SUPPLEMENTARY INFORMATION

by Andrea Lehmann, Katharina Jechow and Cordula Enenkel*

Yeast strains and plasmids. Standard techniques of yeast genetics and molecular biology were used. Chromosomal C-terminal tags were introduced by homologous recombination. To yield GFPS-tagged β5 pSL1190-*PRE2*(β5)-GFPS- *HIS3*-*URA3* was created according to Wendler et al. (2004). For this purpose, the C-terminal 97 codons (without stop codon) of *PRE2* were fused to the codons of Strep II (WSHPQFEK) -tagged GFP. Adjacent to the terminating stop codon *HIS3* and *URA3* markers were inserted followed by 493 bp downstream of the *PRE2* stop codon. The *SalI*-*XhoI*-cut DNA fragment was transformed into mutant and isogenic wild type strains, respectively. The selection of Ura+ His+ transformants yielded cells expressing the GFPS-tagged version of β5 instead of the endogenous protein. The Ump1 deletion was created by chromosomal replacement of the *UMP1* open reading frame by *LEU2* (Ramos et al. 1998). The Blm10 deletion was created by chromosomal replacement of the *BLM10* open reading frame as described (Fehlker et al. 2003). Blm10 over-expression was driven by YEp-derived pTF155 (kindly provided by Joaquin Ortega) in galactose-fermenting WCGa (Iwanczyk et al., 2006). Flag-tagged Rpn4 was expressed from pRS314-RPN4-Flag (Xie and Varshavsky, 2001). The β7 subunit was identified as HA-tagged protein. To express HA-tagged β7 the C-terminal codons of YFR050c (*PRE4* without stop codon) were PCR-amplified, inserted intoYIp5-HA and cut by *BclI* for chromosomal integration. In order to delete amino acid residues 9 to 23 of α4, *PRE6* was modified by silent mutation. The PCR-amplified open reading frames of α4 and α4ΔN including the endogenous promoter were fused to GFP codons and inserted into pRS315. The resulting pRS315-α4-GFPHA and pRS315-α4Δ9-23-GFP were transformed into the respective shuffle strain

(kindly provided by Wolfgang Heinemeyer). Plasmid shuffling was induced by 5-fluororotic acid. Strains are listed in table S1.

GFP-labelling techniques facilitate the analysis of Blm10-associated CP

To facilitate the identification of CP associated with endogenous Blm10, we used a GFPS-tagged version of the β5 subunit. The replacement of endogenous β5 by the GFPS-tagged version was confirmed on chromosomal and protein level by PCR and immunoblot analysis using β5-specific antibodies, respectively. Pulse chase experiments revealed that the processing rates of tagged β5 proproteins agreed with previously reported CP maturation kinetics (Fehlker et al., 2003; Ramos et al., 1998). Glycerol gradient ultracentrifugation confirmed that tagged β5 is fully incorporated. Furthermore, *rpn4Δ* cells expressing tagged β5 instead of the endogenous subunit grew like parental *rpn4Δ* cells suggesting that the tag does not interfere with β5 function. Thus, genetic and biochemical analysis confirmed that the chromosomal replacement of β5 by the GFP-tagged version does not abrogate cell vitality (not shown).

As known from the literature, Blm10 over-expression from a multicopy plasmid behind the strongly inducible *GAL1* promoter resulted in significant CP activation (Iwanczyk et al, 2006; Schmidt et al, 2005). Therefore, we tested whether the same applies to cells expressing CP as GFP-labelled protein complexes. Indeed, Blm10-CP reconstituted from GFP-labelled CP and Blm10 showed enhanced peptide hydrolyzing activity. Again, peptide cleavage activity of CP appeared to be inhibited, when $Blm10_2$-CP were reconstituted (**Fig. S2A**).

Surprisingly, $Blm10_2$-CP was inefficiently formed in cells over-expressing Blm10. One explanation for this finding was that Blm10 over-expression is detrimental for cell viability (Fehlker et al, 2003; Iwanczyk et al, 2006), most likely because increasing amounts of Blm10 compete with RP for binding to CP and hence deplete the pool of RP-associated CP. To test

this, we resolved CP populations by native PAGE of total cell extracts. Wild type cells grown in complete medium harbour Blm10-CP-RP, RP-CP, Blm10-CP and free CP (Schmidt et al, 2005). For unknown reasons RP-CP-RP configurations are only formed upon growth in rich medium but not in complete medium. Upon over-expression of Blm10, the vast majority of CP was found within Blm10-CP, whereas the pool of RP-CP was strongly reduced. Both, the induced Blm10-CP formation and the depletion of RP-CP might account for the toxicity of Blm10 over-expression (**Fig. S2B**).

Description of the native PAGE for a mini gel system (preferentially Biorad)

A 1.5 mm thick 3.5 – 6% native PAGE gel (10 well comb) without stacking gel was poured by a gradient mixer. 1X native PAGE buffer P is a 1:5 dilution of 5X P: 0.45 M Tris base, 0.45 M boric acid, 10 mM $MgCl_2$ pH 8.1 ~ 8.4.

The 3.5% gel consists of 3.67 ml H_2O, 1.1 ml 5X P, 0.640 ml acrylamide (4K-solution, 30%, mix 37.5:1; Applichem), 27.5 µl 200 mM ATP, 27.5 µl 200 mM DTT, 27 µl 10% ammonium persulfate and 2.7 µl TEMED.

The 6% gel consists of 1.72 ml H_2O, 1.5 ml glycerol, 1.1 ml 5X P, 1.1 ml acrylamide (ibid), 27.5 µl 200 mM ATP, 27.5 µl 200 mM DTT, 22 µl 10% ammonium persulfate and 2.2 µl TEMED.

For each sample, 250 µg protein in buffer TB was loaded. The sample contained 5% glycerol and traces of bromine phenol blue. The gel was run in 1X P containing 2 mM ATP and 2 mM DTT over night at 45 Volt in the cold.

Supplementary Table S1: Strains used in this study

Name	genotype
CEY200	*MAT***a** *his3-11,15 leu2-3,112 ura3-52 BLM10-GFPHA-HIS3-URA3*[1]
CEY201	*MAT***a** *his3-11,15 leu2-3,112 ura3-52 PRE2-GFPS-HIS3-URA3*
CEY202	*MAT***a** *his3-11,15 leu2-3,112 ura3-52 PRE2-GFPS-HIS3-URA3 blm10Δ::HIS3*
CEY203	*MAT***a** *his3-11,15 leu2-3,112 ura3-52 can GAL PRE2-GFPS-HIS3-URA3 YEp-GAL1-BLM10* (pFT155)
CEY204	*MAT***a** *his3-11,15 leu2-3,112 ura3-52 PRE2-GFPS-HIS3-URA3 ump1Δ::LEU2*
CEY205	*MAT***a** *his3-11,15 leu2-3,112 ura3-52 PRE2-GFPS-HIS3-URA3 blm10Δ::HIS3 ump1Δ::LEU2*
CEY206	*MAT***a** *his3-11,15 leu2-3,112 ura3-52 PRE2-GFPS-HIS3-URA3 ump1Δ::LEU2 pre4::YIp5-PRE4-HA*
CEY207	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL PRE2-GFPS-HIS3-URA3 blm10Δ::kanMX*[2]
CEY208	*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL PRE2-GFPS-HIS3-URA3 blm10Δ::kanMX*[2]
CEY209	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GAL PRE2-GFPS-HIS3-URA3 rpn4Δ::kanMX*[2]
CEY210	*MAT***a** *his3-Δ200 lys2-801 leu2-3,112 trp1-1 ura3-52 PRE2-GFPS-HIS3-URA3*[3]
CEY211	*MATα ΔN-α3 his3-Δ200 lys2-801 leu2-3,112 trp1-1 ura3-52 PRE2-GFPS-HIS3-URA3*[3]
CEY212	*MATα ΔN-α7 his3-Δ200 lys2-801 leu2-3,112 trp1-1 ura3-52 PRE2-GFPS-HIS3-URA3*[3]
CEY213	*MATα ΔN-α3/α7 his3-Δ200 lys2-801 leu2-3,112 trp1-1 ura3-52 PRE2-GFPS-HIS3-URA3*[3]
CEY214	*MAT***a** *his3-11,15 leu2-3,112 ura3-52 pre6Δ::HIS3* [pRS315-PRE6-GFPHA]
CEY215	*MAT***a** *his3-11,15 leu2-3,112 ura3-52 pre6Δ::HIS3* [pRS315-PRE6ΔN-GFPHA]
CEY216	*MAT***a** *blm10Δ::kanMX pre6Δ::HIS3* [pRS315-PRE6ΔN-GFPHA]
CEY217	*MATα ΔN-α3/α7 blm10Δ::TRP1 PRE2-GFPS-HIS3-URA3*
CEY219	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PRE2-GFPS-HIS3-URA3*[4]
CEY218	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pac3/poc3::HPHMX4 pac4/poc4::KANMX4 PRE2-GFPS-HIS3-URA3*[4]
CEY219	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2* [5]
CEY220	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2 pac1/pba1Δ::kanMX pac2/pba2Δ::natMX* [5]

References:

1) Lehmann et al. 2002
2) EUROSCARF
3) Groll et al., 2000
4) LeTallec et al. 2007
5) Li et al. 2007

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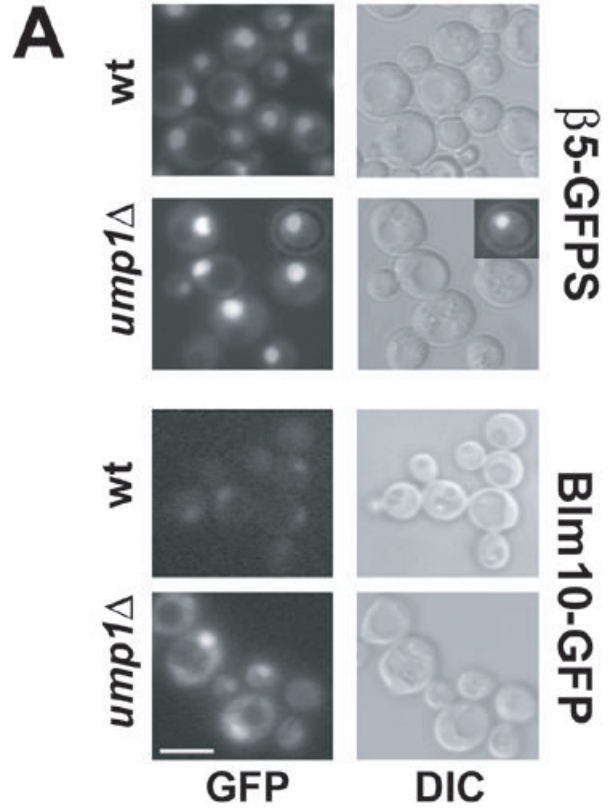
Supplementary Figure Legends

Fig. S1. Blm10 expression is augmented by Rpn4 and strongly induced in *ump1Δ* cells. (**A**) Wild type and *ump1Δ* cells with primarily nuclear localized GFP-tagged β5 or Blm10 (Fehlker *et al*, 2003) were monitored by direct fluorescence microscopy (GFP) and Nomarski optics (DIC). Digital images were taken with same exposure times. The insert shows nuclear 4,6-diamidino-2-phenylindole-staining of a yeast cell, as visualized by UV light and superimposed Nomarski optics. Scale bar, 3 μm. (**B**) Protein levels of Blm10, β5-GFPS, Rpn2 (base RP) and Rpn11 (lid RP) of wild type (lane 1), *ump1Δ* (lane 2), *blm10Δ* (lane 3) and *blm10Δ ump1Δ* (lane 4) cells were detected by immunoblot. Unprocessed pro-β5 and mature β5 are found in *ump1Δ* cells as described (Ramos *et al*, 1998). (**C**) Rpn4 levels of wild type and *ump1Δ* cells were analyzed by Western blot. Blm10 levels were determined in wild type, *rpn4Δ* and *blm10Δ* cells. Kar2 is used as loading control.

Fig. S2. (**A**) **Blm10-CP and Blm10$_2$-CP are reconstituted with GFP-labelled CP**. Affinity-purified CP with endogenous Blm10 (lane 1), CP with over-expressed Blm10 (lane 2), and

purified Blm10 (lane 3) were resolved by SDS-PAGE / Coomassie blue staining. The asterisk marks the heat shock protein hsp70. Blm10 and GFP-labelled CP were incubated in a 1:1 molar ratio and resolved by native PAGE followed by GFP imaging and in-gel activity assay using the chromogenic peptide Suc-LLVY-AMC (lane 4). Purified CP (lane 5) and Blm10 (lane 6) were run as controls. Ten molar excess of Blm10 allows the reconstitution of $Blm10_2$-CP (lanes 7 and 8). (**B**) **Blm10 over-expression results in single-capped Blm10-CP and the depletion of RP-associated CP**. Wild type, *blm10Δ* and Blm10-over-expressing cells with GFP-labelled CP were grown in synthetic medium to logarithmic phase. Fresh cell extracts were subjected to native PAGE. CP were visualized by GFP imaging (lanes 1 to 3). RP-associated CP and Blm10-associated CP were identified by Western blot using RP- and Blm10-specific antibodies (anti-RP blot not shown; lanes 4 to 6). CP configurations are assigned.

Fig. S3. CP activities were determined by means of the ratio of the AMC / GFP pixel intensities within the $Blm10_2$-CP, Blm10-CP and CP bands from wild type lysates resolved by native PAGE (Fig. 2G, upper panel). The pixel intensities of the GFP moieties were counted and are a measure of the CP amount. The pixel intensities of the chromophores (AMC) cleaved off the peptide substrate Suc-Leu-Leu-Val-Tyr-AMC are a measure of the CP activity. The average of the pixel intensities of the chromophores (AMC) were referred to the average pixel intensities of the GFP moieties resulting in arbitrary units. Background was subtracted. Three samples / native PAGEs yielded similar results.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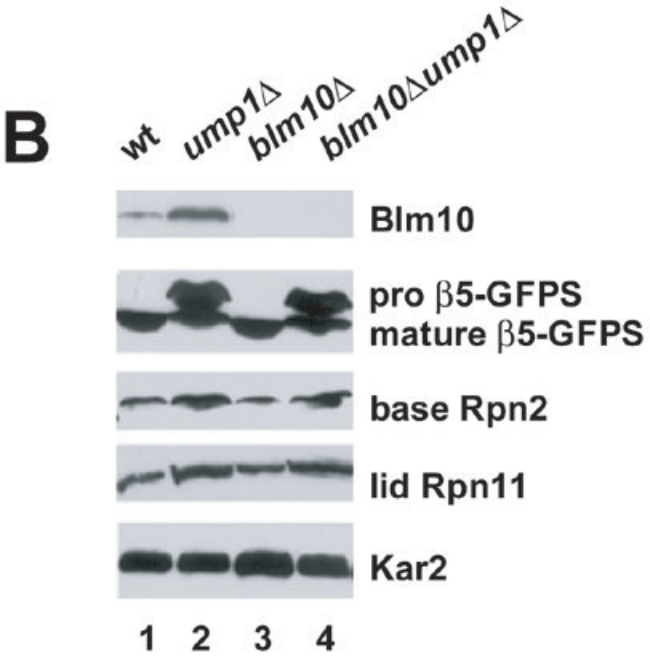
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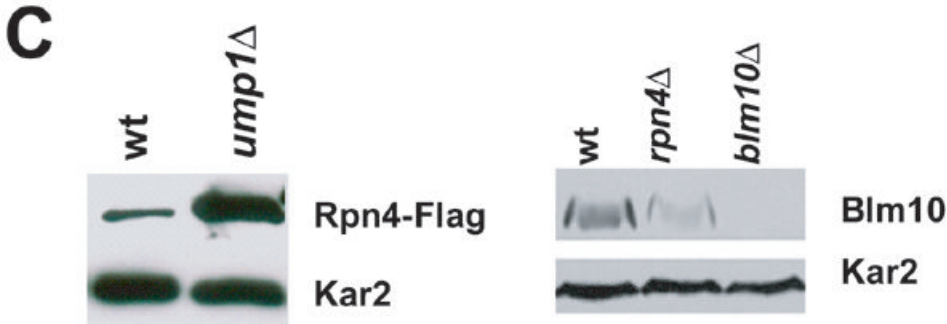
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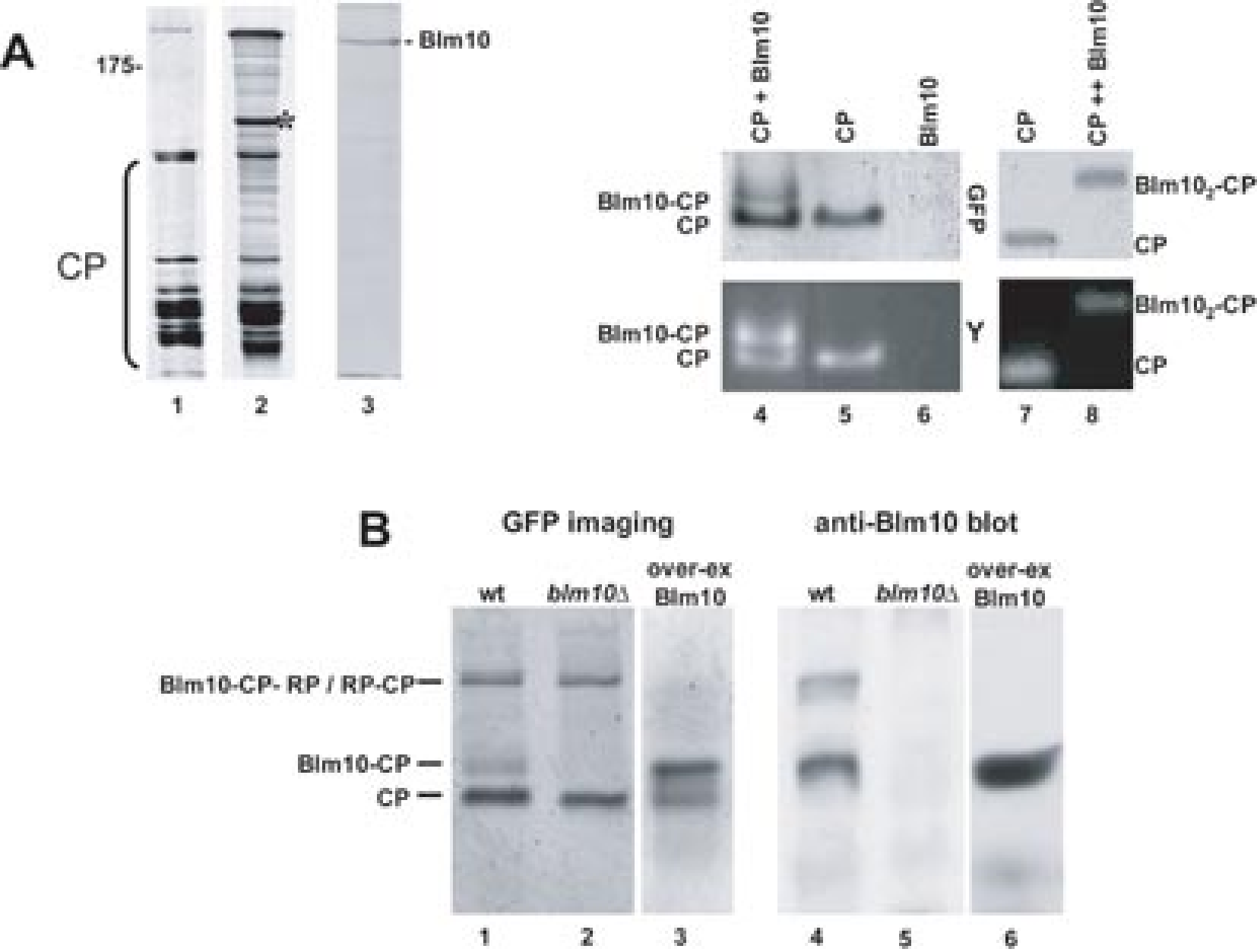
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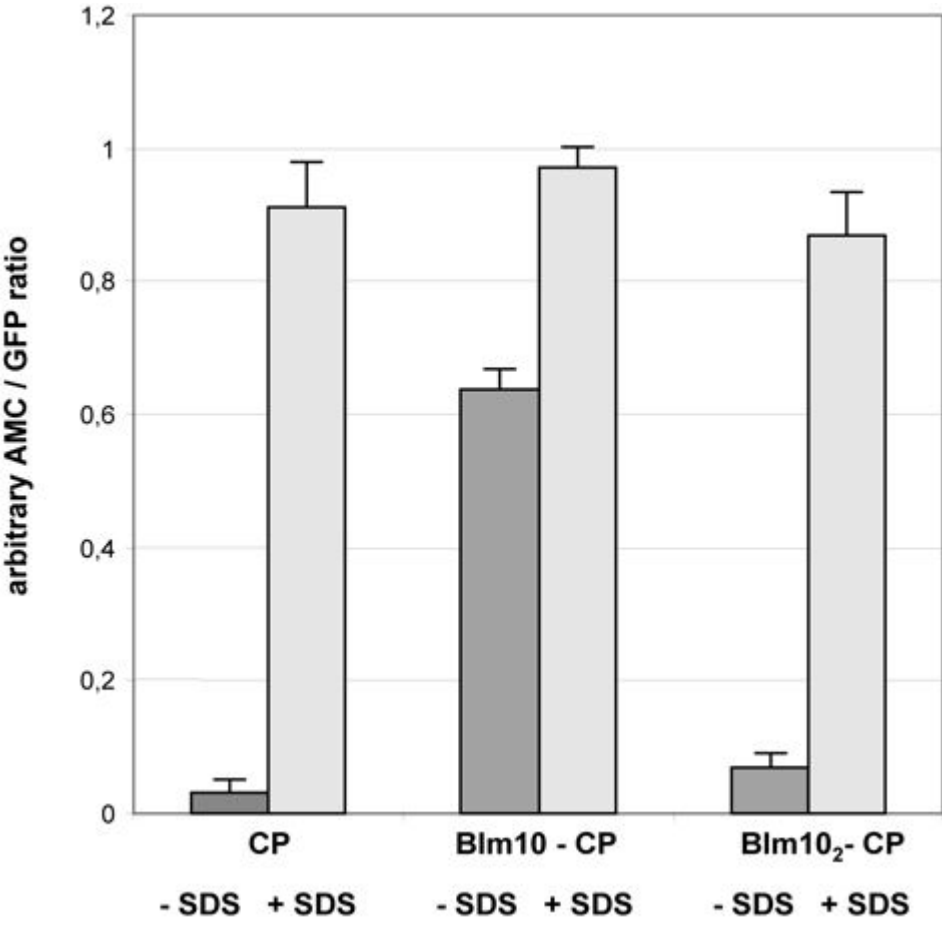
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- SDS + SDS
- SDS + SDS