Extracellular modulation of TREK-2 activity with nanobodies provides insights into the mechanism of K2P channel regulation

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Abstract

Potassium channels of the Two-Pore Domain (K2P) subfamily, *KCNK1-KCNK18*, play crucial roles in controlling the electrical activity of many different cell types and represent attractive therapeutic targets. However, the identification of highly selective small molecule drugs against these channels has been challenging due to the high degree of structural and functional conservation that exists not only between K2P channels, but across the whole K^+ channel superfamily. To address the issue of selectivity, we generated camelid antibody fragments (nanobodies) against the TREK-2 (*KCNK10*) K2P K^+ channel and identified selective binders including several that directly modulate channel activity. Crystal structures of these nanobodies in complex with TREK-2 also reveal insights into their mechanisms of activation and inhibition via binding to the extracellular loops and Cap domain, as well as their suitability for immunodetection. These tools therefore provide important insights into TREK channel gating and a more selective approach to the modulation of K2P channel activity via their extracellular domains.

Introduction

Ion channels control or contribute to the electrical activity and function of almost every living cell. Their selective pharmacological manipulation therefore offers wide-ranging approaches to plant, animal and human health (1). In particular, the regulation of K^+-selective ion channels presents therapeutic potential for a wide variety of human disorders including epilepsy, arrhythmias and various other cardiovascular diseases and neurological conditions, especially the control of pain. Significant effort has therefore been expended in the identification of drugs that target specific types of K^+ channels for the treatment of such disorders (2, 3).

However, although there are over 80 different K^+ channel genes in the human genome, most have a similar architecture in their pore region because this highly conserved structural feature facilitates the selective permeation of K^+ as well as some of the conserved gating mechanisms they employ. As a consequence, the identification of highly selective small molecule agonists and/or antagonists that exhibit no cross-reactivity with other K^+ channels is challenging (1, 4).

Two-Pore Domain K^+ channels (K2P) are a distinct subset of K^+ channels where each gene encodes a subunit with two pore-forming domains that dimerise to create a single pseudotetrameric K^+ selective channel across the membrane (5). These channels underlie the background K^+ currents that control the membrane potential in many different cell types where their activity is often regulated by diverse stimuli (6). In particular, the TREK subfamily of K2P channels (TREK-1, TREK-2 and TRAAK) are regulated by a wide variety of physical and chemical stimuli. This polymodal regulation allows them to integrate cellular electrical activity with a diverse range of cellular signalling pathways (2, 7).

A number of structures now exist for members of the TREK subfamily that has allowed detailed gating models to be proposed (4, 8-11). Like many other K^+ channels, movement of the pore-lining helices, in particular the M4 helix appears important for the regulation of TREK channel gating. However, unlike many other K^+ channels these movements do not constrict a lower 'bundle-crossing-like' gate at the cytoplasmic entrance to the pore. Instead, the channel appears to be gated primarily by much smaller structural changes in the selectivity filter with the 'M4 Up' conformation supporting a more active state of the channel than the M4 down conformations. However, there is evidence that the channel can support K^+ conduction in both conformations (12, 13). Recent evidence suggests that the movement of M4 couples to the filter gate via changes in the network of interactions that support the selectivity filter in its activated state (14).

The expression of TREK channels within the central and peripheral nervous systems (7) and their involvement in processes such as pain perception, neuroprotection, and anaesthesia makes them attractive therapeutic targets (2, 15). Recent approaches have focused on the development of TREK channel agonists as potential analgesics and several lead compounds have been identified that target different elements of the gating mechanism within the selectivity filter of the channel (4, 11, 16). However, although such small molecules have proven to be highly effective agonists, and much is now known about their mechanism of action, they often exhibit cross reactivity within the TREK subfamily. Also, in many cases, these agonists activate other classes of K^+ channel thereby limiting their potential use (4). Other approaches to target these channels may therefore be required.

For some classes of ion channels, the adaptation of channel toxins and the development of RNA-based antagonists has been successful in achieving greater target selectivity

(17, 18), but perhaps the most promising approaches involve biologics that exploit protein-protein interactions for target recognition (19-22). Many antibody-based therapies are now in clinical use for the treatment of a variety of disorders and have a number of potential benefits compared to traditional approaches although their identification and development can be costly (23).

Nanobodies are single-domain (V_{HH}) antibodies derived from camelid heavy chain antibodies (24). Their name derives from their small size and when compared to small molecule inhibitors, have certain advantages such as high target specificity and low toxicity leading to fewer off-target effects (25). In addition, their small size and long complementarity determining region 3 (CDR3) loops enable them to access cavities or clefts in proteins that are inaccessible to antibodies (26).

They can also exhibit greater stability and solubility than conventional antibodies, so when combined with their relative ease of production and purification, this makes them ideal candidates for many therapeutic strategies where small molecule approaches have stalled (27), as well as equivalent detection surrogates for antibodies in immunocytochemistry (28). They are also particularly useful probes to study structure-function relationships and other properties of ion channels and membrane proteins in general (29).

In this study we have generated a range of nanobody binders to the TREK-2 K2P channel, several of which exhibited highly specific regulation of channel activity. The insights provided by these nanobodies highlight new mechanistic approaches to the study of K2P channels and their role *in vivo*, and expand the potential of nanobodies as therapeutic agents.

Results and Discussion

Generation of TREK-2 nanobodies. Nanobodies were produced as part of the Nanobodies4Instruct program in a method described by Pardon et al. (30). Briefly, purified human TREK-2 protein, identical to that used in previous structural studies (9) was used to elicit an immune response in llamas. The resulting immune repertoire was isolated and positive clones selected by phage panning and screening against the same TREK-2 protein. Overall, a panel of 29 unique nanobodies were produced (SI Appendix Fig S1) and these were further examined for their ability to produce a peak shift on size exclusion chromatography (SEC) to determine which nanobodies were capable of forming tight complexes (**Fig 1A**). These tight binders were considered more suitable for subsequent structural and/or functional applications.

Screening for functional effects. To determine possible functional effects of the nanobodies on channel activity, human TREK-2 channels were expressed in *Xenopus* oocytes and currents measured before and after extracellular addition of nanobody (0.5 μM). An increase (or decrease) in current size by more than 50% was used as the initial threshold in this screen. The results are shown in **Fig 1A** and reveal that from this initial panel, three exhibited clear functional modulation of TREK-2: nanobody CA10761 inhibited channel activity, whilst CA10767 and CA10776 both activated TREK-2. Functional effects of these nanobodies were also consistent with their tight binding as determined in the SEC shift assay (**Fig 1A**). These modulatory nanobodies were then selected for further structural studies. Another tight binder that exhibited no functional effect (CA10758) was also included.

Nanobody binding to the extracellular Cap domain. Structures of these four nanobodies in complex with TREK-2 were then determined by X-ray crystallography using approaches similar to those previously described (4, 9) and were resolved

between 2.4 Å and 3.6 Å resolution. For clarity these nanobodies were also renamed to reflect their functional effect (**Fig 1B** and SI Appendix Fig S2 and Table S1).

All four of these nanobodies exhibited binding to the extracellular Cap domain of TREK-2 (**Fig 1B**). Interestingly, the functionally inactive binder only interacted with the extracellular tip of the Cap domain whilst the modulatory nanobodies interact with both the Cap and extracellular loops much closer to the selectivity filter where the primary gate is located. In one case (CA10767), binding was also observed to the intracellular face as well as the Cap domain. The properties of these structures and the functional effects of each modulatory nanobody are described in more detail below.

Immunodetection of TREK-2 expression at the cell surface. Previous studies have demonstrated the usefulness of nanobodies for immunodetection as well as in various immunoassays and other nanobody-based protein targeting strategies (19, 28). The structures determined above show that these TREK-2 nanobodies bind to the extracellular Cap domain indicating that immunodetection of TREK-2 at the cell surface should be possible. To examine this, we chose the functionally inactive nanobody CA10758 which interacts with the most exposed tip of the Cap domain. This nanobody is hereafter referred to as Nb-Binder-58 (or Nb-58).

Many K2P channels exhibit N-linked glycosylation to their extracellular surfaces that may affect nanobody binding (31). However, only one glycosylation site exists within TREK-2 in the extracellular loop that precedes Pore-Helix-1 (PH1). None of the four chosen nanobodies interact with, or near this loop. Instead, Nb-Binder-58 binds as a dimer to the very tip of the Cap domain where it interacts with several residues that are unique between TREK-2 and the closely related TREK-1 channel (**Fig 2A**).

To determine whether this nanobody can detect TREK2 expression at the cell surface,

Nb-Binder-58 was fused to the mVenus fluorescent protein and applied to HEK-293 cells expressing TREK-2 tagged with the mCherry fluorescent protein. **Fig 2B** shows overlapping signals that are not observed on cells expressing TREK-1, nor for any other K2P channel (SI Appendix Fig S3). A similar, functionally inactive nanobody (CA10757: Nb-Binder-57 or Nb57) that exhibits almost complete sequence identity with Nb-Binder-58 is also capable of similar immunodetection of TREK-2 (**Fig 2B** and SI Appendix Figs S1 & S3). This indicates that both these nanobodies represent useful tools for the specific immunodetection of homomeric TREK-2 channels.

A partially-selective activatory nanobody. The first activatory nanobody (CA10767) hereafter referred to as Nb-Activator-67 (or Nb67), binds to the side of the Cap domain (**Fig 3A**) with all three CDR loops interacting with the external face of the Cap helices. However, these interactions are not symmetrical and on one side the EH1 Cap helix is partially unwound at the midpoint (residues 107-109; **Fig 3C**). The structure also reveals a nanobody bound to the intracellular side where it occludes the entrance to the inner cavity (**Fig 3A**). However, upon closer examination of the crystal structure this 'intracellular' interaction appears to result from tetramerisation of the nanobody within the unit cell that may drive the crystal packing and the resulting non-canonical structure (SI Appendix Fig S4A). Nevertheless, to rule out any functional effects of this intracellular interaction we examined the effect of this nanobody on TREK-2 currents in excised patches where it could be applied to the intracellular surface of the membrane. No functional effect on TREK-2 activity was observed even at 10 μM intracellular application (SI Appendix Fig S4B). This is in marked contrast to its clear activatory effect when applied extracellularly (**Fig 1A**). Further examination revealed that Nb-Activator-67 rapidly increased channel activity ~3-4 fold with nanomolar efficacy (EC_{50}= 101 ± 12 nM) when perfused onto oocytes expressing human TREK-2 (**Fig 3B**).

To address its selectivity, we examined effects on other K2P channels. No functional modulation was observed on either TALK-2 or TRAAK. However, an inhibitory effect was observed on the closely related TREK-1 channel at high concentrations of nanobody >1μM (**Fig 3B**). This opposite effect initially appeared interesting because of the opposite effects of extracellular pH on the activity of TREK-2 and TREK-1, and the role of the extracellular Pore/TM loops in this process (32). However, Nb-Activator-67 does not appear to contact these loops and instead only interacts with the extracellular face of the Cap domain resulting in an unwinding of the EH1 Cap helix on one side (**Fig 3C**). Intriguingly, both M4 helices in this TREK-2 structure are in the Up conformation consistent with a more activated state of the channel (**Fig 3A**), although this could also be due to its interactions with the nanobody bound to its intracellular surface. Full occupancy of ion binding sites within the filter of TREK channels is also usually associated with an activated state of the channel (9, 33), and in this structure all the sites appear occupied, albeit in an asymmetric fashion due to unwinding of the Cap on one side (**Fig 3A**). Although it is tempting to associate the effect of unwinding of EH1 with the activatory effects of Nb-Activator-67, the crystal packing interactions make interpretation of this particular structure challenging. Due to the additional lack of specificity between TREK-1 and TREK-2, the mechanism of activation by this nanobody was not investigated further. However, its robust activatory effect on recombinant homomeric TREK-2 channels, especially at lower concentrations, indicates that this nanobody may remain a useful tool in situations where the identity of its target is already known.

A selective activatory nanobody. Unlike Nb-Activator-67, the second activatory nanobody (CA10776, hereafter referred to as Nb-Activator-76 or Nb76) exhibited highly selective activation of TREK-2 with an efficacy of 412 ± 14 nM and had no effect on TREK-1 or other K2P channels tested, even when used at high concentration (**Fig 4A**

& SI Appendix Fig S4C). The structure of this nanobody in complex with TREK-2 reveals that the CDR loops interact with the side of the Cap domain involving residues at the intersubunit interface (**Fig 4B, C**). In particular, W98 on CDR3 of the nanobody inserts into the intersubunit groove between helices in the Cap domain of TREK-2 to form an interaction with E128 (**Fig 4C**). R53 on CDR2 also helps anchor the nanobody to the Cap helices by H-bonding with Q106 on TREK2 **(Fig 4C)**. Consistent with this, we found that mutation of either of these residues on Nb-Activator-76 (W98A or R53A) severely impaired its activatory effect (**Fig 4A**).

Nb-Activator-76 also contacts the extracellular P2-M4 loop. In particular, N56 on CDR2 interacts with N292 on the P2-M4 loop of TREK-2 (**Fig 4D**). This extracellular loop has previously been implicated in the regulation of filter-gating by external H^+ (32). The importance of this interaction with the P2-M4 loop is confirmed by mutation of N56 on Nb-Activator-76 which reduces its activatory effect, (**Fig 4A**). Consistent with an activated state, both M4 helices in this structure are also in the Up conformation (**Fig 4F)**. The role of ions in gating the filter is unclear because crystals of sufficient quality could only be obtained using a crystallisation solution containing Ba^{2+} which contributes to the crystal contacts. However, this does not change the selectivity filter in comparison to the Up-state conformation (PDB ID 4BW5).

ML335 is a small molecule TREK channel agonist that binds within the ‘K2P Modulator Pocket’, a cryptic binding site created by displacement of the top of M4 where its interactions with the P2-M4 loop and Pore-Helix-1 (PH1) are proposed to stabilise TREK-2 in a more conductive conformation (11, 16, 34). It is therefore highly significant that interaction of Nb-Activator-76 with the P2-M4 loop also induces a displacement in the top of the M4 helix similar to that seen in the ML335-bound structures of TREK-2 (**Fig 4E**). Furthermore, it has been reported that the reorientation of a tryptophan residue

at the top of M4 (W306 in TREK-2) also correlates with the activated state of TREK channels (8, 10, 35) and that any hydrophobic moiety occupying this space is capable of stabilising this activated state (10). In this nanobody-bound structure W306 sidechains in both helices are in the inward orientation and occupy this space. But unlike other TREK structures where this M4 tryptophan has rotated inwards, here the adjacent residues on PH1 (F164) also flips orientation to interact with the top of M4 to stabilise this displacement (**Fig 4F**). This demonstrates a mechanism whereby movement of the M4 helix may communicate with PH1 to influence the selectivity filter gate and highlights the importance of this 'K2P modulator pocket' in channel gating (36).

An inhibitory nanobody with dual effects. The inhibitory nanobody, CA10761, hereafter referred to as Nb-Inhibitor-61 (or Nb61), rapidly inhibited TREK-2 currents (IC_{50} = 685 ± 18 nM; **Fig 5A**). Its structure in complex with TREK-2 indicates only one nanobody bound per asymmetric unit, but this appears to result from its crystal packing where interactions between the Cap domains prevent binding of a second nanobody to the opposite side (**Fig 5B).**

The position of Nb-Inhibitor-61 on the side of the Cap domain allows it to form extensive interactions between the Cap helices and CDR1, as well as with the β5 sheet and β5-β6 loop. In addition, its long CDR3 loop forms multiple interactions with the top of M3 and the M3-PH2 loop (**Fig 5C**) as well as additional interactions with the top of M2 and the P2-M4 loop. However, the most striking feature of this long CDR3 loop is that it also inserts a lysine residue (K103) deep within the extracellular ion exit pathway that emerges just below the Cap domain (**Fig 5E-F**). Overall, the position of this nanobody obstructs most of this ion exit pathway with the remaining gap being only 1.35 Å wide compared to > 4 Å in the *apo* state. Intriguingly, the position of this positively-charged lysine within the ion exit pathway is reminiscent of the way some toxins block K^+

channels via a “cork in a bottle” or “plug” type mechanism (37). It also shares similarities with the ‘finger in the dam’ mechanism of TREK channel block by Ruthenium Red where interaction of this the positively-charged inhibitor with the ‘keystone inhibitor site’ below the Cap domain directly blocks K^+ permeation (7, 36, 38). Unlike these other toxins, K103 does not insert far enough into the pathway to interact directly with ions exiting the filter, but it clearly has the ability to impede K^+ movement through this exit pathway and so we examined its role further.

A critical role for Lysine 103 in channel inhibition. This lysine appears to make no specific H-bonding or charged interactions within the ion exit pathway and mutation to a serine (K103S) resulted in a marked reduction in its inhibitory effect (**Fig 5A**). We also examined other substitutions at this position (K103R, K103N and K103D). Consistent with a role for a positively charged residue at this position, the K103R mutant produced similar, if not greater, inhibition whilst neutral or negative substitutions (K103N and K103D) resulted in a markedly reduced inhibitory effect (**Fig 5A**). We also examined the effect of this nanobody on TREK-2 activity at the single channel level (SI Appendix Fig S5). Due to the low intrinsic open-probability of TREK-2, only a qualitative comparison was made between the behaviour of multi-channel patches with or without Nb-Inhibitor-61 included in the pipette solution. This revealed a marked suppression of TREK-2 activity via reduction in the durations of bursts of openings and increase in the time spent in the long-closed states. This is consistent with the idea that this nanobody acts as a slow blocker resulting in a complete cessation of K^+ flux through the channel.

Possible allosteric effects of Nb-Inhibitor-61 Together, these results with Nb-Inhibitor-61 support the contribution of a direct toxin-like (or ‘finger in the dam’) block of ion permeation by the nanobody (38). However, the bifurcation of the ion exit pathway means that such a mechanism would also require nanobody binding to both sides of

the Cap domain. There are no obstructions to the binding of a second nanobody on the other side, but due to crystal packing only one is observed in this structure. It is therefore possible that other, allosteric mechanisms may also contribute to its inhibitory properties. For example, multiple effects are also seen in some toxins where, in addition to direct pore block, interactions with the external pore loops result in allosteric modulation of channel gating (39, 40). For Nb-Inhibitor-61, this may arise through the extensive contacts it makes with the M3-PH2 loop as well as with the P2-M4 loop and M2/M3 helices. Consistent with such an allosteric effect, the M4 helix of the subunit that interacts with this nanobody is in the Down conformation (**Fig 5B**). Furthermore, the relative orientation of side chains for both W306 on M4 and F164 on PH1 are reversed compared to their positions the Nb76/TREK-2 structure, and its interaction with the P2-M4 loop pulls the top of M4 closer to PH1 compared to either Up or Down conformations (**Fig 6**). However, all 4 ion binding sites in the filter appear to be occupied and no obvious structural changes are seen in this region. Thus, although additional allosteric effects of this nanobody on the filter gate cannot be excluded, Lysine 103 clearly contributes to channel inhibition by directly blocking the ion exit pathway.

Summary and Conclusions

In this study, we describe the generation and characterisation of a panel of nanobody binders to the TREK-2 K2P channel resulting in one inhibitor, two activators and other nanobodies suitable for immunodetection of TREK-2. One activator in particular (Nb-Activator-76) elicits highly selective activation of TREK-2. These nanobodies therefore not only provide useful probes for analysing the structure and function of homomeric TREK-2 channels, but also identify a new class of highly specific extracellular modulators that can be further investigated for therapeutic potential.

Ever since the first structures of K2P channels were resolved over a decade ago the

role of their large extracellular Cap domain has remained unclear (33, 41). It was initially suggested this domain may have evolved to protect the selectivity filter of K2P channels from the small peptide toxins that often target other members of the superfamily of tetrameric cation channels. However, the complex domain swap involving the M1 helix required to assemble this extracellular domain suggests this is an unlikely strategy, especially as examples of toxin-resistance in ion channels often only require single mutations in the extracellular toxin binding site (42).

Nevertheless, the Cap domain is a defining feature of K2P channels, and whatever its purpose, it clearly makes toxin block of the pore much less likely. It is therefore significant that we have identified a nanobody in which the longer CDR3 loop inserts a lysine sidechain directly into the extracellular ion exit pathway in a manner analogous to some K^+ channel toxins. In the case of TREK channel inhibition by Ruthenium Red, this small positively charged inhibitor is able to access deeper into the exit pathway to block permeation directly above the filter, whereas it may not be possible for this CDR3 loop to insert deep enough to access the keystone Inhibitor site, even if it were longer. Nevertheless, this inhibitory nanobody is highly effective and its mechanism of action likely involves both allosteric and direct blocking effects on permeation (13). Also, the structure of this TREK-2/nanobody complex, and those of the others solved in this study, reveal that the Cap domain is highly immunogenic and provides a good scaffold on which nanobodies can anchor themselves to influence TREK channel function.

In the case of the two activators, the first (Nb-Activator-67) is a highly effective probe for the study of homomeric TREK-2 when the identity of the target is known, but uncertainties about this crystal structure make interpretations of its mechanism of action difficult. However, the second activator (Nb-Activator-76) provides important insights into the mechanism of TREK channel activation and demonstrate how the extracellular

P2-M4 loop and rearrangements at the top of the M4 helix play a critical role in the regulation of channel gating. Overall, these rearrangements expand current models for TREK channel gating via the K2P modulator site to suggest that outward displacement of the top of M4 changes the contacts between M4, the P2-M4 loop and Pore-Helix-1 to activate the selectivity filter gate, whereas displacement in the other direction results in the opposite effect and inhibition. Similar to that proposed for other TREK channel modulators (8, 10, 16), this may also involve reorientation of the W306 and F164 side-chains, or their equivalents (**Fig 6**). This model is also consistent with recent observations that covalent attachment of ML335 derivatives within the K2P modulator site via a conserved serine on PH1 can irreversibly activate TREK channels (34).

However, the mechanism by which nanobody binding is then coupled to changes in ion occupancy within the filter and the dynamics of K^+ permeation remain unclear. A limitation of this study is that the relative dynamics of these specific regions are probably critical for the regulation of gating, but most structures, including those in this study are determined in high [K^+] (>100 mM). This often results in high ion occupancy within the filter in such crystal structures, whereas the physiological situation is obviously very different and difficult to compare.

The nanobody ‘binders’ that have no functional effects are also likely to be useful tools for the immunodetection of TREK-2, although future studies will be required to demonstrate the sensitivity required for detection *in vivo*. It is also known that TREK-2 can form heteromeric channels with either TREK-1 or TRAAK, and nanobodies capable of detecting such combinations would be extremely powerful for dissecting the role of such heteromeric channels *in vivo*. It is therefore significant that the domain swap involved in assembly of the Cap domain generates an intersubunit interface that forms extensive contacts with nanobodies in these structures and which would be unique in

any heteromeric channel combinations; thus, if binders can differentiate between these interfaces in homomeric TREK channels, then it may be possible to generate similar nanobodies that are uniquely selective for heteromeric K2P channels.

Thus, although ion channels remain underexploited as antibody drug targets, this study demonstrates that the unique nature of the Cap domain combined with the role of the extracellular loops in K2P channels allows nanobodies to be considered as an exciting alternative to small molecules as highly-selective probes to K2P channel structure-function studies, and of TREK channels in particular. This therefore expands the potential of nanobodies as effective therapeutics and their role in future drug development.

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Author contributions

S.J.T., E.P.C. and K.E.J.R. conceived/designed the principal elements of the study. All authors generated, analyzed or interpreted data or generated materials. E.P. and J.S. generated nanobody reagents. S.J.T, A.C., J.S. and K.E.J.R drafted the manuscript and all authors contributed to the final version.

Conflicts of interest

The authors declare no competing financial interest.

Data Availability

All data within this study are included in the article and/or SI Appendix. The atomic coordinates and structure factors for the nanobody bound complexes of TREK-2 have been deposited in the Protein Data Bank.

Methods

Nanobody generation, expression and purification: Nanobodies were generated as part of the Nanobodies4Instruct program using the method described by Pardon *et al.* (28). Briefly, detergent-solubilised purified human TREK-2 protein (Gly67 to Glu340), identical to that used in previous structural studies (9) was used to elicit an immune response in llamas. The resulting immune repertoire was isolated and cloned into the pMESy4 phage display vector. Positive clones were selected by phage panning and screening against the same TREK-2 protein. A total of 29 unique nanobody clones were identified. The sequence alignments shown in SI Appendix Fig S1 are highlighted using TEXshade. Periplasmic expression and purification were modified from Pardon *et al*. (30). Briefly, relevant nanobodies in the pMESy4 vector were transformed into the WK6 strain of *E. coli*. 10 ml overnight cultures were then used to inoculate master cultures (1L Terrific Broth, 4ml Glycerol, 0.1 % w:v glucose). These were then grown at 37 °C shaking at 180 rpm with expression induced by 0.5mM IPTG when OD_{600} reached 0.8. Growth was then continued overnight at 28 °C. Cells were harvested by spinning at 5000g for 20 minutes at 4 °C and the pellets resuspended in ice-cold resuspension buffer (in mM: 200 Tris, 0.5 EDTA, 500 Sucrose). To disrupt the periplasm, cells were kept on ice and shaken for 1 hour in resuspension buffer, followed by a rapid 3-fold dilution with a 5mM $MgCl_2$ solution and then shaken for a further hour. The periplasm was then recovered by spinning at 15000 *g*, for 15 mins at 4 °C. His-tagged nanobodies were then purified by addition of Ni-NTA resin to the supernatant and incubating at 4 °C for 2.5 hours. Following washing with Wash Buffer 1 (in mM: 50 Sodium Phosphate, 1000 NaCl, pH7.0) and Wash Buffer 2 (in mM: 50 Sodium Phosphate, 1000 NaCl, pH6.0), nanobodies were eluted in Elution buffer (in mM: 50 Sodium Phosphate, 150 NaCl, 300 Imidazole, pH 7.0, and the imidazole rich solution exchanged into either Gel Filtration Buffer (200 mM KCl 20 mM HEPES pH7.5, for screening experiments), ND96

(in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, 2.5 Na Pyruvate, for Two-Electrode Voltage Clamp studies), or extracellular buffer (in mM: 140 NaCl, 10 TEA-Cl, 5 KCl, 3 MgCl, 1 $CaCl_2$,10 HEPES, pH 7.4, for patch clamp electrophysiology) using PD10 columns. Purity was assessed by SDS-PAGE. An initial assessment of nanobody binding to TREK-2 was done with size exclusion chromatography. Each nanobody was mixed with purified TREK-2 in a 2:1 molar ratio and applied to a Sepax SRT SEC-300 column, pre-equilibrated in Gel Filtration buffer. Out of the 29 initial nanobodies, 16 co-eluted with TREK-2 and produced a visible shift of the 280 nm TREK-2 peak, compared to TREK-2 injected without nanobody.

Crystallography and Data Collection Human TREK-2 protein (residues Gly67 to Glu340), identical to that used in our recent structural studies was expressed and purified as previously described (4, 9) and relevant nanobodies were prepared as described above. For Nb67 and Nb61 a TREK-2 glycosylation mutant (TREK-2^{glyco}) was used; this was purified in an identical manner and contained the following substitutions (N149Q, N152Q and N153Q; none of these sites form contacts with the nanobody). TREK-2 protein was mixed with the nanobody in a 1:2 TREK-2:nanobody molar ratio prior to crystallisation. Crystals formed in vapour diffusion drops (TREK-2-Nb58: 0.1 M glycine pH 10.0, 0.2 M NaCl, 31% v/v polyethylene glycol (PEG) 1000; TREK-2^{glyco}-Nb67: 0.1 M Tris pH 8.0, 65% v/v 2-methyl-2,4-pentanediol; TREK-2-Nb76: 0.1 M HEPES pH 7.0, 0.3 M $BaCl_2$, 39% PEG 400), apart from the TREK-2^{glyco}-Nb61 complex crystals, which grew in Lipidic Cubic Phase (MAG7.9 with 3:2 lipid:protein ratio, 0.1 M Na acetate pH 4.5, 0.3 M KCl, 23% v/v PEG 400). Vapour diffusion crystals were grown at 4°C and LCP crystals at 20°C for 1-12 weeks. Crystals were screened, and X-ray diffraction data collected on beamline I24 (Diamond Light Source) at 100K. Data were recorded at a wavelength of 0.9686Å on a Pilatus3 6M detector from single crystals using 0.1 or 0.2° oscillation and a beam-size of 20x20μm (30x30μm for TREK-2-Nb76).

Data were processed, reduced and scaled using XDS (43) and AIMLESS (44). All datasets exhibited moderate to strong diffraction anisotropy and were further processed using STARANISO (http://staraniso.globalphasing.org) to apply an anisotropic diffraction cut-off to the merged data as well as structure amplitude estimation and anisotropic correction (see Supplemental Table S1 for resolution limits of data and completeness). Structures were phased by molecular replacement using the 4BW5 (TREK-2 ^{glyco}-Nb67 and TREK-2-Nb76), 4XDJ (TREK-2 ^{glyco}-Nb61) or 4XDK (TREK-2-Nb58) structures as starting models for TREK-2 (9) and 5HVG (Nb58 and Nb76), 5UKB (Nb67) or 5IMK (Nb61) for the nanobodies. Structures were manually built with COOT and refined with BUSTER (2.10.4; Global Phasing Ltd). Models were refined with LSSR restraints (45) and a single translation/libration/screw model group was refined for each protein chain.

Immunodetection of TREK-2: TREK-2 and other human K2P channels were tagged with mCherry by removal of the stop codon and in frame fusion of mCherry to the C-terminus using a $(Gly\text{-}Ser)_2$ linker and then cloned into the pFAW vector suitable for expression in HEK293 cells. Nanobodies were tagged by in frame fusion of mVenus at the C-terminus using a Gly-Ser linker, and also then cloned into the pFAW vector. The nanobody-mVenus fusions were then transfected into HEK293 cells resulting in secretion of tagged nanobodies into the media. This media was then harvested after 96 hours and used directly for immunodetection of mCherry-tagged K2P channels that had transfected into HEK293 cells at least 36hrs previously.

Electrophysiology: For microinjection of oocytes, mRNA was *in vitro* transcribed from the wild-type human TREK-2 gene (*KCNK10*) in the pFAW oocyte expression vector. Each oocyte was injected with ~0.2ng of RNA and Two-Electrode Voltage Clamp recordings then performed as previously described (12). Briefly, after injection of mRNA,

oocytes were incubated for 20–24 h at 17.5°C and measured in ND96 buffer at pH 7.4 (96 mM NaCl, 2 mM KCl, 2 mM MgCl2, 1.8 mM $CaCl_2$, 5 mM HEPES). Unless otherwise stated, for the dose-response measurements, oocytes were perfused with increasing concentrations of purified nanobody in ND96 solution and currents recorded using a 400 ms voltage step protocol from a holding potential of -80 mV delivered in 10 mV increments between -120 mV and +50 mV and 800 ms ramp protocols from -120 to +50 mV. Unless otherwise described, all results shown are reported as mean ± standard deviation and obtained with oocytes from at least 3 independent batches. For the initial screening experiments nanobody was pipetted directly into the bath chamber in gel filtration buffer and current recorded using a 800 ms ramp from -50 to +80 mV. For the measurement of intracellular effects, nanobodies were applied to the inside of giant excised inside-out membrane patches. The intracellular solution had the following composition (in mM): 120 KCl, 10 HEPES, 2 EGTA and 1 Pyrophosphate (pH adjusted with KOH/HCl) with a pipette solution (in mM): 120 KCl, 10 HEPES and 3.6 $CaCl_2$ (pH 7.4 adjusted with KOH/HCl). The recording of TREK-2 channel activity was then performed as previously described (4). For single channel recordings, the pipette solution contained (in mM) 116 NMDG, 4 KCl, 1 $MgCl_2$, 3.6 $CaCl_2$, 10 HEPES (pH 7.4); whilst bath solution contained (in mM) 120 KCl, 1 NaCl, 2 EGTA, 10 HEPES (pH 7.3). The currents were filtered at 1 kHz and sampled at 10 kHz.

Figure Legends

Figure 1. ***Identification of functionally-active nanobody binders for TREK-2*** **(A)** Whole cell currents recorded (at +40mV) from oocytes expressing TREK-2 in the presence of a panel of nanobodies generated against TREK-2. Currents in the presence of extracellular nanobody (0.5 µM) were normalised to those in its absence. A threshold of 50% activation or inhibition was set in this screen (dotted lines). Tight binders within this panel were also identified with an SEC-shift assay (shown in red). Asterisks mark the functionally active nanobodies plus an inactive tight binder chosen for further investigation. **(B)** Crystal structures of the three functionally active nanobodies in complex with TREK-2. The nanobodies are shown in purple as surface representations whilst TREK-2 is shown in cartoon (green and gold). For CA10767 (Nb-Activator-67) the third 'intracellular' nanobody in the unit cell is shown in grey. A structure of the functionally inactive binder, CA10758 (Nb-Binder-58) is also shown in complex with TREK-2. Note in this case that the nanobody binds as a dimer to the top of the Cap domain away from the extracellular TM/Pore loops that influence channel gating.

Figure 2. ***Nb-binder-58 interacts with the apex of the Cap domain.*** **(A)** Crystal structure of Nb-binder-58 (Nb58) in complex with TREK-2. This functionally inactive tight binder interacts as a dimer with the tip of the Cap domain only. The right-hand panel shows an expanded view of the interactions involved and shows the C123 disulphide between chains at the apex of the Cap. D120 and V122 are unique in TREK-2 compared to other TREK channels. **(B)** Immunodetection of TREK-2 at the cell surface. TREK-2-mCherry fusions were expressed in HEK293 cells and stained with either Nb58 (or the highly related Nb57) fused to mVenus. Overlay of these fluorescent signals shows clear binding at the extracellular surface. No overlapping signals are seen in untransfected controls or with other K2Ps (see SI Appendix Fig S3).

Figure 3. ***Extracellular activation of TREK-2 by Nb-Activator-67*****. (A)** Crystal structure of TREK-2 in complex with Nb-Activator-67 (Nb67). Nanobodies bind to each side of the Cap domain and on one side (green subunit) the Cap helix unwinds. A third Nb67 (grey) is also found in the asymmetric unit bound to the intracellular surface. These unusual features may arise from

tetramerisation of Nb67 within the crystal (see SI Appendix Fig 4A) **(B)** Dose-response relationships showing extracellular activation of TREK-2 channel activity by Nb67. Whole-cell TREK-2 currents were recorded at +40 mV and perfused with different concentrations of Nb67. Currents were normalised to those recorded prior to the application of nanobody. No activation of TRAAK or TALK-2 K2P channels was observed, but for the related TREK-1 up to 50% inhibition of channel activity was seen at concentrations of Nb67 >1µM. **(C)** Expanded view of the interaction of Nb67 with the two sides of the Cap domain. The interaction with $Nb67_A$ (Left) shows no distortion of the helices, whereas interaction with $N67_B$ on the opposite side results in unwinding of $EH1_B$ (residues E113, E116 and D120 are unique in TREK-2 vs TREK-1). Right: the interaction of N108 with $NB67_B$ differs on the side where $EH1_B$ is unwound. This interaction does not occur with $Nb67_A$.

Figure 4 Selective extracellular activation of TREK-2 by Nb-Activator-76

(A). Dose-response relationships showing activation of TREK-2 channel activity by Nb-Activator-76 (Nb76). Whole-cell TREK-2 currents were recorded at +40 mV and perfused with different concentrations of Nb76. The effect of mutating different residues in Nb76, which interact with TREK-2, are shown. The curves shown for the R53A and W98A mutant nanobodies were fitted by hand. **(B)** Crystal structure of TREK-2 in complex with Nb76 bound to both sides of the Cap and the P2-M4 loop. **(C)** Interactions with the side of the Cap. W98 on CDR3 inserts between Cap helices of different TREK-2 subunits to form an interaction with E128 on EH2 of TREK-2, whilst R53 on Nb76 interacts with Q106 on EH1 of a different subunit of TREK-2. Mutation of either W98 or R53 on Nb76 reduces its activatory effect. **(D)** Expanded view of the interaction of N56 on CDR3 with N292 on the P2-M4 loop. Mutation of N56 also reduces activation by Nb76. **(E)** Alignment with TREK-2 Up state (4BW5 in Grey) highlights displacement of the top of M4 and the inward rotation of W306. This is accompanied by outward rotation of F164 on Pore-Helix-1 (PH1). The right-hand panel rotates this view through 90° to better visualise the relative reorientation of W306 and F164, and the outward displacement of M4. This expands the K2P modulator site behind the selectivity filter (red asterisk).

Figure 5 Extracellular inhibition of TREK-2 by Nb-Inhibitor-61. (A) Dose-response relationships showing inhibition of TREK-2 channel activity by Nb-Inhibitor-61 (Nb61). Whole-cell TREK-2 currents were recorded at +40 mV and perfused with different concentrations of Nb67. Also shown are the effect of different mutations of K103 in Nb61. **(B)** The crystal structure unit cell reveals only one Nb61 bound to TREK-2; packing interactions between the Cap domains within the crystal prevent binding to the other side. **(C)** Expanded view of the major interactions of Nb61 with the top of M3 and the M3-PH2. Additional interactions with the P2-M4 loop and M2 are also observed **(D)** Expanded view of interactions between Nb61 and the side of the Cap domain. **(E)** Surface view showing obstruction of the extracellular ion exit pathway by Nb61 (in purple). K103 within the obstructed pathway is also marked (in red). (**F**). Expanded view of the location of K103 within the ion exit pathway. K^+ ions are shown as purple spheres and the arrows indicate the direction of K^+ flow through the ion exit pathway that is obstructed by K103 on CDR3 of Nb61.

Figure 6 Activation and Inhibition by opposite movement of the top of M4. Alignment of the TREK-2/Nb76 and TREK-2/Nb61 complexes with the TREK-2 Up state show that activation by Nb76 is associated with outward movement of the top of M4 and the P2-M4 loop whilst inhibition by Nb61 involves movement in the opposite direction (red arrows). The location of the K2P modulator site behind the selectivity filter is indicated by the asterisk.

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Figure 1

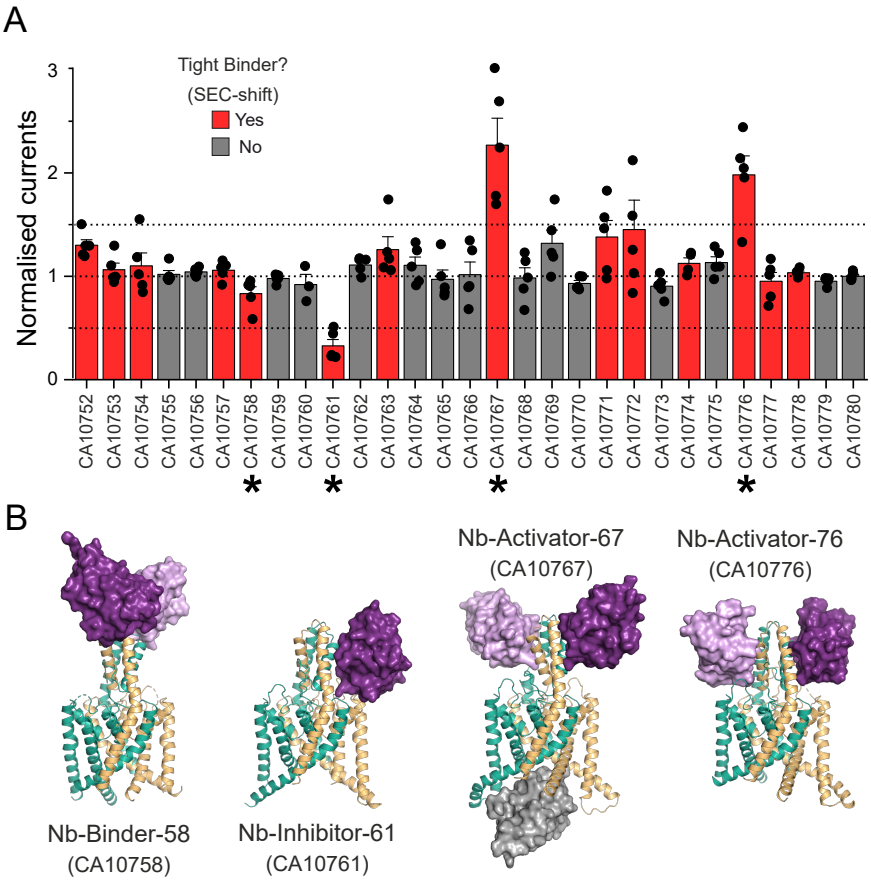
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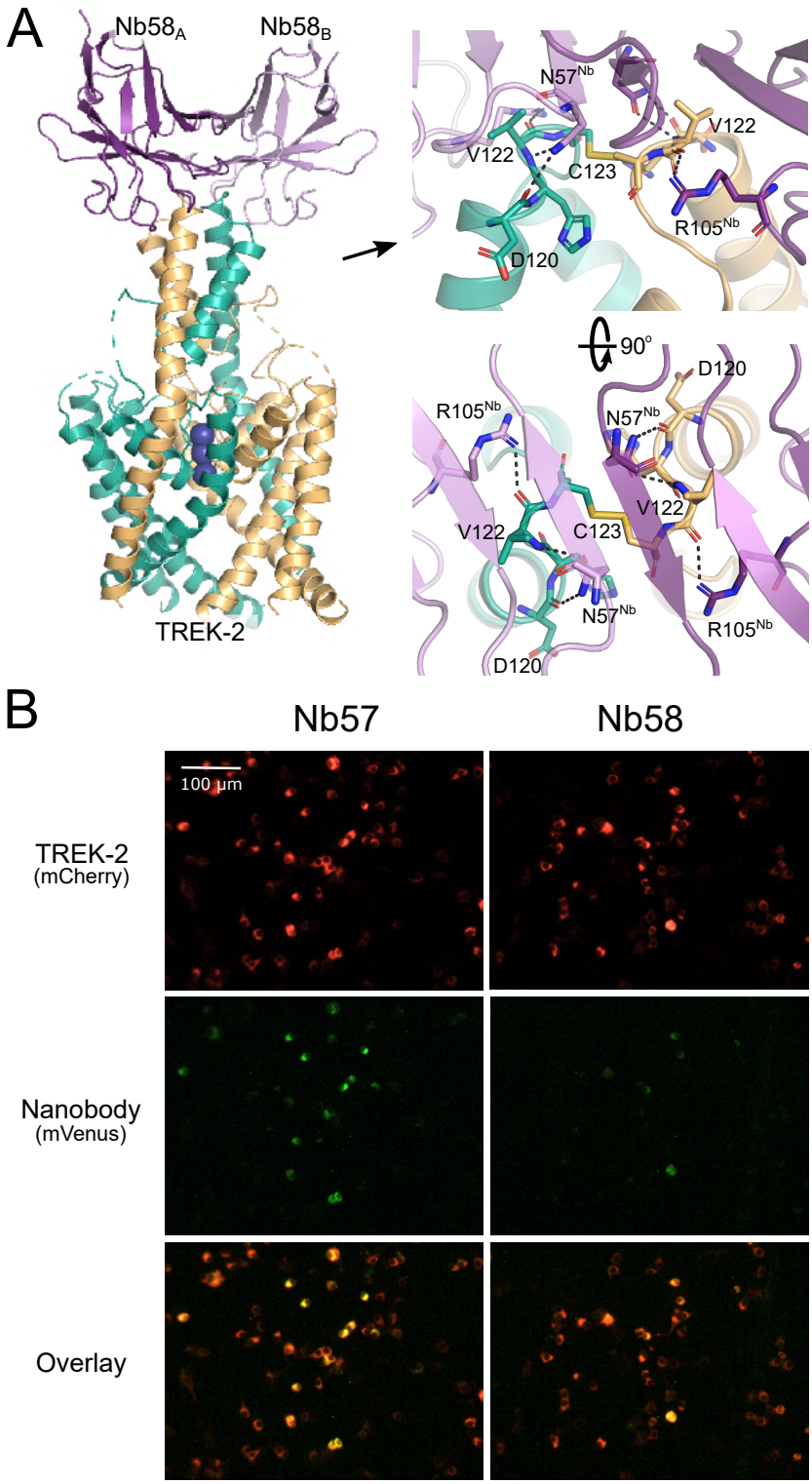
Figure 2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Overlay

Figure 3

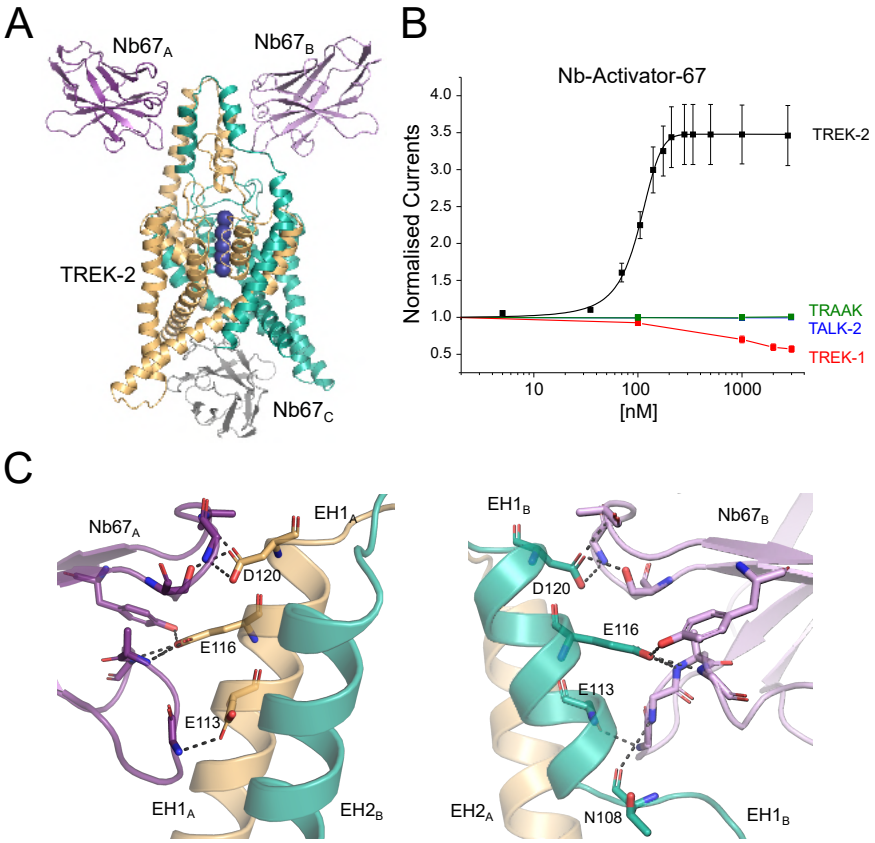
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Figure 4

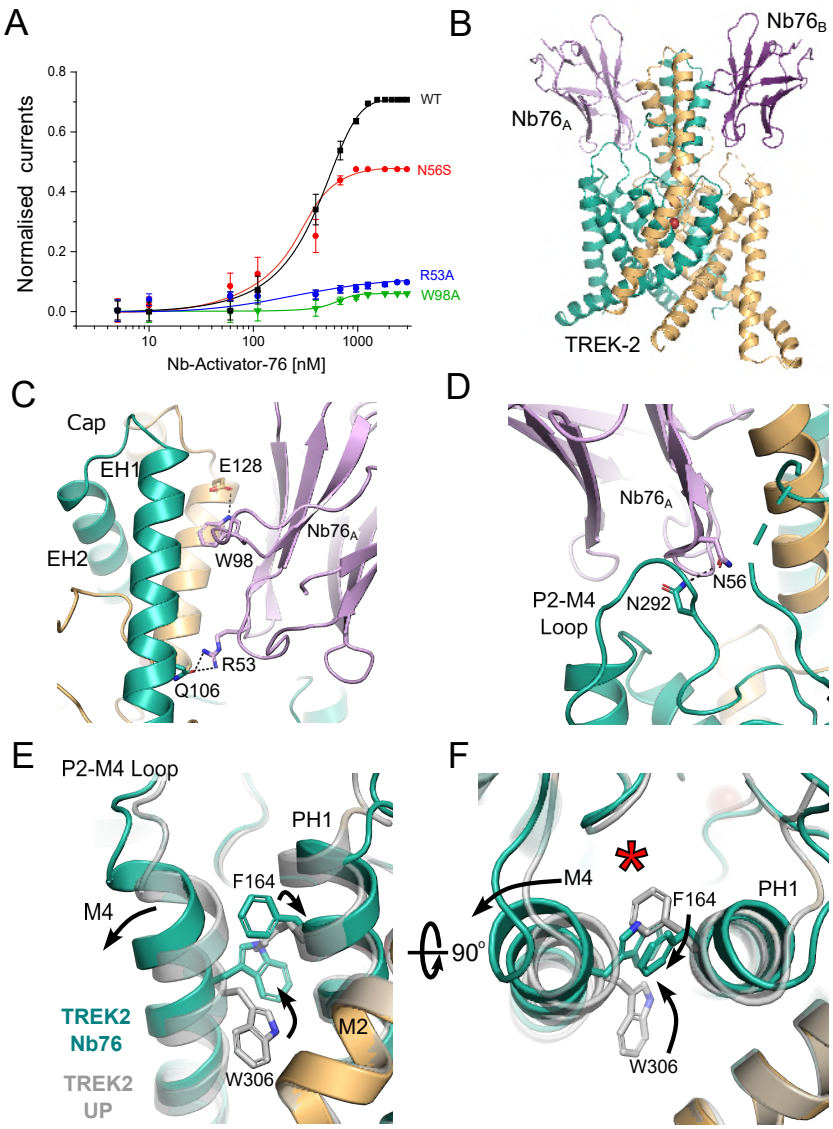
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Figure 5

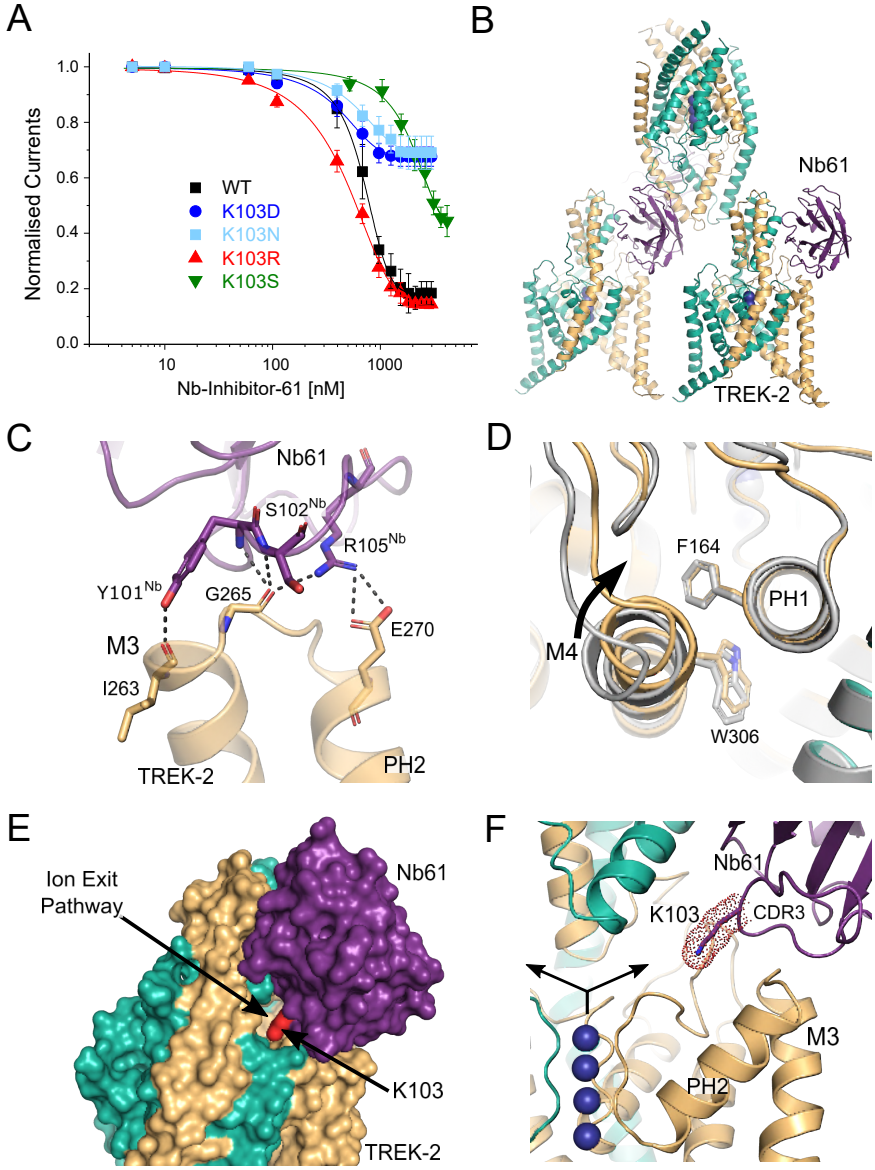
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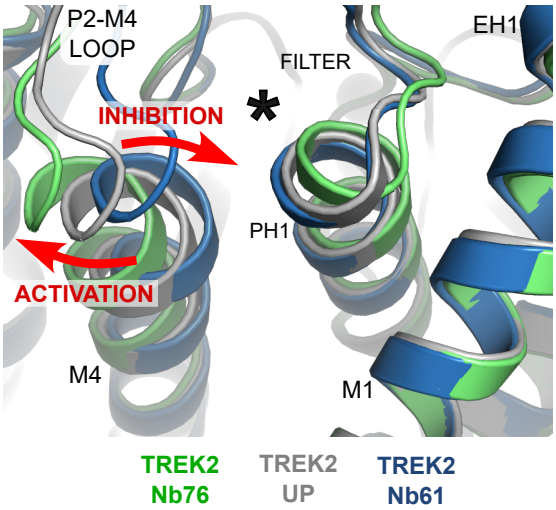
Figure 6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M4
FILTER
PH1
EH1
M1
TREK2
Nb76
TREK2
UP
TREK2
Nb61