Conserved Mode of Interaction between Yeast Bro1 Family V Domains and YP(X)nL Motif-Containing Target Proteins

1 **Conserved mode of yeast Bro1 family V domains for interaction with YP(X)nL**

2 **motif-containing target proteins**

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14 **Running Head**: V-domain analyses of yeast Bro1 and Rim20

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20   **Abstract**

21   Yeast Bro1 and Rim20 belong to a family of proteins, which possess a common architecture

22   of Bro1- and V-domains. Alix and HD-PTP, mammalian Bro1 family proteins, bind YP(X)nL (n = 1

23   ~ 3) motifs in their target proteins through their V domains. In Alix, the Phe residue, which is located

24   in the hydrophobic groove of the V domain, is critical for binding to the YP(X)nL motif. Although

25   the overall sequences are not highly conserved between mammalian and yeast V domains, we show

26   that the conserved Phe residue in the yeast Bro1 V domain is important for binding to its

27   YP(X)nL-containing target protein, Rfu1. Furthermore, we show that Rim20 binds to its target

28   protein Rim101 through the interaction between the V domain of Rim20 and the YPIKL motif of

29   Rim101. The mutation of either the critical Phe residue in the Rim20 V domain or the YPIKL motif

30   of Rim101 affected the Rim20-mediated processing of Rim101. These results suggest that the

31   interactions between V domains and YP(X)nL motif-containing proteins are conserved from yeast to

32   mammalian cells. Moreover, the specificities of each V domain to their target protein suggest that

33   unidentified elements determine the binding specificity.

34

35   Introduction

36   Yeast Bro1 belongs to a family of related proteins that share a common architecture

37   comprising an N-terminal Bro1 homology domain and a following V domain (Fig. 1A). Bro1/Vps31

38   was originally isolated as one of the vacuolar protein targeting mutants and later classified as class E

39   vps mutants (1, 2). Bro1 is reported to function as an accessory factor for Endosomal Sorting

40   Complex Required for Transport (ESCRT) apparatus in the multivesicular bodies (MVB) pathway (3,

41   4). The ESCRT apparatus, which comprises four complexes (ESCRT-0, I, II, and III), is responsible

42   for the sorting of ubiquitinated membrane proteins into MVBs for degradation in the

43   lysosome/vacuole (5). Bro1 is directed to endosomes by the association of the Bro1 domain with

44   ESCRT-III subunit Snf7 (6), and was reported to regulate the membrane-scission activity of

2

45 ESCRT-III (7). Moreover, Bro1 binds to the deubiquitinating enzyme Doa4 through its C-terminus

46 region, recruits Doa4 to endosomes, and activates Doa4 (8, 9). Doa4 plays a role in the recovery of

47 ubiquitins from ubiquitinated cargoes just prior to the invagination of the cargo protein-enriched

48 membranes; therefore, it maintains cellular ubiquitin homeostasis in yeast (10). Intriguingly, we

49 revealed that Bro1 also binds to Rfu1 (a regulator for free ubiquitin chains) through its V domain and

50 recruits Rfu1 to endosomes (11). Rfu1 also has a function to maintain ubiquitin homeostasis by

51 inhibiting Doa4 activity (12). Bro1 has an additional region called the Pro-rich region (PRR), which

52 was reported to bind Rsp5, a major ubiquitin ligase for ubiquitinating cargo proteins (13).

53   Rim20, another Bro1 family protein in yeast, functions in the pH-responsive pathway (14,

54 15). The pathway has been intensively studied in the fungi *Aspergillus nidulans* and the yeast

55 *Saccharomyces cerevisiae* (16). In this pathway, Rim101, a transcription factor, is processed through

56 the proteolytic removal of its C-terminal region in response to alkaline pH. The processed Rim101

57 then regulates the expression of alkaline-responsive genes, resulting in the adaptation to alkaline

58 conditions (17). During this activation process, Rim20 is required for the proteolytic cleavage of

59 Rim101 along with other factors such as Rim13, Rim9, Rim21, Dfg16, Rim8, and several ESCRT-I,

60 -II, and -III factors (18-20). Rim20 appears to function as an adaptor by directly binding to Rim101

61 and several ESCRT components such as Snf7 (18, 19). Recently, it was reported that the events of

62 the Rim101 pathway, after alkaline conditions, occurred on the plasma membrane (21, 22).

63   The mammalian Bro1 homolog, apoptosis-linked gene 2 interacting protein X (Alix),

64 functions in ESCRT-mediated budding of enveloped viruses and membrane abscission in cytokinesis

65 (3, 5). During the process of virus budding, cellular ESCRT machineries are hijacked by the viruses

66 to facilitate their release from the cell membrane. Like Bro1, Alix has three main domains, Bro1, V,

67 and PRR. The PRR of Alix was shown to directly bind multiple proteins such as the Tsg101 (yeast

68 Vps23) or CEP55 (23, 24). In addition, PRR keeps Alix in an autoinhibited conformation (25, 26).

69 Although Alix has not been reported to function in the sorting of ubiquitinated cargoes such as the

70   EGF Receptor, His-domain protein tyrosine phosphatase (HD-PTP), another member of Bro1 family

71   proteins is required for EGF receptor sorting to the MVB (27).

72   The Alix V domain is about 320 amino acids (aa) long, forming the structure of two

73   trihelical bundles taking the shape of the letter V. It has been studied extensively for its interaction

74   with YP(X)nL motif-containing viral and cellular proteins (28, 29). The Alix V domain binds to the

75   YP(X)nL motif-containing late domains of retrovirus such as HIV-1, equine infectious anemia virus

76   (EIAV), and Ebola, and appears to play an important role in virus budding (30, 31). A hydrophobic

77   pocket on the second arm of Alix V was identified as a region for binding to the YP(X)nL motif

78   peptide (28, 29). Particularly, the Phe residue in the pocket plays a critical role in the interaction with

79   YP(X)nL motif, and F676D is an inactivation mutation of Alix V in binding. As for cellular proteins,

80   Alix V was shown to bind to the YPX(3)L motif of G-protein coupled receptor, protease-activated

81   receptor 1 (PAR1), to mediate the ubiquitin-independent sorting of PAR1 (32). In yeast, Bro1 and

82   Rim20 V domains were shown to have a very similar structure to the Alix V domain, albeit they have

83   a low sequence similarity(33). Recently, the V domains of Alix, HD-PTP, Bro1, and Rim20 are

84   shown to bind to ubiquitins, particularly to K63-linked ubiquitin chains (33-35), leading to the

85   proposal that V domains are ubiquitin receptors. The ubiquitin binding regions within the V domains

86   were reported to be different from the YP(X)nL binding region.

87   Because amino acid sequences are not highly conserved between Alix and yeast V domains

88   (11–13% amino acid identity for Alix and Bro1 V domains, Sup Fig. 1), the interaction of the yeast V

89   domain with a YP(X)nL motif-containing protein is overlooked (33). Recently, we showed a direct

90   interaction between a region containing the YPEL motif of Rfu1 and the V domain of Bro1 (11). In

91   this study, we observed that a region containing a critical Phe residue that is reported to bind to

92   YP(X)nL motif of the target proteins in Alix is relatively conserved in the V domains of Alix,

93   HD-PTP, Bro1, and Rim20 (Fig. 1). Therefore, we tested whether the yeast V domain's interaction

94   with YP(X)nL motif-containing target proteins could be analogous to mammalian V domains. We

4

95   examined the interactions between the Bro1 V–Rfu1 and Rim20 V–Rim101 by focusing on the

96   conserved Phe residue in the V domains of Bro1 and Rim20.

97

98   Experimental Procedures

99   **Media.** Yeast strains were grown in YPAD medium (1% yeast extract, 2% Bacto–Peptone, 2%

100   glucose, and 0.002% adenine), in synthetic complete medium (SD; 0.67% yeast nitrogen base and

101   2% glucose supplemented with amino acids) or synthetic casamino medium (SC; 0.67% yeast

102   nitrogen base, 2% glucose, and 0.5% casamino acids). If necessary, tryptophan, uracil, or adenine

103   was added. For microscopy studies, 0.02% adenine was added.

104

105   **Yeast strains and plasmids.** A list of the yeast strains and plasmids used in this study are provided

106   in Sup. Tables 1 and 2, respectively. Plasmid pGST2-Alix (360–702) was obtained from Addgene.

107   Plasmid expressing N-terminally myc-tagged Rim20 under the control of a *RIM2*0 promoter was

108   created as follows. Two kinds of DNA fragments, F and B, were amplified using a *RIM20* plasmid as

109   a template and two sets of primers, RIM20–up875–BamHI,

110   AATTAGGATCCACGTTGTATATTTTCAATCTGGAAAGTAA and RIM20–BtsI-AS,

111   GTTCACTCATGTCACACTGCCTGGATCTCC; RIM20–BtsI–Myc-sense,

112   AATTGCAGTGTGACATGGAACAAAAGCTTATTTCTGAAGAAGACTTGATGAGTGAACTGC

113   TTGCCATTCC and RIM20–Down–XhoI-AS,

114   AATTCTCGAGCTGTTGTCTAAAGGCGAAACTACGATGAAG, respectively. The obtained F and

115   B fragments were cut with BamHI–BtsI and BtsI–XhoI, respectively. The two fragments were ligated

116   to the BamHI–XhoI vector portion of pRS315.

117

118   **Immunoblotting.** Preparation of whole-cell extracts and immunoblot analysis were performed as

119   previously described (36). In western blotting, blots were incubated with a mouse anti-GFP

120   monoclonal antibody (Roche), an anti-HA antibody (HA.11, COVANCE), or an anti-yeast PGK

121   antibody (Molecular Probes, Eugene, OR), followed by horseradish peroxidase (HRP)-conjugated

122   anti-mouse IgG (#NA931V, Amersham) and then visualized using an ECL-plus reagent (Amersham).

123   To detect GST, an HRP-conjugated anti-GST antibody (Wako) was used. A rabbit anti-yeast Bro1

124   antibody was described previously (11).

125

126   **Recombinant protein purification**. MBP-Rfu1 and MBP-fusions of the Rfu1 mutants were purified

127   as previously described (12). Recombinant GST, GST–Bro1, or the various GST–Bro1 mutants were

128   purified using glutathione chromatography as recommended by the manufacturer (GE Healthcare).

129   Recombinant proteins were eluted with 20 mM glutathione, 50 mM Tris HCl (pH 8.0), and 2 mM

130   DTT; dialyzed against 50 mM Tris HCl (pH 7.5), 100 mM NaCl, and 10% glycerol; and then stored

131   at −80°C.

132

133   *In vitro* **binding between various MBP-fused proteins and GST-fused proteins**. Binding

134   experiments were performed as previously described (11).

135

136   **Microscopy**. FM4-64 (Molecular Probes, Inc.) staining was performed as previously described (37).

137   Cells were imaged at room temperature using a confocal microscope (LSM780; Carl Zeiss) equipped

138   with an αPlan-Apochromat 100× oil objective lens. Images were processed using the LSM image

139   browser, and the brightness and contrast were adjusted using Adobe Photoshop CS4.

140

141   **Detection of HA–Rim101**. Logarithmically growing cells in SC-Ura Leu or SC-Ura (pH 4.0)

142   medium were harvested by centrifugation and re-suspended in the same medium, SC-Ura Leu or

143   SC-Ura (pH 8.0). After incubating for 20 min at 30°C, trichloroacetic acid (TCA) was added to make

144   a final concentration of 6% and the mixture was kept on ice for 20 min. Cells collected by

145   centrifugation were suspended in a urea buffer (50 mM Tris–HCl pH 7.5, 5 mM EDTA, 6 M urea,

146   1% SDS, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride) and were disrupted by vortexing with

147   glass beads. Obtained cell lysates were cleared by centrifugation and the total protein concentrations

148   were determined using the DC protein assay (Bio-Rad, Richmond, CA). The cell lysates were

149   incubated with a Laemmli SDS sample buffer at 65°C for 15 min and were subjected to SDS-PAGE

150   and western blotting. To detect HA-tagged Rim101 and Myc-tagged Rim20, membranes were

151   blocked with 1% skimmed milk and then immunoblotted with an anti-HA antibody (12CA5) or an

152   anti-myc antibody (9E10), respectively. The membranes were then treated with an anti-mouse IgG

153   secondary antibody conjugated with horseradish peroxidase (NA931; GE Healthcare) and developed

154   with ECL Prime (GE Healthcare). To detect actin, an anti-actin monoclonal antibody (C4; ICN,

155   Aurora, OH) and IRDye-conjugated anti-mouse IgG antibody (Rockland, Gilbertsville, PA) were

156   used. The signals were detected by using the infrared imaging system Odyssey (LICOR, Lincoln,

157   NE).

158

159   Results

160   **Effect of Phe687 of Bro1 on the interaction with Rfu1**. We noticed that the Phe residue and the

161   following Tyr as well as several neighboring residues were conserved around this region in Bro1 and

162   Rim20 V domains of *S. cerevisiae* and the Bro1 V domain of *Naumovozyma castellii* whose crystal

163   structure was resolved (33) (Fig. 1B). This suggests that this region in the V domains of Bro1 and

164   Rim20 may have similar functions as the Alix V domain.

165   In Bro1, Phe687 is the corresponding Phe residue. First, we examined the effect of the Phe

166   687 mutation in the Bro1 V domain for the binding of Rfu1 *in vitro* (Fig. 2A). In the previous work,

167   we found that recombinant MBP–Rfu1 specifically bound to the recombinant GST-fused Bro1 V

168   domain (11). We observed that the binding activity of GST–Bro1-V (F687A) to MBP–Rfu1 was

169   drastically reduced (Fig. 2A). Additionally, we examined the binding ability of mutants whose

7

170 mutations were closely located to F687; F677A, D680A, and L681A. The binding of Bro1 V

171 (F677A) was moderately reduced, but not as much as that of F687A Bro1 V and D680A. L681A

172 double mutations had no effects.

173   Next, we investigated whether the Bro1 F687 residue functioned in the interaction with

174 Rfu1 *in vivo*. Immunoprecipitation analysis, using lysates from cells expressing Rfu1-3 × Flag plus

175 myc-tagged Bro1 or myc-tagged Bro1 (F687A), were performed using anti-Flag. Myc-tagged Bro1,

176 but not myc-tagged Bro1 (F687A), was specifically precipitated with Rfu1-3 × Flag (Fig. 2B). These

177 results indicated that Bro1 Phe687 played a critical role in the Bro1–Rfu1 interaction both *in vitro*

178 and *in vivo*.

179   The Rfu1 localization at endosomes largely depends on Bro1; Rfu1–GFP is mainly diffusive

180 in the Δ*bro1* mutant, and the Rfu1 mutant in which the YPEL motif was changed to AAEL showed

181 impaired endosomal localization (11). We, therefore, examined the effect of the F687A mutation on

182 the localization of Rfu1–GFP fusions in yeast. First, we found that Bro1–GFP and Bro1

183 (F687A)–GFP were similarly observed, mainly at the class E compartments in Δ*vps4*Δ*bro1* cells

184 (data not shown). Next, the localization of Rfu1–GFP expressed under the *RFU1* promoter was

185 examined in Δ*rfu1*Δ*vps4*Δ*bro1* cells expressing either a wild-type or F687A Bro1. Rfu1–GFP

186 fluorescence was present at foci that overlapped with FM4-64 stained class E compartments in

187 Bro1-expressing Δ*vps4*Δ*rfu1* cells (Fig. 3A, B). In contrast, the localization of Rfu1–GFP at class E

188 compartments was reduced in Bro1 (F687A) expressing cells. The accumulation of Rfu1–GFP was

189 slightly reduced, probably due to its impaired binding to Bro1 (F687A) because a reduction in the

190 accumulation of Rfu1 was previously observed in the Δ*bro1* mutant (Fig. 3C) (11).

191 **Rim20 and Rim101 interaction through the V domain of Rim20 and the YPKIL motif in**

192 **Rim101**. Next, we looked for different interactions between V domains and YP(X)nL

193 motif-containing proteins. Rim20, another V-domain-containing protein, is required for Rim101p

194 processing by direct binding to Rim101 (14, 38). Xu and Mitchell showed that the C-terminus region

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195 containing PEST-like sequences in Rim101 was sufficient for binding to Rim20 (14). Within the

196 C-terminus region of Rim101, there is a YPKIL motif close to the C-terminus end that matches with

197 the consensus YP(X)nL (n = 1–3) motif (39). This motif is located downstream of the cleavage site

198 of Rim101. In addition, about the C-terminal half (353–661aa) of Rim20, that corresponds to its

199 V-domain, was reported to bind to Rim101 (14).

200   To test whether the Rim101–Rim20 interaction was mediated by the Rim20's V-domain and

201 the Rim101's YPKIL motif, we assessed the interaction in the *in vitro* binding experiment. We made

202 recombinant MBP–Rim101-C, a fusion of MBP with the 125 aa of the C-terminal region of Rim101,

203 and checked whether MBP–Rim101-C bound to recombinant GST–Rim20 V (330–661aa) *in vitro*

204 (Fig. 4). As expected, we observed an efficient interaction between the two proteins (Fig. 4, lane 2).

205 When the conserved Rim20 Phe623 was substituted by Ala in GST-Rim20 V, the binding ability was

206 significantly lost (Fig. 4, lane 3). Moreover, we observed that the interaction between GST–Rim20V

207 and MBP–Rim101-C (Y620A, P621A), in which the YPKIL motif was changed to AAKIL, was

208 significantly lost (Fig. 4, lane 5). These results suggest that the interaction between Rim20 and

209 Rim101 is mediated by the Rim20 V domain and the YPKIL motif of Rim101, and the conserved

210 Phe in the Rim20V is critical for the interaction.

211   Next, we investigated the role of Phe623 in the Rim20 V domain by examining the

212 processing of Rim101 *in vivo* (Fig. 5). N-terminally myc–tagged or non-tagged Rim20 or Rim20

213 (F623A) were expressed in the Δ*rim20* mutant together with HA-tagged Rim101. Under acidic

214 conditions (pH 4), the intact full length of Rim101 is a major form; whereas, under alkaline

215 conditions (pH 8), Rim101 undergoes proteolytic processing that removes the C-terminal region of

216 Rim101 (15). As previously reported (14), the processing was defective in the Δ*rim20* mutant. When

217 wild-type Rim20 or myc-tagged Rim20 were introduced to the Δ*rim20* mutant, Rim101 processing

218 became normal. In contrast, Rim101 processing was defective in the Δ*rim20* mutant expressing

219 Rim20 (F623A) or myc-tagged Rim20 (F623A). We observed that the amino acid change of F623A

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220   did not affect the stability of myc-Rim20 (F623A), and its level was similar to that of myc-Rim20

221   (Fig 5 A).

222   In addition, we investigated the effect of the YPKIL mutation of Rim101 on its processing

223   by expressing HA-Rim101 or HA-Rim101 (Y620A, P621A) in Δ*rim101* cells, and observed that

224   proteolytic processing was defective in cells expressing Rim101 (Y620A, P621A) (Fig. 5B). Because

225   the mutants in the Rim101 pathway show sensitivity to LiCl-containing medium (20, 40), LiCl

226   sensitivity was examined (Fig. 5C). Cells expressing Rim101 (Y620A, P621A) showed a marginal

227   but significant sensitivity, indicating that the active form of Rim101 was not efficiently produced

228   from Rim101 (Y620A, P621A). These results indicated that the interaction between the Rim20 V

229   domain and the YPKIL motif of Rim101 was important for their biological function.

230   **Specificity of the V domain–YP(X)nL interaction.** The V domains of Bro1 and Rim20 are

231   structurally similar; however, their physiological roles have been reported to be different. It was

232   reported that the Δ*bro1* mutant showed normal Rim101 processing (14), and the involvement of

233   Rim20 in MVB sorting has not been reported. We therefore suspected that there were specificities for

234   their interactions. To test the idea, we examined whether Rfu1 bound to the Rim20 V domain or

235   whether Rim101 bound to Bro1 V (Fig. 6). We observed that under the conditions that MBP–Rfu1

236   bound to Bro1 V, MBP–Rfu1 did not bind Rim20 or the Alix V domains. Similarly, MBP–Rim101-C

237   bound to Rim20 V but did not bind to Bro1-V or Alix V. These results suggest that there are more

238   unidentified determinants for the specific interaction between V domains and YPX(n)L motifs.

239

240

241   Discussion

242   In this study, we showed that the conserved Phe residue in the V domains of yeast Bro1 and Rim20

243   plays an important role in binding to the YP(X)nL-motif of their target proteins, Rfu1 and Rim101.

244   The results suggest that the yeast Bro1 and Rim20 V domains bind to their target proteins in a similar

245   way as mammalian Alix V does, indicating that V-domains are YP(X)nL-motif binding domains

246   from yeast to mammals. Therefore, results from yeast Bro1 family V domain studies will not only

247   contribute to our understanding of the cellular events in yeast, but may also be informative in our

248   understanding of the interactions between mammalian Bro1 family proteins and their

249   YP(X)nL-containing target proteins such as virus proteins.

250   In addition, we showed that there are specificities of each V domain with its target protein;

251   the C-terminal region of Rim101 specifically binds to the Rim20 V domain, but not to the Bro1 and

252   Alix V domains. Likewise, Rfu1 only binds to Bro1 V but not to Rim20 V domains in our *in vitro*

253   assay. These results suggest that there must be more unidentified sequence or structural determinants

254   of the interaction between V domains and their cognate YP(X)nL motif-containing partners (e.g.,

255   particular sequence or structures). Indeed, there are many proteins that possess YP(X)nL motifs in a

256   cell, but only a subset of them seem to bind to the V domains. For example, the YPFL motif of Doa4

257   does not bind to the Bro1 V domain, instead this motif binds to the C-terminal region of Bro1 (8).

258   Although we do not have any hints for the determinants, an intensive mutagenesis approach may

259   give us a clue to understand the nature of the specificity. In any case, a structural analysis of the Bro1

260   V domain-YPEL peptide or the Rim20 V domain-YPKIL peptide will be needed to define the precise

261   mechanism of the interaction. Moreover, finding more Bro1 V domain-binding proteins and

262   comparing their sequences or structures with those of Rfu1 or Alix V binding proteins may give us

263   some ideas of the specificities.

264   Bro1 is required for Rfu1 to function at endosomes (11). Rfu1 is involved in ubiquitin

265   homeostasis because in Δ*rfu1* cells, monomer ubiquitin is increased and unanchored ubiquitin chains

11

266 or small ubiquitin species decreased (11, 12). We expected that Δ*bro1* cells expressing Bro1 (F687A)

267 would show a similar ubiquitin profile to that of the Δ*rfu1* mutant, but they did not show obvious

268 aberrant profiles (data not shown). The reason was unknown, but it may be that the residual binding

269 of Rfu1 to Bro1 (F687A) *in vivo* is enough to support ubiquitin homeostasis, although other

270 possibilities cannot be excluded.

271   Alix has been reported to have a flexible structure (25, 26). The PRR was reported to fold

272 back and inhibit V domain binding to viral proteins. The V domain appears to take a closed

273 conformation in the presence of PRR and an open conformation in the absence of the PRR. Alix was

274 also reported to have a dimer structure via its V domain (41). Moreover, binding of ubiquitins to Alix

275 V was shown to induce oligomerisation of the V domain (35). Thus, conformational change of Bro1

276 family proteins as a whole as well as its V domain seem to be regulated in complex ways in a cell,

277 and the Bro1 family studies using tractable yeasts would be suitable to reveal such complex

278 mechanisms.

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285 Figure 1. Bro1 family proteins

286 A. Schematic organization of Bro1, Rim20, Alix, and HD-PTP. Bro1, V domain, and PRR (proline

287 rich region) are indicated. PTP, phosphatase domain. B. Conservation of putative YP(X)nL binding

288 regions of the V domains of *Saccharomyces cerevisiae* (sc) Bro1, *Naumovozyma castellii* (nc) Bro1,

289 Rim20, human (hs) Alix, and HD-PTP. Alignments of scBro1 vs. ncBro1, scRim20 vs. Alix, and

290 HD-PTP vs. Alix were generated by Clustal W. Alignments of nsBro1 and hsAlix were made by

12

291 structural comparisons using DaliLite. These alignments were then assembled. Arrow indicates the

292 critical Phe residue in the Alix V domain and the corresponding Phe in other Bro1 family proteins.

293

294 Figure 2. Impaired binding of Bro1 (F687A) to Rfu1

295 A. Impaired binding of Bro1 V (F687A) to MBP–Rfu1 *in vitro*. MBP or MBP–Rfu1 were mixed

296 with GST, GST–Bro1 V, or the noted GST–Bro1 V mutants and the proteins were isolated with

297 amylose resin. Samples were examined by immunoblot analysis using anti-GST (top panel) and

298 anti-MBP antibodies (bottom panel). B. Impaired association of Bro1 (F687A) to Rfu1-3 × Flag *in*

299 *vivo*. Lysates of *Δbro1* cells (lane1)*, or Δbro1 RFU1-3xF* cells harboring a plasmid expressing

300 C-terminally myc-tagged Bro1 or Bro1 (F687A) (lanes 2 and 3) were immunoprecipitated with

301 anti-Flag. The resulting immune-complexes (IP) were analyzed by immunoblot using anti-Flag and

302 anti-myc.

303

304 Figure 3. Impaired endosome localization of Rfu1–GFP in Bro1 (F687A)-expressing cells

305 A. GFP and FM4-64 fluorescence and DIC microscopy of Rfu1–GFP in *Δvps4Δrfu1Δbro1* cells

306 expressing Bro1 or Bro1 (F687A). Arrowheads indicate the class E compartments. Scale bar, 5 μm

307 for upper panels, 2 μm for lower panels. B. Quantification of Rfu1–GFP foci in A. Cells containing

308 GFP foci around the vacuolar membrane were counted (n = 50 cells in each experiment), and mean

309 values of three independent experiments are shown. Standard errors (SE) are shown as bars. C.

310 Rfu1–GFP expression as determined by the anti-GFP immunoblot analysis in (A). Anti-GFP

311 immunoblot (top), anti-Bro1 immunoblot (middle), and anti-phosphoglycerate kinase (PGK)

312 immunoblot (bottom), a control for protein loading.

313

314 Figure 4. Binding of the Rim20 V domain to the Rim101 YP(X)nL motif *in vitro*

315 MBP or MBP–Rim101-C or MBP–Rim101-C (Y620A, P621A) were incubated with GST,

316 GST–Rim20 V, or GST–Rim20-V (F623A), and the proteins were isolated with amylose resin. GST,

317 GST–Rim20V, or GST–Rim20V (F623A) are indicated by arrows. Anti-GST immunoblot for pull

318 down samples (top), anti-MBP immunoblot for pull down samples (middle), and anti-GST

319 immunoblot for input (bottom).

320

321 Figure 5. Effects of Rim101 or Rim20 mutation on HA-Rim101 processing

322 A. Effects of Rim20 (F623A) mutation on HA-Rim101 processing. HA-tagged Rim101 was

323 expressed in Δ*rim20* cells harboring a vector or expressing Rim20, Rim20 (F623A), myc-tagged

324 Rim20 or myc-tagged Rim20 (F623A), at the indicated pH of 4 or 8. Processed (p.f.) and

325 unprocessed forms (u.f.) of HA-Rim101 are indicated. Anti-HA immunoblot analysis (top), anti-myc

326 immunoblot for myc-tagged Rim20 or Rim20 (F623A) (middle), and anti-actin blot, used as a

327 loading control (bottom). B. Effect of HA-Rim101 (AAKIL) mutation on processing. Δ*rim101* cells

328 harboring a vector or plasmids expressing HA-Rim101 or HA-Rim101 (AAKIL) were tested. C. Li

329 sensitivity. Cells were diluted and spotted on SC-Ura plates and YPD containing 0.3M LiCl and

330 incubated for 3 days.

331

332 Figure 6. V domain specificity against YP(X)nL motif-containing proteins

333 MBP, MBP–Rfu1, or MBP–Rim101 were incubated with GST, GST–Bro1V, GST–Rim20 V, or

334 GST–Alix V, and the proteins were isolated with amylase resin. GST-tagged samples were examined

335 by immunoblot analysis using anti-MBP and anti-GST antibodies. Anti-GST immunoblot for

336 pull-down samples (top), GST–Rim20 V and GST–Bro1V of pull-down samples are indicated by

337 arrows. Anti-MBP immunoblot for pull down samples (middle), anti-GST immunoblot for input

338 (bottom).

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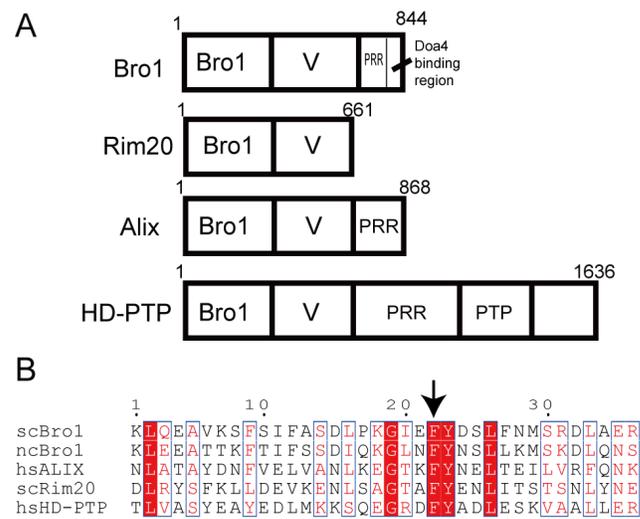
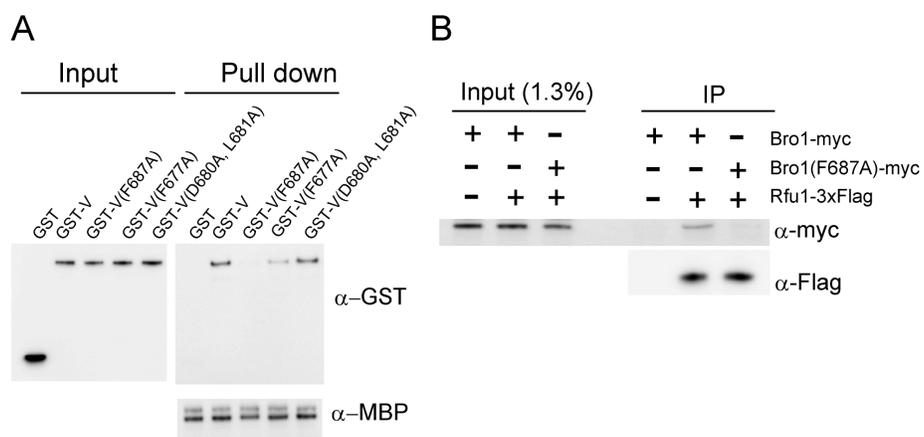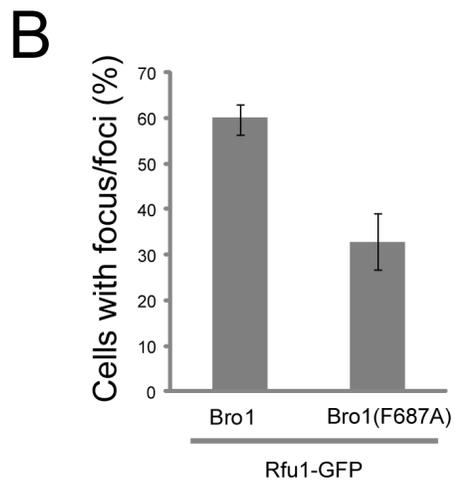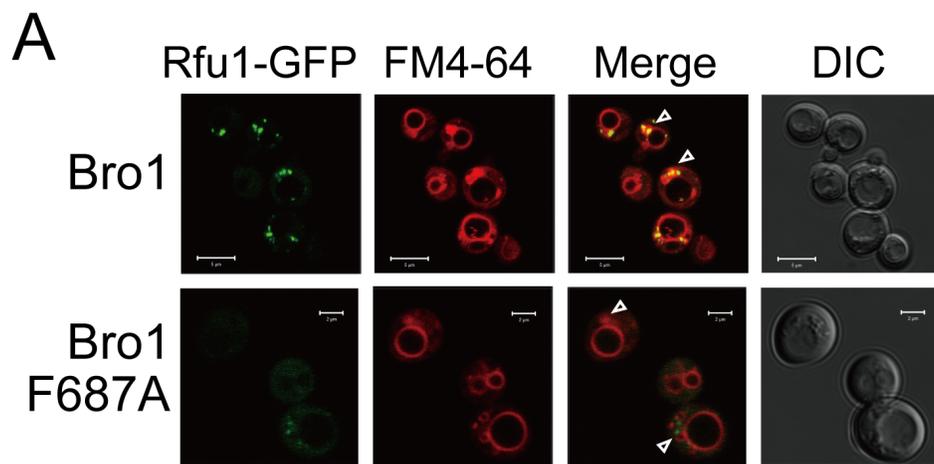444

445

Figure 1

A

B

Figure 2

A

Input   Pull down

GST
GST-V
GST-V(F687A)
GST-V(F677A)
GST-V(D680A, L681A)
GST
GST-V
GST-V(F687A)
GST-V(F677A)
GST-V(D680A, L681A)

α–GST

α–MBP

B

Input (1.3%)   IP

+	+	−		+	+	−	Bro1-myc
−	−	+		−	−	+	Bro1(F687A)-myc
−	+	+		−	+	+	Rfu1-3xFlag

α-myc

α-Flag

Figure 3

A

	Rfu1-GFP	FM4-64	Merge	DIC
Bro1				
Bro1 F687A				

B

Cells with focus/foci (%)

Bro1   Bro1(F687A)

Rfu1-GFP

C

Bro1   F687A

anti-GFP

anti-Bro1

anti-PGK

Figure 4

Pulldown
α-GST

← GST-Rim20V

Pulldown
α-MBP

Input
α-GST

← GST-Rim20V or
GST-Rim20V(F623A)

← GST

MBP-Rim101C   MBP-Rim101C
(Y620A, P621A))

Lane labels: GST, GST-Rim20V, GST-Rim20V(F623A), GST, GST-Rim20V, GST-Rim20V(F623A)

Figure 5

Figure 6

	1	2	3	4	5	6	7	8	
	−	−	−	−	−	+	−	−	MBP
	+	+	+	+	+	−	−	−	MBP-Rim101
	−	−	−	−	−	−	+	+	MBP-Rfu1
	+	−	−	−	−	−	−	−	GST
	−	+	−	−	−	+	+	−	GST-Rim20 V
	−	−	+	−	−	−	−	−	GST-Rim20 V (F623A)
	−	−	−	+	−	−	−	+	GST-Bro1 V
	−	−	−	−	+	−	−	−	GST-Alix V

Supplementary Table 1. Strains used in this study

Name	Genotype	Source/Reference
W303	MATα ade2-1 can1-100 his3-12,16 leu2-3,112 trp1-1 ura3-1	Rothstein
Y795	W303α, Δrfu1::KanMX Δvps4::LEU2	Kimura et al., 2014
Y1009	W303α; RFU1-3xFLAG::RFU1	This study
Y1119	W303α; RFU1-3xFLAG::RFU1, Δbro1::HIS3	This study
Y1118	W303α, Δrfu1::KanMX Δvps4::LEU2, Δbro1::HIS3	This study
Y1140	W303α; Δbro1::HIS3	This study
rim101Δ101	MATa ura3-52 leu2Δ1 his3Δl200 rim101::HIS3	This study
FM201	MATa ura3-52 leu2Δ1 his3Δ200 trp1Δ63 rim20::HIS3	Hayashi et al, 2005

Supplementary Table S2   Plasmids used in this study

Plasmids	Name	Proteins expressed	Characteristis	Source/References
pGCU10	GAL1p-GFP-tADH1	GFP	URA3, CEN	Kimata et al., (1997)
E798	YCplac22-RFU1p-RFU1-GFP	Rfu1-GFP	TRP1, CEN	This study
E744	pRFU1p-RFU1(1-200)-GFP	RFU1(1-200)-GFP	URA3, CEN	This study
pMALP2X	MBP	MBP		
E382	MBP-Rfu1(1-200)	MBP-Rfu1(1-200)		Kimura et al., (2009)
E827	MBP-RIM101(501-625)	MBP-Rim101(501-625)		This study
	MBP-RIM101(501-625,Y620A, P621A)	MBP-Rim101(501-625,Y620A, P621A)		This study
pGEX4T-3		GST		GE
E779	pGEX-Bro1-V Comp	GST-Bro1-V (361-720)		Kimura et al., (2014)
E831	pGEX-Bro1-V Comp(F687A)	GST-Bro1-V (361-720)F687A		This study
E829	pGEX-Bro1-V Comp(F677A)	GST-Bro1-V (361-720)F677A		This study
E828	pGEX-Bro1-V Comp(D680A, L681A)	GST-Bro1-V (361-720)D680A, L681A		This study
E838	pGEX-Rim20 V	GST-Rim20-V(330-661)		This study
E842	pGEX-Rim20 V(F623A)	GST-Rim20 V(330-661, F623A)		this study
	pGST2-Alix (360-702)	GST-Alix V(360-702)		Addgene
E813	pRS316-BRO1	Bro1	URA3, CEN	This study
E837	pRS316-BRO1(F687A)	Bro1(F687A)	URA3, CEN	this study
E846	pRS316-BRO1myc	Bro1-myc	URA3, CEN	This study
E851	pRS316-BRO1(F687A)myc	Bro1(F687A)-myc	URA3, CEN	This study
E847	pRS315-RIM20	Rim20	LEU2, CEN	This study
E854	pRS315-RIM20(F623A)	Rim20(F623A)	LEU2, CEN	This study
E848	pRS315-mycRIM20	Myc-Rim20	LEU2, CEN	This study
E855	pRS315-mycRIM20(F623A)	Myc-Rim20(F623A)	LEU2, CEN	This study
E860	LRIM101m1HApRS416	HA-RIm101	URA3	This study
E861	pRS416-HA-RIM101(Y620A, P621A)	HA-RIm101(Y620A, P621A)	URA3	This study