Discovery of new targets for therapeutic interventions in ARSACS disease

Introduction. Autosomal-recessive spastic ataxia of Charlevoix-Saguenay (ARSACS), a neurological disorder first described during the late 1970s within the French-Canadian population, is the second most common form of recessive ataxia worldwide[1–3]. In the Charlievoix and Sanguenay-Lac Saint Jean regions of Quebec, Canada, the carrier frequency of the pathogenic c.8844delT SACS mutation was estimated to be 1:22 while the disease incidence was 1:1,932[4]. Cases of ARSACS have since been reported worldwide in over 20 countries[3]. Although the clinical symptoms of ARSACS typically include early-onset and progressive cerebellar ataxia, lower-limb pyramidal spasticity, and peripheral sensorimotor neuropathy, increasing numbers of ARSACS patients with different SACS mutations demonstrate more varied symptoms[2,3]. *SACS*, the gene responsible for ARSACS (located on chromosome 13q12.12), encodes sacsin, a 4,579 amino acid protein that is expressed in the central nervous system and localizes to the cytoplasm and mitochondria of cells[5–7]. The exact function of sacsin remains unclear, with potential roles in protein degradation and chaperone assisted protein folding. Knockout/knockdown of sacsin in cell culture and mouse models demonstrate cellular phenotypes observed in ARSACS fibroblasts and lead to a number of cellular changes: mitochondrial abnormalities, disruption of neurofilament organization as well as defective autophagy[7–10]. Conversely, mitochondrial dysfunction and dysregulation of molecular chaperones, autophagy and neurofilament organization are connected to neurodegenerative diseases[11–15]. Indeed, the pivotal roles that molecular chaperones play in neurodegeneration are increasingly appreciated, specifically as key mediators of proteostasis in the ubiquitin-proteasome system, endoplasmic reticulum-associated degradation, and different autophagic pathways (including chaperone-mediated-, micro-, and macro-autophagy, and organelle-specific processes)[13].

To gain insights into the function of sacsin and identify potential protein targets for drug repurposing and development to treat ARSACS disease, we proposed to apply unbiased proteomic approaches to uncover the proteins interacting with sacsin protein and the signaling networks (i.e. post-translational modifications) affected by deletion of the SACS gene in brain cells (**Aim 1**). Next, we integrated these data together using cutting-edge computational approaches to identify the cellular signaling pathways and kinases regulated by the SACS gene (**Aim 2**). Lastly, we will use chemoinformatics to map the protein targets in these pathways to drugs and compounds for testing in cell culture assays (**Aim 3**). We will focus on drugs that are FDA-approved, as drug repurposing provides an expedited path from discovery to translational impact for this debilitating and poorly-studied condition. As a testament to our proteomics-chemoinformatics approach, our recent studies[16,17] have utilized proteomics to identify host cellular processes hijacked by SARS-CoV-2 and successfully translated this information to rationally select repurposed antiviral drugs and compounds that target these hijacked host processes, with at least one of these drugs now in clinical trials. Here, we apply a similar approach to ARSACS disease.

Aim 1: Map global landscape of sacsin protein complexes and signaling control.
We proposed to map the global regulation of post-translational modifications by sacsin **(Aim 1.1.)**. Post-translational modifications, including ubiquitination and phosphorylation, are important regulators of virtually all cellular processes, including autophagy, chaperone activity, neurofilament organization, and mitochondrial activity. Notably, several kinases have been directly linked to autophagy and mitochondrial function[18,19]. Phosphorylation-mediated signaling is mediated by protein kinases, which have been extensively used as drug targets due to their dysregulation in a wide variety of human diseases, including cancer, immunological, inflammatory, degenerative, metabolic, cardiovascular and infectious diseases. In addition, perturbation of kinases can directly regulate the ubiquitin system, as the two are highly intertwined[20].

Here, we performed proteome-wide quantitative profiling of PTMs associated with the loss of sacsin. Loss-of-function mutations are thought to be the primary cause of ARSACS; consistently, *SACS* knock-out mice recapitulate many of the clinical symptoms in ARSACS[7,9,21]. We therefore used SACS-/- cells. Specifically, we acquired SACS-/- HEK293 and SH-SY5Y neuroblastoma cells (a friendly gift from Justin M. Wolter, UNC at Chapel Hill) and validated the absence of sacsin by western blotting (**Fig. 1A**). We next performed global

proteomics to profile protein abundance, phosphorylation, and ubiquitination in WT vs SACS-/- cells in triplicate. Samples were processed using high-resolution data-independent acquisition (DIA) liquid chromatography (LC)-MS using label-free methods[22]. Peptide and protein identification, quantification, and phosphorylation site localization was performed using the Pulsar search engine integrated into Spectronaut[23] and fold-changes and p-values of SACS-/- compared to WT cells calculated using the MSstats statistical package[24]. From this analysis we quantified several thousand proteins, and modification sites and categorized them by their quantitative differences between WT and SACS-/- cells (**Fig. 1B, C**). To gain insights in the biological mechanisms underlying these differences in protein and modification sites regulation we selected protein/modification sites with |fold-change| >2.0 and with $p < 0.05$ and performed gene ontology enrichment analysis and KEGG pathway analysis to identify biological mechanisms that are differentially regulated between WT and SACS-/- HEK293 cells (**Fig. 2**). Global proteomic analysis is currently underway in WT and SACS-/- SH-SY5Y neural cell lines.

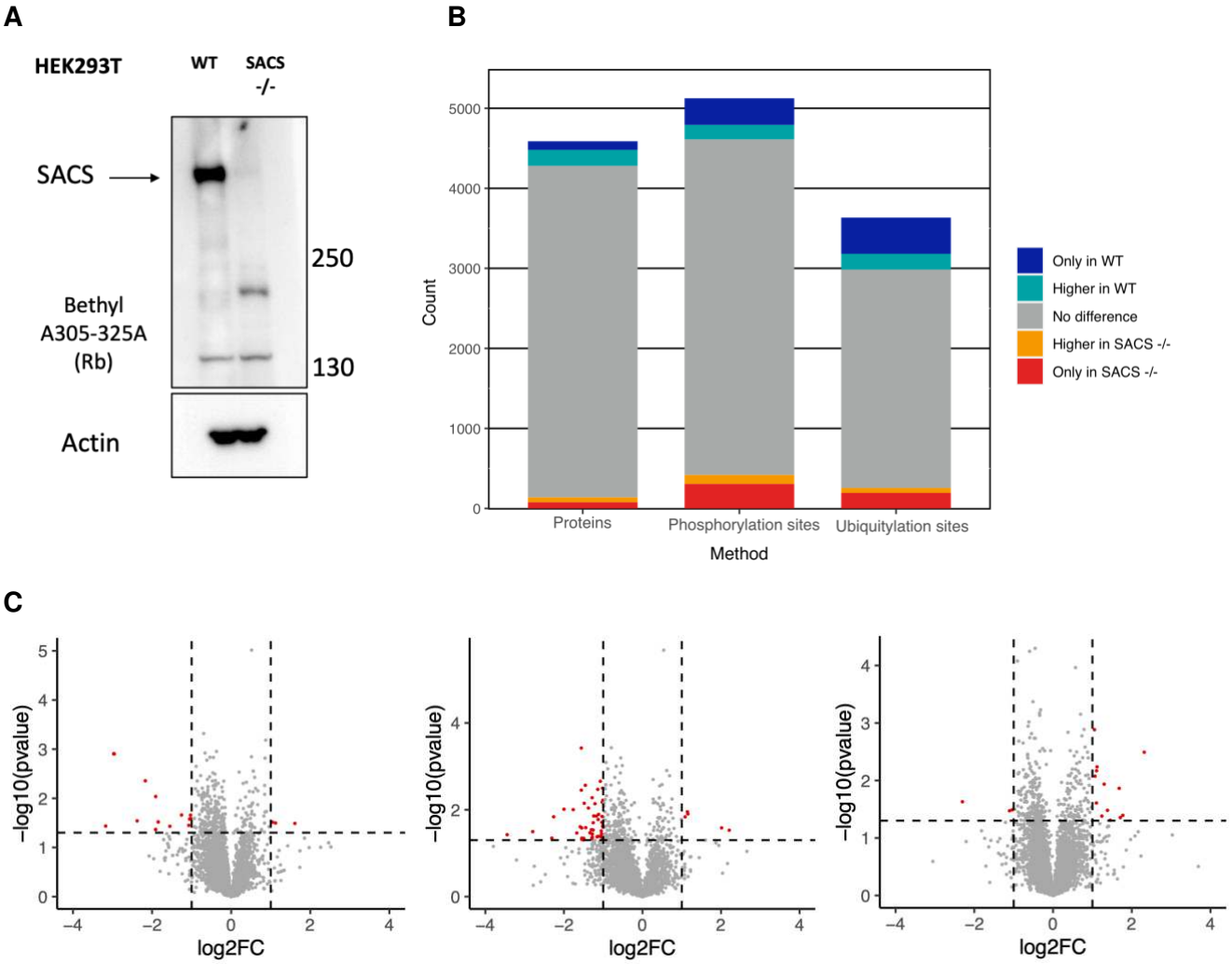

Figure 1. Validation of sacsin protein expression in WT or SACS -/- HEK293T cells. (A) The Bethyl A305-325A antibody was used for sacsin detection. (B) The number of quantified proteins, phosphorylation sites, and ubiquitylation sites detected in our global proteomics profiling experiment (from (C)). Each data type is categorized based on the

quantification between cell lines for proteins/sites that were detected in only one cell type, significantly higher in a given cell type ($p < 0.05$), or which displayed no quantitative difference between cell types ($p > 0.05$). (C) Volcano plots of protein abundance (left), phosphorylation (middle) and ubiquitylation (right) in WT vs SACS –/- HEK cells.

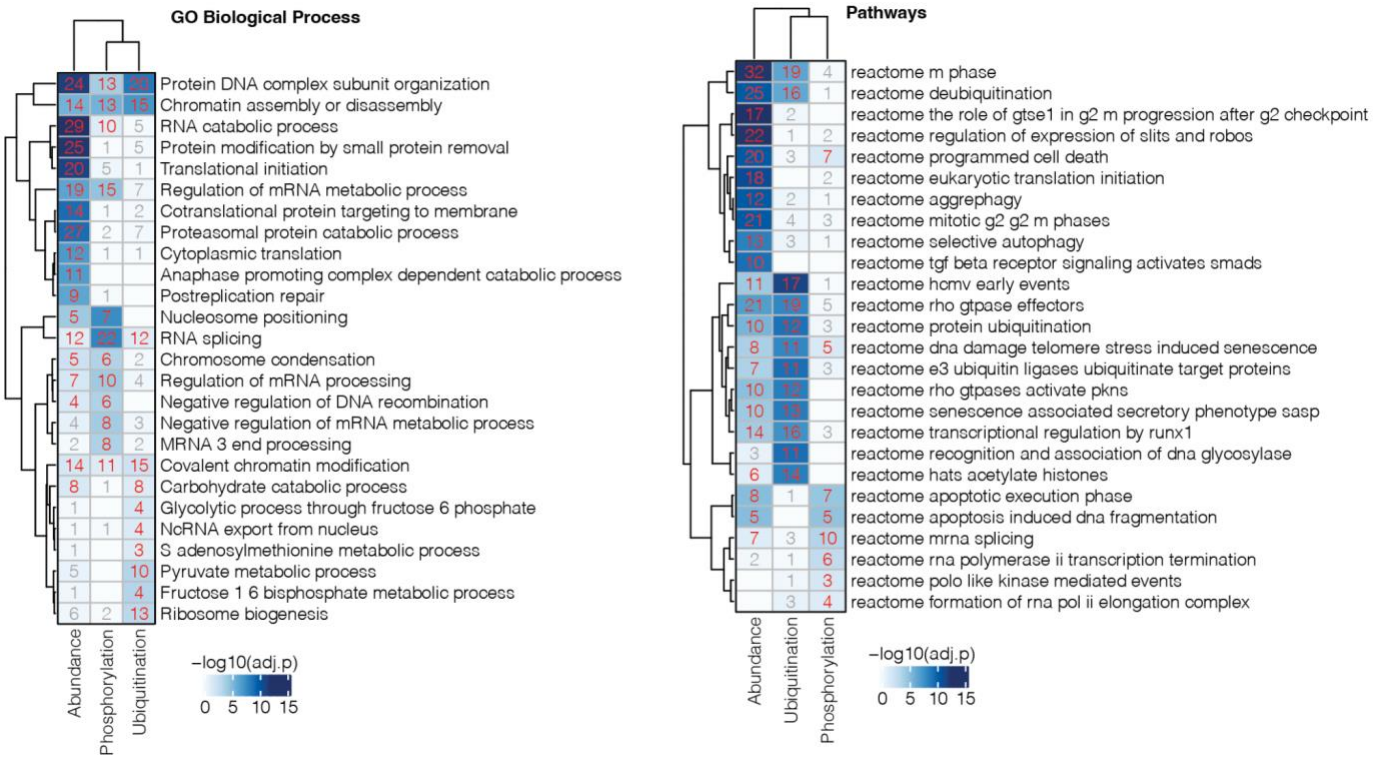

Figure 2. Gene ontology analysis of biological processes (left) and KEGG pathway analysis (right) of proteins that are differentially regulated between WT and SACS-/- cells. The number of differentially regulated proteins is indicated for each term in each data type, with red text indicating statistical significance.

From this analysis we find several ontology terms that point towards differential regulation of cell cycle regulation (e.g. reactome m phase), the global translation and degradation of proteins (e.g. translational initiation, proteasomal protein catabolic process), and cell death (e.g. reactome programmed cell death). These ontology terms will guide the future selection of functional assays to identify quantitative phenotypes distinguishing WT and SACS -/- cells, and can provide markers for experiments aimed at reversing the negative effects of sacsin loss of function.

To identify cellular mechanisms that underlie the perturbations to PTMs by SACS deletion (**Aim1.1**), we next mapped the protein-protein interaction (PPI) networks of sacsin in SH-SY5Y using affinity purification mass spectrometry (AP-MS). In parallel, AP-MS will be performed on HEK293T cells, the workhorse proteomic cell line extensively used in our laboratory[17,25–27]. First, we tested different commercially-available anti-sacsin antibody for endogenous immunoprecipitation followed by AP-MS. We used two different antibodies: Bethyl A305-325A and abcam ab181190. SH-SY5Y cells were resuspended in lysis buffer (50 mM Tris pH7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40 with Protease/phosphatase inhibitor mix) and further lysed by sonication (20% amplitude, 4x 15 sec pulse) on ice. The cleared lysates (from 3 million cells) were incubated with either antibody (10 ug) for 4 hrs at 4C and mixed with 20 uL of Protein A magnetic beads for overnight incubation at 4C. Beads were serially washed with a lysis buffer containing 0.1%, 0.05%, or 0.01% NP-40. Proteins bound to beads were

digested with LysC in 8M urea buffer for 2 hrs and with trypsin in 2M urea buffer overnight at 37C. Cleaved peptides were desalted with C18 column and analyzed by MS.

PPIs were scored against SACS-/- cells using the SAINT scoring pipeline[28] and high-confidence interacting proteins mapped to known protein complexes and pathways. From this experiment we detected 26 high-confidence PPIs (**Fig. 3**), and find that both antibodies resulted in detection of a highly similar set of PPIs, with some PPIs being only detected by one antibody (e.g. VDAC3 only detected by the Bethyl antibody). Gene ontology analysis revealed a significant enrichment for actin cytoskeleton proteins (9 proteins, FDR 1.2×10^{-5}) including DAPK1 and DAPK3, which are both known to regulate apoptosis and cytoskeletal reorganization, with DAPK3 also being involved in muscle contraction. Additionally, we detect mitochondrial porin proteins (VDAC1 and VDAC3, FDR 0.04), as well as NES which is required for proper brain and eye development. We will integrate these results with the global proteomics experiments described above to guide the selection of functional assays that differentiate SACS-/- from WT cells, likely focusing on measurements of cytoskeletal organization and mitochondrial function.

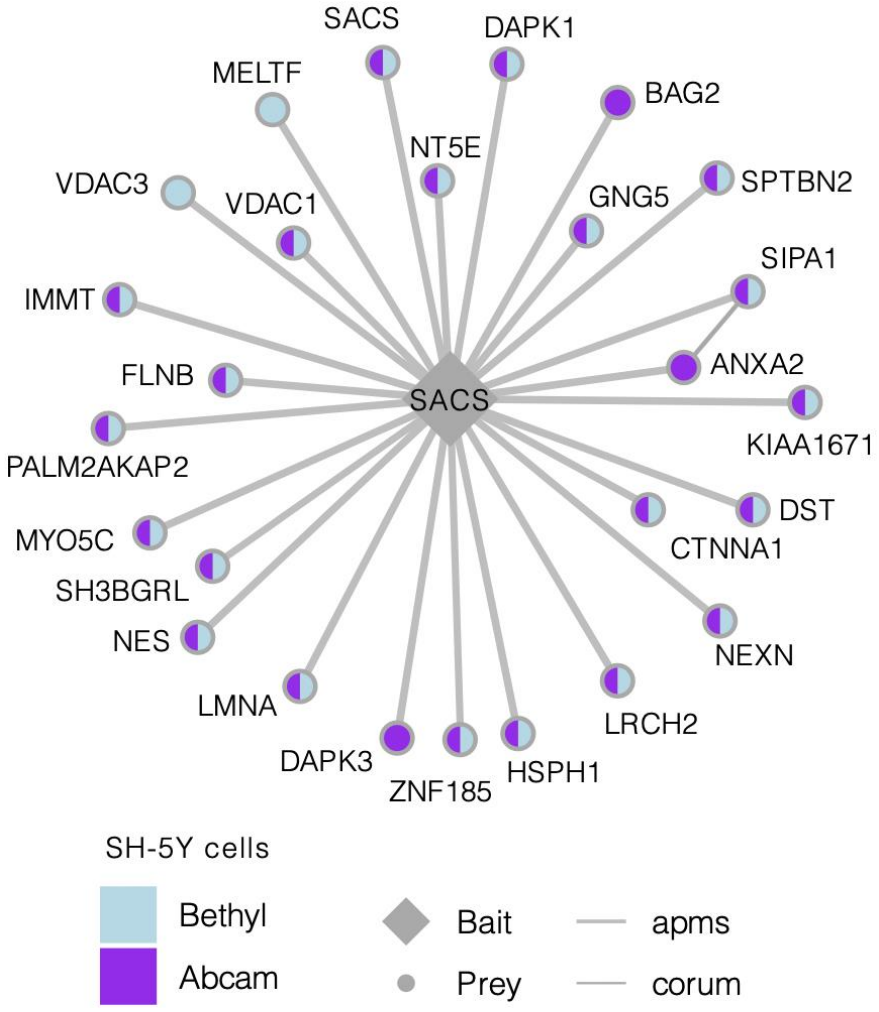

Figure 3. Proteins (preys) found to interact with sacsin (bait) from SH-SY5Y cells using two different antibodies (Bethyl or Abcam). Known connections between preys from protein complexes described in the CORUM database are also indicated.

Aim 2: Computationally identify kinases and pathways controlled by sacsin protein.

We proposed to use a bioinformatics-based statistical approach to quantify kinase activity[13,19] differences between the WT and SACS-/- cell lines (**Aim 2.1**) and delineate the molecular networks (i.e. "pathways") regulated by sacsin (**Aim 2.2.**). We first quantified differential kinase activity in WT and SACS-/- cells (**Aim 2.1**). Kinase activities were estimated using known kinase-substrate relationships[29]. The resource comprises of a comprehensive collection of phosphosite annotations of direct substrates of kinases obtained from six databases, PhosphoSitePlus, SIGNOR, HPRD, NCI-PID, Reactome, and the BEL Large Corpus, and using three text-mining tools, REACH, Sparser, and RLIMS-P. Kinase activities were inferred as a Z-score calculated using the mean log2FC of phosphorylated substrates for each kinase in terms of standard error (Z = [M - u] / SE), comparing fold changes in phosphosite measurements of the known substrates against the overall distribution of fold changes across the sample. A p-value was also calculated using a two-tailed Z-test method. This statistical approach has been previously shown to perform well at estimating kinase activities[30,31]. From this analysis we find a selection of kinases to be activated or inactivated in sacsin k.o. Cells (**Fig. 4**)

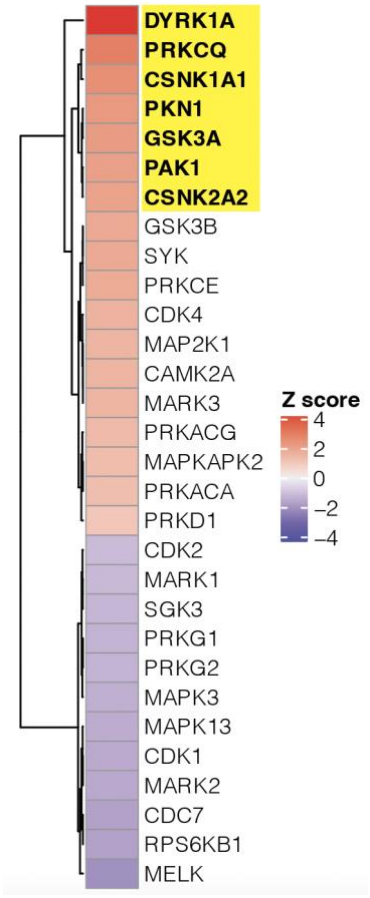

Figure 4. Analysis of changes to kinase activities in cells lacking sacsin as compared to healthy control cells. The Z score indicates the inactivation (blue) or activation (red) of kinases in SACS-/- cells. Kinases with significant changes in activation are highlighted in yellow.

The most highly activated kinase observed was DYRK1A (dual-specificity tyrosine phosphorylation regulated kinase 1A) is a member of the serine/threonine kinase family that is located in chromosome 21, a critical region for Down syndrome (DS). In neural progenitors DYRK1A is mostly localized to the nucleus and is crucial for cell cycle progression from G1 to S phase to produce the proper number of neurons in the developing neocortex. However, overexpression of DYRK1A promotes cell cycle exit and has inhibitory effects on cell proliferation and neuronal differentiation. In differentiated neurons, DYRK1A shifts its localization to dendrites and overexpression of DYRK1A in dendrites decreases dendritic growth, filopodia length and synapse formation. Phosphorylated substrates of DYRK1A mostly consist of transcription factors and chromatin regulators. Among those substrates, cAMP responsive element-binding protein 1 (CREB1), forkhead box protein O1 (FOXO/FKHR) and Histone H3 are involved in regulating gene expression by DYRK1A. Thus, phosphorylation of several proteins by DYRK1A is involved in diverse pathways such as cell differentiation and apoptosis, gene transcription, cytoskeleton organization and cell cycle regulation. Overexpression of DYRK1A contributes to the cognitive impairments in Down Syndrome and is involved in neurodegenerative diseases like Alzheimer's disease, Parkinson's diseases, Huntington's disease, and brain tumors. Therefore, DYRK1A is an attractive target to develop therapeutic drugs. One of the widely used DYRK1A inhibitors is the selective ATP competitive inhibitor Harmine. This β-carboline alkaloid inhibitor binds to DYRK1A ATP-binding pocket and potently inhibits DYRK1A activity. Another potent and relatively selective inhibitor, Leucettine L41, is able to cross the blood-brain barrier and reduces amyloid- beta induced oxidative stress and cell death. Consequently, L41 could prevent amyloid-beta induced memory impairment and neurotoxicity in the hippocampus, which is important for Alzheimer's disease treatment. Therefore, DYRK1A inhibitors could be evaluated for the treatment opportunities of ARSACS disease in the future. Additionally, we observe activation of CSNK1A1 and CSNK2A2, which are subunits of casein kinase 1 and casein kinase 2, respectively. The drug silmitasertib targets casein kinase 2 and is currently in clinical trials for the treatment of cancer and COVID-19.

In progress. We are currently integrating PTM and PPI data (**Aim 2.2**) to identify druggable targets (**Aim 3.1**) and evaluate compound efficacy in vitro (**Aim 3.2**). Functional assays will be established based on ontology terms identified in our proteomic data sets, likely focusing first on measurements of cytoskeletal organization and mitochondrial function with FDA-approved drugs being prioritized for evaluation of functional restoration.

Future Directions. The efficacy of identified drugs/compounds can be tested *in vivo* using sacsin$^{-/-}$ mice as a model for ARSACS with early onset of ataxia-like motor coordination abnormalities. Candidate drug efficiencies can be evaluated using coordination, balance and muscle strength tests along with histology analysis of cerebellar purkinje neurons, spinal motor neurons and calf muscles. Successful drug candidates defined from *in vivo* studies may provide new therapeutic options to treat ARSACs disease and inspire future human clinical trials.

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