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Tid1/Rdh54 translocase is phosphorylated through a Mec1- and Rad53-dependent manner in the presence of DSB lesions in budding yeast

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Abstract

Saccharomyces cerevisiae cells with a single double-strand break (DSB) activate the ATR/Mec1-dependent checkpoint response as a consequence of extensive ssDNA accumulation. The recombination factor Tid1/Rdh54, a member of the Swi2-like family proteins, has an ATPase activity and may contribute to the remodeling of nucleosomes on DNA. Tid1 dislocates Rad51 recombinase from dsDNA, can unwind and supercoil DNA filaments, and has been implicated in checkpoint adaptation from a G2/M arrest induced by an unrepaired DSB.

Here we show that both ATR/Mec1 and Chk2/Rad53 kinases are implicated in the phosphorylation of Tid1 in the presence of DNA damage, indicating that the protein is regulated during the DNA damage response. We show that Tid1 ATPase activity is dispensable for its phosphorylation and for its recruitment near a DSB, but it is required to switch off Rad53 activation and for checkpoint adaptation. Mec1 and Rad53 kinases, together with Rad51 recombinase, are also implicated in the hyper-phosphorylation of the ATPase defective Tid1-K318R variant and in the efficient binding of the protein to the DSB site. In summary, Tid1 is a novel target of the DNA damage checkpoint pathway that is also involved in checkpoint adaptation.

Keywords

Double Strand Break (DSB); DNA recombination; checkpoint adaptation; DNA damage

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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1. Introduction

In *Saccharomyces cerevisiae* cells, formation of one irreparable DSB elicits a robust activation of Rad53 kinase, a central player of the DNA damage checkpoint pathway, and a transient cell cycle block in metaphase (reviewed in [1]). Rad53 is activated through phosphorylation by the upstream kinase Mec1, which is recruited to 5'-to-3' resected DSB ends [1]. Rad53 phosphorylation can be analyzed by Western blotting, and the phosphorylation is commonly used as a biochemical marker to test activation of the Mec1-induced DNA damage checkpoint pathway. It has been observed that the checkpoint signaling is switched off 12–15 hours after the formation of one irreparable DSB. Concomitantly, Rad53 becomes dephosphorylated and the cell cycle can restart in the presence of a damaged chromosome [2]. This phenomenon is called checkpoint adaptation and it has also been observed in other eukaryotic organisms in response to various types of DNA damage and replication stress [3]. Interestingly, checkpoint adaptation has been suggested to promote uncontrolled proliferation of cancer cells, and may play a role in the development of therapy-resistance tumours. Therefore, a better understanding of the mechanisms and factors involved in checkpoint adaptation is a relevant goal in cancer biology, and it may be useful to develop novel therapeutic strategies. Notably, PLK1-like kinases promote checkpoint adaptation in multicellular eukaryotes [3], and specific PLK1 inhibitors are in clinical trials for cancer therapy [4].

Budding yeast has proven to be an ideal system for the study of activation and inactivation of the DNA damage checkpoint and, in particular, for analysis of checkpoint adaptation in the presence of a single irreparable DSB lesion. A single DSB can be induced at a specific locus through the conditional overexpression of HO endonuclease. By using this genetic system, several proteins have been implicated in checkpoint adaptation in yeast [1]. Among these factors is Tid1 (also called Rdh54), a member of the Swi2-like family, which includes proteins having dsDNA-dependent ATPase activity that are able to translocate along a DNA molecule, thus contributing to nucleosome remodeling around the DSB site. Moreover, these factors can supercoil and unwind DNA and promote D-loop formation and branch migration in homologous recombination processes [5]. A number of *in vitro* and *in vivo* data indicate that Tid1 dissociates Rad51 recombinase from dsDNA, thus preventing the accumulation of toxic Rad51-DNA intermediates and also ensuring that a sufficient amount of Rad51 will be available for DSB repair and recombination [6]. Tid1 shares some molecular functions and mechanisms with the Swi2-like homologs Rad54 and Usl1. However, they likely have distinct functions, as indicated by the distinct phenotypes of the corresponding mutants [6].

Tid1 plays major role in meiotic recombination, while it is involved in minor pathway in mitotic recombination, specifically in a diploid [7,8]. Interestingly, Tid1 has been involved in checkpoint adaptation from a G2/M arrest induced by an irreparable DSB [2]. To further address the functional role of Tid1 in cells responding to DSB and in checkpoint adaptation, we tested whether Tid1 protein is post-translationally regulated in the presence of an irreparable DSB. We found that Tid1 is phosphorylated by the Mec1 and Rad53 kinases, similar to other factors such as Srs2, Rad51, Sae2, and Cdc5 involved in turning off Rad53 during checkpoint adaptation [9,10,11,12]. Therefore, Tid1 belongs to a heterogeneous family of factors which are targets of the DNA damage checkpoint pathway, and are involved in silencing the checkpoint response in the presence of one irreparable DSB.

2. Materials and Methods

Yeast strains

All strains are derivatives of JKM background (*MATa* or *MATa*, *hmlΔ::ADE1*, *hmrΔ::ADE1 ade1-100*, *trp1Δ::hisG*, *leu2-3*, *leu2-112*, *lys5*, *ura3-52*, *ade3::GAL::HO*),

generously provided by J. Haber (Brandeis University, Waltham, Boston). Y454 was obtained by integration of the 3XHA tag at the C-end of the *TID1* locus by the one-step PCR system [13]. Standard genetic procedures for transformation and tetrad analysis were followed to construct the various strains. Y841 was obtained by integrating of NvuI-digested pHK255 plasmid into the *TID1*-3XHA locus in Y454 strain. After pop-out by treatment with 5-FOA, the integration of the *tid1*-K318R mutation was confirmed by sequencing analysis. In previous papers [14,15], the K318R mutation was indicated as K352R due to the annotation of an upstream ATG start site. We verified by DNA sequencing the ATG start codon in the genetic background used here (data not shown). Y522 (*MATa, mec1Δ, TID1*-3XHA) was obtained by crossing Y454 with Y138 (*MATa, mec1Δ*); Y876 (*MATa, mec1Δ, tid1*-K318R-3XHA) was obtained by crossing Y841 with Y138 (*MATa, mec1Δ*); Y962 (*MATa, rad53*-K227A, *TID1*-3XHA) was obtained by crossing Y454 with Y677 (*MATa rad53*-K227A); Y966 (*MATa rad53*-K227A *tid1*-K318R-3XHA) was obtained by crossing Y841 with Y677 (*MATa rad53*-K227A); Y741 (*MATa, rad51Δ, TID1*-3XHA) was obtained by crossing Y623 (*MATa, TID1*-3XHA) with Y608 (*MATa, rad51Δ*); Y873 (*MATa, rad51Δ, tid1*-K318R-3XHA) was obtained by crossing Y841 with Y736 (*MATa, rad51Δ*); Y1768 (*MATa, dun1Δ, TID1*-3XHA) was obtained by crossing Y623 with Y1741 (*MATa, dun1Δ*); Y1769 (*MATa, dun1Δ, tid1*-K318R-3XHA) was obtained by crossing Y869 (*MATa tid1*-K318R-3XHA) with Y1741; Y967 (*MATa rad53*-K227A *rdh54*-K318R-3XHA) was obtained by crossing Y 869 with Y677; Y1771 (*MATa chk1Δ rad53*-K227A *rdh54*-K318R-3XHA) was obtained by crossing Y967 with Y1061 (*MATa chk1Δ*). Y811 was obtained by integration of the 3XHA tag at the C-end of the *TID1* locus by the one-step PCR system in YMV80 background [16]. All the strains used in this work are haploid; moreover, all the *mec1Δ* strains also have the *smf1Δ* mutation, to keep cells viable.

Western Blot Analysis

The TCA protein extraction and the Western blot procedures have been previously described [17]. Rad53 and Tid1-3XHA proteins were analysed using Mab.EL7 [17], and 12CA5 monoclonal antibodies, respectively.

Immunoprecipitation analysis

Tid1-3XHA protein was immunoprecipitated with the 12CA5 monoclonal antibody using a standard procedure. 2×10^9 cells were resuspended in 400 microliters of lysis buffer (50 mM Tris-HCl, 50 mM NaCl, 60 mM β -glycerolphosphate, 1 mM DTT, 1mM Sodium Orthovanadate, 1 % NP40, supplemented with protease inhibitor cocktail (Roche)), and disrupted with glass beads by Fast Prep (MPBio). Crude extracts were incubated for 2 hours at 4°C in the presence of magnetic beads (Dynal), which were pre-incubated with the 12CA5 antibody. The beads were then washed with lysis buffer and resuspended with 30 microliters of Laemmli sample buffer. The immunoprecipitates were analysed by Western blot using 12CA5 (anti-HA) and anti-phospho-S/T-Q motifs antibody (Cell Signaling).

Cell synchrony and Flow Cytometry

Cells were pre-synchronized in G1 with α -factor (2 μ g/ml) and then released in fresh medium. Cells were arrested in G1 and G2 /M with α -factor (10 μ g/ml) or nocodazole (15 μ g/ml), respectively. DNA content was analyzed by FACSCalibur (Bekton-Dickinson) and Cell- Quest software (Bekton-Dickinson).

In Vitro Dephosphorylation Assay

Crude extracts were prepared as described [18], and resuspended in λ phosphatase buffer with or without 4000 U of λ phosphatase (Biolabs). Samples were incubated 30 min at 30°C and resuspended in Laemmli sample buffer.

Chromatin Immunoprecipitation Analysis (ChIP)

ChIP analysis was performed as described previously [19]. Multiplex PCRs were carried out by using primer pairs complementary to DNA sequences located 1 kb from the HO-cut site at MAT (DSB) and to DNA sequences located 66 kb from MAT (CON). Gel quantitation was determined using the Quantity One program (Biorad). The relative fold enrichments of DSB-bound protein were calculated as follow: $[\text{DSB_IP}/\text{CON_IP}]/[\text{DSB_input}/\text{CON_input}]$, where IP and Input represent the amount of PCR product in the immunoprecipitates and in input samples before immunoprecipitation, respectively.

3. Results

3.1. Tid1 is phosphorylated in response to DSB formation

In *Saccharomyces cerevisiae*, it has been shown that Srs2, Rad51, Sae2 and Cdc5 factors are involved in DNA damage checkpoint inactivation and adaptation, and that they are regulated through Mec1-dependent phosphorylation in the presence of DSB lesions [9,10,11,12]. We tested whether Tid1, a DNA translocase required for checkpoint adaptation, was specifically modified in response to a single irreparable DSB. To visualize Tid1 on Western blots of crude yeast protein extracts, we initially inserted a 3XHA tag sequence at the C-terminal of the *TID1* gene. The sensitivity of haploid cells carrying the *TID1::3XHA* allele to various DNA damaging agents (the alkylating agent methyl methanesulfonate (MMS), UV irradiation, bleocin, camptothecin) was similar to that of the wild type strain (data not shown), indicating that the presence of the 3XHA tag at the C terminal of Tid1 does not affect the functionality of the protein.

We then tested the stability of Tid1 and the electrophoretic mobility of the protein throughout the cell cycle. Protein samples were prepared from yeast cells, blocked in G1 phase by α -factor treatment and released into fresh medium with or without the ribonucleotide reductase inhibitor hydroxyurea (HU, 0.2M) or the DNA alkylating agent MMS, 0.02% (Figure 1). As expected, the HU-treated cells remained blocked into S-phase because of the deprivation of dNTPs pools, and the MMS-treated cells delayed DNA replication because of the activation of the DNA damage checkpoint, while untreated cells progressed synchronously through S phase and the mitotic transitions, as shown by the FACS profiles (Figure 1 A, C and E). In parallel, cells were grown for 6 hours in medium containing galactose to overproduce the HO endonuclease and induce the formation of a single irreparable DSB at the MAT locus and, as a consequence, cells activated the DNA damage checkpoint and blocked in G2/M cell cycle phase [2]. Protein samples were prepared at the indicated time points and analysed by Western blotting using anti-HA antibodies (Abs) to visualize Tid1 and anti-Rad53 Abs to test activation of DNA damage checkpoint signaling. We found that Tid1 is a stable protein throughout the cell cycle, showing a modest increase in its level in early S-phase, likely linked to increased gene expression during this cell cycle phase. In the experimental conditions used here, we did not observe any Tid1 electrophoretic mobility shift either in untreated or in HU- and MMS-treated cells (Figure 1B, D, F). Surprisingly, we found that Tid1 migrated as a doublet band in protein samples prepared from cells in which one irreparable DSB was induced by HO overexpression ([DSB] lane in Figure 1B, D). The altered electrophoretic mobility of the upper band was reverted by *in vitro* treatment of the protein sample with phosphatase, indicating that Tid1 was modified by phosphorylation (Figure 1G). Interestingly, we also found that Rad53 phosphorylation, which is used as a biochemical marker for Mec1-dependent checkpoint activation, did not always correlate with Tid1 phosphorylation; in fact, Rad53 is phosphorylated in HU- and MMS-treated cells, while Tid1 is not modified in such conditions (Figure 1D, F). Tid1 has been identified in a proteome wide screening for proteins phosphorylated in the presence of MMS [20]; however, we can not see this

modification by Western blotting (Figure 1F); therefore, we believe that the Tid1 phosphorylation we observed in the presence of one irreparable DSB should be different in respect to the one identified by mass spectrometry in the presence of MMS, suggesting the idea that Tid1 would be regulated through distinct mechanisms in the presence of different types of DNA damage.

Moreover, we found that the Tid1 protein, immunoprecipitated from cells with one irreparable DSB, was recognized by anti-phosphorylated S/T-Q motifs specific antibodies (Figure 1F). It is known that ATM/Tel1 and ATR/Mec1 kinases preferentially phosphorylate S/T-Q motifs in response to DNA damage [21], suggesting that they could be likely involved in Tid1 phosphorylation following a single irreparable DSB formation. Interestingly, in the presence of MMS it was reported that Tid1 is phosphorylated at the S51 residue [20], which is not a SQ motif, supporting the idea that MMS-induced and DSB-induced Tid1 phosphorylation could be different.

3.2. Analysis of the HO-induced Tid1 Phosphorylation

It is known that one irreparable DSB is processed and activates a full Mec1-dependent checkpoint pathway in G2-blocked cells, but not in G1-blocked cells [22,23], thus we tested Tid1 and Rad53 phosphorylation in cells treated with α -factor or nocodazole to block the cell cycle in G1 or G2 phases, respectively. We found that Tid1 is not phosphorylated after one irreparable DSB induced in G1-blocked cells, while its phosphorylation is accumulated in G2-blocked cells, following the formation of one irreparable DSB and the activation of Rad53 (Figure 2A). We then analysed Tid1 phosphorylation in a specific genetic background (YMV80), where the conditional overproduction of HO nuclease induces the formation of one DSB that can be repaired through a single strand annealing process (SSA). As previously reported [16], in these cells the accumulation of long ssDNA tails at the resected DSB elicits a robust Rad53 phosphorylation, which is reverted after the DSB has been completely repaired (Figure 2B). Similarly to Rad53, we found that Tid1 is phosphorylated during the prolong SSA process (Figure 2B), and its phosphorylation disappears during the checkpoint recovery, accordingly with the idea that Tid1 protein is a target of the DSB-induced checkpoint signaling.

3.3. Phosphorylation of the ATPase-inactive Tid1 protein variant

The ATPase activity of Tid1 is essential for its function during recombination and checkpoint adaptation [15,24]. We thus tested whether Tid1 ATPase activity was required for the DSB-induced phosphorylation of the protein. To this purpose, the site specific K318R mutation, which is known to abrogate the ATPase activity of the catalytic domain of Tid1 [15], was introduced into the *TID1::3XHA* locus. As expected [24,25], *tid1*-K318R cells were sensitive to MMS and did not adapt to a single irreparable DSB (data not shown).

HO endonuclease was overproduced in wild type and *tid1*-K318R cells to induce the formation of one irreparable DSB, and the phosphorylation state of both Tid1 and Rad53 proteins was analysed by Western blotting at several time points after the DSB formation (Figure 3A). We found that in wild type cells, the HO-induced Tid1 phosphorylation parallels the Mec1-dependent activation of Rad53 and disappears at late time points during checkpoint adaptation. Similar to what was observed in wild type cells, the Tid1-K318R protein variant started to be phosphorylated 3 hours after HO induction. However, at longer time points, essentially all the protein was modified and it remained phosphorylated until the end of the experiment, mirroring the extend of Rad53 activation, which was not dephosphorylated in the adaptation-defective *tid1*-K318R cells. Moreover, the electrophoretic mobility shift of the phosphorylated Tid1-K318R variant at 6–9 hours after

the HO induction appeared slightly higher than that observed for the wild type protein, while both proteins had the same electrophoretic mobility in undamaged conditions.

The results described above prompted us to test whether Mec1, the main protein kinase responding to DSB lesion in budding yeast, is required for Tid1 phosphorylation. We deleted the *MEC1* gene both in *TID1::3XHA* and *tid1-K318R::3XHA* strains, also carrying deletion of *SML1* gene to keep the cells viable. The HO gene was overexpressed in these strains to induce the formation of one irreparable DSB and samples were taken at various time points to analyse the phosphorylation state of both Tid1 and Rad53. As shown in Figure 3B and C, the HO-induced modification of the wild type Tid1 and the Tid1-K318R variant are completely abrogated in *mec1Δ* cells, supporting the notion that Tid1 is regulated through Mec1-dependent phosphorylation in the presence of DSB lesions.

We then analysed Tid1 phosphorylation in G2-blocked cells treated with 4-Nitroquinoline-1-oxide (4NQO, an oxidizing agent that also acts as UV mimetic) and zeocin (a DSB inducing agent). Wild type and *mec1Δ* cells were treated with nocodazole to block cell cycle in G2 phase, then 4 μg/ml 4NQO or 10 μg/ml zeocin were added to the cultures, as indicated in Figure 3D. We found that both Tid1 and Tid1-K318R variant were phosphorylated in a Mec1-dependent manner in the presence of 4NQO and zeocin in G2-blocked cells, suggesting that Tid1 is phosphorylated in the presence of multiple DNA lesions induced by zeocin and 4NQO treatments. Moreover, as 4NQO not only causes DNA adducts similarly to UV, but can also induce the formation of DSB lesions, it is possible that the Tid1 phosphorylation we observed in 4NQO-treated cells may be due to DSBs as well. Accordingly with previous results with the HO-induced DSB in Figure 2A, we also found that the mobility shift of the Tid1-K318R variant in the presence of 4NQO and zeocin is higher than that seen in the wild type protein.

In summary, the Tid1 ATPase activity, although essential for its functional role in recombination and checkpoint adaptation, is not required to promote its DSB-induced phosphorylation. Moreover, in agreement with previous evidence showing that the ATPase activity of Tid1 is essential to restart cell cycle progression in the presence of one irreparable DSB [24], we found that this activity is required to mediate Rad53 inactivation during checkpoint adaptation.

3.4. Genetic requirement for the Tid1 phosphorylation

We further tested whether Rad53, which is one of the main protein kinases activated by Mec1, and Dun1, that is a protein kinase activated by Rad53 itself [1], were required for Tid1 phosphorylation. We deleted the *DUN1* gene and generated the kinase inactive *rad53-K227A* allele both in *TID1::3HA* and *tid1-K318R::3HA* strains. The HO gene was overexpressed in these strains to induce the formation of one irreparable DSB and samples were taken at various time points to analyse the phosphorylation state of both Tid1 and Rad53. As shown in Figure 4A, the HO-induced modification of wild type Tid1 and the Tid1-K318R variant are not affected in *dun1Δ* cells. Interestingly, the *rad53-K227A* mutation does not affect the HO-induced phosphorylation of the wild type Tid1 protein (Figure 4B). However, the hyper-phosphorylation state of the Tid1-K318R variant is not seen in the *rad53-K227A* mutant, and the phosphorylation state of the Tid1-K318R protein variant was similar to that observed for the wild type protein. These results, together with previous findings in Figure 1, suggest that both Mec1 and Rad53 kinases are involved in the HO-induced Tid1 phosphorylation, but the Rad53 contribution becomes evident only when the ATPase-inactive Tid1-K318R variant is analyzed, raising specific questions on the functional regulation of Tid1, as it will be discussed below. We also tested whether Chk1, which is another kinase activated by Mec1 [1], was responsible for the residual Tid1 phosphorylation observed in the double mutant *rad53-K227A tid1-K318R* cells. To this aim,

we generated the deletion of *CHK1* gene in the *rad53-K227A* cells, carrying the *tid1-K318R::3HA* allele, and induced one irreparable DSB as in previous experiments. We found that the residual phosphorylation of the Tid1-K318R variant is still present in the triple mutant *tid1-K318R rad53-K227A chk1Δ* (Figure 4C), further supporting the idea that it could be mediated by Mec1.

It is known that Tid1 is recruited to a DSB through its interaction with Rad51 [14]. We thus tested whether Rad51 had any contribution to the HO-induced Rdh54 phosphorylation. Again we overexpressed the HO gene to induce the formation of one irreparable DSB in wild type and *rad51Δ* cells, carrying the *TID1::3HA* or *tid1-K318R::3HA* alleles. As shown in Figure 4D, the DSB-induced phosphorylation of wild type Tid1 is not completely compromised in the absence of Rad51, although it appeared to be delayed. Interestingly, we found that hyper-phosphorylation of the Tid1-K318R variant is impaired in *rad51Δ* cells, with a residual phosphorylation migrating as the wild type protein (Figure 4D). This result recapitulates what we found when Rad53 activity was impaired by the *rad53-K227A* mutation, suggesting that Rad53 and Rad51 may contribute to a common mechanism required for Tid1 hyper-phosphorylation. Moreover, we noticed that Rad53 phosphorylation is persistent in the double mutant *tid1-K318R rad51Δ*, accordingly with the notion that the deletion of *RAD51* gene does not rescue the checkpoint adaptation defect of *tid1* mutant cells [24,26].

3.5. Mec1-checkpoint mediates recruitment of Tid1 near to a DSB

We have shown that the Mec1-dependent checkpoint mediates Tid1 phosphorylation in the presence of one DSB. However, activation of the Mec1 and Rad53 kinases is not enough *per se* to induce such Tid1 phosphorylation since this protein is not modified in response to DNA replication stress induced by HU and MMS treatments (Figure 1). Moreover, Tid1 hyper-phosphorylation requires the Rad51 recombinase (Figure 4C), which also mediates Tid1 recruitment to the DSB lesion [14]. One possibility is that Tid1 is phosphorylated at the DSB site, and this modification may somehow reinforce the binding of the protein to DNA. We thus tested by chromatin immunoprecipitation (ChIP) whether the Mec1 kinase was also implicated in Tid1 loading near a single irreparable DSB. To this end, we recovered Tid1 protein from sheared chromatin prepared from formaldehyde crosslinked cells, at different time points after DSB formation (Figure 5). Quantitative multiplex PCR was then used to monitor the co-immunoprecipitation of DNA fragments located either 1Kb (DSB) or 66Kb (CON) from the HO-cut site. PCR analysis of the CON site was used as a control of the background signal, as previously described [19]. Firstly, we found that the Tid1-K318R is recruited to the DSB site (Figure 5), indicating that the ATPase activity is dispensable for recruitment of the protein. Interestingly, we noticed that the loading of the Tid1-K318R variant was significantly higher than that of the wild type protein, especially at late time points after the induction of the DSB, and this slight accumulation may be due to an impairment of the catalytic defective protein variant to move along DNA molecules, as showed by previous *in vitro* assays [27].

Next we tested the binding to one DSB of both Tid1 and Tid1-K318R to a DSB in *mec1Δ* cells. As shown in Figure 5, we found that loading of Tid1 near the DSB is significantly reduced in *mec1Δ* cells. This defect is particularly evident for the Tid1-K318R variant, whose binding is normally higher compared to the wild type protein. Therefore, the Mec1-dependent checkpoint contributes, together with Rad51, to mediate the Tid1 recruitment. Among various possibilities, we favour the idea that Tid1 protein is recruited to the DSB through its interaction with Rad51, as was previously showed by others [14]. Then the Tid1 function at the DSB site may be regulated through Mec1 and Rad53 phosphorylation events, which may affect its ATPase and translocase activities. Identification and characterization of the specific Tid1 phosphorylated sites will shed light on this mechanism.

4. Discussion

The Tid1 protein of *Saccharomyces cerevisiae* cells has been implicated in several molecular pathways including DNA recombination, DSB repair and checkpoint adaptation. It has been reported that Tid1 is an *in vitro* target of the meiotic checkpoint kinase Mek1 [28], and that it is phosphorylated in the presence of DNA alkylating agent MMS [20]; however, regulation of Tid1 protein has been poorly studied. Here we show that Tid1 is phosphorylated in the presence of one DSB and, based on the different electrophoretic mobility shifts (see Figure 1), this phosphorylation appears to be different from that observed in MMS-treated cells [20]. In our experimental conditions, the DSB ends are extensively resected and a long ssDNA filament is generated. As a consequence, Mec1 and Rad53 kinases are activated and the DNA damage checkpoint signaling contributes to block cell cycle progression in metaphase. Supporting this model, we did not observe Tid1 phosphorylation after one DSB induced in G1-blocked cells (Figure 2A), in which DSB ends are not efficiently processed and Mec1-signaling is not fully active [22,23]. Moreover, Tid1 phosphorylation decreases both during checkpoint recovery and adaptation, in response to one repairable and irreparable DSB (Figures 2B and 3A). Indeed, our genetic and biochemical evidences support the idea that the DSB-induced Tid1 phosphorylation is mediated by the Mec1 and Rad53 kinases. Accordingly to the protein sequence, Tid1 has several potential Mec1 and Rad53 phosphorylation motifs, suggesting that Tid1 protein can be directly phosphorylated by both Mec1 and Rad53. In agreement with this possibility, we found that in response to one irreparable DSB, immunoprecipitated Tid1 protein is recognized by specific antibodies directed against phosphorylated S/T-Q motifs (Figure 1F), which are often phosphorylated by ATM/Tel1, ATR/Mec1 and Rad53 [20,21,29]. We do not know yet which Tid1 sites are phosphorylated, although we are trying to address this issue by mass spectrometry analysis of the sites phosphorylated *in vivo* in response to DSBs.

It is known that ATPase activity is essential for the functional role of Tid1 in DSB repair, recombination and checkpoint adaptation [14,15,24]. However, here we have shown that the ATPase activity of Tid1 protein is dispensable for its initial recruitment near an irreparable DSB and that the same is true for Mec1-dependent Tid1 phosphorylation. We also observed that the ATPase defective Tid1-K318R variant is heavily hyper-phosphorylated, and this modification is abrogated in *rad53* and *rad51* mutants. It is tempting to speculate that the Tid1-K318R variant, after being loaded near the lesion through the interaction with Rad51, remains in a “frozen” and Rad53-mediated hyper-phosphorylated state. In this condition the catalytic-defective Tid1-K318R variant cannot translocate along the DNA filament [27], and we observed a slightly higher accumulation at the DSB site (Figure 5). However, our results cannot exclude the possibility that the wild type Tid1 protein may become phosphorylated by Rad53-mediated reactions. In fact, because of its ATPase activity, Tid1 would likely translocate along the DNA in a highly dynamic way, perhaps undergoing a faster kinetic loading and dislodging from the DNA, making Rad53-dependent Tid1 phosphorylation very transient and, therefore, very difficult to be detected by Western blotting. Our results raise the possibility that phosphorylation of Tid1 protein is relevant to mediate its efficient loading near the DSB site, and the identification and mutation of the specific Tid1 phosphorylated sites will shed light on this mechanism. Alternatively, or in addition, as the phosphorylation and binding to DSB of Tid1 protein are not completely abrogated in *rad51Δ* cells (Figure 4 and [14]), we could also hypothesize a two-step Tid1 phosphorylation mechanism. Firstly Mec1 phosphorylates Tid1 at the DSB site and then after Tid1 has been engaged in a Rad51-dependent intermediate on the DNA, it is phosphorylated by Rad53. It is possible that Mec1- and/or Rad53-dependent phosphorylation mediates a functional interaction between Tid1 and Rad51, which, in turn, may mediate efficient binding of Tid1 near the DSB. Interestingly, in meiotic yeast cells, a Mek1-dependent phosphorylation of Rad54 (a Tid1 homologue), affects its interaction with

Rad51 and meiotic recombination outputs [28], suggesting that a fine regulation of Rad51 protein complexes is a fundamental step to modulate DNA recombination and DSB repair. In addition, *rad51Δ* cells, as well as *tid1Δ* and *tid1Δ rad51Δ* mutants, do not adapt to a single irreparable DSB [24], suggesting that the functional interaction between Tid1 and Rad51 is important to recruit Tid1 onto irreparable DSB, allowing the checkpoint adaptation process. Indeed, we found that the hyper-phosphorylation of Tid1-K318R variant is abrogated in *rad51Δ* cells, despite the fact that Rad53 phosphorylation is persistent (Figure 4D), according to previous evidences [24,26], showing checkpoint adaptation defect in the *tid1Δ rad51Δ* double mutant cells. The functional role of Tid1 in checkpoint adaptation is mediated by its ATPase/translocase activity and may be linked to its capacity to remodel nucleosomes and dislocate Rad51 and/or other factors from dsDNA [5]. Indeed, it is known that specific nucleosome modifications mark several kilobases around a DSB and, surprisingly, a chromosome-wide spreading of Rad51 molecules was described starting from a persistent DSB [30].

In conclusion, we have shown that Tid1 is a stable protein throughout the cell cycle, and is phosphorylated in the presence of one irreparable DSB through Mec1 and Rad53 kinases. The accumulation of the phosphorylated state correlates with the binding of the protein to the DSB site, which is mediated by Mec1 and Rad53 kinases and Rad51 recombinase [14]. The ATPase activity of Tid1 is dispensable for the recruitment to the DSB and the phosphorylation of the protein, but it is necessary for the functional roles of Tid1 at the DSB site. Further biochemical and genetic analyses will be required to fully clarify the functional regulation of Mec1- and/or Rad53-dependent phosphorylation of Tid1, and to understand its role in checkpoint adaptation.

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Abbreviations

DSB	double strand break
MMS	methyl methanesulfonate
HU	hydroxyurea

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Highlights

The ATPase/translocase Rdh54 is phosphorylated in the presence of one irreparable DSB.

Rdh54 phosphorylation is abrogated in *mec1* Δ cells.

ATPase activity is dispensable for Rdh54 phosphorylation.

Rad53 and Rad51 contribute to mediate hyper-phosphorylation of Rdh54-K318R variant.

Mec1 kinase promotes Rdh54 binding near one irreparable DSB.

Fig 1. Analysis of Tid1 protein with or without DNA damage throughout the cell cycle
(A–F) An exponentially growing culture of the strain Y454 (wild type, *TID1*-3XHA) was grown in YEP+raffinose and pre-synchronized in G1 by α -factor (α F) treatment and released from the G1 block in fresh medium with/without 0.2 M HU or 0.02% MMS. (A, C, E) Samples were taken at the indicated time points to test FACS profiles. (B, D, F) Protein extracts were prepared and analysed by Western blot using 12CA5 (anti-HA) and Ma.EL7 (anti-Rad53) antibodies. Moreover, one part of the original culture was also split and treated with 2% galactose for 6 hours to induce the overexpression of HO and the formation of one irreparable DSB; as a consequence, cells remained blocked in G2/M cell cycle phase (data not shown). Protein extract, labelled [DSB], was analysed in the same gels (Figure 1B and D). (G) Protein extracts obtained after DSB formation (similarly to the lane [DSB] in Figure 1B and D) were treated with λ phosphatase before gel electrophoresis and tested with

12CA5 (anti-HA) antibody. (H) Exponentially growing culture of the strain Y454 (wild type, *TID1*-3XHA) was grown in YEP+raffinose and synchronized at G2/M transition by nocodazole. Half of the culture was treated with galactose for 6 hours to ensure DSB formation. Tid1-3XHA was isolated by immunoprecipitation with anti HA antibody and phosphorylation on SQ/TQ motifs tested by Western blot with anti pSQ/pTQ antibody (Cell Signaling).

Fig 2. Kinetic analysis of Tid1 phosphorylation in the presence of one HO-induced DSB throughout the cell cycle

(A) An exponentially growing culture of the JKM-background strain Y454 (wild type, *TID1*-3XHA) was grown in YEP+raffinose. Half of the culture was treated with α -factor (α F) to block cell cycle in G1 phase; the other part was treated with nocodazole (N) to block the cell cycle in G2 phase. 2% galactose was added (time zero) to induce the formation of one irreparable DSB and samples were taken at the indicated time points. (B) An exponentially growing culture of the YMV80-background strain Y811 (wild type, *TID1*-3XHA) was grown in YEP+raffinose. 2% galactose was added (time zero) to induce the formation of one repairable DSB and samples were taken at the indicated time points. Protein extracts were prepared and analysed by Western blot using 12CA5 (anti-HA) and Ma.EL7 (anti-Rad53) antibodies.

Fig 3. Mec1-dependent phosphorylation of the wild type Tid1 and the ATPase-defective Tid1-K318R variant

(A) Exponentially growing cultures of the JKM-background strains Y454 (wild type, *TID1-3XHA*) and Y841 (*tid1-K318R-3XHA*) were grown in YEP+raffinose. 2% galactose was added (time zero) to induce the formation of one irreparable DSB and samples were taken at the indicated time points. (B) Exponentially growing cultures of the JKM-background strains Y454 (wild type, *TID1-3XHA*), Y841 (*tid1-K318R-3XHA*), Y522 (*mec1Δ*, *TID1-3XHA*) and Y876 (*mec1Δ*, *tid1-K318R-3XHA*), were grown in YEP +raffinose. 2% galactose was added (time zero) to induce the formation of one irreparable DSB and samples were taken at the indicated time points. (C) Exponentially growing cultures of the same strains described in (B), were grown in YPD and treated with nocodazole (N) to block cell cycle in G2 phase. Each culture was split in 3 parts and treated for 30 minutes with/without 4 μ g/ml 4-Nitroquinoline-1-oxide (4NQO) or 10 μ g/ml zeocin. Samples were taken at the indicated time points to determine FACS profiles. Protein extracts were prepared and analysed by Western blot using 12CA5 (anti-HA) and Ma.EL7 (anti-Rad53) antibodies.

Fig 4. Rad53 and Rad51 mediate Tid1 phosphorylation in the presence of one HO-induced persistent DSB

(A–D) Exponentially growing cultures of the JKM-background strains Y1768 (*dun1Δ*, *TID1*-3XHA), Y1769 (*dun1Δ*, *tid1*-K318R-3XHA), Y962 (*rad53*-K227A, *TID1*-3XHA), Y966 (*rad53*-K227A *tid1*-K318R-3XHA), Y841 (*tid1*-K318R-3XHA), Y1771 (*rad53*-K227A, *chk1Δ* *tid1*-K318R-3XHA), Y741 (*rad51Δ*, *TID1*-3XHA), Y873 (*rad51Δ*, *tid1*-K318R-3XHA), were grown in YEP+raffinose. 2% galactose was added (time zero) and samples were taken at the indicated time points. Protein extracts were prepared and analysed by Western blot using 12CA5 (anti-HA) and Ma.EL7 (anti-Rad53) antibodies.

Fig 5. Recruitment of Tid1 protein to a HO-induced persistent DSB

YEP+raffinose nocodazole-arrested cell cultures of the JKM-background strains Y454 (wild-type, *TID1*-3XHA), Y841 (*tid1*-K318R-3XHA), Y522 (*mec1Δ*, *TID1*-3XHA), Y876 (*mec1Δ*, *tid1*-K318R-3XHA), were transferred to nocodazole-containing YEP+raffinose +galactose (time zero), to induce the formation of one irreparable DSB. Cells were collected at the indicated time points and then subjected to chromatin immunoprecipitation analysis. The graph represents the results from four independent experiments.