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Fission yeast Mcl1 interacts with SCF^{Pof3} and is required for centromere formation

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Abstract

The fission yeast S-phase regulator Mcl1, an orthologue of budding yeast Ctf4, is an interacting protein of DNA polymerase α and an important factor to ensure DNA replication and sister chromatid cohesion. Deletion of this protein results in severe cohesion defects, however, the function and cellular role of this protein remains elusive. In this study we isolate Mcl1 as an interaction partner of the F-box protein Pof3, which is a component of the ubiquitin ligase complex SCF^{Pof3}. Comparing the phenotypes of cells lacking *pof3*$^+$ or *mcl1*$^+$ we find a broad overlap including the accumulation of DNA damage and activation of the DNA damage pathway. Importantly, we identity a novel, specific role for Mcl1 in the transcriptional silencing and the localisation of CENP-A at the centromeres.

Keywords: CENP-A; Checkpoint; Gene silencing; Fission yeast; Heterochromatin; SCF ubiquitin ligase

Ubiquitin-dependent proteolysis is implicated in the regulation of a wide variety of cellular processes [1]. The final step in the transfer of ubiquitin to target proteins is catalysed by ubiquitin ligases, which also confer the substrate specificity of the reaction. SCF (Skp1/Cullin/F-box) complexes, a family of these E3 ligases, are involved in the ubiquitylation and degradation of cell cycle regulators, transcription factors, and components of other important pathways [2]. SCF complexes consist of an unaltered core of Cullin1, Rbx1/Roc1/Hrt1, and Skp1, which can bind a number of different F-box proteins. These proteins act as substrate receptors and each F-box protein has a limited number of target proteins that can be recruited for ubiquitylation.

In the fission yeast *Schizosaccharomyces pombe* 16 F-box proteins are known to date [3]. Pof3 (Pombe F-box 3) has previously been described as an important factor for DNA integrity [4]. Cells lacking Pof3 show reduced growth rate and elongated cell shape due to activation of the DNA damage checkpoint. This checkpoint is required for cell survival as deletions of the genes of the checkpoint kinases *rad3*$^+$ or *chk1*$^+$ are lethal in Δ*pof3* cells. Furthermore, deletion of *pof3*$^+$ results in chromosome missegregation, shortened telomeres, and importantly, derepression of the transcriptional silencing at the telomeric and centromeric regions. These phenotypes point to a role of Pof3 in the maintenance of chromatin structure and integrity [4]. However, the pathways which are influenced by Pof3 are currently unknown.

Recent reports suggest a role of the Pof3 orthologue Dia2 in budding yeast in DNA replication [5,6]. Cells lacking *DIA2* were shown to enter S-phase prematurely and Dia2 binding to replication origins possibly prevents reinitiation of replication [5]. It is also suggested to ensure replication fork passage through regions of damaged DNA [6]. This fits with the fact that in a large-scale approach, several components of the replication fork were copurified with Dia2 including Mcm2, 3, 5, and Ctf4 [7]. Ctf4 directly binds Pol1 during S-phase and is therefore seen as a Pol1

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accessory factor [8,9]. Deletion of Ctf4 results in a delay in cell cycle as well as in severe cohesion defects and chromosome missegregation.

Similar phenotypes were also observed for the fission yeast orthologue Mcl1 [10–12]. As described for Ctf4, Mcl1 also physically interacts with Pol1 and synthetic lethal interactions with several replication components further underline the role of Mcl1 in this process. The protein localises to the chromatin in G1 and S-phase but not in G2, again suggesting a direct function at the chromatin during S-phase. The requirement for Rad3 demonstrates that cells lacking *mcl1*$^+$ depend on the DNA damage checkpoint for survival. Δ*mcl1* cells also exhibit the defects in cohesion and chromosome segregation, however the underlying mechanism of Mcl1 function remains elusive. In this study we show that Mcl1 interacts physically and genetically with the F-box protein Pof3. The deletion of each gene gives rise to partially overlapping phenotypes, suggesting the possibility that they might modulate each other's function.

Materials and methods

Strains, media, and genetic methods. Yeast strains used in this study are listed in Table 1. Standard methods were followed as described [13–15]. Yeast two-hybrid screening was performed according to manufacturer's instruction (Clontech Co.) using *pof3*$^+$ as bait. Full-length *pof3*$^+$ and *skp1*$^+$ ORFs were amplified by PCR and cloned into the *Sma*I/*Pst*I sites of pAS2-1 or the *Nde*I/*Bam*HI sites of pGAN6, respectively. Mcl1 was PCR-amplified from a cDNA library and introduced into pGAN6 using *Eco*RI/*Pst*I.

Western blotting and immunoprecipitation. For protein extracts cells were harvested, washed, resuspended in 1 ml H_2O and after addition of 150 μl of 1.85 M NaOH incubated for 10 min on ice. Proteins were precipitated using 150 μl of 50% trichloric acid for 10 min and then resuspended in sample buffer according to cell number. Immunoprecipitation procedures were followed as described previously [16].

Pulse field gel electrophoresis. Preparation of genomic DNA and pulse field gel electrophoresis (PFGE) were performed as described before [17].

Light microscopy. Fluorescence microscopy was performed using a Zeiss Axioplan microscope, a chilled video charge-coupled device camera (C4742-95; Hamamatsu Photonics), and the Volocity 3.1 software (Improvision Inc.), and processed using Adobe Photoshop.

Results

Pof3 interacts with the S-phase regulator Mcl1

To gain further insight into the function of Pof3, we performed a yeast two-hybrid screen to identify Pof3-interacting proteins. Using the full-length Pof3 as bait, we screened 2.7×10^6 colonies and isolated 16 candidate plasmids, which were able to confer growth on plates lacking histidine specifically in the presence of Pof3. Sequencing revealed that 12 of these plasmids contained the known Pof3-binding partner Skp1 and therefore served as positive controls for the screening conditions. Two of the plasmids encoded a previously not described open reading frame and two corresponded to an N-terminal truncated version of Mcl1. Cloning full-length Mcl1 into the pGAN6 vector confirmed that Pof3 and Mcl1 interact, as cells could only grow in the absence of histidine when both plasmids were present (Fig. 1A).

To confirm this interaction in *Schizosaccharomyces pombe* cells we performed immunoprecipitation experiments (Fig. 1B and C). Strains carrying either Pof3-13myc or GFP-Mcl1, the latter under the control of the thiamine-repressible *nmt1* promoter, or both were grown in YE5S medium, harvested, and immunoprecipitation was performed from cell extracts using either anti-myc (Fig. 1B) or anti-GFP antibody (Fig. 1C). Western blot analysis of

Table 1
Strain list

Strains	Genotypes	Derivations
513	*h*$^-$*leu1ura4*	Our stock
TP108-3D	*h*$^+$*leu1ura4his2*	Our stock
YAP300	*h*$^+$*leu1ura4ade6his7pof3::ura4*$^+$	Our stock
YAP8-1	*h*$^-$*leu1ura4 kan-nmtP3-GFP-mcl1*$^+$	This study
YAP45-1	*h*$^-$*ura4ade6kan-nmtP3-GFP-mcl1*$^+$*pof3*$^+$*-13myc-kan*	This study
YAP21-1	*h*$^+$*ura4ade6pof3*$^+$*-13myc-ura4*$^+$	This study
SKP471	*h*$^-$*leu1ura4pof3::kan*	Our stock
YAP81	*h*$^+$*leu1ura4his2mcl1::hph*	This study
YAP12	*h*$^+$*leu1ura4mcl1::kan*	This study
YAP9-1	*h*$^-$*leu1ura4 kan-nmtP3-mcl1*$^+$	This study
YAP224	*h*$^-$*leu1ura4his7ade6kan-nmtP3-mcl1*$^+$*pof3::ura4*$^+$	This study
YAP50	*h*$^+$*leu1ura4his2mcl1*$^+$*-GFP-kan*	This study
YAP60	*h*$^+$*leu1ura4his2ade6pof3::ura4*$^+$*mcl1*$^+$*-GFP-kan*	This study
AL182	*h*$^-$*leu1ura4kan-nmtP3-GFP-fbh1*$^+$	This study
YAP98	*h*$^-$*leu1ura4ade6kan-nmtP3-GFP-fbh1*$^+$*pof3::ura4*$^+$	This study
YAP100	*h*$^+$*leu1ura4his2kan-nmtP3-GFP-fbh1*$^+$*mcl1::hph*	This study
SK1862	*h*^{90}*leu1ura4-DS/Eade6-210his3-d1 otr1(SphI)::ade6*$^+$*ura4*$^+$*-tel2L*	Robin C. Allshire
SKP494-9	*h*^{90}*leu1ura4-DS/Eade6-210his3-d1pof3::kan otr1(SphI)::ade6*$^+$*ura4*$^+$*-tel2L*	Our stock
YAP39	*h*^{90}*leu1ura4-DS/Eade6-210his3-d1mcl1::kan otr1(SphI)::ade6*$^+$*ura4*$^+$*-tel2L*	This study
SP38	*h*$^-$*leu1ura4cnp1*$^+$*-GFP-lys1*$^+$	Kohta Takahashi
YAP68	*h*$^+$*leu1ura4ade6cnp1*$^+$*-GFP-lys1*$^+$*pof3::kan*	This study
YAP57	*h*$^+$*leu1ade6his2cnp1*$^+$*-GFP-lys1*$^+$*mcl1::kan*	This study

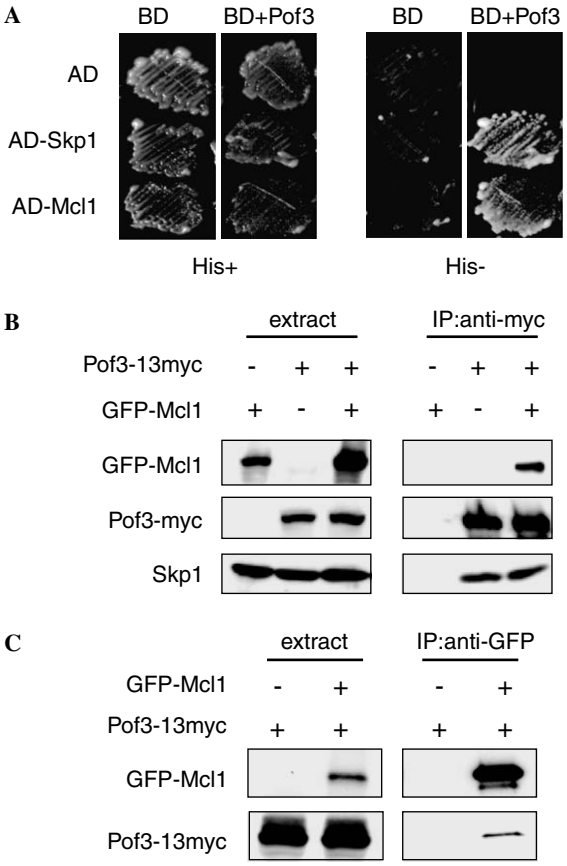

Fig. 1. Pof3 interacts with Mcl1. (A) Yeast cells AH109 carrying plasmids pAS2-1 (BD) or pAS2-1-Pof3 (BD-Pof3) were transformed with pGAN6 (AD), pGAN-Skp1 (Skp1) or pGAN-Mcl1 (Mcl1) and grown in the presence or absence of histidine for 3 days. (B,C) Cells expressing Pof3-13myc, GFP-Mcl1 or both were grown on YE5S and immunoprecipitation using anti-myc antibody (B) or anti-GFP-antibody (C) were performed. Precipitated proteins were separated on SDS–PAGE and detected by immunoblot using anti-GFP, anti-myc, and anti-Skp1 antibodies.

the precipitates showed that the band corresponding to GFP-Mcl1 could only be detected in the samples containing Pof3-13myc, demonstrating that purification of GFP-Mcl1 is dependent on its interaction with the F-box protein. A substantial amount of Skp1 also copurified with Pof3-13myc (Fig. 1B), again serving as an internal control. The same was true for the immunoprecipitation of GFP-Mcl1 (Fig. 1C), in which Pof3-13myc could only be precipitated with GFP antibodies in the presence of GFP-Mcl1 but not in its absence. We therefore concluded that Mcl1 is indeed a binding partner of Pof3.

Genetic interaction of Pof3 and Mcl1

To investigate the biological consequences of the interaction between Pof3 and Mcl1, we analysed the effects of deletion or overexpression. Deletion of Pof3 has been shown to cause cell cycle delay due to activation of the DNA damage checkpoint, chromosome missegregation, and desilencing of heterochromatin regions [4]. Loss of Mcl1 was also described to result in cell cycle arrest and chromosome missegregation [10–12]. To identify the effects of loss of both genes, spores from heterozygous diploid cells containing deletions of *pof3* (kanamycin) and *mcl1* (hygromycin) were analysed by tetrad dissection (Fig. 2A). More than 20 tetrads were dissected, but no colonies were found that contained both markers, showing that deletion of $pof3^{+}$ and $mcl1^{+}$ is synthetic lethal. Spores containing double deletions were able to germinate but cells terminally arrested with elongated cell shape after a few cell divisions (Fig. 2A, inset).

We also tested the phenotypes of overexpression of Mcl1 in the absence of Pof3. Wild-type as well as Δ*pof3* cells containing Mcl1 under the control of the $nmt1^{+}$ promoter were grown on plates in the presence or absence of thiamine.

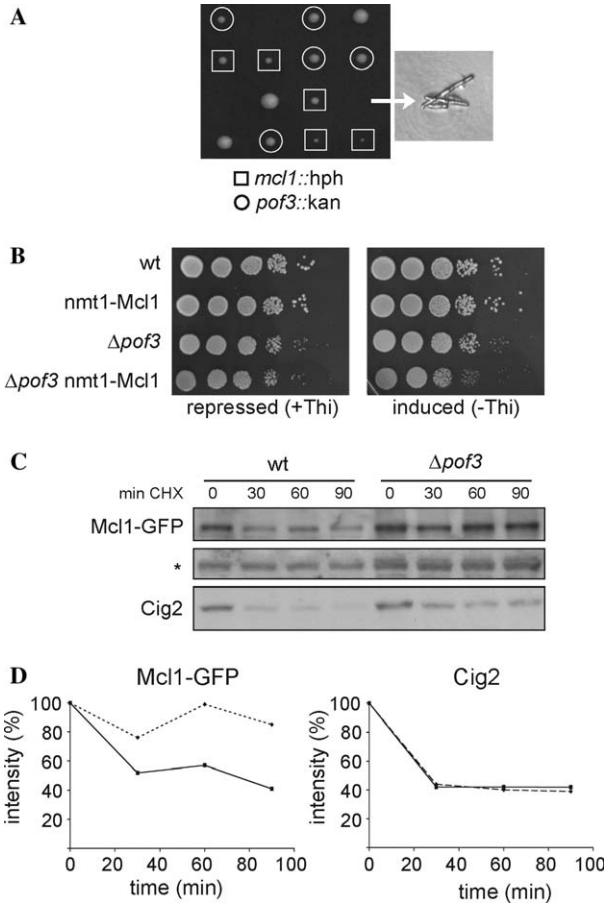

Fig. 2. Genetic interactions of Pof3 and Mcl1. (A) Tetrads from heterozygous dipoid (*pof3*::*kan mcl1*::*hph*) were dissected on YE5S plates and incubated at 27 °C for 6 days. Marker distribution was determined by replica plating to YE5S plates containing G418 or hygromycin B. (B) Strains 513 (wt) YAP9-1 ($nmt1\text{-}mcl1^{+}$), YAP300 (Δ*pof3*), and YAP224 ($nmt1\text{-}mcl1^{+}$, Δ*pof3*) were spotted onto EMM plates with or without thiamine and incubated for 3 days at 30 °C. (C,D) YAP50 (Mcl1-GFP) and YAP60 (Mcl1-GFP Δ*pof3*) were grown to exponential growth phase and treated with 100 μg/ml cycloheximide for the time indicated. Cells were harvested and extracts were analysed by SDS–PAGE and immunoblotted using anti-GFP and anti-Cig2 antibodies. *Indicates a cross reaction of the anti-GFP antibody, which serves as loading control. Band intensities (black lines for wt and dotted lines for Δ*pof3*) of Mcl1-GFP (D, left) or Cig2 (right) were quantified. Intensity at time point 0 was set to 100%.

As described, Δ*pof3* cells showed slower growth than wild-type cells. Overexpression of Mcl1 (Fig. 2B, -Thi) did not show any significant effect on growth of wild-type cells. However, colony size was severely reduced in cells lacking Pof3. These data show that Δ*pof3* cells are more susceptible to the effects of Mcl1 overexpression. As Pof3 is part of an E3 ligase complex, this toxicity points to the possibility that Mcl1 could be a substrate of Pof3.

We next tested the stability of Mcl1-GFP in the presence or absence of the F-box protein. Cells growing in exponential growth phase were treated with cycloheximide and samples were taken before addition of the drug (time 0) and at the indicated time points after treatment (Fig. 2C). Protein extracts were prepared and Mcl1-GFP and Cig2 (the S-phase cyclin) detected by Western blotting. In wild-type cells, Mcl1 levels decreased to about 50% over the 90 min of cycloheximide treatment. However, in Δ*pof3* cells the intensity of the Mcl1-GFP bands was more stable, indicating that degradation of Mcl1 is decreased in the absence of Pof3. Cig2 (lower panel), as an unstable protein and substrate of $SCF^{Pop1/Pop2}$ [18], served as an internal control for the experiment (Fig. 2C and D). This result indicates that the half-life time of Mcl1 is increased in the absence of Pof3.

Deletions of Mcl1 and Pof3 cause accumulation of DNA damage

Deletions of either *pof3*$^+$ or *mcl1*$^+$ activate the DNA damage checkpoint and analysis of temperature sensitive *mcl1*$^+$ mutants by PFGE has demonstrated the presence of broken DNA in these cells [11]. To verify whether this type of DNA damage can also be observed in Δ*pof3* cells, we used GFP-Fbh1, a protein known to form foci in the presence of double strand breaks in living cells [19]. Wild-type, Δ*pof3*, and Δ*mcl1* cells carrying GFP-Fbh1 were examined by fluorescence microscopy (Fig. 3A). Fbh1 foci were detected in 30% of Δ*pof3* and 50% of Δ*mcl1* cells while no foci formation could be detected in the wild-type strain, providing strong evidence for the presence of DNA damage.

To visualise the presence of broken DNA directly, PFGE was performed in exponentially growing wild-type, Δ*mcl1* or Δ*pof3* cells (Fig. 3B, lanes 1–3). As controls, wild-type cells treated with 20 μg/ml phleomycin or 12 mM hydroxyurea were included (lanes 4 and 5). The presence of smaller DNA fragments, indicative of broken DNA, was observed in the phleomycin treated control and in both Δ*pof3* and Δ*mcl1* cells but not in wild-type cells. Together with the foci formation of GFP-Fbh1, this shows that deletion of either *mcl1*$^+$ or *pof3*$^+$ causes accumulation of DNA damage in the cell.

Deletion of Mcl1 results in derepression of transcriptional silencing and delocalisation of Cnp1

Deletion of *pof3*$^+$ causes loss of the transcriptional silencing at the centromeres and the telomeres. We therefore examined whether the deletion of *mcl1*$^+$ might result in a

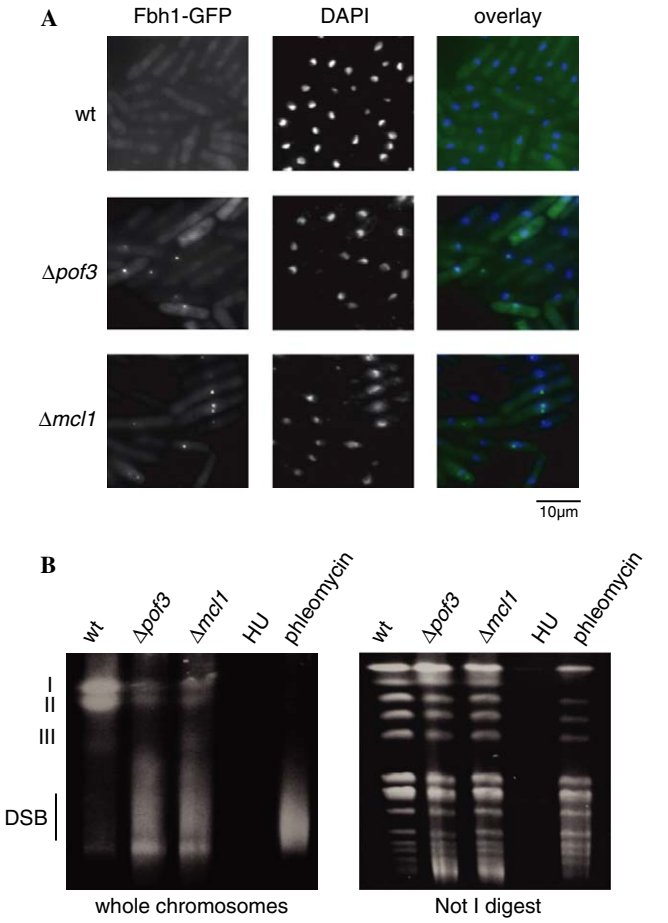

Fig. 3. Deletion of Mcl1 or Pof3 causes accumulation of DNA damage. (A) AL182 (wt), YAP98-1 (Δ*pof3*), and YAP100 (Δ*mcl1*) expressing Fbh1-GFP were grown in YE5S medium, fixed, stained with DAPI, and analysed by fluorescence microscopy. (B) 513 (wt), YAP300 (Δ*pof3*), YAP12 (Δ*mcl1*), and 513 treated with 20 μg/ml phleomycin or 12 mM hydroxyurea were harvested, spheroplasted, and prepared for PFGE. DNA was visualised by ethidium bromide staining.

similar phenotype. Initially, we tested the sensitivity of wild-type, Δ*pof3*, and Δ*mcl1* cells to the HDAC inhibitor Trichostatin A. Cells were spotted in serial dilutions onto plates containing 50 μg/ml of the substance (Fig. 4A). After 3 days of incubation at 27 °C, wild-type cells showed robust growth on the drug-containing plate while colony formation of cells lacking Pof3 or Mcl1 was severely impaired.

To further address this point, we used yeast strains containing the marker genes *ura4*$^+$ and *ade6*$^+$ in the telomeric (*Tel 2L*) and the *otr* region of the centromere, respectively [20]. Cells were spotted on plates lacking uracil as well as on YE plates and on plates containing 5-fluoroorotic acid (FOA), which is toxic for cells expressing *ura4*$^+$ (Fig. 4B). In the wild-type strain both markers, *ura4*$^+$ and *ade6*$^+$, were transcriptionally repressed and cells were therefore unable to grow in the absence of uracil, formed red colonies on YE media, due to the lack of adenine, and grew well on plates containing FOA. Δ*pof3* cells in contrast were able to form colonies in the absence of uracil and showed white colonies on YE plates again demonstrating the loss of transcriptional repression. Interestingly,

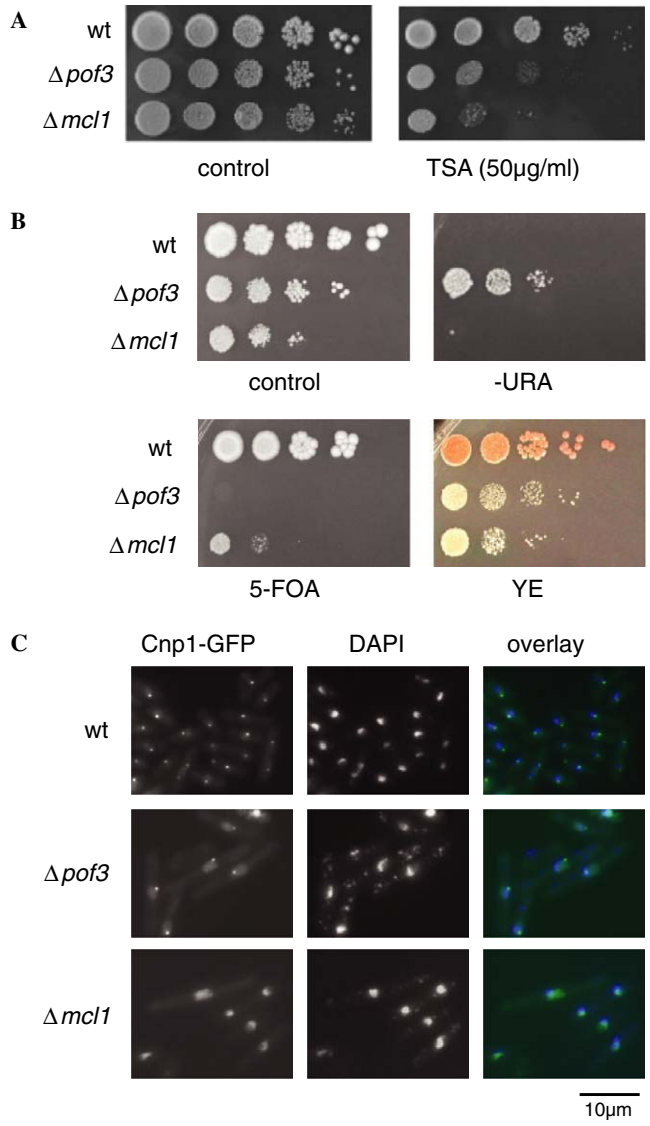

Fig. 4. Deletions of *pof3*$^+$ or *mcl1*$^+$ cause defects in transcriptional repression of heterochromatin regions. (A) Serial dilutions of 513 (wt) YAP300 (Δ*pof3*), and YAP12 (Δ*mcl1*) cells were spotted onto YE5S plates with or without 50 μg/ml Trichostatin A and incubated for 3 days at 27 °C. (B) FY1862, SKP494-9 (Δ*pof3*), and YAP39 (Δ*mcl1*) cells were spotted in serial dilution onto YE5S (control), EMM-ura (-URA), YE5S containing 1 mg/ml 5-FOA or YE plates and incubated for 4 or 6 (-URA) days at 30 °C. (C) SP38 (wt), YAP68 (Δ*pof3*), and YAP57 (Δ*mcl1*) cells growing on YE5S were fixed, stained with DAPI, and Cnp1-GFP localisation was analysed by fluorescence microscopy.

Δ*mcl1* also formed white colonies on YE media, indicating that transcriptional silencing in the centromeric region is impaired. However, repression of gene expression at the telomeric region seemed intact, as cells were unable to grow in the absence of uracil but formed colonies on FOA plates (Fig. 4B). This result reveals an involvement of Mcl1 in establishment or maintenance of heterochromatin in the centromeric but not the telomeric region.

To identify whether defects in gene silencing at the centromere were caused by the same mechanism in Δ*pof3* and Δ*mcl1* cells, we tested the localisation of several markers of heterochromatin and kinetochore formation. While only slight changes of GFP-Swi6 (orthologue of HP1) or Chp1-GFP dots could be detected (data not shown), the dot-like pattern of Cnp1-GFP (orthologue of CENP-A) was totally lost in Δ*mcl1* cells and the GFP signal was distributed throughout the nucleus (Fig. 4C, bottom panels). This dramatic effect could not be observed in Δ*pof3* cells, where the nuclear background of Cnp1-GFP was slightly higher, but the dots were clearly visible (middle panels). These findings indicate that the defects in heterochromatin formation in Δ*mcl1* and Δ*pof3* might not be caused by the same mechanisms. Moreover it points to a novel role of Mcl1 in the localisation of the core centromere protein Cnp1.

Discussion

In this study, we identify the Pol1 accessory factor Mcl1 as a Pof3 binding protein. This is consistent with recent observations that suggest a role of the Pof3-orthologue Dia2 in replication and protection of replication fork stability [5,6,21]. We show that deletions of Mcl1 or Pof3 result in similar phenotypes. Lack of either of these proteins leads to the accumulation of DNA damage under normal growth conditions. For both strains the presence of broken DNA could be detected in PFGE (Fig. 3B) and the formation of foci of repair proteins was observed (Fig. 3A). For Mcl1 it is suggested that this DNA damage is due to a defect in the processing of Okazaki fragments during replication [11], while for Pof3 this phenotype was observed for the first time. As in Δ*mcl1* cells it could be the consequence of S-phase defects.

The function of Pof3 as an F-box protein suggests that it might recruit Mcl1 for degradation and indeed the stability of Mcl1-GFP is increased in the absence of Pof3. However, the relatively long half-life of Mcl1 even in wild-type cells suggests that only a subset of Mcl1 might be degraded in a localisation-dependent and/or cell-cycle specific manner. The relatively weak levels of coimmunoprecipitation from cell extracts compared to the robust interaction of the binding partners in the two hybrid system support the idea that only a subset of the proteins interact in living cells. Mcl1 is a constitutively nuclear protein that localises to the chromatin during G1 and S-phase [10]. Since Pof3 also localises to the chromatin (S.K. and T.T., unpublished results), it might have a role in specifically degrading Mcl1 at these certain stages, thereby regulating the composition of the replication fork. The Mcl1 orthologue Ctf4 is described to prevent Spt16, a component for the FACT complex, from binding to Pol1 [9,22], a step, which might be a potential target of Pof3 function. As deregulation of replication can give rise to genome instability, this would be a possible scenario for the development of Δ*pof3* phenotypes.

However, the degradation of Mcl1 is not very rapid and we cannot exclude that Mcl1 stabilisation in Δ*pof3* mutants is an indirect effect. In vivo ubiquitylation assays failed to reveal Pof3-dependent Mcl1 ubiquitylation (data not shown). The observation that deletion of both *mcl1*$^+$ and *pof3*$^+$ is lethal seems to contradict a simple idea that

accumulation of Mcl1 causes the phenotypes of Pof3. The alternative possibility is that Mcl1 acts as a scaffolding or targeting factor that brings Pof3 in proximity to the replication fork, where it might find its substrates.

Our results provide evidence that Mcl1 like Pof3 is required for the transcriptional repression of the outer repeats of the centromeric region (Fig. 4). However, in contrast to the F-box protein, it does not seem involved in general mechanisms of heterochromatin formation or maintenance, as no defects in the transcriptional repression of telomeric regions were detected in the absence of Mcl1. The mechanisms of Pof3 and Mcl1 function in silencing seems to be generally different, as deletion of *mcl1*$^{+}$ but not *pof3*$^{+}$ causes delocalisation of the histone 3 variant Cnp1, which is the hallmark of the core centromeric region. Failure to incorporate Cnp1 into the chromatin during replication might give rise to the disturbed centromere structure and the cohesin defects, which are observed in Δ*mcl1* cells [10–12]. Desilencing of centromeric regions together with defects in chromosome segregation has been shown for mutants of the protein kinase Hsk1/Dfp1 [23], an important regulator of S-phase events. It is therefore possible that the role of Mcl1 in chromosome missegregation, centromere desilencing, and Cnp1 loading involves directly its properties as an S-phase regulator and is independent of Pof3 function. Perhaps Pof3 and Mcl1 share some functions, but also play separate roles in genome integrity control. Further experiments will be required to delineate the biological relationship between these proteins.

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