Degradation of centromeric histone H3 variant Cse4 requires the Fpr3 peptidyl-prolyl cis-trans isomerase

Kentaro Ohkuni¹, Rashid Abdulle, and Katsumi Kitagawa²

Center for Childhood Cancer, The Research Institute, Nationwide Children’s Hospital
Columbus, OH 43205 USA.

¹Present address: Genetics Branch Center for Cancer Research, National Cancer Institute, National Institute of Health, Bethesda, MD 20889, USA

²Corresponding Author
Running Title: Fpr3 regulates Cse4 proteolysis

Key Words: Fpr3, Cse4, Psh1, isomerization, protein degradation

To whom correspondence should be addressed:
Katsumi Kitagawa, Ph.D.
Center for Childhood Cancer
The Research Institute at Nationwide Children’s Hospital
700 Children’s Drive
Columbus, OH 43205
Phone: 614-355-2632
Fax: 614-722-5895
Email: Katsumi.Kitagawa@nationwidechildrens.org
Abstract (around 50 words)

The centromeric histone H3 variant Cse4 in *S. cerevisiae* is polyubiquitylated and degraded in a proteasome-dependent manner. We report here that the proline isomerase Fpr3 regulates Cse4 proteolysis. Structural change in Cse4 by Fpr3 might be important for the interaction between Cse4 and the E3 ubiquitin ligase Psh1.
Accurate chromosome segregation during mitosis and meiosis is a critical event in the transfer of genetic information to daughter cells. Loss or gain of chromosome is associated with cancer development and genetic disease (Holland and Cleveland 2009; Yuen et al. 2005). The centromere-kinetochore complex is required for faithful segregation. We previously used an in vitro kinetochore assembly system and identified the FK506 binding protein Fpr3 as a novel protein that associates with CEN DNA (Ohkuni and Kitagawa 2011). Fpr3 was also isolated by affinity purification of the Dsn1-Flag tagged central kinetochore protein (Akiyoshi et al. 2010). These data strongly suggested that Fpr3 has a role in centromere or/and kinetochore function. In this study, we investigated the mitotic function of Fpr3.

FPR3 and FPR4 encode two related prolyl isomerases that share 46% identity (Arevalo-Rodriguez et al. 2004). Fpr4 regulates histone H3 lysine 36 methylation and gene activity by its proline isomerase activity (Nelson et al. 2006). To characterize the function of Fpr3 and Fpr4, we first generated null mutants of the nonessential FPR3 and FPR4 genes, respectively (Table S1) (Arevalo-Rodriguez et al. 2004; Dolinski et al. 1997). Single deletion strains (fpr3Δ and fpr4Δ) and double deletion strains (fpr3Δ fpr4Δ) did not show obvious growth phenotypes such as temperature or benomyl sensitivities (Figure S1). We also examined chromosome stability in fpr3Δ or fpr4Δ strains by a colony color assay (Figure 1A). Both fpr3Δ and fpr4Δ single deletion strains showed a moderate chromosome missegregation phenotype (chromosome fragment loss: 0.2% in fpr3Δ, 0.15% in fpr4Δ). Because Fpr3 is associated with centromeres (Akiyoshi et al. 2010).
2010; Ohkuni and Kitagawa 2011) and because Fpr3 and Fpr4 directly interact with histone H3 (Nelson et al. 2006), we tested a possibility that Fpr3 and/or Fpr4 regulate a centromeric histone H3 variant Cse4. Interestingly, we found that the endogenous protein level of Cse4 was increased in fpr3Δ, fpr4Δ, and fpr3Δ fpr4Δ cells (Figure 1B). This result suggests that Fpr3 and Fpr4 have a role in regulating the Cse4 protein level in vivo.

To examine whether Fpr3 and Fpr4 regulate Cse4 proteolysis, we performed a protein stability assay (Figure 2). Cse4 was transiently induced from a GAL1 promoter by the addition of galactose. Then, glucose was added to stop CSE4 transcription, and cells were collected over 80 minutes. The level of Cse4 was determined by quantitative western blotting. As expected, deletion of FPR3 or FPR4 moderately stabilized Cse4 protein levels in vivo (Figure 2A and 2B). The level of the stabilization in fpr3Δ cells was higher than that in fpr4Δ cells. It has been also previously reported that deletion of PSH1, which is an E3 ubiquitin ligase, moderately stabilized the Cse4 protein level (Hewawasam et al. 2010; Ranjitkar et al. 2010). The level of the stabilization in fpr3Δ cells was higher than that in psh1Δ cells (Figure 2C, 2D and S2). Moreover, double deletion mutant, fpr3Δ psh1Δ, showed the dramatically increased protein stability of Cse4 (Figure 2C and 2D). In all, these results strongly support the idea that Fpr3 and Fpr4 regulate the protein level of Cse4.

FPR3 encodes a peptidyl-prolyl cis-trans isomerase (PPIase) (Benton et al. 1994; Shan et al. 1994) that is involved in the meiotic recombination checkpoint pathway (Hochwagen et al. 2005; Macqueen and Roeder 2009). We next tested whether the peptidyl-prolyl cis-trans isomerase enzymatic activity is important for the Cse4 protein stability. We performed the Cse4 protein stability assay using two Fpr3 catalytic domain
point mutants (W363L and F402Y) that have lost PPIase activity (Hochwagen et al. 2005). The two PPIase dead mutations caused a stabilization of Cse4 protein level in vivo, suggesting that the Fpr3 proline isomerase activity is required for Cse4 protein stability (Figure 3A and 3B).

We next aimed to identify the target proline related to the Cse4 protein stability. There are five proline sites in Cse4, which are P53, P98, P100, P134 and P157. We generated yeast strains bearing each mutation (proline to valine) in Cse4 (Table S1) and analyzed the stability of Cse4. Only the P134V mutation has a clear effect on Cse4 stabilization (Figure 3C). This result suggests that P134 may be the target of Fpr3 isomerization.

Psh1 ubiquitinates Cse4 at the following lysine residues in its C terminus - K131, K155, K163, and K172 (Hewawasam et al. 2010). As well, Cse4 directly interact with Psh1 via RING finger domain that is a hallmark of many E3 ligases (Ranjitkar et al. 2010). Because P134 is located close to those ubiquitylation sites, we tested whether deletion of FPR3 or FPR4 influence the interaction between Cse4 and Psh1. We performed coimmunoprecipitation and western blotting using Psh1-myc tagged strains. Psh1-myc tagged protein physically interacts with Cse4 in wild-type cells. Interestingly, deletion of FPR3 or FPR4 diminishes the interaction between Cse4 and Psh1-myc (Figure 4A and 4B). This data indicates that FPR3 and FPR4 regulate the Cse4-Psh1 interaction.

Given that the interaction of Cse4 with Psh1-myc is diminished in fpr3Δ cells, we predicted that mutation of P134V in Cse4 also influences the Cse4-Psh1 interaction. As expected, the interaction between cse4-P134V and Psh1-myc was also diminished.
(Figure 4C and 4D). Taken together, these data suggest that structural change between cis and trans form of P134 in Cse4 is essential for the Cse4-Psh1 interaction (Figure 4E).

We note that cse4-P134V mutants do not show any benomyl sensitivity or chromosome missegregation phenotype (data not shown). These phenotypes are also consistent with that of psh1Δ cells (Yuen et al. 2007). The phenotypic data also support the idea that Fpr3 regulates the Psh1-dependent Cse4 proteolysis. As Psh1 prevents Cse4 localization at noncentromeric regions (Hewawasam et al. 2010; Ranjitkar et al. 2010), Fpr3 might also prevent Cse4 interaction with noncentromeric regions. However, we found that Cse4 is accumulated at CEN (Figure S3), suggesting that Fpr3 might regulate Cse4 levels at the centromere.

Recently, it has become apparent that the Doa1/Ufd3 (WD repeat protein) is also required for the ubiquitination and proteolysis of Cse4 (Au et al. 2013). Doa1 regulates cellular levels of ubiquitin (Zhao et al. 2009). They propose that Doa1-mediated ubiquitination of Cse4 might be regulated by other ubiquitin ligases than Psh1 (Au et al. 2013). Interestingly, Doa1 is one of the targets of Fpr3 (Collins et al. 2007). We have shown that the stabilization level of Cse4 in fpr3Δ psh1Δ cells is much higher than that in fpr3Δ (Figures 2C and 2D), implying that Fpr3 might regulate the proteolysis of Cse4 in both Psh1-dependent and Psh1-independent manners.

Loss of Fpr3 causes chromosome missegregation, but not benomyl sensitivity (Figure 1A and S1). Interestingly, a high copy of FPR3 suppress the Glc7 overexpression lethality and the temperature sensitivity of ipl1-1 mutant (Ghosh and Cannon 2013). Deletion of Fpr3 showed a synthetic sickness phenotype with ipl1-2 mutant (data not shown). The opposing Ipl1 (Aurora kinase B) and Glc7 (Protein phosphatase 1: PP1)
activities ensure a bipolar attachment to the spindle (LAMPSON and CHEESEMAN 2011). Thus, Fpr3 might be also involved in another mechanism in the centromere function such as bipolar attachment.

In summary, we characterized the mitotic function of Fpr3, which was identified by our in vitro kinetochore assembly system. Fpr3 has a role in Cse4 protein stability. Fpr3 PPIase dead mutants stabilized Cse4 protein, suggesting that the isomerization activity of Fpr3 is necessary for the Cse4 proteolysis. The interaction between Cse4 and Psh1 was diminished in fpr3Δ mutant. Furthermore, a mutation on P134, a target of Fpr3 isomerization, reduced the Cse4-Psh1 interaction. Thus, we propose that the structural change between cis and trans form in Cse4 is important for the interaction with E3 ubiquitin ligase Psh1 (Figure 4E).
Acknowledgments

We thank Vivien Measday and members of the Kitagawa lab for helpful discussions and comments on the manuscript. This work was supported by National Institutes of Health Grant GM68418.
Figure Legends

Figure 1 Fpr3 regulates Cse4 protein level in vivo. (A) The fpr3Δ or fpr4Δ strain displays a moderate chromosome missegregation phenotype. Chromosome loss rate in null mutants was determined by half sector analysis, as previously described (OHKUNI et al. 2008). Wild-type (Y14): 3 half-sector colonies/6,421 total colonies; fpr3Δ (Y2249, Y2250, and Y2251): 17/8598; fpr4Δ (Y2252, Y2253, and Y2254): 14/10,105. P value (chi-squared test): WT vs fpr3Δ 0.012; WT vs fpr4Δ 0.072. (B) Increased protein level of Cse4 in fprΔ cells. Equal cell numbers of log phase cells, grown in SRaf medium, were visualized by western blot analysis with anti Cse4 and anti α-tubulin antibodies. Cse4 and α-tubulin protein levels were overlayed and quantitated by the Odyssey Imaging System. Cse4 protein levels were normalized by the amount of α-tubulin. Isogenic yeast strains were wild-type (YPH499 and YPH500), fpr3Δ (Y2243), fpr4Δ (Y2245), fpr3Δ fpr4Δ (Y2247 and Y2248).

Figure 2 Deletion of FPR3 stabilizes Cse4 protein level in vivo. Cse4 was induced from a GAL1 promoter by the addition of galactose for 2hrs. Glucose was added and cells were collected at the time point. Equal cell numbers were visualized by western blot analysis with anti Cse4, or anti Cdc28. We used the Odyssey Imaging System to detect and quantify the signals. (In detail, see File S1) (A and B) Isogenic yeast strains were wild-type (Y2255), fpr3Δ (Y2256), and fpr4Δ (Y2257). (C and D) Isogenic yeast strains were wild-type (Y2255), fpr3Δ (Y2256), psh1Δ (Y2258), and fpr3Δ psh1Δ (Y2340). Error bars represent SE of two independent experiments.
**Figure 3** Fpr3 isomerization activity is necessary for the Cse4 proteolysis. (A and B) PPIase dead mutants stabilize the Cse4 protein level *in vivo*. We constructed plasmids harboring PPIase dead mutations (W363L and F402Y) (Table S2). The protein stability assay was performed as described in Figure 2. Isogenic yeast strains were *FPR3* (Y2259), Vector (Y2260), *fpr3 W363L* (Y2261) and *fpr3 F402Y* (Y2262). Error bars represent SE of two or three independent experiments. (C) Mutation of P134 does stabilize the Cse4 protein level *in vivo*. There are five proline sites in Cse4 (P53, P98, P100, P134, and P157). We constructed plasmids harboring proline to valine mutation (Table S2). The protein stability assay was performed as described in Figure 2. Isogenic yeast strains were *Cse4* (Y2255), *cse4 P53V* (Y2263), *cse4 P98V* (Y2264), *cse4 P100V* (Y2265), *cse4 P134V* (Y2266) and *cse4 P157V* (Y2267).

**Figure 4** Fpr3 regulates the Cse4-Psh1 interaction. (A and B) Interaction between Cse4 and Psh1 was diminished in *fpr3Δ* cells. The indicated strains were grown to log phase, lysed, and anti-myc immunoprecipitations were performed as previously described (OHKUNI et al. 2008). Total and the immunoprecipitated fraction (IP) were subjects to SDS-PAGE, and Western blots were used to detect Cse4 and myc-tagged Psh1. We used the Odyssey Imaging System to detect and quantify the signals. Isogenic yeast strains were untagged (YPH500), Psh1-myc (Y2280), Psh1-myc *fpr3Δ* (Y2281), Psh1-myc *fpr4Δ* (Y2282), Psh1-myc *fpr3Δ fpr4Δ* (Y2283). Error bars represent SE of two independent experiments. Significant difference, p = 0.0089 (WT vs *fpr3Δ*). (C and D) P134V mutation in Cse4 diminishes the Psh1 interaction. Anti-myc immunoprecipitation assay and the quantification were performed as described in Figure 4 A and B. Isogenic
yeast strains were *Cse4* (Y2284) and *cse4 P134V* (Y2285). Error bars represent SE of two independent experiments. Significant difference, *p* = 0.0134. (E) A model for the role of Fpr3 in the Cse4 proteolysis. Psh1 is the E3 ubiquitin ligase that targets Cse4. Four lysine sites (K131, K155, K163, and K172) were ubiquitinated by Psh1. P134 close to the αN-helix (136-147) (*Keith et al.* 1999) might be the target of Fpr3 isomerization. We propose that the structural change in Cse4 from *cis* to *trans* or from *trans* to *cis* is important for the Cse4 degradation by Psh1. It is not known which form of Cse4 is ubiquitinated. The N-terminal domain (black) and the histone fold domain (red) of the α N, α 1, α 2, and α 3-helices are indicated (*Keith et al.* 1999).


Dolinski, K., S. Muir, M. Cardenas and J. Heitman, 1997 All cyclophilins and FK506 binding proteins are, individually and collectively, dispensable for viability in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A **94**: 13093-13098.


Hewawasam, G., M. Shivaraju, M. Mattingly, S. Venkatesh, S. Martin-Brown et al., 2010 Psh1 is an E3 ubiquitin ligase that targets the centromeric histone variant Cse4. Mol Cell **40**: 444-454.


Figure 1

**A**

Loss per 1000 divisions

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>fpr3Δ</th>
<th>fpr4Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative intensity</td>
<td>1.0</td>
<td>1.5</td>
<td>1.9</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>fpr3Δ</th>
<th>fpr4Δ</th>
<th>fpr3Δ fpr4Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cse4</td>
<td>1.0</td>
<td>1.5</td>
<td>1.9</td>
<td>2.2</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>1.0</td>
<td>1.5</td>
<td>1.9</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Relative intensity

Figure 1
AB

Cdc28

0 min 20 min 40 min 60 min 80 min

Raffinose

0 min 20 min 40 min 60 min 80 min

Cse4

Cdc28

Wild-type

After addition of glucose

fpr3Δ

After addition of glucose

fpr4Δ

After addition of glucose

Cse4

Cdc28

Wild-type

fpr3Δ

psh1Δ

fpr3Δ psh1Δ

Relative intensity (%)

0 min 20 min 40 min 60 min 80 min

Time after addition of glucose (min)

Relative intensity (%)

0 min 20 min 40 min 60 min 80 min

Time after addition of glucose (min)
Figure 4