Linking DNA replication checkpoint to MBF cell-cycle transcription reveals a distinct class of G1/S genes

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Reprogramming gene expression is crucial for DNA replication stress response. We used quantitative proteomics to establish how the transcriptional response results in changes in protein levels. We found that expression of G1/S cell-cycle targets are strongly up-regulated upon replication stress, and show that MBF, but not SBF genes, are up-regulated via Rad53-dependent inactivation of the MBF co-repressor Nrm1. A subset of G1/S genes was found to undergo an SBF-to-MBF switch at the G1/S transition, enabling replication stress-induced transcription of genes targeted by SBF during G1. This subset of G1/S genes is characterized by an overlapping Swi4/Mbp1-binding site and is enriched for genes that cause a cell cycle and/or growth defect when overexpressed. Analysis of the prototypical switch gene *TOS4* (Target Of SBF 4) reveals its role as a checkpoint effector supporting the importance of this distinct class of G1/S genes for the DNA replication checkpoint response. Our results reveal that replication stress induces expression of G1/S genes via the Rad53-MBF pathway and that an SBF-to-MBF switch characterizes a new class of genes that can be induced by replication stress.

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Introduction

A cell division cycle is initiated during the G1 phase of the cell cycle by the transcriptional induction of genes that drive transition into S phase. In human cells, G1/S transcriptional regulation depends on the E2F family of transcription factors and their co-regulators, the transcriptional co-repressor pRB family members (Frolov and Dyson, 2004; Cobrinik, 2005; Chen *et al*, 2009). The importance of G1/S transcriptional regulation for controlled cell-cycle progression is illustrated by the fact that E2F-dependent cell-cycle transcription is misregulated in nearly all tumour types (Stevens and La Thangue, 2003).

In budding yeast, G1/S transcription depends on two transcription factor complexes, SBF and MBF (Breden, 2003; Wittenberg and Reed, 2005). The SBF and MBF complexes comprise a shared regulatory subunit, Swi6, and the DNA-binding subunits Swi4 and Mbp1, respectively. Swi4 and Mbp1 recognize distinct DNA sequence elements, SCB (Swi4 Cell-cycle Box) and MCB (Mbp1 Cell-cycle Box), respectively, which define SBF and MBF target promoters. SBF is a potent transcriptional activator, while MBF behaves primarily as a transcriptional repressor (Amon *et al*, 1993; Bean *et al*, 2005; de Bruin *et al*, 2006). During the cell cycle, SBF and MBF are bound and regulated by the transcriptional co-repressors, Whi5 and Nrm1, respectively (Costanzo *et al*, 2004; de Bruin *et al*, 2004, 2006).

Once G1/S transcription is activated, cells progress into S phase and initiate DNA replication. During DNA replication, cells are particularly vulnerable to accumulate genomic instability, as replication forks are prone to stall and collapse when encountering replication blocks or damaged DNA templates (Lopes *et al*, 2001; Tercero and Diffley, 2001). To properly replicate the genome and avoid DNA damage, cells rely on the DNA replication checkpoint. In budding yeast, the DNA replication checkpoint is coordinated mostly by the PI3-like sensor kinase Mec1 and by the effector kinase Rad53, orthologues of human ATR and CHK2/CHK1, respectively (Segurado and Tercero, 2009). The replication checkpoint protects cells from irreversible DNA damage by delaying mitotic entry and, among other responses, initiating a transcriptional programme to deal with stresses in the replication process (Zhou and Elledge, 1993; Allen *et al*, 1994; Gasch *et al*, 2001; Zhao *et al*, 2001).

Genome-wide expression analysis in budding yeast has revealed that hundreds of genes are regulated in response to genotoxic stress (Fu *et al*, 2008). The best understood mechanism by which the DNA replication checkpoint regulates replication stress-induced transcription is via inactivation of the transcriptional repressor Crt1 (Huang *et al*, 1998). Upon replication stress, Rad53 phosphorylates and activates the downstream checkpoint kinase Dun1, which in turn phosphorylates the transcriptional repressor Crt1. Phosphorylated Crt1

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becomes inactive and no longer binds its target promoters leading to the transcription activation of DNA damage response (DDR) genes. However, the Rad53–Dun1–Crt1 regulatory circuit represents only a small part of the checkpoint transcriptional response and is not conserved in higher eukaryotes.

In order to establish how the transcriptional response results in the accumulation of proteins during replication stress, we utilized a quantitative proteomic approach. From this study we found that upon genotoxic stress, apart from Crt1 targets, expression of several G1/S cell-cycle genes are strongly up-regulated. We show that MBF transcripts are up-regulated in response to replication stress via Rad53-dependent, but Dun1-independent, inhibition of the MBF co-repressor Nrm1. Detailed analysis of the putative SBF-only target *TOS4* (Target Of SBF 4) revealed an SBF-to-MBF switch at its promoter that allows its Rad53-dependent transcriptional induction upon replication stress. This led to the identification of a specific subset of G1/S genes that are likely targeted by SBF during G1 and undergo an SBF to MBF switch at the G1/S transition. These genes are characterized by an overlapping SBF and MBF-binding motif (SCB and MCB, respectively), which likely results in a competition for SBF/MBF binding and might be the basis of the SBF-to-MBF switch. This new set of G1/S genes is regulated differently in many ways as compared with G1/S target genes containing non-overlapping SCB and MCB motifs in their promoter. These switch G1/S genes are enriched for genes that cause a cell cycle and/or growth defect when overexpressed and include genes important for the checkpoint response. These findings define the mechanism of the regulation of cell-cycle genes in response to DNA replication stress and reveal a novel mode of transcriptional regulation via the coordinated action of SBF and MBF at distinct stages of the cell cycle.

Results

Expression of G1/S genes is induced during replication stress

Treating cells with hydroxyurea (HU), a specific inhibitor of ribonucleotide reductase, effectively decreases dNTP levels, resulting in the stalling of DNA replication forks and activation of the DNA replication checkpoint response. In order to establish which proteins accumulate in response to DNA replication stress, we carried out quantitative proteomics analysis comparing the protein levels of cells before and after HU-induced replication stress (Dataset 1 in Supplementary data). As expected, we detected an increase in the abundance of the well-established replication stress-induced proteins Rnr3 and Hup1, which are under the control of the Crt1 transcription repressor (Figure 1A). In addition, we found increased abundance of the MBF-only target Rnr1 as well as of other G1/S proteins, including Mcd1 (putative MBF-only target), Tos4 (putative SBF-only target) and Ndd1 (SBF-only target), in response to replication stress. Increase on Tos4 protein levels during replication stress was also validated by western blot analysis (Supplementary Figure S1). To establish whether the accumulation of these proteins during DNA replication stress is the result of transcriptional induction, we carried out a gene expression analysis during the cell cycle in untreated and HU-treated cells (Figure 1B). Treatment with other genotoxic agents (MMS and CPT) has been shown to give similar results

(Wittenberg laboratory, personal communication). This analysis confirms previous observations showing transcriptional induction of *RNR3* and *HUG1* strictly in response to DNA replication stress. It also shows that expression of the G1/S genes *TOS4*, *RNR1* and *MCD1* is periodic during an unperturbed cell cycle, peaking at the G1/S transition and up-regulated during S phase in response to replication stress. Transcription of *NDD1* was not increased during replication stress (Supplementary Figure S2), revealing that the abundance of Ndd1 protein in response to replication stress is under an alternative mode of regulation.

Together, these data show that both the transcript and protein levels of the G1/S cell cycle regulated genes *TOS4*, *MCD1* and *RNR1* are induced in response to DNA replication stress. The fold induction observed for these proteins in our proteomic analysis is among the most drastic changes observed in the proteome, supporting that G1/S transcription is strongly impacted upon replication stress. Moreover, the transcriptional regulation of G1/S genes is different from that of Crt1 targets, whose transcription is not induced in a normal cell cycle but strongly up-regulated in response to DNA replication stress.

Replication stress induces expression of G1/S genes via a Rad53-dependent but Dun1-independent pathway

In response to DNA replication stress, Dun1-dependent phosphorylation inactivates the transcriptional repressor Crt1 leading to induction of its targets, such as *RNR3* and *HUG1*. To establish whether the induction of the G1/S genes *TOS4*, *MCD1* and *RNR1* during replication stress depends on Dun1, we measured their transcript levels during the cell cycle and in response to HU treatment in wt and *dun1Δ* cells. As previously shown, the induction of the Crt1 targets *RNR3* and *HUG1* in response to replication stress depends on Dun1 (Figure 1C, upper panels). We also show that G1/S targets remain cell cycle regulated and replication stress induced in *dun1Δ* cells, revealing that their expression is Dun1 independent (Figure 1C, upper panels). Upstream of Dun1 functions the Rad53 protein kinase. To determine if the expression of *TOS4*, *MCD1* and *RNR1* is dependent on Rad53, the experiment above was carried out in *rad53Δ* cells. Since Rad53 is an essential gene, the experiment was done in an *sml1Δ* background, which suppresses the lethality of Rad53 inactivation. The expression analysis establishes that, as expected, the induction of Crt1 targets is Rad53 dependent (Figure 1C, lower panels). It also shows that the induction of *TOS4*, *MCD1* and *RNR1* depends on Rad53. Overall, these data show that the induction of the G1/S cell cycle regulated genes *TOS4*, *MCD1* and *RNR1*, in response to replication stress is Rad53 dependent, but is independent of Dun1.

Targets of MBF, but not SBF, are up-regulated in response to replication stress

To determine if transcriptional induction of G1/S genes upon replication stress is a general feature of G1/S genes or is specific for MBF and/or SBF-only targets, we determined the cell-cycle expression levels of other well-established G1/S transcripts, two SBF targets (*SVS1* and *CLN2*) and one MBF target (*CDC21*), in untreated and HU-treated conditions. Our analysis revealed that whereas the SBF targets *SVS1* and *CLN2* are repressed in a timely manner, the MBF target *CDC21* is induced in response to DNA replication stress

Figure 1 Replication stress-induced reprogramming of gene expression includes transcriptional up-regulation of G1/S genes. **(A)** Proteomic analysis of changes in protein abundance following replication stress. Cells were arrested in G1 and released for 20 or 120 min in media containing 100 mM HU. Rad53 is activated at ~25 min after release from G1 arrest. Shown results are the average ratio (120/20 min) for each of the 2862 proteins identified and quantified in the experiment. **(B)** Relative mRNA levels of indicated genes in cells synchronized by α -factor arrest and release, in the presence or absence of HU. Transcript levels are represented as a fold over 0 min for the Crt1 targets and a fold over max. unt. 100% for the G1/S targets. **(C)** Relative mRNA levels of indicated genes in wild-type, *dun1* Δ or *sml1* Δ /*rad53* Δ cells. Expression levels, at indicated time points (min), are presented as fold induction of levels detected in time 0. For samples treated with HU, 100 mM HU was added 20 min after release from α -factor arrest. Levels of mRNA were quantified by RT-qPCR and normalized to *ACT1*.

similarly to what we observed for *RNR1*, *MCD1* and *TOS4* (Figure 2A). Furthermore, expression of *CDC21* during replication stress was found to depend on Rad53 but not on Dun1 (Figure 2B). These data reveal that MBF-dependent, but not SBF-dependent, cell-cycle transcripts are induced in response to DNA replication stress in a Rad53-dependent manner. Interestingly, *TOS4* was initially identified as a target of SBF (Iyer *et al*, 2001); however, its pattern of expression during replication stress suggests that *TOS4* is likely regulated by MBF during S phase.

SBF is replaced by MBF at the promoter of *TOS4* during the G1/S transition

To determine if *TOS4* is regulated by MBF during any stage of the cell cycle, we performed ChIP analysis, pulling down myc-tagged Swi4 and Mbp1 proteins and examining their binding to the *TOS4* promoter at different time points after release from G1 arrest. As shown in Figure 3A, we found that

Swi4 binds the *TOS4* promoter in G1, but once cells progress into S phase, Swi4 leaves the *TOS4* promoter and Mbp1 binds it. These findings suggest that SBF and MBF regulate *TOS4* expression in a mutually exclusive manner at different stages of the cell cycle, a feature that differentiates *TOS4* from G1/S genes regulated by SBF-only (i.e., *SVS1*), MBF-only (i.e., *CDC21*) or dual-regulated SBF and MBF (i.e., *CLN1*). This transcription factor switching at G1/S promoters during the cell cycle has not been observed in yeast before but resembles the mode of regulation in human cells for the E2F transcription factors at G1/S promoters (Dimova and Dyson, 2005; Moon and Dyson, 2008).

TOS4 transcription is activated by SBF during G1 and repressed by MBF during S phase

Previous work has shown that whereas SBF functions primarily as a transcriptional activator during the G1 phase of the cell cycle, MBF functions as a transcriptional repressor

Figure 2 Expression of canonical MBF and SBF targets during replication stress. (A) Relative mRNA levels of the canonical SBF targets *SVS1* and *CLN2*, and of the canonical MBF target *CDC21* in cells synchronized by α -factor arrest and release, in the presence or absence of HU. Transcript levels are represented as a percentage of highest level (100%) observed in untreated samples. (B) Relative *SVS1*, *CLN2* and *CDC21* mRNA levels in wild-type, *dun1Δ* or *sml1Δ/rad53Δ* cells. The experiment was performed as described in Figure 1C.

outside of G1 (Amon *et al*, 1993; Bean *et al*, 2005; de Bruin *et al*, 2006). To determine the role of MBF and SBF in controlling the expression of *TOS4* during the cell cycle, we analysed the pattern of *TOS4* transcription in wild-type cells and in cells lacking either Mbp1 (MBFA) or Swi4 (SBFA). The transcription profile of the well-established SBF-only target, *SVS1*, MBF-only target, *CDC21* and, the dual-regulated SBF and MBF target, *CLN1* were used as controls (Bean *et al*, 2005; de Bruin *et al*, 2006; Eser *et al*, 2011). As expected, deletion of either *MBP1* or *SWI4* had no effect on the periodic expression of *SVS1* and *CDC21*, respectively. In turn, as previously shown, deletion of *SWI4* abrogated the transcriptional induction of *SVS1* and its periodicity and deletion of *MBP1* abolished periodicity and de-represses expression of *CDC21* throughout the cell cycle (Figure 3B). Surprisingly, *TOS4* transcription pattern showed characteristics of both *SVS1* and *CDC21* genes (Figure 3B). Our data show that deletion of *SWI4* significantly decreases *TOS4* peak expression levels during G1/S, confirming that *TOS4* is indeed under the control of SBF (Figure 3B). In contrast, while *TOS4* transcript levels in *mbp1Δ* cells were comparable to wild-type levels during G1, it was significantly de-repressed outside of G1. Finally, we show that *CLN1*, a dual-regulated SBF and MBF target, behaves like an SBF target, loosing its transcriptional periodicity in *swi4Δ* cells. Together, these results reveal that while SBF is important for the transcriptional activation of *TOS4* during G1, MBF represses *TOS4* expression outside of G1.

During G1, Swi4 competes with Mbp1 for binding to the *TOS4* promoter

Our findings show that *TOS4* is regulated by SBF during G1 and by MBF outside of G1. SBF and MBF targets are traditionally defined by the presence of SCB (CRCGAAA) and MCB (ACGCGN) DNA regulatory elements in the promoter sequences, respectively. In line with our observations, the *TOS4* promoter contains both an MCB and SCB element (Figure 4A). Interestingly, the sequence of the MCB and SCB elements partially overlaps, which could represent the basis of a competition mechanism between SBF and MBF binding for the *TOS4* promoter. This feature is markedly different from the classical dual-regulated SBF and MBF target *CLN1* that contains SCB and MCB regulatory elements that do not overlap. To examine if Swi4 and Mbp1 compete for binding to the *TOS4* promoter, we carried out ChIP experiments pulling down myc-tagged Swi4 and Mbp1 proteins in wt and *mbp1Δ* and *swi4Δ* non-synchronized cell cultures, respectively. Figure 4B shows that the *TOS4* promoter is mainly bound by Swi4; however, whereas inactivation of Mbp1 has no effect on Swi4 binding, in the absence of Swi4, a significant increase in binding of Mbp1 to the *TOS4* promoter can be observed. The increase in Mbp1 binding to the *TOS4* promoter in the absence of Swi4 is mainly the result of enhanced binding during the G1 phase of the cell cycle, when SBF normally binds (Figure 4C). These data indicate that Swi4 competes with Mbp1 for binding to the *TOS4* promoter during G1 of the cell cycle. These results suggest that the cell cycle-

Figure 3 An SBF-to-MBF switch at the promoter of the G1/S gene *TOS4*. (A) ChIP analysis for Swi4-myc or Mbp1-myc at the *TOS4*, *SVS1*, *CDC21* and *CLN1* promoters during the G1 and S phase of the cell cycle. Analysis was performed in G1 synchronized cells released from α -factor arrest and samples were taken at the indicated intervals. (A) Enrichment levels were accessed by qPCR and are presented as a percentage of highest obtained level in the separate Swi4 ChIPs and Mbp1 ChIPs after normalization to whole cell extract (WCE) levels. Average value and error bars are derived from biological replicates. (B) Relative mRNA levels of indicated genes during the cell cycle in wild-type, *swi4Δ* and *mbp1Δ* cells. Cells were synchronized in G1 by α -factor, released from the arrest and collected at the indicated times after release. Levels of mRNA were quantified by RT-qPCR and normalized to *ACT1*. Transcript levels are represented as a percentage of highest level (100%) observed in wild-type cells.

dependent switch between SBF and MBF at the promoter of *TOS4* is governed by a competition between Swi4 and Mbp1 for the overlapping MCB/SCB motifs present in the *TOS4* promoter.

During S phase, the replacement of SBF by MBF on the *TOS4* promoter is likely an active process

During G1/S transition, active Clb/CDK inhibits the ability of Swi4 to bind its target promoters and shuts off SBF-dependent transcription. To determine if MBF replaces SBF at switch promoters during S phase simply because SBF leaves promoters, or if MBF actively replaces SBF we have carried out a cell-cycle experiment using cells that overexpress a hyperstable form of the Clb/CDK-specific inhibitor Sic1 (*sic1Δp*) in order to keep Clb/CDK inactive. It has been shown that under these conditions SBF-only genes remain active whereas MBF-only genes are still inactivated in Nrm1-dependent manner (de Bruin *et al*, 2006). Therefore, if MBF replaces SBF at switch promoters during S phase simply because SBF leaves promoters in a Clb/CDK-dependent manner, switch genes should remain active in the absence of Clb/CDK activity. However, if MBF actively removes SBF to replace it from switch gene promoters during S phase, switch genes should still be repressed in the absence of Clb/CDK activity. As expected, our data show that overexpression of Sic1Δp had no effect on the periodic expression of MBF-only targets (i.e., *RNR1*) but increases the expression of SBF-only targets (i.e., *CLN2*) and dual-regulated SBF and MBF targets (i.e., *CLN1*).

Interestingly, *TOS4* expression was shown to be inactivated in a timely manner even in the absence of Clb/CDK activity (Figure 4D). This result suggests that MBF can actively replace SBF at switch genes promoters during S phase.

Rad53 mediates induction of *TOS4* and other MBF targets via inactivation of the co-repressor Nrm1

Our findings show that due to an SBF-to-MBF switch at the G1/S transition, expression of *TOS4* during S phase is repressed by MBF. Upon replication stress, Rad53 de-represses *TOS4* expression. Previous studies have shown that transcriptional repression of MBF targets, as cells exit G1 phase, depends on a negative feedback loop involving the transcriptional co-repressor Nrm1 (de Bruin *et al*, 2006). Nrm1, an MBF target itself, accumulates during the G1/S transition and binds to MBF at promoters to repress MBF-dependent transcription. The role of Nrm1 in the regulation of MBF target genes positions it as a potential target through which the replication checkpoint could activate MBF transcription at the G1/S transition. We therefore investigated whether Nrm1 function is regulated in response to DNA replication stress induced by HU treatment.

We reasoned that if Nrm1 is inactivated as part of the checkpoint response then MBF transcripts levels in *nrm1Δ* cells should resemble those of HU-treated cells. In addition, HU treatment of *nrm1Δ* cells should not further induce MBF-dependent genes. Our expression analysis shows that both assumptions hold true. MBF targets, including *TOS4*, are de-

Figure 4 SCB/MCB overlapping motifs at G1/S promoters provides the rationale for SBF and MBF competition. (A) Schematic representation of overlapping SBF (SCB) and MBF (MCB) DNA-binding motifs identified in the *TOS4* promoter element (−232 to −214 from the ATG start codon). Grey shade represents SCB/MCB overlapping region. (B) ChIP analysis for Mbp1-myc and Swi4-myc at the *TOS4* promoter in wild-type, *swi4Δ* and *mbp1Δ* cells. Analysis was performed in asynchronous cells and enrichment levels were accessed by qPCR and are shown as a percentage of WCE signal. Untagged cells were included as negative control. Average value and error bars are derived from experimental triplicates. (C) ChIP analysis for Mbp1-myc at *TOS4* promoter in wild-type and *swi4Δ* cells. Experiment was performed as described in Figure 3A. (D) Relative mRNA levels of indicated genes during the cell cycle in wild-type, and cells expressing *sic1Δp* from the inducible *GAL1* promoter. Cells were synchronized in G1 by α -factor, released from the arrest as detailed in Materials and methods and collected at the indicated times after release. Levels of mRNA were quantified by RT-qPCR and normalized to *ACT1*. Transcript levels are represented as a percentage of highest level (100%) observed in wild-type cells. Budding index (% budded cells) was measured as an indicator of synchrony. Average value and error bars are derived from biological triplicates.

repressed during S phase in *nrm1Δ* cells comparable to levels observed in HU-treated wild-type cells (Figure 5). In addition, HU treatment of *nrm1Δ* cells does not significantly induce MBF transcripts. As expected, the Crt1 target *HUG1* is still induced in response to HU treatment in *nrm1Δ* cells. Furthermore, whereas Nrm1 is able to bind and repress the MBF-dependent *TOS4*, *RNR1* and *CDC21* promoters during S phase, HU-induced DNA replication checkpoint activation results in loss of Nrm1 from these promoters (Supplementary Figure S3). Overall, these data suggest that Nrm1 is likely inactivated by the replication checkpoint resulting in dissociating from MBF at promoters and the induction of G1/S genes.

Overexpression of *Tos4* halts progression of the cell cycle

Taken together, our results reveal that *TOS4* expression is under an elaborate transcriptional regulation that involves SBF and MBF, as well as replication checkpoint signalling. To

address the functional relevance of controlling *TOS4* expression, we tested the effect of *TOS4* overexpression by ectopically expressing it from a *GAL1*-inducible system. FACS analysis of wild-type and *Tos4* overexpressing cells revealed that overexpression of *Tos4* results in a significant delay in cell-cycle progression after release from an α -factor-enforced G1 arrest (Figure 6A). This result reveals that *TOS4* is a dosage-sensitive gene whose overexpression delays S-phase progression.

Tos4 is essential for cell survival during replication stress in the absence of the checkpoint protein kinase Dun1

The strong Rad53-dependent up-regulation of *Tos4* transcription in response to replication stress and the potential role of *Tos4* in promoting cell-cycle arrest suggest that *Tos4* is an effector of the replication checkpoint. To determine the relevance of *Tos4* during replication stress, we tested the sensitivity of *tos4Δ* cells to HU treatment. As shown in

Figure 5 Nrm1 represses expression of MBF targets during unperturbed S phase but not during replication stress. Relative mRNA levels of indicated genes in wild-type and *nrm1Δ* cells in the presence or absence of HU. For HU treatment, 100 mM HU was added 20 min after release from α -factor arrest. Levels of mRNA were quantified by RT-qPCR, normalized to *ACT1*, and presented as fold induction of levels detected in time 0 in wild-type cells. Budding index (% budded cells) was measured as an indicator of synchrony.

Figure 6B, *tos4Δ* cells are not sensitive to HU. However, we found that in cells lacking the downstream checkpoint kinase Dun1, *Tos4* plays an essential role in cell viability in the response to replication stress, as *tos4Δdun1Δ* cells are highly sensitive to HU. This result is consistent with our finding that transcriptional regulation of *Tos4* is Dun1-independent, suggesting that *Tos4* represents a branch of the replication checkpoint that is parallel to the Dun1-mediated response. In addition, microscopy analysis of cells expressing *Tos4*-GFP demonstrates that *Tos4* rapidly accumulates in the nucleus upon HU treatment, consistent with its potential role in the DNA replication checkpoint (Supplementary Figure S4). Collectively, these findings support a role for *Tos4* as a replication checkpoint effector.

The FHA domain of *Tos4* mediates an interaction with the Rpd3L and Set3 HDAC complexes to promote cellular tolerance to replication stress

Tos4 is an FHA (ForkHead-associated) domain containing protein of unknown function. FHA domains are protein-protein interaction motifs originally identified in the ForkHead cell-cycle transcription factors but are also found in checkpoint proteins, including Rad53 and Dun1 (Mohammad and Yaffe, 2009). In order to test whether the FHA domain of *Tos4* mediates its role during replication stress response, we generated point mutations in two residues predicted to be critical for the ability of FHA domains to mediate protein-protein interactions (Figure 6C). Mutation of the FHA domain of *Tos4* in cells lacking Dun1 resulted in a HU sensitivity compared with that of *tos4Δdun1Δ* cells (Figure 6D), suggesting that the role of *Tos4* during replication stress is dependent on the ability of its FHA domain to interact with other protein(s). Protein abundance of the FHA mutant is not affected when compared with wt *Tos4* (Supplementary Figure S5). Using quantitative mass spectrometry, we compared the set of proteins that specifically interact with wild-type *Tos4* but not with a *Tos4* mutant containing an impaired FHA domain (Supplementary Figure S6; Dataset 2 in Supplementary data). Wild-type *Tos4* was found to specifically interact with several components of the histone deacetylase (HDAC) complexes Rpd3 and Set3 (Figure 6E). These data confirm previous mass spectrometry results that identified *Tos4* in Rpd3 and Set3 pull downs (Shevchenko *et al*, 2008). Our results show that this interaction is lost in the *Tos4* FHA mutant. These HDACs have been shown to play an important role in the transcriptional response to various environmental stresses and more specifically in the regulation of transcription in the DDR (Sharma *et al*, 2007; Alejandro-Osorio *et al*, 2009). *Tos4*'s interaction with these HDAC complexes suggests a possible role for *Tos4* in modulating HDAC activity in response to replication stress.

Because *Tos4* accumulates in response to replication stress and binds, through its FHA domain, to HDAC complexes, we hypothesized that if *Tos4* is required for HDAC activity during replication stress, inactivation of the interacting HDAC components should have no effect or a negative effect on cell viability in response to replication stress in the absence of *Tos4* and Dun1. In contrast, if *Tos4* interferes with HDAC function in response to replication stress, this should suppress the HU sensitivity of *tos4Δdun1Δ* mutants. Deletion of the catalytic subunits *RPD3* or *HST1* was found to suppress the sensitivity of *tos4Δdun1Δ* cells to HU treatment, suggesting a potential role for *Tos4* in counteracting the activity of these HDACs components (Figure 6F). Since the FHA domain of *Tos4* is essential for its binding to the HDAC complexes, these findings suggest an important role for *Tos4* in regulating HDAC function during replication stress. Collectively, these results suggest that *Tos4* is an effector of the replication checkpoint that couples Rad53 signalling to the regulation of HDACs function during replication stress.

Overlapping SCB/MCB motifs characterizes a distinct group of G1/S genes

Our results reveal that an SBF-to-MBF switch regulates the expression of *TOS4*, an effector of the replication checkpoint. Analysis of the promoter of other G1/S genes found to be strongly up-regulated by HU in our proteomic screen revealed

Figure 6 Tos4 plays an important role in the replication stress response via interaction with HDACs. **(A)** FACS analysis of cells overexpressing *TOS4* during S phase. Cells were synchronized in G1 by α -factor arrest and released from arrest in 2% galactose 1% raffinose for the indicated interval. **(B)** HU sensitivity of indicated strains. Four-fold serial dilutions were spotted on plates and grown for 2–3 days at 30°C. **(C)** Schematic representation of Tos4 protein displaying its FHA domain and the position of residues that were mutated to disrupt FHA domain function. **(D)** HU sensitivity of indicated strains. Four-fold serial dilutions were spotted on plates and grown for 2–3 days at 30°C. **(E)** Network of Tos4 FHA-interacting proteins during replication stress. According to available information in the Biogrid Database (<http://thebiogrid.org/>), the identified interacting proteins were grouped into two distinct complexes represented by the Rpd3 and Set3 HDACs. Grey lines represent previously known interactions between the different subunits that comprise both HDACs. Dotted line indicates the interactions identified in this work. (*) Peptides for Hst1 were not detected in the MS experiment, but the interaction was validated by co-IP (see Supplementary Figure S6). **(F)** HU sensitivity of indicated strains. Four-fold serial dilutions were spotted on plates and grown for 2–3 days at 30°C.

that the promoter of *MCD1* also contains overlapping SCB/ MCB motifs (Figure 7A). Mcd1 is an essential subunit of cohesin that prevents chromosome segregation and whose overexpression arrests cells in G2/M (Sopko *et al*, 2006). We further confirmed that, like *TOS4*, *MCD1* expression is also regulated by an SBF-to-MBF switch (Figure 7B–D). Next, we sought to determine how common the SBF-to-MBF switch regulation is for G1/S targets. To identify putative SBF/MBF switching promoters, we analysed the promoter sequences of the 200+ SBF and MBF target genes identified in the Iyer ChIP-chip study and the Simon *et al* (2001) and Harbison *et al* (2004) data sets (Iyer *et al*, 2001) for the presence of overlapping SCB/MCB motifs. We classified a target promoter to contain an overlapping SCB/MCB sites as when an SCB and MCB sequence overlap, maintaining both consensus binding sequences (SCB; CNCGAAA and MCB; ACGCG). The analysis of Iyer ChIP-chip data set identified 27 SBF/MBF target promoters with one or more overlapping SCB/MCB motifs (Supplementary Table SI). Analysis of the two data sets from the Young laboratory (Simon *et al*, 2001; Harbison *et al*, 2004) identified 32 and 22 genes containing overlapping SCB/MCB motifs, respectively but did not identify the founding switch

gene member *TOS4*. Since our study shows that *TOS4* is a genuine SBF/MBF target and cell cycle regulated gene, we used the list from the Iyer data set for the initial study of this new group of G1/S genes. A more comprehensive list of potential switch genes from all three data sets, containing 44 genes is included (Supplementary Table SI). Interestingly, the majority of these promoters were designated as SBF targets, with a few for both and none as MBF-only targets. We selected six of the targets containing overlapping SCB/MCB motif to further test by ChIP analysis if they are indeed regulated by SBF/MBF switch. As shown in Figure 7E, in all cases we were able to confirm the switch. Next, we analysed the combined genome-wide overexpression data sets from studies by Sopko *et al* (2006) and Yoshikawa *et al* (2011) for total genes tested, G1/S transcripts (based on Iyer *et al*, data set) and switch genes. In line with our hypothesis, the switch genes are significantly enriched for genes that cause a cell cycle delay or growth defect when overexpressed (Figure 7F). Finally, we also analysed the HU transcriptional regulation of 27 switch genes defined in this work. Our result shows that 2/3 of the tested switch genes are up-regulated upon HU treatment (Supplementary Table SI; Supplementary Figure S7). Taken

Figure 7 SBF-to-MBF switch at the promoter of other G1/S genes. (A) Schematic representation of overlapping SCB and MCB motifs identified in the *MCD1* promoter element (–336 to –329 from the ATG start codon). Grey shade represents SCB/MCB overlapping region. (B) ChIP analysis for Swi4-myc or Mbp1-myc at the *MCD1* promoter during the G1 and S phase of the cell cycle. Experiment was performed as described in Figure 3A. (C, D) Relative mRNA levels of *MCD1* during the cell cycle in (C) wild-type, *swi4Δ* and *mbp1Δ* cells and (D) wild-type and cells expressing *sic1Δp* from the inducible *GAL1* promoter. Experiments were performed as described in Figures 3B and 4D, respectively. (E) ChIP analysis for Swi4-myc or Mbp1-myc at the promoter of the indicated genes. Analysis was performed in G1 synchronized cells released from α -factor arrest and samples were taken at the indicated intervals. G1: 0 min, G1/S: 30 min and S phase: 60 min after release. Enrichment levels were accessed by qPCR and are presented as the ratio between Swi4-myc/Mbp1-myc percentages after normalization of each ChIP for its corresponding maximum value. (F) Percentage (%) of dosage-sensitive genes as compared with the number of genes tested in the combined genome-wide overexpression studies of Sopko *et al* (2006) and Yoshikawa *et al* (2011) for the indicated gene categories. The number of genes tested for overexpression in each of the categories is indicated in parenthesis.

together, these data suggest that the SBF to MBF switch mechanism defines a new group of G1/S genes enriched for dosage-sensitive genes and/or replication stress-induced genes.

Discussion

Here, we report that increase in the expression of G1/S cell-cycle genes is a prominent part of the transcriptional response to replication stress, and we uncover the mechanisms governing such transcriptional up-regulation. We found that replication stress induces transcription of MBF, but not SBF targets in a Rad53-dependent manner likely via inactivation of the MBF co-repressor Nrm1. A more comprehensive study, which establishes the molecular mechanism of this regulation, has been carried out in the Wittenberg

laboratory (Travesa *et al*, 2012). We show that this pathway functions in parallel to the Rad53–Dun1-dependent inhibition of the Crt1 repressor, a pathway that mediates induction of DDR genes (Figure 8A). Furthermore, we uncover a novel SBF-to-MBF switch mechanism that allows G1/S genes targeted by SBF during G1 to be regulated by MBF once cells progress into S phase, which allows regulation by the replication checkpoint (Figure 8B). To our knowledge, this is the first evidence of SBF/MBF switching during the cell cycle at a G1/S promoter. The importance of this mechanism of regulation is illustrated by our detailed analysis of the ‘switch gene’ *TOS4*, which shows the importance of its induction in response to replication stress and its toxicity when overexpressed. We propose that the SBF-to-MBF switch is a mechanism of transcriptional control that allows checkpoint

Figure 8 (A) Parallel pathways for the replication checkpoint-dependent transcriptional regulation in budding yeast. Upon replication stress, Rad53 induces the expression of DDR genes via Dun1/Crt1 pathway and de-represses transcription of MBF-dependent targets required for replication stress response. (B) Regulation of *TOS4* as a prototypical example of SBF-to-MBF subunit switch. *TOS4* transcription is regulated via subunit switching and replication checkpoint signalling. During G1, SBF binds to *TOS4* promoter and activates transcription. Upon entry into S phase, SBF dissociates from the *TOS4* promoter in a Clb/CDK1-dependent manner leaving *TOS4* promoter available for binding of MBF and its co-repressor Nrm1. In response to replication stress, Nrm1 dissociates from MBF in response to replication checkpoint activation leading to transcriptional de-repression of *TOS4*. (C) Proposed importance of SBF-to-MBF subunit switching. The SBF/MBF switch on G1/S promoters provides a fail-safe mechanism that prevents loss of periodicity and hyperaccumulation of dosage-sensitive genes during G1 in case of MBF loss of function. (*) Indicate loss of periodicity in case of MBF 'malfunction'.

activation but prevents loss of periodicity and potential hyperaccumulation of dosage-sensitive proteins in case of cell-cycle transcription factor failure (Figure 8C). Overall, our work demonstrates how the coordinated action of SBF and MBF during the cell cycle allows for the crosstalk between transcription of G1/S genes and the replication checkpoint upon replication stress.

MBF as a link between replication checkpoint signalling and transcriptional regulation of G1/S genes

We found that the accumulation of MBF targets upon replication stress involves Rad53-dependent regulation of the MBF co-repressor Nrm1. Similar observations have been made, using a genome-wide transcriptional approach, by the Wittenberg laboratory (Travesa *et al*, 2012). Our findings provide evidence for this regulation to be conserved between the distantly related budding and fission yeast, *Schizosaccharomyces pombe*. In fission yeast, the G1/S transcription depends on a single heterodimeric transcription factor complex, which is, based on its homology to budding yeast, also called MBF (Lowndes *et al*, 1992; Caligiuri and Beach, 1993; Whitehall *et al*, 1999). However, in addition to Nrm1, the transcriptional repressor Yox1 is also required to confine transcription to the G1 phase (Aligianni *et al*, 2009). It has been shown that in response to replication stress, MBF-dependent transcription is activated via checkpoint protein

kinase Cds1 phosphorylation-dependent inactivation of both Nrm1 and Yox1 (de Bruin *et al*, 2008; Caetano *et al*, 2011; Gomez-Escoda *et al*, 2011; Purtil *et al*, 2011). Overall, our study shows that the mechanism that governs the remodeling of the cell-cycle transcriptional programme by the DNA replication checkpoint is conserved between these distantly related yeasts.

SBF-to-MBF switch genes; a new group of G1/S genes

Detailed analysis of the transcriptional regulation of the G1/S gene *TOS4* allowed us to uncover a prototypical SBF-to-MBF switch at a promoter gene in yeast. We speculate that overlapping SCB and MCB elements at gene promoters is likely the basis for the switch, only allowing MBF or SBF to be bound at any given time. The presumed higher binding affinity of SBF for the SCB element would allow SBF to compete for the binding during G1. On the other hand, our data show that switch genes are inactivated in a timely manner even in the absence of Clb/CDK activity, suggesting that MBF actively replaces SBF at switch promoters during S phase. In addition, the data show that the non-overlapping target gene *CLN1* remains active when Clb/CDK activity is inhibited. This together with *CLN1* being repressed in *mbp1Δ* cells during S phase and in response to HU treatment (Supplementary Figure S8) and being bound by MBF throughout the cell cycle shows a clear difference in regulation between an dual-regulated SBF and MBF target (i.e., *CLN1*) and 'switch' genes (i.e., *TOS4* and *MCD1*), establishing the latter as a new group of G1/S genes.

Rationale for an SBF-to-MBF switch for the regulation of G1/S genes

Analysis of 200+ G1/S promoters, identified by ChIP-chip (Iyer *et al*, 2001), resulted in the identification of a total of 27 genes with an overlapping SCB/MCB motif, suggesting that SBF/MBF switching might be a common mode of regulation. SBF- and MBF-regulated genes appear to be segregated based upon their role in the cell. Whereas the majority of essential G1/S genes are regulated by MBF (Iyer *et al*, 2001) non-essential genes involved in the timing or efficiency of cell-cycle events are more likely to be regulated by SBF. This way, MBF malfunction results in viable cells, constitutively expressing essential genes, while SBF malfunction will not result in uncontrolled cell proliferation with low expression of genes involved in cell cycle timing. So when is regulation by an SBF-to-MBF switch advantageous? We speculate that G1/S genes that need to be induced in response to replication stress, but whose constitutive expression is detrimental to cell-cycle progression, would benefit from a regulation by a SBF/MBF switch. For example, the SBF/MBF switch allows *TOS4* to behave as a replication stress-inducible gene during S phase but avoids constitutive high expression and hyperaccumulation of *Tos4* in case MBF function is compromised (Figure 8C). Consistent with this notion, we show that *Tos4* has an important role in the checkpoint response and that overexpression of *Tos4* delays cell-cycle progression. Analysis of the cluster of genes with overlapping SCB/MCB motifs revealed that overexpression of most of those genes has been reported to result in cell cycle delay and/or growth defect. Moreover, analysis of transcription induction upon HU treatment showed that this group of G1/S switch genes is enriched by genes that are

up-regulated during replication stress conditions. Taken together, the results reveal that SBF-to-MBF switch preferentially regulates dosage-sensitive genes, and we propose that most of the genes controlled by the switch play important roles during replication stress response, as shown here for *Tos4*, and previously for *Mcd1* and *Swe1* (Strom *et al*, 2004; Enserink *et al*, 2006; Liu and Wang, 2006; Covo *et al*, 2010). Further functional analysis of the other genes regulated by the SBF/MBF switch, most of which are currently annotated as putative genes, may reveal other dosage-sensitive genes and/or yet undescribed checkpoint effectors.

Rad53-MBF-Tos4 pathway; a novel branch of the replication checkpoint

Tos4 was originally identified as an SBF target and a putative transcription factor suggested to repress pheromone response genes during the cell cycle and in the presence of α -factor (Horak *et al*, 2002). Several lines of evidence presented here support that *Tos4* is an effector of the replication checkpoint. First, the expression of *Tos4* is strongly up-regulated during replication stress in a Rad53-dependent manner. Second, following replication stress, *Tos4* rapidly accumulates in the nucleus. Third, overexpression of *Tos4* causes a cell cycle delay. Fourth, *Tos4* contains an FHA domain, commonly found in checkpoint proteins, that is required for its function. Furthermore, we found that *Tos4* plays a parallel role to *Dun1* in the replication checkpoint. This is supported by the findings that transcription of *TOS4* is regulated in a *Dun1*-independent manner and that *tos4Δdun1Δ* cells exhibit a much stronger HU sensitivity as compared with the single deletes. Together, our findings establish that the Rad53-MBF-Tos4 pathway represents a novel branch of the replication checkpoint (Figure 8A). While the exact role of *Tos4* remains unclear, the ability of *Tos4* to interact with the Rpd3 and Set3 HDAC complexes and the importance of this interaction for the survival of cells in response to replication stress suggests that *Tos4* functions to couple replication checkpoint signaling to the regulation of HDAC function.

Comparisons with mammalian G1/S transcription

Our work suggests that G1/S transcription factor switching assures that malfunction of any one transcription factor does not result in complete loss of periodicity (Figure 8C). In mammalian cells, switching of the E2F transcription factors at G1/S promoters during the cell cycle seems to be the standard (Dimova and Dyson, 2005; Moon and Dyson, 2008), suggesting that loss of periodicity is detrimental. In line with this, the high frequency of genetic alterations in genes involved in E2F-dependent G1/S transcriptional regulation found in human tumour cells indicates an important role for proper regulation of the E2F system in the prevention of tumour development (Stevens and La Thangue, 2003).

In addition, we uncovered in yeast a simple but elegant mechanism by which checkpoint activation can override the G1/S transcriptional programme by directly regulating a transcriptional repressor. In human cells, the main regulator of DNA damage-inducible genes in G1 is the transcription factor p53, a target of both ATM and Chk2 (Carr, 2000). However, the transcriptional response induced by the DNA

replication checkpoint in S phase is regulated largely through a p53-independent mechanism that has yet to be fully elucidated. Interestingly, recent data suggest that, in response to DNA replication stress, like in yeast, G1/S transcription, regulated by the E2F family of transcription factors, is maintained at high levels in a Chk1-dependent manner (Cosetta Bertoli and RAM de Bruin, unpublished data).

If, as preliminary data suggest, remodelling of the G1/S cell-cycle transcriptional programme by the DNA replication checkpoint proves to be conserved from yeast to man, this would represent the largest group of co-regulated genes among DNA replication stress-induced targets and, consequently, is likely to comprise an important mechanism for the avoidance of genomic instability in human cells. It will be important to establish the precise mechanism of this control in human cells and the importance of this regulation for genome integrity in eukaryotes.

Materials and methods

Yeast strains and plasmids

Strains used in this work were generated by standard genetics methods and derived from S288C (*MATa*, *ura3-52*, *trp1-63*, *his3-200*), MBS164-YPH499, congenic to S288C (*MATa*, *ura3-52*, *leu2Δ1*, *trp1-63*, *his3-200*, *lys2ΔBgl*, *hom3-10*, *ade2Δ1*, *ade8*, *arg4Δ*, *sml1::TRP bar1::HIS3*) or 15Daub (*MATa*, *ade1*, *leu2-3*, *112 his2*, *trp1-1*, *ura3Δns*, *bar1Δ*) unless otherwise stated. All plasmids and yeast strains used in this study are described on Supplementary data (Supplementary Tables SII and SIII, respectively). Strains and plasmids are available upon request.

Cell synchronization

Mating pheromone arrest synchrony experiments were also carried out as described (Stuart and Wittenberg, 1995). In the experiments involving strains carrying *GAL-sic1Δp*, an overnight culture grown in 2% raffinose 0.2% glucose was used to inoculate a 2% raffinose culture. This culture was grown for 2 h before mating pheromone was added. Two hours after mating pheromone addition, 2% galactose was added and 2 h later cells were released from the G1 arrest in 2% galactose medium and samples were taken.

SILAC labelling of yeast and mass spectrometry analysis

For mass spectrometry experiments, cells were grown in (–) Arg (–) Lys dropout media ('light' version complemented with normal arginine and lysine; 'heavy' version complemented with lysine ¹³C6, ¹⁵N2 and arginine ¹³C6, ¹⁵N4) (see Supplementary data for details). Proteins were digested with trypsin, peptides were desalted, dried, reconstituted in 80% acetonitrile and 1% formic acid and fractionated by hydrophilic interaction chromatography (HILIC) as previously described in Albuquerque *et al* (2008). Fractions were dried, reconstituted in 0.1% trifluoroacetic acid and analysed by LC-MS/MS using 125 μM ID capillary C18 column and an Orbitrap XL mass spectrometer coupled with and Eksigent nano-flow system. Database search and analysis was performed as previously described in Ohouo *et al* (2010).

Reverse transcriptase (RT) and quantitative (q)PCR

Total RNA was isolated using the Rneasy kit (Qiagen). The QuantiTech™ SYBR® Green PCR kit (Qiagen) was used for quantitative PCR on ChIP samples and the QuantiTech SYBR Green RT-PCR kit (Qiagen) was used for RT-PCR experiments. Reactions were run on the Chromo-4 qPCR I system (MJ Research) using standard PCR and RT/PCR conditions. Data were analysed using MJ Opticon Monitor Analysis Software 3.0.

ChIP analysis

Chromatin immunoprecipitation was performed as described (Flick *et al*, 2003).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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