq <sup>TM</sup> HS Red Mix)	5.5 µL
Oligo forward (20µM) (gene specific)	0.3125 µL
Oligo reverse (20µM) (gene or marker specific)	0.3125 µL
ddH <sub>2</sub> O sterile	4.875 µL
	-----
Final volume	11 µL total volumen

General PCR programme:

95°C	4'	
95°C	15"	} 35 cycles
55°C	15"	
72°C	30"	
72°C	5'	

The time and the temperature of the annealing step depend on the size of the fragment to be amplified and on the melting temperature of the oligo used in the reaction. The colony PCR products were typically analysed in a 2% agarose/TAE 1X gel.

## 5. GROWTH CONDITIONS, CELL CYCLE ARREST AND HU TREATMENT.

*S. cerevisiae* strains were grown in rich YPDA media at 30°C, unless differently stated, to a final concentration of  $1 \times 10^7$  cells/mL. Strains with mating type a were synchronized in G1 by the addition of synthetic  $\alpha$ -factor pheromone at a final concentration of 5  $\mu$ g/mL. After about 1 hour and 30 minutes in the presence of the pheromone, when more than 90% of cells showed the characteristic shmoo shape  $\alpha$ -factor was washed away from the medium by 2 consecutive centrifugations (3 minutes at 3000 rpm). Cells were then resuspended in new YPDA medium or fresh YPDA with the specific HU concentration.

## 6. SERIAL DILUTIONS AND SPOT ASSAYS.

Cells were grown in 110  $\mu$ L of YPDA at 30°C (unless differently stated) on 96-multiwell plates over night to reach stationary phase (plateau). 10 fold serial dilutions were plated on YPDA medium or YPDA containing HU and Methyl Methane Sulfonate (MMS) at the indicated concentrations and incubated at 30°C for 48 or 72 hours.

## 7. TCA PROTEIN EXTRACTION.

10 mL of a  $1 \times 10^7$  cells/mL culture were collected in 15 mL falcon tube, spinned 3 minutes at 4000 rpm, resuspended in 2 mL of 20% TCA (TriChloroacetic Acid), transferred in a 2 mL eppendorf tube and frozen at  $-20^{\circ}\text{C}$  or centrifuged 1 minute at maximum speed. The pellet was resuspended in 100  $\mu\text{L}$  of 20% TCA and glass beads were added till covering the liquid phase; in order to break cells, the tubes were vortexed 3 minutes at maximum speed and 200  $\mu\text{L}$  of 5% TCA were added to the mixture to have 300  $\mu\text{L}$  of final 10% TCA. The liquid phase was transferred in a new 1.5 mL eppendorf tube using a 1 mL pipette and, after a 10 minutes centrifugation at 3000 rpm, the pellet was resuspended in 100  $\mu\text{L}$  Laemli Buffer 1X plus 50  $\mu\text{L}$  of 1M Tris Base to neutralize the acid pH. Samples were resuspended by vortex and boiled at  $95^{\circ}\text{C}$  for 3 minutes, after a 10 minutes centrifugation at 3000 rpm, the supernatant was transferred into a new 1.5 mL eppendorf tube and loaded directly on a SDS-PAGE or conserved at  $-20^{\circ}\text{C}$ .

## 8. SDS-PAGE AND WESTERN BLOT ANALYSIS.

This technique consists on protein separation according to their molecular weight. The separation was performed in denaturing conditions on a polyacrilamide matrix with specific percentages of acrylamide and bisacrylamide according to the size of the protein analysed: the bigger the protein is, the lower percentage of acrylamide/bisacrylamide in the gel was used. Unless differently stated, 10% acrylamide and 0.13% bisacrylamide gels were used. The proteins run in SDS-PAGE Running buffer through which an electric field was applied and then transferred from the gel to a porous nitrocellulose filter through electric transfer in Transfer buffer. The quality of the transfer was checked by Ponceau S staining. The coloured filter was washed with 1% Tween-TBS 1X (T-TBS) and highly reactive protein epitopes were blocked for 1 hour at RT with 4% milk solution in TBS 1X-0.2% TritonX-100. After blocking, the filter was incubated for 2 hours at RT with a milk solution containing the specific primary antibody (12CA5 for HA epitope, V5 for PK epitope, FLAG and MYC antibodies for the corresponding tags) at the appropriate dilution. After incubation with the primary antibody, the filter was rinsed and washed twice 10

minutes with T-TBS 1X and incubated for 1 hour at RT with the secondary antibody (anti-mouse-IgG unless differently stated) conjugated to alkaline peroxidase. After incubation with the secondary antibody and 2 washes in T-TBS 1X, the filter was incubated for 1 minute in a substrate solution for the chemoluminescent reaction (Amersham™ ECL™ Western Blotting Detection Reagents by GE Healthcare). The filter was then exposed to photographic films and developed.

## 9. NI-NTA AFFINITY CHROMATOGRAPHY (HIS-PULL DOWN).

In Pull Down assays, a bait protein is tagged and captured on an immobilized affinity ligand specific for the tag thereby generating a "secondary affinity support" for purifying other proteins that interact with the bait protein. We used this technique to pull down ubiquitylated proteins using as bait protein a ubiquitin tagged with Histidine (<sup>His</sup>Ub) and as immobilized ligand a Nickel Sepharose resin (Ni Sepharose™ 6 Fast Flow by GE Healthcare) which has affinity for HIS-tagged proteins. For our pull down experiments total ubiquitin conjugates were isolated from strains carrying an His7-tagged ubiquitin under regulatory control of the copper metallothionein (CUP1) promoter (Stelter & Ulrich, 2003). Episomal plasmids bearing URA3 gene (YEplac195) was used to overexpress His7-tagged ubiquitin, while an empty plasmid served as control. Total cell extracts were prepared under denaturing conditions and the purified ubiquitylated proteins were then analysed by SDS-polyacrylamide gel electrophoresis and western blotting using the specific monoclonal antibody against the protein of interest.

A 100 mL culture was grown at 30°C to a final concentration of  $1 \times 10^7$  cells/mL in selective -URA medium in order not to lose the plasmids. Cells were synchronised in G1 with  $\alpha$ F and the expression of <sup>His</sup>Ub was induced since the beginning of the experiment by adding 1  $\mu$ L of 100mM CuSO<sub>4</sub> per 1 mL of culture. After 2-hour treatment with 200 mM HU in the presence of CuSO<sub>4</sub>, cells were collected in 50 mL falcon tubes and centrifuged 2 minutes at 3000 rpm. After a wash with cold water, the pellet was transferred into an O-ring tube and spinned 10 seconds at maximum speed. The pellet was then resuspended by vortex in 500  $\mu$ L of 12% TCA, spinned again, resuspended in 500  $\mu$ L of 1M Tris-HCl pH 8.0 and spinned for a third time. After

removing the supernatant, the obtained pellet was frozen at  $-80^{\circ}\text{C}$  for at least 30 minutes. Without defreezing the pellet, we added 250  $\mu\text{L}$  of freshly prepared Buffer A supplemented by Protease Inhibitor (IP) 1X was added together with 500  $\mu\text{L}$  of glass beads. Cells were broken in the fast-prep machine at power 6 for 40 seconds. Once checked the breakage, the supernatant was recovered in 1.5 mL eppendorf tubes and 750  $\mu\text{L}$  of Buffer A + IP 1X was added to each tube. After a 10-minute centrifugation at maximum speed, the supernatant was collected and quantified using a spectrophotometer. Samples were normalized to the lowest concentrated one and 17  $\mu\text{L}$  (+ 17  $\mu\text{L}$  of SSR 2X) from the whole cell extract were used as INPUT sample for the western blot analysis. Meanwhile 50  $\mu\text{L}$  of Ni resin were washed twice with 900  $\mu\text{L}$  of Buffer A (1 minutes at 3400 rpm) in pre-lubricated Costar tubes. Finally, the normalized samples were incubated with the resin O/N on a wheel at RT in the presence of 15mM imidazole. The day after the tubes were spinned 1 minutes at 3400 rpm, the supernatant carefully removed and the resin was washed as follow: 900  $\mu\text{L}$  of freshly prepared Buffer C, twice 900  $\mu\text{L}$  of Buffer A supplemented with 2mM imidazole and three times 900  $\mu\text{L}$  of Buffer C. Each wash was followed by a 10-minute incubation on a wheel at RT and a spin of 1 minute at 3400 rpm. The washed resin was then resuspended in 25  $\mu\text{L}$  of SSR 2X and proteins were eluted in the buffer by shaking at 600 rpm for 5 minutes at  $95^{\circ}\text{C}$ . Samples were centrifuged 5 minutes at maximum speed and the supernatant was collected to be stored at  $-20^{\circ}\text{C}$  or directly loaded on a 7,5% acrylamide SDS-PAGE gel.



Table 1: Strains used in this study

Strain	Number	Genotype	Reference
WT W303	RB718	<i>MATa, his3-11,15, leu2-3,112, trp1-1, ura3-1</i>	Lab collection
<i>bul2Δ</i>	RB1070	<i>MATa, W303 bul2::His3MX6</i>	This study
<i>rsp5-1</i>	RB1263	<i>MATa, W303, rsp5-1</i>	This study
<i>rsp5-25</i>	RB389	<i>MATa, W303, rsp5-25, SUP4o::CAN1-HIS3::sup4</i>	This study
<i>bul1Δ</i>	RB1142	<i>MATa, W303, bul1::NatMX4</i>	This study
<i>bul1Δ bul2Δ</i>	RB1207	<i>MATa, W303, bul1::NatMX4, bul2::His3MX6</i>	This study
<i>ear1Δ</i>	RB2296	<i>MATa, W303, ear1::KanMX6</i>	This study
<i>tre1Δ</i>	RB2301	<i>MATa, W303, tre1::TRP1</i>	This study
<i>art3Δ</i>	RB2303	<i>MATa, W303, art3::TRP1</i>	This study
<i>art5Δ</i>	RB2376	<i>MATa, W303, art5::KanMX6</i>	This study
<i>art10Δ</i>	RB2383	<i>MATa, W303, art10::KanMX6</i>	This study
<i>ubp2Δ</i>	RB863	<i>MATa, W303, ubp2::KanMX6</i>	This study
<i>bul2Δ ubp2Δ</i>	RB1369	<i>MATa, W303, bul2::NatMX4, ubp2::KanMX6</i>	This study
<i>bul1Δ bul2Δ ubp2Δ</i>	RB1688	<i>MATa, W303, bul1::NatMX4, bul2::His3MX6, ubp2::KanMX6</i>	This study
Mec1-HA ev	RB620	<i>MATa, W303, MEC1-HA-LEU2, YEplac195</i>	This study
Mec1-HA Ub	RB604	<i>MATa, W303, MEC1-HA-LEU2, YEplac195-CUP1-HIS7-Ubi</i>	This study
Ddc2-Myc ev	RB589	<i>MATa, W303, DDC2-13Myc-TRP1, YEplac195</i>	This study
Ddc2-Myc Ub	RB590	<i>MATa, W303, DDC2-13Myc-TRP1, YEplac195-CUP1-HIS7-Ubi</i>	This study
Smc1-PK ev	RB1128	<i>MATa, W303, SMC1-9PK-KanMX6, YEplac195</i>	This study
Smc1-PK Ub	RB1129	<i>MATa, W303, SMC1-9PK-KanMX6, YEplac195-CUP1-HIS7-Ubi</i>	This study
Smc3-PK ev	RB1131	<i>MATa, W303, SMC3-9PK-KanMX6, YEplac195</i>	This study

Smc3-PK Ub	RB1132	<i>MATa, W303, SMC3-9PK-KanMX6, YEplac195-CUP1-HIS7-Ubi</i>	This study
Scc1-HA ev	RB1275	<i>MATa, W303, SCC1-3HA-TRP1, YEplac195</i>	This study
Scc1-HA Ub	RB1277	<i>MATa, W303, SCC1-3HA-TRP1 YEplac195-CUP1-HIS7-Ubi</i>	This study
Scc3-MYC ev	RB1987	<i>MATa, W303, SCC3-13MYC-TRP1 YEplac195</i>	This study
Scc3-MYC Ub	RB1989	<i>MATa, W303, SCC3-13MYC-TRP1 YEplac195-CUP1-HIS7-Ubi</i>	This study
Smc1-PK <i>rsp5-25</i> ev	RB1575	<i>MATa, W303, SMC1-9PK-KanMX6, rsp5-25, SUP4-o::CAN1-HIS3::sup4 YEplac195</i>	This study
Smc1-PK <i>bul1Δ bul2Δ</i> ev	RB1578	<i>MATa, W303, SMC1-9PK-KanMX6, bul1::NatMX4, bul2::His3MX6, YEplac195</i>	This study
Smc1-PK <i>rsp5-25</i> Ub	RB1577	<i>MATa, W303, SMC1-9PK-KanMX6, rsp5-25, SUP4-o::CAN1-HIS3::sup4 YEplac195-CUP1-HIS7-Ubi</i>	This study
Smc1-PK <i>bul1Δ bul2Δ</i> Ub	RB1580	<i>MATa, W303, SMC1-9PK-KanMX6, bul1::NatMX6, bul2::His3MX6, YEplac195-CUP1-HIS7-Ubi</i>	This study
Smc3-PK <i>rsp5-25</i> Ub	RB2362	<i>MATa, W303, SMC3-9PK-KanMX6, rsp5-25, SUP4-o::CAN1-HIS3::sup4, YEplac195-CUP1-HIS7-Ubi</i>	This study
Smc3-PK <i>bul1Δ bul2Δ</i> Ub	RB2009	<i>MATa, W303, SMC3-9PK-KanMX6, bul1::NatMX4, bul2::His3MX6, YEplac195-CUP1-HIS7-Ubi</i>	This study
Scc1-HA <i>rsp5-25</i> Ub	RB2366	<i>MATa, W303, SCC1-3HA-TRP1, rsp5-25, SUP4-o::CAN1-HIS3::sup4, YEplac195-CUP1-HIS7-Ubi</i>	This study
Scc1-HA <i>bul1Δ bul2Δ</i> Ub	RB1694	<i>MATa, W303+, SCC1-3HA-TRP1, bul1::NatMX4, bul2::His3MX6, YEplac195-CUP1-HIS7-Ubi</i>	This study
Smc1-PK <i>mec1Δ</i> Ub	RB2312	<i>MATa, W303, SMC1-9PK-KanMX6, mec1::KanMX6, sml1::His3MX6,</i>	This study

		<i>YEplac195-CUP1-HIS7-Ubi</i>	
Smc3-PK <i>mec1</i> $\Delta$ Ub	RB2549	<i>MATa, W303, SMC3-9PK-KanMX6, mec1::KanMX6, sml1::His3MX6, YEplac195-CUP1-HIS7-Ubi</i>	This study
Scc1-HA <i>mec1</i> $\Delta$ Ub	RB2311	<i>MATa, W303, SCC1-3HA-TRP1 mec1::KanMX6, sml1::His3MX6, YEplac195-CUP1-HIS7-Ubi</i>	This study
<i>smc1-259</i>	RB1952	<i>MATa, W303, smc1-259</i>	This study
<i>smc3-42</i>	RB997	<i>MATa, W303, smc3-42</i>	This study
<i>scc1-73</i>	RB976	<i>MATa, W303, scc1-73</i>	This study
<i>scc3-1</i>	RB2115	<i>MATa, W303, scc3-1</i>	This study
<i>smc3-42 bul1</i> $\Delta$ <i>bul2</i> $\Delta$	RB1680	<i>MATa, W303, smc3-42, bul1::NatMX4, bul2::His3MX6</i>	This study
<i>smc1-259 bul1</i> $\Delta$ <i>bul2</i> $\Delta$	RB1954	<i>MATa, W303, smc1-259, bul1::NatMX4, bul2::His3MX6</i>	This study
<i>smc3-42 ubp2</i> $\Delta$	RB1469	<i>MATa, W303, smc3-42, ubp2::KanMx6</i>	This study
<i>smc1-259 ubp2</i> $\Delta$	RB2342	<i>MATa, W303, smc1-259, ubp2::KanMX6</i>	This study
<i>cdc48-3</i>	RB2368	<i>MATa, W303, cdc48-3</i>	This study
<i>cdc48-3 bul1</i> $\Delta$ <i>bul2</i> $\Delta$	RB2044	<i>MATa, W303, cdc48-3, bul1::His3MX6, bul2::NatMX4</i>	This study
<i>cdc48-3 ubp2</i> $\Delta$	RB2468	<i>MATa, W303, cdc48-3, ubp2::KanMX6</i>	This study
<i>cdc48-3 smc1-259</i>	RB2507	<i>MATa, W303, cdc48-3, smc1-259</i>	This study
<i>cdc48-3 smc3-42</i>	RB2369	<i>MATa, W303, cdc48-3, smc3-42</i>	This study
Smc1-PK <i>pdr5</i> $\Delta$ Ub	RB2332	<i>MATa, W303, SMC1-9PK-KanMX6, pdr5::TRP1, YEplac195-CUP1-HIS7-Ubi</i>	This study
Smc3-PK <i>pdr5</i> $\Delta$ Ub	RB2481	<i>MATa, W303, SMC3-9PK-KanMX6, pdr5::TRP1, YEplac195-CUP1-HIS7-Ubi</i>	This study
Scc1-HA <i>pdr5</i> $\Delta$ Ub	RB2346	<i>MATa, W303, SCC1-3HA-TRP1, pdr5::TRP1, YEplac195-CUP1-HIS7-Ubi</i>	This study
<i>rad61</i> $\Delta$	RB2458	<i>MATa, W303, rad61::HPH</i>	This study
<i>rad61</i> $\Delta$ <i>cdc48-3</i>	RB2461	<i>MATa, W303, rad61::HPH, cdc48-3</i>	This study
<i>GAL-RAD61</i>	RB2451	<i>MATa, W303, RAD61::His3MX6-GAL1-HA-RAD61</i>	This study
<i>GAL-RAD61 cdc48-3</i>	RB2454	<i>MATa, W303, RAD61::His3MX6-GAL1-HA-RAD61, cdc48-3</i>	This study

<i>eco1-1</i>	RB2517	<i>MATa, W303, eco1-1</i>	This study
<i>bul1Δ bul2Δ eco1-1</i>	RB2539	<i>MATa, W303, bul1::NatMX4, bul2::His3MX6, eco1-1</i>	This study
<i>cdc48-3 eco1-1</i>	RB2513	<i>MATa, W303, cdc48-3, eco1-1</i>	This study
<i>rad61Δ bul1Δ bul2Δ</i>	RB2553	<i>MATa, W303, rad61::HPH, bul1::NatMX4, bul2::His3MX6</i>	This study
<i>rad61Δ upb2Δ</i>	RB2532	<i>MATa, W303, rad61::HPH, upb2::KanMX6</i>	This study
<i>eco1-1 upb2Δ</i>	RB2541	<i>MATa, W303, eco1-1, upb2::KanMX6</i>	This study

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