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Journal

The Journal of Physiology, 600(9)

ISSN

0022-3751

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Publication Date

2022-05-01

DOI

10.1113/jp282403

Peer reviewed

Controlling Ion Channel Function with Renewable Recombinant Antibodies

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Abbreviations

mAb: monoclonal antibody
V_H: immunoglobulin heavy chain variable domain
V_L: immunoglobulin light chain variable domain
scFv: single chain variable fragment
V_HH: immunoglobulin heavy chain only variable domain
nAb: nanobody
TRAIL: tumor necrosis factor-related apoptosis-inducing ligand
CaM: calmodulin
CDI: Ca^{2+}-dependent inactivation
APD: action potential duration
I_A: transient voltage-sensitive K^+ current
hERG: human ether-a-go-go related gene
I_{Kr}: delayed rectifier K^+ current
CF: Cystic fibrosis
CFTR: cystic fibrosis transmembrane regulator
NBD1 and NBD2: nucleotide binding domains of CFTR
LoF: loss of function

Abstract

Selective ion channel modulators play a critical role in physiology in defining the contribution of specific ion channels to physiological function and as proof of concept for novel therapeutic strategies. Antibodies are valuable research tools that have broad uses including defining the expression and localization of ion channels in native tissue, and capturing ion channel proteins for subsequent analyses. In this review, we detail how renewable and recombinant antibodies can be used to control ion channel function. We describe the different forms of renewable and recombinant antibodies that have been used and the mechanisms by which they modulate ion channel function. We highlight the use of recombinant antibodies that are expressed intracellularly (intrabodies) as genetically-encoded tools to control ion channel function. We also offer perspectives of avenues of future research that may be opened by the application of emerging technologies for engineering recombinant antibodies for enhanced utility in ion channel research. Overall, this review provides insights that may help stimulate and guide interested researchers to develop and incorporate renewable and recombinant antibodies as valuable tools to control ion channel function.

Introduction

Selective ion channel modulators play a critical role in defining the contribution of specific ion channels to physiological function and as proof of concept for novel therapeutic strategies. Ion channel researchers have long made extensive use of toxins from venomous animals or natural products from plants as naturally occurring ion channel modulators. Synthetic small molecules have also been extensively employed to modulate ion channel function. A small subset of these exhibit selectivity sufficient to modulate a single, molecularly defined ion channel type (Alexander *et al.*, 2021). As prominent examples, κ-dendrotoxin can be used to selectively modulate K^+ channels containing the Kv1.1 α subunit (Robertson *et al.*, 1996), and protoxin-2 selectively inhibits the human Nav1.7 Na^+ channel (Schmalhofer *et al.*, 2008). However, in most cases the selectivity of naturally occurring and synthetic small molecule ion channel modulators is broader than an individual ion channel subtype. For example, α-dendrotoxin similarly impacts Kv1.1, Kv1.2 and Kv1.6 containing K^+ channels (Robertson *et al.*, 1996) and dihydropyridines modulate all L-type Cav1 (Cav1.1-Cav1.4) Ca^{2+} channels (Alexander *et al.*, 2021).

Antibodies have emerged as an important class of pharmacological agents due to their exquisite binding selectivity, and that they can be raised against a specific region of a target protein. Antibodies represent the fastest growing sector of FDA-approved therapeutics (Mullard, 2021), although there are currently no anti-ion channel antibodies in clinical use. Nevertheless, the principle that ion channels can be effectively targeted with antibodies that impact function is demonstrated clinically by autoimmune disorders whose etiology is due to ion channel modulating antibodies, including those that target the central nervous (Kleopa, 2011; Li *et al.*, 2019), cardiovascular (Qu *et al.*, 2019), and neuromuscular (Huang *et al.*, 2019) systems. Here we provide a perspective on the growing use and future potential of renewable, recombinant antibodies and antibody mimetics as selective modulators to control the function of specific ion channels.

Ion channel modulating antibodies

Antibodies that modulate voltage-gated ion channel function have a long track record of important uses in ion channel research [reviewed in (Dallas *et al.*, 2005; Sun & Li, 2013)]. Molecular cloning of ion channels facilitated the generation of antibodies specific for particular ion channel subtypes using synthetic peptide or recombinant protein immunogens. Many of the early studies employed polyclonal antibodies to modulate ion channel function. This class of antibodies is now recognized to have limitations related to research reproducibility, in part due to their being complex collections of many different antibody clones whose representation can vary between different batches of antisera and/or purified preparations. In addition, they are obtained in finite quantities and are not truly renewable, as immunization of different animals with the same immunogen can often yield antisera with very different properties (Bradbury & Pluckthun, 2015a). For these reasons, monoclonal antibodies or mAbs offer enhanced attributes in terms of research reproducibility as renewable and homogeneous preparations of a single antibody. Recombinant mAbs offer numerous additional advantages including allowing for antibody engineering to generate forms of antibodies that do not exist in nature but that have substantial advantages over conventional antibodies (Trimmer, 2020). Here we will focus on ion channel modulation by renewable forms of antibodies, including mAbs, recombinant antibodies in their various engineered forms, and antibody mimetics. These include ion channel binding antibodies that intrinsically exert functional effects on ion channels, as well as those that do not, but that can be used to deliver a functional adduct to their target ion channel to impact function.

Forms of renewable and recombinant antibodies that have been used to control ion channel function

Monoclonal antibodies

Monoclonal antibodies or mAbs are a renewable form of “conventional” (non-recombinant) antibody secreted from hybridoma cells. A hybridoma is the product of the fusion of a non-antibody producing myeloma cancer cell and an antibody producing B cell (most often a spleen cell) isolated from an immunized animal. Hybridomas retain the attribute of cancer cells to multiply indefinitely in cell culture, and the ability of B cells to produce a single antibody. Hybridomas are amenable to cell culture at any scale and can be reliably cryopreserved and recovered years or even decades after their original development. As such mAbs are advantageous in being a reliably renewable form of a homogenous population of antibodies of defined characteristics.

Recombinant antibodies

Recombinant antibodies are genetically encoded and typically produced in transfected mammalian cells from recombinant plasmids although other expression systems (*e.g.*, E. coli, yeast, *in vitro* translation, viruses, etc.) can also be used. Recombinant mAbs were initially developed in the 1980s and have been used primarily as therapeutics, which are a rapidly growing sector of the modern pharmaceutical industry (Buss *et al.*, 2012; Chiu & Gilliland, 2016). While the use of recombinant antibodies in basic biomedical research has been more limited, it is expanding in part due to advances in recombinant DNA techniques that have greatly reduced the time and effort needed for their development and subsequent engineering. There is also a growing recognition of the substantial advantages of recombinant antibodies as research reagents, in conforming to best practices in research transparency, rigor and reproducibility (Bradbury & Pluckthun, 2015a; Bradbury & Pluckthun, 2015b; Trimmer, 2020). These include: 1) unambiguous identification and definition of recombinant antibodies as produced in expression systems via DNA sequencing of the expression plasmids; 2) more reliable and less variable expression; 3) easier and more reliable dissemination as DNA sequences and as plasmids; and 4) permanent and absolute archiving as DNA sequence. Recombinant antibodies also allow for subsequent engineering to enhance their utility.

Forms of antibodies

Antibodies are immunoglobulin proteins, mostly commonly immunoglobulin G or IgG, formed as a heterotetramer of two identical heavy or H chains and two identical light or L chains (**Figure 1A**). The intact IgG antibody is a dimer of H+L heterodimers, with each H+L dimer having the capacity to bind to the target antigen. As such intact IgG antibodies are bivalent, with identical antigen binding sites on each arm (**Figure 1A**). This feature enhances antibody binding beyond the inherent affinity of each antigen binding site through avidity effects, which comprise the combined strengths of antibody binding through the two antigen binding arms. The variable domains of the heavy and light chains (V_H and V_L) are located at the N-terminus of each subunit and form the antigen binding region, determining target affinity and specificity. Antibody fragments retaining the variable domains can maintain the binding characteristics of the source antibody and have advantages over intact IgG antibodies due to their smaller size. Some forms, including monomeric single chain variable fragments or scFvs (Bird *et al.*, 1988; Huston *et al.*, 1988), can be produced as a single polypeptide chain. Their design employs a flexible linker to join the V_H and V_L regions that are on separate chains of an IgG in a single polypeptide (**Figure 1A**). The successful generation of scFvs can be complicated by several factors, including that the N-terminus of the second V region within the scFv (the V_L domain in Figure 1A) is attached to the C-terminus of the leading V region (the V_H domain in Figure 1A) *via* a flexible linker instead of being free as it is in a full-length IgG molecule. This and other factors yield a variable success rate when attempting to convert any particular IgG into an scFv (Schaefer *et al.*, 2010), although there are numerous strategies for circumventing these problems [*e.g.*, (Tiller & Tessier, 2015; Chiu *et al.*, 2019) and many others]. In certain cases, scFvs are fused to Fc domains to generate scFv-Fc fusions (**Figure 1A**) in which the Fc domain can facilitate folding of the fused V domains, has a smaller size than a conventional IgG at ≈100 kD, and provides a bivalent structure that enhances binding through avidity effects as in conventional IgGs.

In addition to these H + L chain IgGs present in all mammals, all camelid mammals also produce unique H chain only IgGs (**Figure 1B**) (Muyldermans, 2013). In this case the V_H domain (termed a V_HH domain to distinguish it as being from a heavy chain only IgG) comprises the entire antigen binding domain and can function autonomously as a single domain antibody or nanobody (**Figure 1B**). Nanobodies (nAbs) have numerous advantages (Muyldermans, 2013), starting with their small size of 15 kD (≈1/10 of an IgG antibody). As naturally occurring single chain antibodies they are typically more stable than engineered forms such as scFvs. Moreover, nAbs bind to antigens in a manner distinct from conventional H + L IgGs (Muyldermans, 2013), increasing the diversity of the repertoire of antibodies available to researchers. As discussed below, single chain scFvs and nAbs also have distinct advantages in being amenable to expression in the cytoplasm of mammalian cells as genetically-encoded intracellular antibodies or intrabodies. This contrasts with IgGs that require H + L chain assembly that only occurs efficiently in the lumen of the ER.

Antibody mimetics

Other forms of designer proteins have also been developed that can yield the high affinity and selective binding of antibodies in alternative protein scaffolds. Among these are monobodies, designer binding proteins built on the scaffold of a human fibronectin type III domain or FN3 (Koide *et al.*, 1998; Koide *et al.*, 2007). Monobodies are versatile modular building blocks for creating protein tools with sophisticated functionalities. The FN3 scaffold used to construct monobodies is small (≈10 kD) and stable without disulfide bonds (Koide *et al.*, 1998), and as such monobodies are typically fully functional when expressed in the reducing environment of the cytoplasm of mammalian cells, making them particularly suitable as intrabodies [e.g., (Gulyani *et al.*, 2011; Gross *et al.*, 2013; Son *et al.*, 2016)]. Monobodies are typically isolated from high complexity phage libraries using display technology and are often subjected to *in vitro* affinity maturation to obtain monobodies of suitable affinity, as was done to isolate and further refine monobodies targeting the Fluc fluoride ion channel (Stockbridge *et al.*, 2014).

Ion channel function can be modulated by diverse mechanisms

There are numerous mechanisms of action that have been defined for the wide array of ion channel modulators (Alexander *et al.*, 2021). These primarily depend on the site at which the modulators bind on the ion channel. The level of the macroscopic ionic current "I" permeating through the population of a particular ion channel in the cell membrane is described by the equation $I=nP_oi$. This equation illustrates that this whole cell macroscopic current "I" is the product of "n", the number of that particular ion channel in the cell membrane, "P_o", the probability that any given channel in this population is in its open (versus closed or inactivated) state, and "i" the amount of current that passes through any single open channel of that particular type. To use ion channel inhibitors as an example, there are those that reduce "n", for example by triggering internalization of channels from the cell membrane. Inhibitors that impact P_o, termed gating modifiers, can reduce the number of open channels by stabilizing channels in their closed or inactivated gating states. There are also inhibitors termed pore blockers that physically block the ion conductance pathway, thereby reducing the "i" of individual channels to zero. The growing availability of ion channel structures with modulators bound has provided detailed information on the structural basis of these mechanisms of action. For example, the Na^+ channel gating modifier Dc1a binds to the S3-S4 voltage sensing domain (Shen *et al.*, 2018). The Na^+ channel pore blocker tetrodotoxin (Shen *et al.*, 2018) and the Ca^{2+} channel pore blocker verapamil (Tang *et al.*, 2016) bind within the selectivity filter within the ion channel pore, while larger pore blocking inhibitors, such as charybdotoxin bound to Kv1.2 K^+ channel (Banerjee *et al.*, 2013) and μ-conotoxin KIIIA bound to Nav1.2 Na^+ channel (Pan *et al.*, 2019), are too large to enter the pore, but have small projections that insert into the selectivity filter. Other ion channel modulators act allosterically, for example the L-type Ca^{2+} channel inhibitor amlodipine binds to a site distinct from either the voltage sensor or pore but its binding allosterically remodels the pore to prevent ion flux

(Tang *et al.*, 2016). Presumably ion channel modulating antibodies have the potential to exert their action by analogous mechanisms.

Renewable recombinant antibodies that modulate ion channel function

Antibodies against external domains of ion channels

Antibodies are not able to cross biological membranes. As such, only those antibodies that bind to the channel's extracellular domains will be able to bind to and modulate ion channels when added to the extracellular solution. The pore forming and voltage-sensing principal subunits of voltage-gated ion channels stand out among plasma membrane proteins in having little of their structure accessible to the extracellular space. Moreover, external domains involved in highly conserved ion channel functions, such as voltage sensing and pore domains, often exhibit a high degree of structural conservation between different ion channels and do not represent prime targets for subtype-selective modulators (Wulff *et al.*, 2019). However, there are numerous modulating antibodies that have been developed against ion channel extracellular domains (Table 1 and **Figure 2**).

A set of mAbs were generated against the extracellular turret or E3 segment of the S5-S6 linker domain of Kv10.1 or Eag1 K^+ channel (Gomez-Varela *et al.*, 2007). Of these mAb56 reduced Kv10.1 currents when applied to intact cells. The inhibition is use-dependent, in that it requires voltage-dependent channel activation. mAb56 has an EC_{50} of ≈70 nM for inhibition of Kv10.1 and has no detectable impact on the related Kv10.2 (Eag2) or Kv11.1 (HERG) channels. The mechanism of mAb56 mediated inhibition is not known. However, as structural studies indicate the mAb56 epitope is not within the ion permeation pathway, mAb56 is unlikely to block the pore itself. A second mAb (mAb62) was generated against an adjacent site in this same region (Gomez-Varela *et al.*, 2007). This mAb selectively binds to Kv10.1 but does not inhibit function. An scFv, "scFv62", developed from mAb62 (Hemmerlein *et al.*, 2006) was then "armed" by its

recombinant fusion to the TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) protein. TRAIL binds to five different receptors and upon binding induces apoptosis. By combining the Kv10.1 targeting function of scFv62 and the apoptogenic function of the TRAIL moiety, the scFv62-TRAIL fusion protein selectively induced apoptosis in Kv10.1-expressing prostate cancer cells (Hartung *et al.*, 2011). The same group subsequently generated llama nAbs against the same region of Kv10.1 and showed that when fused to TRAIL these nAbs also induced apoptosis in a variety of Kv10.1 cancer cells (Hartung *et al.*, 2020). These studies show the utility of using both anti-ion channel scFvs and nAbs to restrict TRAIL-induced apoptosis to cells expressing the target ion channel.

It is also possible to use antibodies that bind specifically to an external domain of ion channel without inhibiting function but that could then be “armed” to elicit ion channel-specific modulation. This was accomplished for the anti-Kv4.2 mAb K57/1, generated against the S1-S2 extracellular linker domain of Kv4.2 (Rhodes *et al.*, 2004; Menegola & Trimmer, 2006). This mAb has no detectable impact on Kv4.2 function but when conjugated to porphyrin selectively targeted porphyrin-mediated photoablation to Kv4.2 channels (Sack *et al.*, 2013).

An innovative approach including producing a full-length Kv1.3 immunogen in the protozoan *Tetrahymena thermophila* was used to develop externally acting scFv-Fc fusions (see Figure 1 for a schematic of an scFv-Fc fusion) that inhibit Kv1.3 K^+ channels (Bednenko *et al.*, 2018). Individual splenocytes from chickens and llamas were incorporated into gel encapsulated microenvironments (Mettler Izquierdo *et al.*, 2016), and subjected to display technologies to isolate individual splenocytes producing anti-Kv1.3 antibodies whose V_H and V_L domain sequences were then PCR amplified and used to generate scFv-Fc fusions. As a complementary approach, V_H and V_L domains PCR amplified from peripheral blood lymphocytes obtained from immunized llamas were used to construct scFv cDNA libraries that were subjected to phage display to isolate anti-Kv1.3 llama scFvs that were converted into scFv-Fc fusions. These chicken and llama scFv-Fc fusions were tested for their ability to inhibit Kv1.3 currents when applied at

400 nM (≈40 μg/mL) to a stably transfected Kv1.3 cell line; nine chicken and one llama scFv-Fc were identified that inhibited Kv1.3 function. Concentration dependence measurements revealed an IC_{50} of 6 nM for one of the chicken scFv-Fc inhibitors, and 109 nM for the llama scFv-Fc (Bednenko *et al.*, 2018). These IC_{50} values are somewhat comparable to many ion channel blocking neurotoxins (Alexander *et al.*, 2021) and could likely be improved with further engineering (e.g., *in vitro* evolution).

Antibody mimetics in the form of monobodies have also been used to modulate the function of an ion channel. In this case monobodies were isolated against bacterial Fluc fluoride ion channels from two different species of bacteria (Stockbridge *et al.*, 2014), named Ec2 and Bpe (Stockbridge *et al.*, 2013). Monobodies were first isolated from complex synthetic libraries using phage display technology against magnetic beads coated with one or the other purified recombinant Flu channel protein. This was followed by a step in which gene shuffling (Koide *et al.*, 2012) was used to generate a second generation mini-library derived from the initial Fluc binders. Monobodies in this mini-library were expressed on the surface of yeast followed by labeling with fluorescent Fluc proteins and flow cytometry isolation of fluorescently-labeled yeast cells. This resulted in isolation of a set of eight monobodies, seven of which were found to inhibit F^- current through Fluc channels in planar bilayers. Detailed binding analyses of monobody mb(Bpe-L3) or L3 yielded a K_d=58 nM. Subsequent analyses employing L3-mediated inhibition of homodimeric Fluc channels in planar bilayers supported that the Fluc monomers are assembled in a unique antiparallel structure (Stockbridge *et al.*, 2014). Subsequent studies support that monobodies inhibit Fluc channels by blocking the ionic pore (Turman *et al.*, 2015). By strategically employing the lower affinity (≈200 μM) monobody S8, it was possible to determine the structure of the Bpe-S8 complexes with only a single monobody bound (McIlwain *et al.*, 2018). The structure supported that the mechanism of Fluc inhibition by S8 was through a “cork-in-bottle” mechanism of physical occlusion of the ionic pore (McIlwain *et al.*, 2018). These studies support that a purely

in vitro development strategy can lead to isolation of protein ligands in the form of recombinant antibody mimetics that are capable of controlling ion channel function.

Targeting intracellular domains with ion channel modulating antibodies

The studies detailed in the previous sections show that by employing diverse approaches it is possible to develop antibodies that can be used to selectively modulate the function of specific ion channels by binding to the extracellular domains of ion channels. However, it remains that the pore-forming subunits of many voltage-gated ion channels have a relatively small fraction of their structure accessible from the extracellular compartment, presenting challenges to reliably developing antibodies that modulate ion channels from the outside. By contrast, many of these voltage-gated ion channels have a significant portion of their mass present within the intracellular compartment. This presents an opportunity to develop antibodies that modulate ion channels by binding their cytoplasmic domains. Modulation of ion channels by proteins that bind intracellular domains of pore-forming subunits is an established principle in biology with deep physiological ramifications. Some channels are critically dependent on association with a cytosolic auxiliary subunit for their functional maturation. Pore-forming α_1 subunits of Cav1/Cav2 Ca^{2+} channels (Buraei & Yang, 2010) and of Kv1 (Shi *et al.*, 1996) and Kv4 K^+ channels (Shibata *et al.*, 2003) require association with cytosolic auxiliary subunits to traffic to the cell surface in most cells. In addition, they and other proteins modulate channel gating properties including increasing open probability, controlling inactivation and voltage-dependence of activation. Calmodulin (CaM) binds to the C-termini of Cav1/Cav2 channels and enables feedback regulation of channel activity by Ca^{2+} ions (Lee *et al.*, 1999; Peterson *et al.*, 1999). In the heart, Ca^{2+} binding to resident CaM on Cav1.2 mediates Ca^{2+}-dependent inactivation (CDI) of inward L-type Ca^{2+} current which is important for controlling cardiac action potential duration (APD). Preventing CDI leads to abnormally long APs, and this mechanism likely contributes to arrhythmogenesis in patients with inherited mutations in CaM (Alseikhan *et al.*, 2002; Limpitikul *et al.*, 2014). Voltage-dependent

inhibition of presynaptic Cav2.1-Cav2.3 channels is mediated by $G_{\beta\gamma}$ subunits released from activated G-protein coupled receptors, which contributes prominently to the analgesic effects of opiates (Raingo *et al.*, 2007). Together, these examples underscore the potential for a rich diversity of ion channel modulation that may be possible to obtain with antibodies that bind intracellular domains of voltage-gated ion channels. While to a large extent that potential remains untapped, there are several studies that demonstrate the utility of this approach to control ion channel function (**Figures 3 and 4**).

Intracellular ion channel modulating monoclonal antibodies

Antibodies targeting the cytoplasmic C-terminus of the Kv2.1 K^+ channel have been used as selective inhibitors of Kv2.1 function. Among voltage-dependent K^+ channels, Kv2.1 has an extremely long cytoplasmic C-terminus (>440 amino acids). As first shown for a rabbit polyclonal antibody (Murakoshi & Trimmer, 1999) and subsequently for mouse mAbs K89/34 and D4/11 (Guan *et al.*, 2007; Bishop *et al.*, 2015), antibodies targeting the last 17 amino acids of the large (≈440 a.a.) C-terminus are selective inhibitors of Kv2.1 (**Figure 3**) and do not block the highly related Kv2.2 or other Kv channels (Murakoshi & Trimmer, 1999). The mechanism of action of these antibodies is not known, although there are extensive interactions between the Kv2.1 N- and C-termini (Bentley *et al.*, 1999; Mohapatra *et al.*, 2008), the nature of which changes during voltage activation of the channel (Kobrinsky *et al.*, 2006). It is thus possible that binding of the bivalent antibodies to two subunits within the channel tetramer prevents conformational changes within the intracellular domains that occur during activation gating. The anti-Kv2.2 mAb N372B/60 targeting the cytoplasmic C-terminus is also inhibitory (Bishop *et al.*, 2015). These antibodies represent the only selective inhibitors of Kv2.1 and Kv2.2 as even the most selective neurotoxins and drugs, such as Guangxitoxin-1E (Herrington *et al.*, 2006) and Ry796 (Herrington *et al.*, 2011), respectively, affect Kv2.1 and Kv2.2 similarly.

Zamponi, Turner and colleagues employed mAbs against the auxiliary cytosolic KChIP subunits of Kv4 channels (Rhodes *et al.*, 2004) to define their functional role in neurons. They found that transient K^+ currents (I_A) in cerebellar stellate cells are enhanced in the subthreshold voltage range by T-type Ca^{2+} channel-mediated Ca^{2+} influx (Anderson *et al.*, 2010). They observed a biochemical interaction between Kv4 channels, which mediate a major form of I_A in neurons, and Cav3 channels, which underlie T-type Ca^{2+} currents. The Kv4 channels present in the co-immunoprecipitated complex contained associated KChIP3 cytoplasmic auxiliary subunits. KChIPs bind Ca^{2+} (An *et al.*, 2000), raising the possibility that KChIP3 played a role in translating the T type channel Ca^{2+} influx into increased I_A. They reconstituted the Cav3-Kv4 complex in heterologous cells and found that KChIP3 was in fact needed for T-type channel modulation of Kv4.2-based I_A, which was primarily due to effects on shifting the voltage-dependence of inactivation towards more positive potentials, thereby increasing the I_A window current. They showed that introducing the anti- "pan-KChIP" mAb K55/82 or the anti-KChIP3-specific mAb K66/38 into stellate cells *via* a patch pipet mimicked blocking T-type channels in altering the native I_A (**Figure 3**) such that they now resembled Kv4 currents in heterologous cells lacking KChIP expression. Specific mAbs against KChIP1 (K55/7), KChIP2 (K60/73) or the anti-KChIP3 mAb that had been denatured by boiling were without effect. Thus, the use of these anti-KChIP mAbs allowed for a definition of the specific role of KChIP3 in the absence of KChIP-specific small molecule pharmacology.

The same set of mAbs was used to define the role of KChIP3-dependent Ca^{2+}-mediated modulation of I_A underlying gain of stellate cell firing in response to repetitive stimulation (Anderson *et al.*, 2013), and to block Kv4- and Ca^{2+}-dependent EPSP boosting induced by SNX-sensitive Cav2.3 R-type Ca^{2+} channels in CA1 pyramidal neurons (**Figure 3**) (Wang *et al.*, 2014). The mechanism of action of these mAbs has not been detailed, as to how a mAb targeting a cytoplasmic auxiliary subunit could selectively eliminate the functional effects of that auxiliary subunit on channel function without impacting the overall expression or function of the channel.

Regulating ion channels using genetically encoded intracellular antibodies or intrabodies

The human ether-a-go-go-related gene (hERG) encodes a voltage-gated K^+ channel that gives rise to the delayed rectifier K^+ current (I_{Kr}) which is important for normal cardiac AP repolarization. Decreased cardiac I_{Kr} due to either loss-of-function (LoF) mutations in hERG or block of the channel by drugs causes long QT syndrome with an attendant increase in the risk of lethal ventricular arrhythmias and sudden cardiac death. Morais-Cabral and colleagues (Harley *et al.*, 2016) immunized chickens with purified intracellular hERG PAS domain and generated an scFv phage-display library by amplifying V_L and V_H regions of IgY mRNA obtained from bone marrow and spleen. Panning using purified hERG PAS domain as bait identified several scFv binders including scFv 2.10 and scFv 2.12 which bound the hERG PAS domain with a 1:1 stoichiometry and K_ds of 254 nM and 4.1 μM, respectively. In voltage-clamp studies using heterologously expressed hERG, scFv 2.12 introduced *via* the patch pipette accelerated the kinetics of activation and recovery from inactivation, whereas scFv2.10 slowed the time course of inactivation onset. Both scFv 2.10 and scFv 2.12 caused a ≈2-fold increase in recombinant I_{Kr} (**Figure 4**) elicited either by square test pulses or cardiac AP voltage clamp. These results were recapitulated in cardiomyocytes derived from human induced pluripotent stem cells where scFv 2.10 and scFv 2.12 both increased I_{Kr} in voltage-clamp experiments, and shortened APD measured in current clamp (Harley *et al.*, 2016). These recombinant scFvs offer further potential for use as genetically encoded intrabodies.

Cystic fibrosis (CF) is an autosomal recessive genetic disorder caused by LoF mutations in the chloride ion channel, cystic fibrosis transmembrane regulator (CFTR). Loss of CFTR function leads to disruption of ionic and water transport in epithelial cells in organs including the lungs, pancreas, and intestines. CF patients suffer from impaired airway mucus clearance, recurrent bacterial infection and inflammation that damage the lungs leading to poor quality of life and premature death. Although CFTR functions as an ion channel, structurally, it belongs to the ATP-

binding cassette transporter superfamily that have 12 transmembrane helices and two nucleotide binding domains (NBD1 and NBD2) in the cytoplasm (Liu *et al.*, 2017). The most common CF mutation is a deletion of phenylalanine 508 (F508del) in NBD1 of CFTR, which causes protein processing defects that lead to CFTR retention and degradation in the endoplasmic reticulum, destabilization of channels that do make it to the cell surface, and ultimately a dramatic reduction in F508del surface density (Cutting, 2015). The F508del mutation decreases the thermal stability of NBD1 and compromises its interactions with NBD2 and the transmembrane domain. Immunization of llamas with purified human NBD1 that had three mutations introduced to stabilize the protein (2PT-NBD1) yielded several nAbs that bound 2PT-NBD1 with high affinity and strongly stabilized the protein, as inferred from increases in the melting temperature measured in thermal shift assays (Sigoillot *et al.*, 2019). Remarkably, three of these nAbs (D12, T2a and T27) stabilized F508del-2PT-NBD1 to the same extent as the 2PT-NBD1 mutations. Crystal structures of NBD1-nAb complexes were used to confirm the mode of binding and deduce the mechanism of stabilization (Sigoillot *et al.*, 2019). While the potential for the NBD1-targeted nAbs to rescue F508del trafficking was not evaluated in this study, these *in vitro* studies provide proof of concept for using nAbs to stabilize the structure of an unstable ion channel pathogenic variant and suggest their potential for future use as genetically encoded intrabodies.

Rescuing trafficking-deficient ion channels by targeted deubiquitination

In addition to CF, LoF mutations in other ion channels is a common mechanism in diverse rare diseases. For example, LoF mutations in hERG and KCNQ1 give rise to long QT syndrome 1 and 2 (LQT1 and LQT2), respectively; LoF mutations in Nav, KCNQ2/KCNQ3, and in Cav2.1 give rise to a variety of neurological disorders including epilepsy, ataxia, hemiplegic migraines, epileptic encephalopathies, and intellectual disability (Spillane *et al.*, 2016). Like CF, impaired channel surface density due to compromised trafficking or stability in the plasma membrane is a prominent mechanism underlying LoF for many ion channel pathogenic variants. Thus, a general

approach that could stabilize functional expression of trafficking-deficient ion channels could be potentially harnessed for therapy in many different diseases. Ubiquitination of ion channels typically results in their internalization and/or degradation, and mutant trafficking-deficient ion channels such as F508del often have increased ubiquitination (Meacham *et al.*, 2001; Okiyoneda *et al.*, 2010). Thus, deubiquitinating trafficking-deficient mutant ion channels would potentially result in their functional rescue. Achieving this through inhibiting or deleting particular E3 ligases is problematic because of the widespread role of these proteins in cellular protein homeostasis. To selectively remove ubiquitin from target proteins without disturbing global proteostasis, engineered deubiquitinases (enDUBs) comprised of a nAb fused to the catalytic domain of a deubiquitinase have been developed (Kanner *et al.*, 2020). In proof-of-concept experiments, enDUBs featuring a GFP-targeting nAb fused to catalytic domains of distinct DUBs selectively stripped ubiquitin from and rescued the cell surface density of mutant YFP-tagged KCNQ1 and CFTR channels expressed in heterologous cells (Kanner *et al.*, 2020). In a cellular model of long QT1 generated by adenoviral-mediated expression of a human disease KCNQ1 mutation (G589D-YFP) in adult guinea pig ventricular myocytes, expression of GFP-targeted enDUB restored outward slowly activating delayed rectifier K^+ current, and normalized action potential duration (Kanner *et al.*, 2020).

To test the generality of the approach and enable targeting of endogenous channels, the previously discovered CFTR NBD1 nAb T2a (Sigoillot *et al.*, 2019) was armed with the catalytic domain of the ubiquitin-specific protease USP21, creating enDUB-U21$_{CF.T2a}$ (Kanner *et al.*, 2020). In combination with the pharmacological corrector (lumacaftor) and potentiator (ivacaftor) (Van Goor *et al.*, 2009; Van Goor *et al.*, 2011), enDUB-U21$_{CF.T2a}$ significantly rescued whole cell currents from Fischer Rat Thyroid epithelial cells stably expressing F508del channels (**Figure 4**). Moreover, in combination with lumacaftor, enDUB-U21$_{CF.T2a}$ expression resulted in a synergistic rescue of apical expression of endogenous F508del in patient-derived primary bronchial epithelial cells cultured in the air-liquid interface to WT CFTR levels (Kanner *et al.*, 2020).

Down-regulating ion channel functional expression by targeted ubiquitination

Removing ion channels from the cell surface is a potential mechanism for potently inhibiting ionic currents by decreasing *n*. Because ubiquitination of membrane proteins typically causes their intracellular retention and degradation, it is possible that an approach employing nAb-mediated targeted ubiquitination would offer a general mechanism for developing novel inhibitors for ion channels. In proof-of-concept studies, the catalytic domain of the U-Box E3 ubiquitin ligase CHIP (C-terminus of the Hsp70-interacting protein) was fused to an anti-GFP nAb, creating nanoCHIP. When co-expressed with YFP-tagged KCNQ1, nanoCHIP abolished current by promoting intracellular retention of the channel (Kanner *et al.*, 2017). Surprisingly, nanoCHIP did not lead to frank degradation of KCNQ1-YFP, even though current was abolished. By contrast, arming the GFP nAb with the catalytic HECT domain of the Nedd4L E3 ubiquitin ligase (generating nanoNedd) led to both current ablation and increased KCNQ1 degradation. Thus, the nature of the E3 ligase catalytic domain in the nAb fusion construct strongly impacts the effects on KCNQ1 stability. Interestingly, targeting nanoCHIP to KCNE1-YFP was also effective in preventing surface trafficking of KCNQ1, indicating the approach can be used to eliminate ion channel complexes from the surface membrane by targeting auxiliary subunits.

The generality of this concept was further explored in studies focused on high-voltage-activated Ca^{2+} (Cav1/Cav2) channels. Following immunization of llamas with purified auxiliary β_1 and β_3 subunits several nAbs against Cavβ subunits were isolated. One of these, nb.F3, indiscriminately bound all four Cavβ isoforms (β_1-β_4) with 1:1 stoichiometry and high affinity (K_d for β_1 = 12 nM). Co-expressing recombinant Cav2.2 or Cav1.2 channels with nb.F3 revealed that this nAb was completely inert with respect to modulating channel function. However, attaching Nedd4L catalytic HECT domain to nb.F3 generated a construct that eliminated functional expression of Cav1/Cav2 channels reconstituted in HEK293 cells (**Figure 4**). Moreover, viral-mediated expression of nb.F3-HECT in either primary cardiac myocytes or dorsal root ganglion

neurons eliminated high-voltage-activated Ca^{2+} currents from these cells. Given its efficacy in inhibiting Cav1/Cav2 channels by targeting auxiliary Cavβ subunits, nb.F3-HECT was named Cav-aβlator (Morgenstern *et al.*, 2019). These studies reveal the promise of employing genetically-encoded intrabodies to selectively modulate the function of specific ion channels in living cells.

Summary and future perspectives

The studies detailed above support the premise that the research community would greatly benefit from a concerted effort to develop a toolbox of widely available renewable recombinant antibodies targeting ion channels. These antibodies could then be evaluated by researchers as direct modulators of ion channel function, and/or armed with various functionalities to obtain selective modulation of specific ion channels in native cells. The examples above provide ample proof of the effectiveness of these approaches.

To begin to address the need for renewable recombinant antibodies for ion channel research, efforts are underway to convert mAbs into recombinant form. This includes the anti-Kv2.1, anti-Kv4.2 and anti-KChIP mAbs used in the studies detailed above. Some of these efforts employed cloning of RT-PCR amplified heavy and light chain variable ("V_H" and "V_L") domain sequences into a mammalian expression plasmid (Crosnier *et al.*, 2010), followed by sequencing of V_H and V_L domains (Andrews *et al.*, 2019). More recent efforts have employed a high-throughput Illumina-based sequencing platform to sequence the V_H and V_L domain sequences directly from hybridomas and publicly posting these sequences on the NeuroMabSeq website (neuromabseq.ucdavis.edu). These V_H and V_L domain sequences can then be used to develop R-mAb and scFv expression plasmids. In many cases plasmids encoding recombinant antibodies are being made available through the open access plasmid repository Addgene, including R-mAbs (Andrews *et al.*, 2019) and nAbs (Dong *et al.*, 2019) against ion channels and ion channel-associated proteins.

The utility of expressing recombinant antibodies as intrabodies in native cells should also increase with continued advancements in gene transfer techniques in both *ex vivo* and *in vivo* environments. As genetically encoded tools, intrabody expression can be directed by a wide array of cell-type specific promoters, recombinant viruses and other available tools that allow researchers to achieve enhanced specificity of cellular targeting. Emerging technologies for engineering recombinant antibodies to have photo- or chemo-switchable function offers additional promise for future research. Light-activated “optobodies” have been developed by expressing split N- and C-terminal fragments of nAbs, with each fragment fused to a photoswitchable dimerization domain that led to the reconstitution of the entire nAb structure and function in response to light (Yu *et al.*, 2019). Intrabodies with inducible function have also been generated by intentionally engineering improperly folded nAbs fused to a photoswitchable light–oxygen–voltage domain that can allosterically drive the light-induced folding of the nAb and recovery of function (Gil *et al.*, 2020). Incorporation of photocaged amino acids into nAbs has also been used to generate optobodies (Joest *et al.*, 2021). An alternate approach is to use chemical or light stimulation to induce the coupling of an intrabody to a specific functional protein, in this case an E3 ubiquitin ligase to yield stimulus-dependent target protein degradation (Deng *et al.*, 2020). Employing such approaches to generate inducible functionality yielding “on demand” function will yield increased temporal precision of intrabody-mediated ion channel modulation. Together, a future combining increased availability of renewable recombinant antibodies with these emerging techniques for directing their expression and function will open new avenues of ion channel research.

Additional information section

Competing Interests: H.M.C is co-founder of Stablix, Inc. J. S. T. declares no competing interests.

Author contributions: Both authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. Both persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding: This work was supported by NIH grants R01 HL121253 and R01 HL122421 (to H.M.C.), and U24 NS109113 to (J.S.T.).

Acknowledgements: We thank all our talented and dedicated colleagues in the Colecraft and Trimmer labs who have contributed to these research directions.

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Figure legends

Abstract figure legend. Two different approaches for controlling ion channel function using renewable recombinant antibodies. On the left, an externally applied intact IgG antibody (purple) binds to an extracellular domain of an ion channel (light blue) to control ion channel function. On the right, a genetically-encoded intrabody, in this example a camelid nanobody (green) fused to an effector molecule (reddish brown) binds to an intracellular auxiliary subunit of an ion channel (dark blue) to control ion channel function.

Figure 1. Forms of antibodies used to control ion channels. Panel A. Left. A typical mammalian IgG antibody. Light chains comprise one variable (V_L, pink) and one constant (C_L, light grey) domain. Heavy chains comprise one variable domain (V_H, red) and three constant (C_H1–3, dark grey) domains. The antigen binding surface is formed by the assembled V_L and V_H domains. Typical mammalian H + L chain IgGs can be miniaturized to various forms. These include single chain variable fragments or ScFvs (middle), which can be tethered to V_H domains to yield scFv-Fc fusions. Panel B. Camelid HC-only IgGs exist as a homodimer of two identical H chains. The antigen-binding surface is contained within a single V_HH domain, which can function autonomously as a nanobody (nAb).

Figure 2. Extracellular antibody-mediated control of ion channels. Schematic depicts from left to right the anti-Kv10.1 mAb56 (dark blue) mediating inhibition of the Kv10.1 K^+ channel (tan), the scFv62-TRAIL fusion (the anti-Kv10.1 scFv62 is in light purple, TRAIL is in pink) mediating apoptosis of Kv10.1-expressing cancer cells, the K57/1-porphyrin conjugate (the anti-Kv4.2 mAb K57/1 is in purple, porphyrin is in red) mediating reactive oxygen species (ROS)-induced photoablation of the activity of the Kv4.2 K^+ channel (light blue), and the anti-Fluc L3 monobody (gold) mediating inhibition of the Fluc F^- channel (green).

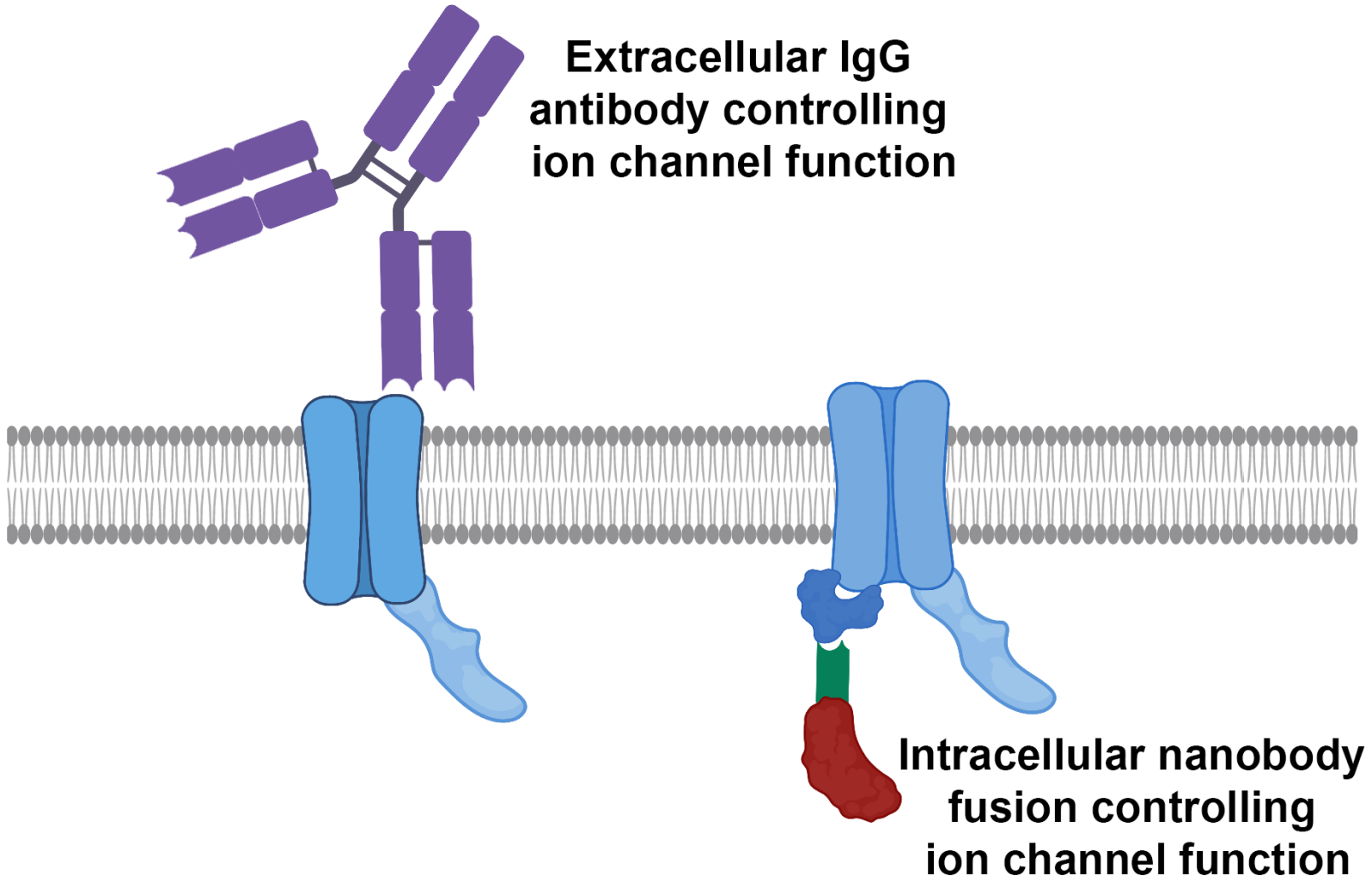
Figure 3. Intracellular antibody-mediated control of ion channels. Schematic depicts from left to right the anti-Kv2.1 mAb K89/34 (dark green) mediating inhibition of the Kv2.1 K^+ channel (light green), the anti-KChIP3 mAb K66/38 (purple) mediating inhibition of the Cav2/Cav3 Ca^{2+} channel (light orange) mediated Ca^{2+}-dependent modulation of Kv4.2/KChIP3 K^+ channels (Kv4.2 in light purple, KChIP3 in dark purple).

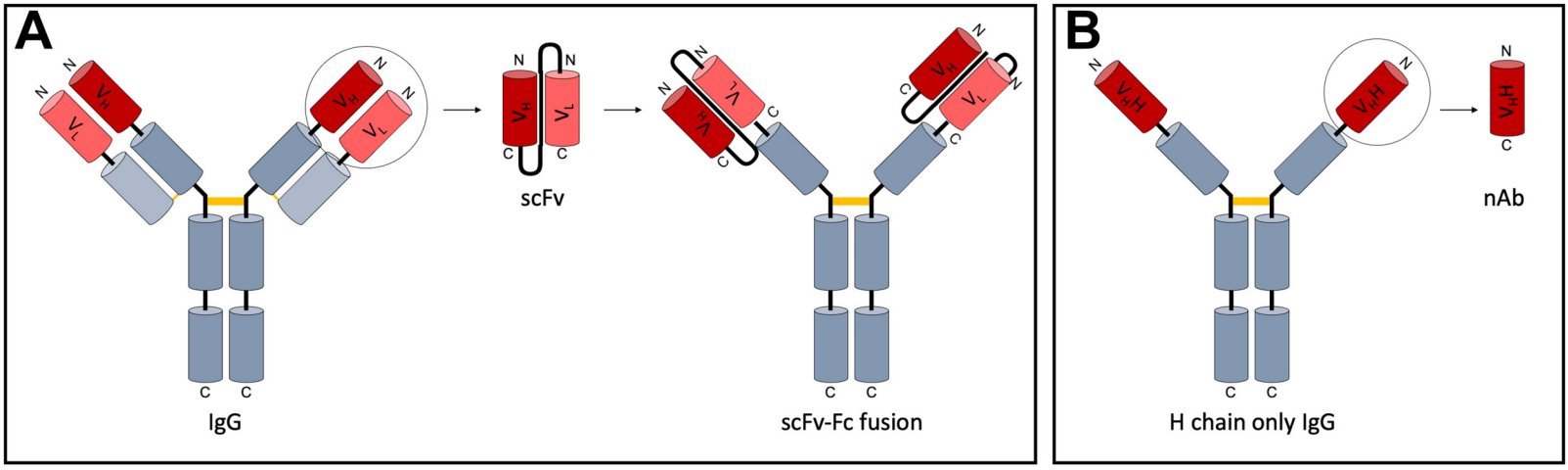
Figure 4. Genetically-encoded intrabody-mediated control of ion channels. Schematic depicts from left to right the anti-hERG scFvs 2.10 and 2.12 (light purple) mediating inhibition of the hERG K^+ channel (light green), the T2a-USP21 fusion (the anti-CFTR nanobody T2a is in gold, the USP21 deubiquitinase catalytic domain is in dark blue) catalyzing the deubiquitination of ΔF508 mutant CFTR channels leading to their enhanced surface expression (purple), and the nb.F3 nAb-Nedd4L fusion (the anti-Cavβ nanobody nb.F3 is in green, the catalytic HECT domain of the Nedd4L E3 ubiquitin ligase Nedd4L is in reddish-brown) catalyzing the ubiquitination of the Cav1.2/Cavβ Ca^{2+} channel leading to their inhibition (the Cav1.2 α1 subunit is in light blue, and the Cavβ subunit is in dark blue).

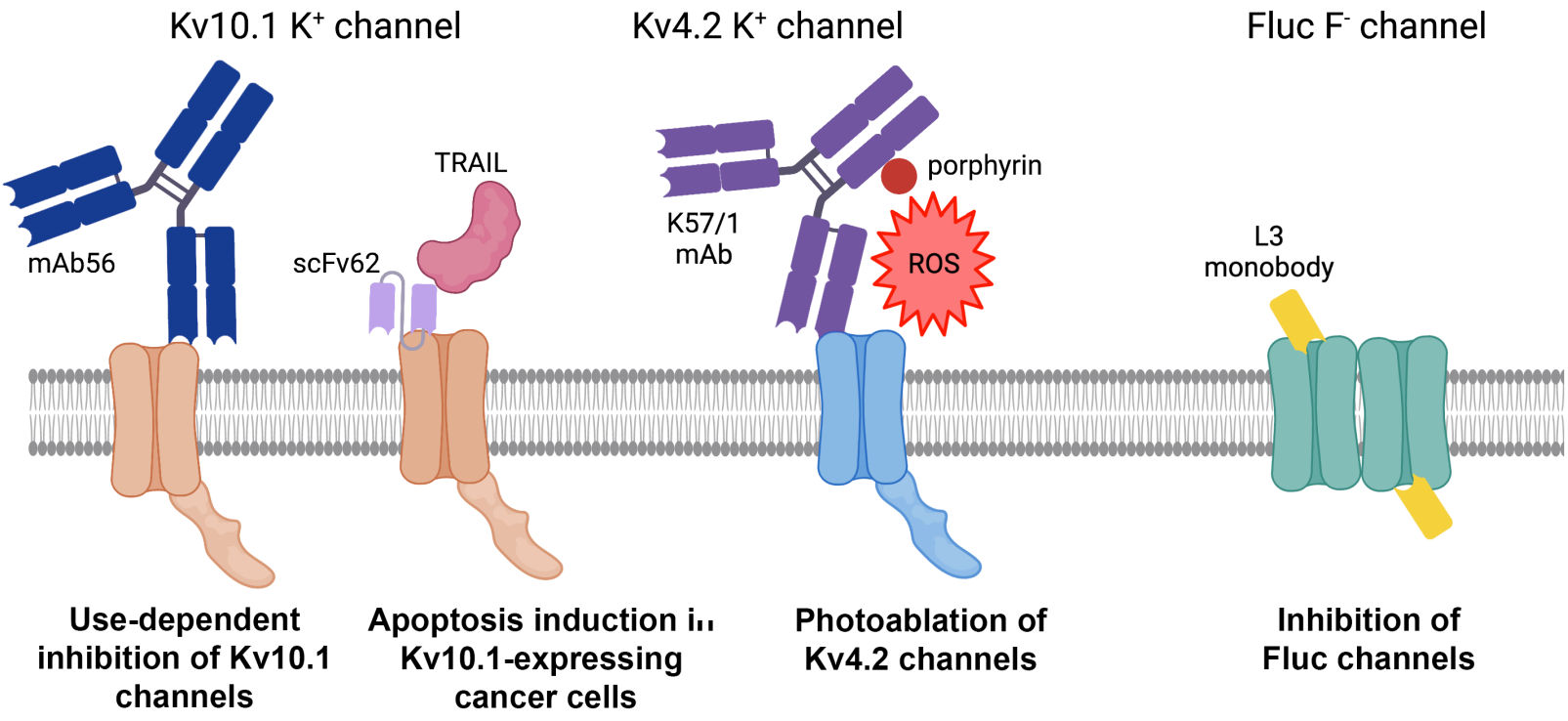
Table 1. Examples from each class of renewable and recombinant antibodies controlling ion channel function

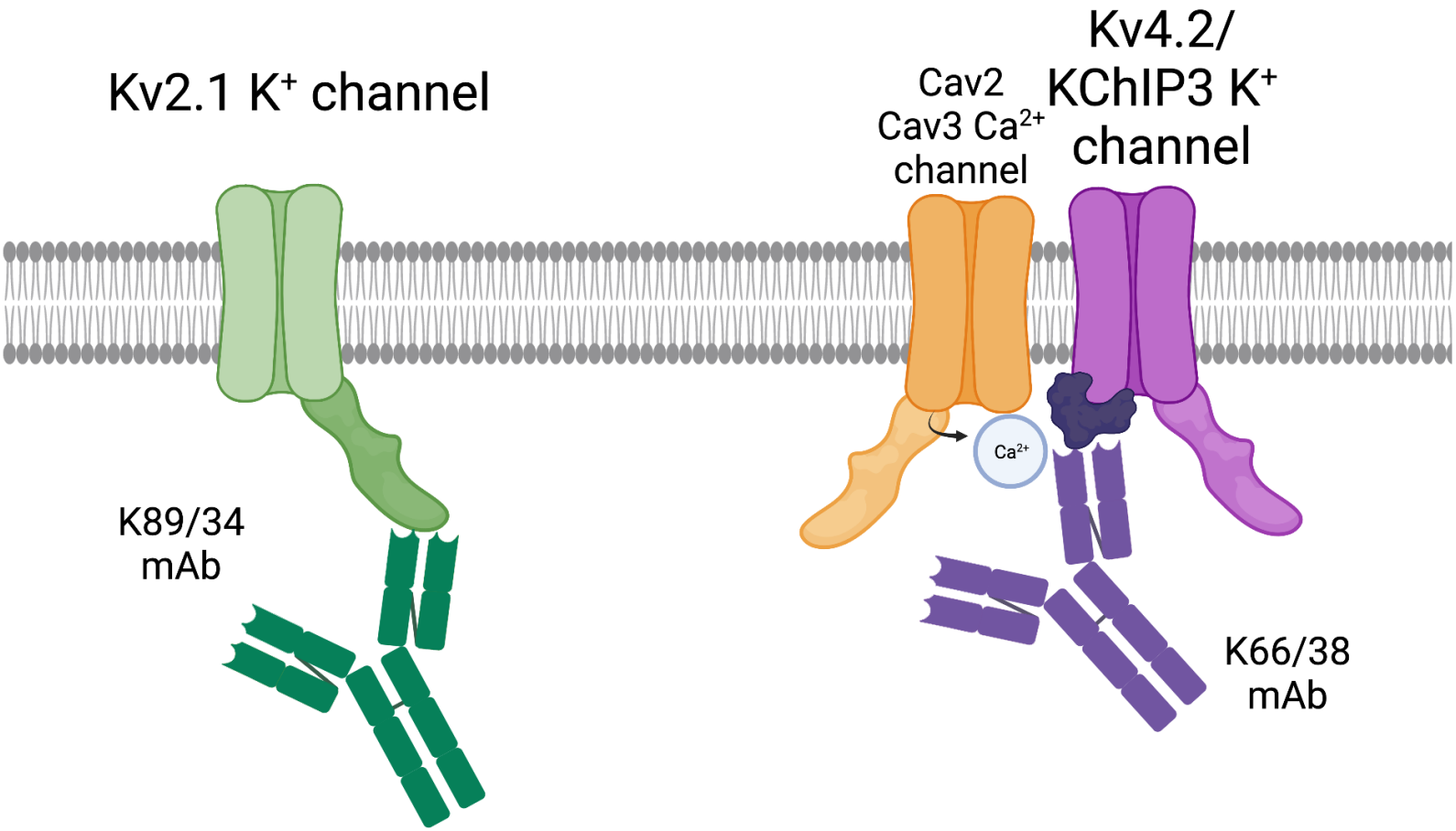
Target ion channel	Antibody name and form	Intrinsic Activity	Added Activity	Impact
Extracellular mechanism of action				
Kv10.1 K^+ channel	mAb56 mouse mAb	Inhibitor	NA	Use-dependent inhibition of Kv10.1 channels
Kv10.1 K^+ channel	scFv62/ scFv from mouse mAb	None	Fused to TRAIL	Apoptosis induction in Kv10.1-expressing cancer cells
Kv4.2 K^+ channel	K57/1 mouse mAb	None	Fused to porphyrin	Light-induced inhibition of Kv4.2 channels
Kv1.3 K^+ channel	Various chicken and llama scFv-Fc fusions	Inhibitor	NA	Inhibition of Kv1.3 channels
Fluc F^- channel	mb(Bpe-L3) "L3" monobody	Inhibitor	NA	Inhibition of Fluc channels
Intracellular mechanism of action				
Kv2.1 K^+ channel	K89/34 mouse mAb	Inhibitor	NA	Inhibition of Kv2.1 channels
Kv2.2 K^+ channel	N372B/60 mouse mAb	Inhibitor	NA	Inhibition of Kv2.2 channels
KChIP3	K66/38 mouse mAb	Inhibitor	NA	Inhibition of Ca^{2+} -dependent regulation of Kv4.2 channels
Pan-KChIP	K55/82 mouse mAb	Inhibitor	NA	Inhibition of Ca^{2+} -dependent regulation of Kv4.2 channels
hERG	scFv 2.10 chicken scFv	Activator	NA	Slowed onset of inactivation of hERG channels. Shorten APD in cardiomyocytes.
hERG	scFv 2.12 chicken scFv	Inhibitor	NA	Accelerated activation and recovery from inactivation of hERG channels. Shorten APD in cardiomyocytes.

CFTR	Various llama nAbs	Stabilizer		Stabilize F508del NBD1 domain
YFP-tagged KCNQ1 and CFTR	Anti-GFP nAb llama nAb	None	Deubiquitinase	Enhanced surface expression of GFP-tagged KCNQ1 and CFTR
YFP-tagged KCNQ1	Anti-GFP nAb llama nAb	None	CHIP E3 ubiquitin ligase	Reduced surface expression of GFP-tagged KCNQ1
YFP-tagged KCNQ1	Anti-GFP nAb llama nAb	None	Nedd4L E3 ubiquitin ligase	Reduced surface expression and enhanced degradation of YFP-tagged KCNQ1
Pan-Cavβ	nb.F3 llama nAb	None	Nedd4L E3 ubiquitin ligase	Reduced functional expression of Cav1/Cav2 channels


Extracellular IgG
antibody controlling
ion channel function
Intracellular nanobody
fusion controlling
ion channel function

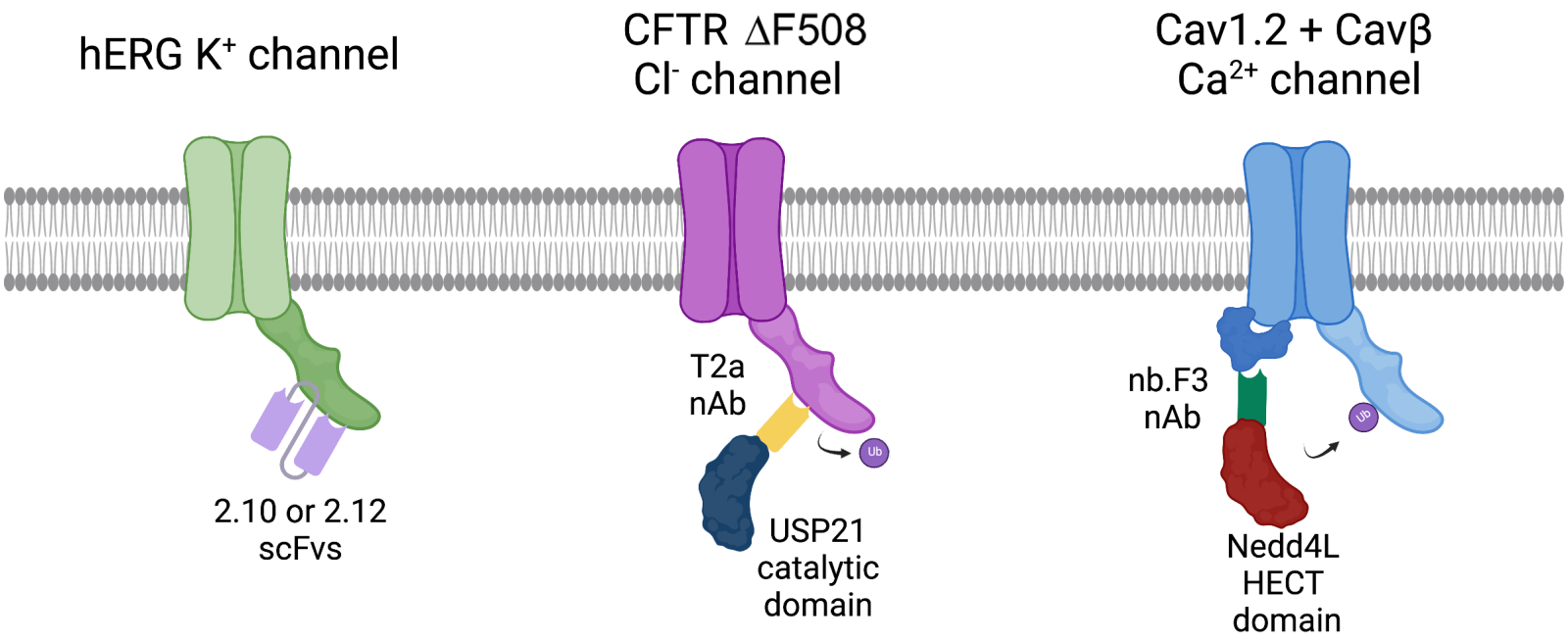

A
B
IgG
scFv
scFv-Fc fusion
H chain only IgG
nAb


Kv10.1 K+ channel
Kv4.2 K+ channel
Fluc F- channel
mAb56
TRAIL
scFv62
K57/1
mAb
porphyrin
ROS
L3
monobody
Use-dependent inhibition of Kv10.1 channels
Apoptosis induction in Kv10.1-expressing cancer cells
Photoablation of Kv4.2 channels
Inhibition of Fluc channels



Inhibition of Kv2.1 channels

Inhibition of Ca^{2+}-dependent modulation of Kv4.2 channels



Enhance activity of hERG channels

Enhance surface expression of CFTR ΔF508 channels

Inhibition of Cav1.2 channels