Methods 57 (2012) 227-233

Contents lists available at SciVerse ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Conditional inactivation of replication proteins in fission yeast using hormone-binding domains

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ARTICLE INFO

Article history: Available online 5 April 2012

Communicated by Marcel Méchali

Keywords: β-estradiol Conditional allele GINS Hsp90 Hormone-binding domain Schizosaccharomyces pombe

ABSTRACT

The fission yeast *Schizosaccharomyces pombe* is a useful model for analysing DNA replication as genetic methods to allow conditional inactivation of relevant proteins can provide important information about S-phase execution. A number of strategies are available to allow regulation of protein level or activity but there are disadvantages specific to each method and this may have limitations for particular proteins or experiments. We have investigated the utility of the inducible hormone-binding domain (HBD) system, which has been described in other organisms but little used in fission yeast, for the creation of conditional-lethal replication mutants. In this method, proteins are tagged with HBD and can be regulated with β -estradiol. In this article, we describe the application of this method in fission yeast, specifically with regard to analysis of the function of GINS, an essential component of the eukaryotic replicative helicase, the CMG complex.

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1. Introduction

Isolation of conditional alleles is a standard method for investigating protein function in yeasts. In fission yeast, a number of strategies are available for conditionally regulating the activity of a target protein. The most common method is to generate temperature-sensitive mutants. However, not every gene can be mutated to confer temperature-sensitivity on its product and it is rarely possible to predict which residues can be changed to inactivate the protein at high or low temperature. Another method which has been successfully applied to some proteins in fission yeast is to inactivate the target protein by fusing it with a temperaturesensitive degron [1-4]. Recognition of the degron at higher temperatures by a ubiquitin ligase results in targeted degradation of the fusion protein via the ubiquitin-proteasome pathway. An auxin-inducible degron (AID) system has also recently been developed but as with temperature-sensitive degrons, not all replication factors modified with the AID are effectively depleted [5]. Another frequently used approach is to regulate the level of the target protein by replacing the native promoter with a regulatable one. The most commonly used promoter for this purpose is nmt1 (no message in thiamine), which is repressed in the presence of thiamine [6]. However, this inactivation system is less useful for stable proteins and there is some promoter leakiness in the presence of thiamine. Also, the reactivation of expression after promoter-repression is slow. Recently, use of the uracil-inducible promoter *urg1* has been reported to allow rapid induction and repression of transcription, potential advantages over *nmt1* [7].

The alternative method presented here is based on fusing the target protein with a β -estradiol hormone-binding domain (HBD) that can allow conditional inactivation. Previous work in other organisms has described that proteins tagged with HBD can be regulated with β -estradiol [8–10]. In the absence of β -estradiol, the un-liganded HBD is in a complex with heat-shock protein 90 (Hsp90), which can inactivate the tagged protein by steric hindrance. When β -estradiol binds to HBD, it displaces Hsp90 and thus can reactivate the tagged protein (Fig. 1A) [11].

Here, we describe application of the HBD system in *Schizosaccharomyces pombe*, and its use in the analysis of essential replication proteins. This method has been applied to analysis of the function of the GINS complex (see also [12,13]), where inactivation of the fusion protein is rapid and reversible. We also discuss limitations of this method and possible strategies to improve the system.

2. Materials and methods

2.1. Growth conditions

General fission yeast genetic methods, culture conditions and standard growth media were as described before [14]. β -estradiol





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Fig. 1. β -estradiol-dependent conditional alleles of GINS subunits: (A) Schematic representation of the regulation of an HBD-fusion protein (protein X). The HBD with the hormone-reversible Hsp90 complex can function to post-translationally regulate protein X's function. Inactivation is mediated by steric hindrance on Hsp90 binding. (B) Serial dilutions of *S. pombe psf1-HBD* (P1519) or *psf2-HBD* (P1520) strains were spotted onto YES medium containing different concentrations of β -estradiol as indicated. (C) Morphology of *psf1-HBD* cells grown in YE3S containing different concentrations of β -estradiol. Cells were stained with DAPI and viewed under phase contrast using a fluorescence microscope. Bar = 10 µm (D) Growth rates of *psf1-HBD* and *psf2-HBD* strains. *S. pombe* cultures were monitored over a period of 8 h. The growth rate of *psf1-HBD* or *psf2-HBD* cells was compromised after withdrawal of β -estradiol at 4 h.

(Sigma E2758) was made as a 10 mM stock in ethanol and stored at -20 °C. The hormone was used at 125–200 nM in yeast extract (YES) or Edinburgh minimal medium (EMM) for growth of HBD mutants. Nitrogen starvation was carried out using EMM lacking NH₄Cl (EMM–N).

into the *psf1*⁺ locus.

2.2. Construction of HBD mutants

S. pombe strains were generated by one-step PCR-mediated gene targeting [15] or were transformed with a linearised DNA fragment, constructed from plasmid cloning, to give an epitope-tagged strain under the control of the endogenous promoter. Strains used were: P138 *ade6-M210 leu1-32 ura4-D18* h⁻; P1519 *psf1-HBD-kanMX6* h⁻; P1520 *psf2-HBD-kanMX6* h⁺; P1597 *psf1-HBD-kanMX6 ade6-M210 leu1-32 ura4-D18* h⁻; P1697 *psf2-HBD-kanMX6 ade6-M210 leu1-32 ura4-D18* h⁻; P1907 *psf2-HBD-YFP-kanMX6*; P1968 *psf2-HBD-kanMX6 psf3-YFP-ura4⁺*; P2001 *mcm10-YFP-HBD-ura4⁺*; P2003 *pol1-HBD-YFP-kanMX6*; *Sp536 mcb1-HBD-natMX6* h⁻.

2.2.1. Psf1-HBD and Psf2-HBD

A pFA6a–HBD–kanMX6 plasmid for C-terminal HBD tagging was constructed by replacing the GFP region in pFA6a–GFP(S65T)–kanMX6 with the estrogen hormone-binding domain (HBD) [12]. The *psf1*⁺ gene and *psf2*⁺ genes were tagged with the HBD by one-step PCR-mediated gene targeting using the plasmid pFA6a–HBD–kanMX6 as a template [12,13]. Yeast transformants were selected for using YES plates containing 100 µg/ml G418 plus β -estradiol.

2.2.2. Psf1-HBD-YFP and Psf2-HBD-YFP

Construction of $psf2^+$ -HBD-YFP has been previously described [13]. To construct $psf1^+$ -HBD-YFP, a $psf1^+$ fragment was amplified using oligos 5'-Apal-psf1C (5'-tttgggcccgtttggggaatcgatcaataaattaattc-3') and 3'-Xhol-psf1C (5'-tttccgagtaacttagccaaaaaaccttggg caatgag-3') and the resulting fragment was subcloned into Apal

2.2.3. Pol1–HBD–YFP

To construct $pol1^+$ -HBD-YFP, a pol1 fragment was cut from pSMRY2 + Pol1 [13] and the resulting fragment was subcloned into *Apal* and *Xhol*-cut pSMRY2-HBD + Psf2, to give pSMRY2-HBD + Pol1. pSMRY2-HBD + Pol1 was cleaved with *Aarl* to direct integration at the $pol1^+$ locus.

and XhoI-cut pSMRY2-HBD + Psf2, to give pSMRY2-HBD + Psf1.

pSMRY2-HBD + Psf1 was cleaved with EcoRI to direct integration

2.2.4. Cdt1-HBD

Cdt1 was C-terminally tagged with HBD by amplifying a $cdt1^+$ fragment with oligos 5'-Sall-cdt1C (5'-tttgtcgacgctattaccccatgttt-tactattc-3') and 3'-Smal-cdt1C (5'-tttgtcgggtagaattgaagaatagtgatgcta-3'), and inserting the fragment into Sall and Smal-cut pFA6a-HBD-kanMX6, to give pFA6a-HBD-kanMX6 + Cdt1. This resulting plasmid was linearised with SnaBl to tag the endogenous $cdt1^+$ gene with HBD.

2.2.5. Mcm10-YFP-HBD

To construct $mcm10^+$ -YFP-HBD, an mcm10 fragment was cut from pSMUC2 + mcm10 [17] and the fragment was inserted into *Apal* and *Smal*-cut pSMUY2-HBD, to give pSMUY2-HBD + mcm10. The resulting plasmid was linearised with *Spel* to tag the endogenous $mcm10^+$ gene with YFP-HBD.

2.2.6. Mcb1-HBD

The $mcb1^+$ gene was tagged with the HBD by one-step PCRmediated gene targeting using the plasmid pFA6a–HBD–natMX6 as a template [12,13]. Transformants were selected for using YES plates containing 200 µg/ml ClonNAT plus β-estradiol.



Fig. 2. Inactivation of Psf1–HBD and Psf2–HBD causes defects in DNA replication: (A) A *psf1–HBD::kanMX6* strain (P1519) was grown in EMM plus 125 nM β -estradiol to log phase then transferred to EMM–N (+ β -estradiol) for 16 h at 25 °C. Cells were released from the block by resuspending in EMM + N in the presence (+est) or absence (-est) of β -estradiol. The data in the "-est + est" panel relate to cells that were initially released into EMM + N in the absence of estradiol, and 125 nM β -estradiol was added at 4 h. (B) A similar experiment was performed with a *psf2–HBD* strain (P1520). (C) Relative cell viability of *psf1–HBD* strain was examined during the arrest and re-addition of β -estradiol. (D) Cells from the experiments shown in (A, B) were fixed with 70% ethanol, stained with DAPI and examined by microscopy.

3. Analysis of HBD phenotypes

3.1. HBD-dependent regulation of GINS subunits in fission yeast

To develop the HBD regulatory system in the fission yeast, a C-terminal tagging vector pFA6a–HBD–kanMX6 was constructed [12]. We explored whether the HBD could confer conditionality on Psf1 and Psf2, two of the four subunits of the fission yeast GINS complex. GINS is an essential component of the CMG (Cdc45–MCM–GINS) complex, the eukaryotic replicative helicase complex [18–20]. The *psf1–HBD* and *psf2–HBD* mutant cells were analysed by spot assays on YE3S medium containing different concentrations of β -estradiol, and cell morphology was examined by microscopy at the indicated concentrations. Plate assays show that *psf1–HBD* and

psf2–HBD strains grew similarly to wild-type cells on plates containing 100 nM–100 μ M β -estradiol, but grew poorly in the absence of the hormone (Fig. 1B) and division rates of *psf1–HBD* or *psf2–HBD* cells slow down after washing out 125 nM β -estradiol (Fig. 1D). Mutant cells also show elongated morphology at a limiting level of β estradiol (Fig. 1C) consistent with incomplete DNA replication (see the following sections). Based on these observations, β -estradiol was used at 125–200 nM for subsequent experiments.

3.2. Inactivation of GINS by the HBD system causes cell-cycle arrest in S-phase

Earlier studies have shown that the fission yeast temperaturesensitive *psf2^{ts}* and *psf3^{ts}* mutants are defective in DNA replication



Fig. 3. Nuclear localisation of Psf2 is lost in the absence of β -estradiol: (A) *psf2–HBD–YFP* (P1907) and wild-type cells (P138) were serially (10-fold) diluted and spotted onto YE3S plates containing 125 nM or no β -estradiol and incubated at indicated temperatures for 2–3 days. (B) A *psf2–HBD–YFP* (P1907) strain was arrested in G1 and released from the block in the presence or absence of β -estradiol. At time points indicated, samples were analysed for DNA content. (C) *psf2–HBD–YFP* cells (P1907) show nuclear localisation of Psf2 when they were grown in YE3S containing β -estradiol, but after 1 h growth at 36 °C in medium lacking hormone, nuclear localisation of the Psf2 fusion protein is lost. (D) Western blotting analysis of Psf2–HBD–YFP levels from cells grown in medium containing (+est) or lacking (–est) β -estradiol. (E) When *psf2–HBD–YFP* cells were arrested in S-phase using 12 mM HU, withdrawal of β -estradiol caused nuclear localisation of Psf2 to be lost. Bar = 10 µm.

[21,22]. To determine whether a similar phenotype is observed with HBD inactivation of GINS, psf1–HBD and psf2–HBD strains were grown in EMM medium containing 125 nM β -estradiol until in log phase. Log phase cells were transferred to EMM–N medium for 16 h, followed by washing twice in sterile water and released from the G1 block in the presence or absence of β -estradiol. Samples were taken over a 5 h time course and the DNA content of cells was monitored by flow cytometry. Control cells with β -estradiol (fusion protein "active") enter S-phase around 3 h after re-feeding and DNA replication is largely completed by 5 h (Fig. 2A and B, +est). However, the cells released from the starvation block in the absence of β -estradiol (fusion protein "inactive") show a block to DNA replication (Fig. 2A and B, –est), consistent with a role of the GINS complex in DNA replication. During the arrest, cells do not enter an untimely mitosis (Fig. 2D), suggesting that the S phase checkpoint is activated. To explore the reversibility of the arrest caused by inactivation of GINS subunits, cells were initially released from a G1 arrest in the absence of β -estradiol. As shown



Fig. 4. Effects of Psf1/Psf2 HBD inactivation on nuclear localisation of GINS complex: (A) A *psf2–HBD psf3–YFP* strain (P1968) was arrested in G1 by nitrogen starvation and released into the cell cycle in the presence or absence of β -estradiol. Samples of cells were taken at indicated time points for direct fixation and examination by fluorescence microscopy. (B) Cellular localisation of Psf2–YFP before and after Psf1 inactivation. A *psf1–HBD* strain expressing Psf2–YFP (P1597) was grown in β -estradiol-containing medium (+est) then transferring to medium lacking β -estradiol for 5 h (–est). Cells were fixed directly and examined by fluorescence microscopy. Bar = 10 μ m.

in Fig. 2A or B (-est, + est), the cells remained arrested with a 1C DNA content during the time of inactivation. After the 4 h incubation in the absence of hormone, β -estradiol was added to the culture. DNA replication was resumed on re-addition of β -estradiol within 1 h, and replication was largely completely in 2 h (6 h time point), suggesting that the estradiol–HBD regulation is rapidly reversible (Fig. 2A and B, –est, +est). The arrested and released cells retained high viability as shown in Fig. 2C.

3.3. Loss of nuclear GINS–HBD–YFP in the absence of β -estradiol

To investigate the mechanism of GINS subunit regulation by HBD, their cellular localisation was examined by tagging HBDtagged proteins with YFP. We were unable to construct a haploid psf1-HBD-YFP strain, possibly as the C-terminal region of Psf1 is important in mediating protein-protein interactions [23], but a haploid *psf2–HBD–YFP* strain is viable in the presence of β -estradiol at 32 °C and inviable in its absence. This effect is more dramatic when cells were grown at 37 °C (Fig. 3A) suggesting that Hsp90 binding is more inhibitory at higher temperatures. The psf2-HBD-YFP strain showed arrest of DNA replication in the absence of B-estradiol (Fig. 3B) as with the *psf1-HBD* or *psf2-HBD* mutant cells. In the presence of β-estradiol, Psf2-HBD-YFP is constitutively nuclear during the vegetative cell cycle (Fig. 3C, +est), as seen with Psf2-YFP [13,24], but after withdrawal of β-estradiol, nuclear localisation of the protein is lost within 1 h (Fig. 3C, -est). This occurs in the absence of any effect on protein levels (Fig. 3D) Similar results were obtained using the heterozygous *psf1–HBD–YFP/psf1*⁺ diploid strain (results not shown).

We also investigated whether the HBD system regulates nuclear localisation of Psf2–HBD–YFP when the GINS complex is first allowed to bind to chromatin. *psf2–HBD–YFP* cells were first arrested in HU for 2 h in the presence of β -estradiol; under these conditions GINS subunits are chromatin associated at replication forks. The cells were then incubated in HU for 2 h in the absence of the hormone at 36 °C and fixed for microscopic analysis. The fluorescence intensity of nuclear Psf2–HBD–YFP was greatly reduced in cells after withdrawal of β -estradiol, compared to that in the presence of hormone (Fig. 3E). This result suggests that chromatin-bound *Psf2–HBD–YFP* is lost from DNA in the absence of hormone.

3.4. Inactivation of GINS–HBD compromises nuclear localisation of the GINS complex

Genetic and biochemical studies in yeasts suggest that GINS subunits interact [22,25,26]. Earlier results showed that nuclear localisation of Psf1–HBD–YFP or Psf2–HBD–YFP is lost after with-drawal of β -estradiol, implying that the other subunits may not remain as a complex. To determine whether the sub-cellular localisation of Psf3 is affected by Psf2 inactivation, a *psf2–HBD* strain containing Psf3–YFP was isolated. In the presence of β -estradiol, Psf3 localised in the nucleus as expected (Fig. 4A, + est). However, inactivation of Psf2–HBD resulted in loss of Psf3–YFP nuclear localisation (Fig. 4B, - est), suggesting that that the chromatin binding of GINS is lost and the entire complex is delocalised under these conditions. A similar result was seen in *psf1–HBD psf2–YFP* cells: inactivation of Psf1 also caused some delocalisation of Psf2–YFP in the absence of β -estradiol (Fig. 4B, - est).



Fig. 5. Characterisation of cdt1–HBD, pol1–HBD, mcb1-HBD and mcm10–HBD mutants: (A) Serial dilutions of a cdt1–HBD–kanMX6 (P1895) and wt (P1625) strains were plated onto minimal plates without β -estradiol and incubated at different temperatures as indicated. (B) Serial dilutions of mcb1–HBD–natMX6 (Sp536) and wt (P1625) strains were plated as in (A). (C) Cellular localisation of Pol1–HBD–YFP in the presence or absence of β -estradiol. pol1–HBD–YFP (P2003) cells were initially grown in EMM medium containing 125 nM β -estradiol at 32 °C, and then transferred to 36.5 °C with or without β -estradiol for 2 or 5 h as indicated. (D) Cellular localisation of Mcm10–YFP–HBD in the presence or absence of β -estradiol. Cells were treated as in B except a mcm10–YFP–HBD strain (P2001) was examined. Bar = 10 μ m.

3.5. Effect of HBD fusion on regulation of other replication proteins in fission yeast

The β -estradiol-regulated HBD provides a rapid and reversible system to regulate the function of GINS subunits in fission yeast. Therefore, it would be useful if the HBD regulation system could be broadly applied to other proteins that are involved in fission yeast cell cycle regulation. Four mutant strains with other HBD-tagged replication factors were constructed to examine their sensitivity or cellular localisation in response to β -estradiol. The factors examined were Cdt1, a key protein required for Mcm2–7 chromatin binding [27], the Mcm binding protein Mcb1 [28,29], Pol1, the catalytic subunit of DNA Pol α [30,31], and Mcm10 [17].

A strain expressing HBD-tagged Cdt1 is viable in the presence of β -estradiol. A spot assay showed that the *cdt1–HBD* strain was hardly sensitive to lack of β -estradiol (Fig. 5A) at 32 °C but showed more growth retardation in the absence of β -estradiol at 36 °C, as did the *psf2–HBD–YFP* strain. In contrast, no significant growth defect was observed for strains *mcb1–HBD*, *pol1–HBD–YFP* and *mcm10–YFP–HBD* in the absence of β -estradiol (Fig. 5B and data not shown). Since no growth defect was apparent for *pol1–HBD–YFP* and *mcm10–YFP–HBD* strains, we tested whether the nuclear localisation of Pol1 or Mcm10 can be regulated by the HBD system. Fluorescence microscopy showed that the nuclear localisation of Pol1–HBD–YFP is reduced after withdrawal of β -estradiol for 2 h at 36.5 °C (Fig. 5C), suggesting that the HBD-fusion protein is destabilized or delocalized under these conditions. The nuclear

localisation of Mcm10–YFP–HBD is not significantly affected by withdrawal of β -estradiol (Fig. 5D).

4. Discussion

We show here that in a limited survey of six replication proteins, the HBD system can be used to conditionally inactivate just two, the Psf1 and Pst2 subunits of the GINS complex. In the presence of β estradiol, Psf2–HBD–YFP and Psf1–HBD–YFP are constitutively nuclear during the vegetative cell cycle, but following withdrawal of β -estradiol, the proteins become delocalised over the cell in 1 h. These changes in cellular localisation occur in the absence of any effects on protein levels. Inactivation of Psf2–HBD results in a loss of nuclear localisation of Psf3–YFP, suggesting that the chromatin binding of GINS is impaired and the entire complex is delocalised. A similar result was observed in *psf1–HBD psf2–YFP* cells. The success of HBD inactivation for GINS subunits contrasts with the lack of inactivation of Cdt1, Mcb1, Mcm10 and Pol1, and may reflect the fact that interaction of GINS with other replication proteins in the replisome makes it especially sensitive to steric inhibition.

4.1. Effective inactivation of GINS conditional alleles by using HBD

It is shown here that GINS subunits Psf1 and Psf2 can be effectively regulated by fusion to the HBD. The HBD-tagged Psf2 subunit seems to arrest more tightly compared to the available temperature sensitive *psf2*^{ts} mutant [21]. However, in the *psf2–HBD* strain, there is still some DNA replication at the later time points. During the replication arrest caused by GINS inactivation, the mutant cells retain high viability suggesting that the GINS complex can be reversibly inactivated.

4.2. Cellular delocalisation of HBD-fusion proteins and limitations of HBD regulation

In the absence of β -estradiol, there is rapid loss of nuclear localisation of Psf2–HBD–YFP. Similarly, a lack of β -estradiol also causes loss of Psf1–HBD–YFP nuclear localisation in a heterozygous diploid strain that contains *psf1*⁺. These results suggest that efficient inactivation of GINS function by HBD may occur via regulation of its nuclear localisation. The relocalisation of GINS subunits in the absence of β -estradiol could occur as a result of an interaction with Hsp90/Sw01 since it has been reported that Hsp90/Sw01 is mainly cytoplasmic [32,33]. If either the GINS complex or Hsp90/Sw01 shuttle between the nucleus and the cytoplasm, in the absence of β -estradiol the formation of an Hsp90–GINS complex could result in a shift in localisation of GINS to the cytoplasm.

To see whether this relocalisation is a general feature of HBD regulation in *S. pombe*, another two replication factors, Pol1 and Mcm10, were tagged with HBD and their nuclear localisation was examined in the absence of β -estradiol. Pol1–HBD–YFP appears to be weakly delocalised from the nucleus in the absence of β -estradiol, although this strain does not depend on β -estradiol for viability. However, western blotting would be required to verify whether their expression levels are affected by the absence of β -estradiol. Given that Swo1/Hsp90 in fission yeast is predominantly localised in the cytoplasm [32], in future work it may be possible to improve the inactivation of nuclear proteins fused to HBD by over-expressing Swo1 in the nucleus.

5. Conclusions

The HBD system can be a useful tool to regulate the function of proteins in fission yeast. This system results in rapid and reversible inactivation of the target protein and has the advantage that a temperature shift is not involved. Analysis of GINS subunits suggests that HBD regulation may, at least in some cases, act through loss of nuclear localisation of HBD-fusion proteins, although further work is needed to establish whether this applies to other HBDinactivated proteins. The HBD system may be particularly suitable in situations where protein function can be blocked by inhibiting protein–protein interactions.

Acknowledgements

We are grateful for help and advice on use of the HBD system from Beata Grallert. This work was performed with financial assistance from Cancer Research UK (CC and SK) and the Scottish Universities Life Sciences Alliance (JS and SM).

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