Title:

How does Hsp90 function in RNAi-dependent heterochromatin assembly?

Authors:

Hiroaki Kato[1], Kosuke Okazaki[1¶] and Takeshi Urano[1]

Institutional Addresses:

1: Department of Biochemistry, Shimane University School of Medicine, Izumo, Shimane 693-8501, Japan

¶: Current address: KNC Laboratories Co. Ltd. Kobe, Hyogo 651-2271, Japan

Email Addresses:

Hiroaki Kato: hkato@med.shimane-u.ac.jp

Kosuke Okazaki: okazakimed@gmail.com

Takeshi Urano: turano@med.shimane-u.ac.jp

Correspondence:

Hiroaki Kato

Department of Biochemistry, Shimane University School of Medicine

89-1 Enya-cho, Izumo 693-8501, Japan

Tel: +81-853-20-2126; Fax: +81-853-20-2125

Email: hkato@med.shimane-u.ac.jp

ORCID: 0000-0001-9591-1875

Abstract:

Heat-shock protein 90 (Hsp90) was recently identified as a silencing factor required for RNA interference (RNAi)-dependent heterochromatin assembly in the fission yeast *Schizosaccharomyces pombe*. As Hsp90 is known to contribute to the formation of small RNA-containing effector complexes, it would be expected that Hsp90 is also involved in the RNAi pathway in fission yeast. However, upon investigation, we found it very difficult to determine how Hsp90 modulates RNAi-dependent heterochromatin assembly in the cell. A lack of detectable small interfering RNAs in *hsp90* mutant cells prevented us from

examining the role of Hsp90 in the siRNA loading in the cell. In addition, deletion of genes encoding co-chaperones for Hsp90 appears not to affect RNAi-dependent pericentromeric silencing. One possible approach for elucidating the role of Hsp90 in RNAi-dependent heterochromatin assembly is the use of forward genetic screens to identify novel factors linking Hsp90 with other known RNAi factors. Here, we discuss the benefits of conducting further screenings and present some technical hints to help identify new factors.

Keywords:
Hsp90, RNAi-dependent heterochromatin assembly, fission yeast, forward genetics

Introduction:

Heterochromatin, a transcriptionally inert chromatin structure, was considered to be so tightly packed as to prevent transcription. However, paradoxically, more than a decade of research into fission yeast heterochromatin has revealed that transcription itself plays an important role in heterochromatin assembly, particularly in RNA interference (RNAi)-dependent heterochromatin regions (Allshire and Ekwall 2015, Alper, et al. 2012, Holoch and Moazed 2015, Martienssen and Moazed 2015, Zukowski and Johnson 2018) (**Fig. 1**). The successive identification of two subunits of RNA polymerase II as silencing factors via two independent forward genetic screening studies (Djupedal, et al. 2005, Kato, et al. 2005) paved the way for the current understanding of cotranscriptional assembly of heterochromatin in which RNAi processes the nascent long non-coding transcripts for targeting chromatin modifiers in *cis*. As an image of a self-enforcing loop for RNAi-dependent heterochromatin assembly was developed early in these studies, integrating novel factors into that image could enhance our understanding of the underlying mechanism (Motamedi, et al. 2004, Sugiyama, et al. 2005).

The loading of double-stranded small RNAs onto Argonaute protein and release of the passenger strand are key steps in the formation of functional small RNA-containing effector complexes that are central to the function of RNAi (Iki, et al. 2010, Miyoshi, et al. 2010, Yoda, et al. 2010). Previous studies

established that heat-shock protein 90 (Hsp90) is essential for the formation of effector complexes in every species examined (Iki, et al. 2010, Iwasaki, et al. 2010, Miyoshi, et al. 2010, Woehrer, et al. 2015), thus suggesting that Hsp90 is also involved in the RNAi pathway in fission yeast, despite the lack of confirmatory evidence. In a recent study published in *Epigenetics & Chromatin*, we demonstrated the requirement for the heat-shock chaperones Hsp90 and Mas5, a nucleocytoplasmic Hsp40 family protein, in the assembly of RNAi-dependent heterochromatin in the fission yeast *Schizosaccharomyces pombe* (Okazaki, et al. 2018). The identification of a missense (R33C) mutant allele of Hsp90 (*hsp90-A4*) in a forward genetic screening was surprising, as we intended to identify transcription-related factors in that screening.

Colony color matters:

As previously reported (Kato, et al. 2013, Okazaki, et al. 2018), we carefully assessed the color of colonies of silencing-defective mutants harboring the *ade6*$^{+}$ marker gene in the pericentromere (*otr1R(SphI)::ade6*$^{+}$) and a deletion allele of *ade6* in the endogenous locus (*ade6*Δ*::kanMX* or *ade6-DN/N*) (Ekwall, et al. 1997). In these genetic backgrounds, wild-type strains form red colonies, as red pigment is produced as a result of marker gene silencing. Mutations in canonical heterochromatin factors (e.g., the histone methyltransferase Clr4) lead to derepression of *ade6*$^{+}$, which in turn leads to the formation of dark pink colonies (**Fig. 2A**). In contrast, cells with mutations in a subunit of RNA polymerase II (*rpb2-m203*), in subunits of mediator complex (*med8-K9* and *med31-H1*), or in the RNA polymerase II–associated histone chaperone Spt6 (*spt6-K20*) form bright pink colonies (Kato, et al. 2005, Kato, et al. 2013, Oya, et al. 2013). We identified a missense mutation in TATA box-binding protein-associated factor 6 (*taf6-85*), suggesting that Taf6 is also involved in pericentromeric silencing (H. Kato, unpublished results). All of the abovementioned factors are related to RNA polymerase II–driven transcription, and associated mutants were selected based on the formation of bright pink colonies. In this sense, identification of Hsp90 in the same line (as a bright pink colony-forming mutant) was interesting.

Although we identified Hsp90 as a novel silencing factor, how this

chaperone contributes to RNAi-dependent heterochromatin assembly remains unclear. We demonstrated that the *hsp90-A4* mutation causes a decrease in the level of Argonaute protein Ago1 and weakening of the intercellular interaction between Ago1 and Arb1, a subunit of the non-chromatin–associated Argonaute complex ARC (Buker, et al. 2007, Okazaki, et al. 2018). However, as the small interfering RNA (siRNA) corresponding to the pericentromeric repeats was undetectable in *hsp90-A4* cells, we could not determine its intracellular role in the loading and passenger-strand removal of siRNA. In addition, we did not detect any physical interaction between Hsp90 and known RNAi factors (Okazaki, et al. 2018), suggesting that supposed interactions, if they occur at all, are transient. We hypothesized that co-chaperones of Hsp90 might be involved (Ishida, et al. 2013, Martinez, et al. 2013, Olivieri, et al. 2012, Pare, et al. 2013, Preall, et al. 2012, Xiol, et al. 2012); however, deletion of the genes encoding three potential co-chaperones (Sti1, Aha1, and Wis2) did not lead to pink colony formation (**Fig. 2B**). Therefore, details regarding the role of Hsp90 in RNAi-dependent heterochromatin assembly remain elusive.

One potential approach for finding clues that would help elucidate the role of Hsp90 in RNAi-dependent heterochromatin assembly is the use of forward genetic screens to identify novel factors linking Hsp90 with known heterochromatin assembly factors. Technically, the mating type of the wild-type strain being mutagenized should be h^-, because the silencing derepression phenotype is epigenetically more stable in h^- than h^{+N} in many cases (H. Kato, unpublished results). Although the reason for the genetic linkage between epigenotype stability and the mating-type locus remains unknown, using h^- strains has been empirically successful. Genetic alterations caused by mutagens (e.g., ultraviolet light and ethyl methanesulfonate) can be easily identified by means of whole-genome sequencing. Once one has raw next-generation sequencing data, a web-based tool known as Mudi can be used to perform almost all bioinformatic analyses to identify relatively small candidate mutations (i.e., SNPs and indels) (Iida, et al. 2014). When the viability of cells at the mutagenesis step is 50%, the resultant mutant genome will contain only about 10 mutations exhibiting high mapping quality. Most mutations would be genetically diluted after three rounds of backcrossing if they do not contribute to

the phenotype.

The most important thing may be to carefully assess the color of mutant colonies. In our experience, selecting bright pink mutants led to the identification of novel factors that can be distinguished from canonical factors, mutants of which form dark pink colonies. If the color of a given mutant strain's colonies appears white at first glance but then is revealed to be a shade of very faint pink, it is worthy of further examination. However, mutants that form completely white colonies (as white as strains that express *ade6*$^{+}$ from its endogenous locus) should be excluded, as they cannot always be explained according to Mendel's law. We once thoroughly investigated such a white mutant, in which gross rearrangement had occurred between chromosomes 1 and 3 and that was confirmed by pulsed-field gel electrophoresis and genetic linkage analyses (H. Kato, unpublished results). In addition, phenotype-associated mutations are not always SNPs or indels (Kato, et al. 2013). Thus, one should be prepared for some unexpected difficulties when conducting forward genetic screens. Nevertheless, a merit of the forward genetic approach is that it covers genes that are essential for cell growth. Indeed, the genes encoding Rpb2, Med8, Taf6, and Hsp90 are essential, and they are usually not covered by reverse genetic approaches.

Concluding remarks:

As RNAi-dependent heterochromatin assembly appears to be a cotranscriptional event, dozens of factors (including many involved in canonical RNA polymerase II–driven transcription and cotranscriptional events) may play important roles in the process. Thus, it is possible that Hsp90 and other molecular chaperones contribute to the modulation of still-unknown factors that regulate the intracellular heterochromatin assembly process. Note that other aspects of Hsp90-related biological processes (Gopinath and Leu 2017) and epigenetic regulation (D'Urso and Brickner 2017, Xue and Acar 2018) should also be taken into account. Here, we discussed some technical hints for researchers desiring to conduct further forward genetic screens to identify novel factors involved in the process. We hope these tips will help the research community to further our understanding of how heterochromatin is assembled in fission yeast, which is a good model

organism for investigating eukaryotic epigenetic regulation.

Notes:

Acknowledgements:

HK was supported by JSPS KAKENHI grant numbers JP26116513 and JP18K05556. KO was supported by a Grant-in-Aid for JSPS Research Fellows (no. JP13J07740).

Conflict of Interest:

The authors declare that they have no conflict of interest.

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Figure 1. How does Hsp90 function in RNAi-dependent heterochromatin assembly?

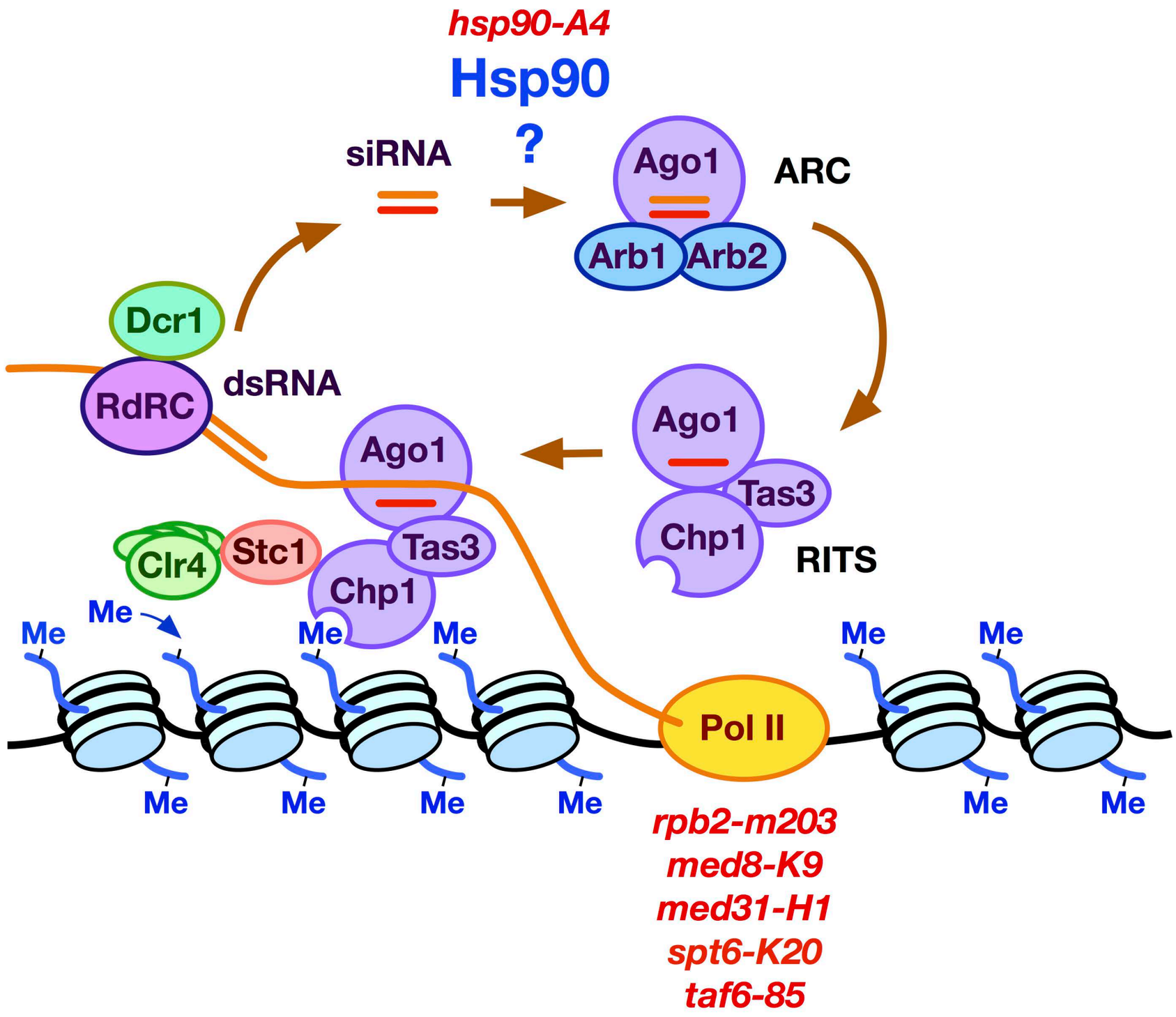
In fission yeast, assembly of RNAi-dependent heterochromatin is considered to occur cotranscriptionally. Long non-coding RNAs transcribed by RNA polymerase II are processed to generate double-stranded siRNAs, which are loaded onto the Argonaute protein Ago1 in the ARC complex. The siRNA-containing complex releases the passenger strand of the siRNA and changes its subunit composition to form the chromatin-associated effector complex RITS, which recruits histone methyltransferase Clr4, an enzyme that methylates histone H3 at Lys9. Biochemical studies in other species suggest that Hsp90 plays an important role in the loading of siRNA in the effector complex. However, as siRNA cannot be detected in *hsp90-A4* mutant cells, we cannot examine the expected cellular function of Hsp90. Thus, the role of Hsp90 in RNAi-dependent heterochromatin assembly remains unclear. Most mutants that form bright pink colonies (colored in red and italicized) isolated in our

forward genetic screens are related to RNA polymerase II–driven transcription. This suggests that Hsp90 also is involved in the cotranscriptional regulation of heterochromatin assembly. This image is a modified version of an image in an article by Okazaki, et al. (2018) (https://epigeneticsandchromatin.biomedcentral.com/articles/10.1186/s13072-018-0199-8) licensed under a Creative Commons Attribution 4.0 international license (https://creativecommons.org/licenses/by/4.0/legalcode).

Figure 2. Color of heterochromatin mutant colonies

(A) Non-canonical heterochromatin mutants that form bright pink colonies. (B) Null mutations in co-chaperones Sti1, Aha1, and Wis2 and in type II Hsp40 Psi1 did not form pink colonies. Cells were serially diluted, spotted on normal YES plates and YES plates containing limited amounts of adenine (low adenine) and incubated at 30°C for 3 days. All strains were h^- and harbored an *ade6*$^+$ marker gene in the pericentromere (*otr1R(SphI)::ade6*$^+$) and a deletion allele of *ade6* in the endogenous locus. Note that the color of the *hsp90-A4* and *mas5*Δ mutants was faint pink (not completely white), which is difficult to discern from the photographs. Some of the photographs in panel B are from an article by Okazaki, et al. (2018) (https://epigeneticsandchromatin.biomedcentral.com/articles/10.1186/s13072-018-0199-8) licensed under a Creative Commons Attribution 4.0 international license (https://creativecommons.org/licenses/by/4.0/legalcode).

Figure 1


hsp90-A4
Hsp90
?
siRNA
Ago1
ARC
Arb1
Arb2
Dcr1
RdRC
dsRNA
Ago1
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Chp1
RITS
Clr4
Stc1
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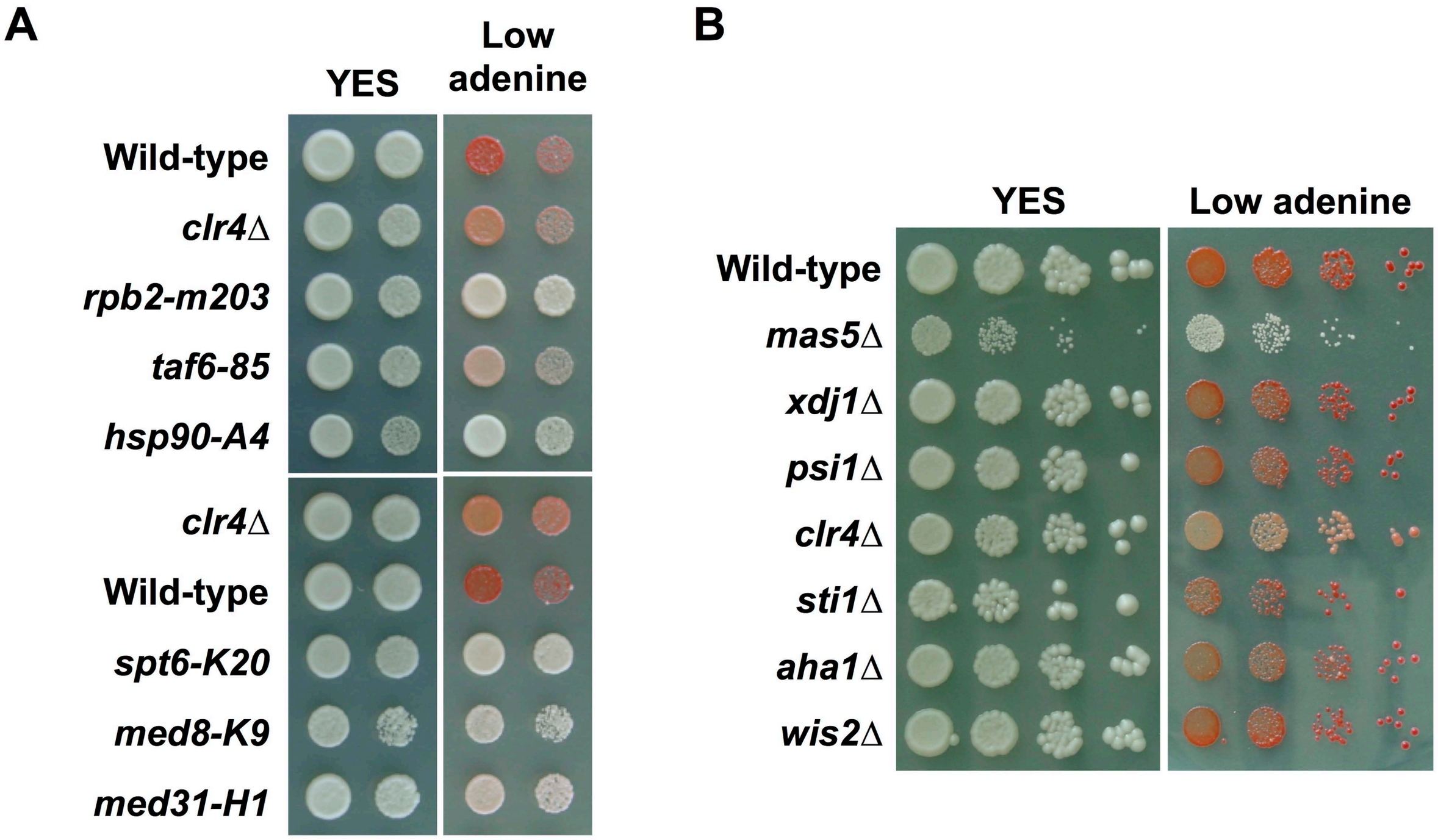
med31-H1
spt6-K20
taf6-85

Figure 2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Low adenine
Wild-type
clr4Δ
rpb2-m203
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hsp90-A4
clr4Δ
Wild-type
spt6-K20
med8-K9
med31-H1
B
YES
Low adenine
Wild-type
mas5Δ
xdj1Δ
psi1Δ
clr4Δ
sti1Δ
aha1Δ
wis2Δ