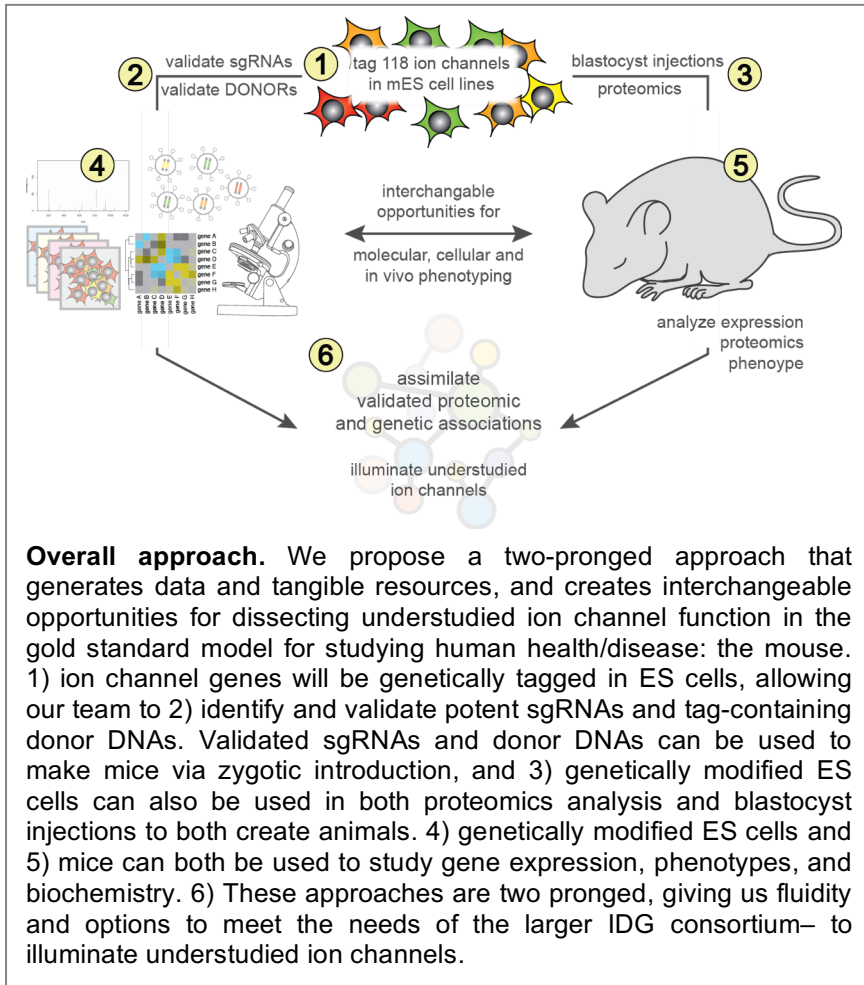


SIGNIFICANCE

The fundamental basis for understanding human development and disease is rooted in genetics. For a large number of diseases including channelopathies such as cystic fibrosis, a single gene causes a disorder whose inheritance can easily be traced on a family tree. In these monogenic diseases, genetic modifiers can alter disease severity, for example, the transmembrane CFTR-interacting transporter family member SLC26A9 reduces survival in CFTR deficient mice (for review³). There are many such examples that underscore the complexity of genetic interaction in human disease. In fact, for it is generally accepted that in the majority of common diseases, multiple genes interact in such complex ways that it is nearly impossible to determine which genes are the causative alleles. Although these complex disorders often cluster in families, they do not have a clear-cut pattern of inheritance. Examples of these conditions include Alzheimer's and Parkinson's disease, cardiovascular disease, cancer, diabetes, a number of birth defects, and numerous psychiatric disorders. Even



Overall approach. We propose a two-pronged approach that generates data and tangible resources, and creates interchangeable opportunities for dissecting understudied ion channel function in the gold standard model for studying human health/disease: the mouse. 1) ion channel genes will be genetically tagged in ES cells, allowing our team to 2) identify and validate potent sgRNAs and tag-containing donor DNAs. Validated sgRNAs and donor DNAs can be used to make mice via zygotic introduction, and 3) genetically modified ES cells can also be used in both proteomics analysis and blastocyst injections to both create animals. 4) genetically modified ES cells and 5) mice can both be used to study gene expression, phenotypes, and biochemistry. 6) These approaches are two pronged, giving us fluidity and options to meet the needs of the larger IDG consortium— to illuminate understudied ion channels.

redundancies present challenges to studying families of genes (channels, GPCRs, channels, etc.). However, few studies have taken approaches to meet the challenge. In part this is due to the difficulties in co-ablation of more than one gene in cells or organismal models. In addition, the uncertainty in choosing which phenotype(s) to survey in a double knockout cell line or organism greatly increases the numbers of possible genetic and phenotypic experiments to evaluate. Given the time and cost in studying a double knockout mouse (for example), one must choose carefully. Conventional genetics approaches are just too cumbersome.

This proposal aims to illuminate the understudied ion channels in a function-first approach. Importantly we used proteomics and bioinformatics to build models for ion channel interaction pairs that are then validated in vivo using mouse models. It is notable that approximately 10% of the most highly cited channels in 2009 garnered 50% of the research activity¹⁵. For example, after David Julius (UCSF, our advisory board member) discovered the TRPV1 in 1997, almost 10% of ion channel research focused on TRPV1¹⁵. In fact, the lowest 60% of ion channels garnered only 12% of the citations, even though many understudied channels have proven to have significant disease associations^{15,16}. Our work will help fill this void. We believe researchers value first-and-foremost seeing an in vivo phenotype and our overall plan is centered on this activity.

in familial inherited cancers harboring high penetrance alleles (e.g. Brca, Pten, Apc, Rb1) it is generally accepted that multiple genetic 'hits' are required to promote full-blown disease. As identifying the causative gene combinations among the thousands of genes in the genome is as difficult as picking a combination lock with hundreds of thousands of possible combinations, determining the genetic composition of complex diseases represents the major challenge to genetic researchers. For this practical reason, many researchers have focused on studying a single disease gene at a time in cells and laboratory model organisms like the laboratory mouse. This approach is largely inconsistent with how genes act in disease pathways that have evolved functional redundancies and complex interaction.

Among the disease gene families, ion channels dominate, constituting some 1.2% of annotated human genes. Ion channels from large and diverse families can serve redundant functions^{9,10}. In addition, disrupting one channel function may induce compensatory changes of other channels¹¹⁻¹⁴. In addition, genetic

INNOVATION

The heart of this proposal is to overturn the existing one-gene-at-a-time paradigm for studying human disease in mouse models. We propose to leverage our recent advances in cell culture based epistatic gene interaction technologies with *in vivo* mouse model experiments to develop a high-throughput CRISPR based platform. If successful, we will unlock the hidden combinations of genes in human complex diseases. At a large-scale, targeting multiple genes to place an unknown gene in a functional known pathway is innovative. In addition, we apply approaches for tagging endogenous alleles using CRISPR based homologous insertion and use similar cutting-edge published approaches. These technologies are scalable and give us room to expand in collaborative endeavors across the kinase and Gpr IDG centers.

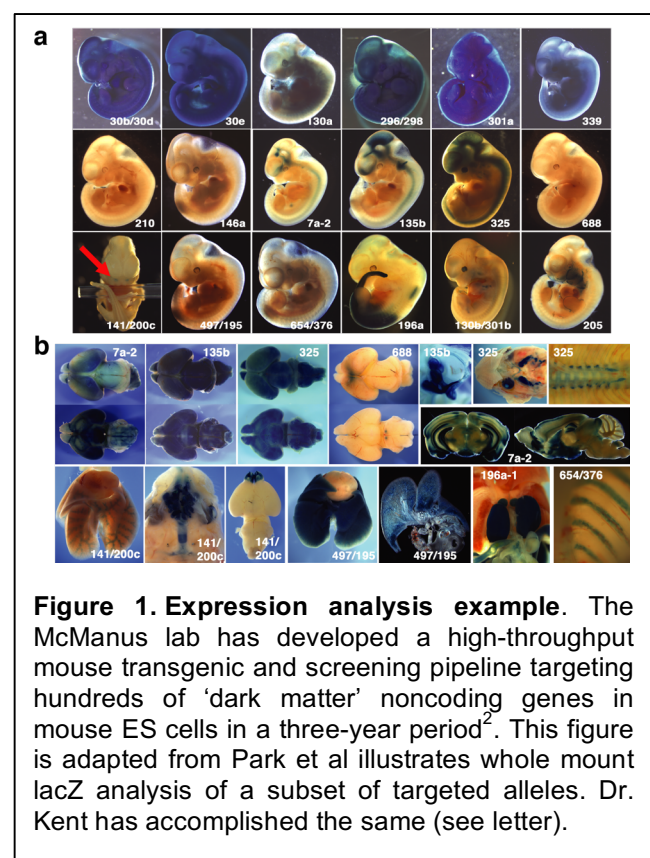
APPROACH

AIM 1. Establish functional roles for underexploited ion channels.

Expression analysis of ion channels. A major challenge in the ion channel field is to probe the expression of channels *in vivo* contexts. The field is hampered by the lack of effective antibodies, which is especially problematic for understudied genes that have not been adequately studied by the community. To systematically study expression in a native physiological background, propose a complement of two approaches: antibody generation (monoclonal first and polyclonal as a backup) and endogenous reporter tagging. In the case of antibodies, we will QC commercially available reagents to identify which reagents are most needed (prioritizing no antibody available, then poor/non-specific antibody). We will outsource production to a facility with a strong track record, a national monoclonal antibody-generating resource funded by the NIH/NINDS located at UC Davis (<http://neuromab.ucdavis.edu>). Immunohistochemical stains will be performed in our own histology processing room, or outsourced to a Core at UCSF (run by Scott Kogan, see letter).

Antibodies validated by Western blot, IHC, and FACs will be transferred to the RDOC along with the necessary optimized staining protocols. Antibody validation will include co-stains with CRISPR cells not expressing the ion channel (see below text). These protocols are routine for our collective labs and Cores and we estimate that we may complete most antibody projects in the first three years, although we expect to accomplish the more difficult ones throughout the project period.

The endogenous gene tagging represents a strategic opportunity, where we can more quickly and definitively use tagged cells to validate sgRNAs (described herein). In this approach, we tag the endogenous locus using a powerful CRISPR epitope (GFP, GFP11, and sfCherry11 tags) knockin strategy developed by Dr. Bo Huang lab^{17,18} (UCSF, see letter). That said, we reserve the opportunity to employ lacZ as a transcriptional readout when GFP fails to give expression adequate signal, since we have found it to be a very sensitive and robust measure of gene expression² (**Figure 1**). For most ion channels, GFP is our tag of choice because it shows minimal nonspecific binding to mammalian cell proteins, can be quantitatively depleted from cell extracts, and allows the integration of biochemical protein interaction data with *in vivo* measurements using fluorescence microscopy¹⁹, and opens up the possibility of performing functional



interrogation of live cells. Moreover, GFP fusion contexts have been validated in ion channels with no deleterious effect²⁰. Importantly all of these reporter tools are available with certain ion channel alleles better suited for particular tags. Our overall plan is to introduce these tags into 118 channel alleles and using Huang lab protocols, we have already generated DNA Donor tag constructs in a high-throughput approach using 96 well plates. Alleles will be validated using sequencing of the targeted locus.

We see targeting ES cells as a strategic opportunity. First, it will allow us to assess the targeting efficiency for each construct at each of the 118 loci. Second, it will give us the opportunity to begin proteomic work in primary embryonic stem cell lines during the first year. Third, we will generate reporter lines to identify the most

efficient CRISPR sgRNAs. Fourth, ES cells can be used to generate mice, which introduces a tangible alternative strategy. And fifth, understudied ion channels are expressed in embryonic stem cells, and their ability to be differentiated into specific cell types opens up many interesting directions for researchers. Taken

together our research will provide significant resources that will be pipelined to the IDG RDOC during the first year. Having the expertise and resources in both embryonic stem cell modification and zygotic modification gives us ample options to meet the needs of the IDG initiative. To this end, we have included a letter from our UCSF collaborators Drs. Stephen Altschuler and Lani Wu who bring an incredibly quantitative high-content imaging platform to this table, opening up the possibility of performing screens using these tagged lines, should the science and IDG NIH leadership guide us in this direction.

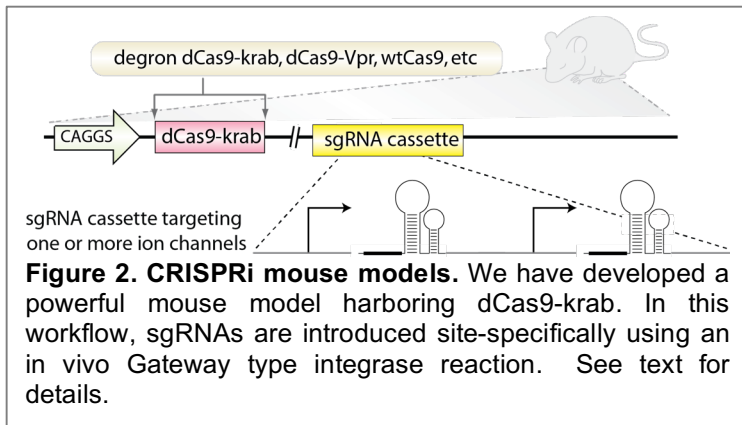


Figure 2. CRISPRi mouse models. We have developed a powerful mouse model harboring dCas9-krab. In this workflow, sgRNAs are introduced site-specifically using an in vivo Gateway type integrase reaction. See text for details.

Ion channel high-throughput loss-of-function mouse screen. Many groups have usurped the ‘wild type’ wtCas9 based CRISPR systems for genome editing, and we have significant experience in creating large numbers of CRISPR indels in mice. However, this is explicitly not our prioritized approach. We reserve wtCas9 as a backup, instead focusing on a newer catalytically dead variant called dCas9, which represents an advance over wtCas9²¹. Like wtCas9, the use of dCas9 is a preferred embodiment given its dominant nature

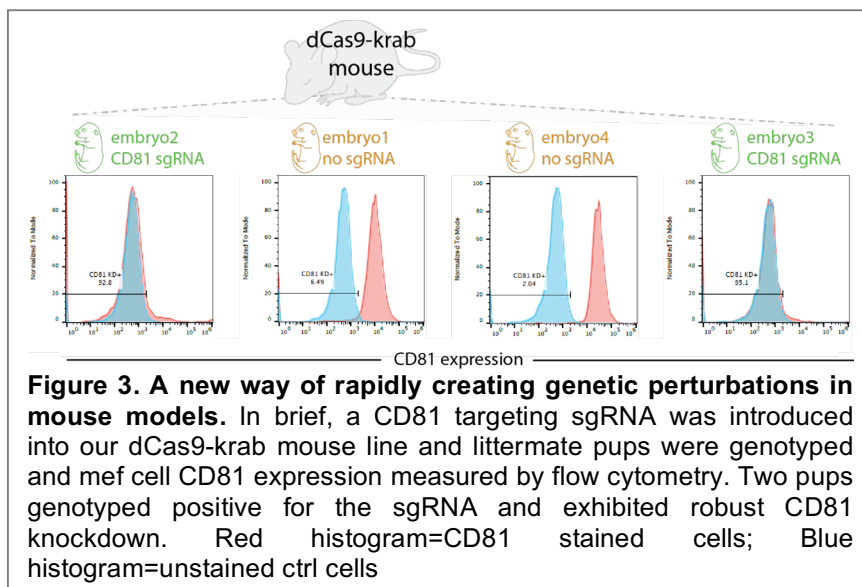


Figure 3. A new way of rapidly creating genetic perturbations in mouse models. In brief, a CD81 targeting sgRNA was introduced into our dCas9-krab mouse line and littermate pups were genotyped and mcf cell CD81 expression measured by flow cytometry. Two pups genotyped positive for the sgRNA and exhibited robust CD81 knockdown. Red histogram=CD81 stained cells; Blue histogram=unstained ctrl cells

(i.e. any animal containing both the dCas9 and an sgRNA will lead to gene silencing. However, dCas9 is cleaner and not subject to wtCas9-centric indel variations, saving time and animal costs²¹. Since dCas9 technology published several years ago it has been used in many studies, in single, double, and higher order gene perturbation. It exhibits better specificity, reproducibility, and exceptionally robust activity²¹. Relevant to this proposal, CRISPR tools surpass decades of work in the field that centered on more limited technologies and allow for a more rapid systematic genetic modification at a lower cost, without compromising precision and variability.

To date, we have made a battery of Cas9 derivatives (**Figure 2**), and center our attention on an extremely effective dCas9-krab line in a C57BL/6J background. These lines contain a Cas9 transgene driven by the ubiquitous CAGGS promoter and are integrated into safe harbor loci. Most of our site-specific targeting technologies focused on delivery to the *Hipp11* safe harbor locus, but we have a ROSA26 system available for use, particularly for our proposed goal of creating sgRNAs insertions and multi-component systems (which we have already validated, see **Figure 3** with other data not shown).

Additional opportunities. The dCa9-krab mice that we are using are constitutive, meaning that dCas9 silences the target gene in all cells and developmental stages. Although temporal inducibility is not essential for this project, we see it as an area that will help advance the current crop of Cas9 systems to allow targeting in adult animals in both space and time.

Direct zygotic transformation as a secondary approach. We have developed turnkey protocols for introducing sgRNA cassette transgenes site-specifically directly into mouse zygotes, using *in vivo* site-specific integrase technology. This technology involves co-introduction of Phi31 integrase and a Donor DNA construct containing the sgRNA(s) of interest into mouse blastocysts. In addition to conventional microinjection, we are adopting a zygotic electroporation protocol used as an SOP in the Lloyd and Marson group (see letters). In these experiments, the integrase recognizes a specific target site (an attP sequence located in a safe harbor locus²²) engineered in the genome and efficiently integrates the Donor DNA. This is a highly efficient process. Nearly every injection results in a genetically modified animal containing the sgRNA of interest. As these do not use wtCas9, we do not have to screen through animals to identify transgenes that have the 'correct indel'; instead, positive PCR based genotyping of the successfully integrated dCas9 sgRNA is sufficient to move the animal through the pipeline.

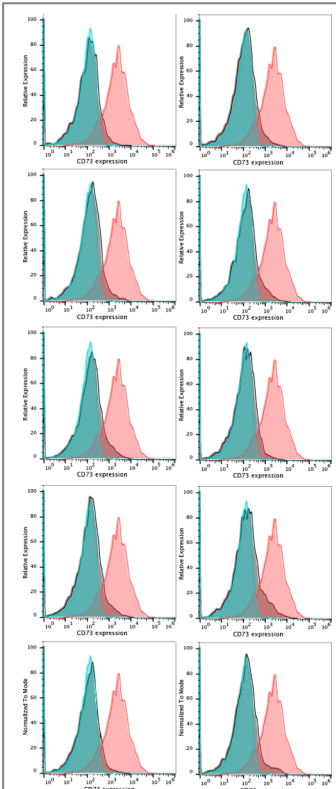


Figure 4. Potent knockdown of CD44. This FACS plot depicts analysis of MEFs harboring ten different sgRNA sequences designed against the mouse CD44 promoter. Red=stained positive ctrl; green=unstained ctrl; overlapping grey=CD44 sgRNA containing cells.

Maximizing efficiency and expediting the workflow. To expedite the generation of large numbers of ion channel deficient animals, we co-injected multiple sgRNA vectors, yielding animals each harboring a different sgRNA construct. We will adopt this strategy, anticipating that a single microinjection (or electroporation) can produce a litter of eight animals, most of which will contain an sgRNA construct targeting a specific ion channel. In this way, we anticipate that around a dozen serial electroporations will yield CRISPRi animals for all underexplored ion channels (i.e., we would exclude Donor DNAs that have been successful at each serial electroporation round). Note our goal is explicitly not to create straight knockout animals for individual ion channels, which is the mission of the KOMP program (see Lloyd letter). Instead, our goal is to conduct pairwise combinations, linking understudied ion channels to: 1) interaction genes identified in the proteomics experiments (**Aim 2**), and 2) interaction genes identified through bioinformatics analysis with Butte and Monarch (see Monarch letter). We have already generated a priority list. We anticipate uncovering missed phenotypes from understudied ion channels so as to gain better information related to polygenic disease. Most critically, we expect that the double CRISPRi data will provide functional information about the proteomic and bioinformatics data, essentially validating observations in the most physiologically relevant setting possible—*in vivo*. Based on our planned workflow we aim to co-ablate each ion channel with approximately 3-5 different genes prioritized from the proteomics and bioinformatics platforms.

Additional opportunities. Note that dCas9-krab mice can be used in settings where the sgRNA is introduced by virus (for review^{23,24}). We retain this as a possible endeavor. For example, our colleagues in the Diabetes Center at UCSF, Drs. Christian Vaisse and Allison Xu (see letter), routinely perform stereotactic injections and have had success in using dCas9 mice and AAV in the study of hypothalamic control of feeding. Dr. Vaisse has collaborated with our colleague Dr. Nadav Ahituv (UCSF) with whom we have collaborated and published and whose lab is in the same building as Drs. Basbaum, Ptacek, and Jan. Dr. McManus runs a ViraCore at UCSF and has the capacity to create AAV and retrovirus for these types of experiments. This work will broaden the utility of dCas9 based technologies.

sgRNA perturbation. Dr. McManus will contribute his expertise with CRISPR based technologies. He has developed powerful double sgRNA systems under

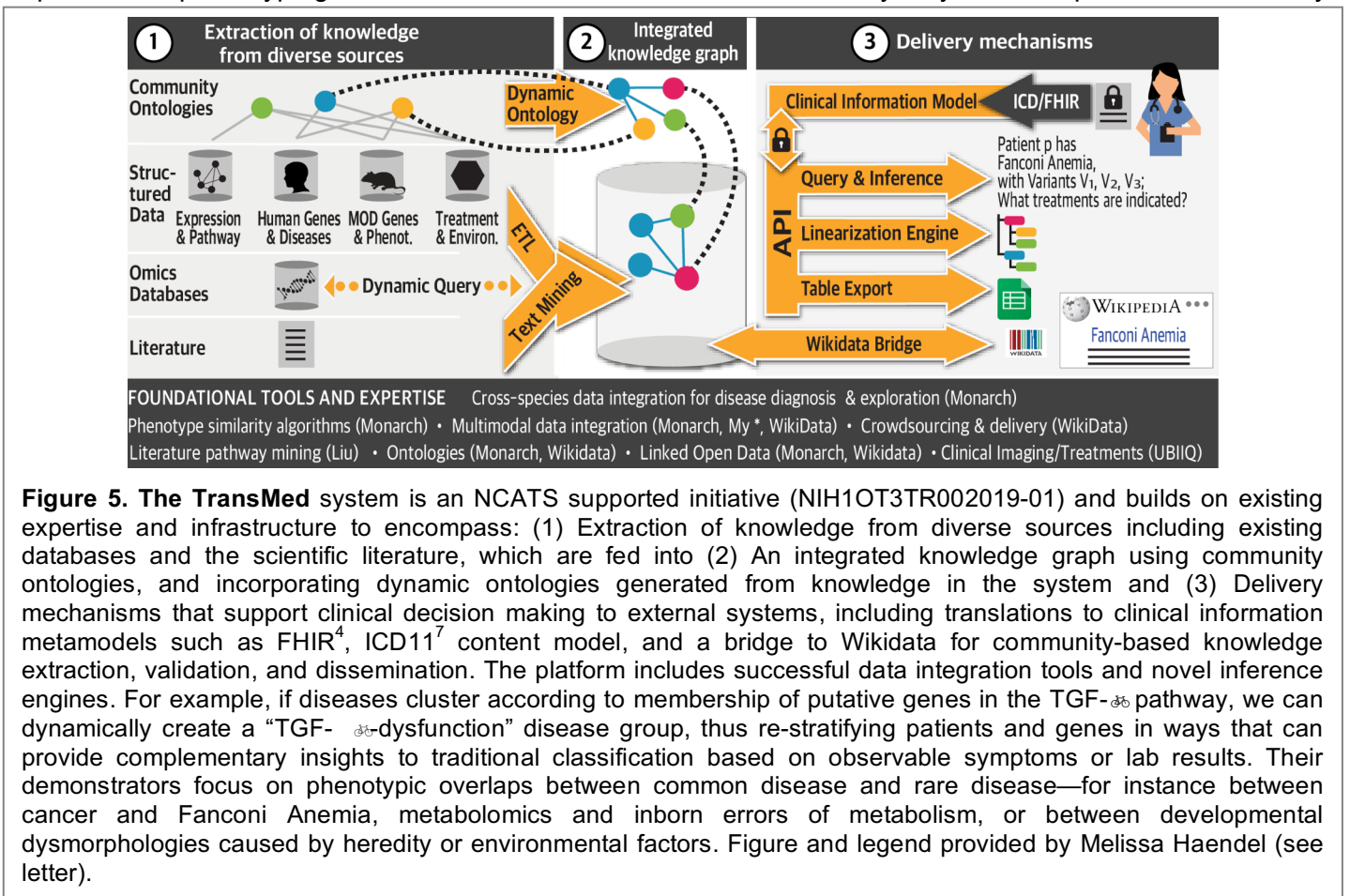
support of his IDG kinome project (Dr. McManus has submitted a separate application to this IDG FOA where he advances his scalable double technologies to uncover deeper information on understudied kinome biology). His group has adopted double sgRNA systems to conduct genome-wide screens, and has a paper currently in revision describing the double sgRNA platform and its application to dissect pathways in a directional manner. In as yet unpublished work, he has used it to simultaneously co-target and also knockdown many different cell surface genes in mouse tissue and created transgenic sgRNA dCas9 mice (**Figure 3**).

Sequence design. One of the challenges in the CRISPR field has been to identify sgRNA sequences that potently perturb the target gene. Drs. McManus and Weissman at UCSF have each generated an established set of validated sgRNAs targeting several thousand genes and vastly improved second generation genome-scale libraries²⁵. Thus far, most of the tested sgRNAs are active, with some cases reflecting 100% of the

sgRNAs tested (**Figure 4**). The take home message is that we have developed a very efficient pipeline, where most sgRNAs work quite well. Nonetheless, we will validate each sgRNA in mouse ES cells as described above (**Ion channel tagging** subsection). Designing and validating potent sgRNAs is a routine procedure in the McManus lab.

Target gene prioritization. Our goal is to create double sgRNA CRISPR mice targeting ion channel genes and their interacting partners. As controls, we will include the singly targeted ion channels, allowing us to quantitate the degree of interaction between cohorts. Identification of biochemical and genetically interacting partners will be identified through bioinformatics and proteomic studies described at the end of the proposal. We have initiated a collaboration with the Monarch Initiative team (see letters) which will work closely with Bin Chen (Butte lab) to help prioritize targets for co-perturbation in ES cells and mouse models. The Monarch Initiative is an integrative data and analytic platform connecting phenotypes to genotypes across species²⁶. Both Bin Chen and Melissa Haendel (see letter) have analyzed all druggable genes under this IDG initiative and prioritized a list of immediate target gene pairs based on the culmination of data integrative approaches in line with the scope of the Monarch Initiative. Although we expect this prioritization list will change over time, our pipeline is in place. Our plan is to synergize the Monarch/TransMed team, the Butte team, and the IDG KMC to prioritize targets, and integrate proteomic, bioinformatic, and phenotypic data throughout the pipeline. With the NIH, KMC and other IDG members, we will align priorities with overall IDG objectives.

Mouse line phenotyping. The principal investigators of this proposal have collectively nearly 100 years of experience in phenotyping mouse models for human disease, and nearly 50 years of experience in the study of



ion channels in mouse models. Clearly this team is well suited to create and phenotype animals relevant to the goals of this ion channel initiative. As a major program endeavor is to phenotype mouse models for understudied ion channels in a high-throughput manner, we bring on board Dr. Kent Lloyd who heads the UCD Mouse Biology Program (MBP) high-throughput mutant mouse phenotyping. Dr. Lloyd brings deep knowledge in the production and phenotyping of animals and brings an established high-throughput phenotyping pipeline to our project. We will take advantage of Dr. Lloyd’s high-throughput phenotyping program for male and female cohorts of juvenile early adult homozygous mice (**Figure 6**, top half) and for late adult mice (**Figure 6**, bottom half). Embryo lethal and subviable mouse lines are phenotyped as embryos during development (**Figure 7**).

During the first phase of his phenotyping project, Dr. Lloyd's center completed phenotyping of 833 mutant lines and this number is growing at a rate of ~7 lines/week plus controls, a rate exceeding that needed to complete 1,050 mutant lines within their 5-year project. Their Consortium can phenotype all of the proposed number of lines without significant expansion or modification of their existing and currently active phenotyping pipelines. Our goal is to phenotype ion channel CRISPRi mice at a rate of ~100 mice/month. These mice will be created in their mouse facility and/or our UCSF mouse facility. A few solvable strategic issues will need to be resolved, namely courier by mouse truck (UCD is an hour away) and barrier (the UCSF and UCD barrier facilities operate at a similarly high level of pathogen exclusion to ensure the prevention of adventitious disease). We do not anticipate problems, as UCD handles these strategic issues regularly during the course of their collaborative endeavors with other institutions. Nonetheless, if problems arise, all double mice will be produced at the UCD facility, using our technologies and the protocols described above. With regards to data transfer, both UCD and UCSF have high standard LIMS systems for data capture, management and QC, and are well positioned to maintain and continue to support high quality phenotyping. Data will be shared on a daily to weekly basis, led by Dr. Bin Chen in the Butte lab.

Phenotyping pipeline. Phenotyping will be conducted in two phases. The first phase will be the juvenile for the young adult (4-16 weeks of age), and embryo for the embryo lethal/neonatal subviable.

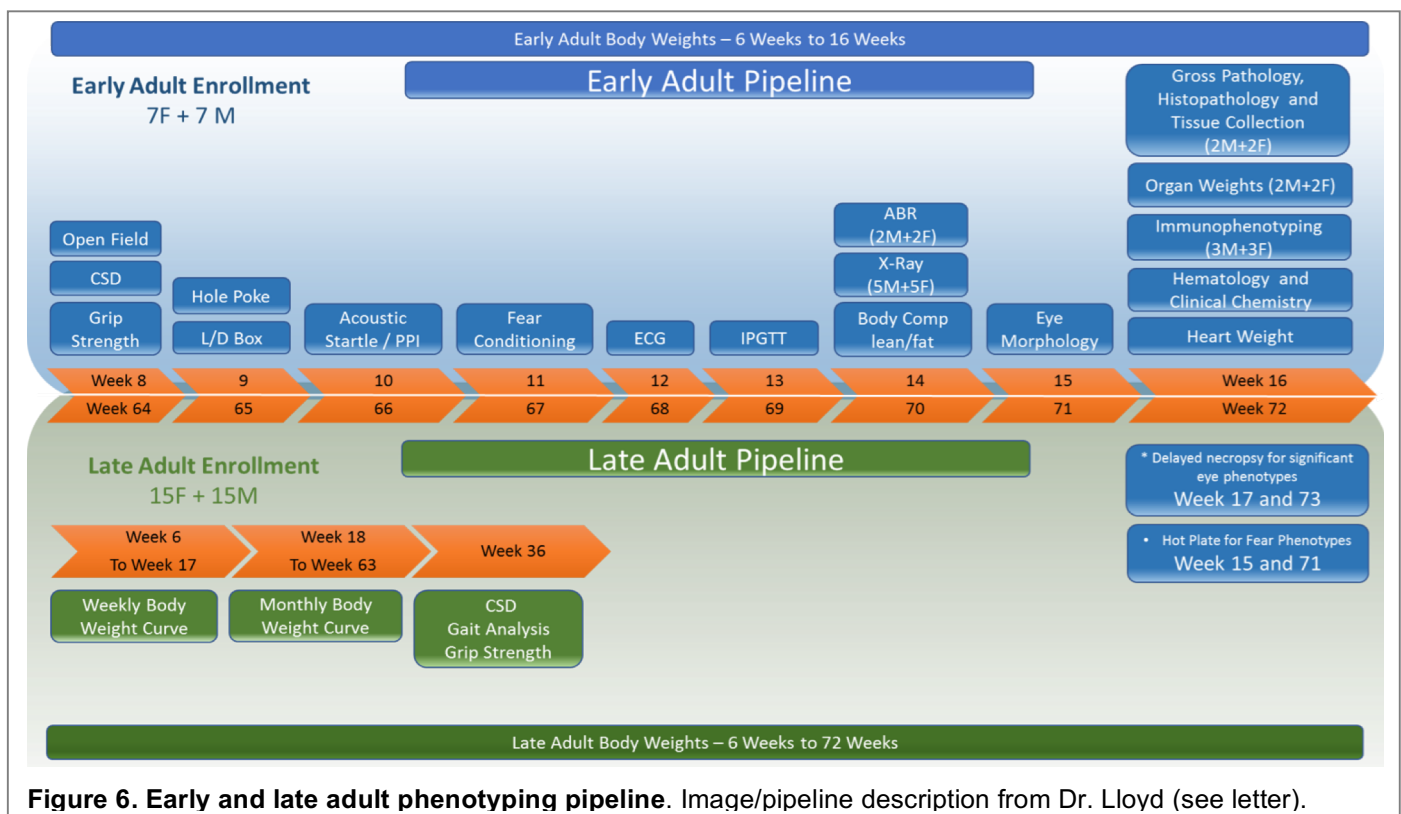
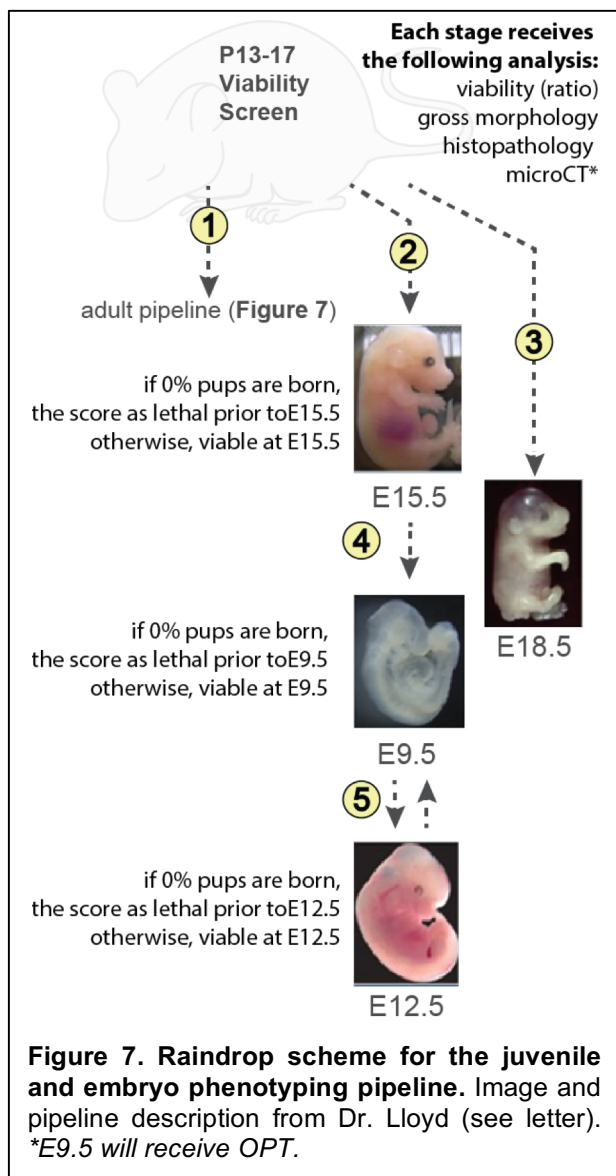


Figure 6. Early and late adult phenotyping pipeline. Image/pipeline description from Dr. Lloyd (see letter).

Juvenile Phenotyping. Figure 6 describes the basic tests we will do, although we may add additional tests or modify/drop tests as necessary to achieve the overall goals of the program. The SOPs we will follow are described at IMPReSS (www.mousephenotype.org/impress). Open field, electrocardiogram (ECG), juvenile LacZ staining and insulin blood level are all available in the pipeline. In addition, the juvenile pipeline includes a cognition (learning and memory) test and fear conditioning. UCD will equip and implement this test and use it on all juvenile mice. All equipment and personnel are in place to complete all of these tests. These tests will be complemented by more specific tests conducted by the Basbaum, Ptacek, and Jan labs.

Adult Phenotyping. In phase 2 we plan to enroll all of the 118 CRISPRi ion channel lines into the Late Adult Pipeline. There is a substantial body of scientific literature showing that many human diseases, including hypertension and cardiovascular disease, Type 2 Diabetes, cancer, and degenerative diseases (e.g. musculoskeletal, neurological) increase in prevalence with aging. The high-throughput ENU aging phenotyping screen at MRC Harwell in the UK has found that 30% more phenotypes were detected at 1 year compared with 4 months of age²⁷. In addition to detecting new late onset phenotypes revealed by aging, the Adult Pipeline will serve to assess the replicability of the phenotypes detected in the same lines as juveniles. The adult pipeline is depicted with respect to the juvenile pipeline in Figure 6.

We will produce and enroll 12 male and 12 female mice per line into the Juvenile and Adult Pipeline. When all 24 mice complete the in-life portion of the Juvenile Pipeline as described above, 3 males and 3 female mutants will be euthanized for necropsy, tissue collection and fixation for histopathology, harvesting spleens for flow cytometry, and whole blood collection for the clinical blood chemistry and hematology tests. Besides three mice of each sex will be used for necropsy, flow cytometry, and fixation for histology for the Juvenile Pipeline. We will collect blood from the 9 surviving mice of each sex for clinical blood chemistry and hematology. Animal Care guidelines allow taking blood volume equivalent to 1% of body weight every 2 weeks. We will draw venous blood at week 16 and week 18 for surviving mutants (and WT mice) to have enough plasma and whole blood to complete the required clinical blood chemistry and hematology tests for the mice that will be aged and meet the sample number requirements for statistical analysis of these test results at 16 weeks. To this end we have included a letter of support from the UCD Metabolomics center (Dr. Oliver Fiehn, see letter).



The 9 male and 9 female mice in the Adult Pipeline will be weighed monthly, visually checked daily by animal care staff, and their health status assessed during routine cage changing. The extra 2 mice of each sex enrolled in the Adult Pipeline should ensure we have the minimum required animals (7M and 7F) at 52 weeks of age to repeat the 8-week panel of phenotyping tests. If 2 of each sex, or 4 or more of one sex, of mice per line die or are euthanized as per our IACUC mandated guidelines prior to 52 weeks of age, then the entire line will be entered immediately into the Alternate pipeline. Between 52 and 59 weeks of age, the mice will go through the entire 8-week panel of mandatory tests. We will also perform histopathology on 2 males and 2 female mice from ALL lines completing the Adult Pipeline to maximize the opportunity to detect abnormalities not detected by the clinical tests in these valuable lines.

In terms of controls, WT littermates (2 male and 2 female) will be enrolled for each ion channel line in the Adult Pipeline. This will be in addition to the control mice that are processed and euthanized at the end of the Juvenile Pipeline. We used the method described by Bate and Karp²⁸ and calculated the optimum number of reference controls for a study design with many experimental groups, in this case the ion channel CRISPRi lines, compared against a common control.

Pipeline considerations. Part of the analysis for inclusion/exclusion of tests is a cost-benefit evaluation where cost includes not just the reagent or equipment costs, but also personnel time. The benefits included robustness of the data and ability to detect mutant effects. There are some short-comings in the existing UCD pipeline, including the lack of cancer-related outcomes. However, we bring on board Alan Balmain who fills this void and has run a mouse cancer program for decades (described below). However, overall, when viewed from the perspective that this is a high-throughput multi-system screen designed to produce reliable

and reproducible phenotype data, the resulting pipeline is economical, well-reasoned, efficient, and generates a significant amount of new gene-function information. This is the primary reason we chose Dr. Kent Lloyd as a key collaborator. If we were to set up this pipeline de novo, we estimate the expected cost would be 216% higher, not to mention the delays due to setup time and training of personnel.

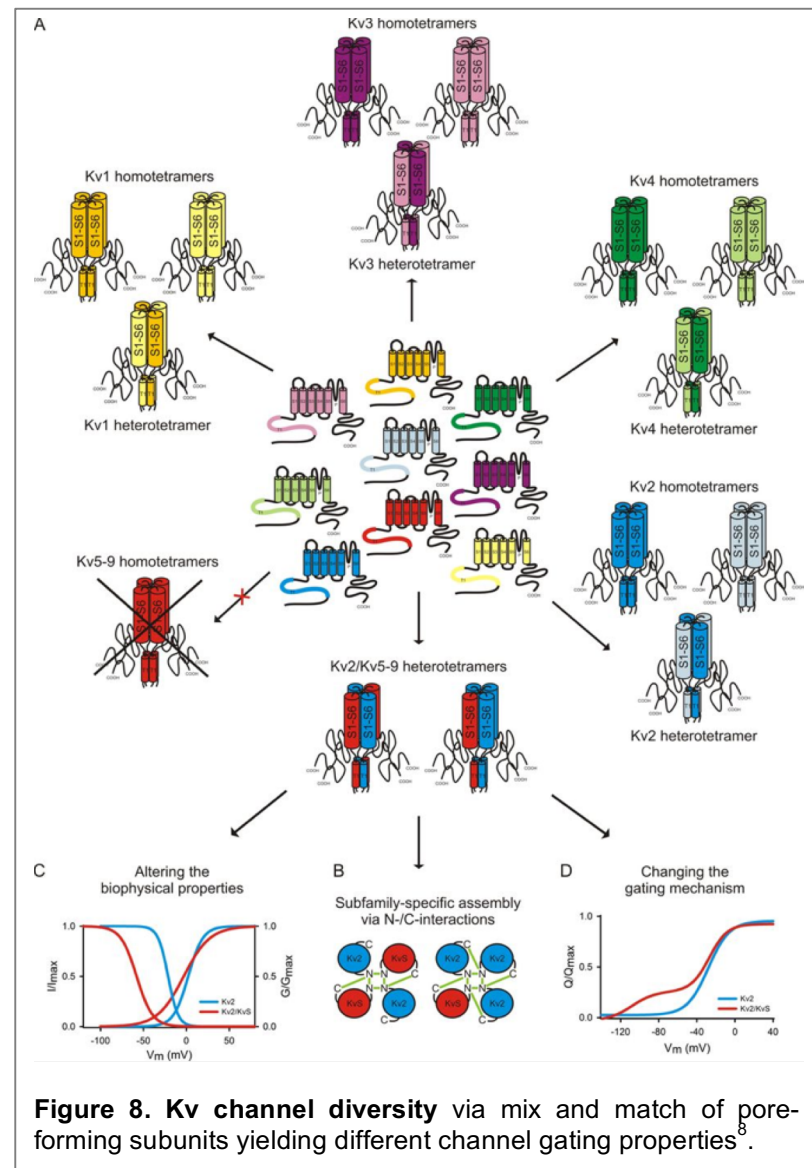
Additional opportunities. In addition to the above described pipeline, we may conduct a proteomic analysis of a subset of mice, prioritizing those having defined phenotypes first. Dr. Christoph Borchers (CentreUVic/Genome BC Proteomics Center) is a collaborator of Dr. Lloyd (see letter) and has developed multiplexed human and mouse proteomics kits for precise, highly sensitive quantitation of 500 proteins in mouse plasma, and for 324 proteins across mouse heart, lung, and brain tissue²⁹. We propose to use these

assays for our juvenile and adult phenotyping pipelines using 3 male and 3 female mouse lines, euthanized for tissue collection at weeks 16 and 59. These experiments will be conducted with the Lloyd and Krogan groups, although we may ask Dr. Borchers to assist if needed. Mass spectrometry based biomarker discovery, verification, and validation is a high impact and developing area and Dr. Borchers kits will facilitate phenotypes discovery in these models. To expand our phenotyping to lipids, primary metabolites, biogenic amines, bile acids, eicosanoids, and very small fatty acid, we have enlisted support of the UCD metabolomics core (Dr. Oliver Fiehn, see letter). As per Dr. Lloyd and his interactions with Dr. Borchers, these technologies will deliver an -Omics approach to phenotyping that is cost effective, highly informative, novel, compatible with the established pipeline, and reliable for differentiating 'normal' from 'abnormal' and help prioritize which animals to follow up with additional phenotyping analysis.

Additional phenotyping. Drs. Jan, Basbaum, Ptacek, and Balmain bring diverse advanced phenotyping skills that most closely relate to known ion channel health measures. Their groups bring a strong ion channel expertise to the program which are highly relevant to the double ion channel perturbation strategies. Dr. Lily Jan is studies the functional relationship between ion channels from large and diverse families that can serve redundant functions^{9,10}. Because disrupting one channel function may induce compensatory changes of other

channels¹¹⁻¹⁴, it is more likely to detect mutant phenotypes due to reduction of the activities of two channel genes at once, when compared to the traditional approach of mutating one channel gene at a time. Take voltage-gated potassium (Kv) channels as an example, there are 40 human genes encoding Kv subunits¹⁰ including 15 understudied channel genes (KCNAB6/Kv1.6, KCNA7/Kv1.7, KCND1/Kv4.1, KCNG2/Kv6.2, KCNG3/Kv6.3, KCNG4/Kv6.4, KCNV1/Kv8.1, KCNS1/Kv9.1, KCNS2/Kv9.2, KCNS3/Kv9.3, KCNH6/Kv11.2, KCNH7/Kv11.3, KCNH8/Kv12.1, KCNH4/Kv12.3). Kv channel density and function may be modulated by association with subunits such as KCNAB2 and KCNAB3 for Kv1 channels and KCNIP1 and KCNIP4 for Kv4 channels³⁰ – all four are understudied. Homo- or hetero-tetramers assembled by members within each of the Kv1/2/3/4/7/10/11/12 subfamilies and hetero-tetramers formed by Kv2 and Kv5-9 subfamily members are functional; different channel compositions confer overlapping channel functionalities (Figure 8)⁸. Not surprisingly, these channels may have redundant functions or provide compensatory activities. This project is ideally suited to disentangle these effects in a quantitative manner.

Indeed, ablating KCND2/Kv4.2 gene causes compensatory increases of other potassium channels in cortical pyramidal neurons¹² and preoptic GABAergic neurons^{13,14} with behavioral consequences. Likewise, ablating TASK1 in the two-pore domain potassium



channel family containing KCNK4/7/12/16, which are under studied, leads to up-regulation of channels in the GABA_A receptor family including GABRA5/6, GABRB1, GABRG1/3, GABRP and GABRR1/3 that are also under studied. These findings underscore the importance of co-ablating multiple channel genes to scrutinize overlapping functions. Roughly half of the under studied channel genes for this proposed study correspond to potassium channels and chloride channels for inhibition of neuronal excitability whereas others such as

calcium channels and acetylcholine receptors are excitatory, though enhancing excitability of excitatory neurons could have effects opposite to that of enhancing excitability of inhibitory neurons. Such considerations are incorporated in our experimental design.

Dr. Jan has significant experience in performing electrophysiological measurements of channel functions in the hippocampus (synaptic plasticity and modulation³¹⁻³³) and hypothalamus (energy and thermal homeostasis^{34,35}). We will compare neurons in brain slices from CRISPR and sibling wildtype mice, and

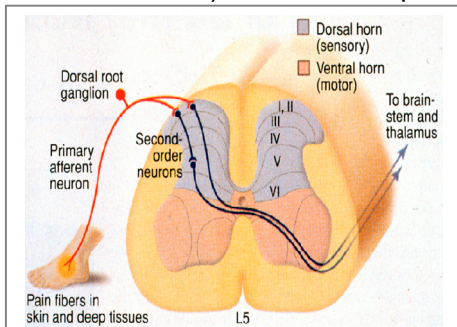


Figure 9. Afferent input to the spinal cord and a nociceptor cell body in the dorsal root ganglion (DRG). See text.

specifically analyze the threshold, waveform, and firing patterns of action potentials— as well as modulation and plasticity of synaptic potentials. This will provide a more granular measure of channel loss-of-function phenotypes and yield mechanistic data. Electrophysiological studies will be guided by the behavioral phenotypes characterized by Dr. Lloyd and others. The choice of brain regions will also be guided by channel expression patterns as revealed by examining mice with GFP11 fusion channels. With these lines, it will be possible to survey channel expression globally and then examine channel expression in subcellular compartments such as the axon or dendrites of a specific neuronal type. Based on Dr. Jan’s experience, a readily detectable fluorescent reporter for channel expression is invaluable in highlighting brain regions with high channel expression – even for small nuclei not typically ‘on the radar’ for expression surveys.

Pain and itch. Dr. Basbaum is an expert in the relationship between ion channels and pain and itch. There are two major chronic pain conditions, nociceptive pain produced by tissue injury and neuropathic pain following nerve damage. When there is tissue injury nociceptors, which are the primary sensory neurons that normally respond only to noxious, pain provoking mechanical, thermal and chemical stimuli, are sensitized. Now even innocuous stimuli, such as light touch, movement of the limbs or exposure to warm or cool temperatures can provoke intense pain.

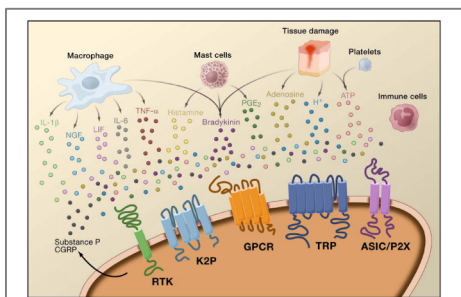


Figure 10. cyclooxygenase-mediated production of prostaglandins that act directly on nociceptors. See text.

Figure 9 illustrates afferent input to the spinal cord and a nociceptor cell body in the dorsal root ganglion (DRG). Although cyclooxygenase-mediated production of prostaglandins that act directly on nociceptors and lower their activation threshold is a major contributor to this peripheral sensitization process, recent studies have implicated a host of immune cell-derived mediators (**Figure 9**). Even pH changes that occur in the biochemical milieu of inflammation can dramatically alter the properties of the nociceptor. Unfortunately, to date only a very limited number of targets through which nociceptors are activated and sensitized have been identified. Many of these, e.g. voltage gated sodium and calcium channels, acid sensing ion channels and a variety of neurotransmitter receptors (e.g. 5HT) are members of very large

families. The understudied genes include many members of these families. The Basbaum laboratory will study the consequence of deletion of these channels on pain processing, using a battery of behavioral assays. The battery includes tests of acute mechanical and heat pain, using the von Frey and Hargreaves’ (**Figure 11**) tests,

respectively. He also uses these tests to study tissue injury-induced hypersensitivity and will routinely test motor function using the rotarod apparatus (**Figure 11**).

In contrast to nociceptive/inflammatory pain, neuropathic pain (e.g. postherpetic neuralgia diabetic neuropathy) are largely unresponsive to opioids and non-steroidal anti-inflammatory drugs.

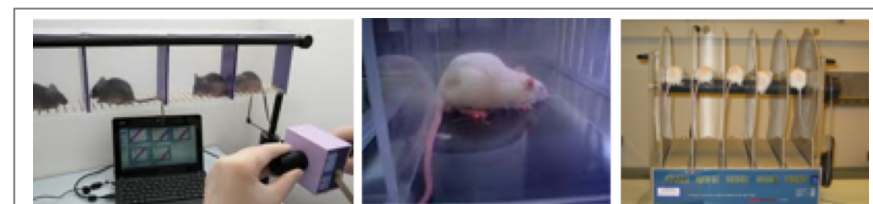


Figure 11. Principle tests to measure acute mechanical and heat pain. von Frey (left), Hargreaves’ (center, and rotarod (right) test.

When there is neuropathic pain, the biology of the sensory neurons, whose peripheral axons have been damaged, undergoes dramatic changes, with a host of new genes induced. In fact, up 1200 genes have been identified as being altered after nerve injury, but very few of these genes have been studied, and even fewer implicated in the generation and persistence of neuropathic pain. Here again the Basbaum laboratory is ideally positioned to determine the extent to which these minimally studied channels

contribute to the development of a major chronic pain condition. His laboratory has published extensively using preclinical traumatic injury and chemotherapy-induced neuropathic pain models. Of particular interest is the profound nerve injury-induced mechanical hypersensitivity that results from inappropriate engagement of pain circuits by sensory neurons that normally respond to innocuous stimulation. Here the hypersensitivity is mediated by a central sensitization process in which pain transmission circuits in the spinal cord (and at higher levels of the neuraxis) are hyperactive, in a manner comparable to the hyperactivity that occurs in epileptic cortex. Identifying novel channels that come into play during the central sensitization processes is an important step in the development of novel pharmacotherapies for the management of chronic pain.

Sleep and ion channels share established phenotypic interconnections but are not well explored, particularly for the understudied ion channels and their redundancies and relationships to other gens. Sleep and its circadian alignment to the solar day is critical for health. Disruption of sleep leads to increased risks of many cancers, autoimmune disorders, psychiatric disorders, metabolic disorders, etc. The circadian clock is a cell autonomous molecular feedback loop which regulates timing of many physiological processes. A great deal has been learned about the clock through genetic experiments in flies, mice, and humans. Sleep homeostasis is a related but distinct phenomenon that allows us to override the circadian clock, for example, after periods of sleep deprivation. Little is known about the genetic and molecular basis for sleep. However, it is clear that circadian clock function and sleep, both phenotypes emanating from the brain, must ultimately be transduced as electrical signals to effect behaviors. Given the growing recognition of the role of channels in sleep behavior³⁶⁻⁴⁰, we will phenotype mice from the collection for measures of both circadian and sleep behaviors. We will record running wheel behavior in both 12 hr. light:12 hr. dark (LD 12:12) cycles and under constant darkness (DD). This will allow us to calculate circadian period (τ) and the period of activity during each 24 hour day (α) in each mouse. We can also use additional testing such as EEG monitoring to study sleep and sleep architecture in mutants of particular interest⁴¹⁻⁴⁵.

Protocol for mouse behavioral phenotyping. Wheel-running is a gold standard test to assess voluntary activity and circadian behavior of mouse. This system is not invasive and not labor intensive, and thus quite effective for screening. 8-10 week-old male mice of knockout mice and wild-type littermates will be individually housed in a cage equipped with a wheel with free access to food and water. First, mice will be kept in light:dark 12hr:12hr condition for entrainment for at least 1 week and then released to constant darkness. 2 weeks after constant darkness, mice will be given by 30-min (usually 15 min to 60 min) light pulse at subjective early night to introduce a phase shift of behavioral rhythms. 2 weeks after the first light pulse, mice will be given a second light pulse at subjective late night. Mouse behavior will be continuously recorded for all experimental paradigms using Clocklab (Actimetrics). Basic circadian phenotype listed below will be evaluated. Daily activity and activity profile (phase angle of behavioral rhythms) will be determined wheel-running counts. Alpha (active period), rho (inactive/resting period) and response to light pulse (phase-shift) will be determined by daily activity onset and offset. Chi-square periodogram analysis provides information of amplitude of behavioral rhythms and free-running period in constant darkness.

Cancer studies. The cancer therapeutic landscape has been changed by the development of targeted drugs that inhibit only specific gene products that act as cancer drivers. Although these drugs have increased patient survival, benefit is usually transient, and each drug in isolation rarely results in long term responses or cures. In almost all cases, patients relapse due to drug resistance resulting from mutation of the target gene or rewiring of signaling pathways. A priority in cancer research is therefore to develop approaches to prediction of rational combinations of drugs that may circumvent development of resistance.

It has been suggested that cancer might, in fact, be loosely termed a channelopathy⁴⁶, playing important roles in cell proliferation, migration, apoptosis and differentiation. Analysis of the transcriptional networks and mutational landscapes for individual target genes will allow us to identify networks of genes that function in the same or similar pathways, providing an opportunity to identify combinations of “druggable” genes that when inhibited have a higher probability of leading to network collapse, and consequently to greater therapeutic effect. The Balmain laboratory has developed network analysis tools to help understand cancer susceptibility and tumor signaling, and have recently applied similar approaches to analysis of the transcriptional networks surrounding target genes during the evolution of metastasis. This includes inactivating or activating these genes in normal and tumor cell lines and in mice, using CRISPR approaches based on transgenic mice or viral delivered sgRNAs. These are standard approaches in the Balmain lab. Any large population of aging mice will exhibit tumors naturally, and we anticipate this as a very real possibility for the understudied channels. For this reason we will monitor each animal during the course of phenotyping, examining for cancer development. Depending on the proteomic and bioinformatic data, we may mate animals to tumor sensitizers including tumor

suppressors, or introduce sgRNAs against these genes using lentiviral transduction. It is difficult to propose a specific course of action without having the details but we have the tools and expertise to steer the research in the appropriate directions.

A large number of ion channels can be found as recurrently mutated in human cancers, including the understudied ion channels. However scant data illuminates their role(s) in cancer despite the signs that point to functional roles. Moreover, the understudied ion channels have very little information about their functions in normal tissue or disease states. We will work closely with Bin Chen in the Butte lab, along with Monarch and IDG team members to carry out network analysis of these genes in a range of normal tissues for which large scale data sets are already available from our mouse backcross populations. Tissues for which data are already available include mammary gland, lung, dorsal and tail skin, and brain. We will apply this same approach broadly to identify potential functions of the understudied ion channels in multiple normal tissues, and interrogate the changes in network architecture that take place during development of benign and malignant tumors. We anticipate that this approach will lead to the functional annotation of many of these unknown genes, and may identify cancer target genes that can be used in combination with each other, or with existing approved drugs, for cancer therapy.

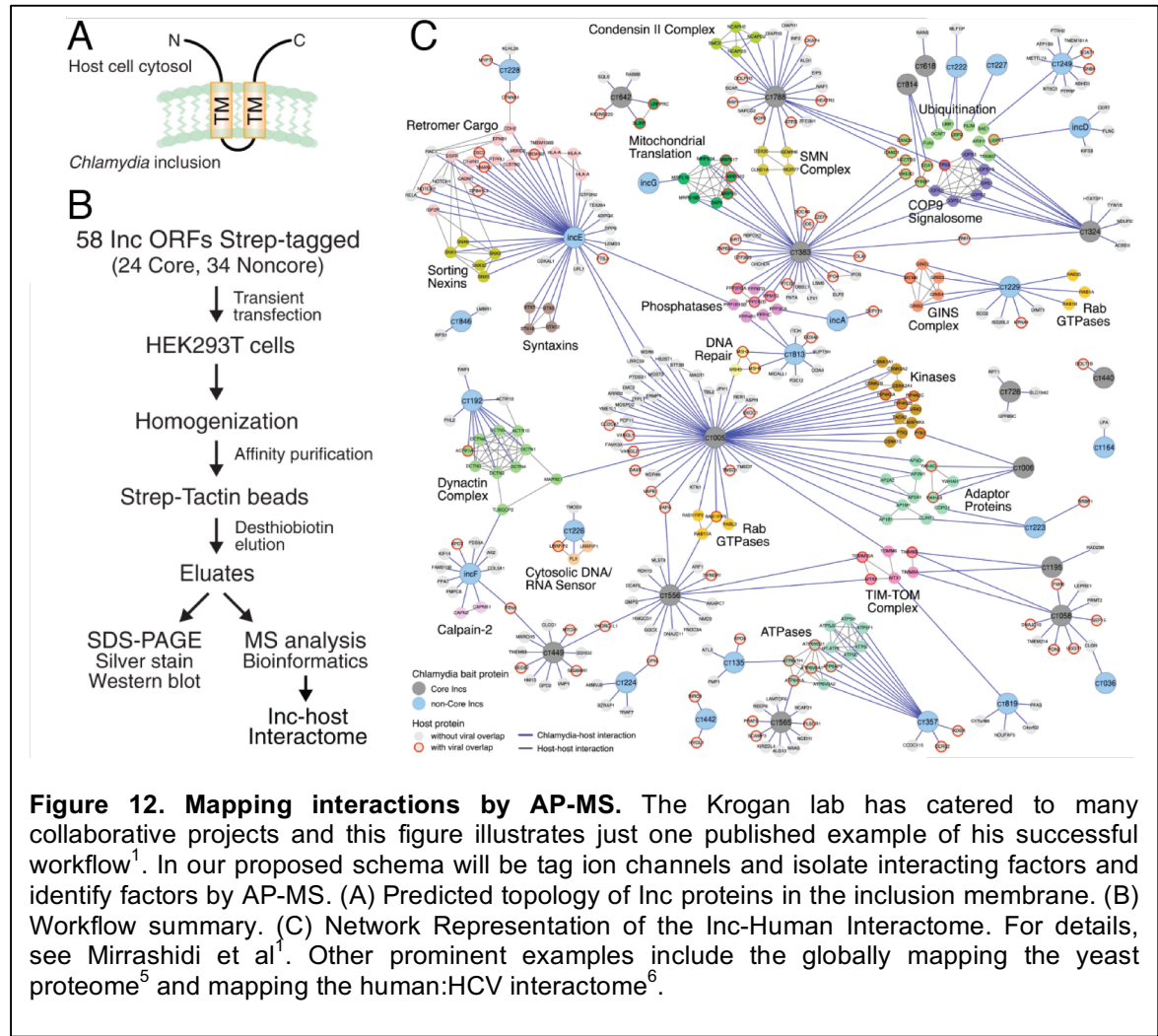
Aim 2: Capture the compendium of understudied ion channel proteomic interactions.

Ion channels are 'more than a pore' as they exist in multi-protein complexes, often exhibiting interaction with a variety of signaling and scaffolding proteins. These protein:protein interactions influence ion channel function, and many ion channel functions are largely independent of their ion-conducting roles which may include interactions with other genes⁴⁷. Analysis of the ion channel interactome has yielded new insights into the composition of ion channel complexes yet large numbers of ion channels remain understudied and little is known about the protein composition and regulatory apparatus. This Aim will be executed in parallel with the first Aim, and although both Aims are not dependent on each other, they are nearly inextricably linked given proteomics perfectly complements the genetics and phenotyping approaches.

The channels studied in this project are likely to display a range of interactions with both scaffolding and substrate proteins. We have enlisted a collaboration with Dr. Nevan Krogan (UCSF) who has excellent experience to help us identify the compendium of understudied ion channels. Dr. Krogan led a project that systematically identified protein complexes in the model organism, *Saccharomyces cerevisiae*, through an affinity tagging-purification/mass spectrometry strategy (AP-MS). This work led to the characterization of 547 complexes, comprising over 4000 proteins, and represents the most comprehensive protein-protein interaction map to date in any organism. His systems are scalable and more than capable for identifying the proteomic interactions for 118 understudied ion channel genes. We leverage his AP-MS technology to enable an unbiased systematic proteomic analysis to map targetable understudied ion channel relationships to human disease using cell lines and mouse models. In this proposal, we use mouse embryonic stem cells as a workhorse cell line, given their value in functional studies including differentiation, and importantly they can derive transgenic mice as a backup. That said, we have the bandwidth and capability to grow, genetically modify, record action potentials, and conduct the full suite of biochemical and phenotypic analysis on nearly any cell line and will remain flexible to the needs of this program. It is worth noting that ion channels are abundantly expressed in embryonic stem cells, and their differentiated progeny opens up many directions for downstream proteomic analysis.

With Dr. Krogan we will assimilate and slightly modify a standard workflow that he has used successfully in many projects, where ion channels will be tagged and affinity purified for mass spec detection (**Figure 12**). For strategic reasons we prefer to use the CRISPR epitope knockin strategy developed by Dr. Bo Huang lab^{17,18} (UCSF, see letter), and reserve Strep-Tactin based strategies only if challenges exist for targeting the specific ion channel allele. Given that Dr. Huang's knockin strategy allows us to study expression in live cells, it is a preferred embodiment for the studies in Aim 2, where we trace native ion channel expression in primary tissues. This approach allows direct monitoring of ion channel expression in cell lines during CRISPRi and CRISPRa strategies. This will save time and costs compared to antibody based detection. Thus, building upon Dr. Krogan's existing high-throughput collaborator and existing scalable platforms, we are readily equipped for the proposed targeted ion channel PPI aim. Data emanating from his MS pipeline will be analyzed by his staff, in collaboration with Dr. Bin Chen. The Chen/Butte team will work with investigators on this grant, the NIH, and the IDG Knowledge Management Center to prioritize channels for further study.

Proteomic analysis of CRISPR repression of tagged cell lines. To rapidly develop a robust data resource characterizing the cellular effects of the understudied channelome, we will employ precise, flexible genetic models and well-proven proteomic approaches using the established workflows. These experiments will use CRISPR technology; which brings in the *S. pyogenes* CRISPR Cas9 technologies expertise of Dr. McManus who was an investigator in the first phase of the IDG initiative. He will contribute his expertise in CRISPR technologies and has significant experience using *S. pyogenes* CRISPR Cas9. Using validated sgRNAs against each understudied channel, we will generate stable CRISPR mouse ES cell lines with each sgRNA in the epitope tagged cell lines. Dr. Gordan in the Krogan lab (see Krogan letter) has provided us with their



standardized workflow text using our tagged cell lines: after cell lysis, anti-epitope tag antibody beads will be used to enriched protein complexes associated with the tagged channel. Lysates will then be digested into peptides and analyzed with the mass spectrometry and computation approaches above. Their group developed the MiST (Mass Spectrometry Interaction Statistics) software, which will be used to assign relative confidence to individual understudied

channel protein-protein interactions and negative control (i.e., CRISPRi line). IPs will be used for each condition to further assess potential false-positive interactions⁴⁸. The Krogan Lab has applied this standard operating procedure to uncover interactions with other membrane proteins (**Figure 12**).

The proteomic approaches described above will provide a comprehensive resource of tangible data describing the signaling output of poorly understood channels. Based on input from other members of the organizing center, further targets will be identified for detailed characterization that may aid in the interpretation and mechanistic analysis planned for later aims. Guided by potential disease association, subtype classification, and signaling network information, we will prioritize targets in a resource generating pipeline. This pipeline includes characterization of cell- and tissue-specific protein expression patterns in native environments and antibody production when needed. As these data and reagents are generated and validated, the Butte lab will work with team members to channel these entities to the RDOC during the course of the project. In this way, resources will become available during the entire course of the project.