

# Progress Report

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## Introduction

Owing to its sheer size (4579 amino acids), thorough biochemical characterization of the SACS protein remains lacking or limited to small domains in the protein. However, much can be learned about SACS if there was a highly selective tool to enable its purification thus allowing detailed structural and biochemical characterization of its function, as well as to investigate its subcellular localization accurately and in a variety of model cell lines.

To this end, we set out to tag the *SACS* gene in the chromosome. We used CRISPR/Cas9 technology to introduce a fluorescent tag at the 3' end of the coding sequence of *SACS* (C-terminus of the protein). This has been a very difficult task, and we had to devise many different strategies to finally accomplish our goal. We provide evidence of the verification of successful chromosomal tagging of *SACS* and a brief outline of future work that should eventually allow us to isolate the full length protein and, subsequently, determine its structure.

## Methods and results

After attempting many different approaches, the method described below eventually allowed us to endogenously tag the *SACS* gene at its 3' end.

Over the course of several months we assembled a vector that encodes a 1 kbp-long left homology arm to the 3' end of the tenth exon of *SACS* (minus STOP codon), flexible linker, tobacco etch virus (TEV) cleavage sequence, monomeric Clover3 (a brighter version of GFP), flexible linker, 3 x FLAG tag and a 6 x HIS tag (**Figure 1**).

We *in vitro* synthesized two sgRNAs using the PAM-out configuration to improve editing efficiency. Both sgRNAs were loaded onto purified *Staphylococcus pyogenes* Cas9, also *in vitro*. The genomic editing vector described above was cleaved *in vitro* using two different commercially available purified Cas9 proteins (**Figure 2**). Finally, the vector encoding the tagging construct along with sgRNA-loaded SpCas9 were transfected into HEK 293T cells using Dharmafect Duo, designed for nucleic acid and protein transfection.

Cells were allowed to incubate for 2 days in 15 mm diameter silicone chambers in a 10 cm diameter plate. Afterwards, chambers were removed, and cells were allowed to expand. Cells were harvested near confluence, and ~ 2 million cells were sorted using fluorescence-activated cell sorting (FACS). Gating was set in such a way to only allow genome-edited cells with fluorescence signature above that of WT cells to be selected.

Of the sorted cell pool, we allowed three colonies to grow. Each colony was picked and expanded individually to ensure clonal purity. Genomic DNA was extracted from each of the three cell lines. A pair of primers were designed with the forward primer binding to the sequence of exon 10 of *SACS* and the reverse primer sequence to bind within the tagging sequence (**Figure 1**). PCR was used on WT and the three genome-edited cell lines (**Figure 3**). Of the three tested colonies, two showed PCR amplicons that accurately match the expected size. We chose colony B (henceforth termed 293T-SACS-mCl3-B) to further confirm genomic editing. We used 5 more primer pairs that cover most of the inserted sequence. Of the four primer pairs tested, three successfully produced a PCR amplicon from both the tagging vector as well as 293T-SACS-mCl3-

B, but not WT 293T cells.

Successful PCR reactions were gel-purified and extracted and sent to SickKids TCAG DNA sequencing facility. Using Sanger sequencing, we fully verified the DNA sequence from the genomic DNA of 293T-SACS-mCl3-B near the 3' end of exon 10 of SACS demonstrating that the designed tag was successfully integrated into the genome ensuring in-frame seamless translation of SACS tagged with mCl3.

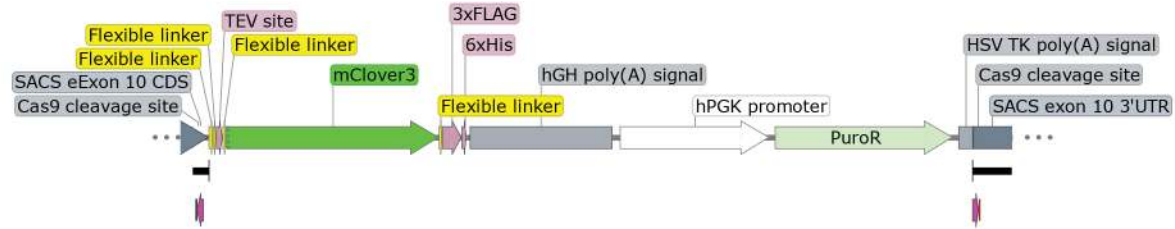
Subsequently, we prepared a large culture of the above cell line. Using a variety of buffers and lysis conditions, we found that lysates prepared using triton X-100 showed the expected fluorescence profile when excited at 475 nm and emission measured at 495-600 nm. Fluorescence emission spectra match that expected for mCl3 fluorescence, providing further evidence of the expression of mCl3-tagged SACS in HEK293T cells (**Figure 4**).

### **Future directions:**

We are currently optimizing protocols for detection and purification of SACS-mCl3 protein. Although HEK 293T cells are often used in biotechnology for protein production and purification, SACS endogenous level of expression remains low in these cells. Therefore, we will attempt to optimize protein production. For example, improvement of SACS-mCl3 production can be achieved by varying culture conditions or media type culture density. We will also attempt to visualize SACS-mCl3 in HEK 293T cells using confocal microscopy. The above outlined work will provide the necessary platform for large scale purification of SACS-mCl3, an important prerequisite for its eventual structural and *in vitro* characterization that we are pursuing.

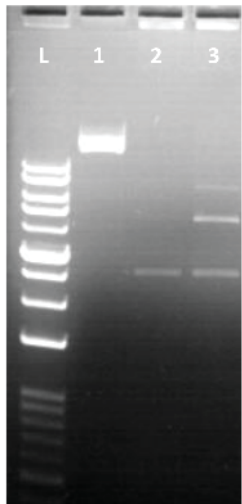
In terms of manuscripts, we hope to write our first manuscript in early 2021 in which we describe the endogenous fluorescent tagging of SACS and the characterization of the localization of SACS under different stress conditions using various imaging techniques. A second manuscript in late 2021 will describe the purification of full length SACS and its initial structural characterization.

**Figure 1**



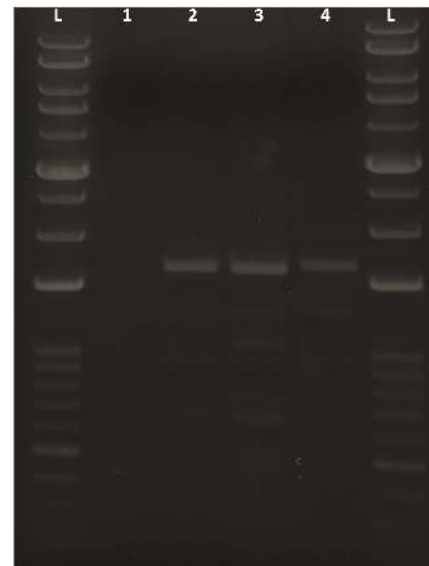
**Genomic locus after editing with mCl3 repair template**

**Figure 2**



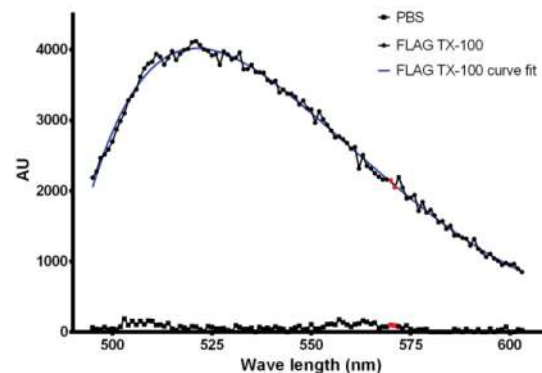
*In vitro* cleavage of SACS genomic editing vector (lane 1, uncut vector) after incubation with Cas9/sgRNAs ribnucleoprotein from NEB (lane 2) and Sigma-Aldrich (lane 3). L is molecular weight DNA ladder.

**Figure 3**



PCR amplicon from 293T cells WT (lane 1), colony B (lane 2), colony C (lane 3) and colony D (lane 4). L is molecular weight DNA ladder.

**Figure 4**



**Fluorescence scan of cell lystae from 293T-SACS-mCl3-B cells solubilized with Triton X-100 or PBS control.**