Control of S-phase progression in response to stress

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Caminante, son tus huellas
el camino y nada más;
Caminante, no hay camino,
se hace camino al andar.
Al andar se hace el camino,
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.
Caminante no hay camino
sino estelas en la mar.

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SUMMARY

This thesis describes a key mechanism to prevent genomic instability provoked by transcription-replication conflicts (TRCs) in response to stress.

In response to stress, several signalling pathways such as the Stress Activated Protein Kinases (SAPKs) are activated to ensure a coordinated response to stress acting on cellular metabolism, gene expression, protein translation and cell cycle. In response to multiple internal and external stresses, yeast cells activate a common transcriptional response known as the Environmental Stress Response (ESR) that permits them to survive and adapt to the new situation.

The massive activation of transcription that occurs upon stress can be problematic in S phase, when DNA is being replicated. In an unperturbed S phase, cells try to minimize transcription-replication interactions by using several strategies, to prevent conflicts that can lead to genomic instability. However, stress-dependent transcription can be induced unexpectedly at any moment of the cell cycle and therefore cells need a dedicated mechanism to prevent TRCs and genomic instability under these circumstances.

Previously, we described a mechanism to prevent TRCs and genomic instability in response to osmostress in *S. cerevisiae*. We showed that upon osmostress, the Hog1 SAPK is activated and phosphorylates Mrc1, a protein of the replication complex. Phosphorylation of Mrc1 delays replication progression, and prevents transcription-associated recombination (TAR) and genomic instability (Duch et al., 2013a). However, the ESR transcriptional

response is also activated by other stresses rather than osmostress. The aim of this thesis was to explore whether the mechanism that we previously identified is a general mechanism to prevent TRCs in response to different stresses beyond osmostress.

In this thesis, we show that the Mrc1-mediated mechanism is a general mechanism that prevents TAR and genomic instability upon heat, oxidative and low glucose stresses. In an unbiased kinome screening, we identified Mpk1, Psk1 and Snf1 as kinases able to phosphorylate the same three sites of Mrc1 that we previously reported as Hog1-sites. Furthermore, we characterized how Mpk1, Psk1 and Snf1 play an analogous role to Hog1 in response to heat, oxidative and low glucose stresses respectively. Finally, we also found that Mrc1 function is not restricted to environmental stress but it also plays a role upon other internal stresses that compromise cell fitness and induce the ESR response, such as the slow growth or genomic instability itself.

Therefore, this thesis establishes the basis of a general mechanism, which we named “Mrc1 transcription-replication safeguard mechanism (MTR)” that protects genomic integrity from unscheduled transcriptional outbursts triggered by either environmental or internal stresses.

Aquesta tesis descriu un mecanisme clau per a evitar la inestabilitat genòmica causada pels Conflictes entre la Transcripció i la Replicació (TRCs) en resposta a estrès.

Múltiples vies de senyalització, com les Proteïnes Quinasa Activades per Estrès (SAPKs), s'activen per assegurar una resposta coordinada a l'estrès actuant sobre el metabolisme cel·lular, l'expressió gènica, la traducció de proteïnes i el cicle cel·lular. Alhora, en resposta a diversos estressos tant d'origen intern com extern, les cèl·lules de llevat activen una mateixa resposta transcripcional que es coneix com la Resposta a l'Estrès Ambiental (ESR) que els hi permet sobreviure i adaptar-se a la nova situació.

L'activació transcripcional massiva que es dona en resposta a estrès pot ser problemàtica en fase S, quan l'ADN està alhora sent replicat. Durant la fase S, les cèl·lules minimitzen la interacció entre la transcripció i la replicació mitjançant diverses estratègies per evitar conflictes que podrien causar inestabilitat genòmica. Malgrat això, l'estrès pot activar la transcripció en qualsevol fase del cicle cel·lular i, per tant, les cèl·lules necessiten tenir un mecanisme dedicat a prevenir els TRCs i la inestabilitat genòmica.

Anteriorment, vam descriure un mecanisme dedicat a prevenir els TRCs i la inestabilitat genòmica en resposta a estrès osmòtic en *S. cerevisiae*. Vam mostrar com, en resposta a estrès osmòtic, la SAPK Hog1 s'activa i fosforila Mrc1, una proteïna del complex de replicació. La fosforilació de Mrc1 alenteix la replicació i, a més a més, preveu la Recombinació Associada a la Transcripció (TAR) i la inestabilitat genòmica (Duch et al., 2013a). A part de l'estrès

osmòtic, altres estressos activen la resposta transcripcional ESR. Per això, l'objectiu principal d'aquesta tesis consisteix en explorar si el mecanisme que havíem identificat amb anterioritat era en realitat un mecanisme general que permet prevenir els TRCs en resposta a diversos estressos més enllà de l'estrès osmòtic.

En aquesta tesis demostrem que el mecanisme descrit per Mrc1 és un mecanisme general que preveu el TAR i la inestabilitat genòmica en resposta a estrès tèrmic, oxidatiu i a baixa glucosa. Alhora, en un cribratge de totes les quinases del llevat, hem identificat les quinases Mpk1, Psk1 i Snf1 com a quinases capaces de fosforilar Mrc1 en els mateixos residus que anteriorment havíem descrit com a residus de Hog1. Aquestes quinases tenen la funció anàloga a Hog1 però en resposta a estrès tèrmic, oxidatiu i a baixa glucosa respectivament. Finalment, també mostrem com aquesta funció de Mrc1 no es limita a les respostes als estressos ambientals, sinó que també és important davant situacions que comprometen la estabilitat cel·lular i alhora indueixen la ESR, com per exemple en cèl·lules amb creixement lent o amb inestabilitat genòmica basal.

Per tant, aquesta tesis estableix la base d'un mecanisme general, que proposem anomenar "Mrc1 transcription-replication safeguard mechanism (MTR)", que manté l'estabilitat genòmica davant l'activació massiva i no programada de la transcripció causada tant per estressos ambientals com per estressos cel·lulars interns.

PREFACE

Genomic instability is a hallmark of cancer in human cells. However, there is lack of knowledge on the causes of genomic instability and how to prevent or revert their harmful effects. Cells are often confronted with external and internal insults that lead to genomic instability. Essential processes such as DNA replication or transcription can be extremely dangerous in the absence of dedicated control and repair mechanisms. One of the consequences of miss-regulated replication or transcription is the occurrence of the transcription-replication conflicts (TRCs). TRCs occur when the transcriptional machinery or the transcriptional byproducts (DNA torsional stress, R-loops...) interfere with the normal progression of the replication machinery and impair DNA replication, which lead to genomic instability.

Cells have evolved to minimize TRCs, but still they must face them in many circumstances. For instance, there are long genes whose transcription takes longer than one cell cycle and therefore they have high risk to experience TRCs. Additionally, when cells trigger adaptive responses that involve massive activation of transcription, if those responses occur during S phase, then the conflicts between RNA and DNA polymerases might cause TRCs. Yeast cells experience massive changes in transcription in response to environmental stress, even during S phase. Similarly, human cells change their gene expression pattern during cell differentiation or upon viral infections. This unscheduled transcriptional can interfere with replication during S phase and cause TRCs if it is not properly regulated. However, still not much is known regarding the

mechanisms of cells to prevent TRCs upon outbursts of transcription.

It has been shown in *S. cerevisiae* that osmostress induce a fast transcriptional reprogramming of hundreds of stress-responsive genes all over the genome. This adaptive transcriptional induction can cause TRCs during S phase. In this regard, we described a mechanism that is activated in response to osmostress that specifically delays replication progression to prevent trancription-associated recombination (TAR) and genomic instability (Duch et al., 2013a).

In this thesis we show that the mechanism previously described for osmostress is a general mechanism to prevent genomic instability upon unexpected transcriptional outbursts caused by different stresses in yeast. Our results indicate that the phosphorylation of Mrc1 prevents genomic instability triggered by the TRCs upon heat, oxidative and low glucose stresses as well as in mutant cells that have basal transcriptional activation of stress-responsive genes. We believe that the results shown in this thesis will be useful to further study the causes and consequences of the TRCs upon stress and their impact on genomic instability in higher eukaryotes.

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INTRODUCTION

1. YEAST RESPONSES TO STRESS

1.1 *Saccharomyces cerevisiae* as a model organism

Yeasts are used as a model organism to study the biology of eukaryotic cells. The yeast *Saccharomyces cerevisiae* has been used with research purposes because of its unbeatable characteristics (Duina et al., 2014; Hoffman et al., 2015). Similarly to bacteria, yeasts are unicellular organisms that can be easily cultured. They have a short generation time of around 90 minutes at an optimal growth temperature of 30ºC. Of note, these yeasts are non pathogenic and therefore do not require special safety measures. Their life cycle alternates between haploid and diploid forms, although both forms can be maintained in culture. Yeasts are unicellular eukaryotes and their genomes share high evolutionary conservation with those of higher eukaryotes. For years, yeasts have been used to study the functions of mammalian genes by complementation assays. This assays allowed the discovery of functional conservation between mammalian and yeast genes, as well as to discover the functions of many genes and signaling pathways. Many of the processes initially discovered and studied in yeast such as transcription regulation, stress signaling transduction or cell cycle regulation are conserved in higher eukaryotes and therefore allow for translational studies based on the knowledge acquired from yeast.

One of the most precious characteristics of *S. cerevisiae* is that its genome can be easily manipulated in the laboratory. Unlike other organisms, the integration of external DNA into the yeast genomes is performed almost exclusively via homologous recombination and

therefore the integration of plasmids or cassettes can be easily and precisely directed to specific genomic *loci* via the addition of homologous tails.

Since the early 90's, yeasts have been a precious tool for genetic studies of eukaryotic cells. The full genomic sequence of *S. cerevisiae* was published in 1996 (Goffeau et al., 1996) and became the first eukaryotic genome to be fully sequenced, which established *S. cerevisiae* as a referent model organism. It was estimated that the genome of *S. cerevisiae* contains ~6000 genes. Another precious tool that has helped to understand gene function in yeast are the collections of the gene-disruption mutants such as the Knock-Out (KO) collection (Giaever et al., 2002; Wach et al., 1994; Winzeler et al., 1999) or the TAP-tagged ORFs collection to express and purify proteins from yeast (Ghaemmaghami et al., 2003). Extensive study has led to the functional characterization of around 80% of yeast genes (Dujon, 2010), which strongly facilitates studies of gene characterization in higher eukaryotes. Altogether, yeast studies have helped to understand several biological mechanisms that regulate gene expression, protein synthesis, signaling pathways and cell cycle progression.

1.2 Adaptive responses to environmental stress

Yeasts are constantly forced to cope with acute and extreme changing environmental conditions. From water or nutrient depletion to the presence of extreme temperatures or toxins in their media, environmental stress affects the growth and survival of yeast cells (Hohmann and Mager, 2003). Therefore, yeasts have evolved sophisticated adaptive responses to survive and adapt in the wild.

Generally, when a cell is subjected to stress, its first reaction is to stop cell cycle to mount an appropriate defense to the stress (Brauer et al., 2008). The defense to stress must then be rapid but transient, as the cell must eventually restart cell cycle (López-Maury et al., 2008).

The adaptive responses to environmental stress in *S. cerevisiae* are integral and able to control all aspects of the cell physiology. Generally, defense responses result in cell growth arrest and, at the same time, induce massive changes in transcription, translation and post-translational protein modifications, which change protein abundance, localization, activity and/or function. Thus, cells are able to detect the presence of different stresses and to mount proportional responses via the activation of internal signaling pathways (Figure 1).

In yeast, several signaling pathways have been described to control the molecular responses to stress. Moreover, albeit mammalian cells are not exposed to such continuous changes in their environments, several of the signaling kinases described for yeast are conserved in mammals and have been shown to regulate similar molecular responses to environmental insults.

Several signaling pathways regulate molecular mechanisms to survive and adapt to changes in nutrient availability and environmental stress. Good examples are the cAMP-dependent Protein Kinase (PKA), mTOR and MAPK signaling pathways. PKA and mTOR are highly conserved pathways across all eukaryotes and are considered master regulators that mediate the switch between

anabolic to catabolic states of the cell and to regulate all aspects of cell physiology in accordance with nutrient availability (Fuller and Rhodes, 2012; Hall, 2008; Manning et al., 2002; Stephan et al., 2009).

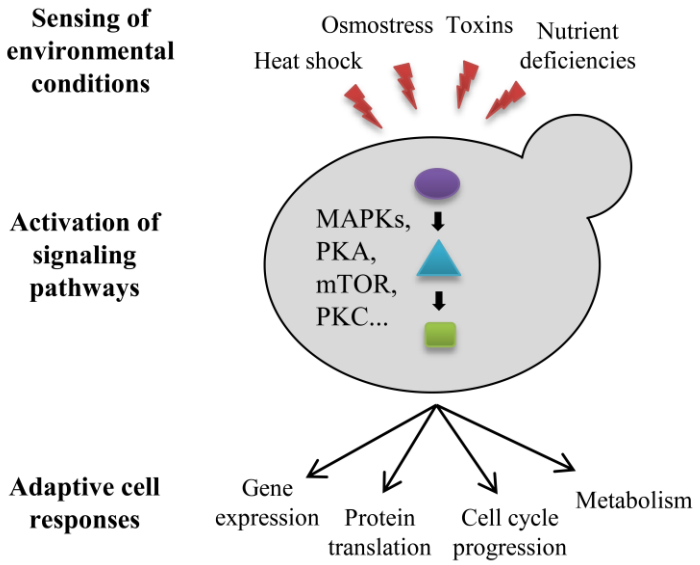


Figure 1. Cells respond to environmental stress via the activation of multiple signaling pathways that regulate several aspects of the cell physiology. Environmental stress such as heat shock, osmostress, the presence of toxins or changes in nutrient availability are sensed and lead to the activation of signaling pathways that regulate all aspects of the cell physiology to ensure cell survival and adaptation.

Of note, in yeast, the nutrients are not only the substrates required for cell growth but also the signals to promote it. The PKA pathway is mostly regulated by direct nutrient availability in yeast while, analogously, it is regulated by growth factors in mammals (Fuller and Rhodes, 2012). Other relevant signaling pathways are the Mitogen-Activated Protein Kinase (MAPK) pathways that control

cell physiology in response to diverse environmental signals (Kyriakis and Avruch, 2001, 2012). The distinctive characteristic of all MAPK pathways is their organization in sequential modules of three consecutive protein kinases. In brief, the upstream detection of the external signal leads to the activation of the MAPKKK, which in turn phosphorylates and activates the MAPKK, which ends up phosphorylating and activating the final effector MAPK (Figure 2; Widmann et al., 1999).

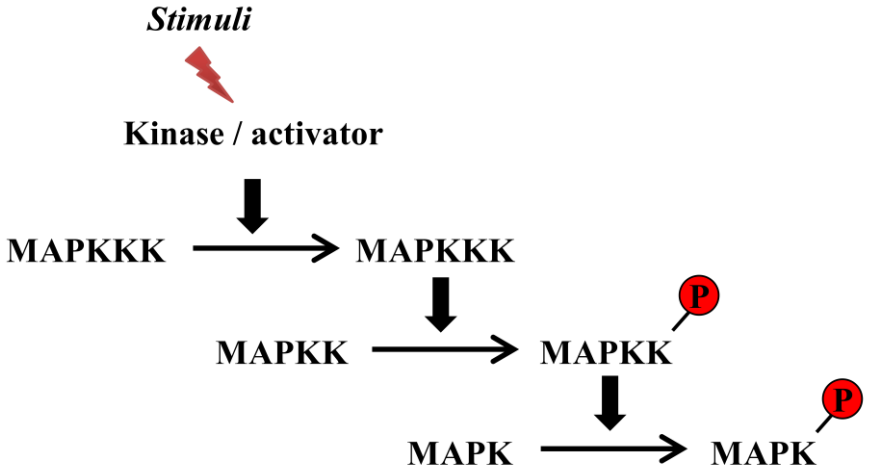


Figure 2. Schematic diagram of a canonical MAP kinase module. Each MAPK module is composed of three MAP kinases. Each MAPK phosphorylates and activates the downstream kinase until the final MAPK is activated.

There are 5 well-known MAPK pathways in *S. cerevisiae*; the filamentous growth or pseudohyphal development pathway (Kss1), the mating pheromone response (Fus3), the spore wall assembly pathway (Smk1), the cell wall integrity pathway (Mpk1/Slt2) and the HOG pathway (Hog1) (Chen and Thorner 2007).

Specifically, a family of Stress-Activated MAPKs (SAPKs) is specialized in the responses to environmental stress, such as the

Mpk1/Slt2 and Hog1 SAPKs. SAPKs are highly conserved in all eukaryotes. For example, the mammalian p38 SAPK is a highly conserved structural and functional homologue of the yeast Hog1 SAPK, and the same happens between the mammalian Erk1/2 SAPK and the yeast Mpk1/Slt2 SAPK (Galcheva-Gargova et al., 1994; Goshen-Lago et al., 2016; Han et al., 1994). Overall, MAPKs have ubiquitous substrates in the cell to regulate metabolism, protein synthesis, gene expression and cell cycle progression (Chang and Karin, 2001).

The first barrier that yeasts possess to protect from environmental stress is the cell wall. The cell wall is mostly made by polysaccharides and it gives yeasts their shape and rigidity. However, the cell wall is not a static armor, but instead it is involved in complex processes such as cell morphogenesis and cell-cell recognition (Levin, 2011). The stability of the cell wall is crucial for yeasts and cells have evolved a specific signaling pathway to protect their cell wall and to transmit signals through it. The Cell Wall Integrity (CWI) pathway is dedicated to sense the weakening of the wall and also to signal for the presence of environmental stress such as severe hypo- osmo or heat shocks and activate molecular responses to ensure cell adaptation. Essentially, the CWI signaling pathway orchestrates compensatory changes to ensure adaptation via the activation of the Protein Kinase C (PKC) and eventually of the effector Mpk1/Slt2 MAPK (Fuchs and Mylonakis, 2009; Levin, 2005; Smits, Kapteyn, Van den Ende, and Klis, 1999).

1.3 Transcriptional responses to stress

The cellular responses to stress generally involve a deep reorganization of the cellular transcriptional program. When cells sense the presence of stress they promote the activation of specific transcriptional programs. Selected sets of genes are induced or repressed depending on each type of stress. There are multiple transcription factors (TFs) involved in the activation of specific transcriptional responses to different stresses.

For instance, yeast cells grown at temperatures higher than 37º-39ºC induce the Heat-Shock Response (HSR) that modifies several aspects of cell membrane permeability, protein stability and gene expression to promote survival and adaptation (Morano et al., 2012). The activation of mainly three TFs drives the transcriptional response to heat stress: Hsf1, Msn2 and Msn4. Hsf1 is a nuclear protein that binds the Heat-Shock Elements (HSEs) located in the promoter regions of heat-responsive genes to induce their transcription. Msn2 and Msn4 are two partially redundant TFs that shift from the cytoplasm to the nucleus in response to multiple stresses and bind the Stress-Responsive Elements (STREs) located in the promoter regions of the stress-responsive genes to promote their transcription (Görner et al., 1998; Martínez-Pastor et al., 1996; Schmitt and McEntee, 1996; Wieser et al., 1991).

Moreover, cells accumulate Reactive-Oxygen Species (ROS) as a consequence of internal cell metabolism or by exposure to exogenous factors such heavy metals, ultraviolet (UV) irradiation, herbicides or air pollutants that strongly damage membranes, proteins and DNA (Gille and Sigler, 1995; Halliwell and Cross,

1994; Liang and Zhou, 2007; Xu et al., 2011). In response to oxidative stress, cells activate the so-called Oxidative Stress Response (OSR) to induce the activation and production of detoxifying enzymes and protect the cell. The OSR is controlled by the action of several TFs i.e. Yap1, Skn7, Hsf1 and Msn2/Msn4. Interestingly, stress-responses to heat and oxidative stresses share many similarities, as heat stress is known to indirectly cause oxidative stress (Morano et al., 2012).

Another well-known transcriptional-stress response is mediated by the HOG pathway, which is essential to survive to osmostress. In response to osmostress, cells activate osmostress-dependent genes by the action of several TFs such as Hot1, Sko1 and Msn2/Msn4 as outlined in section 1.4 (Capaldi et al., 2008; de Nadal et al., 2011).

Many other stress-responses to specific stresses have been described in budding yeast and most of them involve the regulation of gene expression by several TFs and chromatin regulators.

1.3.1 The Environmental stress response (ESR)

Despite the specificity of the transcriptional programs that are activated upon different environmental stress, it is known that the Msn2/Msn4 TFs are activated in response to most of them and control the induction of a common set of stress-responsive genes that represent the so-called Environmental Stress Response (ESR) (Gasch et al., 2000). Originally, the ESR was described as a common transcriptional response to several environmental stress such as osmo, heat or oxidative stress and also in situations of nutrient deprivation (Causton et al., 2001; Gasch et al., 2000).

However, further studies have shown that the ESR is induced in response to a plethora of external and also internal stimuli, such as DNA damage (Gasch et al., 2001).

The activation of the ESR reorganizes the transcription of about 10-15 % (~500-900 genes) of all yeast genes, where ~250-300 genes are induced-ESR genes and ~300-600 genes are repressed-ESR genes (Causton et al., 2001; Gasch et al., 2000, 2001; Ho and Gasch, 2015). As described before, most of the stress responses orchestrated by the distinct signaling pathways require the switch from anabolic to catabolic metabolism, which prevents energy waste in times of need. Accordingly, the ESR switches the cell into a protective state by modifying the transcription of different sets of genes (Ho and Gasch, 2015). In one hand, a set of defensive genes encoding elements such as ROS detoxifying enzymes, chaperones or glycolysis enzymes are induced (iESR) to promote cell protection and repair. On the other hand, genes coding for ribosomal proteins and general anabolic processes are repressed (rESR), which leads to a delay on proliferation and permits the recycling of the transcription and translation machineries to prioritize the expression of iESR genes (Ho and Gasch, 2015; Nadal-Ribelles et al., 2012).

Despite the relevance of the Msn2/4 TFs in the stress response, these TFs are not essential for cell viability in response to mild stress. However, the ESR response shows a cross-protection effect. The cross-protection consist in that the activation of the ESR in response to a specific stress increases cell resistance to secondary stress of different nature, and usually permits adaptation to a more severe stress (Berry and Gasch, 2008; Berry et al., 2011).

Multiple signaling pathways regulate the ESR in response to stress, mainly by acting on the localization or activity of the Msn2 and Msn4 TFs. Under optimal cell proliferation and cell growth, high PKA and mTOR activities keep the ESR repressed by inhibiting the activity of the TFs Msn2 and Msn4. The ESR is a key pathway in yeast that needs to be strictly regulated to ensure cell survival, since the ESR inhibits cell growth and its basal activation is strongly incompatible with proper cell growth. Correspondingly, the essential function of the protein kinase PKA is to prevent the activation of the ESR by inhibiting the activity of the Msn2/4 TFs (Morano et al., 2012). Upon environmental stress, PKA and mTOR activities are turned off relieving its inhibitory action on the Msn2/4 TFs, which then shuttle into the nucleus to drive the transcription of more than 80 % of the iESR genes (Jacquet et al., 2003). However, there are subsets of genes of the ESR that can be induced by alternative TFs independently of Msn2 and Msn4 in response to a specific stress (Rep et al., 1999). For example, the Hsf1 TF can also induce some ESR genes in response to heat or oxidative stress and also Hot1 induces a subset of ESR genes in response to osmostress. Another signaling pathway that regulates the ESR responses is Yak1, a protein kinase that antagonizes PKA and inhibits cell proliferation. Activation of Yak1 is dependent, at least partially, on the inhibition of the PKA activity. Yak1 promotes ESR transcription via Msn2/4 and Hsf1 activation in response to several environmental stress including heat shock, oxidative stress and nutrient starvation (Lee et al., 2008). Moreover, upon glucose starvation, the high activity of the kinase Snf1 causes the re-

phosphorylation of Msn2, which induces its nuclear exclusion and inhibits the ESR to promote cell adaptation (Mayordomo et al., 2002; De Wever et al., 2005). Additionally, the HOG pathway also regulates the activation of the ESR by modulating several TFs such as Msn2/4, Hot1 and Sko1 in response to osmostress (Capaldi et al., 2008; Proft and Struhl, 2002).

1.4 Osmostress and the HOG pathway

Yeast cells face constant changes in their environment. For example, the accumulation of rainwater or the ripening of fruits causes tremendous changes in the osmotic pressure known as osmostress. Osmostress (hyper-osmotic and hypo-osmotic) can be described as changes in the activity of free water (thermodynamically available) or the water potential of the medium. Hyper-osmotic shock (from now on: osmostress) occurs when the activity of free water or the external water potential decreases, causing massive water outflow from cells to the external medium provoking cell shrinkage. Water outflow and cell shrinkage occurs within seconds of the osmostress, and therefore cells must activate immediate adaptive responses to counteract these effects and ensure cell survival (Hohmann, 2002; Mager and Varela, 1993).

The High Osmolarity Glycerol (HOG) pathway is a SAPK pathway essential for survival upon osmostress (Brewster and Gustin, 2014; Brewster et al., 1993). The HOG pathway is activated in response to osmostress via two independent branches, the SHO1 branch and the SLN1 branches. The SHO1 branch activates the Ste11 MAPK kinase kinase (MAPKKK), which in turn can be activated by the Ste20 MAPK kinase kinase kinase (MAPKKKK) while the SLN1

branch activates the Ssk2/Ssk22 MAPKKKs. Both branches converge at the level of the Pbs2 MAPK kinase (MAPKK) and, finally, lead to the activation of the Hog1 MAPK (Dan et al., 2001; Maeda et al., 1995; Posas and Saito, 1997; Posas et al., 1996; Raitt et al., 2000).

Exposure of cells to osmostress leads to rapid activation of Hog1, which regulates all aspects of cell physiology including cell metabolism, gene expression, protein translation, and cell cycle progression (de Nadal et al., 2002). Within minutes in the presence of osmostress, the HOG pathway promotes a metabolic adaptation to balance the osmotic pressure of the cell with the environment. It increases the accumulation of osmolytes such as glycerol and threalose inside the cell to compensate the osmotic pressure and revert water outflow (Albertyn et al., 1994; Klipp et al., 2005; Proft and Struhl, 2002). The most immediate actions of Hog1 to promote glycerol accumulation are to close the Fps1 glycerol channel and phosphorylate glycolytic enzymes which redirects the carbon sources to promote glycerol production (Dihazi et al., 2004). Then, Hog1 rapidly shifts into the nucleus where it regulates the expression of many osmostress-dependent genes. Hog1 controls gene expression by acting at several levels of mRNA biogenesis; it regulates the activity of several stress-related TFs such as Msn2/4, Sko1 and Hot1. Additionally, Hog1 associates to DNA indirectly through the TFs and it recruits the transcription machinery, as well as it controls nucleosome positioning and eviction to regulate osmostress-transcription by regulating several chromatin remodelers and histone modifying enzymes (Alepuz et al., 2003;

Capaldi et al., 2008; Mas et al., 2009; Nadal-Ribelles et al., 2012; de Nadal et al., 2004). Moreover, Hog1 also promotes the translation of a specific subset of mRNAs in response to osmostress, when translation is globally inhibited, although the mechanism is still poorly understood (Warringer et al., 2010).

Finally, one of the most important functions of SAPKs such as Hog1 is to regulate cell cycle progression in response to environmental stress (Correia et al., 2010). Upon osmostress, Hog1 has been shown to regulate G_1, S and G_2 phases of the cell cycle (Duch et al., 2012; Zapater et al., 2005). During G_1, osmostress causes a transient cell cycle delay mediated by Hog1, which prevents degradation of the Sic1 CDK inhibitor (CKi) through its direct phosphorylation and down regulation of G_1-cyclin expression (Escoté et al., 2004). CKis negatively regulate different cyclin-CDK complexes to regulate cell cycle progression. A similar scenario occurs in G_2, where Hog1 promotes the stabilization of the Swe1 CKi through the phosphorylation of the protein Hsl1 and the down regulation of M-cyclins (Clotet et al., 2006). Therefore, in G_1 and G_2, Hog1 regulates cell cycle control elements to mount adaptive responses before S and M phases. Also, during S phase, osmostress-activated Hog1 delays replication progression via the phosphorylation of Mrc1, a protein of the replisome progression complex (RPC) to prevent genome instability (Yaakov et al., 2009; Duch et al., 2013a).

2. CELL CYCLE

2.1 The Eukaryotic cell cycle

The cell cycle comprises a series of events that leads to cell division. These events include cell growth, DNA duplication and cell division in two cells. *S. cerevisiae* cells divide asymmetrically in a process known as budding. The mother cell buds to divide into a mother and a daughter cell. At birth, the daughter cell (bud) is smaller than the mother and has a longer subsequent cell cycle as it needs to reach an optimal volume prior to start a new cell cycle (Hartwell, 1974; Herskowitz, 1988; Johnston et al., 1977). The mitotic cell cycle can be divided into two main phases: interphase and Mitosis. Interphase starts when cells are born. During interphase, cells increase their volume and duplicate their DNA. Specifically, interphase is composed of two gap phases, Gap 1 (G_1) and Gap 2 (G_2) where cells grow in size and prepare the necessary elements for the following phases. G_1 and G_2 phases are separated by DNA synthesis (S phase). After G_2, cells enter into Mitosis (M phase), when they divide their previously duplicated components into two new cells. In turn, Mitosis is organized in four differentiated steps (prophase, metaphase, anaphase and telophase) and ends up with cytokinesis (Figure 3).

The most important events of the cell cycle are DNA replication and segregation of replicated chromosomes (Mitchison et al., 1997; Nurse, 2001). In eukaryotes, these events are temporarily separated, and occur in S and M phases respectively. To ensure that each daughter cell receives a complete genome, each step of the cell cycle must be strictly controlled to ensure that they occur in the

correct sequence and only once per cell cycle. Importantly, there are specific points of control, which are called cell cycle checkpoints, where cells verify that processes have been correctly performed or otherwise are corrected before proceeding through cell cycle. (Tyers, 2004).

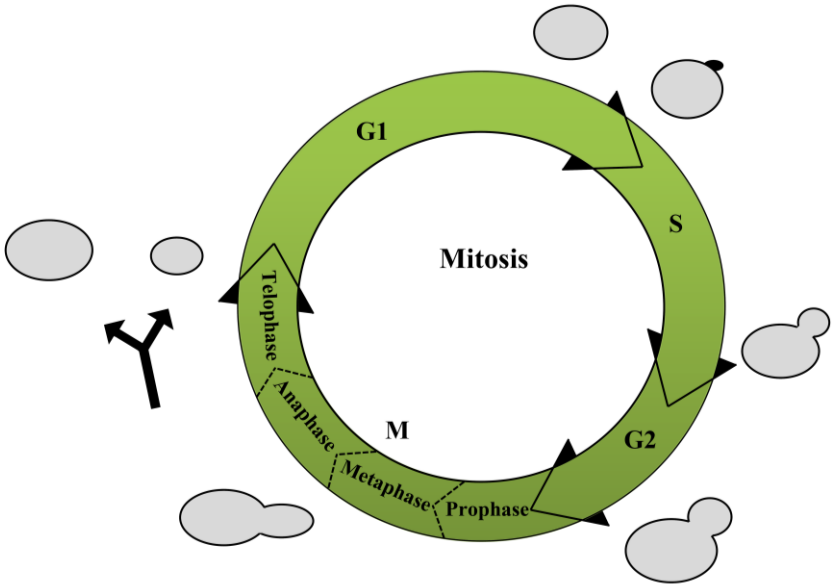


Figure 3. Schematic diagram of the mitotic cell cycle in budding yeast. Diagram shows the three steps of interphase: Gap 1 (G_1), Synthesis (S phase), Gap 2 (G_2) followed by mitosis (M phase). Examples of a budding yeast cell growing, budding and dividing are shown corresponding with each cell cycle phase.

2.2 Control of the cell cycle

Cells possess multiple mechanisms that adequate cell cycle progression to internal and external signals. Missregulation of cell cycle control can lead to aberrant DNA duplication or wrong distribution of DNA into the daughter cell resulting in mutated or

unviable progeny. For this, multiple layers of control exist to prevent or repair any error occurring during the cell cycle.

The cyclin-dependent kinases (CDKs) are the major regulators of cell cycle progression and they coordinate the activation of the sequential steps that lead to cell cycle completion (Hartwell and Weinert, 1989; Nurse, 2001). In budding yeast, the only essential CDK that drives cell cycle is known as Cdc28, and it has homologs in all studied eukaryotic organisms such as Cdc2 in *S. pombe* or Cdk1 in mammals (Draetta et al., 1987). The protein levels of Cdc28 do not change much during cell cycle, and its activity and specificity relies mainly on its association to specific cyclins, which are regulatory proteins whose expression oscillate during cell cycle (Mendenhall and Hodge, 1998). Paul Nurse, Leland Hartwell and Tim Hunt received the Nobel Prize in Physiology or Medicine in 2001 for their discoveries of the existence, function and regulation of CDKs. Two main groups of cyclins exist in budding yeast: the G_1 cyclins (Cln) and the B-type cyclins (Clb). In G_1, Cln1, Cln2 and Cln3 associate with Cdc28 to promote the G_1/S transition (START) (Aldea et al., 2007; Skotheim et al., 2008). In S phase, Clb5 and Clb6 associate with Cdc28 to promote DNA replication. Later, during mitosis, Clb1, Clb2, Clb3 and Clb4 are the last to associate with Cdc28 to promote all mitotic events (Fitch et al., 1992). At the end of mitosis, cells need to degrade M-cyclins to inhibit Cdc28 kinase activity, allowing the activation of the Cdc14 phosphatase, which is essential to exit Mitosis (Bouchoux and Uhlmann, 2011; Glotzer et al., 1991; Queralt and Uhlmann, 2008; Sanchez-Diaz et al., 2012; Visintin et al., 1998). Cyclins are regulated in a temporary

cascade, where the accumulation of the previous cyclin promotes the expression/stability of the next one (Figure 4).

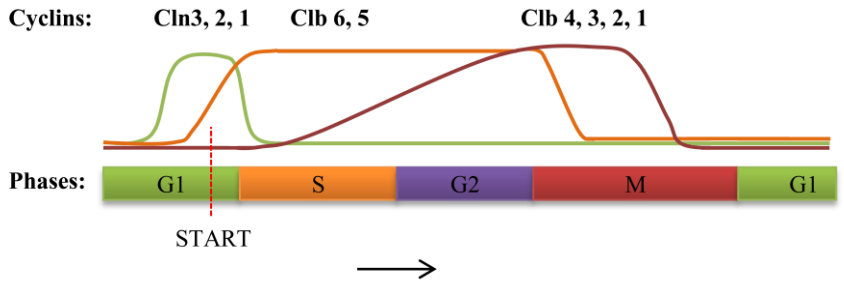


Figure 4. Cyclin oscillation during cell cycle. During G_1, yeast cells accumulate Cln3, Cln2 and Cln1. At the G_1/S transition, Clb6 and Clb5 start accumulating to promote S-phase progression and are not get degraded until the M-cyclins Clb4, Clb3, Clb2 and Clb1 drive the G_2/M transition and passage through mitosis. To finish cell division and to promote the entry into a new cell cycle, cells must degrade all cyclins and inhibit CDK activity.

In a regular cell cycle, CDK activity fluctuation allows to timely control different aspects of DNA replication and cell division. For instance, at the beginning of the G_1 phase, the CDK activity is very low which allows the recognition of the origins of replication (Araki, 2010; Evrin et al., 2009; Remus et al., 2009). During S phase, the high CDK activity phosphorylates and activates origins (licensing) to promote replication and, simultaneously, it prevents the recognition of new origins, which is essential to avoid the re-replication of DNA (Schwob and Nasmyth, 1993; Tanaka et al., 2007). Therefore, the cascades of cyclin-CDK complexes drive cell cycle progression and establish the basic machinery to control cell cycle.

There is a second layer of control that corresponds to the TFs involved in cyclin expression and to the CDK inhibitors. Multiple genes involved in the G_1/S transition are controlled by the TFs SBF and MBF. SBF is composed of Swi4 (DNA-binding) and Swi6, and it is required to activate the G_1/S transcripts during G_1. On the other hand, MBF is composed of Mbp1 (DNA-binding) and Swi6, and it is required to repress the G_1/S transcripts out of G_1. These TFs coordinately regulate the expression of many cell cycle-regulated genes to promote progression trough G_1 and the G_1/S transition (Futcher, 2002; Nasmyth, 1996).

Several CKi exist in budding yeast, such as Far1, Sic1 and Swe1. Far1 inhibits G_1-CDK complexes and arrests cells in G_1 in response to mating pheromone (McKinney and Cross, 1995; Peter et al., 1993). This is extremely important, as mating between haploid cells only occurs between cells in G_1 phase. Sic1 is expressed at the end of the M phase and its levels remain high until the G_1/S transition, when Cln1/Cln2-Cdc28 and Clb5/Clb6-Cdc28 complexes promote its degradation. Sic1 binds and inhibits S-CDK complexes to prevent premature S-phase entry (Lengronne and Schwob, 2002; Schwob et al., 1994). Swe1 is expressed during S phase until the M phase, when M-CDK promotes its degradation. Swe1 phosphorylates Cdc28 to inhibit M-CDK complexes during the G_2-M transition (Asano et al., 2005). Interestingly, several signaling pathways have been shown to target CKIs in order to regulate cell cycle. For instance, as mentioned before, the Hog1 SAPK phosphorylates and stabilizes Sic1 to prevent G_1/S transition upon

osmostress (Escoté et al., 2004) and, also, it promotes the accumulation of Swe1 in G_2 upon osmostress (Clotet et al., 2006).

The checkpoint pathways constitute another layer of cell cycle regulation (Hartwell and Weinert, 1989). Cell cycle checkpoints are surveillance signal transduction pathways that monitor and control the major events of the cell cycle. Cells size, genome integrity, chromosome segregation and nutritional status are monitored by specific checkpoint pathways to coordinate cell cycle events accordingly. One example would be the canonical DNA damage checkpoint (DDC) pathway, which is activated in response to DNA damage (Zhou and Elledge, 2000). The presence of DNA damage is sensed and this signal leads to the activation of the upstream kinase Mec1 (ATR). Then, Mec1 is assisted by the mediator protein Rad9 to phosphorylate and activate the effector kinase Rad53 (Chk2), which arrests cell cycle and promotes DNA repair. One of the most important checkpoint pathway controls the G_1/S transition, also known as START, which is a point of no return of the cell cycle (restriction point in mammals). During G_1, the cell size increases and this is monitored to prevent passage trough START before cells reach an optimal volume (Ferrezuelo et al., 2012; Hartwell and Unger, 1977; Johnston et al., 1977; Jorgensen and Tyers, 2004; Leslie, 2011). Yeast cells can stop before START while waiting for more nutrients or non-hostile environments but, once cells cross START, they are committed to enter S phase and complete the next cell cycle (Johnson and Skotheim, 2013; Johnston and Singer, 1983). Therefore, multiple checkpoints exist to control crucial steps

of cell cycle and to adequate them to nutritional status or environmental stress.

2.3 The S phase and Mrc1

During S, cells replicate their DNA (Longhese et al., 2003). In all eukaryotic chromosomes, DNA replication starts simultaneously from several origins of replication distributed all along the genome. The activation of DNA replication is a multistep process that starts in G_1 with the assembly of the pre-replication complexes (pre-RCs) (Araki, 2010; Evrin et al., 2009; Remus et al., 2009). However, origin firing occurs only in S phase when these are activated by two essential S-phase kinase activities (S/CDK and DDK) (Sheu and Stillman, 2006; Tanaka et al., 2007; Zegerman and Diffley, 2007). In the S phase, the coordinated activities of Dbf4-Cdc7 (DDK) and Clb5/Clb6-Cdc28 (S-CDK) complexes allows the recruitment and activation of proteins to form an active Cdc45-MCM-GINS (CMG) replicative helicase (Figure 5; Schwob and Nasmyth, 1993; Tanaka et al., 2007; Yeeles et al., 2015).

Two functional CMG helicases start unwinding the DNA in opposite directions from the replication origins. Once the CMG is assembled, many other proteins are recruited to form two RPCs. The RPC includes Pol α, Mrc1, Csm3, Tof1, Ctf4, FACT and Topo I (Gambus et al., 2006). Pol δ and PCNA also localize at the forks but do not co-purify with the other RPC elements (Yeeles et al., 2017; Yu et al., 2014). In eukaryotic cells replication is achieved by three DNA polymerases (Pol α, ε, and δ). Pol α is the primase required to initiate replication from both leading and lagging strands (Foiani et al., 1997). Pol ε replicates the leading strand while Pol δ replicates

the lagging strand, however, recently it has been proposed that Pol δ replicates the leading strand before Pol ε engaging (Pursell et al., 2007; Yeeles et al., 2017). The FACT complex is a histone chaperone that promotes chromatin remodeling to permit replication (Formosa, 2012; Reinberg and Sims, 2006). The PCNA complex increases Pol ε and Pol δ processivity (Chilkova et al., 2007; Yeeles et al., 2017). Among the other proteins of the RPC, Tof1, Csm3 and Mrc1 have been reported to be involved in the stability of the replication complex upon DNA obstacles and other insults that can alter the normal progression of the replication fork (Alcasabas et al., 2001; Bando et al., 2009; Katou et al., 2003; Szyjka et al., 2005; Tourrière et al., 2005).

Mrc1 was initially described as the Mediator of the Replication Checkpoint pathway. In response to replication stress, Mrc1 mediates the activation of the protective DNA-replication checkpoint (DRC) pathway. By a still unclear mechanism, phosphorylation of Mrc1 by Mec1 promotes the recruitment, auto-phosphorylation and full activation of the kinase Rad53 by Mec1 (Alcasabas et al., 2001; Osborn and Elledge, 2003). Rad53 is the effector kinase of the DRC pathway that stabilizes replication forks, delays late origin firing and promotes activation of repair mechanisms to reverse replication stress (Quivy and Almouzni, 2003). The proteins Csm3 and Tof1 help to recruit Mrc1 to the fork and are also involved in replication and checkpoint signaling. Altogether, Mrc1, Tof1 and Csm3 form the MTC fork protection complex (Bando et al., 2009; Katou et al., 2003; Lewis et al., 2017; Uzunova et al., 2014). Eventually, cells must recover from

checkpoint activation and restart cell cycle. Interestingly, the degradation of Mrc1 facilitated by the $SCF^{(Dia2)}$ complex promotes checkpoint recovery and cell cycle restart (Buser et al., 2016; Chaudhury and Koepp, 2016; Fong et al., 2013; Maculins et al., 2015).

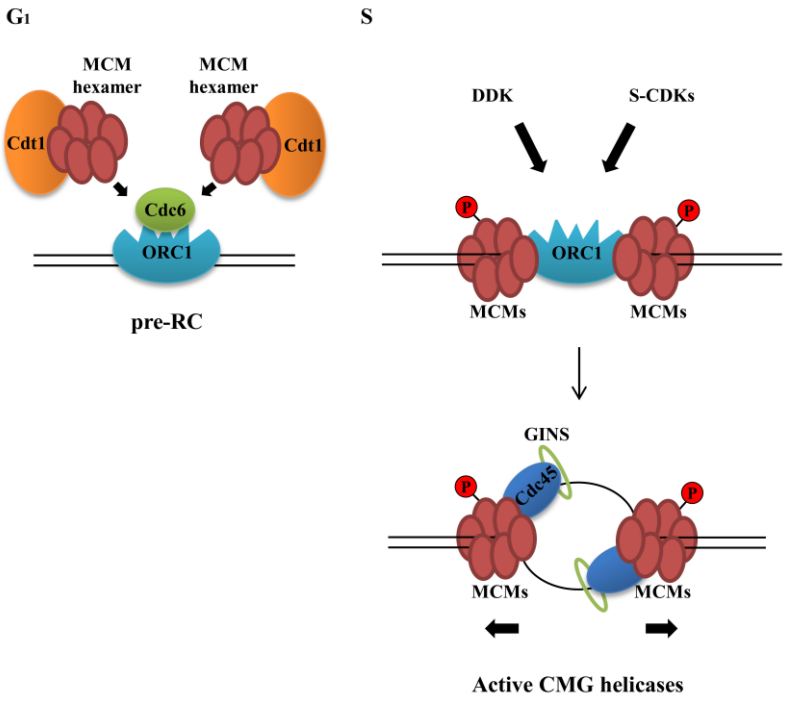


Figure 5. Two-step activation of the replication origins. In G_1, loading of two MCM hexamers on DNA leads to the formation of pre-Replication (pre-RC) complexes, which is assisted by the proteins Orc, Cdc6 and Cdt1. Later, in S phase, DDK and S-CDK kinases promote the loading of GINS and Cdc45 factors which eventually lead to the activation of the replicative CMG helicase. Multiple factors are involved in the activation of the CMG helicase and origin firing.

Mrc1 has other important roles in addition to those regarding the activation of DRC. Mrc1 associates with Pol ε and CMG helicase

and it is a key determinant of the rate of DNA replication (Gambus et al., 2006; Lou et al., 2008; Nedelcheva et al., 2005; Yeeles et al., 2017). Also, it has been proposed that Mrc1 might prevent the uncoupling between polymerase and helicase functions (Labib, 2008; Lou et al., 2008), although more recent work claims that uncoupling is prevented by the direct contact of the leading strand polymerase and helicase, similarly to what occurs in bacteria (Sengupta et al., 2013). In the lagging strand, Ctf4 binds to MCMs and DNA polymerase α and it has been shown to directly prevent the uncoupling between helicase and polymerase activities (Gambus et al., 2009). In any case, *mrc1* cells are viable but progress slowly through S phase and accumulate genomic instability both in the absence or the presence of external insults and display activation of the DDC pathway (Tourrière et al., 2005).

As commented previously, our group reported a novel function of Mrc1 to protect genomic integrity upon osmostress via the phosphorylation of Mrc1 by Hog1. We showed that the N-terminal phosphorylation of Mrc1 by Hog1 upon osmostress leads to an S-phase delay to prevent transcription-associated recombination (TAR) and genomic instability (Duch et al., 2013a). Therefore, Mrc1 plays several important functions to regulate replication progression in response to internal (replication stress) and external (osmostress) signals, to prevent genomic instability (Figure 6).

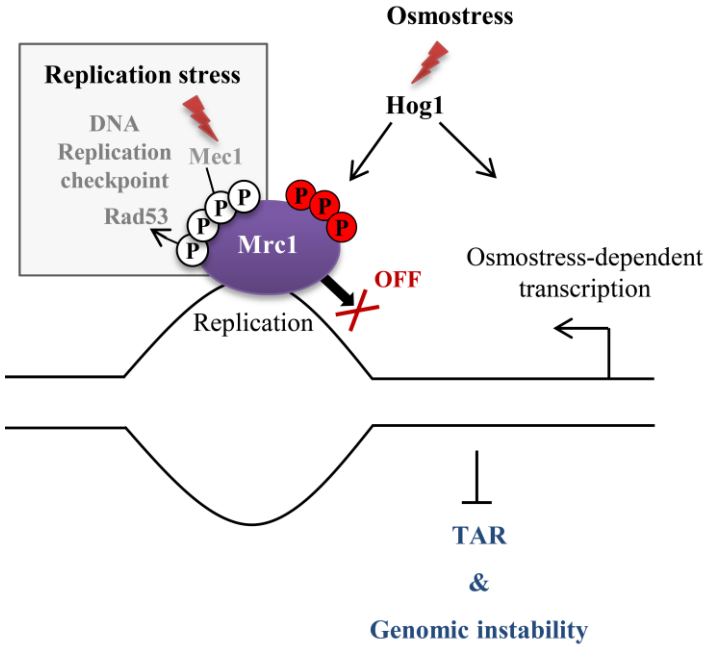


Figure 6. Mrc1 integrates signals from replication stress and osmostress to regulate replication progression and prevent genomic instability. Mrc1 is independently phosphorylated in different residues in response to internal (replication stress) and external (osmostress) stimuli, which regulates S-phase progression and prevents genome instability. Upon osmostress, Mrc1 phosphorylation delays replication progression and prevents transcription-associated recombination (TAR) and genomic instability.

2.4 Transcription-Replication Conflicts (TRCs)

Multiple obstacles can interfere with replication in a regular S phase, which may lead to replication stress. Replication stress is any impediment that interferes with the normal activity or progression of the replication fork. The prototypical cause of replication stress is a decrease on the levels of dNTPs that can be induced by drugs such as hydroxyurea, and even transcription can be a major source of replication stress. Transcription can induce replication stress directly interfering with the replication machinery via the ongoing

transcription machinery bound to DNA or by the generation of indirect obstacles such as topological stress or RNA:DNA hybrids (R-loops) (Hamperl and Cimprich, 2016). Therefore, transcription and replication must be strictly coordinated to prevent genomic instability caused by the transcription-replication conflicts (TRCs). TRCs can be in a head-on or a co-directional orientation (Figure 7). During S phase, replication is initiated from multiple origins of replication distributed all along the genome and, simultaneously, transcription remains active forcing transcription and replication machineries to share DNA as substrate.

Albeit both types of TRCs can induce severe genomic repercussions, the specific consequences vary with the relative orientation of the transcription and replication machineries, as head-on encounters tend to be more dangerous. All organisms have evolved different strategies throughout evolution to prevent TRCs and preserve genomic integrity. For example, prokaryotic genomes cannot avoid TRCs but have evolved to favor co-directional over head-on conflicts to reduce genome instability. They have a circular chromosome whose replication originates from a single bidirectional origin of replication. Of note, there is a bias in the direction of transcription of most essential genes in these genomes to promote only co-directional encounters between transcription and replication machineries (Brewer and Fangman, 1988; Guy and Roten, 2004; Kunst et al., 1997). Interestingly, experiments done in bacteria, where some highly expressed genes (such as the rDNA genes) have been turned around to face replication in a head-on orientation, have shown that head-on conflicts generate devastating

consequences, impair rDNA transcription, induce genome instability and cause cell death (Srivatsan et al., 2010). Therefore, even TRCs cannot be avoided in bacteria, these cells have evolved a system to maintain genome stability by regulating the direction of transcription.

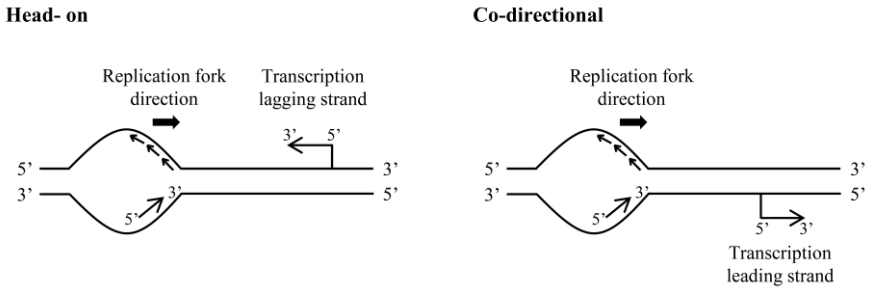


Figure 7. Head-on and co-directional TRCs. Transcription and replication can encounter each other in a head-on (opposite) or a co-directional (same) orientation.

In eukaryotes, DNA replication initiates from hundreds of origins that fire all over the genome in a timely controlled manner. Still the full map of origins is unknown in most eukaryotes, especially in higher eukaryotes, but it is clear that not all origins fire in every cell cycle (dormant origins), and not all of them fire simultaneously (early and late origins) (Raghuraman et al., 1997; Santocanale et al., 1999; Woodward et al., 2006). The cell cycle in eukaryotes with different phases temporally restricts TRCs to the S phase, as replication is only active in this phase. Therefore, transcription in G_1, G_2 and M phases can occur without the risk of originating TRCs. Remarkably, genes that replicate early in S phase tend to be transcribed later in this phase and vice versa, suggesting a temporal separation between replication and transcription also during S phase

(Meryet-Figuiere et al., 2014). However, still DNA polymerase takes longer to travel through open reading frames (ORFs) of highly transcribed genes, suggesting an interference between RNA polymerase II (RNAPII) transcription and replication complexes (Azvolinsky et al., 2009; Tuduri et al., 2009). There are certain locations in the genome where TRCs seem unavoidable. For instance, the longest human genes can take longer than one cell cycle to be transcribed, and therefore replication and transcription irremediably travel simultaneously through these genes, which have a high risk to experience TRCs. Interestingly, some of these long genes overlap with sequences prone to breakage known as common fragile sites (CFSs) (Helmrich et al., 2011; Le Tallec et al., 2014).

It is not clear whether eukaryotic genomes are also organized to prevent global head-on conflicts as occurs in prokaryotic genomes. Albeit there is not an apparent bias in transcriptional direction to prevent head-on conflicts in *S. cerevisiae*, in the human genome, replication originated from dormant origins in response to replication prevents an increase on the rate of head-on conflicts, suggesting a preference for co-directional TRCs in an unperturbed S phase (McGuffee et al., 2013; Petryk et al., 2016). Albeit the orientation of transcription and replication is generally random in yeast, it is known that some specific regions are protected from head-on TRCs. For instance, the replication-fork barriers (RFB) are a specific structure present in the rDNA locus that prevents head-on TRCs. The rDNA locus is located in the nucleolus and consists of a track of multiple repeats of the 35S and 5S rRNA-encoding genes, which are constantly transcribed to produce all the ribosomes of the

cell. Each rDNA repeat contains its own origin of replication and an RFB sequence, which is bound by the protein Fob1 causing a polar block of replication (Figure 8; Brewer et al., 1992; Linskens and Huberman, 1988). Therefore, this protein structure permits rDNA genes to be transcribed during the S phase without the risk of experiencing head-on TRCs. Other strategies to prevent head-on TRCs have also been described for sites such as those for tRNA genes (Labib et al., 2007).

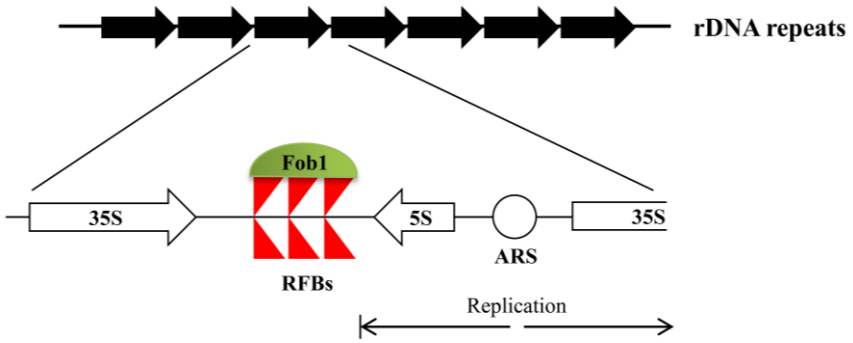


Figure 8. Replication-fork barrier (RFB) of the yeast rDNA locus. Replication of each repeat originates from their origin in both directions. The forks stop at the Fob1-bound RFB to prevent head-on conflicts between replication and transcription of the 35S or 5S ribosome subunits of the following repeat.

In prokaryotes, problems such as replication fork stalling or even removal of replication elements from DNA can be solved by replacing the essential components to resume replication. In eukaryotes, origin licensing is a two-step process that initiates in G_1 (low CDK) and finishes in S phase (high CDK) to prevent re-replication. Therefore, eukaryotes cannot afford removal of the replisome, as it would be irreplaceable until the following cell cycle. To stabilize replication forks and regulate the activation of

the accessory origins, eukaryotic cells activate the DRC pathway that alleviates TRCs by the neutralization of topological stress and the removal of R-loops (Barlow et al., 2013; Casper et al., 2002; Gómez-González et al., 2009, 2011).

2.4.1 TRCs induced by stress

Transcription is a dynamic process that changes constantly during the life of a cell to guarantee the adaptation to changing circumstances. For instance, as described previously, cells activate dedicated transcriptional programs to adapt and survive to internal and external stresses. Therefore, cells have a high risk to suffer TRCs when stress-transcription is activated in S phase.

A good example of a mechanism to prevent TRCs induced by stress responses is the mechanism that we described upon osmostress (Duch et al., 2013a). Upon osmostress, the Hog1 SAPK promotes the activation of hundreds of genes that hinder genome stability when activated during S phase. Moreover, we showed that Hog1 phosphorylates Mrc1, which delays replication progression and prevents transcription-associated recombination (TAR) and genomic instability. Correspondingly, a mutant of Mrc1 with the residues T169, S215 and S229 mutated to Alanine (*mrc1*^{3A}) bypassed Hog1 phosphorylation and was unable to delay S-phase progression, resulting in TAR and genomic instability in response to osmostress. We also saw that *mrc1*^{3A} cells required proficient DDC pathway to survive upon stress, as deletion of the kinase Mec1 rendered the mutant cells sensitive to osmostress.

Therefore, Mrc1 is a key protein in the regulation of DNA replication that is required to maintain an adequate fork rate. Accordingly, Mrc1 is also crucial to respond to signals from replication stress (Mec1) and osmostress (Hog1) to protect genomic integrity. In this thesis, we studied whether other environmental stress in addition to osmostress, and cellular insults that induce transcriptional outbursts, required Mrc1 to prevent TRCs and genomic instability.

OBJECTIVES

Our group is interested in understanding the cell response to environmental stress. In this thesis, we aimed to study whether the role of Mrc1 in preventing genomic instability upon osmostress was a general mechanism to prevent transcription-replication conflicts and genomic instability upon outbursts of transcription that occur during S phase.

Specifically, the main objectives of this PhD were:

1. Characterization of the Mrc1 mechanism upon heat and oxidative stresses to prevent genomic instability.

2. Identification of novel kinases other than Hog1 able to phosphorylate Mrc1 in response to different stresses.

3. Study of alternative sources of transcriptional outbursts that rely on Mrc1 to prevent genomic instability.

RESULTS AND DISCUSSION

Duch A, Canal B, Barroso SI, García-Rubio M, Seisenbacher G, Aguilera A, et al. Multiple signaling kinases target Mrc1 to prevent genomic instability triggered by transcription-replication conflicts. Nat Commun. 2018 Dec 25;9(1):379. DOI: 10.1038/s41467-017-02756-x

RESULTS AND DISCUSSION

The interaction between replication and transcription that occurs during S phase is inevitable. Some aspects of this interaction are beneficial for the cell while others have damaging consequences. For instance, it has been proposed that changes in the chromatin mediated by transcriptional activation facilitate firing of replication origins (Barlow and Nussenzweig, 2014). In contrast, replication forks often are blocked as a consequence of ongoing transcription. These are known as transcription-replication conflicts (TRCs) and lead to genomic instability, a hallmark of cancer (Bermejo et al., 2012; Duch et al., 2013b; García-Muse and Aguilera, 2016; Hamperl and Cimprich, 2016). In eukaryotes, transcription is active in all phases of cell cycle but replication is only activated during S phase, which temporarily restricts TRCs. Cells have mechanisms to prevent TRCs and repair their effects. As described in the introduction, prokaryotic genomes are organized to favour co-directional TRCs as most of their essential genes are encoded in the leading strand (Merrikh et al., 2012; Srivatsan et al., 2010). In contrast, in higher eukaryotes, there is no obvious bias for co-directional TRCs but cells have evolved sophisticated mechanisms to spatially and temporally regulate gene expression and coordinate it with replication. Nevertheless, the coordination of these crucial processes is still poorly understood.

Although some mechanisms to prevent TRCs have been described, not many are known to be dedicated to stress responses (Duch et al., 2013b; Hamperl and Cimprich, 2016). Unscheduled transcription can be activated during S phase by diverse cellular insults such as

environmental stress. In response to stress, cells activate a common environmental stress response (ESR) but also induce specific transcriptional programs that differ in every stress. *Causton et al.,* proposed that the sum of stress-responsive genes correspond to up to 60% of all yeast genes. Therefore, stress-responses cause global rearrangements of gene transcription that, during S phase, can induce TRCs and genomic instability. We previously described a mechanism that prevents TRCs and genomic instability in response to outbursts of transcription induced by osmostress (Duch et al., 2013a). In response to osmostress, the Hog1 SAPK is activated and phosphorylates the sites T169, S215 and S229 of Mrc1, which causes a delay of replication and prevents transcription-associated recombination (TAR) and genomic instability. The non-phosphorylateble mutant of Mrc1 (*mrc1*^{3A}) bypasses Hog1 phosphorylation upon osmostress, is unable to block cells in S phase and accumulates TAR and genomic instability.

Remarkably, Mrc1 and also other proteins of the RPC such as the DNA polymerase have a dual role in the replication fork. They promote replication progression in undisturbed conditions and they are also involved in the activation of the DNA replication checkpoint (DRC) in response to replication stress. The localization of those checkpoint proteins at the RPC facilitates the fast *in situ* detection and repair of the damage caused by replication stress. We believe that the function of Mrc1 in delaying DNA replication and preventing TRCs upon osmostress also benefits from its key localization at the fork. However, it is important to highlight that the functions of Mrc1 as mediator of the DRC and its function as a

Hog1 target upon osmostress are fully independent from each other and can be genetically separated. Two mutants of Mrc1 clearly allow the separation of these functions; the *mrc1*^{AQ} (17 Mec1-sites S/T-Q mutated to Alanine) and the *mrc1*^{3A} (T169, S215 and S229 S/T-P sites mutated to Alanine) mutants. In contrast to *mrc1*^{3A}, which bypasses the S-phase delay upon osmostress but it is fully proficient to delay S phase upon HU (Duch et al., 2013a), the mutant *mrc1*^{AQ} fails to activate the DRC pathway in the presence of HU but it is fully able to delay S phase upon osmostress (Supplementary Figure 1D-E; Duch et al., 2013a; Lou et al., 2008). Correspondingly, a *mrc1*$^{AQ+3A}$ mutant is deficient in both DRC and osmostress-dependent S-phase delays (Supplementary Figure 1D-E). Interestingly, the three mutants *mrc1*^{AQ}, *mrc1*^{3A} and *mrc1*$^{AQ+3A}$ replicate as wild type cells in unstressed conditions, unlike *mrc1* cells that replicate to half of the speed of wild type cells (Szyjka et al., 2005; Tourrière et al., 2005; Yeeles et al., 2017). Thus, the three functions of Mrc1 in replication progression, DRC and osmostress are independent from each other, and suggest that Mrc1 could be an integrator of multiple stress signals to adequate the replication speed to changing environments.

With the aim of exploring whether the role of Mrc1 in preventing genomic instability upon osmostress was a general mechanism, we analyzed the role of Mrc1 upon stresses other than osmostress that cause sudden outbursts of transcription. Initially, we chose to study heat and oxidative stresses (Causton et al., 2001; Gasch et al., 2000). As shown in Figures 1A and 1B, we found that Mrc1 was phosphorylated in response to heat and oxidative stresses,

suggesting that the role of Mrc1 to protect genomic instability upon osmostress is conserved also upon those stresses.

Accordingly, we assessed whether the phosphorylation of Mrc1 upon heat and oxidative stresses was also delaying replication progression, as previously shown upon osmostress. To study S-phase progression upon heat and oxidative stresses, DNA replication was assessed. We usually synchronize cells in S phase with a $cdc7^{ts4}$ thermo-sensitive allele. Cells at the restrictive temperature (≥37ºC) degrade Cdc7 and synchronize at the onset of S phase, as Cdc7 is the kinase that conforms the DDK, which is essential for origin firing (Donaldson et al., 1998; Meddle et al., 1985; Wan et al., 2006). However, to study the response to heat stress (37ºC), a new synchronization method had to be established. We used the auxin-inducible (AID) degron system to induce the degradation of Cdc7 with auxin (Nishimura et al., 2009; Tanaka et al., 2015). We tagged the endogenous *CDC7* gene with three copies of the MiniAID tag and integrated a plasmid containing the adaptor protein Tir1 from *Oryza sativa* under the control of the *ADH1* promoter to induce its constitutive expression (Tanaka et al., 2015). With this system, upon the addition of auxin (IAA) to the media, the F-box protein Tir1 binds the AID tag on Cdc7, which leads to the binding of the SCF E3 ubiquitin ligase complex and the E2 ubiquitin-conjugating enzyme to Cdc7 which promotes its degradation by the proteasome. Therefore, the new strain ($cdc7^{AID}$) synchronized at the onset of S phase in response to the addition of auxin and it could be used to analyse replication progression upon heat stress. Altogether, the results shown by FACS, WB and

combing assays in Figures 1C-1H indicated that the phosphorylation of the Hog1-sites of Mrc1 that occured upon heat and oxidative stresses delayed DNA replication.

To adapt and survive to stress, cells massively activate hundreds of stress-dependent genes. In S phase, massive induction of genes can cause TRCs and lead to TAR and genomic instability. Therefore, we studied whether TAR increases upon heat and oxidative stresses. To assess TAR, we transformed cells with an episomal plasmid (modified from Prado and Aguilera, 2005) that contains a *leu2* direct repeat under the control of the stress-responsive *CTT1* promoter, and a yeast origin of replication. The *CTT1* promoter contains several STRE elements and it is activated by the Msn2 and Msn4 TFs in response to osmo, heat and oxidative stresses (Gasch et al., 2000; Marchler et al., 1993; Martínez-Pastor et al., 1996; Schüller et al., 1994; Winkler et al., 1988). We used two variants of the plasmid in which the direction of the *CTT1* promoter changes relative to the origin of replication: the head-on (IN) and the co-directional (OUT) plasmids. The N-terminal phosphorylation of Mrc1 upon heat and oxidative stresses prevented the accumulation of TAR (Figure 2A). Correspondingly, TAR is only observed in cells containing the IN-plasmid, in which putative conflicts would occur in a head-on direction. Moreover, we confirmed that TAR was dependent on stress-activated transcription, as it was prevented in an *msn2msn4* strain (Figure 3A). One important limitation of the TAR assay is that it measures the recombination that occurs in an episomal plasmid and not direct changes in the genome of a cell. Therefore, to further characterize genomic consequences of TRCs,

we analyzed genomic instability by using two independent techniques: a Red-sectoring assay and a Rad52-YFP *foci* assay.

Cells experiencing genomic instability tend to lose parts or whole chromosomes, which can be measured by a Red-sectoring assay (Hieter et al., 1985). To perform the Red-sectoring assay, we used an *ade2* strain that is white while it contains an episomal plasmid that expresses the *SUP11* gene, and becomes red when the plasmid is lost. Therefore, the quantification of colonies that turn red is a measure of plasmid loss, which reflects the levels of chromosome instability in each cell. We performed Red-sectoring assays upon heat and oxidative stresses, and found that the phosphorylation of Mrc1 is essential to prevent the accumulation of red colonies (Figure 2B). This indicated that, accordingly to what was known for osmostress, the phosphorylation of Mrc1 prevents plasmid loss caused by genomic instability also upon heat and oxidative stresses. Analogously, we performed Rad52-YFP *foci* assays to quantify DNA damage upon stress. Rad52 is involved in the repair of DNA double strand breaks, and it localizes in regions of damage to promote repair (Lisby et al., 2003). Therefore, the percentage of cells-containing Rad52-YFP *foci* is a measure of the levels of genomic instability induced by DNA damage. As seen upon osmostress, the phosphorylation of Mrc1 prevented the accumulation of Rad52-YFP *foci* upon heat and oxidative stresses (Figure 2C). Moreover, we quantified Rad52-YFP *foci* in a *msn2msn4* strain and observed that their accumulation is dependent on stress-dependent transcription (Figure 3B). Altogether, these

results indicated that the phosphorylation of Mrc1 prevents TAR and genomic instability upon heat and oxidative stresses.

The accumulation of genomic instability in *mrc1*^{3A} cells upon osmostress does not render cells osmosensitive. However, *mrc1*^{3A} cells require the kinase Mec1 (ATM) to survive upon osmostress (Duch et al., 2013a). Analogously, *mrc1*^{3A} cells were not sensitive to heat and oxidative stresses, but showed a growth defect in combination with *MEC1* (ATR) mutation (Figure 2D). Mec1 is the upstream kinase of the DNA-damage checkpoint (DDC) and, therefore, the growth defect seen upon stress of the *mrc1*^{3A}*mec1* mutant suggested the need for an intact DDC pathway to survive upon heat and oxidative stresses (Longhese et al., 1998; Tercero and Diffley, 2001; Zhou and Elledge, 2000). Taken together, these results indicated that the Mrc1 mechanism previously described for osmostress is indeed a general mechanism to prevent genomic instability caused by TRCs in response to osmo, heat and oxidative stresses and we proposed to call it "Mrc1 transcription-replication safeguard mechanism (MTR)".

Hog1 phosphorylates three N-terminal residues of Mrc1 in response to osmostress (Duch et al., 2013a). However, Hog1 is not activated in response to heat or oxidative stress (Supplementary Figure 3A). To discover additional kinases targeting Mrc1, we performed an unbiased kinome screening, and found that six kinases (Hog1, Mpk1, Psk1, Snf1, Pho85 and Ste20) specifically phosphorylated the T169, S215 and S229 of Mrc1 *in vitro* (Figure 4A). One of the kinases found in the screening was Hog1, which served as an internal control and validated the screening. The kinases Mpk1 and

Psk1 that also appeared in the screening are known to regulate responses to heat and oxidative stresses respectively and, therefore, we chose them as potential candidate kinases to regulate the MTR safeguard mechanism upon those stresses.

Mpk1 is the SAPK of the cell wall integrity (CWI) pathway and it is involved in the response to heat stress (Hahn and Thiele, 2002; Kamada et al., 1995; Mattison et al., 1999). To study the role of Mpk1 in the MTR mechanism upon heat stress, we used a deletion mutant of *MPK1* (*mpk1*). The deletion of Mpk1 is viable at 25ºC, but cells are very sensitive to any cell wall-damaging agent. The sensitivity of *mpk1* strain can be osmotically compensated by growing them in media supplemented with 1M sorbitol. Addition of 1M sorbitol activates Hog1. However, we observed that if cells were continuously maintained in 1M sorbitol, they adapted and did not display activation of the stress response. Therefore, all the experiments that involve the *mpk1* strain were performed in sorbitol-containing media. Taken together, we showed that the Mpk1 SAPK interacts with and phosphorylates Mrc1 to delay replication progression and prevent genomic instability upon heat stress (Figures 4B-4F). Therefore, we concluded that while the Hog1 SAPK mediates the MTR safeguard mechanism upon osmostress, Mpk1 mediates the MTR upon heat stress.

Psk1 is a PAS-domain containing kinase involved in energy flux and protein synthesis that has been shown to protect cells against oxidative stress (Cardon et al., 2012; Grose et al., 2007, 2009; Huang et al., 2014). However, little is known about its specific role in the oxidative stress response. Here we showed that Psk1 is

activated in response to oxidative stress (Figure 5A and 5C), and it is able to phosphorylate Mrc1 (Figure 5C and 5D). Deletion of Psk1 prevents the delay of replication upon oxidative stress, and results in an increase of TAR and genomic instability (Figures 5B-5G). Therefore, we concluded that Psk1 is the kinase that mediates the MTR safeguard mechanism upon oxidative stress.

Interestingly, the kinase Snf1 was also identified in the screening. Snf1 is the yeast homolog of the mammalian AMP-dependent kinase (AMPK) and it leads the transcription and metabolic switch activated when cells are grown in non-glucose fermentative sources (Clark et al., 1993; Hedbacker and Carlson, 2008; Thompson-Jaeger et al., 1991). Snf1 is activated in response to low glucose, which is an environmental stress that also induces the ESR similarly to the osmo, heat and oxidative stresses (Supplementary Figure 7; Casamayor et al., 2012; Mayordomo et al., 2002; Thevelein and de Winde 1999; Zaman et al., 2009). Therefore, we studied the role of the MTR safeguard mechanism upon low glucose, and the role of Snf1. Here we showed that cells delay S-phase progression upon low glucose via the phosphorylation of Mrc1 by Snf1 (Figures 6A – 6F and Supplementary Figure 5). Snf1 prevents genomic instability upon low glucose by phosphorylating the Hog1-sites of Mrc1 (Figure 6G). Overall, our data indicated that Snf1 is another kinase able to activate the MTR safeguard mechanism to prevent genomic instability upon transcriptional outbursts induced by low glucose stress.

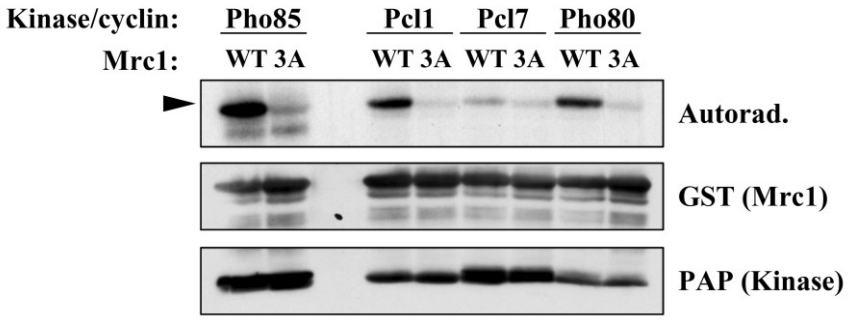
The kinases Hog1, Mpk1, Psk1 and Snf1 have been found to similarly activate the MTR safeguard mechanism in response to

stress. However, there are several differences among those kinases that must be considered. For instance, the Hog1-sites of Mrc1 are canonical MAPK and CDK consensus SP / TP sites (Mok et al., 2010). These sites are consensus sites for Hog1 and Mpk1, but are not optimal for Psk1 (DeMille and Grose, 2013) or Snf1 (Dale et al., 1995; Smith et al., 1999). In this sense, we confirmed in *Duch et al., 2013a* that Hog1 phosphorylates all three sites of Mrc1. However, we have not tested if the single mutants of Mrc1 are phosphorylated by Mpk1, Psk1 or Snf1 and, therefore, we cannot confirm if those phosphorylate one, two or all three sites of Mrc1 *in vivo*. It would be interesting to test this in detail to further characterize this mechanism. Another important difference among these kinases is that Hog1 has a dual role in response to osmostress; it phosphorylates Mrc1 and it induces the ESR. However, the Mpk1, Psk1 and Snf1 kinases phosphorylate Mrc1 but are not required to induce the ESR in response to heat, oxidative or low glucose stress. For instance, Mpk1 regulates the transcription of the CWI genes in response to cell wall damaging insults but it is not involved in the activation of the ESR in response to heat stress (Supplementary Figure 7; Hahn et al., 2004; Verghese et al., 2012). It is reported that activation of the ESR in response to heat stress is mainly mediated by PKA inactivation and activation of the Hsf1 TF (Fuchs and Mylonakis, 2009). Similarly, we showed that Psk1 and Snf1 are dispensable to trigger the ESR upon oxidative stress and low glucose respectively (Supplementary Figure 7), which is known to be mediated by the inhibition of PKA and mTOR pathways upon those stresses (Morano et al., 2012; Becket al., 1999; Görner et al.,

1998). Interestingly, we showed by northern blot that the expression of the canonical ESR gene *CTT1* is even higher in *snf1* cells than in wild type cells in unperturbed conditions, in accordance with Snf1 being a repressor of the Msn2 TF (Lenssen et al., 2005; Mayordomo et al., 2002; De Wever et al., 2005). Overall, we showed that the MTR mechanism is not specific for osmostress but, instead, several kinases are activated upon other stresses that induce unscheduled outbursts of transcription, and converge on Mrc1 to coordinate replication and transcription to prevent genomic instability.

Another kinase found in the screening is Pho85, a cell cycle CDK that regulates cell responses to nutrient levels and links cell cycle progression to environmental conditions (Carroll and O'Shea, 2002; Huang et al., 2007; Jiménez et al., 2013). The results from the screening showed that Pho85 specifically phosphorylates the N-terminal sites of Mrc1 *in vitro* (Figure 4A). However, Pho85 is not an obvious candidate since it is inhibited upon nitrogen or phosphate stress (Jiménez et al., 2013). Nonetheless, Pho85 is a really interesting candidate for its relevance in cell cycle control. We did some experiments beyond the manuscript to further analyze the relationship between Pho85 and Mrc1. First, we studied "which" Pho85 was phosphorylating Mrc1. Pho85 is a cyclin-dependent kinase and, therefore, its association to specific cyclins determines its specificity. There are two main families of cyclins that associate with Pho85: the Pho80 family, which is essentially involved in phosphate/nitrogen metabolism as well as environmental changes and the Pcl1/2 family, which is involved in cell cycle regulation (Jiménez et al., 2013). To narrow down which

cyclin of Pho85 was involved in regulating Mrc1, we selected a representative cyclin of each family and tested their ability to phosphorylate Mrc1 (Pho85/Pho80 and Pho85/Pcl1). In parallel, we also tested the Pcl7 cyclin (Pho85/Pcl7) because it is an interesting cyclin that, although it belongs to the Pho80 family, its expression is cell cycle regulated and peaks during S phase (Lee et al., 2000). We immunoprecipitated TAP-tagged Pho85 or the selected TAP-tagged cyclins and tested Mrc1 phosphorylation by an *in vitro* kinase assay. Strikingly, our results indicated that the representatives of both cyclin families (Pho80 and Pcl1) phosphorylate Mrc1 *in vitro*. Also, albeit to a lesser extent, Pho85 phosphorylated Mrc1 *in vitro* when associated with the cyclin Pcl7 (Extra Figure 1).



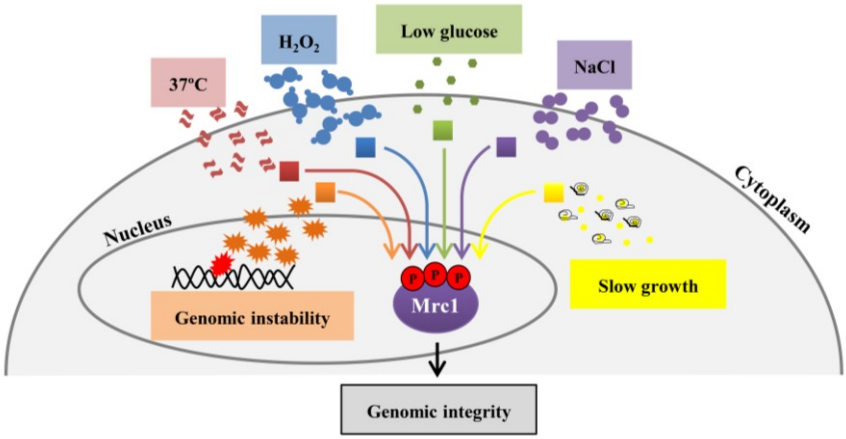
Extra Figure 1. Pho85/Pcl1 and Pho85/Pho80 phosphorylate the N-terminus of Mrc1 *in vitro*. Pho85, Pcl1, and Pho80 immunoprecipitated from the yeast TAP-tag collection phosphorylated the GST tagged N-terminal fragments of Mrc1 and *mrc1*^{3A} purified from *E. coli* in an *in vitro* kinase assay. Pcl7 failed to phosphorylate it.

These results will deserve further study in the near future. Another interesting question was whether Pho85 could phosphorylate Mrc1 in unperturbed conditions as Pho85 has been shown to have basal

activity. If this was the case, Pho85 could be phosphorylating Mrc1 in unperturbed conditions to regulate replication in unperturbed cells to adapt replication speed to, for instance, the available phosphate levels.

There are other physiological and pathological scenarios that compromise cell fitness, cause dramatic changes of transcription and induce the ESR in which cells would also require mechanisms to prevent genomic instability induced by putative TRCs. For instance, it was reported that mutants which grow slowly or mutants that accumulate genomic instability, display compromised cell fitness and induce the ESR response (O'Duibhir et al., 2014; Thorburn et al., 2013; Torres et al., 2007). Therefore, we studied the role of the MTR safeguard mechanism to prevent genomic instability in a slow growing (i.e. *ssn6*) and a genomically unstable (i.e. *rad53sml1*) mutants. The ESR is activated in unstressed conditions in those cells, which moreover display TAR and genomic instability when combined with the *mrc1*^{3A} allele. (Figures 7A and Supplementary Figure 6). Of note, the accumulation of TAR and genomic instability in the *ssn6mrc1*^{3A} and *rad53mrc1*^{3A} mutants was suppressed by deletion of the Msn2 and Msn4 TFs, indicating that, as found upon environmental stress, transcription causes TAR and genomic instability. These results open new and very interesting scenarios in which Mrc1 can play a role in preventing TAR and genomic instability beyond stress, adding new perspectives on the role of Mrc1 responding to outbursts of transcription during S phase. Overall, our results indicated that the MTR safeguard mechanism plays an essential role in protecting genomic integrity

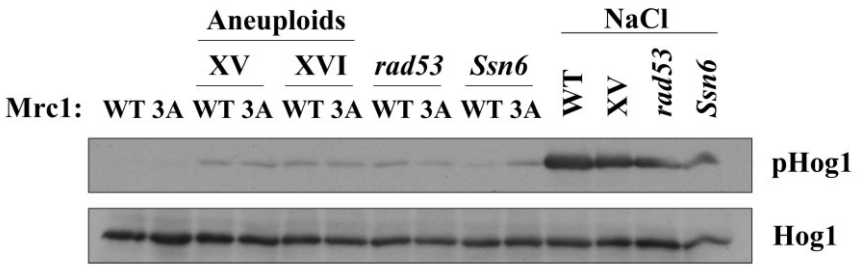
upon unscheduled outbursts of transcription that take place upon stressful conditions, either coming from extracellular or intracellular stress (Extra Figure 2).



Extra Figure 2. Schematic diagram of the MTR safeguard mechanism. Several kinases (coloured □) phosphorylate the N-terminus of Mrc1 upon external and internal insults. Phosphorylation of Mrc1 delays replication and prevents TAR and genomic instability.

We still do not know which kinase/s could mediate the MTR under slow growth or genomically unstable mutants. We cannot discard that some of the canonical SAPK could be active in these conditions that somehow resemble stress. Along this line, we have preliminary data suggesting that Hog1 is activated in these mutant cells and in other genomically unstable strains (aneuploids), albeit to a lesser extent than upon osmostress (Extra Figure 3). We did not test yet whether this low activation of Hog1 is sufficient to induce the MTR or if other kinases are activated in these mutants. On the other hand, there is the formal possibility that we did not identify these kinases due to the lack of activation when we purified them for the

screening protocol. Alternatively, some kinases require the coordination of multiple subunits to be active and therefore they could be inactive in our screening. It would be really interesting to find out kinases that phosphorylate Mrc1 in those complex scenarios.



Extra Figure 3. Hog1 is slightly activated in unstressed conditions in slow growth and genomic unstable strains. Hog1 phosphorylation in aneuploids (disomic strains for chromosomes XV or XVI), *rad53* or *ssn6* strains containing wild type Mrc1 (WT) or $mrc1^{3A}$ (3A). Hog1 phosphorylation upon osmostress (10 minutes 0.4M NaCl) is shown as control. Total Hog1 levels were monitored as loading control.

Of note, *rad53* cells display low levels of genomic instability (Myung et al., 2001), which is precisely one of the main features of mammalian cells at early steps of tumorigenesis. Therefore, if the role of Mrc1 was conserved throughout evolution, it could play an essential role preventing TAR and avoiding high levels of genomic instability in those pre-cancerous cells. The ESR was initially characterized as a common response to several environmental stress in *S. cerevisiae* (Causton et al., 2001; Gasch et al., 2000). However, similar transcriptional responses to stress have been shown in other yeasts. For example, the common environmental stress response (CESR) was described in *S. pombe* and it was shown to share high conservation with the ESR (Mata et al., 2002). In mammals, there is

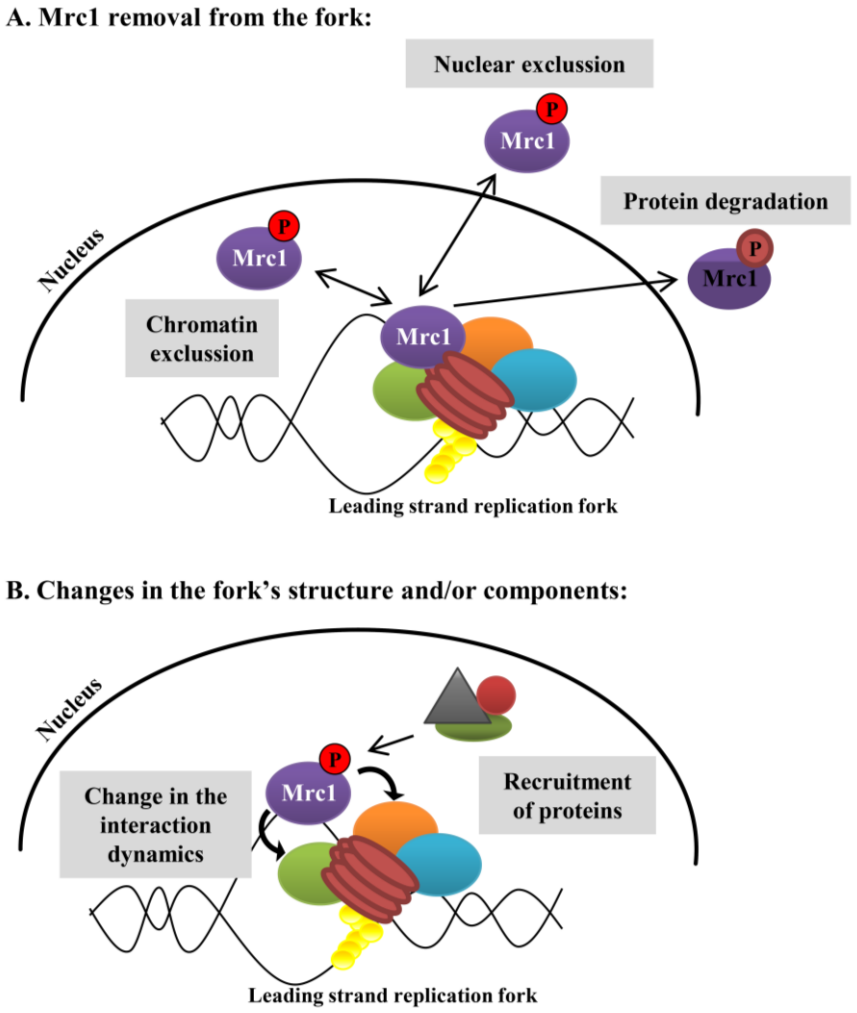
not a clear common set of stress-dependent genes albeit transcriptional responses have been described, for instance, upon heat, oxidative agents, chemotherapy, hypoxia or infectious agents. In spite of that, these transcriptional responses to stress share some features with the yeast ESR. As in yeast, mammalian cells subjected to stress inhibit the expression of the anabolic-promoting genes (Ribosomal, tRNAs…) to reduce energy expenses and release the transcription and translation machineries to permit the expression of the stress-protective genes (Budde and Grummt, 1999; Cairns and White, 1998; Ho and Gasch, 2015; Menendez et al., 2009; Nikulenkov et al., 2012; Wei et al., 2006; Zhai and Comai, 2000). Therefore, changes in transcription to respond to stress is a conserved feature that can endanger genomic instability in many species, and mechanisms to prevent it would be important to prevent genomic instability involved in early tumorigenesis in human cells.

We proposed that the MTR safeguard mechanism works as follows: (1) Mrc1 is phosphorylated upon stress, (2) the phosphorylation of Mrc1 delays replication progression and (3) the delay of DNA replication prevents TAR and genomic instability. However, there are other possible scenarios to be considered. For example, it could be that the phosphorylation of Mrc1, instead of preventing TAR and genomic instability directly, it could stimulate or recruit an unknown activity to repair the consequences of the TRCs. If this was the case, we would not find any difference with the results we have obtained. It would be interesting to study the presence of a repair pathway on the fork that could be recruited or activated in

response to the phosphorylation of Mrc1. Along this line, some candidates exist such as the helicases hRECQL5 (yeast Sgs1) and Rrm3 that associate with the replication fork and are involved in the repair and prevention of TRCs (Felipe-Abrio et al., 2015; Saponaro et al., 2014).

Another key remaining question is whether the delay of replication that is observed upon stress is a direct or indirect consequence of the phosphorylation of Mrc1. Interestingly, two recent papers directly linked Mrc1 to the control of replication speed. *In vitro* replication assays using yeast-purified proteins showed that Mrc1 is essential to achieve full-speed of the replisome (*in vivo* rate) demonstrating a direct role of Mrc1 to promote replication (Yeeles et al., 2017). Also, the fork protection complex integrated by Mrc1, Tof1 and Csm3 was shown to dynamically enter and exit the replication fork in a regular S phase, creating alternate phases of fast and slow replication respectively (Lewis et al., 2017). Therefore, when Mrc1 is not at the fork, replication slows down. Thus, the phosphorylation of Mrc1 in the MTR could delay replication by removing Mrc1 from the fork, either via chromatin exclusion, excluding it from the nucleus or by directly degrading it. Alternatively, the phosphorylation of Mrc1 could delay replication by changing the interaction of Mrc1 with its partners in the fork (Extra Figure 4). Although we still do not have the complete picture of the mechanism behind Mrc1's regulation, we have evidences suggesting that the phosphorylation of Mrc1 could delay replication by altering the interaction of Mrc1 with its partners. We have seen by ChIP that the mutant *mrc1*^{3D} is still loaded on the RPC,

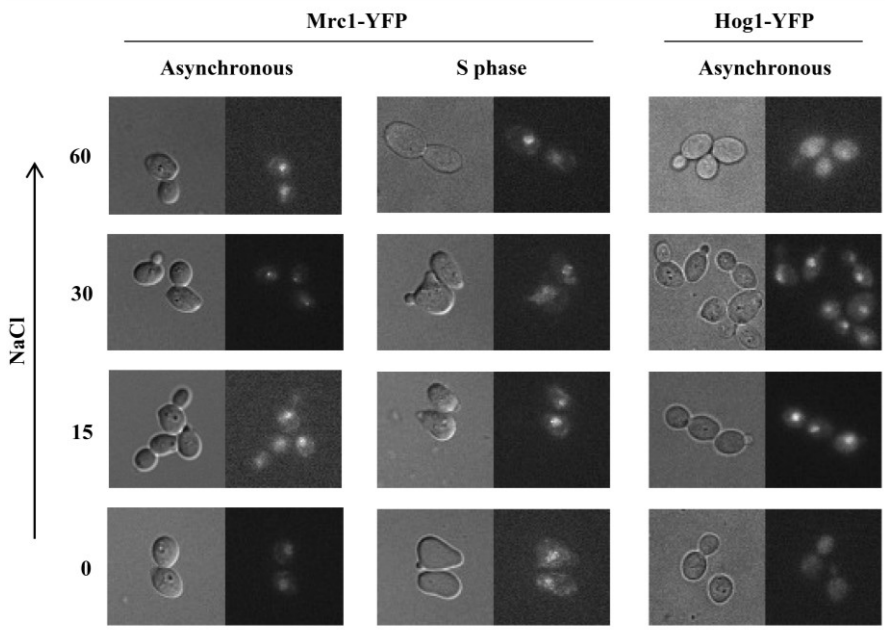
suggesting that phosphorylated Mrc1 remains in the fork (Duch et al., 2013a).



Extra Figure 4. The phosphorylation of Mrc1 could delay replication via multiple mechanisms. (**A**) Mrc1 could be removed from the replication fork by different mechanisms. (**B**) Alternatively, Mrc1 could change its interaction with other proteins to change the fork structure and/or components.

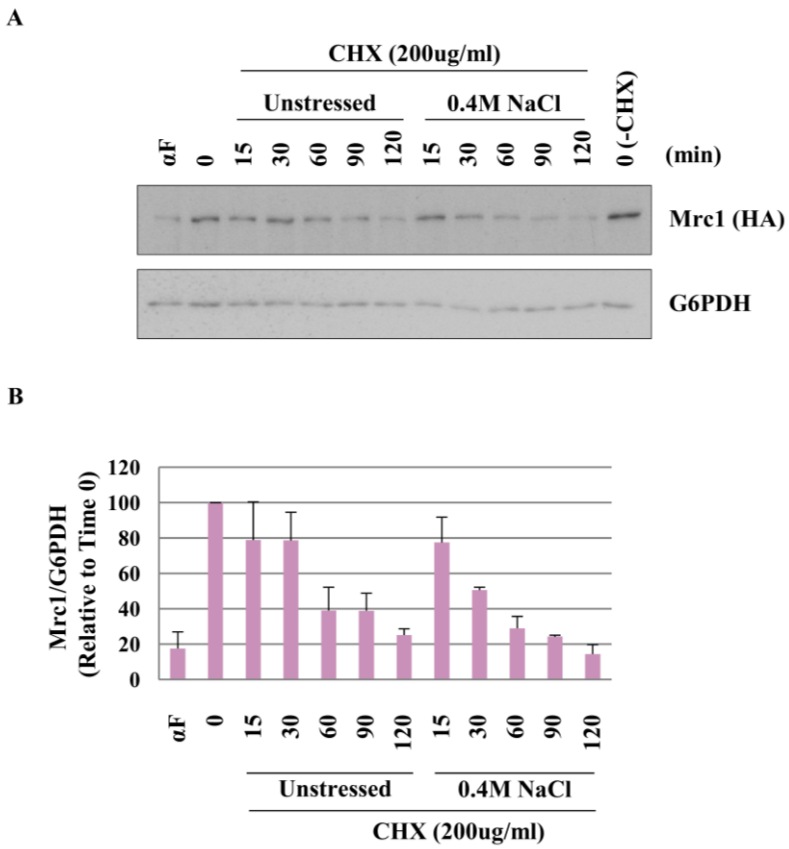
Correspondingly, we found that phosphorylation of Mrc1 does not exclude Mrc1 from the nucleus (Extra Figure 5), and that it neither

induce its degradation (Extra Figure 6). To test the direct effect of the phosphorylation of Mrc1 on replication speed, we could perform an *in vitro* replication assay using purified proteins to compare the rates of replication of Mrc1, *mrc1*^{3A} and *mrc1*^{3D}-containing RPCs.



Extra Figure 5. Mrc1 remains in the nucleus upon osmostress. Cells treated with osmostress were fixed and Mrc1-YFP or Hog1-YFP localization were assessed by microscopy. Mrc1-YFP cultures were synchronized in G_1 with αF and then released into S phase and stressed with 0.4M NaCl **(S phase)**.

Moreover, we showed that phosphorylation of Mrc1 upon osmostress changes its affinity to Pol2 subunit of the DNA polymerase (Duch et al., 2013a). It was proposed that mutations affecting the interaction between DNA Pol2 and Mrc1 cause a delay of replication (Lou et al., 2008), therefore, our observation could explain why stress-dependent phosphorylation of Mrc1 delays DNA replication.



Extra Figure 6. Osmostress does not induce the degradation of Mrc1. Cells were synchronized in G1 with αF (αF) and released into S phase (0). Then, cycloheximide (CHX) was added and the culture was unstressed or stressed with 0.4M NaCl. **(A)** Western blot showing the levels of Mrc1-HA. Glucose-6-phosphate dehydrogenase (G6PDH) was monitored as loading control. **(B)** Average of the quantification of three independent experiments using the image J software.

Major advances have been made to understand how the DNA is organized inside the nucleus and how replication and transcription are regulated. However, it is still not well understood how replication and transcription machineries are organized in the nucleus and whether they form stable replication and transcription

factories. The existence of such factories is supported by several observations and helps to explain the complex coordination between multiple pathways involved in correct replication and transcription (Chakalova et al., 2005; Kitamura, Blow, and Tanaka 2006; Osborne et al., 2004; Saner et al., 2013). For example, some authors have shown by fluorescent labeling of the replication forks that the number of active replication forks far exceeds the number of *foci* observed, suggesting that multiple forks would accumulate into replication factories (Kitamura et al., 2006). Understanding how freely the replication and transcription forks are to move inside the nucleus is crucial to study transcription-replication conflicts. Along this line, a complex spatial organization of the DNA is also exemplified by the existence of the so-called "Higher-Order Chromatin Folding and Topologically Associating Domains" or TAD domains. TADs represent sub-regions of the chromosomes, where processes such as replication and transcription are collectively regulated. Furthermore, TAD borders can be modified in response to stress, causing global changes in gene expression and replication timings (Cubeñas-Potts and Corces, 2015; Ea et al., 2015; Li et al., 2015). Therefore, 4C techniques that help delimit TAD borders and its changes upon stress will be key to study the regions prone to experience TRCs genome-wide.

Finally, there is an intriguing question to take into consideration. Is it possible that the TRCs originated in response to stress are actually beneficial for the cell as they cause mutations that promote evolution? We assumed that mutations occur randomly in the genome, however, mutagenesis rate has been shown to increase in

response to stress, especially in stress-responsive genes (Jack et al., 2015; Rosenberg et al., 2012). It was proposed that, at a population level, higher mutation rate of the stress-responsive genes could increase the chances of survival to deadly new scenarios. It is still unknown whether the increase on the mutagenesis rate is an indirect consequence of the activation of the stress responses, such as the accumulation of TRCs, or if it is an active mechanism that increases genetic variability to ensure the survival of the population to the new environmental conditions. Thus, the amount of "use" of a gene could influence its rate of mutation, which proposes a change in the way we understand random evolution and reaffirm the importance of understanding stress responses and how these broadly modulate the genomes.

Overall, this thesis describes the Mrc1 transcription-replication (MTR) safeguard mechanism as a conserved mechanism to prevent TRCs and genomic instability upon unscheduled outbursts of transcription induced by environmental stress or decreased cell fitness. We have shown that multiple signaling kinases phosphorylate Mrc1 to regulate replication upon several stresses which prevents the accumulation of TAR and genomic instability.

Personal contribution to this work: I have been involved in all the steps of this work except for the DNA combing assays that were performed in collaboration with the laboratory of Dr. Andrés Aguilera.

CONCLUSIONS

The main conclusion of this PhD thesis is that there is a mechanism mediated by Mrc1 required during S phase to delay replication and prevent transcription-associated recombination (TAR) and genomic instability upon unscheduled outbursts of transcription.

More specifically, the results obtained during this PhD thesis lead to the following conclusions:

- Mrc1 is phosphorylated upon environmental stresses such as heat, oxidative and low glucose stresses in the previously reported Hog1-sites.
- Phosphorylation of Mrc1 upon heat, oxidative and low glucose stresses transiently delays S-phase progression.
- Phosphorylation of Mrc1 in response to heat, oxidative and low glucose stresses prevents the accumulation of TAR and genomic instability.
- TAR and genomic instability accumulated in an *mrc1*^{3A} mutant strain upon stress is dependent on active transcription.
- We identified six kinases (Hog, Mpk1/Slt2, Psk1, Snf1, Pho85 and Ste20) that can phosphorylate the N-terminus of Mrc1 *in vitro*.
- Mpk1, Psk1 and Snf1 phosphorylate Mrc1 *in vivo* to delay replication, and prevent the accumulation of TAR and

genomic instability upon heat, oxidative or low glucose stress respectively.

- The role of Mrc1 is not restricted to environmental stress but it is also crucial to prevent TAR and genomic instability by other stimuli in which cell fitness is compromised and ESR is activated, such as slow growth (*ssn6*) and genomic unstable (*rad53*) mutants.

SUPPLEMENTARY ARTICLE

Chang Y-L, Tseng S-F, Huang Y-C, Shen Z-J, Hsu P-H, Hsieh M-H, et al. Yeast Cip1 is activated by environmental stress to inhibit Cdk1–G1 cyclins via Mcm1 and Msn2/4. Nat Commun. 2017 Dec 4;8(1):56. DOI: 10.1038/s41467-017-00080-y

DISCUSSION

Several cyclin-CDK inhibitors (CKis) such as Far1 or Sic1 can modulate the activity of the diverse cyclin-CDKs to control cell cycle progression. In a regular cell cycle, the master Sic1 CKi inhibits the S-CDK complexes to prevent premature entry into S phase. During G_1, cells accumulate enough G_1-CDK complexes to phosphorylate and promote the degradation of Sic1, which allows cell cycle progression. Then, by modulating Sic1 stability, cells can control cell cycle progression (Lengronne and Schwob, 2002). For instance, upon osmostress, the Hog1 SAPK phosphorylates and stabilizes Sic1 to delay G_1/S transition (Escoté et al., 2004).

Recently, the protein Cip1 has been described as a novel CKi able to stabilize Sic1 and prevent premature G1/S transition (Ren et al., 2016). Also, during S phase, Cip1 has been found to accumulate upon replication stress in a Rad53- and Mec1-dependent manner (Zhang et al., 2017). Therefore, Cip1 might be a key regulator of cell cycle in response to several stimuli. Here, we described Cip1 as a G_1-CKi involved in regulating cell cycle progression in response to environmental stress. We showed that the expression of Cip1 is controlled by the Mcm1 TF in a cell cycle regulated manner (peaking in G_1). Overexpression of Cip1 delays G_1 progression as it binds and inhibits the activity of all three G_1-CDK complexes (Cln3, 2 and 1-Cdc28) both *in vitro* and *in vivo*. Specifically, our results indicated that Cip1 inhibits Cln3 *in vivo*, the most upstream G_1 cyclin. The inhibition of Cln3 impairs SBF-dependent transcription which delays Cln1 and Cln2 expression, thus preventing premature G_1/S transition. Interestingly, we showed that

Cip1 expression is also controlled by the activation of the Msn2 and Msn4 TFs in response to osmo, oxidative, low carbon or DNA damage stress, which suggests a role of Cip1 in regulating cell cycle progression upon stress. Correspondingly, Cip1 and Sic1 have redundant effects on cell cycle progression and cell viability upon osmostress. For instance, we showed that the Hog1 SAPK phosphorylates Cip1 *in vitro* and *in vivo* upon osmostress to prevent G_1/S transition, as Hog1 phosphorylation of Cip1 makes it more proficient to bind and inhibit Cln3.

Altogether, this paper extends the knowledge on the Cip1 CKi in a regular G_1 phase as well as it establishes a new link between Cip1 and cell cycle regulation upon osmostress.

Personal contribution to this work: My contribution to this work consisted in the design and execution of the elutriation and FACS experiments shown in Figure 6B and the growth curves shown in Figure 6C and Supplementary Figure 3B.

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LIST OF ABBREVIATIONS

ATM: Ataxia telangiectasia mutated

DDC: DNA-damage checkpoint

DRC: DNA-replication checkpoint

***cdc7^{AID}*:** Cdc7 C-terminally tagged with three copies of the *miniAID* sequence of the auxin-induced degron system

CDK: Cyclin-dependent kinase

CFS: Common fragile site

CKi: Cyclin-CDK inhibitor

CMG helicase: Replicative helicase (Cdc45-MCM-GINS)

CWI pathway: Cell-wall integrity pathway

ESR: Environmental stress response

iESR: genes induced in the ESR

rESR: genes repressed in the ESR

HOG pathway: High osmolarity glycerol pathway

HSE: Heat-shock element

HSR: Heat-shock response

MAPK: Mitogen-activated protein kinase

***mrc1^{3A}*:** Mrc1 with T169, S215 and S229 mutated to alanine

MTR safeguard mechanism: Mrc1 transcription-replication safeguard mechanism

OSR: Oxidative-stress response

PKA: cAMP-dependent Protein Kinase

PKC: Calcium-regulated Protein kinase

RFB: Replication fork barrier

RNAP: RNA polymerase

ROS: Reactive-oxigen species

RPC: Replication progression complex

RNR: Ribonucleotide reductase

SAPK: Stress-activated protein kinase

STRE: Stress-responsive element

TAD: Higher-Order Chromatin Folding and Topologically Associating Domains

TAR: Transcription-associated recombination

TF: Transcription factor

TRC: Transcription-replication conflicts