



**Figure S1: Truncation analysis of the bdSUMO/bdSEN1 substrate/protease pair**

Fusion proteins consisting of a N-terminal maltose-binding protein (MBP), a protease recognition site (PRS; here bdSUMO) and the respective protease (here bdSEN1) harboring truncations at defined positions (A) were expressed in *E. coli*. In this assay, an *in vivo* cleavage of the fusion protein indicates a decent functionality of both the protease recognition site and or the respective protease.

A, General layout of the constructs used in B. Truncations at the indicated positions were introduced by site-directed mutagenesis and verified by DNA sequencing.

B, Analysis of in-vivo cleavage efficiency of substrate/protease pairs harboring defined truncations. Indicated constructs were expressed in *E. coli* for 1.5h at 37°C. Lysates prepared by boiling in SDS sample buffer were analyzed by SDS-PAGE and Coomassie staining. Separate panels show cleavage assays using N-terminally truncated bdSUMO (left panel), N-terminally truncated bdSEN1 (middle panel), and C-terminally truncated bdSEN1 (right panel), respectively. Amino acid numbers refer to the respective full-length proteins.

Note that bdSUMO<sup>83-97</sup> and bdSEN1<sup>288-477</sup> are sufficient for a basal activity of the bdSUMO/bdSEN1 system while proper cleavage and stability of the proteins requires bdSUMO<sup>21-97</sup> and bdSEN1<sup>248-481</sup>.