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ORIGINAL ARTICLE

Distinct associations of the *Saccharomyces cerevisiae* Rad9 protein link Mac1-regulated transcription to DNA repair

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Abstract

While it is known that *Sc*Rad9 DNA damage checkpoint protein is recruited to damaged DNA by recognizing specific histone modifications, here we report a different way of Rad9 recruitment on chromatin under non DNA damaging conditions. We found Rad9 to bind directly with the copper-modulated transcriptional activator Mac1, suppressing both its DNA binding and transactivation functions. Rad9 was recruited to active Mac1-target promoters (*CTR1, FRE1*) and along *CTR1* coding region following the association pattern of RNA polymerase (Pol) II. Hir1 histone chaperone also interacted directly with Rad9 and was partly required for its localization throughout *CTR1* gene. Moreover, Mac1-dependent transcriptional initiation was necessary and sufficient for Rad9 recruitment to the heterologous *ACT1* coding region. In addition to Rad9, Rad53 kinase also localized to *CTR1* coding region in a Rad9-dependent manner. Our data provide an example of a yeast DNA-binding transcriptional activator that interacts directly with a DNA damage checkpoint protein in vivo and is functionally restrained by this protein, suggesting a new role for Rad9 in connecting factors of the transcription machinery with the DNA repair pathway under unchallenged conditions.

Keywords Checkpoint protein recruitment · Mac1 · Metal-regulated transcription · Rad53 · Rad9 · Hir1

Introduction

DNA damage checkpoints are highly conserved molecular mechanisms for the negative control of DNA replication and mitosis when the genome integrity is compromised (Finn et al. 2012; Li and Xu 2016; Longhese et al. 2008; Nair et al. 2017). Checkpoint pathways involve sensing of damage and transmission of a signal from damaged DNA to effector molecules leading to cell cycle regulation and DNA repair or apoptosis (in multicellular organisms) (Ciccia and Elledge 2010; Coutelier and Xu 2019; Harrison and Haber 2006). This dynamic process has been genetically and visually reconstructed in *S. cerevisiae* (Lisby et al. 2004; Pardo et al. 2017).

In this paper, we focus on new properties of the prototype DNA-damage checkpoint protein Rad9, discovered in *S. cerevisiae* 30 years ago in X-ray and UV-irradiation sensitive mutants failing to arrest in G2 stage (Schiestl et al. 1989; Weinert and Hartwell 1988). Its known role is to protect cells from genomic instabilities by delaying progress in the cell cycle, reflected in the increased rates of spontaneous chromosome loss and rearrangements seen in *rad9*Δ cells (Fasullo et al. 1998; Weinert and Hartwell 1990). Rad9 is a key player in the DNA damage response (DDR) pathway, as adaptor/mediator required for efficient signal transmission from sensor to effector kinases at G1/S, intra-S and G2/M phases of the mitotic cell cycle (Moriel-Carretero et al. 2019; Sau and Kupiec 2019; Siede et al. 1993; Weinert and Hartwell 1989) and at initial steps of meiosis (Weber and Byers 1992). More specifically, Mec1/Tel1-hyperphosphorylated Rad9 (Emili 1998) mediates amplification of the initial signal by the activation of Chk1 and Rad53 (Chk2/Cds1)

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protein kinases. While Chk1 is important for cell-cycle arrest in *cdc13*-1 and yku70Delta-induced telomere damage at the G2/M checkpoint (Blankley and Lydall 2004), Rad53 transduces the signal to downstream kinases to regulate DNA repair, replication, fork stabilization, cell-cycle progression, and transcriptional regulation (Emili et al. 2001; Harrison and Haber 2006; Smolka et al. 2007). Oligomerized hyperphosphorylated Rad9 first mediates Mec1-Rad53 interaction for Rad53 phosphorylation (Sweeney et al. 2005) and then acts as a scaffold to catalyze Rad53 *in trans* autophosphorylation (Gilbert et al. 2001; Ma et al. 2006). Rad9 was also previously implicated in another signal linked to DNA repair, the transcriptional response of specific genes involved in DNA repair, replication and recombination at all stages of the cell cycle (Aboussekhra et al. 1996) and later in the excision repair of active genes (Al-Moghrabi et al. 2009).

In response to DNA damage, Rad9 is recruited on chromatin adjacent to damaged sites to be enriched for signal amplification. Its recruitment is accomplished via two different pathways. One is based on its interaction with the Mec1 activator Dpb11 involving two Rad9 key CDK phosphorylation sites, S462 and T474, that bind directly to the Dpb11 N-terminal BRCT repeats 1 and 2 (Pfander and Diffley 2011). The other relies on two histone modifications. Histone H2A C-terminal phosphorylation (γH2A) by Mec1/Tel1 kinases promotes interaction between H2A and Rad9 BRCT phosphopeptide binding domains (Hammet et al. 2007) while histone H3 methylation on lysine 79 (H3K79me) by the Dot1 methyltransferase mediates interactions with the Rad9 Tudor domain folds (Huyen et al. 2004; Toh et al. 2006; Wysocki et al. 2005). Another DNA damage-regulated histone modification was recently detected to affect Rad9 chromatin localization. The phosphorylation of histone H4 Threonine 80 (H4T80ph) by the p21-activated Cla4 kinase promotes the timely recruitment of Rtt107 protein to damaged sites where it displaces Rad9, resulting in termination of the checkpoint signaling cascade and resumption of normal cell growth after DNA damage (Millan-Zambrano et al. 2018). Rad9 phosphorylation and subsequent activation of the DDR checkpoint was also accomplished in the absence of DNA damage, by artificially colocalizing and accumulating the Ddc2-Mec1 and Ddc1-Mec3-Rad17 damage sensors to multimerized LacI binding sites (LacO arrays) in the yeast genome (Bonilla et al. 2008). This emphasized the importance of the initial step of recruitment on chromatin for the activation of the whole pathway.

Although much is known with respect to Rad9 and DDR, scant information exists about its role under non DNA damage-inducing conditions. Rad9 may possess other functions that could impinge on important physiological cellular pathways. It was previously shown that while upon DDR, hyperphosphorylated Rad9 and Rad53 kinase were included in a 560 kDa protein complex (Gilbert et al. 2001), in non-damaged cells, a hypophosphorylated form of Rad9 was found in a larger protein complex (> 850 kDa) of yet unknown function (Gilbert et al. 2003). It is known that, in physiological conditions, Rad9 binds chromatin by recognizing distinct histone methylation marks although its functional role at non-damaged DNA sites remains unclear (Gilbert et al. 2001; Granata et al. 2010; Hammet et al. 2007). Additionally, in a previous genome-wide study, our group found that, in the absence of exogenously induced DNA damage, Rad9 localizes on fragile genomic regions via its direct interaction with the multifunctional transcriptional activator Aft1, thereby ensuring rapid and effective cellular response to possible DNA damage events (Andreadis et al. 2014). Here, we demonstrate Rad9 association with chromatin in the absence of DNA damage, via its direct interaction with different transcription complexes.

In the course of a yeast two-hybrid screen, we have identified Rad9 as a prominent interacting partner of the DNA binding transcriptional activator Mac1. Taking into consideration (a) the unknown function of the Rad9-containing protein complex in undamaged yeast cells, (b) our findings regarding the physical interaction of Rad9 with Aft1 transcription factor and (c) previous biochemical evidence associating unphosphorylated BRCA1, and 53PB1, functional *Sc*Rad9 homologues in mammals, with transcription complexes (Cuella-Martin et al. 2016; Krum et al. 2003; Lane 2004), we have analyzed Rad9 association with Mac1 and its role in transcription.

Homodimerizing Mac1 (Joshi et al. 1999) transcriptionally induces a number of genes whose products are involved in copper uptake under copper depletion conditions (Georgatsou and Alexandraki 1999; Gross et al. 2000; Yamaguchi-Iwai et al. 1997). Mac1 possesses distinct DNA binding and transcriptional activation regions (Serpe et al. 1999) and its functionality is modulated by copper ions (Georgatsou et al. 1997; Jensen and Winge 1998) as well as specific chromatin protein regulators (Voutsina et al. 2019). Our findings on Rad9 involvement in Mac1-driven transcription provide a specific example of a yeast DNA-binding transcriptional regulator that is directly bound and functionally affected by a DNA checkpoint protein. This is a new link between RNA Pol II-dependent transcription and chromatin repair factors, under physiological conditions, possibly implying targeted genomic surveillance.

Results

Mac1 transcriptional activator interacts with Rad9 protein in vivo and directly in vitro

We isolated Rad9 protein as a potentially interacting partner of Mac1 transcriptional activator in a yeast two-hybrid screen followed by β-galactosidase assays of binary

interactions between Rad9 and Mac1 deletion derivatives (Fig. 1a). Mac1 includes a DNA-binding activity mapping to its N-terminal 159 residues, including a cysteine-rich zinc ion-binding sequence and a C-terminal half responsible for transactivation, including two cysteine-rich sequences that bind a total of 8 Cu(I) ions (Graden and Winge 1997). Copper repression of Mac1 is due to an intramolecular interaction between the N- and C-terminal cysteine-rich motifs by the formation of a polycopper cluster (Jensen and Winge 1998). Since a *mac1*Δ strain was previously identified in a genome-wide screen as exhibiting sensitivity to methyl methanesulfonate (MMS) although not to UV irradiation (Hanway et al. 2002), we proceeded with the analysis of the Rad9-Mac1 interaction.

The in vivo Rad9-Mac1 interaction was confirmed by the copurification of chromosomally expressed Mac1-9Myc protein and episomally expressed FLAG-Rad9 from total extracts of *rad9*Δ cells grown under normal non-induction conditions (SC medium-0.25 μM $CuSO4^{-}$), under copper depletion and Mac1 activation (SC plus 100μM BCS), under copper depletion combined with stress conditions (SCBCS plus 0.3 mM H2O2) at which Rad9 is also activated (Flattery-O'Brien and Dawes 1998) and under copper excess for Mac1 repression (SC plus 100 μM $CuSO4^{-}$). We observed Mac1-Rad9 association under all four conditions (Fig. 1b), including in the presence of excess copper ions that should inactivate Mac1 by altering its conformation; this is possibly due to the overexpression of FLAG-Rad9. Chromosomally expressed Rad53-9Myc (in SCBCS H2O2), used as positive control (Vialard et al. 1998) of the assay, also copurified with FLAG-Rad9 (data not shown) while FLAG used as negative control (Fig. 1b) did not interact with Mac1-9Myc protein neither did the irrelevant protein His3-9Myc with FLAG-Rad9 (data not shown).

To determine whether the in vivo detected association reflected direct contacts between the involved proteins, we tested whether bacterially produced GST-fused Mac1 deletion derivatives could associate in vitro with bacterially produced 6His-tagged Rad9 derivatives in the absence of additional yeast proteins. Full-length Mac1 and Rad9 proteins were not efficiently produced in bacteria. GST-NMac1(1–159) and GST-C2Mac1(287–417) interacted with 6His-CRad9 and 6His-BRCTRad9 but not with 6His-NRad9. GST-C1Mac1(159–417), GST-Mac1(41–257) and

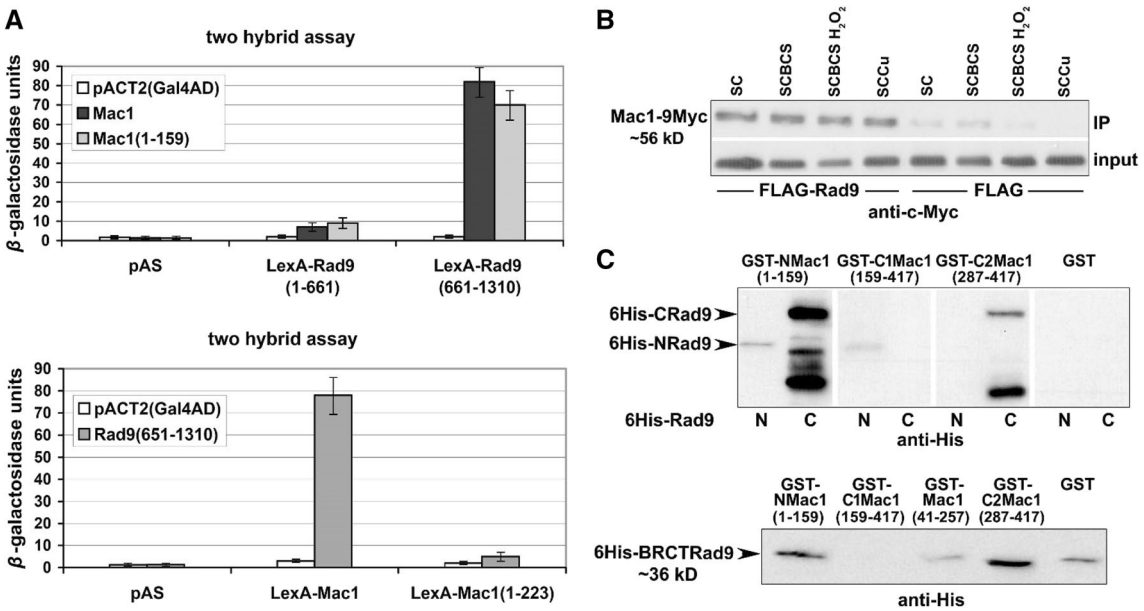

Fig. 1 Mac1 directly interacts with the BRCT domain of Rad9. **a** *β*-galactosidase activity units obtained from L9FT5 cells expressing the indicated hybrid proteins and a *lacZ*-reporter in pJK103 plasmid, shown diagrammatically. The values shown represent the averages and standard errors from three different biological experiments. **b** Coimmunoprecipitation (IP) of Rad9 and Mac1 performed in whole-cell lysates from FT5*rad9*Δ(Mac1-9Myc) cells transformed with pDB20 expressing either FLAG-Rad9 or FLAG epitope alone and grown under four different conditions using monoclonal anti-FLAG for the precipitation and polyclonal anti-c-Myc for immunoblotting. Input lanes contain 20% of the extract before immunoprecipitation. The faint bands seen in the FLAG lanes are due to non-specific binding on the beads. **c** GST-Mac1 derivatives, as indicated, purified from bacterial extracts and equal amounts (examined by PAGE) incubated with purified Rad9 derivatives, similar amounts of N-terminal [6His-Rad9(1-504)], C-terminal [6His-Rad9(505-1310)] (top) and [6His-BRCTRad9(997-1310)] (bottom), for 5 h at 4 °C. Samples were analyzed by SDS-10% PAGE and immunoblotting using anti-6His. Multiple bands observed in lanes are due to Rad9 degradation products. The very faint bands detected by 6His-NRad9 on both GST-NMac1 and GST-C1Mac1 (top panel), are probably due to background binding; they dont represent any predominant interactions as also indicated by the bottom panel results. Numbers of Mac1 and Rad9 derivatives indicate amino acid residues

GST alone did not interact with any Rad9 derivative. Thus, a direct contact between fragments containing the cysteine-rich regions of Mac1 and the BRCT domain of Rad9 was observed (Fig. 1c). The longer C1Mac1(159–417) derivative, although contains all of the C-terminal portion, may have a no favourable conformation to interact with Rad9.

This is a previously unknown direct association. The fact that Rad9 interacted directly with both the N- and C-termini of Mac1 implied that Rad9 might have a role in Mac1 function by interfering with its DNA-binding and/or transactivation domains.

Rad9 is recruited to the *CTR1* and *FRE1* promoters and negatively affects both DNA binding and transactivation functions of Mac1

The identified Mac1-Rad9 association suggested that Rad9 could play a role in transcriptional regulation of Mac1-activated genes. To test this hypothesis, we first performed chromatin immunoprecipitation (ChIP) assays in cells grown under repressing/high copper, non-induction/low copper and induction/copper depletion conditions. Rad9-9Myc protein was specifically recruited to both *CTR1* and *FRE1* promoters and quantitatively increased under induction conditions, following Mac1 occupancy. Recruitment to *CTR1* promoter was completely abolished in a *mac1*Δ strain under all conditions examined. Rad9-9Myc occupied at background levels the coding sequence of the basally expressed *PHO5* (Fig. 2a) and the constitutively transcribed *ACT1* and *TRP3* genes (data not shown). These results further confirmed a Rad9-Mac1 association on Mac1 target promoters under transcription induction conditions.

The fact that Rad9 protein could correlate with Mac1 transcribed genes prompted us to examine the functional importance of Rad9 recruitment on Mac1-regulated promoters. We tested whether Mac1-9Myc recruitment was affected in a *rad9*Δ strain and found that it was increased by more than 50% under induction conditions on both *CTR1* and *FRE1* promoters (Fig. 2b). This implied that Rad9 negatively affects Mac1 binding on DNA. Moreover, Mac1 fused to LexA exhibited about 60% higher transcriptional activation of a LexAop-HIS3-*LacZ* reporter gene in *rad9*Δ compared to wild type cells. This implied that Rad9 negatively affects also Mac1 transactivation function (Fig. 2c). To further confirm this finding, we analyzed *CTR1* (Fig. 2d) and *FRE1* (data not shown) mRNA accumulation levels and found transcription slightly increased (~10%) in *rad9*Δ cells and slightly decreased (~20%) in Rad9 overexpressing compared to wild type cells. The observed effect on Mac1 dependent transcription levels was not strong, however, it was in agreement with our results so far showing that Rad9 is recruited on Mac1 target promoters and interferes with Mac1 function.

Rad9 is recruited to the *CTR1* coding region following the localization pattern of RNA Pol II

To further understand the role of Rad9 on *CTR1* transcription, we investigated its genomic localization in the coding region of *CTR1* gene. We concentrated on *CTR1* since it is simply regulated by Mac1 under the conditions we used and additionally we have previously analyzed the role of other Mac1-interacting factors on that gene (Voutsina et al. 2019). We observed that Rad9 was not only found on the Mac1-regulated promoter but also localized over the entire transcribed region of *CTR1* quantitatively upon induction conditions (Fig. 3a). Rad9 was found at background levels both 5′ to the CuRE (copper regulatory) region on the promoter and 3′ to the stop codon, similarly to its binding over the entire length of the gene under repressing conditions (Fig. 3a) or when Mac1 was absent (data not shown). Moreover, the amount of Rad9 protein was higher in the coding region, compared to that found on the promoter and followed a characteristic quantitative association pattern with apparent stalling of Rad9 close to the 5′end of *CTR1* coding region. This pattern matched the localization pattern of RNA Pol II on *CTR1* gene, examined by three different specific antibodies (Fig. 3b). This RNA Pol II localization pattern is characteristic for the active *CTR1* gene and remained unaffected in a *rad9*Δ strain (data not shown). It was different from that observed for histone H3 that peaked at the end of the gene (Fig. 3c), indicating that nucleosomes do not gather where RNA Pol II and its associated proteins accumulate. Thus, Rad9 associates with the coding region of a Mac1-regulated gene in a manner that correlates with RNA Pol II association and with the transcriptional status of that gene, pointing to a role or an association of Rad9 with transcription.

CTR1 promoter is sufficient for Rad9 recruitment to the heterologous *ACT1* coding region

Given that Mac1 is localized only on the (CuRE region of) *CTR1* promoter (Voutsina et al. 2019), we then asked what brings and keeps Rad9 in the actively transcribed *CTR1* coding region. We entertained several different possibilities. Rad9 could follow (a) RNA Pol II itself or (b) other RNA Pol II associated factors involved in both initiation and elongation of transcription and/or (c) specific chromatin marks. Regarding (a), we were not able to detect any interaction of Rad9 with the RPB1 subunit of RNA Pol II in a coimmunoprecipitation assay (data not shown). Regarding (b), we exchanged the *ACT1* promoter in the genome with the *CTR1* promoter and created a hybrid region where the *ACT1* gene was under the control of the *CTR1* promoter. As mentioned above, Rad9 associates

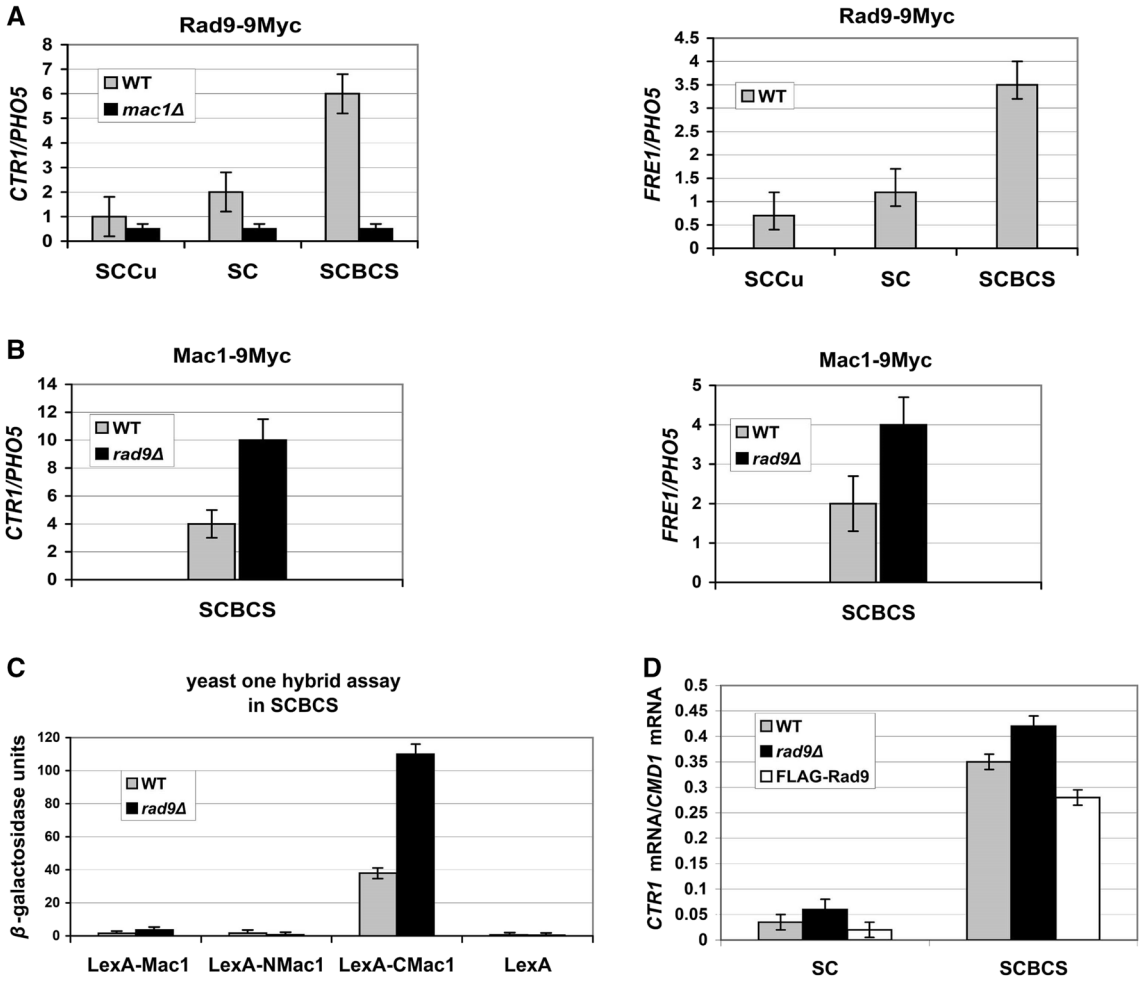

Fig. 2 Rad9 localizes via Mac1 to the *CTR1* and *FRE1* promoters and negatively regulates Mac1 binding and transactivation functions. **a** Rad9 recruitment on *CTR1* (left panel) and *FRE1* (right panel) promoters assayed in wild-type and *mac1*Δ cells, chromosomally expressing Rad9-9Myc, grown in the indicated media and subjected to chromatin imunoprecipitation (ChIP) with anti-c-Myc, followed by real time PCR analysis of the immunoprecipitated (IP) and input DNA using primers specific for *CTR1* (−475/−220) and *FRE1* (−353/−1) promoters and *PHO5* coding region (+1017/+1220). IP efficiency is represented by the ratios: *CTR1* or *FRE1* IP DNA/input DNA/*PHO5* IP DNA/*PHO5* input DNA. The values shown represent the averages and standard errors from three different experiments. **b** Rad9 negative effect on Mac1 binding to *CTR1* (left panel) and *FRE1* (right panel) promoters is shown in wild-type and *rad9*Δ cells, expressing Mac1-9Myc chromosomally, grown in SCBCS, subjected to ChIP with anti-c-Myc and analyzed as described in (**a**). **c** *β*-galactosidase activity units obtained from wild-type and *rad9*Δ cells co-transformed with LexAop-HIS3-*Lac*Z and LexA-Mac1 or LexA expression plasmid and grown in SCBCS. **d** Total RNA extracted from wild type, *rad9*Δ, and wild type cells transformed with pDB20FLAG-*RAD9* (overexpressing FLAG-Rad9) grown in SC and SCBCS was subjected to Northern hybridization analysis using radiolabeled *CTR1*. *CMD1* served as a loading (internal) control. Bands were quantified using the Phosphor Imager and ImageQuant software and bars represent the indicated intensity ratios (normalized mRNA levels). The values shown in all panels represent averages and standard errors from three different biological experiments

with the *ACT1* coding region at background levels. Performing ChIP assays on the hybrid $CTR1_{prom}$-*ACT1* region, we observed that Rad9 associated with the newly embedded *CTR1* promoter, as expected, but also associated with the *ACT1* coding region fused downstream (Fig. 4). Therefore, Mac1-dependent transcriptional initiation was crucial for Rad9 recruitment to the heterologous *ACT1* coding region.

Hir1 protein is partly responsible for Rad9 localization on *CTR1* promoter and coding region

We have previously shown that Hir1 histone chaperone directly interacts with Mac1 on *CTR1* promoter and also localizes in the coding region of *CTR1* gene (Voutsina et al. 2019). Hir1 may play a redundant role in transcriptional elongation of that gene revealed by synthetic mutant interactions (Voutsina et al. 2019) with subunits of the FACT

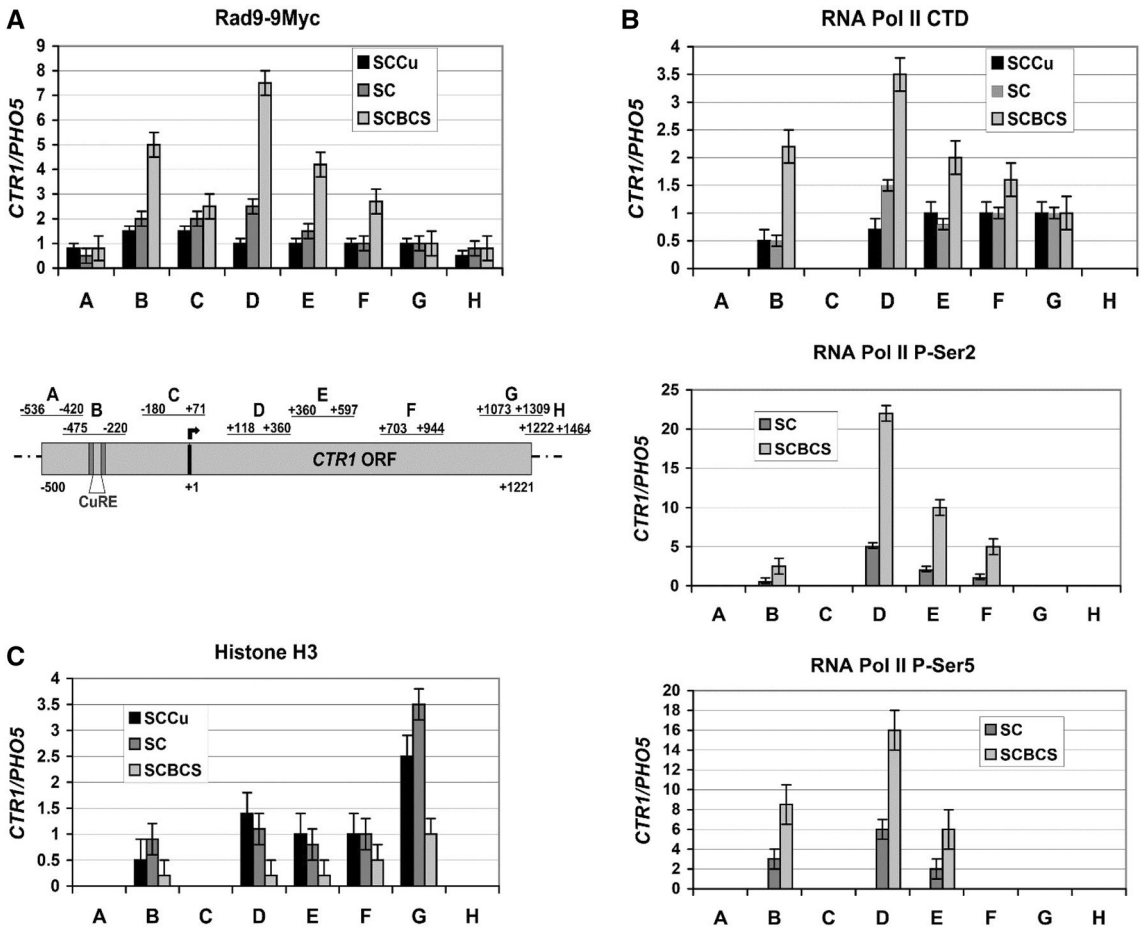

Fig. 3 Rad9 associates with *CTR1* coding region under induction conditions following the localization pattern of RNA Pol II. **a** ChIP assays of Rad9-9Myc in wild type cells grown in repressing (SCCu), non-inducing (SC) and inducing (SCBCS) conditions, performed and analyzed as described for Fig. 2, using anti-c-Myc. A to H correspond to the specified regions of *CTR1* promoter and coding sequence, shown schematically below, analyzed by real-time PCR. **b** ChIP assays of wild type cells, performed and analyzed as described for Fig. 2, using 8WG16, H5 and H14 antibodies specific for examining the localization of RNA Pol II CTD, P-Ser2 and P-Ser-5 respectively at the indicated regions of *CTR1* gene. *CTR1/PHO5* shown values are underestimated due to some (minor) RNA Pol II occupancy on the *PHO5* (+1017/+1220) region. **c** ChIP assays using anti-H3 for examining the localization of histone H3. Bars are shown only in regions with available experimental data

complex that facilitate RNA Pol II transcription elongation (Formosa et al. 2002). Therefore, Hir1 could be a candidate RNA Pol II-associated factor involved in Rad9 localization on the *CTR1* coding region. We tested a possible in vivo association between Rad9 and Hir1 proteins and found them to copurify from total extracts of a *rad9*Δ strain expressing endogenous Hir1-9Myc and episomal FLAG-Rad9 (Fig. 5a). We additionally observed a binary interaction between Gal4AD-Rad9(651–1310) and LexA-CHir1(393–840) in a yeast two-hybrid assay (data not shown). Despite this interaction, deletion of both *RAD9* and *HIR1* genes did not exhibit any synthetic effect on *CTR1* mRNA accumulation, reproducing the slightly reduced levels observed in the *rad9*Δ strain (data not shown). Nevertheless, ChIP analysis showed that Rad9-9Myc recruitment on both *CTR1* promoter and coding region was reduced by 50–70% in a *hir1*Δ strain while conversely, the quantitative localization pattern of Hir1-9Myc was unaffected by the absence of *RAD9* gene (Fig. 5b). Our results indicate that Rad9 associates with the Mac1-interacting protein Hir1 and this is partly responsible for Rad9 quantitative localization in the coding region.

Histone H3K79 dimethylation is not a prerequisite for Rad9 recruitment

In parallel to the above experiments, we checked our third hypothesis, namely whether Rad9 recognizes specific chromatin marks such as methylated histones for its localization in the *CTR1* coding region. One candidate modification was H3K79me2, known to be restricted to euchromatic regions of the genome (Ng et al. 2003) but also recognized by Rad9 at regions of DNA damage where it is either increased or

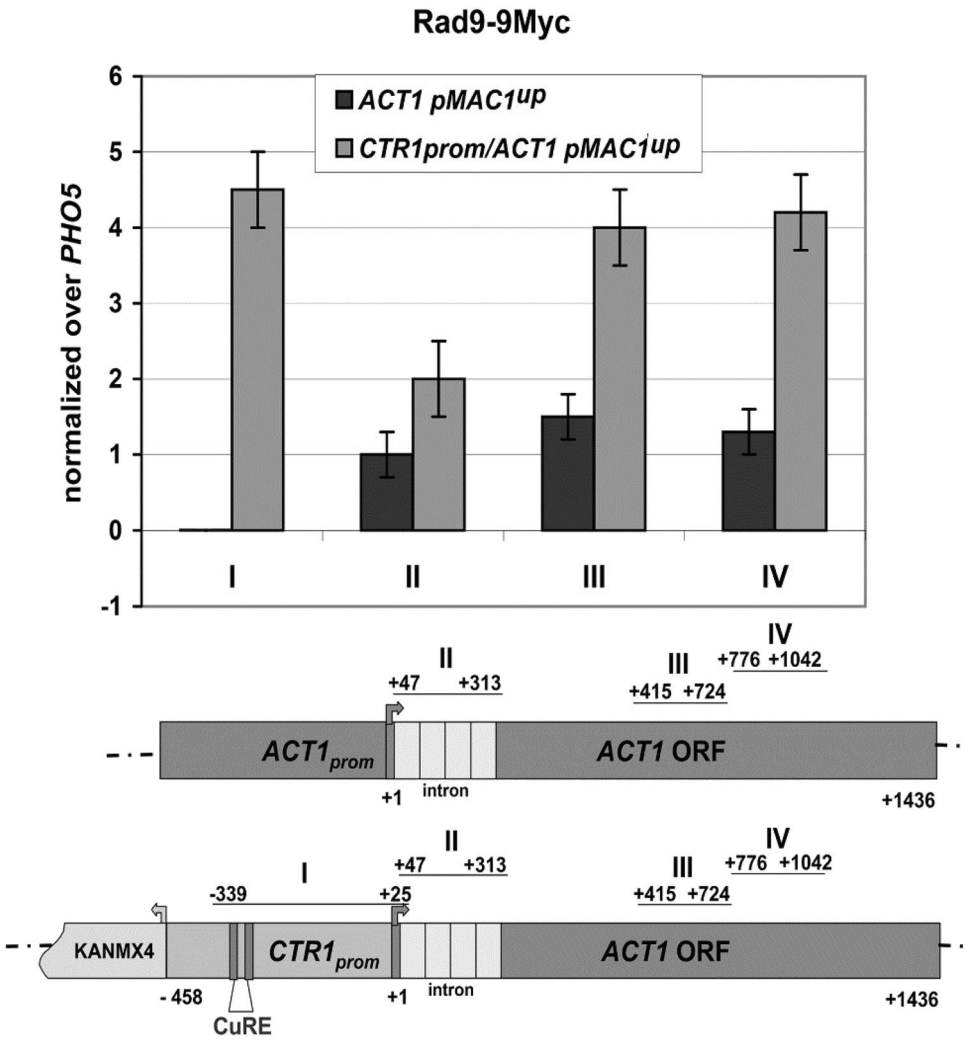

Fig. 4 Rad9 is recruited on the *ACT1* coding region (where Rad9 is not normally localized) via *CTR1* promoter fused upstream. ChIP assays of Rad9-9Myc in wild type cells carrying the chromosomal construction $CTR1_{prom}$-*ACT1* and episomally expressing $Mac1^{up}$, grown in SC medium, performed and analyzed as described for Fig. 2, using anti-c-Myc. I to IV correspond to the specified regions of the *CTR1* promoter and *ACT1* coding sequence, shown schematically below, analyzed by real-time PCR. The fold enrichment was calculated against the *PHO5* coding sequence

revealed locally (Huyen et al. 2004). We performed ChIP analysis on the *CTR1* gene using antibodies that specifically recognize H3K79me2 and found this particular modification localized all over *CTR1* coding region but not on *CTR1* promoter in induction conditions (Fig. 6a). In parallel, we tested H3K36me2, a modification detected on most actively transcribed RNA Pol II genes, known to play an important role in transcriptional elongation (Kizer et al. 2005). H3K79me2 and H3K36me2 were both detected on *CTR1* coding region at comparable levels. However, the levels of those two modifications were entirely different in the *ACT1* coding region. While H3K36me2 was detected at comparable levels with those on the induced *CTR1* gene, H3K79me2 was not detectable (Fig. 6b). Notably, absence of H3K79me2 was also observed in the $CTR1_{prom}$-*ACT1* coding region (Fig. 6b) where we succeeded to artificially transfer Rad9. Our combined results imply that under physiological conditions (SC, in the presence of $Mac1^{up}$) or copper depletion conditions, histone H3K79me2 is not a prerequisite for Rad9 association with an active coding region.

Under copper depletion conditions both cellular Rad9 and Rad53 are hypophosphorylated

Our finding that H3K79me2 is not required for Rad9 association with gene coding regions under the growth conditions we used, is distinct from that described for Rad9 recruitment on chromatin under DDR. It is also known that, in the absence of DNA damaging agents, Rad9 is hypophosphorylated during all phases of the cell cycle, exhibiting a damage independent electrophoretic shift in G2/M that cannot be detected in asynchronous whole-cell extracts (O'Shaughnessy et al. 2006). Rad53 kinase is similarly hypophosphorylated in the absence of DNA damaging agents (Sun et al. 1998). To further investigate whether the copper depletion condition (or active Mac1) used in our experiments (SCBCS) could induce DDR, we examined the phosphor-forms of both Rad9 and its target kinase Rad53. We compared the sizes of Rad9-9Myc and Rad53-9Myc proteins in cells grown under condition typically considered as physiological (SC), under conditions causing DNA damage

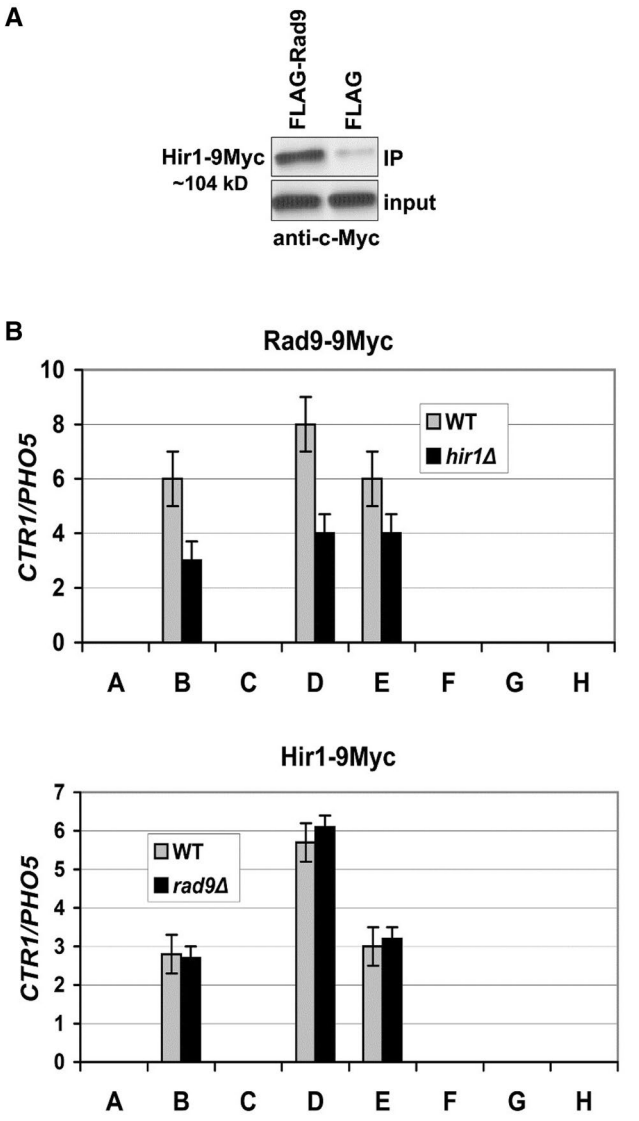

Fig. 5 Hir1 physically interacts with Rad9 and partially affects its localization on *CTR1* promoter and coding region under induction conditions. **a** Coimmunoprecipitation of Rad9 and Hir1 in extracts from yeast cells chromosomally expressing Hir1-9Myc and episomally expressing FLAG-Rad9 (pDB20-FLAG-RAD9) or FLAG epitope alone and grown in SCBCS, was performed as described in Materials and methods using monoclonal anti-FLAG for the precipitation and polyclonal anti-c-Myc for the immunoblotting. **b** ChIP assays of Rad9-9Myc in wild type and *hir1*Δ strains grown in SCBCS, performed and analyzed as described for Fig. 2, using anti-c-Myc. A to H correspond to the specified regions of *CTR1* gene (shown in Fig. 3) analyzed by real-time PCR. The fold enrichment was calculated against the *PHO5* coding sequence. ChIP assays of Hir1-9Myc in wild type and *rad9*Δ strains were similarly analyzed. Bars are shown only in regions with available experimental data

(UV, zeocin), under copper depletion (SCBCS) and under copper excess (SCCu). Both Rad9-9Myc and Rad53-9Myc were detected in their hypophosphorylated forms in cells grown in SC and copper depletion or excess, while in cells treated with UV or zeocin higher molecular weight forms were detected for both (Fig. 7a). This observation confirmed that the copper depletion condition (or active Mac1) used in our experiments is not responsible for inducing detectable DDR, therefore, Rad9 recruitment on *CTR1* chromatin is mechanistically distinct from its recruitment on damaged DNA.

Rad53 is also recruited on the *CTR1* coding region in a Rad9 dependent manner

We found Rad9 to associate with and follow the transcriptional elongation machinery on active *CTR1* gene despite its non-essential, but not insignificant, role in mRNA accumulation. Since under copper depletion, cellular Rad9 and Rad53 were not typically activated as by DNA damage induction, we considered important to further analyze factors of the DNA damage checkpoint in relation to the newly identified type of recruitment on chromatin. We first examined whether Rad53 that acts immediately downstream of Rad9 in the DDR pathway was also localized on the *CTR1* promoter and coding region. Under DDR, Rad53 is known to loosely associate and is not detected on chromatin (Toh et al. 2006). On the other hand, Rad53 physically interacts with Rad9 (Smolka et al. 2006; Vialard et al. 1998) and has also been identified in a dynamic complex with Asf1 histone chaperone (Emili et al. 2001). Asf1 cooperates with Hir1 histone chaperone affecting nucleosome structure (Green et al. 2005) and is also involved in transcriptional initiation and elongation of particular genes (Schwabish and Struhl 2006) implying collaboration between transcription and DNA checkpoint protein components. Although we found no essential role for Asf1 on *CTR1* transcription (data not shown), we were able to detect Rad53-9Myc on the *CTR1* coding region under transcriptional induction. Moreover, Rad53 recruitment was dependent on the presence of Rad9 (Fig. 7b) and undetectable under transcriptional repression (data not shown). Finally, Rad53-9Myc recruitment was also dependent on the presence of Hir1, consistent with our findings for Rad9 (Fig. 7b). We were not able to detect Rad53-9Myc on the *CTR1* promoter quantitatively, possibly due to lower amounts of recruited Rad9. We also examined the recruitment of the Ddc1-13Myc protein on *CTR1* gene in wild type and *rad9*Δ strains and found it at background levels throughout, similarly to its recruitment on *ACTI*, *PHO5* and *TRP3* genes (data not shown). Ddc1 is part of the PCNA-like complex (Rad17-Mec3-Ddc1) required upstream of Rad9 together with the Ddc2-Mec1 complex in DDR (Giannattasio et al. 2003). These results emphasize that both Rad9 and its target essential kinase Rad53 are accumulated on the active *CTR1* gene under physiological non DNA damage-inducing conditions, recruited by and

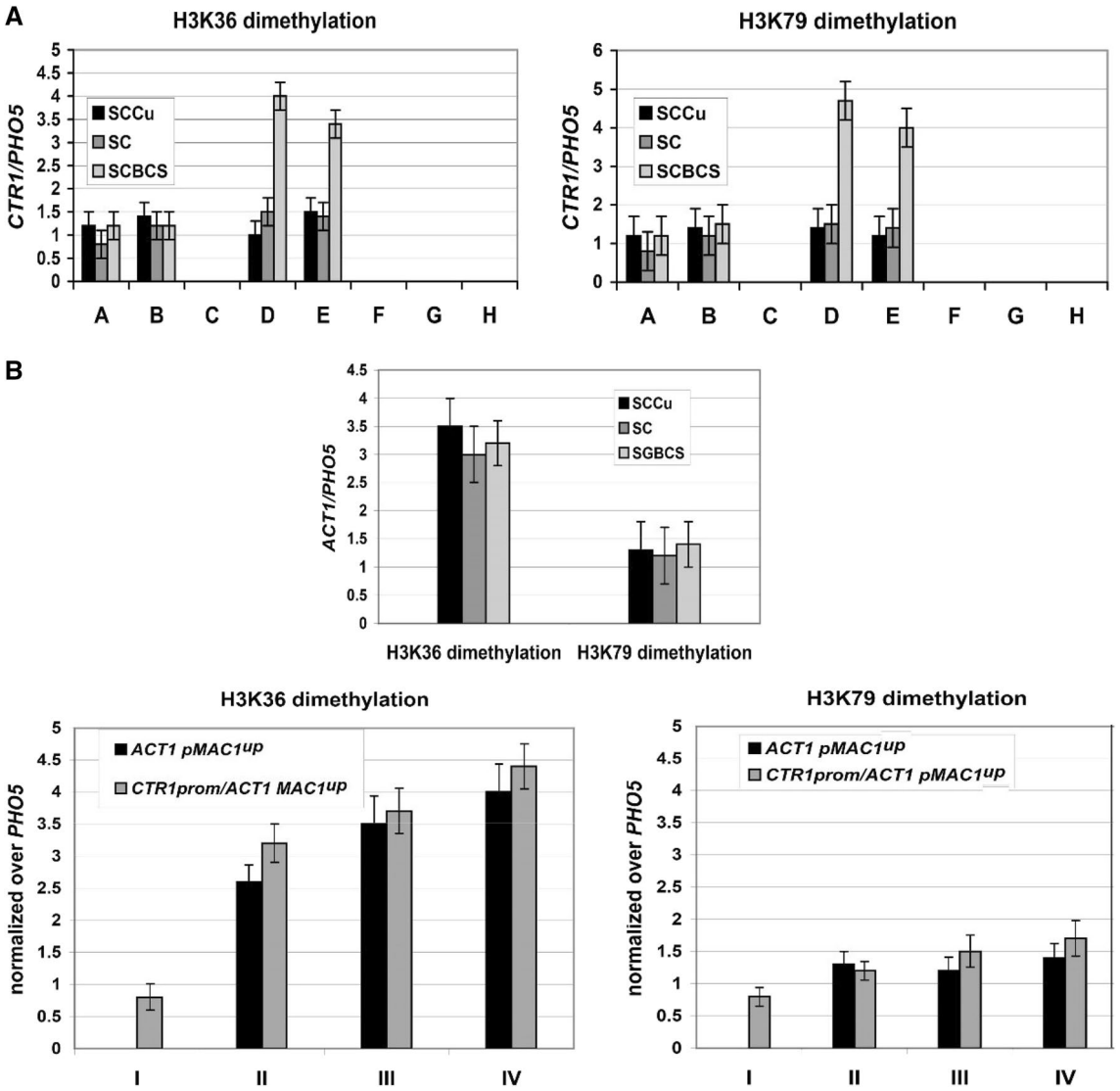

Fig. 6 While H3-K79 dimethylation occurs in the *CTR1* coding region it is not required for Rad9 localization in the coding region of $CTR1_{prom}$-*ACT1*. **a** ChIP assays of wild type cells grown as indicated, performed and analyzed as described on the legend to Fig. 2, using anti-H3-K36me2 and anti-H3-K79me2 and assayed at the indicated regions of *CTR1* gene (shown in Fig. 3) by real-time PCR. **b** ChIP assays of wild type cells analyzed as in (**a**) at the coding region of *ACT1* (+415/+724) (top). ChIP assays of wild type cells carrying the chromosomal construction $CTR1_{prom}$-*ACT1* and episomally expressing $Mac1^{up}$ grown in SC, performed and analyzed as described for Fig. 2, using anti-H3-K36me2 and anti-H3-K79me2 assayed at the indicated regions of $CTR1_{prom}$-*ACT1* gene (shown in Fig. 4) by real-time PCR. The values shown in all panels represent averages and standard errors from three different biological experiments

associated with components of the transcription machinery, distinctly to their recruitment in DDR.

Discussion

In this work, we report novel protein interactions involving the *Sc*Rad9 protein, known as DNA damage checkpoint adaptor and (a) the sequence-specific copper-modulated transcriptional activator Mac1 and (b) the histone chaperone transcriptional regulator Hir1 also known to interact with Mac1 (Voutsina et al. 2019). Our findings provide solid biochemical evidence for Rad9 association with a DNA-binding transactivator on active promoters as well as with initiating and processive RNA Pol II transcription complex. Rad9 was known to be recruited on chromatin only under DNA damage conditions via histone modifications whereas we have demonstrated that, in undamaged cells, it associates with Aft1 transcriptional activator on fragile genomic regions (Andreadis et al. 2014). Here, we show that Rad9 can also interact with chromatin, in the absence of DNA damage, via its direct association with different transcription complexes.

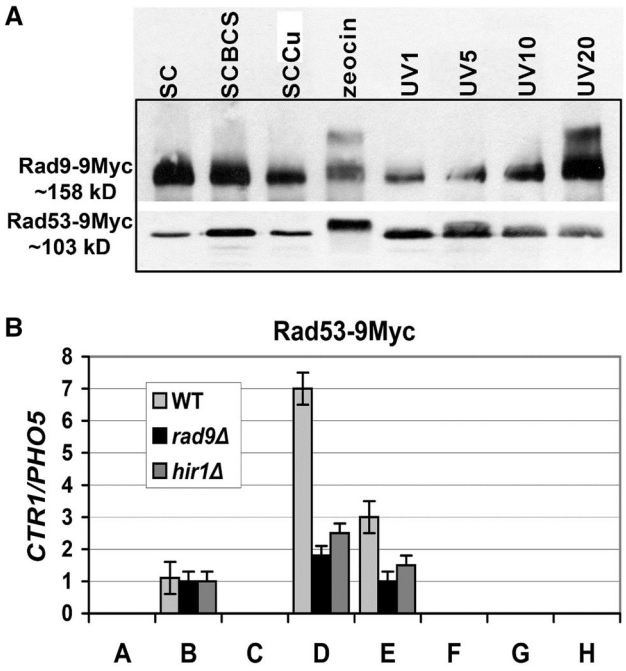

Fig. 7 Rad53 associates with *CTR1* coding region in a Rad9-dependent manner while both Rad9 and Rad53 are hypophosphorylated. **a** Whole-cell lysates purified from equal amount of wild type cells expressing Rad9-9Myc grown under five different conditions [SC, SCBCS, SCCu, zeocin, SCUV (1 to 20)]. Samples were analyzed by SDS-6.5% PAGE containing an acrylamide to bis-acrylamide ratio of 80:1 (O'Shaughnessy et al. 2006) and by immunoblotting using anti-c-Myc antibody. **b** ChIP assays of Rad53-9Myc chromosomally expressed in wild type, *rad9*Δ and *hir1*Δ strains grown in SCBCS, analyzed as described for Fig. 2, using anti-c-Myc. A to H correspond to the specified regions of *CTR1* gene (shown in Fig. 3) analyzed by real-time PCR. Bars are shown only in regions with available experimental data

Moreover, we provide evidence for an association between Rad9 adaptor and Rad53 kinase on chromatin distinct from their known interaction in the cellular response to genotoxic agents. Rad9 protein is another example of multifaceted protein related to DNA damage response (Botchkarev and Haber 2018; Leshets et al. 2018).

Mac1-recruited Rad9 follows the transcription machinery

We have previously shown (Voutsina et al. 2019) that on *CTR1* promoter, Mac1-driven transcription, induced by copper-depletion, depends primarily on the SWI/SNF chromatin remodeler while Ssn6 is a prominent repressor on the non-induced gene expression (SC medium). In that promoter context, Hir1, physically interacting with each of Mac1, Snf2 and Ssn6, is a coregulator of this copper-dependent transcriptional initiation switch and also functionally interacts with the yFACT complex for transcriptional elongation. In addition to these Mac1-recruited coregulators, here we showed that Rad9 was also recruited on Mac1-regulated genes as part of the same protein complex and physically interacted with both Mac1 and Hir1. Moreover, Rad9 localized at higher quantities along the protein-coding region of *CTR1* coincided with transcriptional induction and correlated with the characteristic quantitative association pattern of RNA Pol II. Our data clearly indicate that although Rad9 may not interact directly with the RNA Pol II, it is closely associated to both the initiation and elongation transcription machineries. This was shown directly since the Mac1-dependent transcriptional initiation complex assembled on the ectopically inserted *CTR1* promoter (of the genomic construct $CTR1_{prom}$-*ACT1* coding region) was responsible for Rad9 localization to the *ACT1* coding region, where it is normally not localized. Thus, the first step of Rad9 localization on the promoter depends on Mac1, and its traveling in the coding region requires this initial step. It also requires its association with components of the transcription elongation machinery, since Rad9 recruitment on the *CTR1* coding region was significantly dependent on the presence of Hir1.

Rad9 binds to the active Mac1 transcriptional activator by its $BRCT_2$ domain

Using different assays, we found that Rad9 negatively affected both DNA binding and transactivation functions of Mac1 and this was in agreement with the result that Rad9 interacted with both N- and C-terminal regions of Mac1 in vitro. Moreover, we found the BRCT-containing C-terminal portion of Rad9 involved in these interactions. It is known that Rad9 BRCT domains are essential to mediate its dimerization and phosphorylation at multiple sites as well as its interaction with phosphorylated H2AX, important for its checkpoint function following DNA damage (Bantele and Pfander 2019; Hammet et al. 2007; Soulier and Lowndes 1999; Usui et al. 2009). C-terminal di-BRCT domains are phosphopeptide binding motifs with high affinity for phosphoserine and phosphothreonine residues (Kilkenny et al. 2008; Williams et al. 2004; Yu et al. 2003) whereas it was shown recently that a tetra BRCT N-terminal domain of the yeast Rtt107 protein is engaged in recognizing phospho-free ligands and supporting constitutive genome protection (Wan et al. 2019). Mac1 is known to be phosphorylated in its active form (Heredia et al. 2001) with potential phosphorylation sites within the regions interacting with Rad9. Therefore, Rad9 $BRCT_2$-Mac1 interaction may be a type of heterologous dimerization. Such interactions have been observed for 53BP1 (p53 binding protein 1) and BRCA1 (breast cancer associated 1) proteins which are the functional ScRad9 homologues in mammals, showing low sequence conservation but containing similar tandem C-terminal BRCT motifs (FitzGerald et al. 2009; Mochan et al. 2004; Panier and Boulton 2014; Scully et al. 2004; Stucki and

Jackson 2004; Wu et al. 2010). 53BP1 additionally contains a $Tudor_2$ domain just upstream of the $BRCT_2$ motif (Alpha-Bazin et al. 2005) structurally similar to that of ScRad9 and its *S. pombe* ortholog Crb2 (Lancelot et al. 2007). It is known that 53BP1 $BRCT_2$, along with an inter-BRCT linker, provides a binding site for the DNA-binding domain of the transcriptional activator p53 (Derbyshire et al. 2002). The analogous region of BRCA1 binds BACH1 DNA helicase (Joo et al. 2002) while p53 interacts with both N-terminal and C-terminal regions of BRCA1 (Chai et al. 1999). Therefore, the Rad9-Mac1 interaction most likely reflects a conserved property of these DDR adaptor/mediator molecules. In that respect, it is likely that the Rad9 BRCT2 domain may also serve additional functions, considering the fact that the 53BP1 respective domain is dispensable for the repair activities of 53BP1 protein (Bothmer et al. 2011; Cuella-Martin et al. 2016; FitzGerald et al. 2009; Panier and Boulton 2014; Ward et al. 2006).

Rad9 recruits Rad53 to the actively transcribed *CTR1* gene in the absence of DNA damaging agents

While Rad9 $BRCT_2$ domain is engaged in interactions with Mac1, these Rad9 molecules are unlikely to be involved in DDR. In fact, under the copper-depletion culturing conditions used, detectable cellular Rad9 as well as Rad53 were in their hypophosphorylated form. When Rad9 subsequently localizes to the *CTR1* coding region, it is no longer interacting with Mac1. It is associated with factors involved in transcriptional elongation such as Hir1 but curiously enough also with Rad53 kinase, since Rad9 is absolutely responsible for its recruitment there. According to what is known so far (Pardo et al. 2017; Smolka et al. 2007; Sweeney et al. 2005; Vialard et al. 1998), Rad9 needs to be hyperphosphorylated to recruit Rad53. Also Rad53 copurifies only with the hyperphosphorylated Rad9 complex (van den Bosch and Lowndes 2004). We presume that Rad9 on *CTR1* is not oligomerized, as it is in DDR (Soulier and Lowndes 1999), but, by its interaction in sufficient quantities with components of the transcription machinery, it acquires a conformation appropriate for Rad53 recruitment. Alternatively, Rad9 molecules specifically associated with *CTR1* coding region (as well as with other genes) are locally activated (hyperphosphorylated) due to a local damage signal and able to recruit Rad53. This is rather unlikely since both yeast Rad53 and its mammalian homolog Chk2 were not found on chromatin following DDR (Toh et al. 2006) or even following artificial recruitment and activation of the DNA damage sensors at specific foci in the absence of DNA damage (Soutoglou and Misteli 2008). It is assumed that the activated kinases diffuse away to phosphorylate specific protein targets (Li and Stern 2005). Rad53 has only been seen to transiently localize to bud necks in response to DNA replication stress (Smolka et al. 2006), independently of both DNA and mitotic checkpoints, and to origins of replication being essential for initiation of DNA replication (Dohrmann and Sclafani 2006). Finally, hypophosphorylated form of Rad9 was previously found preferentially retained on bulk chromatin during and after down-regulation of checkpoint signalling by γ-irradiation (Toh et al. 2006).

Therefore, in our culturing conditions, both Rad9 and Rad53 are accumulated on the active *CTR1* gene following a sequel of chromatin transcription-specific components, independent of their known so far roles in DDR. This was further supported by the fact that Rad9 recruited by Mac1 and subsequently localized on the $CTR1_{prom}$-*ACT1* coding region did not seem to rely on the presence of H3K79me2 (Huyen et al. 2004). Interestingly, a different signaling mode during ongoing DNA synthesis, distinct from its canonical one in replication stress, has been recently proposed for Mec1 kinase, implying that another known DDR component has a role in non DDR conditions (Bastos de Oliveira et al. 2015; Corcoles-Saez et al. 2019).

Rad9 holds back Mac1 activity and mediates cross talk between transcription and DNA repair on the *CTR1* gene

We originally assumed that hypophosphorylated Rad9 and Rad53 may be recruited on chromatin of specific genes to play a role in transcription per se, since *RAD9* deletion and overexpression affected *CTR1* transcription in opposite directions by about 10–20%. This slight negative effect could be a non-harmful consequence of Rad9′s presence in the Mac1-organized transcription complexes or could indicate a redundant function of Rad9 in transcription. In the latter case, we still have to uncover the condition where Rad9 is mostly required. Furthermore, the pattern of RNA Pol II on *CTR1* gene remained unchanged when *RAD9* was deleted whereas the combination of *rad9*Δ with mutant factors involved in elongation such as Hir1 or Spt16 did not reveal any synthetic effect on *CTR1* expression or any growth defect (our unpublished observations). We have previously observed a slight growth defect for the *rad9*Δ strain, only in the presence of a-Azauracil (interfering with transcriptional elongation) (Andreadis et al. 2014).

Similarly puzzling findings have been reported for the mammalian *Sc*Rad9 homologues, 53BP1 (the closest one) and BRCA1, concerning their involvement in transcription. Both have characteristics allowing the comparison with Rad9 in terms of cell cycle delay and transcriptional response. Upon DNA damage, they are hyperphosphorylated by ATM and colocalize in DNA repair foci (DiTullio et al. 2002; Fernandez-Capetillo et al. 2002). 53BP1 exhibits a conserved interaction with p53 in vertebrates (Xia et al. 2001) and its involvement in transcription is

under investigation (Cuella-Martin et al. 2016; Derbyshire et al. 2002). It requires both its oligomerization and BRCT domains to bind to p53 and thus promote p53 global transcriptional activity and this is possibly a separate 53BP1 property, independent from its function as a DNA repair factor (Cuella-Martin et al. 2016). BRCA1 also interacts with p53 DNA-binding domain (Chai et al. 1999) and, moreover, via its distinct domains, with other regulators involved in either activation or repression of transcription, affecting diverse cellular responses (Mullan et al. 2006; Sharma et al. 2018; Zhang and Li 2018). Its precise function in the RNA Pol II complex is unclear and several proposed models range from a role in transcription to specific activating and repressing roles in DNA damage-inducible genes (Lane 2004). Furthermore, systematic screening for BRCA1 interacting partners revealed that it is required to prevent or repair DNA damage associated with transcription (Hill et al. 2014). BRCA1 additionally associates with a human SWI/SNF-related chromatin remodeling complex (Bochar et al. 2000). We have not tested such direct interaction between Rad9 and SWI/SNF components but we have detected Snf2 subunit on the *CTR1* promoter, playing a pivotal role on *CTR1* transcription (Voutsina et al. 2019). Also, Snf2 was previously found among several Rad53-dependent in vivo phosphorylation targets (Smolka et al. 2007). Finally, in undamaged cells, hypophophorylated BRCA1 associates with processive RNA Pol II (Mullan et al. 2006) in accordance with our findings for Rad9. Given that the vertebrate ScRad9 homologues have evolutionarily acquired unique protein interaction modules with distinct cell-type specific functions in DDR and transcription, and considering the genome-wide Rad9 localization in actively transcribed regions (Andreadis et al. 2014) and our findings in this work, we presume that Rad9 may exhibit several roles including a role in transcription.

In *S. cerevisiae*, as in other organisms, it has been shown that transcriptionally active genes, as well as non-transcribed upstream control regions of active genes, are preferentially repaired (Terleth et al. 1990; van Hoffen et al. 1993). Furthermore, the transcribed strand of an active gene is repaired faster by TCR machines than the non-transcribed one (Leadon and Lawrence 1992). Still, Rad9 shows no preference to transcribed strands; it was found involved in the removal of photolesions at the same rate from both the transcribed and the non-transcribed strands of the reporter *GAL10* gene (Al-Moghrabi et al. 2001). Considering the non-essential role of Rad9 (and thereof Rad53) in Mac1-dependent transcription, they could, therefore, be involved either in redundant post-initiation functions or in aspects of genomic surveillance that are linked with the RNA Pol II transcriptional machinery.

Regarding genomic surveillance of particular loci prone to DNA damage, we have shown that Rad9 is recruited to fragile genomic regions (transcriptionally active, GC rich, centromeres, meiotic recombination hotspots and retrotransposons) in a manner that depends on the transcriptional activator Aft1 (Andreadis et al. 2014). *CTR1* and *FRE1* are inducible genes, bound by Rad9 and Aft1 (Andreadis et al. 2014) and, moreover, both *CTR1* and *CRR1* (sharing its promoter with *FRE1*) belong to a group of hotspots for meiotic recombination (Gerton et al. 2000), therefore, considered as fragile genomic regions. Therefore, Rad9 could be localized by various specific DNA binding factors on genes or regions prone to accidental or programmed DSBs (mitotic or meiotic hotspots), moving along with the transcription machinery during transcription. Rad53 is also known to be involved in the degradation of excess non-nucleosomal soluble histones (Gunjan and Verreault 2003) possibly playing a direct role in maintenance of chromatin structure (Pan et al. 2006). Rad53 and Hir1 have been placed in the same pathway of telomeric heterochromatin formation, coordinating DNA synthesis and chromatin assembly (Sharp et al. 2005). Our results colocalize Rad9, Rad53 and Hir1 for the first time on the chromatin of an actively transcribed gene, in the absence of DDR. Other yeast chromatin remodelers such as Chd1, Ino80, RSC, SWI/SNF and histone chaperones like Asf1 are all known to be involved in transcription but have been also associated with DNA repair and recombination (Bao and Shen 2007; Liang et al. 2007; Marfella and Imbalzano 2007; Mousson et al. 2007; Osley et al. 2007). Therefore, ScRad9, like BRCA1 and 53BP1, is a multifunctional protein that may mediate a crosstalk between transcription and DNA repair machineries under unchallenged conditions. Being recruited in transcription complexes when defects arise, accidentally or following a cellular program, it may ensure fast response and repair.

Finally, concerning a role in transcription, in our previous Rad9-related genome-wide study (Andreadis et al. 2014) we correlated the genomic binding profile of Rad9 protein with the gene expression profile of a *rad9*Δ mutant, both examined under non-DNA damage-inducing conditions, to assess the level of interplay between Rad9 chromatin binding and gene deregulation upon *RAD9* depletion. This analysis revealed 935 genes bound by Rad9, displaying a trend towards upregulation in *rad9*Δ, as well as 131 genes deregulated by *RAD9* depletion (nearly half of them upregulated and the other half downregulated) of which 31 were bound by Rad9. Very interestingly, the shift towards upregulation, which was noted as a trend for the total Rad9-bound genes, became statistically significant for the Rad9-bound deregulated gene set (one-sided *t* test P-value < 0.05). In other words, the yeast genes that were found both bound by Rad9 and deregulated in *rad9*Δ, seem to be under the direct control of Rad9 which represses their transcription. These genes are enriched in metabolic functions; *CTR1*, also a metabolic gene bound by Rad9 and upregulated in *rad9*Δ, was not found among them probably due to the marginal

effect of Rad9 on its transcription. Still, in accordance with our results here, it provides the opportunity to uncover a possible mechanism by which Rad9 interferes with transcription (Fig. 8). Through distinct associations with specific members of the transcriptional machinery, Rad9 seems to accomplish its goal to "bridle" transcription by constantly holding it in check. In this way, it is rapidly halted in the case of a DNA damage event enabling repair to take place. To test the validity of this mechanism beyond *CTR1*, the precise transcriptional contribution of Rad9 and its possible alternative partners in different contexts and circumstances, taken from the above mentioned gene group, should be studied. Rad9 can be considered as a scaffold protein in various multiprotein complexes and our analysis contributes to the understanding of similar adaptor protein functions.

Materials and methods

Yeast strains and media

FT5(*MATa ura3-52 trp1-Δ63 his3-Δ200 leu2*::*PET56*) was used to generate *rad9*Δ using a *KanMX* disruption cassette, m*ac1*Δ using a *URA3* disruption cassette. FT5(Rad9-9Myc*), mac1*Δ(Rad9-9Myc), FT5(Mac1-9Myc), *rad9*Δ(Mac1-9Myc) and FT5(Rad53-9Myc) and *rad9*Δ (Rad53-9Myc*)* strains were generated by C-terminal tagging the genomic *MAC1, RAD9 and RAD53* genes (Knop et al. 1999). Synthetic complete (SC) medium (0.67% yeast nitrogen base, 20 amino acids, uracil, adenine, 2% glucose, 1.23 μM $FeCl_3$ and 0.25 μM $CuSO_4$; Difco) was supplemented before harvesting the cells for three hours with 100 μM bathocuproine disulfonic acid-Na_2 salt (BCS) as Cu(II) chelator (SERVA) or for two hours with 400 μg/ml zeocin (Invitrogen 46-0509 100 mg/mL) or for 20 min with 100 μM CuSO4.

Plasmids

FLAG epitope was cloned into the *Xho*I-*Not*I site of pDB20 (Becker et al. 1991) and then *RAD9*(1-3930) coding sequence was cloned into the *Not*I site of pDB20FLAG to yield FLAG-Rad9 plasmid. [FLAG-*RAD9* is under the control of the *ADH1* 5′ and 3′ regulatory regions, ensuring an ~ 80X overexpression, further augmented by the 2*μ* element of the vector.] *RAD9* coding sequence was digested into four different fragments and cloned into the *Ava*I-*Eco*RI site of pRSET-A (Invitrogen) to yield 6His-NRad9(1–504) plasmid, into the *Eco*RI-*Hind*III site of pRSET-C (Invitrogen) to yield 6His-C1Rad9(505–1086 bp) and 6His-C2Rad9(505–1310) and into the *Eco*RI-*BamH*I site of pRSET-A to yield 6His-BRCTdomain-Rad9(997–1310). *MAC1* coding sequence was digested into four different fragments and each was cloned into the *Eco*RI-*BamH*I site of pGEX-2T (Pharmacia) to yield GST-NMac1(1–159), GST-C1Mac1(159–417), GST-Mac1(41–257) and GST-C2Mac1(287–417) plasmids, respectively. pRS315-MAC1^{up} derived from pRS316-MAC1^{up} (Jensen and Winge 1998).

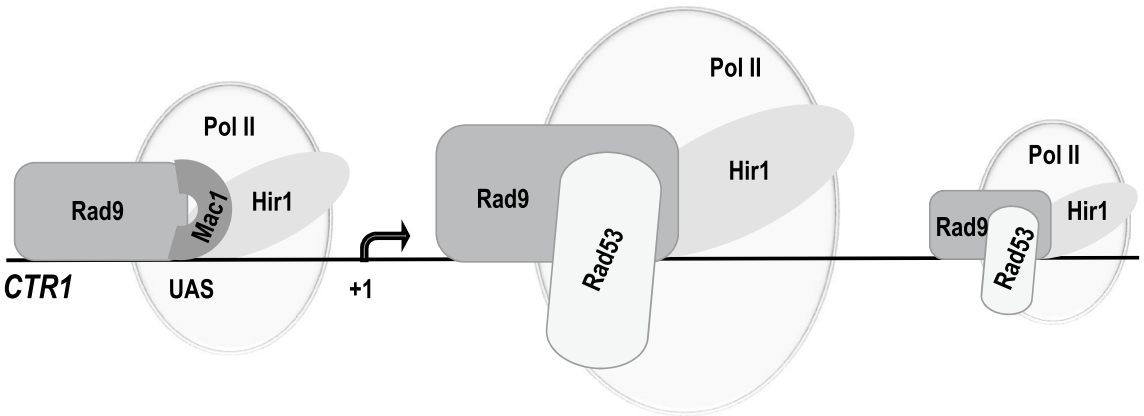

Figure 8

Fig. 8 Model of distinct protein associations on *CTR1* gene that link transcription to DNA repair. On *CTR1* active promoter (low copper conditions), Rad9 interacts physically with both the DNA-binding and transactivation domains of Mac1 as well as with Hir1 coregulator. It then moves to the *CTR1* coding region along with Hir1, following the localization pattern of RNA Pol II, interacting there with its target kinase Rad53, under non DNA damage-inducing conditions. Through its distinct associations with specific transcriptional machinery components, Rad9 constantly holds Mac1-dependent transcription in check so that it is rapidly halted in the case of a DNA damage event, enabling rapid and efficient repair. Differences in the relative size of the same protein depicted in the three shown complexes reflect its respective amount

β-Galactosidase, one- and two-hybrid assays

5 ml of yeast cultures were grown in SC to an OD_{550} of 1.5, diluted and regrown to an OD_{550} of 0.6–1.0 in SC and β-galactosidase activity was measured (Ausubel et al. 1987). Yeast two-hybrid screens were performed against a Gal-4pAD–yeast genomic library (Bilsland et al. 2004) and one- and two-hybrid assays as previously described (Georgakopoulos et al. 2001; Voutsina et al. 2001).

Purification of fusion proteins expressed in *E. coli* and in vitro interaction assays

GST-Mac1 fusion derivatives were co-expressed with *E. coli* thioredoxin to achieve protein solubility (Jensen and Winge 1998) in BL21DE3pLys cells pregrown at 30 °C to an OD_{600} of 0.5 prior to the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). 30 min after IPTG induction, $CuSO_4$ was added to 1.4 mM and cells were grown for 4 h. Harvested cells were washed with 0.25 M sucrose and resuspended in 20 mM NaH_2PO_4 pH 7.5, 250 mM NaCl, 10% glycerol, 5 mM DTT and sonicated. The lysate was clarified at 100,000 *g* for 30 min at 4 °C. Triton X-100 was added to 1% and the extract was purified on a glutathione–Sepharose column (Glutathione Sepharose 4B Amersham Pharmasia 17-0756-01). GST-fusion proteins were eluted by 20 mM glutathione in sonication buffer and monitored by SDS–PAGE. 6His-tagged proteins expressed in BL21DE3pLys, purified by Ni–NTA chromatography (Qiagen), were eluted in 75 mM KCl, 20 mM Tris, pH 8.00, 0.01% NP-40, 0.25% BSA, 250 mM imidazole and protease inhibitors and incubated with glutathione agarose bead-bound GST or GST-fusion protein for 8–12 h, at 4 °C, in 200 µl of the above buffer without imidazole. Beads were washed in the same buffer without BSA and retained proteins were eluted in gel loading buffer and analyzed by SDS–PAGE and immunoblotting using a polyclonal anti-His(G-18) (sc-804; Santa Cruz Biotechnology).

RNA analysis

Total RNA from 30 ml yeast cultures grown to an OD_{550} of 0.6–1.0 in SC medium or initially in SC and subsequently in SCBCS or in SCCu, was extracted using the acid phenol method (Ausubel et al. 1987). RNA samples (40 µg) were resolved on formaldehyde-containing 1.5% agarose gels, transferred to nylon membranes and hybridized with [^{32}P]-labeled probes generated by random priming.

Coimmunoprecipitation assays from yeast cellular extracts

Cells co-expessing 9Myc-tagged proteins and FLAG-tagged proteins were grown to an OD_{550} of 0.8. Proteins were extracted from lysed cells in 50 mM HEPES–KOH, pH 7.5, 5 mM magnesium acetate, 100 mM potassium acetate, 0.1% NP-40, 1 mM NaF, 0.5 mg/ml BSA, 10% glycerol and protease inhibitors. The supernatant was precleared with protein G Sepharose (17-0618-010; GE Health Care) for one hour at 4 °C, then incubated with monoclonal anti-FLAG (F-3165; Sigma) at 4 °C for four hours. Samples were analyzed by electrophoresis on SDS-PAGE and 9Myc-tagged proteins in the immunoprecipitates were detected by immunoblotting using rabbit polyclonal anti-c-Myc (A-14) (sc-789; Santa Cruz Biotechnology). RNA Pol II was detected using a polyclonal rabbit anti-Pol II (C-21) mapping within the tandem repeat domain of the mouse RPB1 (sc-900; Santa Cruz Biotechnology), Secondary antibodies (anti-rabbit 111-035-003; Jackson ImmunoResearch) were visualized by an ECL Western blotting detection kit (Pierce).

ChIP assays

50 ml of yeast cultures grown to an OD_{550} of 0.6–1.0 were used for ChIP assays (Kuo and Allis 1999). Antibodies used were the anti-c-Myc (A-14), rabbit polyclonal antibody for Histone H3 (ab1791; Abcam), anti-H3K36me2 and anti-H3K79me2 (07–274, 07–366; Upstate Biotechnologies). Immunoprecipitated (5/100 µl) and total input DNA (5/1000 µl) were analyzed by quantitative real-time PCR [4 min at 94 °C; 30 s at 94 °C, 30 s at 52 °C, 55 s at 72 °C (27 cycles); 5 min at 72 °C] using SYBR green quantified by a DNA Engine Opticon System (MJ Research) for continuous fluorescence detection. In experiments where mouse monoclonal antibodies (8WG16 MMS-126R, H5 MMS-129R, H14 MMS-134R; Covance Innovative Antibodies) were used to identify RNA Pol II CTD and characterize its phosphorylation state (CTD, P-Ser2, P-Ser-5) (Komarnitsky et al. 2000; Morris et al. 2005), in all buffers NaF was added to 10 mM. Cross linking time was minimized to 5 min. For the H5 and H14 immunoprecipitation, anti-mouse IgM antibodies were preincubated with protein G Sepharose beads for 8 h at 4 °C. To minimize loss of RNA Pol II we washed at minimal stringency (four washes with 10 volumes of ChIP buffer and elution buffer with 0.025% SDS). Oligonucleotide primers were purchased from the Microchemistry Lab at FORTH and MWG-Biotech. All PCR primer sequences are available on request. The polymerases used for PCR were Vent (New England Biolabs) or *Taq* (MINOTECH Biotechnology).

Exchange of *ACT1* promoter with *CTR1* promoter

Replacement of *ACT1* promoter by the *CTR1* promoter in the yeast genome ($CTR1_{prom}$-*ACT1*) was performed by homologous recombination, *CTR1* promoter was first cloned into the *BamH*I site upstream of the KanMX4 gene in pFA6-KanMx4 plasmid. $CTR1_{prom}$-KanMX4 DNA flanked by

45 bp homologous to the regions adjacent to the site of integration was PCR amplified and used to transform wild type Rad9-9Myc strains (Verstrepen and Thevelein 2004). Continuous expression of active Mac1 from pRS315-MAC1^{up} ensured expression of the essential *ACT1* gene. Transformants were selected in the presence of G418-Sulfate (GIBCO) and verified by PCR amplification using primers within *CTR1* promoter and *ACT1* coding sequence.

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