



# Production of K<sub>2p</sub>2.1 (TREK-1) for structural studies

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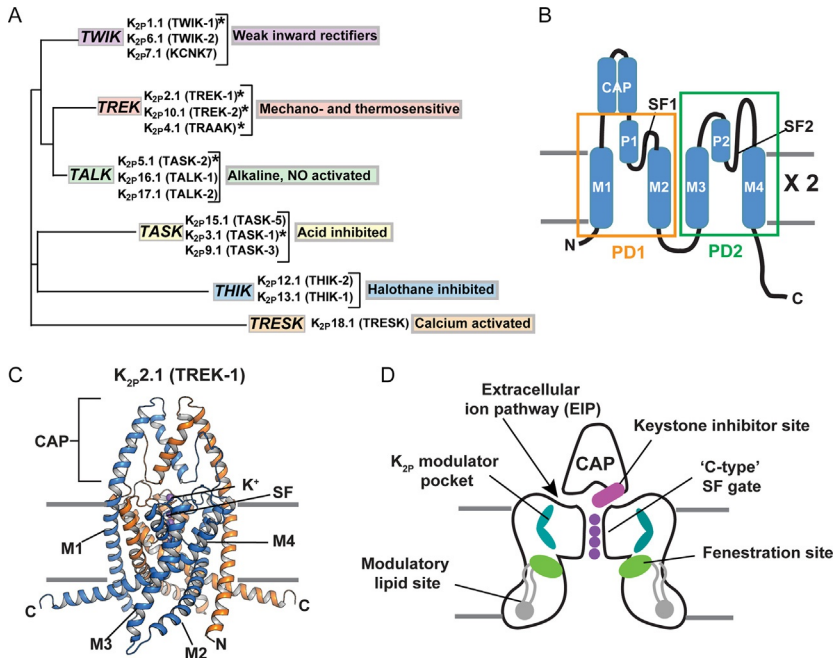
## Abstract

$K_{2P}$  (KCNK) potassium channels form 'background' or 'leak' currents that are important for controlling cell excitability in the brain, cardiovascular system, and somatosensory neurons.  $K_{2P2.1}$  (TREK-1) is one of the founding members of this family and one of the first well-characterized polymodal ion channels capable of responding to a variety of physical and chemical gating cues. Of the six  $K_{2P}$  subfamilies, the thermo- and mechano-sensitive TREK subfamily comprising  $K_{2P2.1}$  (TREK-1),  $K_{2P4.1}$  (TRAAK), and  $K_{2P10.1}$  (TREK-2) is the first to have structures determined for each subfamily member. These structural studies have revealed key architectural features that provide a framework for understanding how gating cues sensed by different channel elements converge on the  $K_{2P}$  selectivity filter C-type gate. TREK family structural studies have also revealed numerous sites where small molecules or lipids bind and affect channel function. This rich structural landscape provides the framework for probing  $K_{2P}$  function and for the development of new  $K_{2P}$ -directed agents. Such molecules may be useful for affecting processes where TREK channels are important such as anesthesia, pain, arrhythmia, ischemia, migraine, intraocular pressure, and lung injury. Production of high quality protein samples is key to addressing new questions about  $K_{2P}$  function and pharmacology. Here, we present methods for producing pure  $K_{2P2.1}$  (TREK-1) suitable for advancing towards these goals through structural and biochemical studies.



## 1. Introduction: $K_{2P2.1}$ (TREK-1), a model polymodal ion channel

$K_{2P}$  (KCNK) potassium channels are members of the voltage-gated ion channel superfamily (Yu, Yarov-Yarovoy, Gutman, & Catterall, 2005) that produce 'background' or 'leak' currents that have a critical role in control of cell excitability in the brain, heart, and peripheral nervous system (Enyedi & Czirjak, 2010; Feliciangeli, Chatelain, Bichet, & Lesage, 2015). There are 15 human  $K_{2P}$  subunits (Enyedi & Czirjak, 2010; Feliciangeli et al., 2015) comprising six subfamilies (Fig. 1A) that are regulated by various stimuli.  $K_{2Ps}$  are named based on their unique subunit architecture. Each subunit bears two pore-forming domains, PD1 and PD2, that comprise two transmembrane helices (M1-M2 and M3-M4) bridged by a pore helix (P1 and P2) and selectivity filter (SF1 and SF2) (Fig. 1B).  $K_{2P}$  subunits dimerize to create a channel in which the pore is intrinsically heterotetrameric due to sequence differences between PD1 and PD2. Unlike voltage-gated or inward rectifier channels,  $K_{2Ps}$  conduct ions over the entire physiological voltage range. Nevertheless, the leak current magnitude can be tuned by diverse inputs including pressure, temperature, extracellular and intracellular pH, lipids, phosphorylation, and agents such as volatile anesthetics and antidepressants.



**Fig. 1** K<sub>2p</sub> channel relationships, architecture, and structural pharmacology. (A) K<sub>2p</sub> channel dendrogram. Subfamilies are indicated. Asterisks indicate structurally characterized K<sub>2ps</sub>. (B) K<sub>2p</sub> subunit diagram. Pore domains 1 and 2 (PD1 and PD2), transmembrane helices (M1–M4), pore helices (P1 and P2), selectivity filters (SF1 and SF2), and CAP domain are indicated. (C) Cartoon diagram of the K<sub>2p</sub>2.1 (TREK-1) structure (PDB:6CQ6) (Lolicato et al., 2017). Chains are colored marine and orange. Potassium ions are purple. Channel elements are labeled as in 'B.' (D) Locations of structurally defined K<sub>2p</sub> small molecule binding sites: Keystone inhibitor site (magenta), K<sub>2p</sub> modulator pocket (cyan), fenestration site (green), and modulatory lipid site (gray). CAP and 'C-type' SF gate are indicated. Potassium ions are shown (purple). Gray lines in (B)–(D) denote the lipid bilayer.

(Felicangeli et al., 2015; Mathie, Al-Moubarak, & Veale, 2010). Various K<sub>2ps</sub> have roles in diverse physiological responses and pathological conditions such as action potential propagation (Brohawn et al., 2019; Kanda et al., 2019), anesthetic responses (Heurteaux et al., 2004; Lazarenko et al., 2010), microglial surveillance (Madry et al., 2018), sleep duration (Yoshida et al., 2018), pain (Alloui et al., 2006; Devilliers et al., 2013; Vivier et al., 2017), arrhythmia (Decher, Ortiz-Bonnin, et al., 2017), ischemia (Heurteaux et al., 2004; Laigle, Confort-Gouny, Le Fur, Cozzone, & Viola, 2012; Wu et al., 2013), cardiac fibrosis (Abraham et al., 2018), depression (Heurteaux et al., 2006), migraine (Royal et al., 2019), intraocular pressure

regulation (Yarishkin et al., 2018), and pulmonary hypertension (Lambert et al., 2018). Although there have been recent advances in defining new  $K_{2P}$  modulators for select  $K_{2Ps}$  (Bagriantsev et al., 2013; Lolicato et al., 2017; Pope et al., 2018; Su, Brown, Wang, & MacKinnon, 2016; Tian et al., 2019; Vivier et al., 2017; Wright et al., 2019),  $K_{2P}$  channel pharmacology remains undeveloped (Dong et al., 2015; Lolicato et al., 2017; Mathie, Veale, Cunningham, Holden, & Wright, 2020; Pope, Lolicato, & Minor, 2020; Schewe et al., 2019; Sterbuleac, 2019). Due to their unusual topology, diverse gating stimuli, and poor pharmacology,  $K_{2Ps}$  have remained the most poorly understood potassium channel class (Bayliss & Barrett, 2008; Enyedi & Czirjak, 2010; Feliciangeli et al., 2015; Honore, 2007; Lesage & Barhanin, 2011).

To date, the structures of six homomeric  $K_{2Ps}$  have been determined (Brohawn, del Marmol, & MacKinnon, 2012; Dong et al., 2015; Li, Rietmeijer, & Brohawn, 2020; Lolicato et al., 2017; Miller & Long, 2012; Rödström et al., 2020). These structures reveal a conserved overall architecture that defines the  $K_{2P}$  family and that is embodied in the representative  $K_{2P}$   $K_{2P2.1}$  (TREK-1) (Fig. 1C). The pore helices and selectivity filter of the membrane-embedded channel pore coordinate a set of four potassium ions on the channel central axis. The CAP domain, a unique structural feature of  $K_{2Ps}$ , forms an arch directly over the channel pore and creates the bifurcated extracellular ion pathway (EIP) from which the ions exit the channel after passing through the selectivity filter (Brohawn et al., 2012; Miller & Long, 2012).

$K_{2P2.1}$  (TREK-1) (*KCNK2*) (Patel et al., 1998) is one of the first well-described polymodal ion channels and a founding member of the  $K_{2P}$  channel family. It is part of the TREK subfamily of polymodal ion channels, comprising  $K_{2P2.1}$  (TREK-1) (Fink et al., 1996; Patel et al., 1998),  $K_{2P10.1}$  (TREK-2) (Bang, Kim, & Kim, 2000; Lesage, Terrenoire, Romey, & Lazdunski, 2000), and  $K_{2P4.1}$  (TRAAK) (Maingret, Fosset, Lesage, Lazdunski, & Honoré, 1999). This  $K_{2P}$  subfamily responds to diverse physical and chemical gating cues including temperature, pressure, pH, and modulatory lipids (Douguet & Honore, 2019; Feliciangeli et al., 2015; Honore, 2007).  $K_{2P2.1}$  (TREK-1) is one of the best-studied  $K_{2Ps}$  (Dedman et al., 2009; Honore, 2007) and has been shown to be important in sensory neuron pain responses (Alloui et al., 2006; Noel et al., 2009), vasodilation (Blondeau et al., 2007; Garry et al., 2007), chronic pain (Noel et al., 2009), migraine (Royal et al., 2019), general anesthetic responses (Heurteaux et al., 2004), depression (Heurteaux et al., 2006), arrhythmias (Decher, Kiper, & Rinne, 2017), intraocular pressure regulation (Yarishkin et al., 2018), and lung

injury (Zyrianova et al., 2020). Thus, it is an excellent candidate for the development of new molecules that could be used to treat a range of diseases (Mathie et al., 2020).

The sensors that enable K<sub>2p</sub>2.1 (TREK-1) to respond to the diverse set of physical chemical gating cues that control its function reside in different parts of the channel. The intracellular C-terminal tail is key to control by temperature (Bagriantsev, Clark, & Minor Jr., 2012; Bagriantsev, Peyronnet, Clark, Honore, & Minor, 2011; Chemin et al., 2005; Maingret et al., 2000), pressure (Bagriantsev et al., 2011; Maingret, Fosset, et al., 1999; Maingret, Patel, Lesage, Lazdunski, & Honore, 1999), intracellular pH (Honore, Maingret, Lazdunski, & Patel, 2002; Maingret, Patel, et al., 1999), lipids such as phosphoinositol (4,5) bis-phosphate (PIP<sub>2</sub>) (Chemin et al., 2005, 2007; Lopes et al., 2005), and phosphorylation (Bagriantsev et al., 2012; Murbartian, Lei, Sando, & Bayliss, 2005; Patel et al., 1998). The extracellular pH sensor for K<sub>2p</sub>2.1 (TREK-1) is a histidine (Cohen, Ben-Abu, Hen, & Zilberberg, 2008; Sandoz, Douguet, Chatelain, Lazdunski, & Lesage, 2009) that resides in the loop between the P1 helix and the CAP domain (Lolicato et al., 2017). Regardless of the point of origin, the gating cues that control K<sub>2p</sub>2.1 (TREK-1) activity converge on the selectivity filter C-type gate and make this channel element the nexus of signal integration and functional control (Bagriantsev et al., 2011, 2012; Lolicato et al., 2017, 2020; Schewe et al., 2016).

The TREK subfamily is the most extensively studied K<sub>2p</sub> family and the only subfamily for which structures of each subtype are known (Brohawn et al., 2012; Dong et al., 2015; Lolicato et al., 2017). These efforts, combined with structure-guided functional studies (Brohawn et al., 2012; Brohawn, Campbell, & MacKinnon, 2013, 2014; Dong et al., 2015; Lolicato et al., 2017, 2020; Schewe et al., 2019) have been essential for developing a mechanistic framework for understanding the function of the TREK subfamily and K<sub>2ps</sub> in general.

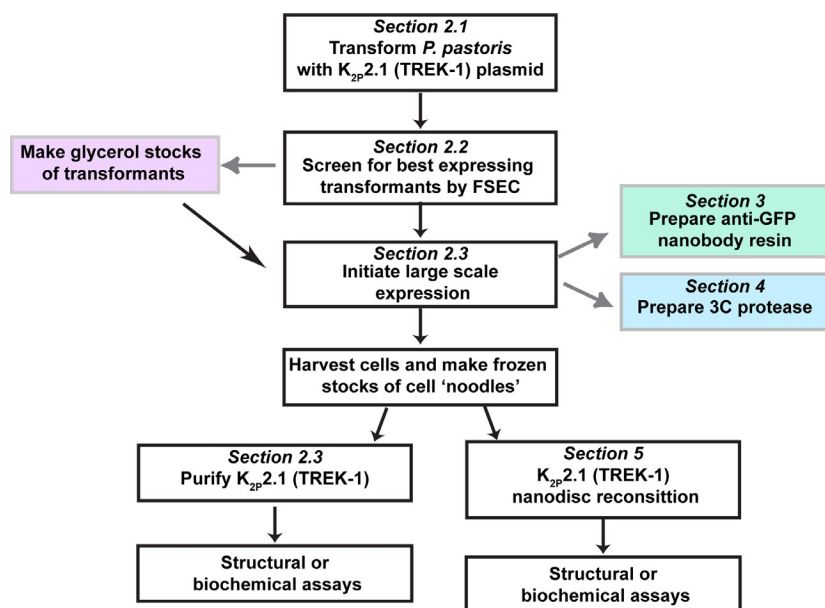
Studies of K<sub>2ps</sub> have been challenged by the lack of good pharmacological tools to manipulate K<sub>2p</sub> function (Mathie et al., 2020; Sterbuleac, 2019). However, recent discoveries of new classes of K<sub>2p</sub> modulators are beginning to define new pharmacological tools directed at this channel family (Bagriantsev et al., 2013; Dadi et al., 2016; Liao et al., 2019; Lolicato et al., 2017; Rodrigues et al., 2014; Rödström et al., 2020; Su et al., 2016; Tian et al., 2019; Vivier et al., 2017). In this regard, a variety of structures of TREK subfamily members complexed with a various modulators (Dong et al., 2015; Lolicato et al., 2017, 2020; Pope et al., 2020) have provided an important step for uncovering the rich polysite framework by

which various chemical agents can activate or inhibit  $K_{2pS}$  (Pope et al., 2020). These studies have revealed modulator binding sites at every layer of the channel, starting from its extracellular side through the portion that interacts with the membrane bilayer inner leaflet (Fig. 1D) and comprising the Keystone inhibitor site (Pope et al., 2020), the  $K_{2p}$  modulator pocket (Lolicato et al., 2017), the Fenestration site (Dong et al., 2015; Schewe et al., 2019), and the Modulatory lipid site (Lolicato et al., 2017). Each site offers a distinct structural environment and mechanism for controlling  $K_{2p}$  function.

Developing a biochemically tractable  $K_{2p2.1}$  (TREK-1) construct (Lolicato et al., 2017) has been the key factor in enabling structural studies of  $K_{2p2.1}$  (TREK-1) (Lolicato et al., 2017, 2020; Pope et al., 2020) and should enable further studies of  $K_{2p}$  pharmacology. Here, we outline the key steps for producing pure  $K_{2p2.1}$  (TREK-1) amenable to both structural and biochemical investigation.

## 1.1 Before you begin

The workflow for producing  $K_{2p2.1}$  (TREK-1) using *P. pastoris* expression is outlined in Fig. 2. Going from the  $K_{2p2.1}$  (TREK-1) clone to identification of candidate transformants takes ~15–20 days. Large scale



**Fig. 2** Workflow for producing  $K_{2p2.1}$  (TREK-1) Corresponding chapter sections are indicated. Gray arrows indicate key reagent preparation steps.

production and purification takes ~8–10 days. Key reagent preparation of anti-GFP nanobody resin and 3C protease are separate workflow steps of ~7 days each that should be initiated at the beginning of large scale expression efforts. Nanodisc incorporation requires ~5 days for production of MSP1E1 and takes ~4 days for the reconstitution. Key resources are listed at the beginning of each protocol. Pause points are also indicated.



## 2. Expression and purification of K<sub>2p</sub>2.1 (TREK-1) using *Pichia pastoris*

The following section presents a general protocol for generating K<sub>2p</sub>2.1 (TREK-1) expressing *P. pastoris* through electroporation.

Expression of mouse K<sub>2p</sub>2.1 (TREK-1) (Lolicato et al., 2017) uses a *P. pastoris* pPICZ (Thermo Fisher Scientific) expression vector containing a construct optimized for expression that includes residues 21–322 and the following mutations: K84R, Q85E, T86K, I88L, A89R, Q90A, A92P, N95S, S96D, T97Q, N119A, S300A, E306A (Fig. 3A). Eleven of these changes are in the CAP domain and together with the truncations at the N- and C-termini, were found to increase expression and sample quality without compromising function (Lolicato et al., 2017). Two mutations remove a phosphorylation site in the C-terminal tail that inhibits function (S300A) (Bagriantsev et al., 2012; Murbartian et al., 2005) and introduce an activating mutation (E306A) (Bagriantsev et al., 2012; Honore et al., 2002), respectively. The construct bears a C-terminal fusion to a 3C protease cleavable green fluorescent protein (GFP) followed by His<sub>10</sub> tag (Fig. 3A). The GFP enables analysis of protein expression by Fluorescence-detection size exclusion chromatography (FSEC) (Kawate & Gouaux, 2006) as well as large scale purification using anti-GFP coupled nanobody beads.

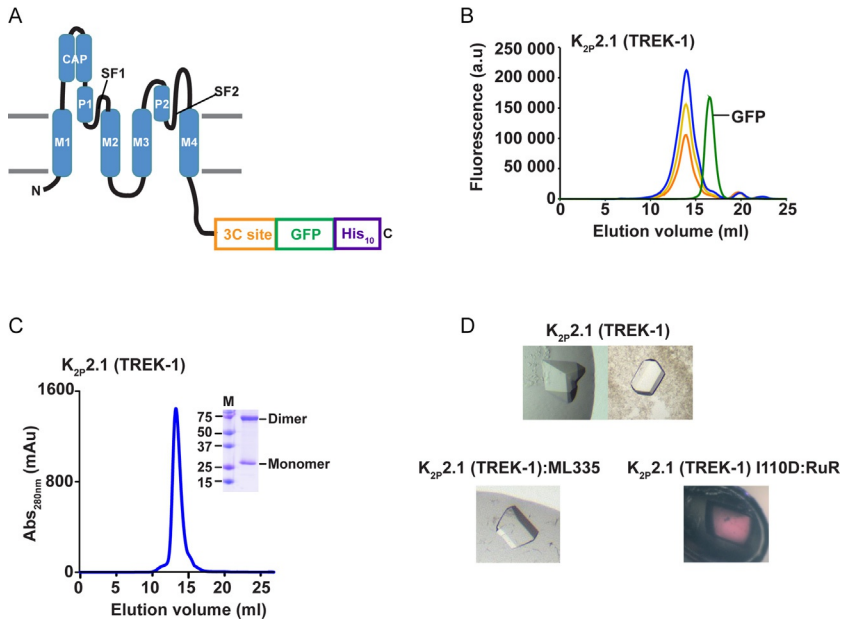
### 2.1 *P. pastoris* transformation by electroporation

#### 2.1.1 Preparing linear DNA for *P. pastoris* transformation

To transform *P. pastoris*, high quality DNA is essential. Plasmid DNA is first prepared with standard Midi prep procedure (Qiagen Plasmid Plus Midi Kit). 100 µg of DNA are then linearized and purified using the procedure below.

#### Materials

- 100 µg pPICZ:mTREK-1<sub>cryst</sub> plasmid DNA (Addgene Plasmid #133269) (Lolicato et al., 2017).
- restriction enzyme *PmeI* (New England Biolabs, R0560S, 500 units).



**Fig. 3** (A)  $K_{2p2.1}$  (TREK-1) expression and analysis a,  $K_{2p2.1}$  (TREK-1) construct diagram. 3C site (orange), GFP (green), and His<sub>10</sub> tag (purple) locations are indicated. Gray lines denote the lipid bilayer. (B) Exemplar FSEC analysis of  $K_{2p2.1}$  (TREK-1)-GFP transformants (blue, yellow, orange) and GFP (green) run on a Superdex 200 column. (C) Exemplar SEC purification of  $K_{2p2.1}$  (TREK-1). Inset shows SDS-PAGE analysis under reducing conditions. (D) Exemplar crystals of  $K_{2p2.1}$  (TREK-1) (Lolicato et al., 2017) (top), the  $K_{2p2.1}$  (TREK-1):ML335 complex (Lolicato et al., 2017), and a complex of ruthenium red (RuR) with the  $K_{2p2.1}$  (TREK-1) I110D mutant (Pope et al., 2020). RuR binding to the channel gives the crystals a magenta color.

- Phenol:Chloroform:Isoamyl Alcohol 25:24:1 saturated with 1 mM EDTA, 10 mM Tris, pH 8.0 (Sigma, P2069-100ML).
- Chloroform-isoamyl alcohol mixture 24:1 (Sigma, 25,666-100ML).
- 3 M Na acetate, pH 5.0.
- pure Ethanol.
- sterile, deionized water.
- 37 °C incubator.
- table-top microcentrifuge.
- nanodrop spectrophotometer.

### Protocol

- (1) Digest 100  $\mu$ g of plasmid DNA with *PmeI* to linearize the vector in a total volume of 200  $\mu$ L and incubate at 37 °C overnight, following manufacturer's instructions.



*Tip:* We recommend that you check 1  $\mu\text{L}$  of the digest by agarose gel electrophoresis to ensure that linearization is complete.

Once the vector is linearized it is purified by a phenol/chloroform extraction as follows:

- (2) Add 1 volume of Phenol:Chloroform:Isoamyl Alcohol 25:24:1 to the linearized DNA, vortex, and then centrifuge at  $3500 \times g$  for 1 min at room temperature. You will have two layers, a lower phenol/chloroform layer and an upper aqueous layer.
- (3) Carefully transfer the upper layer to a new microcentrifuge tube.
- (4) Add 1 volume of Chloroform-isoamyl alcohol mixture 24:1. Vortex and then centrifuge at  $3500 \times g$  for 1 min at room temperature. You will have two layers, a lower chloroform-isoamyl alcohol layer and an upper aqueous layer.
- (5) Carefully transfer the upper layer to a new microcentrifuge tube.
- (6) Add 1/10 volume of 3M Na acetate pH 5.0 and mix well by pipetting. Add 2.5 volumes of an ice-cold ethanol and invert the tube several times to mix. DNA will appear as a white precipitate.
- (7) Centrifuge at  $16,500 \times g$  for 45 min at  $4^\circ\text{C}$  to pellet the DNA. Carefully discard the solution and add 300  $\mu\text{L}$  of 70% ethanol without disturbing the DNA pellet.
- (8) Centrifuge at  $16,500 \times g$  for 20 min at  $4^\circ\text{C}$  to wash the pellet. Carefully pipette off the liquid and allow the tube to air-dry.
- (9) Resuspend the dried DNA pellet in 10–15  $\mu\text{L}$  sterile, deionized water. Measure the concentration of the DNA with nanodrop spectrophotometer. You should have  $\sim 6\text{--}10 \mu\text{g}/\mu\text{L}$  DNA. Use immediately or store at  $-20^\circ\text{C}$ .

**(Pause point)**

## 2.1.2 Preparing *P. pastoris* for electroporation

### Materials

- 30–50  $\mu\text{g}$  linearized pPICZ:mTREK-1<sub>cryst</sub> from [Section 2.1.1](#).
- *Pichia pastoris* SMD1163H (*prb1-*, *pep4-*, *his4+*) cells.
- 1000 mL Rapid-Flow Filter Unit 0.2  $\mu\text{m}$  CN membrane, 75 mm dia (Thermo Scientific Nalgene, 127-0020).
- Yeast Nitrogen Base without Amino Acids (YNB, Sigma, Y0626-1KG).
- Biotin (Sigma, B4501-5G).
- BMG (Buffered Minimal Glycerol) Medium 1 L:

*To prepare:* Make 1 L of  $10 \times$  Potassium phosphate buffer pH 6.0 by making 1 M Potassium phosphate buffer, pH 6.0. Filter sterilize or

autoclave. Make 1 L of 10 × Glycerol by making 10% glycerol solution. Filter sterilize or autoclave. Prepare 1 L of 10 × YNB medium by dissolving 134 g YNB and 4 mg Biotin. Filter sterilize. Add the following to 700 mL autoclaved ddH<sub>2</sub>O and mix well: 100 mL 10 × Potassium phosphate buffer pH 6.0, 100 mL 10 × Glycerol, 100 mL 10 × YNB medium.

- 50-mL conical polypropylene tubes.
- non-baffled 500 mL sterile flask.
- 50 mL resuspension buffer: 600 mM Sorbitol, 100 mM Lithium Acetate, 10 mM DTT, 10 mM Tris-Cl, pH 7.5.
- 200 mL cold (4 °C) sterile 1 M sorbitol (place on ice the day of the experiment).
- 30 °C incubator.
- tabletop centrifuge.

### Protocol

- (1) Grow a 5 mL culture of *Pichia pastoris* SMD1163H (*prb1-*, *pep4-*, *his4+*) in BMG in a 50 mL conical tube at 30 °C overnight.
- (2) Inoculate 100 mL of fresh BMG in a non-baffled 500 mL sterile flask with 0.1–0.5 mL of the overnight culture. Grow overnight at 30 °C to an OD<sub>600nm</sub> = 1.3–1.5.
- (3) Centrifuge the cells at 1500 × *g* for 15 min at 4 °C. Discard the supernatant and resuspend the pellet with 50 mL of resuspension buffer. Incubate cells for 30 min at room temperature.
- (4) Centrifuge the cells at 1500 × *g* for 5 min. Discard the supernatant and resuspend the pellet with 50 mL of ice-cold 1 M sorbitol.
- (5) Repeat Step 4 three times.

*Tip:* It is essential to remove as much salt as possible with these washes to avoid arcing during electroporation.

- (6) Resuspend the pellet in ice-cold 1 M sorbitol for a final volume of approximately 1 mL. Keep the cells on ice and use that day. Do not store cells.

*Tip:* This protocol is based on the protocol from (Wu & Letchworth, 2004). We find the combination of lithium acetate and DTT to be essential for reliable transformation results.

### 2.1.3 Transformation of *P. pastoris* by electroporation

#### Materials

- 2 mL cold (4 °C) sterile 1 M sorbitol.
- 15-mL conical polypropylene tubes.

- Zeocin™ (Invivogen, ant-zn-5p).
- Bio-Rad Gene Pulser Xcell Electroporation System 165-2666.
- 0.2 cm electroporation cuvettes.
- 10 × D (Dextrose) 100 mL (filter sterilized).
- YPD (Yeast Extract Peptone Dextrose) Medium 1 L.  
*To prepare:* Dissolve 10 g yeast extract and 20 g peptone in 900 mL H<sub>2</sub>O. Autoclave the solution. Cool to ~60 °C and add 100 mL of 10 × D.
- YPD (Yeast Extract Peptone Dextrose) plates containing 500 and 1000 µg/mL Zeocin™.  
*To prepare:* Dissolve 1 g yeast extract, 2 g peptone, and 2 g bacto agar in 90 mL H<sub>2</sub>O. Autoclave the solution. Cool to ~60 °C and add 10 mL of 10 × D. Add 50 mg or 100 mg Zeocin™. Pour into petri dishes (~25 mL/100-mm plate).
- 30 °C incubator.
- 30 °C shaking incubator.

### Protocol

- (1) Mix 100 µL of the prepared *P. pastoris* cells from [Section 2.1.2](#) with approximately 50 µg of linearized DNA (in 10–15 µL sterile water) and incubate on ice for 5 min. 100 µL of the *P. pastoris* cells from [Section 2.1.2](#) without linearized DNA should also be used for all of the following steps as a control.
- (2) Add 300 µL of ice-cold 1 M Sorbitol and immediately transfer to an ice-cold 0.2 cm electroporation cuvette.
- (3) Electroporate the cells using the manufacturer's instructions. Electroporation is carried out at 2500 V, 25 µF and 400 Ω resistance using the BioRad Gene Pulser Xcell 165-2666.
- (4) Immediately transfer the cuvette contents to a sterile 15-mL tube containing 700 µL of ice-cold 1 M sorbitol. Incubate at 30 °C without shaking for 1–2 h.
- (5) Add 1 mL of YPD media to each tube. Shake (~200 rpm) the cultures at 30 °C for 2–4 h.
- (6) Plate 200 µL of culture on two YPD plates containing 500 and 1000 µg/mL Zeocin™.  
*Tip.* You can plate the cells in YPD plates containing up to 4 mg/mL of Zeocin. Increasing the antibiotic concentration will enable the selection of colonies with multiple integration events. Such cells may have increased protein production.
- (7) Incubate plates for 3–5 days at 30 °C until colonies form.

- (8) You can store these plates at 4 °C in the dark for 1 week. Colonies from these plates are used for [Section 2.2.1](#).

(Pause point)

## 2.2 Optimization of protein expression

Linearization and transformation of the K<sub>2p</sub>2.1 (TREK-1) plasmid into *P. pastoris* will result in colonies from individual transformants that have varied numbers of integration events. Therefore, it is important to screen multiple transformants in order to identify a strain that produces sufficient quantities of high-quality protein. The following steps are used to identify such clones. Once identified, the glycerol stocks (Step 2 of [Section 2.2.1](#)) can be used to initiate expression.

### 2.2.1 Screening recombinant *P. pastoris* transformants for protein expression

#### Materials

- 50 mL conical polypropylene tubes.
- 1000 mL Rapid-Flow Filter Unit 0.2 μm CN membrane, 75 mm dia (Thermo Scientific Nalgene, 127-0020).
- Yeast Nitrogen Base without Amino Acids (YNB, Sigma, Y0626-1KG).
- Biotin (Sigma, B4501-5G).
- BMG (Buffered Minimal Glycerol) Medium 1 L (see [Section 2.1.2](#)).
- BMM (Buffered Minimal Methanol) Medium 1 L:

*To prepare:* Add the following to 800 mL autoclaved ddH<sub>2</sub>O and mix well:

100 mL 10 × Potassium phosphate buffer pH 6.0.

100 mL 10 × YNB medium.

- glycerol stock solution: BMG with final 15% (v/v) glycerol concentration.
- Zeocin (Invivogen, ant-zn-5p).
- Methanol (Merck, MX0485-7).
- non-baffled 500 mL sterile flask.
- 15-mL conical polypropylene tubes.
- 30 °C shaking incubator.
- tabletop centrifuge.

#### Protocol

- (1) Pick 10 colonies from the plate of [Section 2.1.3](#) and grow a 5 mL cultures of each in BMG medium with an appropriate concentration of zeocin based on the plate of colony origin in a 50 mL conical tube at 30 °C in the dark overnight.

- (2) Inoculate 100 mL of BMG medium culture with an appropriate concentration of zeocin based on the plate of colony origin in a non-baffled 500 mL sterile flask with 1 mL of the overnight culture. Grow at 30 °C in a shaking incubator (250–300 rpm) in the dark for 48 h. You can make glycerol stocks of these cells by centrifuging 10 mL of culture and resuspending them in the same volume of BMG + 15% glycerol. Keep the glycerol stocks at –20 °C for two days and then move them to –80 °C.
- (3) Harvest the cells by centrifuging at 3000 × g for 10 min at room temperature. Decant supernatant and resuspend cell pellet in 100 mL of BMM medium to induce expression using a final concentration of 0.5% (v/v) methanol.
- (4) Add 100% methanol to a final concentration of 0.5% (v/v) methanol every 24 h to maintain induction.
- (5) At each of the times indicated below, transfer 10 mL of the expression culture to a 15 mL conical polypropylene tubes. Centrifuge at maximum speed in a tabletop centrifuge for 5 min at room temperature to pellet the cells. Discard the supernatant. The cells can be frozen at this point and stored at –80 °C. These samples will be used to analyze expression levels and determine the optimal time post-induction to harvest.

*Tip:* Suggested time points (hours): 0, 6, 12, 24 (1 day), 36, 48 (2 days), 60, 72 (3 days), 84, and 96 (4 days).

**(Pause point)**

## **2.2.2 Preparing cell lysates and analysis of protein expression by FSEC**

### **Materials**

- Breaking buffer: 200 mM KCl, 50 mM Tris-Cl pH 8.0.
- DNase I (Roche, 10104159001).
- Phenylmethanesulfonyl fluoride (PMSF, Sigma, P7626–100 g).
- Solubilization buffer: 60 mM DDM, 200 mM KCl, 50 mM Tris-Cl pH 8.0.
- FSEC buffer: 1 mM DDM, 200 mM KCl, 50 mM Tris-Cl pH 8.0.
- Glass beads, 0.5 mm (Spectrum chemicals & laboratory products, 980–19987).
- Vortexer.
- Orbitron Rotator II Model 260250, Boekel Scientific.
- tabletop ultracentrifuge.

## Protocol

- (1) Thaw each cell pellet at room temperature. Resuspend each thawed cell pellet by pipetting up and down in 1 mL of cell breaking buffer having a final concentration of 0.1 mg/mL DNase I and 1 mM PMSF until the suspension is homogeneous. Transfer cells to a 2 mL microcentrifuge tube containing 0.3 g of 0.5 mm glass beads.
- (2) Vortex the mixture for 3 min, then incubate on ice for 3 min. Repeat 2 more times. Alternating vortexing with cooling reduces denaturation of the protein.

*Tip:* *P. pastoris* lysis is challenging due to a very tough cell wall. However, because of the sensitivity of the FSEC assay, we find that the above procedure is sufficient to screen samples.

- (3) To pellet the membrane fraction, transfer the lysed cells to a clean, chilled ultracentrifuge tube. Centrifuge for 30 min at  $100,000 \times g$  and  $4^\circ\text{C}$  and discard the supernatant.
- (4) Resuspend the membrane fraction in the solubilization buffer.
- (5) Transfer the solubilization mixture to a clean, chilled microcentrifuge tube and rotate gently on an orbitron rotator for 2 h at  $4^\circ\text{C}$ . Wash the used ultracentrifuge tube with water to be ready to use again after solubilization step.
- (6) Transfer the solubilized mixture of step 5 to the previously used ultracentrifuge tube and pellet unsolubilized material by centrifugation for 30 min at  $100,000 \times g$  and  $4^\circ\text{C}$ .
- (7) Remove the supernatant from each tube and transfer it to a clean, chilled fluorescence size-exclusion chromatography (FSEC) sample tube.
- (8) Analyze the solubilized membranes fraction by FSEC (Kawate & Gouaux, 2006) to determine which colony and growth time gives the greatest expression.

### (Pause point)

*Tip:* Individual transformants may have different expression levels due to differences in integration events and may not always produce clean protein. Examples of expression level variation are shown in Fig. 3B. You should select the colonies that give a good, symmetric peak shape and that have the highest level of fluorescence signal.

## 2.3 Large scale culture and protein purification

Once transformants have been screened to identify those having the best expression profiles, choose one for large-scale expression (typically 3–6 L).

You can use the glycerol stocks of the cells you made at Step 2 of [Section 2.2.1](#).

*Note:* Before initiating the protein purification protocol ([Section 2.3.2](#)), you will need to prepare both the anti-GFP nanobody beads ([Section 3](#)) and 3C protease ([Section 4](#)).

### 2.3.1 K<sub>2p</sub>2.1 (TREK-1) large scale expression

#### Materials

- Glycerol cell stock of step 2 of [Section 2.2.1](#).
- 1000 mL Rapid-Flow Filter Unit 0.2 μm CN membrane, 75 mm dia (Thermo Scientific Nalgene, 127–0020).
- 6L BMG (Buffered Minimal Glycerol) Medium.

*To prepare:* Add 1600 mL of ddH<sub>2</sub>O each to 3 × 2L bottles. Prepare 900 mL of Potassium phosphate buffer at pH 6.0 by mixing 140.85 g of KH<sub>2</sub>PO<sub>4</sub> and 28.75 g of K<sub>2</sub>HPO<sub>4</sub> and adjusting pH to 6.0 with 5 M KOH. Add 150 mL of this Potassium phosphate buffer to each 2L bottle. Prepare 150 mL of 40% glycerol solution. Filter sterilize. Add 50 mL of this 40% glycerol solution to each 2L bottle. Autoclave the bottles.

Prepare fresh YNB medium by dissolving 102 g of YNB and 30 mg of Biotin in 750 mL. Filter sterilize.

Add 200 mL of this YNB medium to each bottle right before inoculating BMG medium.

- 6L BMM (Buffered Minimal Methanol) Medium:

*To prepare:* Add 1650 mL of ddH<sub>2</sub>O each to 3 × 2L bottles. Add 150 mL of the prepared Potassium phosphate buffer to each bottle. Autoclave the bottles.

Prepare fresh YNB medium by dissolving 102 g of YNB and 30 mg of Biotin in 750 mL. Filter sterilize.

Add 200 mL YNB medium to each bottle right before inoculating BMM medium.

- 2 × autoclaved 1 L cylinders.
- Yeast Nitrogen Base Without Amino Acids (YNB, Sigma, Y0626-1KG).
- Biotin (Sigma, B4501-5G).
- Zeocin (Invivogen, ant-zn-5p).
- non-baffled 250 mL flask.
- non-baffled 3-L flask.
- Shaking incubator.
- centrifuge.

- Methanol (Merck, MX0485-7).
- 50 mL syringe.

### Protocol

- (1) Use a glycerol stock from a colony that had the best expression. Scoop  $\sim 50 \mu\text{L}$  of the glycerol stock and use it to inoculate 30 or 60 mL of BMG with an appropriate concentration of zeocin in a non-baffled 250 mL flask for the growth of 3 or 6 L, respectively. The zeocin concentration depends on which zeocin plate concentration was chosen to make glycerol stock. Grow at  $30^\circ\text{C}$  in a shaking incubator (250–300 rpm) in the dark for 48 h.
- (2) Aliquot 1 L of BMG medium in each non-baffled 3 L flask with the autoclaved 1 L cylinder. Use this 10 mL culture to inoculate 1 L of BMG in a 3 L flask and grow at  $29^\circ\text{C}$  with vigorous shaking (250–300 rpm) for 48 h.
- (3) Harvest the cells using sterile Beckman 1 L centrifuge bottles by centrifuging at  $3000 \times g$  for 6 min at room temperature. (The sterile centrifuge bottles can be readily prepared by washing them with 70% Ethanol and then allowing them to air-dry overnight). To induce expression, decant the supernatant and resuspend the cell pellet of each flask in 1 L of BMM medium. Aliquot 1 L of BMM medium in each non-baffled 3 L flask with the autoclaved 1 L cylinder.
- (4) Add 0.5% v/v of 100% methanol to each flask to start induction. Continue to grow at  $29^\circ\text{C}$  with shaking.
- (5) Add 100% methanol to 0.5% v/v every 24 h until the optimal time of induction is reached as determined from the time course study.

*Note:* The methanol is the only carbon source for the cells. Hence, it is essential to add every 24 h to sustain cell growth until the desired expression time is reached.

- (6) Harvest cells by centrifuging at  $9000 \times g$  for 15 min at  $4^\circ\text{C}$ . Decant the supernatant and transfer the cell pellet to a 50 mL syringe. Make cell noodles by pushing the pellet through a 50 mL syringe into a container of liquid Nitrogen. Store the cell noodles at  $-80^\circ\text{C}$  until ready to process.

**(Pause point)**

### 2.3.2 $K_{2p2.1}$ (TREK-1) purification

#### Materials

- Ball Mill (Retsch model MM400).
- Solubilization buffer: 60 mM DDM (N-dodecyl- $\beta$ -D-maltoside), 200 mM KCl, 100 mM Tris-Cl pH 8.2.



- DNase I (Roche, 10104159001).
- Phenylmethanesulfonyl fluoride (PMSF, Sigma, P7626–100 g).
- Wash buffer: 6 mM DDM, 200 mM KCl, 20 mM Tris-Cl pH 8.0.
- SEC buffer: 1 mM DDM, 200 mM KCl, 20 mM Tris-Cl pH 8.0.
- Ultracentrifuge.
- 250 mL conical tube.
- Sepharose resin coupled to anti-GFP nanobody (see [Section 3](#)).
- 500 mM EDTA pH 8.0.
- 3 M KCl.
- 3C protease (see [Section 4](#)).
- Orbitron Rotator II Model 260250, Boekel Scientific.
- Gel filtration column (Superdex200 10/300 GL) connected to the chromatography system.
- Ultra spin concentrator (Amicon Ultra–15 centrifugal filters, Millipore, 100 kDa cut-off).
- tabletop microcentrifuge.

### Protocol

- (1) Break the frozen cell noodles by milling (Retsch model MM400) for three cycles of 3 min at 25 Hz. Keep the cell noodles and broken cell powder in liquid N<sub>2</sub> for whole process. Broken cell powder can be stored at –80 °C. All subsequent purification steps should be carried out at 4 °C with buffers chilled to 4 °C.

#### (Pause point)

- (2) Resuspend the thawed cell powder in 3 mL of membrane solubilization buffer having a final concentration of 0.1 mg/mL DNase I and 1 mM PMSF for each gram of cell powder. Agitate by vigorous stirring with a magnetic stir bar at 4 °C for 3 h to yield a homogeneous suspension.
- (3) Transfer the solubilization mixture to a clean, chilled ultracentrifuge tube and pellet unsolubilized material by centrifugation for 45 min at 95,800 × g and 4 °C. Decant the supernatant into a 250 mL conical tube.
- (4) Equilibrate Sepharose resin coupled to anti-GFP nanobody (see [Section 3](#)) with 10 column volumes (CV) of SEC buffer. Add the resin to the supernatant and incubate the protein–resin mixture for 3 h at 4 °C with gentle rotation.

*Tip:* 5 mL of Sepharose resin coupled to anti-GFP nanobody is enough to purify K<sub>2p</sub>2.1 (TREK-1) from a 6 L growth.

- (5) Load the mixture onto an empty column and let the resin settle by gravity. Let the liquid flow through and collect the flow-through to check for unbound protein.

- (6) Wash the column with 30 CV of Wash buffer.
- (7) To the remaining 2 CV of Wash buffer add the final concentration of 1 mM EDTA, 200 mM KCl and 3C protease at a target protein:3C protease ratio of 50:1 (w:w) to cleave off GFP and release the protein from the resin.
- (8) Add the 3C buffer of step 7 to the resin and mix with the pipette. Cap the top of the column. Incubate for 12–18 h (or overnight) at 4 °C.
- (9) Collect the flow-through. Wash the resin with 3 CV of SEC buffer and collect the flow-through and combine this with the previous flow-through.
- (10) Equilibrate a gel filtration column (Superdex200 10/300 GL) connected to the chromatography system in 2 CV of size-exclusion buffer following the manufacturer's instructions.
- (11) Concentrate the sample with an ultra spin concentrator (100 kDa cut-off) and centrifuge the sample to be injected onto the gel filtration column for 10 min at  $>10,000 \times g$  and 4 °C.
- (12) Inject the sample onto the gel filtration column. Collect 0.3 mL fractions of the eluate while monitoring at 280 nm.
- (13) Analyze fractions for protein composition via SDS-PAGE followed by Coomassie staining. Pool the fractions from the size-exclusion purification step that contain the pure protein.

*Tip:* Exemplar Superdex 200 chromatogram of purified K<sub>2p</sub>2.1 (TREK-1) and SDS-PAGE analysis is shown in Fig. 3C. Depending on how the SDS sample is treated, you may see some reduced but undissociated dimer as shown in the inset.

(Pause **point**)



### 3. Generation of anti-GFP nanobody Sepharose resin

This section describes the production of anti-GFP nanobody resin using a nanobody described by (Kirchhofer et al., 2010). This reagent is essential for the purification protocol.

#### 3.1 Expressing anti-GFP nanobody in *E. coli*

##### Materials

- anti-GFP nanobody (Kubala, Kovtun, Alexandrov, & Collins, 2010) subcloned in pET24a (Lolicato et al., 2017).
- *E. coli* BL21 (DE3) Gold cells.
- QIAprep Spin Miniprep Kit (Qiagen, 27106).

- heatblock.
- LB medium and ampicillin (Fisher, 69-52-3).
- 37 °C shaking incubator.
- LB agar plates having ampicillin at 100 µg/mL.
- 2 × YT medium:  
    *To prepare:* Add 16 g Bacto Tryptone, 10 g Bacto Yeast Extract, and 5 g NaCl in ~900 mL H<sub>2</sub>O. Adjust pH to 7.0 with 5 N NaOH. Adjust to 1 L with distilled H<sub>2</sub>O.  
    Sterilize by autoclaving.
- baffled 3 L flask.
- Isopropyl β-D-1-thiogalactopyranoside (IPTG, Goldbio, 12481).
- centrifuge.

### Protocol

- (1) Transform *E. coli* BL21 (DE3) Gold cells by heat shock with the vector containing the anti-GFP nanobody gene.
- (2) Prepare purified anti-GFP nanobody vector with standard Mini prep procedure, according to manufacturer's protocol (QIAprep Spin Miniprep Kit).
- (3) Add 1 µL of 10 ng/µL anti-GFP nanobody plasmid to *E. coli* BL21 (DE3) Gold cells and incubate them for 20 to 30 min on ice.
- (4) Put this cell-DNA mixture on a 42 °C heatblock for 45 s and immediately transfer to ice.
- (5) After 5 min add 600 µL LB medium and incubate in a shaker at 37 °C for 30 min. Plate the transformed cells onto LB agar plates having ampicillin at 100 µg/mL. Grow at 37 °C overnight. You can store these plates at 4 °C for 2 to 3 weeks.

#### (Pause point)

- (6) Pick a single colony and inoculate an LB culture of the appropriate size in a shaker flask. 10 mL of inoculating culture should be grown for each liter of medium that will be used in Step 7. Add ampicillin to a final concentration of 100 µg/mL. Place the culture in a shaker overnight at 37 °C and 225 r.p.m.
- (7) Inoculate each liter of 2 × YT medium with 10 mL of the overnight growth culture in a baffled 3 L flask. Add ampicillin to a final concentration of 100 µg/mL.
- (8) Grow the cultures in a shaker at 37 °C and 175–225 r.p.m. When OD<sub>600 nm</sub> reaches ~0.3, shift the temperature set point to 20 °C.
- (9) When OD<sub>600 nm</sub> reaches 0.5–0.6, induce protein expression by adding IPTG to a final concentration of 0.5 mM. Continue to grow the cultures overnight at 20 °C.

- (10) Transfer the cells to Beckman 1 L centrifuge bottles and harvest the cells by centrifuging for 15 min at  $9000 \times g$  and  $4^\circ\text{C}$ .
- (11) Discard the supernatant. The cells can be frozen at this point and stored at  $-80^\circ\text{C}$ .

**(Pause point)**

### 3.2 Anti-GFP nanobody purification

*Tip:* This prep should yield  $\sim 100$  mg of purified anti GFP nanobody from 12 L of culture.

#### Materials

- Buffer A: 300 mM NaCl, 20 mM Imidazole, 10% glycerol, 50 mM Na-phosphate pH 7.5.
- Buffer B: 300 mM NaCl, 500 mM Imidazole, 10% glycerol, 50 mM Na-phosphate pH 7.5.
- SEC buffer: 150 mM NaCl, 50 mM Na-phosphate, pH 7.5.
- DNase I (Roche, 10104159001).
- Phenylmethanesulfonyl fluoride (PMSF, Sigma, P7626-100g).
- 1 M  $\text{MgCl}_2$ .
- Sonic Dismembrator Model 500 (Fisher Scientific).
- Centrifuge.
- Ni-NTA agarose resin (Qiagen, 160015386).
- Gel filtration column (Superdex75 10/300 GL).
- Ultra spin concentrator (Amicon Ultra-15 centrifugal filters, Millipore, 10 kDa cut-off).
- Microcentrifuge.

#### Protocol

- (1) Thaw frozen cell pellets on ice.
- (2) For each liter of cell culture, resuspend the thawed cell pellet in 25 mL of Buffer A supplemented with final concentrations of 2 mM  $\text{MgCl}_2$ , 1 mM PMSF and 0.1 mg/mL DNase I. Agitate by vigorous stirring with a magnetic stir bar at  $4^\circ\text{C}$  until the suspension is homogeneous.
- (3) Lyse the cells by sonication with Sonic Dismembrator Model 500 (Fisher Scientific) for 4 min with 2 s ON/2 s OFF pulses in ice-cold water bath under 34% amplitude of sonication tip model CE CONVERTER 102C (Branson).
- (4) Centrifuge for 1 h at  $125,000 \times g$  and  $4^\circ\text{C}$  to pellet unlysed cells and cellular debris. Collect the supernatant.

- (5) Prepare the 40 mL Ni-NTA agarose resin column. 40 mL Ni-NTA agarose resin is enough for culture anti-GFP nanobody purification. All purification steps should be carried out at 4 °C with buffers chilled to 4 °C.
- (6) Load the supernatant collected onto the column containing the equilibrated 40 mL Ni-NTA agarose resin. Collect and save the flow-through containing the unbound material. The flow-through should not contain the target protein but should be saved until this is confirmed by Coomassie gel.
- (7) Wash the column with 2 CV of 100% Buffer A and 2 CV of 94% Buffer A mixed with 6% Buffer B.
- (8) Elute the protein with 2 CV of 40% Buffer A mixed with 60% Buffer B and collect the peak on the chromatogram. Wash the column with 2 CV of 100% Buffer B.
- (9) Analyze the eluant by SDS-PAGE followed by Coomassie staining.
- (10) Equilibrate a gel filtration column (Superdex75 10/300 GL) connected to the chromatography system in size-exclusion buffer following the manufacturer's instructions.
- (11) Concentrate the protein obtained from affinity column purification with an ultra spin concentrator (Amicon Ultra-15 centrifugal filters, Millipore, 10 kDa cut-off) and centrifuge the sample to be injected onto the gel filtration column for 10 min at  $>10,000 \times g$  and 4 °C.
- (12) Inject the sample onto the gel filtration column. Collect 0.5- to 1-mL fractions of the eluate while monitoring at 280 nm.
- (13) Analyze fractions for protein composition via SDS-PAGE followed by Coomassie staining. Pool the fractions from the size-exclusion purification step that contain the pure protein. The pure proteins can be frozen at this point and stored at -80 °C.

*Tip:* anti-GFP nanobody extinction coefficient is  $27,055 \text{ M}^{-1} \text{ cm}^{-1}$ , MW = 13.78 kD.

**(Pause point)**

### 3.3 Conjugation of anti-GFP nanobody to CNBr Sepharose resin

The quantities shown in this protocol are to make ~100 mL of hydrated GFP nanobody-Sepharose.

#### Materials

- CNBr-activated Sepharose™ 4B (GE Healthcare, 17-0430-02).
- 1 mM HCl.

- Coupling buffer: 0.5 M NaCl, 0.1 M NaHCO<sub>3</sub>, pH 8.3.
- Blocking buffer: 150 mM NaCl, 50 mM Glycine, 50 mM NaH<sub>2</sub>Phosphate, pH 8.0.
- Wash buffer: 150 mM NaCl, 10 mM NaH<sub>2</sub>Phosphate, pH 7.0.
- Storage buffer: wash buffer with 20% (v/v) EtOH.
- 1 L Nalgene rapid-flow filter unit, 0.45 μm aPES membrane, 90 mm diameter (Thermo Scientific, 167–0045).
- 250 mL conical tube.
- Orbitron Rotator II Model 260250, Boekel Scientific.

### Protocol

- (1) Swell 25 g dry CNBr-activated Sepharose™ 4B resin in 250 mL of 1 mM ice-cold HCl. Use a spatula to crush any solids.
- (2) Load the beads onto a 1 L Nalgene filter unit and let the resin settle by gravity. Wash them with 2 L of ice-cold 1 mM HCl by gravity.
- (3) Wash with 2 L of ice-cold Coupling Buffer by gravity. Harvest the washed resin into a 250 mL conical tube.
- (4) Dilute the purified GFP nanobody of Step (13) of [Section 3.2](#) in ice-cold Coupling Buffer to make 100 mL at a concentration of 1 mg/mL. This is the equivalent of reacting ~4 mg of nanobody per g of dry resin.
- (5) Incubate beads of Step (3) with the diluted nanobody of Step (4) and rotate at 4 °C overnight.
- (6) Load the resin-GFP nanobody mixture onto a new 1 L Nalgene filter unit. Save flow-through to check for unbound protein. If conjugation is successful it should be nearly protein free.
- (7) Wash beads with 100 mL Coupling Buffer by gravity.
- (8) Incubate beads in 125 mL of Blocking Buffer in a 250 mL conical tube. Rotate for 2 h at room temperature.
- (9) Load the beads onto a new 1 L Nalgene filter unit and let the resin settle by gravity. Wash beads with 1 L Wash Buffer by gravity.
- (10) Add appropriate amount of Storage Buffer to make a 50% slurry and store at 4 °C.

**(Pause point)**



## 4. Generation of 3C protease

This protocol follows the general description of 3C protease production and purification as described by ([Shaya et al., 2011](#)).

## 4.1 Expressing 3C protease in *E. coli*

### Materials

- *E. coli* BL21 (DE3) pLysS cells.
- pET28\_3C protease bearing a His<sub>8</sub> tag (Shaya et al., 2011).
- QIAprep Spin Miniprep Kit (Qiagen, 27106).
- LB medium, Kanamycin Sulfate (Growcells, MBPC-2300), and Chloramphenicol (Merck, 220551-100 g).
- LB agar plates having Kanamycin at 50 µg/mL and Chloramphenicol at 34 µg/mL.
- 2 × YT medium (see Section 3.1).
- baffled 250 mL flask.
- baffled 3-L flask.
- Isopropyl β-D-1-thiogalactopyranoside (IPTG, Goldbio, 12481).
- 37 °C Shaking incubator.
- heatblock.
- centrifuge.

### Protocol

- (1) Transform *E. coli* BL21 (DE3) pLysS cells by heat shock with the vector containing the 3C protease gene using the general heat shock transformation protocol in Section 3.1.
- (2) Plate the transformed cells onto LB agar plates having Kanamycin at 50 µg/mL and Chloramphenicol at 34 µg/mL. Grow at 37 °C overnight. You can store these plates at 4 °C for 2 to 3 weeks.  
(Pause point)
- (3) Pick a single colony and inoculate an LB culture in a baffled 250 mL shaker flask. 10 mL of inoculating culture should be grown for each liter of medium that will be used in Step 4. Add Kanamycin to a final concentration of 50 µg/mL and Chloramphenicol to a final concentration of 34 µg/mL. Place the culture in a shaker overnight at 37 °C and 225 r.p.m..
- (4) Inoculate each liter of 2 × YT medium with 10 mL of the overnight growth culture in a baffled 3L flask. Add Kanamycin to a final concentration of 50 µg/mL and Chloramphenicol to a final concentration of 34 µg/mL.
- (5) Grow the cultures in a shaker at 37 °C and 175–225 r.p.m.. When OD<sub>600 nm</sub> reaches ~0.4 shift the temperature set point to 30 °C.
- (6) When OD<sub>600 nm</sub> reaches 0.6–0.8, induce protein expression by adding IPTG to a final concentration of 0.4 mM. Continue to grow the cultures overnight at 30 °C.

- (7) Transfer the cells to Beckman 1 L centrifuge bottles and harvest the cells by centrifuging for 15 min at  $9000 \times g$  and  $4^\circ\text{C}$ .
  - (8) Discard the supernatant. The cells can be frozen at this point and stored at  $-80^\circ\text{C}$ .
- (Pause point)**

## 4.2 3C protease purification

The quantities shown in this protocol are to purify 6L culture of 3C protease.

### Materials

- emulsiFlex homogenizer (Avestin, EF-C5).
- 40 mL Ni-NTA agarose resin (Qiagen, 160015386).
- HiTrap Desalting 53 mL column.
- Cation exchange HiLoad<sub>16/10</sub>\_SP\_Sepharose\_HP 20 mL column.
- Lysis buffer: 500 mM NaCl, 50 mM Tris-Cl pH 8.0.
- Wash buffer: 300 mM NaCl, 50 mM Tris-Cl pH 8.0.
- Elution buffer: 300 mM Imidazole, 300 mM NaCl, 50 mM Tris-Cl pH 8.0.
- Desalting column buffer: 50 mM NaCl, 5% glycerol, 50 mM Mes pH 6.0.
- Cation exchange column buffer A: 50 mM NaCl, 5% glycerol, 50 mM Mes pH 6.0.
- Cation exchange column buffer B: 1 M NaCl, 5% glycerol, 50 mM Mes pH 6.0.
- Dialysis buffer: 150 mM NaCl, 20% glycerol, 10 mM EDTA, 50 mM Tris-Cl pH 8.0.
- dialysis tube, 3.5 kDa cut-off (Spectrum Laboratories, Spectra/Por 6 Dialysis Membrane Pre-wetted RC Tubing MWCO: 3.5 kD, 132592).
- DNase I (Roche, 10104159001).
- Phenylmethanesulfonyl fluoride (PMSF, Sigma, P7626-100 g).
- 1 M  $\text{MgCl}_2$ .
- $16\text{G} \times 1$  needles ( $1.6\text{ mm} \times 25\text{ mm}$ , BD PrecisionGlide, 305197).
- ultracentrifuge.

### Protocol

- (1) Thaw frozen cell pellets from 6L culture on ice.
- (2) Resuspend the thawed cell pellet in 100 mL Lysis buffer containing final concentrations of 0.5 mM  $\text{MgCl}_2$ , 1 mM PMSF, and 0.1 mg/mL DNase I. Agitate by vigorous stirring with a magnetic stir bar at  $4^\circ\text{C}$  for about 1 h until the suspension is homogeneous. Filter resuspended cells through the needle of  $16\text{G} \times 1$  ( $1.6\text{ mm} \times 25\text{ mm}$ ) with syringe.



- (3) Break the cells by emulsiflex for three cycles at 15 Kpsi in ice-cold condition.
- (4) Centrifuge for 1 h at 125,000 × *g* and 4 °C to pellet unlysed cells and cellular debris. Collect the supernatant.
- (5) Prepare 40 mL Ni-NTA agarose resin column. All purification steps should be carried out at 4 °C with buffers chilled to 4 °C.
- (6) Load the supernatant collected onto the 40 mL Ni-NTA agarose resin column equilibrated with 2 CV of Wash buffer. Collect and save the flow-through containing the unbound material. The flow-through should not contain the target protein but should be saved until this is confirmed by Coomassie gel.
- (7) Wash the column with 3 CV of Wash buffer.
- (8) Elute the protein with 2.5 CV of Elution buffer.  
*Tip:* Prior to the elution collection, add one elution volume of wash buffer in each tube of the fraction collector. The imidazole will be diluted down to 150 mM, preventing precipitation.
- (9) Collect 4 mL fractions of the eluate corresponding to the protein peak while monitoring at 280 nm, combine them.
- (10) Equilibrate a HiTrap Desalting 53 mL column connected to the chromatography system in a Desalting column buffer following the manufacturer's instructions.
- (11) Load the eluate of step 9 onto the HiTrap Desalting 53 mL column. Collect 20 mL fractions of the eluate while monitoring at 280 nm. You can store the eluate at 4 °C overnight.  
**(Pause point)**
- (12) Equilibrate a Cation exchange HiLoad\_16/10\_SP\_Sepharose\_HP 20 mL column connected to the chromatography system in 2 CV of Cation exchange column buffer A following the manufacturer's instructions.
- (13) Load the eluate of step 11 onto the Cation exchange column.
- (14) Wash the column with 4 CV of Cation exchange column buffer A. Elute the protein with 9 CV of Cation exchange column buffer A with 0 to 100% gradient Cation exchange column Buffer B. Collect 2.5 mL fractions of the eluate corresponding to the protein peak while monitoring at 280 nm, and combine them.  
*Tip:* Elution from the Cation exchange column should give two peaks. They do not run exactly the same, but they both cleave equally, suggesting both peaks contain functional 3C protease.
- (15) Analyze fractions for protein composition via SDS-PAGE followed by Coomassie staining.

- (16) Harvest the appropriate eluate of step 14 in a dialysis tube (3.5 kDa cut-off) and dialyze it with a 4 L ice-cold dialysis buffer at 4 °C overnight.
- (17) Quantify concentration of the purified protein.  
(The theoretical extinction coefficient is  $6085 \text{ M}^{-1} \text{ cm}^{-1}$ , MW = 21.28 kD)
- (18) Aliquot in 200  $\mu\text{L}$ , flash-freeze in liquid N<sub>2</sub>, and store at -80 °C.  
**(Pause point)**



## 5. Reconstitution of K<sub>2p</sub>2.1 (TREK-1) in nanodiscs

Strategies for incorporating K<sub>2p</sub>2.1 (TREK-1) into lipid nanodiscs (Denisov & Sligar, 2016) make use of the dual tags present on the construct (Fig. 3A) to minimize the generation of empty nanodiscs. First K<sub>2p</sub>2.1 (TREK-1) is purified using the His-tag available (and not the GFP tag as mentioned previously). After reconstitution in nanodiscs, the sample is passed through the anti-GFP column to remove empty nanodiscs. Reconstituted K<sub>2p</sub>2.1 (TREK-1) is, then, eluted with on-column 3C cleavage as described previously.

### 5.1 Expressing MSP1E1 protein

#### Materials

- *E. coli* BL21 (DE3) Gold cells.
- MSP1E1 cDNA subcloned in pET28a (plasmid #20062).
- QIAprep Spin Miniprep Kit (Qiagen, 27106).
- LB agar plates having Kanamycin at 50  $\mu\text{g}/\text{mL}$ .
- LB medium and Kanamycin Sulfate (Growcells, MBPC-2300).
- baffled 250 mL flask.
- 37 °C Shaking incubator.
- TB (Terrific Broth) medium:
  - Add 900 mL of deionized water to 24 g of yeast extract, 20 g of tryptone, and 4 mL of glycerol.
  - Stir until the solutes have dissolved.
  - Sterilize by autoclaving.
  - Allow the solution to cool to ~60 °C and add 100 mL of sterile 10 × phosphate buffer (0.17 M KH<sub>2</sub>PO<sub>4</sub>, 0.72 M K<sub>2</sub>HPO<sub>4</sub>).
- baffled 3 L flask.

- coffee filter papers.
- Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG, Goldbio, 12481).
- heatblock.
- centrifuge.

### Protocol

- (1) Transform *E. coli* BL21 (DE3) Gold cells by heat shock with the vector containing the MSP1E1 gene using the heat shock protocol in [Section 3.1](#).
- (2) Plate the transformed cells onto LB agar plates with kanamycin at 50  $\mu$ g/mL. Grow at 37°C overnight. You can store these plates at 4°C for 2 to 3 weeks.

#### (Pause point)

- (3) Pick a single colony and inoculate an LB culture in a baffled 250 mL flask. 10 mL of inoculating culture should be grown for each liter of medium that will be used in Step 4. Add Kanamycin to the culture (final concentration 50  $\mu$ g/mL). Place the culture in a shaker overnight at 37°C and 225 r.p.m..
- (4) Inoculate each liter of TB medium with 10 mL of the overnight growth culture in a baffled 3 L flask. Add Kanamycin to a final concentration of 50  $\mu$ g/mL to the culture. Use paper lid such as coffee filter papers for maximum aeration.
- (5) Grow the cultures in a shaker at 37°C and 175–225 r.p.m. until the optical density at 600 nm reaches 2.
- (6) Induce protein expression by adding IPTG to a final concentration of 1 mM. Continue to grow the cultures for 3 h at 37°C.
- (7) Transfer the cells to Beckman 1 L centrifuge bottles and harvest the cells by centrifuging for 15 min at 9000  $\times$  g and 4°C.
- (8) Discard the supernatant. The cells can be frozen at this point and stored at –80°C.

#### (Pause point)

## 5.2 MSP1E1 purification

### Materials

- emulsiFlex homogenizer (Avestin, EF-C5).
- Ni-NTA agarose resin (Qiagen, 160015386).
- Lysis buffer: 20 mM Phosphate buffer pH 7.4.
- Wash Buffer 1: 0.3 M NaCl, 1% (v/v) Triton X-100, 40 mM Tris-Cl pH 8.0.

- Wash Buffer 2: 0.3M NaCl, 50mM Na-Cholate, 20mM Imidazole, 40mM Tris-Cl pH8.0.
- Wash Buffer 3: 0.3M NaCl, 50mM Imidazole, 40mM Tris-Cl pH8.0.
- Elution Buffer: 0.3M NaCl, 0.4M Imidazole, 40mM Tris-Cl pH8.0.
- Dialysis Buffer: 0.1M NaCl, 0.5mM EDTA, 20mM Tris-Cl pH7.4.
- dialysis tube, 3.5kDa cut-off (Spectrum Laboratories, Spectra/Por 6 Dialysis Membrane Pre-wetted RC Tubing MWCO: 3.5 kD, 132592).
- DNase I (Roche, 10104159001).
- Phenylmethanesulfonyl fluoride (PMSF, Sigma, P7626-100g).
- 1M MgCl<sub>2</sub>.
- 16G × 1 needles (1.6 mm × 25 mm, BD PrecisionGlide, 305197).
- ultracentrifuge.

### Protocol

- (1) Thaw frozen cell pellets on ice.
- (2) Resuspend the thawed cell pellet in Lysis buffer containing final concentrations of 1 mM MgCl<sub>2</sub>, 1 mM PMSF, and 0.025 mg/mL DNase I. 25 mL lysis buffer is enough to resuspend 1 L culture cells. Agitate by vigorous stirring with a magnetic stir bar at 4°C for about 1 h until the suspension is homogeneous. Filter resuspended cells through the needle of 16G × 1 (1.6 mm × 25 mm) with syringe.
- (3) Break the cells by emulsiflex for three cycles at 15 Kpsi in ice-cold condition.
- (4) Centrifuge for 1 h at 125,000 × *g* and 4°C to pellet unlysed cells and cellular debris. Collect the supernatant.
- (5) Prepare the appropriate amount of Ni-NTA agarose resin according to the manufacturer's instructions. All affinity purification steps should be carried out at 4°C with buffers chilled to 4°C.
- (6) Load the supernatant collected onto a 40 mL column containing the equilibrated Ni-NTA agarose resin. Collect and save the flow-through containing the unbound material. The flow-through should not contain the target protein but should be saved until this is confirmed by Coomassie gel.
- (7) Wash the column with 5 CV of Wash buffer 1, 5 CV of Wash buffer 2, and 5 CV of Wash buffer 3.
- (8) Elute the protein with 2.5 CV of Elution buffer and collect the peak on the chromatogram.
- (9) Analyze the fractions for protein composition by SDS-PAGE followed by Coomassie staining.

- (10) Harvest the appropriate eluant in a dialysis tube (3.5 kDa cut-off) and dialyze it with an ice-cold dialysis buffer at 4 °C overnight.
- (11) Analyze the protein by SDS-PAGE followed by Coomassie staining.
- (12) Quantify concentration of the purified protein.  
(The theoretical extinction coefficient is 32430 M<sup>-1</sup> cm<sup>-1</sup>, MW = 27.49 kD)  
**(Pause point)**

### 5.3 K<sub>2p</sub>2.1 (TREK-1) purification for nanodisc reconstitution

#### Materials

- Ball Mill (Retsch model MM400).
- Solubilization buffer: 60 mM DDM, 200 mM KCl, 100 mM Tris-Cl pH 8.2.
- Equilibration buffer: 6 mM DDM, 200 mM KCl, 50 mM Tris-Cl pH 8.0.
- Wash buffer 1: 10 mM imidazole, 6 mM DDM, 200 mM KCl, 50 mM Tris-Cl pH 8.0.
- Wash buffer 2: 30 mM imidazole, 6 mM DDM, 200 mM KCl, 50 mM Tris-Cl pH 8.0.
- Elution buffer: 500 mM imidazole, 6 mM DDM, 200 mM KCl, 50 mM Tris-Cl pH 8.0.
- Desalting buffer: 1 mM DDM, 200 mM KCl, 20 mM Tris-Cl pH 8.0.
- ultracentrifuge.
- 250 mL conical tube.
- Talon Metal Affinity resin (Takara, 635503).
- DNase I (Roche, 10104159001).
- Phenylmethanesulfonyl fluoride (PMSF, Sigma, P7626-100 g).
- 500 mM EDTA pH 8.0.
- 3 M KCl.
- 3C protease (Section 4).
- Orbitron Rotator II Model 260250, Boekel Scientific.
- Disposable chromatography columns 10 mL, Bio-Rad.
- Ultra spin concentrator (Amicon Ultra-15 centrifugal filters, Millipore, 100 kDa cut-off).
- Econo-Pac 10DG Desalting columns (Bio-Rad, 7322010).
- tabletop microcentrifuge.

## Protocol

- (1) Break the frozen cell noodles by milling (Retsch model MM400) for three cycles of 3 min at 25 Hz. Keep the cell noodles and broken cell powder in liquid N<sub>2</sub> for the whole process. Broken cell powder can be stored at -80 °C. All subsequent purification steps should be carried out at 4 °C with buffers chilled to 4 °C.  
**(Pause point)**
- (2) Resuspend the thawed cell powder in 3 mL of membrane solubilization buffer added with final concentrations of 1 mM PMSF and 0.1 mg/mL DNase I for each gram of cell powder. Agitate with a magnetic stir bar at 4 °C for 3 h to yield a homogeneous suspension.
- (3) Transfer the solubilization mixture to a clean, chilled ultracentrifuge tube and pellet unsolubilized material by centrifugation for 45 min at 95,800 × *g* and 4 °C. Decant the supernatant into a 250 mL conical tube.
- (4) Equilibrate Talon resin (5 mL) with Equilibration buffer. Add the resin to the supernatant and incubate the protein-resin mixture for 3 h at 4 °C with gentle rotation.
- (5) Load the protein-resin mixture of Step (4) onto an empty column (Disposable chromatography columns 10 mL) and let the resin settle by gravity. Let the liquid flow through and collect the flow-through to check for unbound protein.
- (6) Wash the column with 5 CV of Wash buffer 1 and 5 CV of Wash buffer 2.
- (7) Elute the protein with 5 CV of Elution buffer. Analyze the eluant for protein composition via SDS-PAGE followed by Coomassie staining.
- (8) Concentrate the protein to 3 mL with an ultra spin concentrator (100 kDa cut-off) and centrifuge the sample for 10 min at >10,000 × *g* and 4 °C.
- (9) Desalt the sample to 4 mL with a desalting buffer following the manufacturer's instructions.
- (10) Quantify concentration of the purified K<sub>2P</sub>2.1 (TREK1)-GFP protein and store at 4 °C until use in reconstitution.  
(The theoretical extinction coefficient is 76,445 M<sup>-1</sup> cm<sup>-1</sup>, MW = 63.30 kD)  
**(Pause point)**

## 5.4 Reconstitution of K<sub>2p</sub>2.1 (TREK-1) in nanodiscs

### Materials

- soybean polar lipid extract (Avanti polar lipids, 541602P-100 mg).
- Bio-Beads SM-2 Adsorbent Media (Bio-rad, 152-8920).
- Reconstitution buffer: 20–30 mM DDM, 200 mM KCl, 20 mM Tris-Cl pH 8.0.
- SEC nanodisc buffer: 200 mM KCl, 20 mM Tris-Cl pH 8.0.
- methanol (MX0485-7, Merck).
- sintered glass funnel and Milli-Q treated water (Millipore).
- 50 mL conical polypropylene tubes.
- sepharose resin coupled to anti-GFP nanobody (Section 3).
- vortexer.
- Sonic Dismembrator Model 500 (Fisher Scientific).
- Orbitron Rotator II Model 260250, Boekel Scientific.
- 3C protease (Section 4).
- Gel filtration column (Superose200 10/300 GL) connected to the chromatography system.
- ultra spin concentrator (100 kDa cut-off, Amicon Ultra-0.5 mL Centrifugal filters, Merck Millipore Ltd.)
- tabletop microcentrifuge.

### Protocol

- (1) Prepare lipid stocks in chloroform (100 mg/2.5 ml) and store at –20 °C in glass vials with Teflon-lined screw caps.
- (2) Transfer the desired amount (20 mg) of chloroform lipid stock into a clean glass tube and dry lipids to a thin film using a gentle stream of nitrogen gas in a fume hood.
- (3) To remove residual solvent, place the tube in a vacuum dessicator under high vacuum overnight.
- (4) Add reconstitution buffer to the dried lipid film. Based on the molecular weight of the lipidic species present in the soybean extract, 20 mg of soybean polar lipid extract resuspended in 0.69 mL of reconstitution buffer yields a 40 mM solution. Vortex the lipid solution vigorously for 10 s and sonicate for 30 s under 13% amplitude of sonication tip model CE CONVERTER 102C (Branson) in ice-cold water bath. Pipette up and down to check if there is any aggregate of lipids. Repeat vortexing and sonication two or three times until no lipid

remains on the walls of the tube. Alternatively use a bath sonicator for 5 min, then vortex for 30s and repeat until the solution is white but transparent.

- (5) Add purified MSP1E1 (Step 12 of Section 5.2) to soybean polar lipid to yield desired lipid:MSP1E1 ratio. Incubate the mixture for 15 min or longer on ice. As described in (Ritchie et al., 2009) the final lipid concentration of the reaction should not be lower than 8mM.

*Tip:* Optimization of the lipid: protein stoichiometry during the self-assembly process is important. Generally, 1:1:100 K<sub>2p</sub>2.1 (TREK-1):MSP1E:lipid is optimal.

- (6) Add purified K<sub>2p</sub>2.1 (TREK-1)-GFP (step 10 of Section 5.3) to the lipid:MSP1E1 of Step (5) to yield desired lipid:MSP1E1:K<sub>2p</sub>2.1 (TREK-1)-GFP ratio. Incubate on ice for 1h.
- (7) Prepare Bio-beads SM-2 in a 50 mL conical tube by suspending in about 30 mL of 100% methanol and gently rotating for 30 min at room temperature. Remove methanol by passing through a sintered glass funnel. Wash the Bio-beads with ~1L of Milli-Q water (Millipore) by flowing through the sintered glass funnel to remove methanol traces. Equilibrate the Bio-beads with ~500mL of SEC nanodisc buffer in the same way. About ~500mg wet Bio-beads SM-2 per mL of lipid-protein mixture solution is needed.
- (8) Add half of the beads to the lipid-protein mixture to remove detergent and rotate gently for one to 2h at 4°C. Transfer the supernatant to a new microcentrifuge tube. Avoid transferring the beads. Add the rest of the beads and rotate gently overnight at 4°C.
- (9) Transfer the supernatant to a new microcentrifuge tube while avoiding transferring the beads. Equilibrate Sepharose resin coupled to anti-GFP nanobody with 10 CV of SEC nanodisc buffer. 0.5mL of resin is enough for the 100µL of 10mg/mL K<sub>2p</sub>2.1 (TREK-1)-GFP. Add the resin to the supernatant and incubate the mixture for 1h at 4°C with gentle rotation.
- (10) Load the mixture of Step (9) onto an empty column and let the resin settle by gravity. Let the liquid flow through and collect the flow-through to check for unbound protein.
- (11) Wash the column with 10 CV of SEC nanodisc buffer.
- (12) Add 3C protease to 2 CV of SEC nanodisc buffer at a target protein to 3C protease ratio of 50:1 (w:w) to cleave off GFP and release the protein from the resin. Add the 3C buffer to the resin and mix with the pipette. Cap the top of the column. Incubate for 12–18h (or overnight) at 4°C.



- (13) Collect the flow-through. Wash the resin with 2 CV of SEC nanodisc buffer and collect the flow-through. Combine this with the previous flow-through.
  - (14) Equilibrate a gel filtration column (Superose200 10/300 GL) connected to the chromatography system in 2 CV of size-exclusion buffer following the manufacturer's instructions.
  - (15) Concentrate the sample with an ultra spin concentrator (100 kDa cut-off) and centrifuge the sample to be injected onto the gel filtration column for 10 min at  $>10,000 \times g$  and 4 °C.
  - (16) Inject the sample onto the gel filtration column. Collect 0.3 mL fractions of the eluate while monitoring at 280 nm.
  - (17) Analyze fractions for protein composition via SDS-PAGE followed by Coomassie staining. Pool the fractions from the size-exclusion purification step that contain the pure protein.
- (Pause point)**



## 6. Summary

The above protocols yield structure-grade preparation of K<sub>2p</sub>2.1 (TREK-1). Such preparations of K<sub>2p</sub>2.1 (TREK-1) and K<sub>2p</sub>2.1 (TREK-1) mutants have yielded diffraction quality crystals of K<sub>2p</sub>2.1 (TREK-1) (Lolicato et al., 2017, 2020) and K<sub>2p</sub>2.1 (TREK-1):modulator complexes (Lolicato et al., 2017, 2020; Pope et al., 2020) suitable for structural studies (Fig. 3D).

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