REPORT Ataxia Charlevoix-Saguenay Foundation

"Biophysical and functional study of Sacsin Trojan fragments as a protein complementation and phenotypic rescue strategy for ARSACS."

Principal Investigator: Javier Santos

Summary

A structural model based on the full-length Sacsin was carefully studied for this project. This phase took longer than we initially expected, but we obtained important results from the analysis that helped us make key design decisions. Four recombinant-large Sacsin fragments were designed considering both structural and functional hypotheses. The protein expression was carried out in BL21 DE3 cells, and two fragments yielded a significant amount of recombinant protein in the soluble fraction. These fragments were purified, and western blotting was performed, indicating the presence of the C-terminal His-tag. Furthermore, the observed molecular weight values were compatible with the expected values (85 and 65 kDa). Fragment A, which includes the repetitive stretch of SIRPT3, was studied using size-exclusion chromatography, and preliminary results indicated a monomer-oligomer equilibrium. We are currently optimizing production and purification protocols, as well as performing cellular transduction experiments using human cell lines. Mass spectrometry analysis is in progress; fragment A was identified correctly. Co-immunoprecipitation experiments, proteomics, and studies will be conducted as soon as possible to determine whether Fragment A can serve as a platform for protein-protein interactions and the Sacsin fragment non-covalent coupling. This ARSACS project is part of a PhD student's research plan in our lab.

ARSACS group at iB3 (School of Exact and Natural Science, Buenos Aires University):

- Naira Rodriguez (PhD student ARSACS, experiments and bioinformatics)
- Marco Mancini (PhD student, bioinformatics)
- Alejandro D. Nadra (researcher)
- Javier Santos (principal investigator)

It is worth mentioning that this project is part of Naira Rodriguez's PhD research plan in our lab. The first months of the scholarship were possible thanks to the ARSACS foundation. After that, Naira won a local PhD scholarship from the local Scientific Council (CONICET) for a period of four years to study Sacsin function.

The ARSACS Seed grant was crucial for our involvement in this pathology and for advancing and building the project. We would like to apply to the ARSACS Foundation for the possibility of obtaining a one-year renewal of the grant to continue the project. The additional time will enable us to complete the original scope of work. Anyway, we are grateful for the support the ARSACS community has provided us since 2024.

Sacsin fragments as a platform for studying protein-protein interactions and structure-function relationships

To understand the role of the highly repeated regions, pathogenic variants were mapped onto the structure, allowing us to identify areas of structural and/or functional importance (**Figure 1**). We found that the mutational variants are spread throughout the Sacsin sequence, including the repetitive segments. This supports the idea that these regions are crucial for the protein's structural integrity and/or biological function.

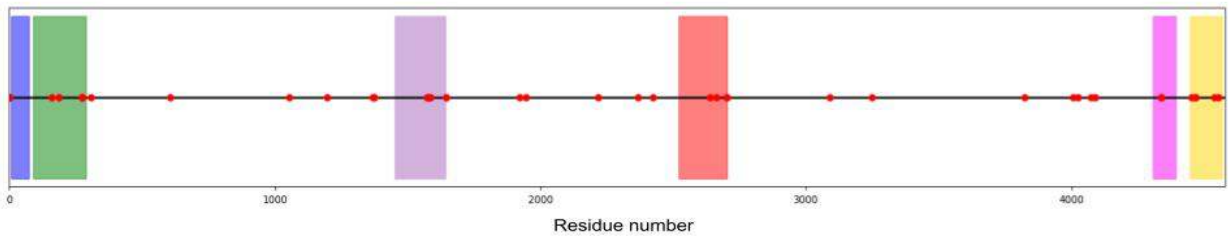

Figure 1. Distribution of pathogenic variants and domains in the Sacsin protein. The ubiquitin-like domain is highlighted in blue, ATPase1 in green, ATPase2 in violet, ATPase3 in red, DNAJ in pink, and HEPN in yellow. The pathogenic variants were mapped on Sacsin. Several mutations are located within the repeats, suggesting that these stretches play structural and/or functional roles.

Design of recombinant fragments.

We designed four large fragments to study the function of Sacsin and the capability of these fragments to interact with each other and with other cellular proteins (**Figure 2**). An N-terminal transactivation peptide (TAT; sequence MRKKRRQRRR) was included in all four designed fragments to facilitate cellular penetration, together with a C-terminal His-tag (HHHHHH) to allow subsequent identification and quantification.

- Fragment A: Corresponds to the repetitive region of the SIRPT3 domain (**Figure 2A**). It has 760 amino acids from Sacsin plus the Trojan, and the His-tag peptides, a molecular weight of 88 kDa.

- Fragment B corresponds to the SIRPT3 stretch, but the repetitive region is replaced by a 12-glycine fragment (**Figure 2B**). This fragment includes ATPase 3, the HEPN domain, and the DNAJ domain. It consists of 1374 amino acids, with a molecular weight of 155 kDa.

- Fragment C corresponds to ATPase 3 of Sacsin (**Figure 2C**). It has 615 amino acids and a molecular weight of 69 kDa.

- Fragment D: This segment includes the ubiquitin-like domain and ATPase 1 (**Figure 2D**). It consists of 583 amino acids and has a molecular weight of 66 kDa.

These fragments are versatile and will assist in exploring various hypotheses. The first one (Fragment A) is located within the repetitive regions and was designed to encode restriction sites, enabling easy integration into other domains. This fragment will be essential for experimentally determining whether the repetitive regions encode information about protein-protein interactions. Our working hypothesis is that these stretches serve as a platform for protein-protein interactions. We aim to examine whether Fragment A can act as a platform for the non-covalent coupling of different Sacsin fragments.

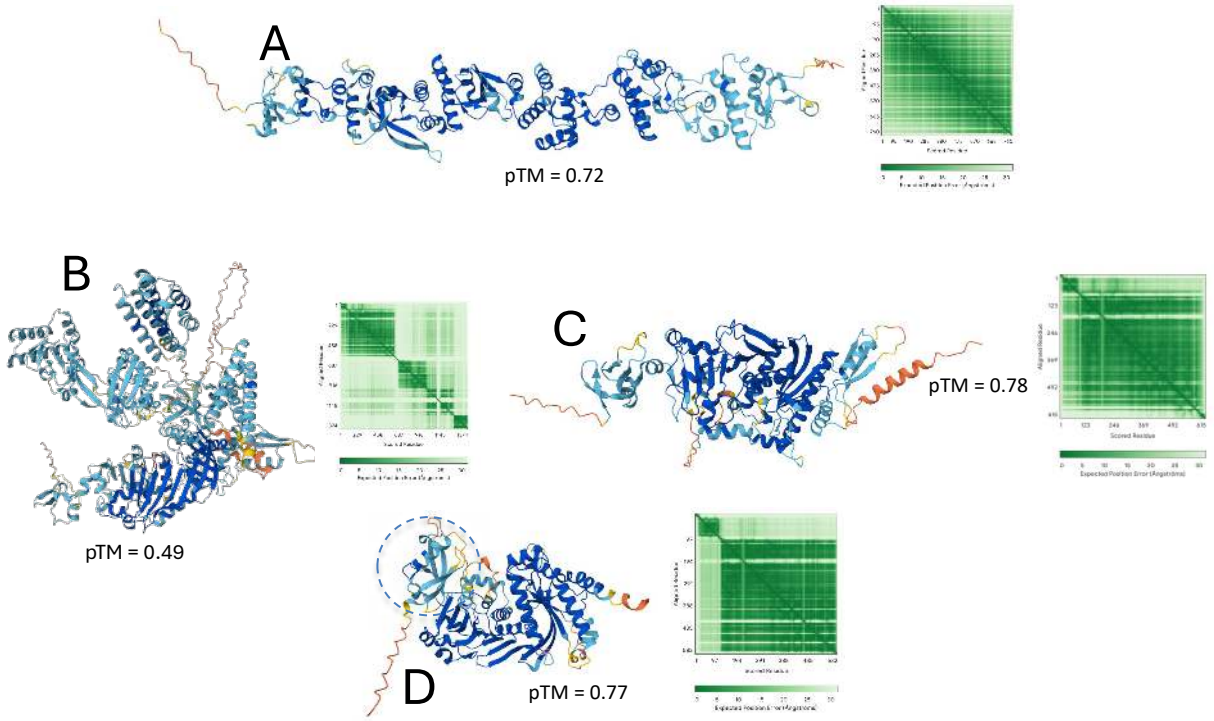

Figure 2. Predicted structures of the designed recombinant fragments using AlphaFold3. **(A)** Predicted structure of fragment A. An organized and linear structure is evident. **(B)** Predicted structure of fragment B. **(C)** Predicted structure of Fragment C. **(D)** Predicted structure of fragment D. The ubiquitin-like domain (UbLD) is indicated with a dashed circle. The predicted template modeling (pTM) score measures the accuracy of the entire structure. A pTM score greater than 0.5 means that the overall predicted fold for the complex may be similar to the true structure [1].

Expression in *E. coli* and purification of the recombinant Trojan fragments.

Protein expression was carried out in *E. coli* BL21 strain. A Western blot assay using anti-His-tag antibodies was performed to evaluate the expression of the four Sacsin fragments (**Figure 3**).

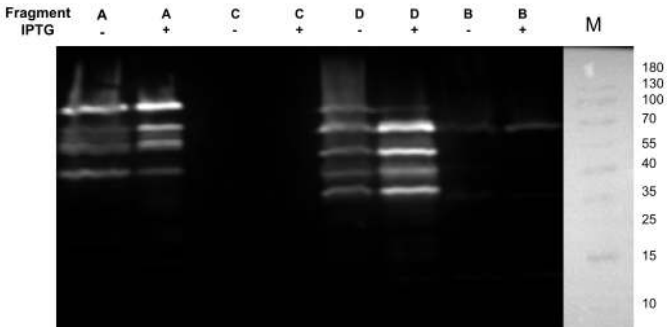

Figure 3. Western blot of the four recombinant Sacsin fragments detected with anti-His antibody. For each Fragment (A, C, D, and B) the following were analyzed: NI, an aliquot of the bacterial culture before induction; I, an aliquot after induction with

IPTG; INS, insoluble fraction; SOL, soluble fraction. L: molecular-weight marker (kDa) shown on the left.

After the initial attempts, we observed that the production of Fragments C and B was extremely low. Nevertheless, only Fragments A and D were obtained in the soluble fraction.

Purification and Characterization of Fragment A

We started trying to purify the soluble fragments from *E. coli* BL21. For that, we first optimized the expression conditions. Fragment A was purified, and protein purity was assessed by SDS-PAGE (**Figure 4**). The protein was stored at –70 °C until use. The purified Fragment A was identified by Western blotting, using an anti-His tag antibody (**Figure 5**). Next, we identify and characterize the recombinant protein by mass spectrometry. The highest-molecular-weight band corresponding to the purified expression product of Fragment A was submitted for LC–MS/MS analysis (**Table 1**). This step was performed to confirm that the band corresponds to Fragment A.

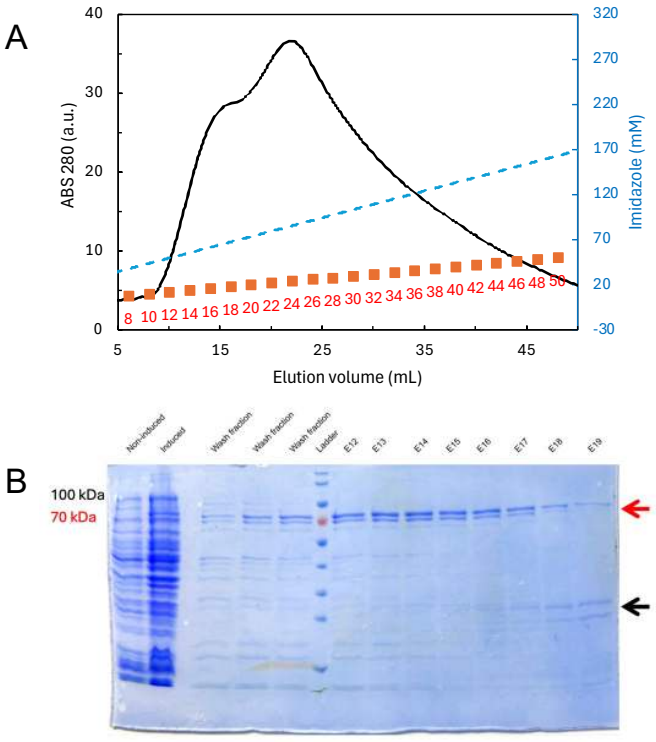

Figure 4. Purification of Soluble Fragment A. (A) Chromatography profile for fragment A purification using a Cytiva HisTrap FF 5 mL column (Cytiva #17525501). (B) SDS-PAGE analysis of the fractions containing the fragment. Black and red arrows indicate contaminants and Sacsin fragment, respectively.

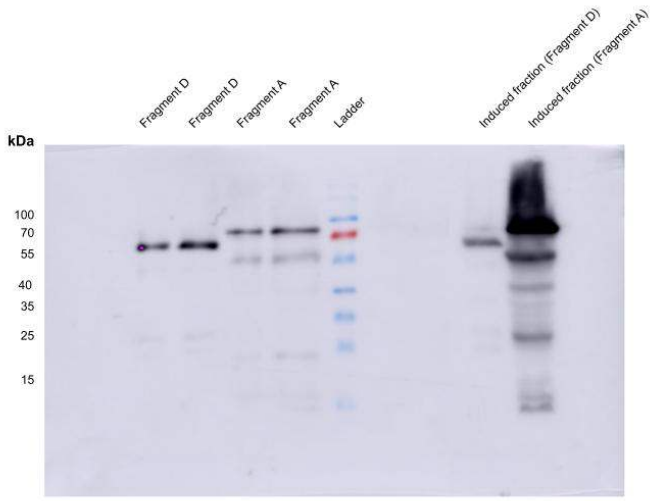

Figure 5. Identification of Purified Sacsin Fragments by (10%) SDS-PAGE and Western blotting. The His-tagged Sacsin fragments were transferred to PVDF and identified with an anti-His-tag antibody. Lanes (left to right): Fragment D (purified), Fragment D (purified), Fragment A (purified), Fragment A (purified), molecular-weight ladder, induced soluble fraction expressing Fragment D, induced soluble fraction expressing Fragment A.

Table 1. Mass spectrometry preliminary results for Fragment A (NR03).

Confidence	Sequence	Modifications	# Protein Groups	# Proteins	# PSMs	Master Protein Accessions	Protein Accessions	# Missed Cleavages	Theo. MH+ [Da]	Charge (by Search Engine): A Sequest HT	m/z [Da] (by Search Engine): A Sequest HT	XCorr (by Search Engine): A Sequest HT
High	LSSAEELSEIKEQLFEKLESLLIIHDANSR		1	1	3	NR03	NR03	2	3441.81115	5	689.16213	6.08
High	LFIPNDFFK		1	1	1	NR03	NR03	0	1140.60881	2	570.80263	1.91
High	VFDISSFADLLSSVLPR		1	1	4	NR03	NR03	0	1866.00074	2	933.50074	4.6
High	DSAFVPLLSCHTANIESPTSILK	1xCarbamidom	1	1	1	NR03	NR03	0	2500.2752	3	834.09243	4.88
High	ETLQNTVDILLHHIFQER		1	1	3	NR03	NR03	0	2206.16149	3	736.05623	5.18
High	FLTTYHELIPSR		1	1	1	NR03	NR03	0	1476.78454	3	492.92819	3.3
High	FTVSANQLVVPEGDVLLPLSLMHIAVFPN AQSDK		1	1	1	NR03	NR03	0	3649.92983	3	1217.30598	4.36
High	EQEGSDLGPQEQLEQVLNMLNVNLDPP LDK	1xOxidation [M	1	1	1	NR03	NR03	0	3378.63696	3	1126.87757	1.87
High	EDQEETKPTFDIVVDTLKDWALLPGTK		1	1	1	NR03	NR03	1	3088.57249	3	1030.18595	1.84
High	DAEENEIEVEGLPLLITLDSVLQTFDAK		1	1	1	NR03	NR03	0	3101.57763	3	1034.5261	1.21
High	FTVSANQLVVPEGDVLLPLSLMHIAVFPN AQSDK	1xOxidation [M	1	1	1	NR03	NR03	0	3665.92474	4	917.22927	3.25
High	YSNILLNCK	1xCarbamidom	1	1	1	NR03	NR03	0	1124.57686	2	562.787	1.54
High	LSSAEELSEIK		1	1	1	NR03	NR03	0	1205.62597	2	603.31207	1.36

Conclusions

Other groups have previously prepared some TAT-derived Sacsin fragments. Among them, the J domain or SacsJ fragment (homologous to Hsp40, corresponding to residues 4316–4420), which interacts with neurofilaments [2]. Additionally, the ubiquitin-like domain (Ubl, residues 2–85), HSP90-like domain (referred to here as ATPase1 or Sr1, comprising residues 89–336), and even Ubl-Sr1 (covering residues 1–339) domains were expressed [3] or the HEPN domain (residues 4441–4579) [4]. Their structures were resolved or analyzed in detail, providing crucial insights into their structural and functional relationships. However, with our project, we have focused on

producing high-molecular-weight fragments. We successfully produced two Sacsin fragments and purified them from the soluble fractions: **Fragment A (the repetitive region of the SIRPT3 domain)** and **Fragment D (which contains the ubiquitin-like domain and ATPase 1 from the SIRPT1 domain)**. We are currently conducting the first transduction experiments with purified Fragment A, which is incubated with human cells to evaluate the translocation of the fragment from the media into the cytosol. We have found some unspecific detection by using the anti-His tag. Therefore, we will modify the detection method for fragments within the cellular context (chemical modification with fluorophores, and small identification tags). As mentioned earlier in the report, we will determine whether Fragment A serves as a platform for protein-protein interactions (much more than a simple linker). This is plausible because this stretch of Sacsin has a high level of structure. In this regard, proteins that interact with Sacsin fragments will be identified via mass spectrometry and a proteomics approach. Co-immunoprecipitation experiments and studies of protein-protein interactions will also be conducted over the next few months.

Scientific publications

Part of the results included in this report were shared in a local Scientific meeting:

-Poster at Argentine Biophysical Society (SAB 2024) Meeting: **Sacsin Fragments to Study Protein Function**. Naira Rodríguez, Marco Mancini, Alejandro Nadra and Javier Santos.

References

1. Xu, J. and Y. Zhang, *How significant is a protein structure similarity with TM-score = 0.5?* Bioinformatics, 2010. **26**(7): p. 889-95.
2. Dabbaghizadeh, A., et al., *The J Domain of Sacsin Disrupts Intermediate Filament Assembly.* Int J Mol Sci, 2022. **23**(24).
3. Menade, M., et al., *Structures of ubiquitin-like (Ubl) and Hsp90-like domains of sacsin provide insight into pathological mutations.* J Biol Chem, 2018. **293**(33): p. 12832-12842.
4. Li, X., et al., *High-Throughput Screening for Ligands of the HEPN Domain of Sacsin.* PLoS One, 2015. **10**(9): p. e0137298.