Sim3 shares some common roles with the histone chaperone Asf1 in fission yeast

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ABSTRACT

An H3/H4 histone chaperone, Asf1, plays an essential role in maintaining genomic stability in many species, including fission yeast. Here, we showed that overexpression of a CENP-A chaperone Sim3 suppressed the temperature sensitive phenotype of asf1–33 and asf1–30 mutants and the defect in chromatin structure, and prevented the accumulation of DNA damage in asf1–33 mutants at high temperatures. Furthermore, asf1–33 and asf1–30 mutants were synthetic lethal. Consistent with this, shutdown of sim3 expression in asf1–33 sim3 double mutants that contained extragenic sim3 resulted in growth retardation. In addition, the asf1–33 mutant displayed sensitivity to thiabendazole and hydroxyurea, which suggests that Sim3 plays a general role in maintaining chromatin structure. Our results suggest a possibility that Sim3 functions as a histone chaperone.

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1. Introduction

Histone chaperones mediate changes in chromatin structure through the deposition and eviction of histones [1]. The structure of chromatin is altered in response to cellular processes that occur in DNA, such as DNA replication, transcription, recombination, and DNA repair. For example, when a gene is expressed in response to certain signals, histone proteins are evicted from the promoter region by histone chaperones to disassemble chromatin for transcription [2]. In addition, when DNA damage is repaired, histone proteins are temporarily evicted from the damaged site by histone chaperones to enable repair factors to function. Then, histone proteins are deposited back onto the repaired sites by histone chaperones after the completion of repair [3].

Histone chaperones are categorized into several groups by their preference for histone proteins: Asf1 (anti-silencing function 1) [4], CAF1 (chromatin assembly factor 1) [5], and HIRA (histone interacting protein A) [5] are considered to be histone H3/H4 chaperones, while Nap1 (nucleosome assembly protein 1) [5] is a histone H2A/H2B chaperone. Histone variants are assembled or disassembled into chromatin by special histone chaperones that are specific for each histone variant. For example, Asf1 generally deposits (and evicts) histone H3/H4 onto chromatin. However, Asf1 does not bind to histone H3 variant CENP-A; instead, HJURP incorporates CENP-A onto centromeres in human cells [6].

Chromosome segregation is a pivotal process required for the transmission of genetic material to daughter cells. When chromosomes segregate, spindle microtubules emanating from SPB (spindle pole bodies) or centrosomes attach to the kinetochores of chromosomes. The kinetochore is composed of large protein complexes and centromeric DNA [7], and its formation is specified by CENP-A [8]. CENP-A is specifically localized to the center of the centromere region, while histone H3 is distributed to all other chromosomal regions. The loading of CENP-A onto centromeres requires many factors including Sim3 and Scm3. Sim3 was identified as a factor that functions in the maintenance of silencing at the center centromeric regions in fission yeast Schizosaccharomyces pombe [9]. Sim3 binds to CENP-A<sup>Cnp1</sup>, and transfers it to a CENP-A<sup>Cnp1</sup> receptor, Scm3 [10], on centromere chromatin. This results in the incorporation of CENP-A<sup>Cnp1</sup> into the center region of centromeres in S. pombe.

Previously, we reported that an asf1 ts mutant (asf1–33) showed defects in bulk chromatin structure and displayed elevated levels of DNA damage, which was associated with activation of the DNA damage checkpoint [11]. Based on these observations, we suggested that fission yeast Asf1 is essential for the maintenance of genomic stability. In addition, we found that overexpression of CENP-A<sup>Cnp1</sup> histone chaperone sim3 suppressed the temperature sensitive phenotype of the asf1–33 mutant. Here, we showed that overexpression of sim3 also suppressed the defects in chromatin structure and prevented the accumulation of DNA damage in the asf1–33 mutant. Furthermore, our results reveal that asf1–33


Asim3 double mutants were synthetically lethal. In summary, our results indicate that CENP-A histone chaperone Sim3 shares some functional characteristics with Asf1 as a histone H3/H4 chaperone in fission yeast.

2. Materials and methods

2.1. Fission yeast media and general manipulations

The yeast strains examined in this study are all listed in Table 1. Each strain was cultured in YES medium (0.5% yeast extract, 3% glucose, 225 mg/liter adenine, histidine, leucine, uracil, and lysine hydrochloride) or EMM medium [12]. Nitrogen-free EMM medium was used to mate h+ and h- strains. Standard methods used for S. pombe were described previously [12].

2.2. Detection of Rad22-GFP foci

Rad22-GFP foci in asf1–33 mutants harboring pREP41 or pREP41-sim3 were observed by a confocal laser scanning microscope (Leica TCS SP-5, Leica Microsystems, Tokyo). The number of Rad22-GFP positive cells was calculated by counting 100 cells.

2.3. Micrococcal nuclease assay

Micrococcal nuclease assay was performed as described previously [13]. Cells were cultured in EMM at 36 °C for 6 h. After incubation, cells were collected by centrifugation. Then, spheroplasts were prepared with Zymolyase 100 T. Chromatin DNA was digested with MNase. Digested chromatin DNA was resolved on a 1.2% agarose gel and was visualized by ethidium bromide staining.

2.4. Construction of a sim3 gene deletion strain and pREP81-sim3

Gene deletion of sim3 was carried out using a PCR-based method [14]. The kanMX6 module was amplified using pFA6a-kanMX6 as a template together with the pFA6a F and pFA6a R primers (Table S1). Fragments approximately 500 bp in length were amplified using sim3d1, sim3d2, sim3d3, and sim3d4 primers, which contained homologous sequences corresponding to the 5' and 3' regions of sim3 that were attached to the ends of the kanMX6 module. The resulting fragments were introduced into S. pombe cells. G418 resistant colonies were selected on YES plates containing G418 (100 μg/ml). Colony PCR (using the primers chkHR12–42 and sim3 check) was performed to confirm the construction of a sim3 strain. For the construction of pREP81-sim3, the sim3 gene was cloned into pREP81 using a gap-repair cloning method [15]. The ORF region of sim3 containing a sequence homologous to pREP81 was amplified by PCR. This fragment, together with BamHI digested pREP81, was introduced into PR110 (h+ leu1–32 ura4–D18), and transformants were selected on EMM-Leu. The plasmids were extracted from the transformants and introduced into Escherichia coli DH5α to amplify the plasmids. The correct construction of the plasmid was confirmed by sequencing using the primers Pntmt1 80 bp F and Tnmt1 80 bp R.

An asf1–33 sim3 strain harboring pREP41-sim3 or pREP81-sim3 was constructed by chromosomal deletion of sim3 from an asf1–33 strain harboring pREP41-sim3 or pREP81-sim3. Proper deletion of sim3 was confirmed by PCR as described above.

3. Results

3.1. Overexpression of sim3 suppressed the defect in chromatin structure and prevented the accumulation of DNA damage in the asf1–33 mutant

We previously reported that overexpression of sim3 suppressed the temperature sensitive phenotype of the asf1–33 mutant in S. pombe [11]. In addition to the role of Sim3 as a CENP-A histone chaperone that transfers CENP-A<sup>sim3</sup> protein to the center region of centromeres (cnt, imr), it has also been reported that Sim3 has an ability to bind histone H3 [17]. Therefore, we hypothesized that Sim3 might be able to replace Asf1 as a H3/H4 histone chaperone. To test this idea, we used an MNase assay to examine the bulk chromatin structure of an asf1–33 mutant that overexpressed sim3. In the asf1–33 mutant harboring the control vector, adding increasing amounts of MNase showed that the bulk chromatin was digested into small units of chromatin. By contrast, the chromatin from the asf1–33 mutant that overexpressed sim3 was resistant to MNase digestion (Fig. 1A), which indicates that sim3 complemented the function of asf1 in maintaining chromatin structure.

To examine further the relationship between asf1 and sim3, the heterochromatin structure at the outer repeats of centromeres in the asf1–33 mutant overexpressing sim3 was analyzed. Heterochromatin exhibits a condensed chromatin structure in % which gene expression is repressed [18]. By monitoring the uro4<sup>+</sup> marker gene inserted at the outer repeats of a centromere, we previously reported that heterochromatin structure at the outer centromeric repeats was disrupted in the asf1–33 mutant [11]. 5-FOA sensitivity was observed as a result of increasing the transcriptional level of the uro4<sup>+</sup> gene at the outer repeats of the centromere in the asf1–33 mutant. To examine whether sim3 would suppress the defect in heterochromatin structure in the asf1–33 mutant, we examined the expression of the uro4<sup>+</sup> gene inserted at the outer repeats of a centromere. We found that overexpression of sim3 suppressed the 5-FOA sensitivity of the asf1–33 mutant (Fig. 1B), and likely its defect in heterochromatin structure.

We previously observed that the asf1–33 mutant accumulates DNA damage that results in the loss of viability at 36 °C [11]. We predicted that the suppression of temperature sensitivity in the asf1–33 mutant by sim3 overexpression was attributable to reduced levels of DNA damage. To determine whether the levels of DNA damage were reduced in the asf1–33 mutant that overexpressed sim3, we analyzed the presence of Rad22-GFP foci in these cells. Rad22 protein involves in DNA repair mediated by homologous recombination. When DNA is damaged, this protein accumulates at the sites of DNA damages and forms foci. Overexpression of sim3 decreased the number of Rad22-GFP foci by about 69% in the asf1–33 mutant at 36 °C (Fig. 1C). Therefore, the levels of DNA...
3.2. Overexpression of sim3 suppressed the temperature sensitivity and chromatin defects of the asf1–30 mutant

We previously reported that the mutant Asf1 protein (Asf1–30), which is encoded by asf1–30, was degraded by the ubiquitin–proteasome system at higher temperatures [19]. The mutation sites of asf1–30 differ from asf1–32, and it was found that asf1–30 displayed a higher degree of temperature sensitivity because of the absence of any detectable Asf1 protein at higher temperatures [19]. To test whether sim3 can suppress the defects of the asf1–30 mutant, we examined the temperature sensitivity of an asf1–30 mutant strain that overexpressed sim3. While the asf1–30 mutant did not grow at temperatures higher than 32°C, sim3 expression clearly restored the growth of the asf1–30 mutant at higher temperatures (Fig. 2A). Repressing sim3 expression by the addition of thiamine in the asf1–30 mutant harboring pREP41-sim3 again resulted in temperature sensitivity. These results indicate that sim3 can replace the function of Asf1 even when Asf1 function is severely impaired.

To exclude the possibilities that overexpressed sim3 might change localization or a protein level of Asf1–30 (or Asf1–33), we observed the localization of Asf1 proteins by indirect immunofluorescence.
microscope and protein levels by western blotting [16]. The results showed that overexpression of \textit{sim3} did not change the localization and protein levels of Asf1–33 and Asf1–30 both at 26°C and 36°C (Supplementary Fig. 1A and B). Particularly, a lower protein level of Asf1–30 at higher temperature was not recovered even when \textit{sim3} was overexpressed. These results indicate that up-regulation of Asf1 proteins or change of the localization was not a reason for suppression of temperature sensitivity of \textit{asf1–30} and \textit{asf1–33} mutants by \textit{sim3}.

We next examined the structure of heterochromatin in the \textit{asf1–30} mutant that overexpressed \textit{sim3}. The \textit{ura4}+ gene in the \textit{otr1} locus is shut off in wild-type cells and is thus not sensitive to 5-FOA. The \textit{asf1–30} mutation restored 5-FOA sensitivity due to the reversion of \textit{ura4}+ gene expression at 30°C (Fig. 2B). However, it is not clear that overexpression of \textit{sim3} can suppress the 5-FOA sensitivity of the \textit{asf1–30} mutant, because it is difficult to separate the effect of overexpression of \textit{sim3} on the temperature sensitivity and the 5-FOA sensitivity in the \textit{asf1–30} mutant (Fig. 2B).

Cellular morphology and phloxin B staining of \textit{asf1–30} cells expressing \textit{sim3} was also assessed (Fig. 2C). We found that the number of stained and elongated cells were reduced in \textit{asf1–30} cells by the overexpression of \textit{sim3}, which supports the idea that \textit{sim3} has a suppressive effect on \textit{asf1–30} cells.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Overexpression of \textit{sim3} suppressed the temperature sensitivity and defect in heterochromatin structure of the \textit{asf1–30} mutant. (A) Overexpression of \textit{sim3} suppressed the temperature sensitivity of the \textit{asf1–30} mutant at 32°C, 34°C, and 36°C. Logarithmically growing \textit{L972}, \textit{SKP620 (\textit{h}− leu1–32 asf1–30-13myc-kanMX6)} cells harboring \textit{pREP41} or \textit{pREP41-sim3} were diluted 5-fold from \(3 \times 10^7\) cells with sterilized water, and spotted onto \textit{EMM (+, - thi)}. Plates were incubated at 26°C, 32°C, 34°C, and 36°C for 3 days. (B) Overexpression of \textit{sim3} suppressed the defect in heterochromatin structure of the \textit{asf1–30} mutant at 30°C, 32°C, 34°C, and 36°C. Logarithmically growing \textit{L972 (\textit{h}+), SKP551–6 (\textit{h}+ leu1–32 ural4-D18 otr1::ura4)} harboring \textit{pREP41}, and \textit{SKP593–35 (\textit{h}+ leu1–32 ural4 asf1–30-13myc-kan')} cells harboring \textit{pREP41} or \textit{pREP41-asf1} or \textit{pREP41-sim3} were diluted 10-fold from \(3 \times 10^6\) cells with sterilized water, and spotted onto \textit{EMM (+, - 5FOA)}. Plates were incubated at 26°C, 32°C, 34°C, and 36°C for 5 days. (C) Overexpression of \textit{sim3} suppressed the elongated cell shape and prevented cell death of the \textit{asf1–30} mutant. \textit{SKP620 (\textit{h}− leu1–32 asf1–30-13myc-kanMX6)} cells harboring \textit{pREP41} or \textit{pREP41-sim3} were stained with phloxin B and observed by microscopy.}
\end{figure}
3.3. \textit{Asf1–33} and \textit{Sim3} double mutants display synthetic lethality

To further investigate the genetic relationship between \textit{asf1} and \textit{sim3}, we tried to construct an \textit{asf1–33 Sim3} double mutant. Strain \textit{YP48 (h+ asf1–33-13myc-hphMX6 leu1–32 ure4-D18)} was crossed with \textit{KT146 (h+ sim3::kanMX6)} and their tetrads were analyzed. Although 19 sets of tetrads were dissected, we were unable to isolate any double mutants that were resistant to both G418 and Hygromycin B.

Fig. 3. \textit{asf1–33} and \textit{Sim3} double mutants were synthetically lethal. (A) \textit{YP48 (h+ asf1–33-13myc-hphMX6 leu1–32 ure4-D18)} was mated with \textit{KT146 (h+ sim3::kanMX6 leu1–32 ure4-D18)} on EMM (without nitrogen) for several days at 26 °C. Then, tetrad analysis was carried out, and sporulated cells were cultured on YES (+G418 and Hygromycin) to select \textit{asf1–33 Sim3} double mutants. However, no G418 and Hygromycin B resistant strains were obtained. (B) The \textit{sim3} gene was disrupted in \textit{YP48 (h+ asf1–33-13myc-hphMX6 leu1–32 ure4-D18)} strains harboring \textit{pREP41-Sim3} or \textit{pREP81-Sim3}. Then, cultures of this strain (\textit{asf1–33 Sim3} mutant harboring \textit{pREP41-Sim3} or \textit{pREP81-Sim3}), \textit{L972 (asf1+)} and \textit{YP48 (asf1–33-13myc-hphMX6 leu1–32 ure4-D18)} were diluted 10-fold from \(3 \times 10^6\) cells with sterilized water, and spotted onto EMM (with or without 2 µMthiamine). Thiamine repressed the expression of \textit{sim3} partly in \textit{pREP41}, and completely in \textit{pREP81}. Each plate was cultured at 26 °C, 30 °C, 34 °C, and 36 °C for 5 days.
3.4. Deletion of sim3 results in TBZ and HU sensitivity

Deletion of sim3 results in TBZ and HU sensitivity. This was observed in the presence of thiamine, strains containing pREP41-sim3. The expression of sim3 gene was regulated by the nmt41 promoter in these double mutants, and the addition of thiamine partially and completely repressed sim3 gene expression in pREP41(nmt41) and pREP81(nmt81), respectively. Without thiamine, an asf1–33 sim3 strain that harbored pREP41-sim3 or pREP81-sim3 grew well at 34 °C. However, the addition of thiamine to the same cells resulted in a growth defect (Fig. 3B). The asf1–33 sim3 strain harboring pREP81-sim3 exhibited a more severe phenotype than the strain harboring pREP41-sim3, which indicated that even in the presence of thiamine, strains containing pREP41-sim3 display residual levels of sim3. Growth defect was observed in the asf1–33 sim3 strain harboring pREP81-sim3 at 34 °C when thiamine was added. Together, these results indicate that sim3 is required for the growth of the asf1–33 mutant.

3.4. Deletion of sim3 results in TBZ and HU sensitivity

We next examined the drug sensitivity of Asim3 cells. We found that Asim3 cells displayed greater sensitivity to a spindle poison, TBZ (thiabendazole), at all temperatures, and to a DNA replication inhibitor, HU (hydroxyurea), at higher temperatures than wild-type cells (Fig. 4). Because Sim3 is a centromere chromatin assembly factor and sim3 mutant cells show defects in chromosome segregation [1], it was a reasonable prediction that Asim3 cells might be sensitive to TBZ. The HU sensitivity of Asim3 cells suggested that there might be defects in S phase progression. However, FACS analysis did not reveal a delay in S phase progression in Asim3 cells (data not shown). The growth defect in Asim3 cells appeared to be more pronounced at lower temperatures (26 °C) than at higher temperatures (30 °C or higher). Therefore, the HU sensitivity of Asim3 cells at higher temperatures might not cause severe defects. In contrast to TBZ and HU, Asim3 cells did not show any sensitivity to a DNA damaging agent, MMS (methyl methanesulfonate). This is in contrast with our previous results showing that the asf1–33 mutant displayed sensitivity to MMS, but not to HU [11].

4. Discussion

Asf1 is a commonly found histone chaperone that predominantly interacts with H3/H4 in many eukaryotes [1,4]. Our analysis of asf1 mutants in S. pombe revealed that asf1 is essential for growth, and that its temperature sensitive mutation resulted in the accumulation of DNA damage and DNA breaks that induced DNA damage checkpoints at restricted temperatures [11]. A gene that encodes a NASP type protein, sim3, was isolated as a high copy suppressor of the temperature sensitive asf1–33 mutant in S. pombe. Sim3 was originally identified as a factor that interacts with CENP-AACnp1 [17], but our analysis suggested that Sim3 plays a role as a H3/H4 chaperone. It is interesting to note that the amino acid sequence and protein structure of Asf1 and Sim3 bear no similarity [17], despite their similar functions.

We showed that the overexpression of sim3 suppressed the defect in bulk chromatin structure (Fig. 1A) and reduced the accumulation of DNA damage in the asf1–33 mutant (Fig. 1C). In addition, the silencing defect at the outer centromeric repeats of the asf1–30 and asf1–33 mutants was also suppressed by overexpressing sim3 (Figs. 1 and 2B). Therefore, Sim3 shares some common roles with Asf1. Since it is reported that Sim3 binds to histone H3 in addition to CENP-AACnp1 [17], our results support the idea that Sim3 can function as a H3/H4 histone chaperone. In addition, we failed to construct an asf1–33 sim3 double mutant; in other words, asf1–33 and sim3 is a synthetically lethal combination. We also found that the sim3 mutant affects silencing at the centromeric region [17], but grows normally at 30 °C. When it was combined with the asf1–33 mutant, sim3 was required for viability, which suggests that sim3 shares an important role with asf1. Since our previous analysis indicated that mutated Asf1–33 proteins were unable to bind histone H3, the ability of the Sim3 protein to bind histone H3 might be required for the survival of the asf1–33 mutant.

In addition to its histone H3 chaperone functions, Asf1 also plays a role in maintaining heterochromatin structure at the centromere. Heterochromatin structure was impaired in the asf1 mutants (either asf1–30 or asf1–33), but was then suppressed by Sim3 overexpression. The role of Sim3 was reported to be restricted to the center of the centromere region, but it seems that Sim3 may potentially act as a silencing maintenance factor at the outer centromeric repeats as well. Sim3 can substitute for Asf1 by incorporating (or removing) histone proteins onto chromatin. We found that the deletion of sim3 resulted in the sensitivity to a spindle poison, TBZ, which is consistent with previous results by Pidoux et al. (Pidoux, Richardson, & Allshire, 2003). In addition, Asim3 cells displayed sensitivity to HU at 30 °C or higher. However, cell cycle progression during S phase was not delayed in Asim3 cells. Therefore, the effect of HU on sim3 cells was limited. As the deletion of sim3 conferred sensitivity to HU and asf1–33 conferred sensitivity to MMS, Sim3 likely plays a greater role in replication, while Asf1

![Fig. 4. Deletion of sim3 caused partial sensitivity to DNA replication inhibitor, HU. Logarithmically growing KT146 (leu1–32 ura4-D18 sim3::kanMX6) cells were diluted 10-fold from 3 x 10^6 cells with sterilized water, and spotted onto YES (No drug, 18 μg/ml TBZ 10 mM HU, 5 mM CPT, 0.0075% MMS) plates. Plates were incubated at 18, 26, 30, and 36 °C for 3 days.](image)
may be more crucial in response to DNA damage. Different roles of Sim3 and Asf1 as a chaperone are envisaged from these drug sensitivity tests.

Scm3 (and its homolog, HJURP) has been shown to be involved in the incorporation of CENP-A to the center region of centromeres in several species, such as *S. pombe* [20], *S. cerevisiae* [21] and humans [6]. Scm3 localizes to the center region of centromeres and incorporates CENP-A into centromeres. Although *sim3* overexpression suppressed the temperature sensitivity of the *asf1–33* mutant, *scm3* overexpression did not (data not shown). Likewise, overexpression of *asf1* did not suppress the temperature sensitivity of the *scm3* mutant (data not shown). Unlike Sim3, which is localized throughout the nucleus (Dunleavy et al., 2007), Scm3 localizes to and functions at only the centromere region. This specific localization of Scm3 may be one reason why we were unable to detect a genetic interaction between *scm3* and *asf1*.

In conclusion, our analysis of *sim3* and *asf1* indicates that Sim3 and Asf1 share some common roles as histone chaperones. Although Asf1 functions as the predominant histone chaperone in fission yeast, we suggest that Sim3 may also play a role as histone chaperone.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.10.020.

**References**


