Supplementary information

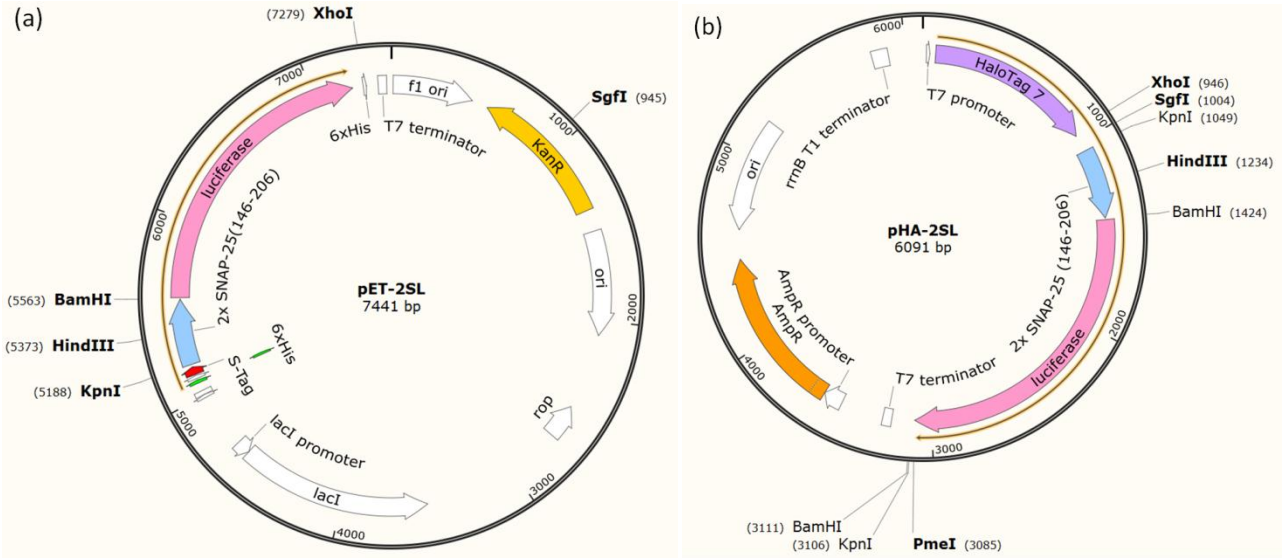

Fig. S1 Restriction digest map of (a) pET-2SL and (b) pHA-2SL showing the location of restriction sites used in constructing the plasmids. These maps were produced with SnapGene Viewer Version 1.1.2 (GSL Biotech LLC.).

Construction of plasmids and expression of fusion proteins

pET-L

Plasmids pET-30c(+) (Merck) and pGEM-luc (Promega) were digested using *Bam*HI and *Xho*I enzymes in Buffer D (Promega), and the digestion products separated by gel electrophoresis on 1% agarose. Bands at 5,383 and 1,716 bp were cut from the gel, purified with Wizard SV Gel and PCR Clean-Up System (Promega) and ligated using T4 DNA ligase in 2X Flexi Ligase Buffer (Promega) to create a ligation product called pET-L.

Single Step (KRX) Competent *E. coli* cells (Promega) were transformed with pET-L. After overnight growth on 1.5 % agar with 30 µg kanamycin/mL, clones were picked out and grown in Terrific Broth (1.2% w/v Bacto-Tryptone, 2.4% w/v Yeast Extract, 0.4% v/v glycerol, 10% v/v 0.89 M Potassium Phosphate pH 7.8, in deionized water) and kanamycin (30 µg/mL). The plasmids were purified using the Miniprep System (Promega), digested with *Xho*I and *Bam*HI restriction enzymes (Promega), and the products identified by gel electrophoresis. Fig. S2 shows *Kpn*I/*Xho*I digestion products of pET-L. The bands for pET-L (lane 2) were at the expected positions for 5,342 and 1,757 bp DNA.

Expression of luciferase was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) and rhamnose (final concentrations: 1 mM and 0.001% respectively) to the cell culture. After several hours incubation at 25 °C, 25 µL BrightGlo Luciferase Assay (Promega) was mixed with 10 µL cell culture in a well of a white 96 micro plate and the luminescence measured. Bioluminescence from these clones was significantly above those of negative controls, demonstrating that the luciferase CDS was in the correct reading frame.

pET-1SL and pET-2SL

Human SNAP-25 protein coding region (Gen Bank #AM393653) was derived from ORF Shuttle Clone OCAAo5051G0517D (Source BioScience LifeSciences, Berlin, Germany). DH10B *E. coli* with pENTR221 containing ORF Shuttle clone AM393653 was grown overnight in LB media with 30 µg/mL kanamycin. The plasmid was purified using PureYield Plasmid Miniprep System (Promega).

For inserting one sequence of SNAP-25 amino acids 146-206 sequence between the *Kpn*I and *Bam*HI restriction sites of pET-L, pENTR221 with AM393653 was amplified by PCR with forward primer 5' GAC TGG TAC CAT GGA TGA AAA CCT AGA G 3' and reverse primer 5' AGT CGG ATC CCA CCA CTT CCC AGC ATC T 3'. This provided the protein coding region for human SNAP-25(146-206) with *Kpn*I and *Bam*HI restriction sites on the 5' and 3' ends respectively.

For inserting two SNAP-25(146-206) sequences between the *Kpn*I and *Bam*HI restriction sites of pET-L, pENTR221 with AM393653 was amplified by PCR with forward primer 5' GAC TGG TAC CAT GGA TGA AAA CCT AGA G 3' and reverse primer 5' CCC CAA GCT TAC CAC TTC CCA GCA TCT T 3', and with forward primer 5' GCC CAA GCT TAT GGA TGA AAA CCT AGA G 3' and reverse primer 5' AGT CGG ATC CCA CCA CTT CCC AGC ATC T 3'.

This provided the protein coding region for SNAP-25(146-206) with *Kpn*I and *Hind*III, and *Hind*III and *Bam*HI restriction sites on the 5' and 3' ends respectively. These PCR products were purified and then digested with *Hind*III in Buffer B (Promega), purified by gel electrophoresis and ligated to form a DNA sequence encoding two SNAP-25(146-206) sequences in series, with *Kpn*I and *Bam*HI restriction site at the 5' and 3' ends respectively. Purified pET-L and inserts encoding one or two sequences of SNAP-25(146-206) were digested with *Kpn*I and *Bam*HI in Multicore buffer (Promega).

pET-L was analyzed by gel electrophoresis and a band at 7,058 bp was cut out and purified using the Wizard SV Gel and PCR Clean Up System (Promega).

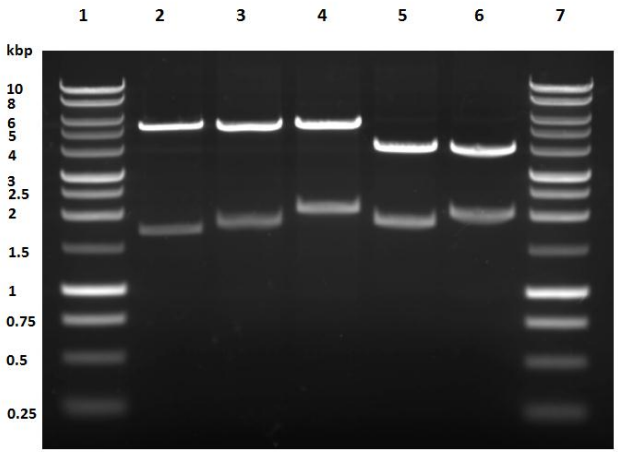

Fig. S2. Gel electrophoresis of plasmids pET-L (lane 2), pET-1SL (lane 3) and pET-2SL (lane 4) after digestion with *Kpn*I/*Xho*I, and pHA-1SL (lane 5) and pHA-2SL (lane 6) after digestion with *Sgf*I/*Pme*I. The gel was 1% agarose, stained with 0.5 µg/mL ethidium bromide. Bench Top 1 kb DNA Ladder (G7541, Promega, lanes 1 and 7).

The digested DNA inserts were purified with the Wizard system and ligated with the 7,058 bp DNA to form products called pET-1SL and pET-2SL (Fig. S1(a) shows the restriction map of pET-2SL).

Single Step (KRX) Competent *E. coli* cells (Promega) were transformed with pET-1SL and pET-2SL. After overnight growth on 1.5 % agar with 30 µg kanamycin/mL, clones were picked out and grown in Terrific Broth (TB) with 30 µg kanamycin/mL. The plasmids were purified using the Miniprep System (Promega), digested with *Xho*I and *Xho*I restriction enzymes, and the products identified by gel electrophoresis. Fig. S2 shows *Kpn*I/*Xho*I digestion products. The bands for pET-1SL (lane 3) are at the expected positions for 5,350 and 1,902 bp, and for pET-2SL (lane 4) are at expected positions for 5,350 and 2091 bp.

Protein expression was induced in Single Step (KRX) competent *E. coli* containing pET-1SL or pET-2SL, and expression of luciferase was induced in the cell culture as for pET-L. The presence of luciferase, detected as for pET-L, was found to increase as the cultures grew, indicating that the luciferase protein coding region was in the correct reading frame for both plasmids.

pHA-1SL and pHA-2SL

To create plasmid pHA-1SL, pET-1SL was amplified by PCR with forward primer 5' AGT GGC GAT CGC CAA ATT CGA ACG CCA GCA CAT GGA CAG CCC AGA TCT GGG TAC C 3' and reverse primer 5' TAC GGT TTA AAC CAA TTT GGA CTT TCC GCC CTT CTT GGC CTT TAT GAG GAT CTC T 3'. This provided a protein coding region for human SNAP-25 amino acids 146-206 and luciferase with *Sgf*I and *Pme*I restriction sites on the 5' and 3' ends respectively.

To create plasmid pHA-2SL, Fig. S1(b), pET-2SL was amplified by PCR with forward primer 5' AGT GGC GAT CGC TAA ATT CGA ACG CCA GCA CAT GGA CAG CCC AGA TCT GGG TAC C 3' and reverse primer 5' TAC GGT TTA AAC CAA TTT GGA CTT TCC GCC CTT CTT GGC CTT TAT GAG GAT CTC T 3'. This provided a protein coding region for two sequences of human SNAP-25 amino acids 146-206 and luciferase with *Sgf*I and *Pme*I restriction sites on the 5' and 3' ends respectively.

The inserts and pFN-18A Flexi Vector (Promega) were digested with *Sgf*I and *Pme*I in Flexi Digest Buffer (Promega) and ligated according to the manufacturer's instructions and Single Step (KRX) Competent *E. coli* cells (Promega) were transformed with the ligation products. After overnight growth on 1.5 % agar with 100 µg/mL ampicillin, clones were picked out and grown in Terriffic Broth with 100 µg/mL ampicillin. The plasmids were purified using the Miniprep System (Promega), digested with *Sgf*I and *Pme*I restriction enzymes (Promega), and the products identified by gel electrophoresis. Fig. S2 shows *Sgf*I/*Pme*I digestion products. The bands for pHA-1SL (lane 5) are at the expected positions for 4,010 and 1,892 bp DNA, and for pHA-2SL (lane 6) are at expected positions for 4,010 and 2,081 bp DNA.

Expression of luciferase was induced as for pET-L, and the presence of luciferase, detected as for pET-L, was found to increase as the culture grew, indicating that the luciferase protein coding region was in the correct reading frame for both plasmids.

Cleavage products of ET-2SL

The conclusion from the western blot analysis of HA-2SL before and after digestion with LC/A1, Fig, 4, was that the rate of cleavage on the polypeptide linker was higher at cutting site '2' than at site '1' (see Fig 1). This was based on the observation that the luciferase-containing digestion product was 62 kDa in size. This observation is supported by the SDS-PAGE analysis of the digestion products shown in Fig. S3(a), in which a single band below 66 kDa is seen after incubation with LC/A1.

To test whether the higher rate of cleavage at cutting site '2' is due to steric hindrance of the HaloTag, an analysis of cleavage products after LC/A1 digestion of a protein construct (ET-2SL) was made. The new construct is identical to HA-2SL except that the 34 kDa HaloTag is replaced with a 3 kDa combined 6xHis and S-tag affinity tag. The protein ET-2SL was expressed in competent KRX *E. coli* cells and after overnight culture, the cells were lysed and the protein isolated from the lysate on an NiNTA column.

After 30 minutes RT incubation with LC/A1, the digestion products were analysed by SDS-PAGE, shown in Fig. S3(b). The educt is above the 66 kDa marker, which is expected for the 79 kDa ET-2SL, and after incubation with LC/A1, this band was replaced with a band just below the 66 kDa band of the marker. This band is assigned to the luciferase digestion product after cleavage at the site closest to the luciferase with an expected size of 62 kDa. As with HA-2SL, a 69 kDa digestion product was not visible.

Two bands, below the 20 kDa and 14 kDa markers, are assigned to the digestion products containing the affinity tags after the linker wss cleaved at cutting sites closest to the affinity tags (10 kDa product) or luciferase, (17 kDa product). As with HA-2SL, the pattern of digestion can only be explained by preferential cleavage at the cutting site closest to the luciferase, rather than at the site closest to the tag. The observation of preferential cleavage at the cutting sites closest to the luciferase with the smaller tag, suggests that the reason for preferential cleavage at site '2' on HA-2SL is other than steric hindrance of the HaloTag.

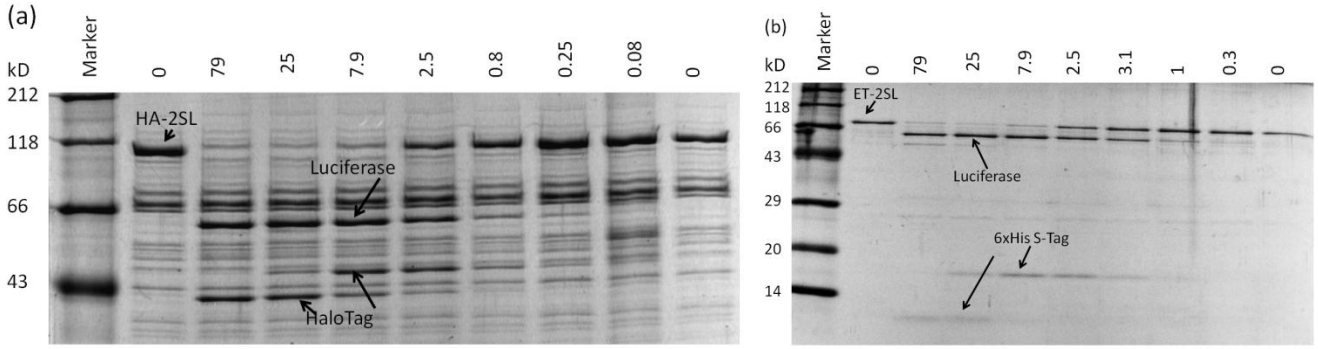

Fig. S3 Reducing SDS PAGE of (a) HA-2SL, and (b) IMAC purified ET-2SL after incubation with LC/A Hydrilysis Buffer with LC/A1 (concentrations in nM). Lanes containing the educt are marked '0' and cleavage products are marked with LC/A1 concentrations (in nM) above each lane. The assignments of the bands are indicated. Gels were 4% stacking and (a) 10% or (b) 15% resolving. The marker is Roti-Mark Standard (T852, Carl Roth).

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