

Chapter 2

Experimental Methods

Unless otherwise stated, chemical reagents were purchased from Sigma-Aldrich and molecular biology reagents and enzymes were purchased from New England Biolabs (NEB). Solution recipes are found in Appendix B.

2.1 Cell Culture

2.1.1 Prokaryotic Cells

Bacterial cell cultures were grown in Luria Bertani (LB) broth and shaken at 37 °C at 250 rpm. *Escherichia coli* (*E. coli*) DH5 α cells (Invitrogen) were used to maintain vectors, BL21(DE3) (Stratagene) cells were used as expression hosts. Glycerol stocks were prepared by resuspending the cell pellet in LB broth with 20 % glycerol. Stocks were stored at –80 °C.

2.1.1.1 Transforming Chemically Competent *Escherichia coli* Cells

DH5 α and BL21 cells were transformed with 1–10 ng of DNA by heat-shock. Two different volumes of transformed cells were spread onto selective agar plates. Non-transformed cells were also spread as a negative control and pUC19-transformed cells were grown on ampicillin-agar plates as a positive control. Positive and negative controls confirmed the transformation competency and antibiotic stock viability, respectively.

2.1.2 *Eukaryotic Cells*

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 1% Penicillin-Streptomycin. Cells were grown at 37°C in 5% CO₂. Cells were passaged when approximately 80% confluency was reached. Old media was removed and cells were washed twice with phosphate-buffered saline solution (PBS). Detachment from the growing surface was achieved by incubation for 5 min at 37°C in 1 mL Trypsin-EDTA prior to gentle agitation to further dislodge cells. Cells were resuspended in 5 mL DMEM and 1 mL of this solution was added to 20 mL of fresh DMEM and incubated at 37°C.

2.1.2.1 Transfection of HeLa Cells

HeLa cells were transfected using either FuGENE (Roche) or Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Both were used in combination with OptiMEM reduced-serum media (Invitrogen). After transfection, cells were incubated at 37°C overnight.

2.1.3 *Apoptosis Assays*

Either MTT (Novagen) or AlamarBlue (Invitrogen) were used as per the manufacturers instructions.

2.2 Molecular Biology

2.2.1 *DNA Quantitation*

DNA concentration was calculated by measuring UV absorbance from 230–320 nm. Reference samples were 5 mM Tris-HCl pH 8. The concentration was calculated using the formula:

$$[\text{dsDNA}] = OD_{260} \times 50 \times \text{dilution factor} \quad (2.1)$$

DNA quality was assessed by comparing the A_{260} and A_{280} values:

$$\text{Quality}_{DNA} = \frac{A_{260}}{A_{280}} \quad (2.2)$$

A value of 1.5–2 was judged as pure.

2.2.2 Plasmid Purification

Plasmid DNA was purified using QIAGEN Plasmid Purification kits (Mini or Maxi). Volumes of buffer used were as recommended by the manufacturer.

2.2.3 Agarose Gels

Agarose gels were made by dissolving agarose powder in TBE buffer to the required concentration (between 0.8–1.5 %). Gels were run in TBE buffer at 100 V and visualised over a UV lamp after staining with Ethidium Bromide or GelRed™ (Biotium). The QIAGEN QIAquick Gel Extraction kit was used to purify DNA from the gel following the manufacturer's instructions.

2.2.4 Restriction Digests and Vector Ligations

Digestion by restriction enzymes was performed as recommended by NEB, adding 0.1 mg/mL Bovine Serum Albumin (BSA) as standard. Double digests were designed to function in the same buffer unless otherwise stated. Digestion time was 1–1.5 h, followed by denaturation at either 65 or 80 °C for 20 min and purification using QIAGEN QIAquick PCR purification columns or gel extraction.

The following formulae were used to calculate the amount of vector and insert required for ligation.

Molecular weight of DNA:

$$Mr_{DNA} = N^{\circ} \cdot \text{bp} \times 660 \quad (2.3)$$

Concentration of DNA in fmoles/μL:

$$C_{DNA}(b) = \frac{\text{mass}_{DNA}(\text{ng}/\mu\text{L})}{Mr_{DNA}} \times 10^6 \quad (2.4)$$

For ligation, 30 fmoles each of vector and insert were added as standard. Incubation with 1 unit T4 DNA Ligase varied from 3 h at room temperature (21 °C) or 4 °C overnight for sticky-ended ligation, to 16 °C overnight for blunt-ended ligations. Ligase was not deactivated before transformation.

2.2.5 PCR, Gradient PCR and Colony PCR

Primers were designed by hand, verified using FastPCR (Primer Digital Ltd.) and synthesised by Eurofins MWG Operon (Ebersberg, Germany). Taq DNA polymerase

was used for analytical PCR, whereas Vent_R DNA polymerase was used when the product was to be sequenced or ligated. PCR generally followed the thermal cycle: initial melting 94 °C for 5 min, then; 30 × 94 °C for 1 min, T_m − 5 °C for 45 s, 72 °C for 1 min, followed by final extension at 72 °C for 5 min. Gradient PCR used a similar cycle with annealing temperature varied from T_m ± 10 °C, and the magnesium concentration varied from 2–6 mM. Colony PCR was performed to identify colonies with the correct ligation product. Selected colonies were picked with a sterile tip, swirled in deionised H₂O and deposited in selective LB Broth for culture. Samples were boiled at 99 °C for 5 min, centrifuged and 10 µL of the supernatant used for PCR. Purification of PCR samples was performed with PCR column purification kits (QIAGEN).

2.3 Gene Synthesis and Subcloning

Primer sequences are provided in Appendix B in the manner and order as they appear in the text.

2.3.1 *Bacterial Expression Plasmids*

To confirm successful subcloning of genes, each vector was sequenced by Eurofins MWG Operon (Ebersberg, Germany) using the Sanger dideoxy sequencing method. To simplify purification a His₆ tag was added to fusion proteins.

2.3.1.1 His₆-Tagged EGFP

A His₆ tag was added to fusion proteins to enable one-step purification. pET-45b(+) (Novagen) encodes an N-terminal His₆ sequence followed by a multiple cloning site (MCS). EGFP was subcloned into pET-45b(+) from pEGFP-C1 (Clontech) using the forward primer *His EGFP FWD*, which adds an MfeI site (underlined, Appendix B) before the EGFP start codon, and the reverse primer *His EGFP REV*, which retains the XhoI site (underlined) present in the MCS. Both insert and vector were digested with MfeI and XhoI and ligated as described to create pET-45b(+)-EGFP (Fig. 2.1).

2.3.1.2 His₆-Tagged EGFP-MT2

To create an inducible EGFP-MT2 fusion protein, two MT genes were subcloned adjacent to one another within the original pET-3d vector before subcloning the whole MT2 domain into pET-45b(+)-EGFP after the EGFP gene.

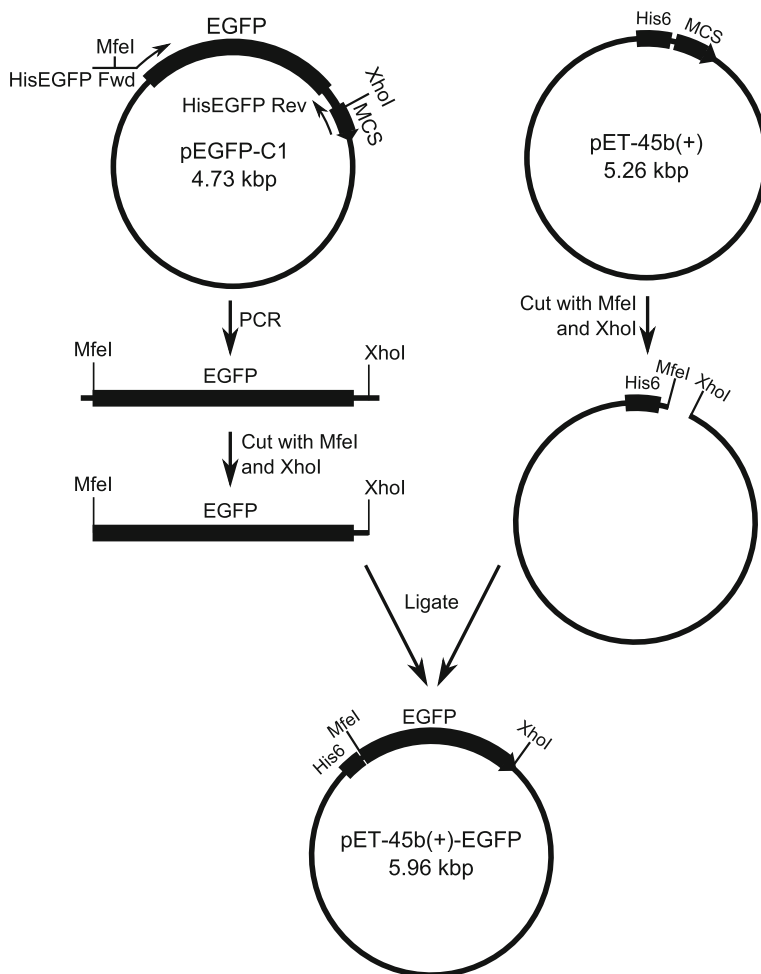


Fig. 2.1 Production of pET-45b(+)-EGFP, encoding a His₆-tagged EGFP fusion protein. PCR was performed with the primers *HisEGFP Fwd* and *HisEGFP Rev*. Both vector and insert were digested with MfeI and XhoI

The mouse MT-1 gene was amplified from pET-3d-MT (a gift from Prof. Winge, University of Utah) by gradient PCR using the forward primer *T7 Prom*, which retains the ribosome binding site, and the reverse primer *pET-3d-MT2 REV*, which adds a BspHI restriction site (underlined) and removes the MT stop codon. The insert was digested with XbaI and BspHI. The vector, pET-3d-MT, was digested with XbaI and NcoI. BspHI and NcoI generate complementary overhangs and ligate with the removal of both recognition sites. The insert was subcloned before the existing MT gene with no linker between the two domains to form pET-3d-MT2 (Fig. 2.2). This procedure was repeated to produce higher-order concatenations as necessary.

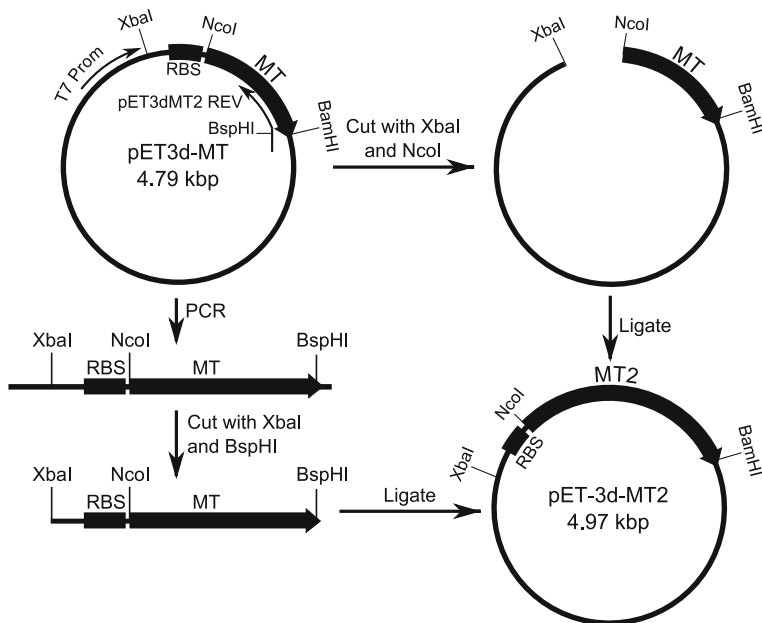


Fig. 2.2 Production of pET-3d-MT2. PCR was performed with the primers *T7 Prom* and *pET3dMT2 REV*. The vector was digested with XbaI and NcoI. The insert was digested with XbaI and BspHI. RBS; ribosome binding site

The MT2 domain was then inserted into pET-45b(+)-EGFP after the EGFP gene. The MT2 domain was amplified from pET-3d-MT2 by PCR using the forward primer *pET45-link-MT2*, which has homology to the DNA sequence preceding the MT2 domain to avoid amplifying only a single MT domain, and adds a BsrGI restriction site (underlined), and the reverse primer *T7 Term*. The insert was digested with BsrGI and BamHI. The vector, pET-45b(+)-EGFP, was digested with BsrGI and BglII. BamHI and BglII generate complementary overhangs and ligate with the removal of both recognition sites, creating pET-45b(+)-EGFP-MT2-C1 (Fig. 2.3).

2.3.1.3 His₆-Tagged EGFP-MT2-SNX1

Purifiable EGFP-MT2-SNX1 (EMS1) for membrane tubulation was achieved by cloning MT2-SNX1 into pET-45b(+)-EGFP to create pET-EGFP-MT2-SNX1 (pET-EMS1). This was achieved by first forming pEGFP-BsiWI-MT2-SNX1 by digesting pEGFP-MT2-C1 and pEGFP-SNX1 (for eukaryotic plasmids please see below) with AgeI and BsrGI prior to gel purification and ligation. This was then digested with BsiWI and Sall to yield sticky ended MT2-SNX1 whilst pET-45b(+)-EGFP was digested with BsrGI and XhoI. These were ligated without purification to yield pET-EMS1.

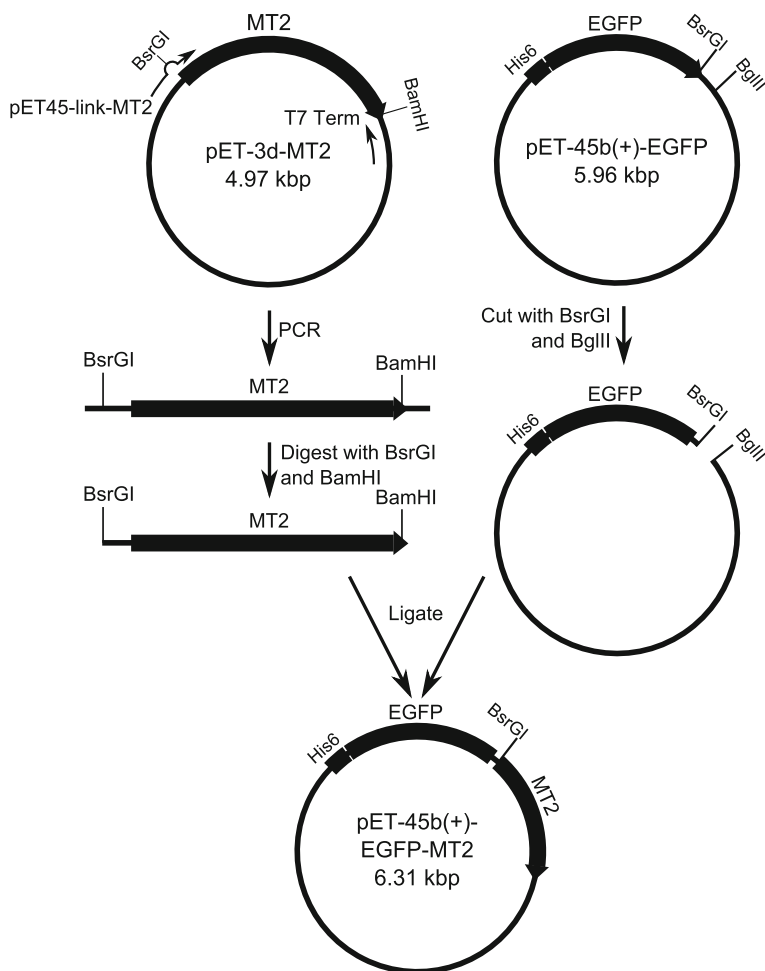


Fig. 2.3 Production of pET-45b(+)-EGFP-MT2, encoding a His₆-tagged EGFP-MT2 fusion protein. PCR was performed with the primers *pET45-link-MT2* and *T7 Term*. The vector was digested with BsrGI and BglII. The insert was digested with BsrGI and BamHI

2.3.2 Design and Synthesis of an MT2 Domain and *pEGFP-MT2-C1*

We required a concatenated MT domain to increase clustering of heavy-metal ions to form electron-dense nanoparticles. However a tandem gene fusion containing two identical sequences is undesirable due to potential homologous recombination and secondary structure elements interfering with PCR and expression. For this reason, a non-identical MT2 domain was designed using Gene Designer (DNA2.0, inc., California) and synthesised by GENEART (Regensburg, Germany) for insertion into

1	M	D	P	N	C	S	C	S	T	G	G	S	C	T	C	T	S	S	C	A
1	ATGGACCCTAATTGTTCTCTACTGGTGGGAGTTGCACTTGTACCTCAAGCTGTGCT																			
21	C	K	N	C	K	C	T	S	C	K	K	S	C	C	S	C	C	P	V	G
61	TGTA AAAA ACTGCAAGTGCACGTCTTGTAAGAAGAGCTGTTGCTCATGTTGCCCAGTAGGG																			
41	C	S	K	C	A	Q	G	C	V	C	K	G	A	A	D	K	C	T	C	C
121	TG TAGCAAATGCGCCCAGGGGTGCGTATGTAAAGGCGCAGCCGACAAATGTACCTGCTGT																			
61	V	M	D	P	N	C	S	C	S	T	G	G	S	C	T	C	T	S	S	C
181	GTCATGGACCCGA ACTGTAGCTGTAGCACTGGCGGGAGCTGTACTTGCACCAGTTCCTGT																			
81	A	C	K	N	C	K	C	T	S	C	K	K	S	C	C	S	C	C	P	V
241	GCCTGCAAGAACTGTAAGTGCACATCATGTAAGAAAAGCTGCTGTTCTTGCTGTCCCGTG																			
101	G	C	S	K	C	A	Q	G	C	V	C	K	G	A	A	D	K	C	T	C
301	GGTTGCAGTAAATGCGCACAGGGCTGTGTATGCAAAGGGGCTGCCGATAAGTGCACCTGT																			
121	C	A																		
361	TGCGCA																			

Fig. 2.4 Sequence of the synthetic MT2 gene and protein. Amino acids are above the corresponding DNA codons

pEGFP-C1 (Fig. 2.4). The protein sequence of MT-1 was placed head-to-tail with no linker spacer. The protein sequence was then back-translated to DNA with the following restrictions; all repeat sequences longer than 10 bp were removed, all RE sites also found in pEGFP-C1 multiple cloning site (MCS) were removed, the BsrGI site found in pEGFP-C1 was moved to 3' end of MT2 but kept in same reading frame, a BsiWI (which has an overhang identical to BsrGI) site was added at 5' end of the MT2 domain (in same frame as BsrGI, for cutting out MT2 domain or swapping EGFP for EYFP, ECFP, etc), and the stop codon of MT was removed. Codon optimization was performed for human cell expression. For insertion into pEGFP-C1, a BbsI site was also added to the 5' end of the insert that produces a 4 bp overhang downstream of the recognition site which was designed to be complimentary to that produced by BsrGI, but forms no recognition site for any restriction endonuclease. The fragment was cloned into pEGFP-C1, digesting the insert with BbsI and BsrGI and the vector with BsrGI with dephosphorylation, concomitantly removing the BbsI site whilst retaining BsrGI (Fig. 2.5).

2.3.3 *pEGFP-MT2-Tagged Proteins*

For eukaryotic expression, EGFP-MT2 was fused to various proteins that localise to specific regions of the cell within expression vectors. In each case, expression was under the control of the human cytomegalovirus (CMV) immediate early promoter.

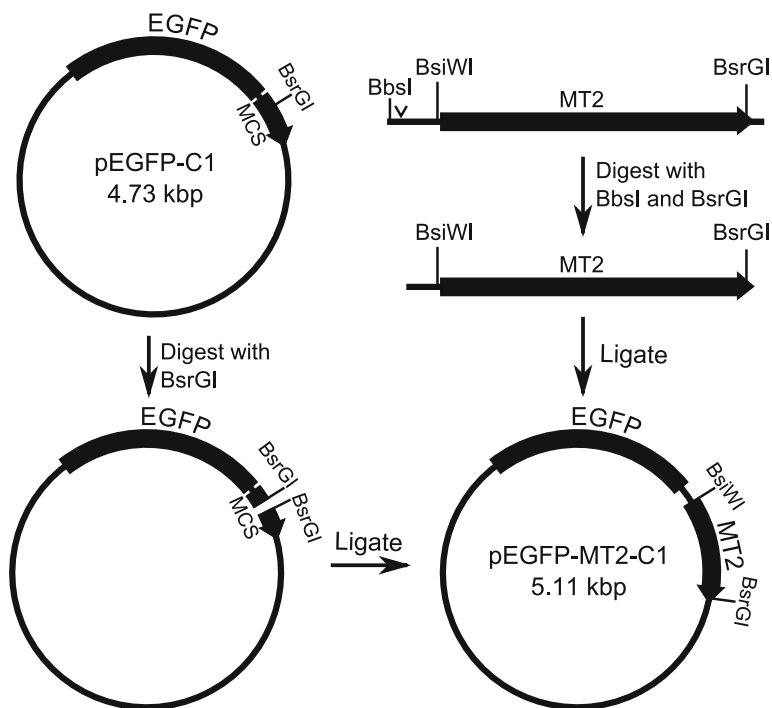


Fig. 2.5 Subcloning strategy to produce pEGFP-MT2-C1. The synthetic MT2 domain was digested with BsrGI and BbsI to leave a complementary overhang to BsrGI. The vector was digested with BsrGI and dephosphorylated before ligation of the fragments

pEGFP-Sec23A (a gift from Dr. David Stephens, University of Bristol) encodes a COPII coat component Sec23A fused to EGFP. pEGFP-Sec23A and pEGFP-MT2-C1 were digested with AgeI and BsrGI prior to purification by gel electrophoresis. The fragments were ligated as described above to form pEGFP-MT2-Sec23A.

pEGFP-SNX1 (a gift from Dr. Jan van Weering, University of Bristol) encodes sorting-nexin 1 (SNX1), which tubulates early endosomal compartments, fused to EGFP. pEGFP-SNX1 was digested with BglII and BsrGI whilst pEGFP-MT2-C1 was digested with BglII at 37 °C for 1 h, before BsiWI was added for a further incubation at 55 °C for 1 h. The fragments were isolated by gel purification prior to ligation to form pEGFP-MT2-SNX1.

pEGFP-tub (Clontech), encoding a fusion protein of EGFP and human α -tubulin, and pEGFP-MT2-C1 were digested with BsrGI and NheI prior to purification by gel electrophoresis and ligation to form pEGFP-MT2-Tubulin.

pCR3.1-Tetherin-GFP (a gift from Dr. George Banting and Mr. Pete Billcliff) encodes human Tetherin HuCD317 with GFP subcloned between bases 246 and 247 (after the glycosylation site and before the coiled-coil region of the protein). EGFP-MT2 was amplified from pEGFP-MT2-C1 using the primers *TethGFP For*

and *TethMT2 Rev*, adding a 3' *SacII* RE site (underlined). The insert was digested with *BsiWI* and *SacII* and the vector with *BsrGI* and *SacII* before purification and ligation to yield pCR3.1-EGFP-MT2-Tetherin.

pLVX-TfnR-GFP (a gift from Dr. Fred Boal, University of Bristol) contains an N-terminal fusion of the human transferrin receptor to GFP. This was digested with *AgeI* and *SmaI*, pEGFP-MT2-C1 was digested with *AgeI* and *AfeI*. These were purified by gel electrophoresis and ligated as described to form pEGFP-MT2-TfnR.

2.3.4 pEGFP-FKBP-Tagged Proteins

pEGFP-FKBP-C1 was produced by amplifying FKBP from pC4-Fv1E, a commercially available vector from Ariad Pharmaceuticals encoding FKBP12(F36V), and subcloning it into pEGFP-C1. The forward primer *FKBP FWD* included a *BbsI* site, which ligates to *BsrGI* with removal of the RE site, a *BsiWI* site, which has an identical overhang to *BsrGI*, and a *SpeI* site, included so that, if required, FKBP could be moved with ease to the N-terminus of EGFP using *NheI*, which has the same overhang. The reverse primer, *FKBP REV*, included a *BsrGI* site to maintain the RE site after subcloning FKBP, and a *SgrAI* site, which shares an overhang with *AgeI* for moving FKBP to the N-terminus if required. The RE sites are underlined in the order that they are described. The PCR product was digested with *BbsI* and *BsrGI*, whilst pEGFP-C1 was digested with *BsrGI* and dephosphorylated with calf intestinal alkaline phosphatase. The digested vector was purified and FKBP ligated in place.

pEGFP-FKBP-tagged variants of the genes described above for MT2-tagging were produced by digesting out EGFP-MT2 with *AgeI* and *BsrGI* and subcloning in EGFP-FKBP with the same REs.

2.4 Protein Expression and Purification

2.4.1 Inducing Protein Expression

pET-45b(+) contains the *T7lac* promoter which requires IPTG for high-level expression. However, in BL21(DE3) cells lacking pLysS there is a low level of background expression. Selective LB was inoculated with 1:40 dilution of overnight culture and grown for 1 h with shaking at 250 rpm.

For expression of His₆-EGFP, IPTG was added to a final concentration of 1 mM prior to incubation at 37 °C for 5 h. Due to the high cysteine content of His₆-EGFP-MT2 high-level expression was undesirable, so after dilution of overnight culture 0.05 mM IPTG was added, followed 30 min later by 0.3 mM ZnCl₂. Cells were then incubated overnight at 20–25 °C. pET-EMS1 expression appeared to be more stable than EGFP-MT2 expression, therefore 1 mM IPTG was used, followed by

0.3 mM ZnCl₂ as before. Expression was continued for 5 h. Cells were harvested by centrifugation at 8000 g for 10 mins.

2.4.2 Purification of His₆-Tagged Protein

His₆-tagged protein was purified using nickel(II)-nitrilotriacetic acid (Ni-NTA)-agarose beads (QIAGEN). Lysis, wash and elution buffer compositions were as described in the QIAexpressionist handbook (QIAGEN).

Purification of His₆-EGFP, EGFP-MT2 and EMS1 followed standard procedures. Briefly, the cell pellet was resuspended in lysis buffer and incubated with lysozyme at 1 mg/ml on ice for 30 min before freeze-thaw twice in liquid nitrogen. The solution was then sonicated for three ten-second bursts, all performed on ice. Clarification by centrifugation gave a bright green supernatant that was then incubated at 4 °C with Ni-NTA resin, with rocking, for 30 mins. The sample was loaded into a column with a frit (20 µm pores) and subject to 2 column washes with wash buffer. Finally, purified protein was eluted with high imidazole concentration. Buffer exchange was performed by concentrating protein in Amicon Ultra-30K spin concentrators (Millipore), resuspending in 25 mM Tris·HCl pH 8, and repeating twice more.

2.4.3 Polyacrylamide Gel Electrophoresis (PAGE)

Samples were run on 4–12 % Bis-Tris polyacrylamide gels at 150 V for 90 min and stained with Coomassie Blue R250 for 10 min. The gel was washed twice with destaining solution, each for 1 hr, prior to a final overnight wash in deionised H₂O.

2.5 Metal-Binding Assays

2.5.1 Mass Spectrometry

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) was used to elucidate the mass-to-charge ratio of purified EGFP-MT2 before and after incubation with heavy-metal salts, and therefore calculate the number of ions bound to the protein. Buffers tested were 0.1 M ammonium acetate, 0.1 M phosphate buffer, PBS, or 25 mM Tris·HCl, all at pH 8. Matrixes tested were 2,5-dihydroxybenzoic acid ±0.1 % formic acid or ±50 % acetonitrile, α-cyano-4-hydroxycinnamic acid, sinapinic acid and 6-aza-thiothymine. Each with or without β mercaptoethanol or TCEP, both at either 1 or 20 mM. This was attempted for each metal complex at cysteine:metal ratios of 10:1, 1:1, 1:10 after 3 h at 37 °C (50 µM protein contains 40 cysteine residues and is equivalent to 2 mM cysteine). The signal-to-noise ratio varied

widely depending on which matrix and buffer was used, as well the concentration of protein and metal. Tris·HCl was optimal, combined with 2,5-dihydroxybenzoic acid (DHB) with 0.1 % formic acid as a matrix. Serial dilutions of protein in matrix were spotted onto a MALDI plate and analyzed using linear-mode on a MALDI 4700 Proteomics Analyzer (Applied Biosystems, California).

2.5.2 Synthesis of Gold Nanoparticles

Gold nanoparticles (AuNPs) were synthesised as described by Haiss et al. [1]. Briefly, a 1 % chloroauric acid ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, w/v) was prepared in deionised water. 90 ml of deionised water was rapidly stirred and 1 ml chloroauric acid added. After 1 min of continuous stirring, 2 ml of 38.8 mM trisodium citrate was injected and stirred for a further minute. Finally, 1 ml of 0.075 % NaBH_4 (w/v) in 38.8 mM trisodium citrate was added and stirred for 5–10 min. The concentration of AuNPs was determined by UV-vis spectrometry as described in [1] and the size determined by DLS and TEM.

2.5.3 Large Unilamellar Vesicles

Vesicles were prepared by sonication and extrusion as described [2]. Briefly, stock palmitoylcholine phosphatidylcholine (POPC) lipids were diluted to 2.5 mg/mL in chloroform:methanol (10:1 v/v) in glass vials and dried under gentle nitrogen current. All traces of solvent were removed by freeze-drying for at least 2 h. The lipids were rehydrated using 1–1.5 mL of protein solution or buffer prior to sonication for 30 min at 40–50 °C. Finally, the solution was extruded 11 times through a polycarbonate membrane perforated with 200 nm pores. Diameter and size distribution of vesicles was confirmed by DLS.

Vesicles were incubated with metal compounds at room temperature for 3 h. For imaging in the TEM, vesicles were either placed directly onto formvar and carbon-coated grids and allowed to air-dry, or incubated with methylcellulose to stabilise the lipids. To coat with methylcellulose, vesicles were pipetted onto formvar and carbon-coated grids and allowed to settle for 5 min. Three washes with deionised water was followed by addition of 1.8 % methylcellulose and 0.3 % uranyl acetate in deionised water on ice. After 5–10 min grids were looped out and excess fluid was wicked away.

2.6 Membrane Tubulation

Membrane tubulation by SNX-BAR proteins requires the presence of phosphatidylinositol-3-phosphate (PI(3)P) as well as other ancillary phospholipids to create microdomains suitable for tubulation. Liposomes were formed at 1 mg/mL from

bovine brain extract (Folch fraction 1, Sigma-Aldrich and Avanti batches were mixed 1:1) dissolved in chloroform and supplemented with 4 % protonated PI(3)P (Avanti polar lipids). PI(3)P was protonated by dissolving the non-protonated lipids in chloroform before removing the solvent under nitrogen and desiccation for 1 h. The lipid film was redissolved in chloroform:methanol:1N HCl (2:1:0.01 v/v) and left for 15 min. These were dried and desiccated as before and washed with chloroform:methanol (3:1 v/v) and twice with chloroform.

Lipid solutions were treated as above and extruded to either 400 or 200 nm in diameter. Purified pET-EMS1 was incubated with AuTM (1:1 gold to cysteine residues) before being added to the liposomes at 17.5 μ M, incubated at room temperature for 10 min and spotted onto carbon-coated EM grids. The liposomes were stabilised with methylcellulose and either left unstained or negatively stained with uranyl acetate before viewing in the TEM.

2.7 SAF Formation

Peptides were synthesised by solid-phase peptide synthesis by Dr. Thomson, University of Bristol, and purified by high-performance liquid chromatography. The sequence of each peptide is shown in Table 2.1. Freeze-dried peptides were rehydrated in deionised water to form stock concentrations at \sim 1 M. Stocks were diluted to a final concentration of 0.1 mM in 10 mM MOPS pH 7.4 and grown for 18 h at 21 $^{\circ}$ C.

Table 2.1 SAF peptide sequences. Sequences start at g and end at f because g–e interactions are between successive heptads

Peptide	Sequence
Heptad repeat	g abcdefg abcdefg abcdefg abcdefg abcdefg abcdef
SAF-p1	K IAALKQK IASLKQE IDALEYE NDALEQ
SAF-p1-I	K IAALKQK IASLKQE IDALEFE NDALEQ
Blunt-p1-I	E IDALEFE NDALEQK IAALKQK IASLKQ
SAF-p2a	K IRRLKQK NARLKQE IAALLEYE IAALQ
Blunt-p2a-I	E IAALLEFE IAALQK IRRLKQK NARLKQ
p1 ₃ -N	Ac-K IASLKQE IDALEYE NDALEQ
p1 ₃ -C	K IAALKQK IASLKQE IDALEY-Am
p2a ₆	Ac-E IAALQK IRRLKQK NARLKQE IAALLEYE IAALQK IRRLKQ-Am

E represents the *p*-iodo-phenylalanine. Ac and Am represent acetylated and amidated termini, respectively

References

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