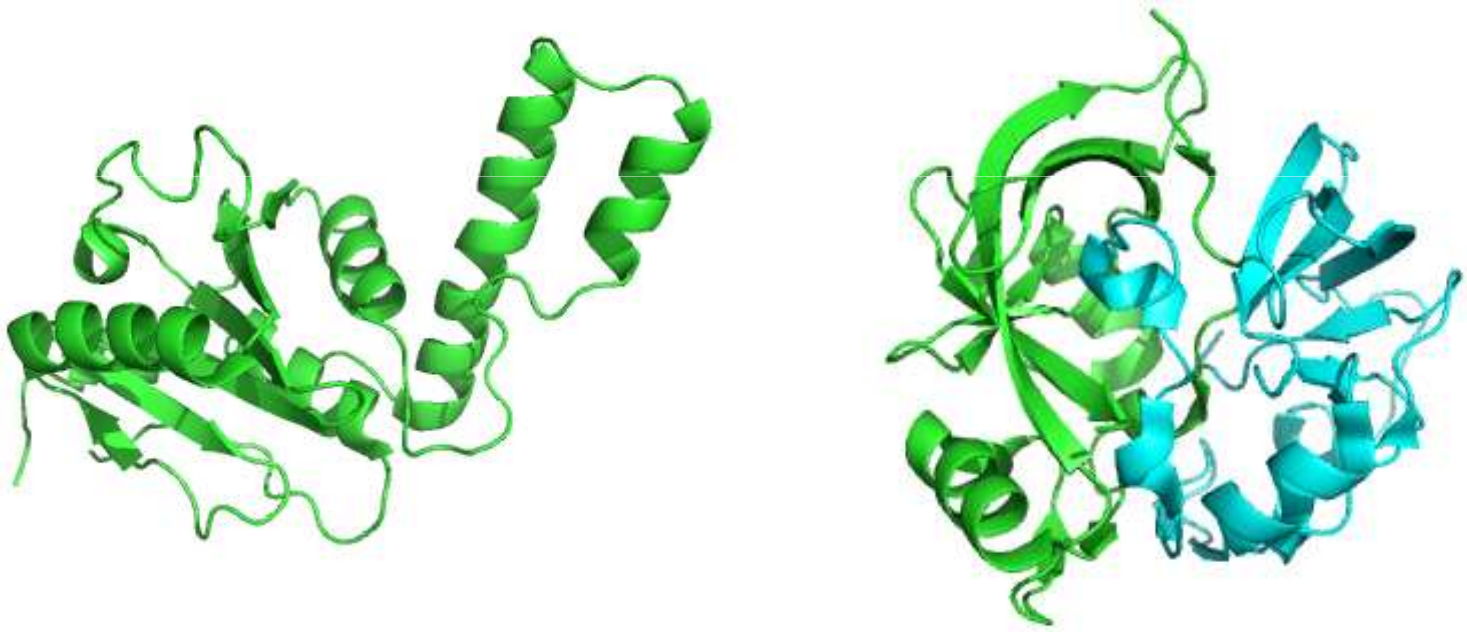
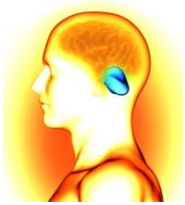
PP2A and spinocerebellar ataxias: a structural approach



Espinocerebellar Ataxias (SCAs):



Are the most common group of ataxias.

Heterogeneous origin (more than 23 different SCAs): mainly **autosomal dominant**.

Many are cathegorized as **polyglutamine diseases**, and the severity of the disease directly correlates with the lenght of the abnormal polyglutamine extension.

The abnormal triplet expansion results in a toxic gain of function leading to **neurodegeneration** (e.g. Atx-1).

Self-association due to misfolding generates **nuclear aggregates** leading to pathogenesis.

Whereas Atx-1 has a wide expression pattern in neurons and peripheral tissues, SCA1 predominantly affects cerebellar regions. This suggests that **additional tissue specific partners** are involved in SCA1 pathology.

LANP (Leu-rich acidic nuclear protein) / ANP32A /PHAP-I

ANP32 family are **nucleus-cytosol** shuttling **phosphoproteins** involved in a variety of processes (signaling, cell proliferation and diferentiation, apoptosis, histone acetylation inhibition, mRNA stability, or microtubule dynamics).

ANP32A interaction with MAP1B regulates neurite generation (Opal, 2003).

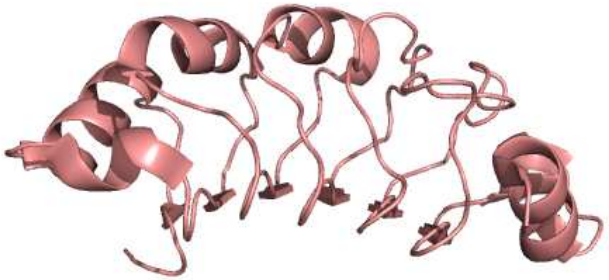
ANP32A is highly expressed in Purkinje cells and interacts with mutant **Atx-1**, suggesting its role in SCA pathology.

Binding to Atx-1 has **low (mM) affinity** but the affinity increases with the lenght of the Atx-1 polyglutamine extension. Additional partners must assist in the formation of the Atx-1 complex.

ANP32A has another major interaction partner: the catalytic subunit of **PP2A**.

It is a heat-stable, very specific and strong **inhibitor (nM) of PP2A** (I1-PP2A).

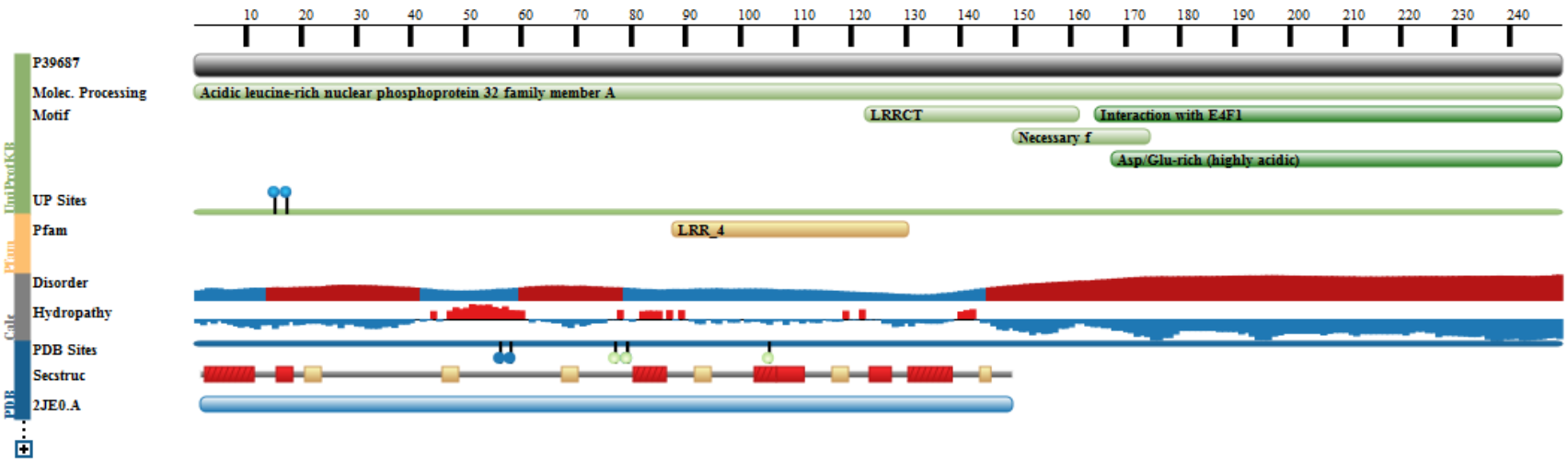
LANP (Leu-rich acidic nuclear protein) / ANP32A /PHAP-I



The structure of the N-terminal part of ANP32A has been solved by **NMR** (1-164) and **X-ray crystallography** (1-149).

Contains 4 N-terminal Leucine-Rich Repeats and a C-terminal polyacidic cluster (~70% of Glu and Asp residues) similar to that found in chromatin binding proteins. The C-terminus is disordered and has not been observed in any previous structure.

Despite being a strong inhibitor of PP2AC, no structure in complex with PP2A has been determined yet.

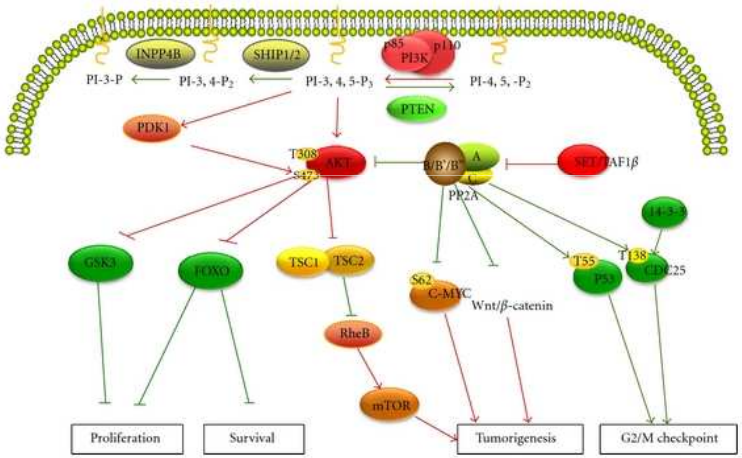


Ser/Thr phosphatase PP2A: biological function

PP2A is a global player in **cell signaling**, with a role in development, control of cell cycle, cell mobility, apoptosis or cytoskeleton dynamics.

Very **conserved throughout eukaryotes**. In some cell types, corresponds to 1% of cellular proteins.

Together with PP1, accounts for **>90% Ser/Thr phosphatase activity** in most tissues.
PP2A dephosphorylates many critical molecules like Akt, p53, c-Myc, or β-catenin.



Impairment of **PP2A activity** leads to abnormal hyperphosphorylation of Tau, a major component of **Alzheimer's Disease** (AD) microfibrillary tangles.

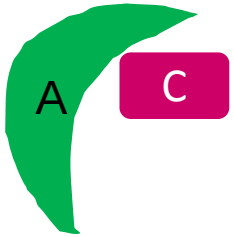
SCA12 occurs as a result of a triplet expansion mutation in PP2R2B, a regulatory subunit from the PP2A holoenzyme.

Ser/Thr phosphatase PP2A:

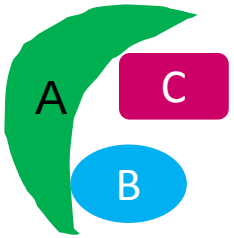
Broad specificity that is narrowed down through the association to >= 4 types of **regulatory subunits,** forming >200 biochemically distinct complexes.

In the cells, PP2A exists in two different forms:

-heterodimeric **core enzyme: A (scaffold) and C (catalytic)**



-heterotrimeric **holoenzyme: core A-C and B (regulatory)**

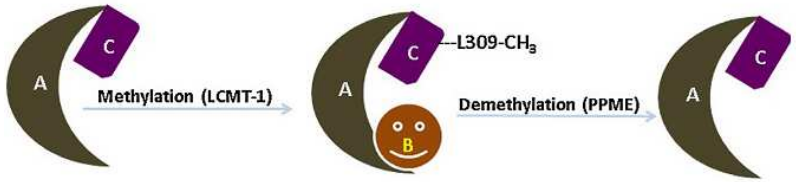


Subunit family	Gene symbol	Isoforms	
C	*PPP2CA*	Cα	
	PPP2CB	Cβ	
A	*PPP2R1A*	Aα	
	PPP2R1B	Aβ	
PR55/B	*PPP2R2A*	Bα	B
	PPP2R2B	Bβ	
	PPP2R2C	Bγ	
	PPP2R2D	Bδ	
PR61/B′	*PPP2R5A*	B′α	
	PPP2R5B	B′β	
	PPP2R5C	B′γ1	
		B′γ2	
		B′γ3	
	PPP2R5D	B′δ1	
		B′δ2	
		B′δ3	
	PPP2R5E	B′ε	
PR72/B″	*PPP2R3A*	B″α1	
		B″α2	
	PPP2R3B	B″β	
	PPP2R3C	B″γ	

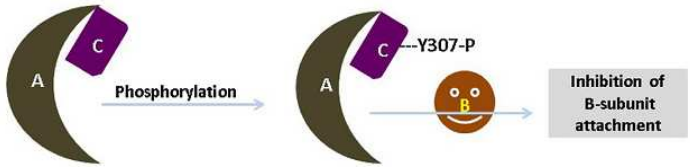
Ser/Thr phosphatase PP2A:

It has a complex regulation at different levels:

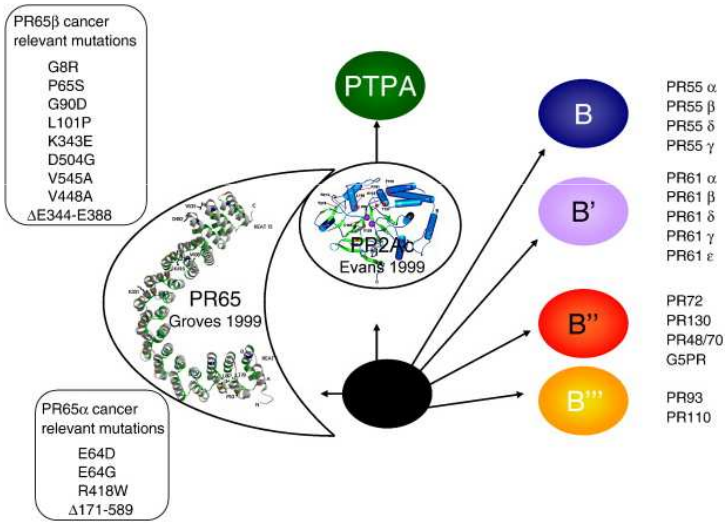
-C-terminal **methylation** by LCMT-1 and **demethylation** by PPME. It affects regulatory subunit affinity.



-Tyr **phosphorylation** inhibiting B subunit attachment



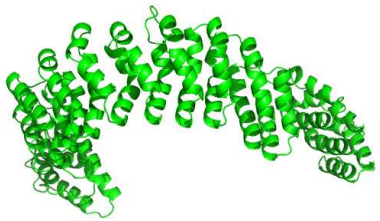
-Binding to **inhibitory partners**



-Other: e.g. regulatory subunit mutations

Structures of Ser/Thr phosphatase PP2A

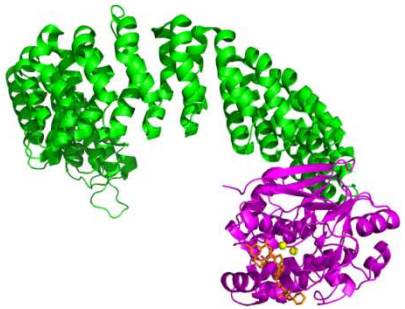
A subunit (scaffold)



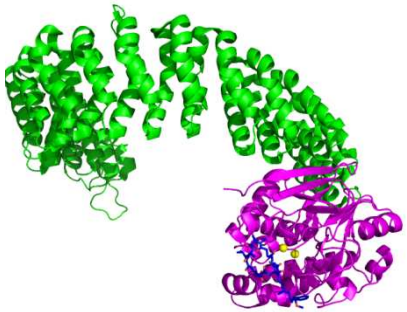
Groves et al. Cell (1999)

PP2A oligomers with Inhibitors

AC plus Okadaic Acid

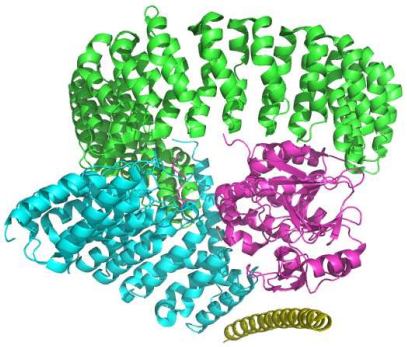


AC with Microcystin



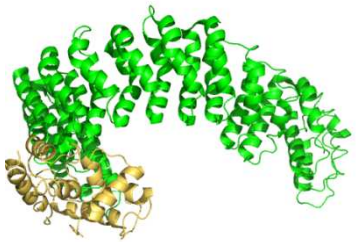
Xing et al. Cell (2006)

AB'C with Shugosin



Xu et al. Mol.Cell (2009)

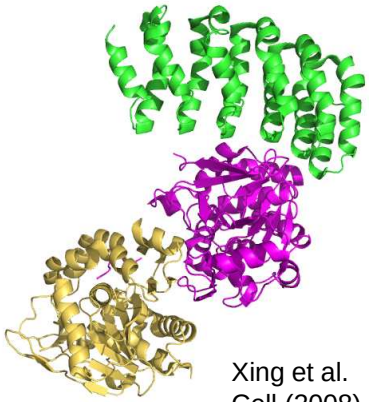
A subunit (scaffold) with SV40 small t-antigen



Cho et al. PLoS Biol. (2007)
Chen et al. NSMB (2007)

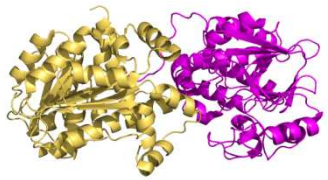
Other regulatory complexes

C subunit with PPME1



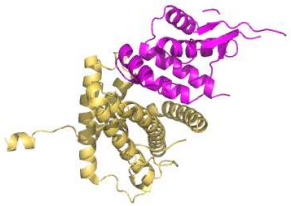
Xing et al. Cell (2008)

C subunit with LCMT1



Stanevic et al. Mol.Cell (2011)

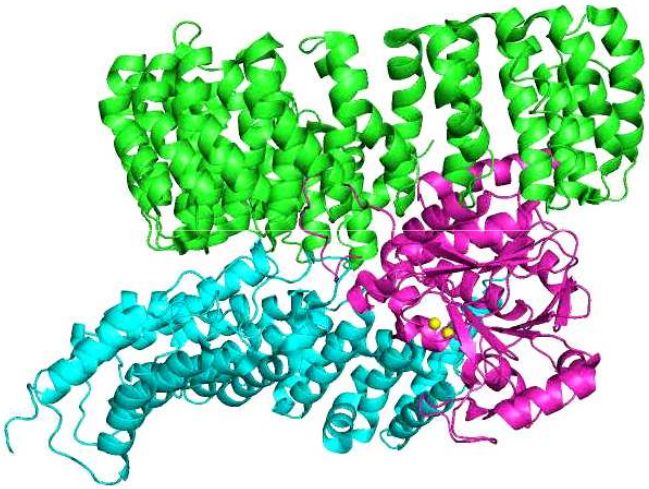
C subunit with Alpha4



Yiang et al. Nat. Commun. (2013)

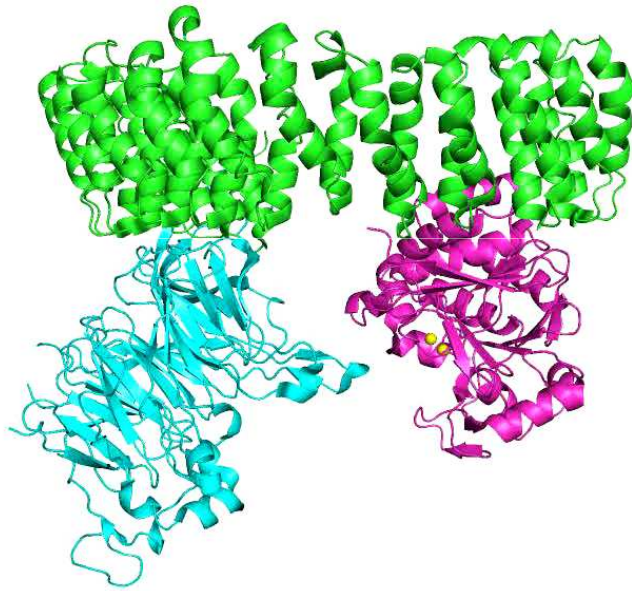
Structures of Ser/Thr phosphatase PP2A

Holoenzyme B' (A-C-B')



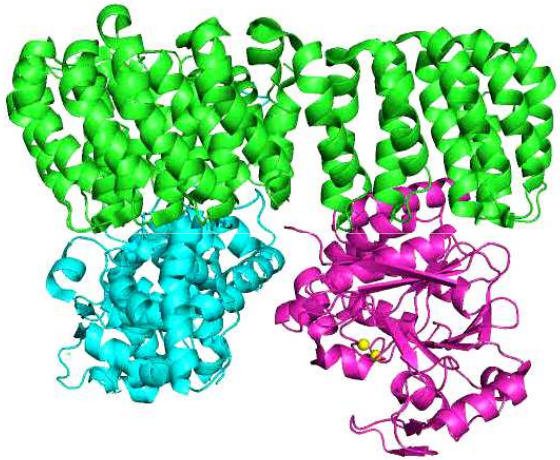
Xu et al. Cell (2006)
Cho et al. Nature (2007)

Holoenzyme B (A-C-B)



Xu et al. Mol.Cell (2008)

Holoenzyme B'' (A-C-B'')



Wlodarchak et al. Cell Res. (2013)

A novel function of Ataxin-1 in the modulation of PP2A activity is dysregulated in the spinocerebellar ataxia type 1
Sanchez et al. Hum.Mol.Gen.,2013

Atx-1 modulates PP2A activity and regulates holoenzyme composition, before SCA1 disease onset

Mutant Atx1:
Decrease in PP2AC-Y307 phosphorylation (enhances its activity)
Reduces PP2A-Bβ expression
Inhibits ANP32A expression levels

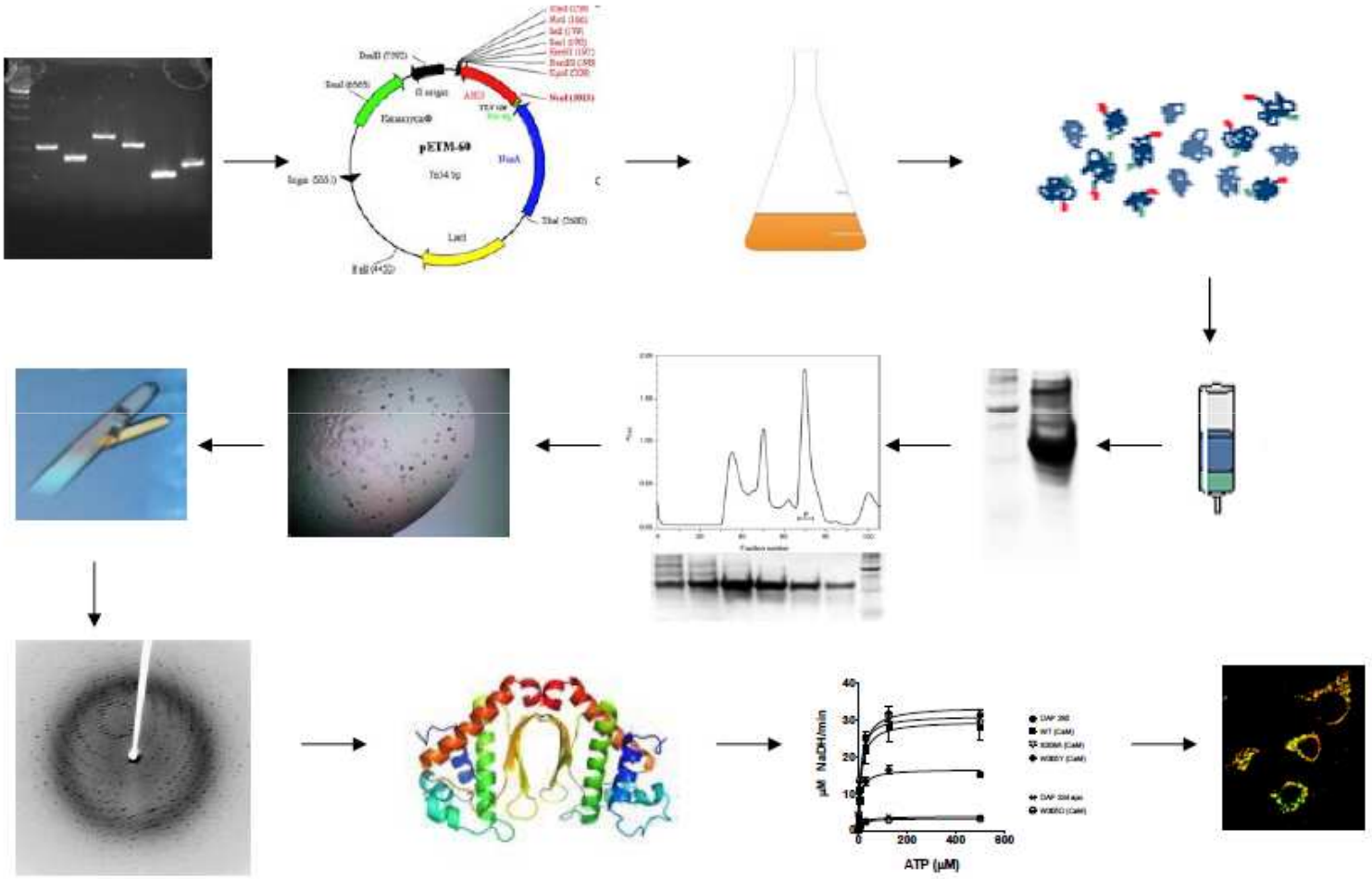
This effects are reversed by overexpression of ANP32A.

Main evidences pointing to a role of PP2A in SCA

- Atx1 interacts with ANP32A
- ANP32A strong (nM) inhibitor of PP2A
- Atx1 (S776) seems to be a target for PP2A
- Triplet expansion in B regulatory subunit in SCA12
- PP2R2Bβ expression restricted to Purkinje cells
- Mutant Atx1 downregulates PP2R2Bβ and ANP32A expression
- Mutant Atx1 decreases Y307 phosphorylation of PP2A
- Alterations in phosphorylation of PP2A-known substrates Erk2 and Gsk3β in SCA1

Aim:
to investigate the formation of a PP2A (core enzyme or A/Bβ/C heterotrimer) in complex with ANP32A

Pipeline X-ray crystallography experiment

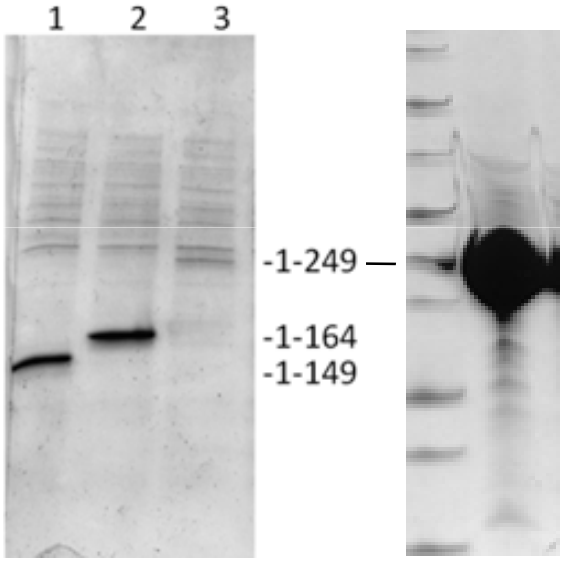


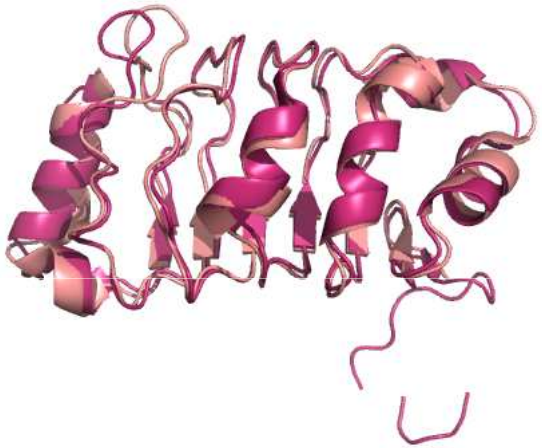
Expression tests ANP32A

ANP32A

Small scale

Big scale

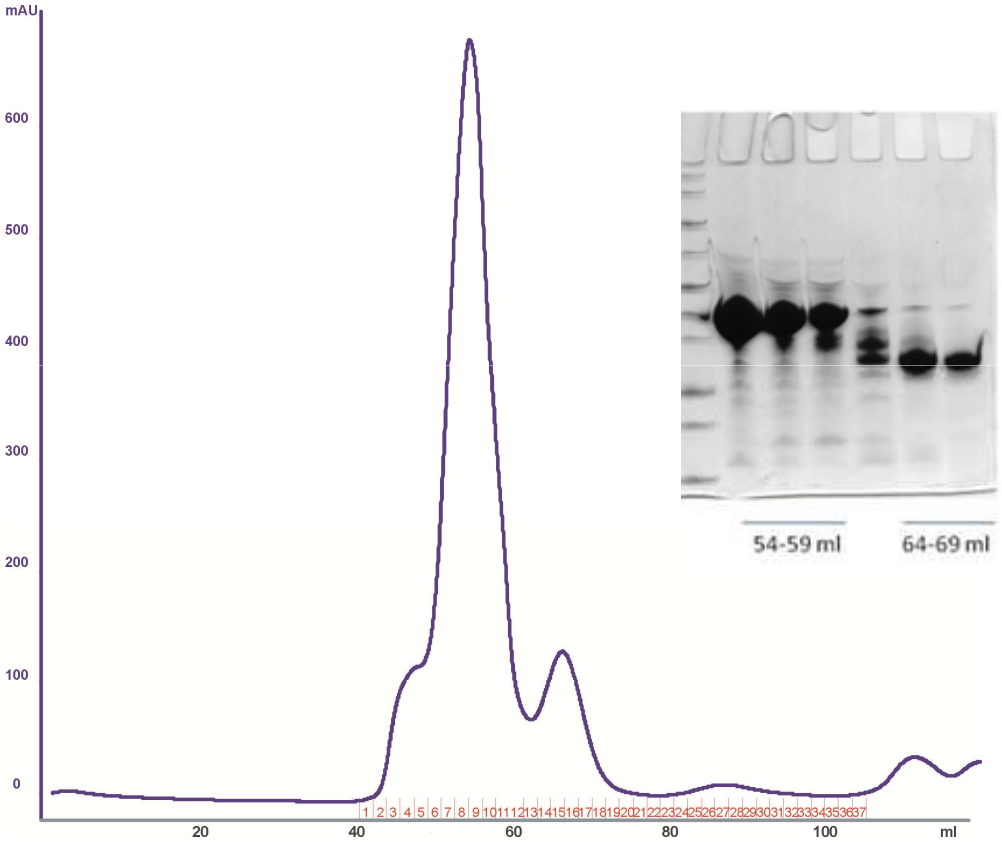




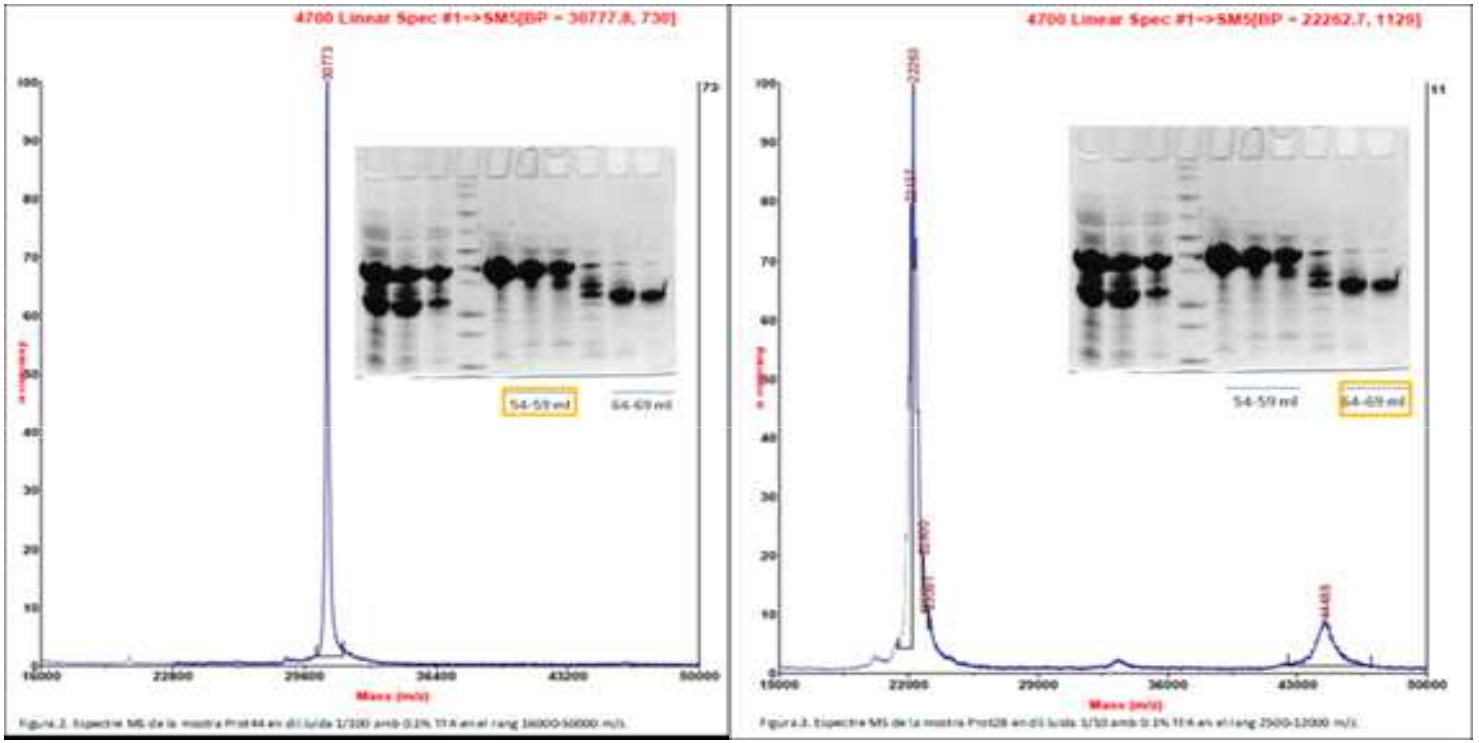
1-149 : X-Ray structure
1-164 : NMR structure
1-249: full lenght

All fragments are soluble and expressed in mid-high yields.

Size exclusion chromatography of His-ANP32A$_{1-249}$



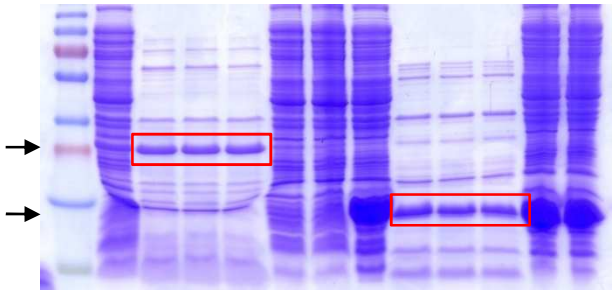
Lower forms expressed correspond to the N-terminal core of His-ANP32A$_{1-249}$




```
  1 MEMGRRIHLE LRNRTPSDVK ELVLDNSRSN EGKLEGLTDE FEELEFLSTI
 51 NVGLTSIANL PKLNKLKKLE LSDNRVSGGL EVLAEKCPNL THLNLSGNKI
101 KDLSTIEPLK KLENLKSLDL FNCEVTNLND YRENVFKLLP QLTYLDGYDR
151 DDKEAPDSDA EGYVEGLDDE EEDEDEEEYD EEGYNDGEVD DEEDKKKKKK
201 KKKKKKKKK
```

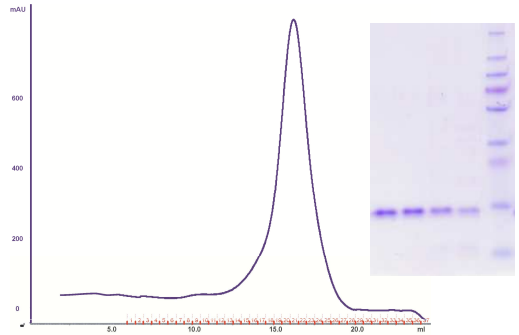
Purification of different forms of untagged ANP32A by thermoprecipitation

Thermoprecipitation 10’ @ 65ºC



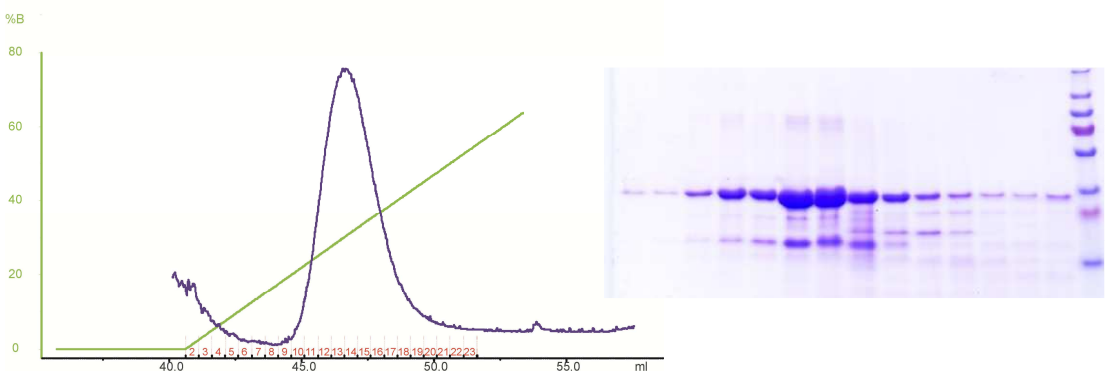
Size Exclusion

ANP_{1-149}

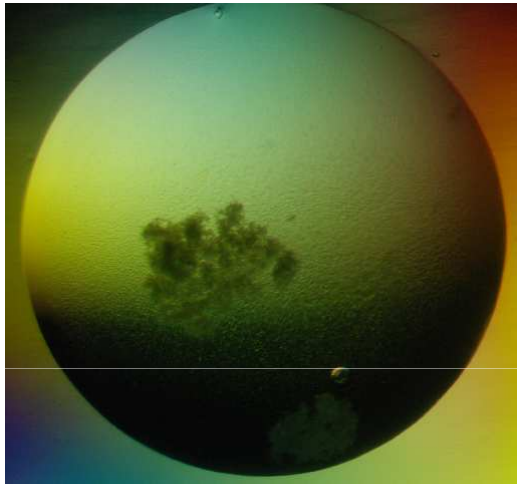


Anion Exchange

ANP_{1-249}



Crystallization trials of untagged ANP$_{1-249}$

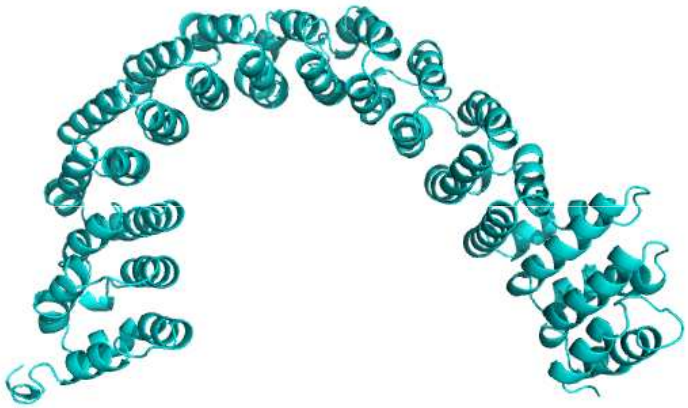


0.2 M Calcium acetate
0.1 M Sodium cacodylate pH 6.5
40 %(v/v) PEG 300

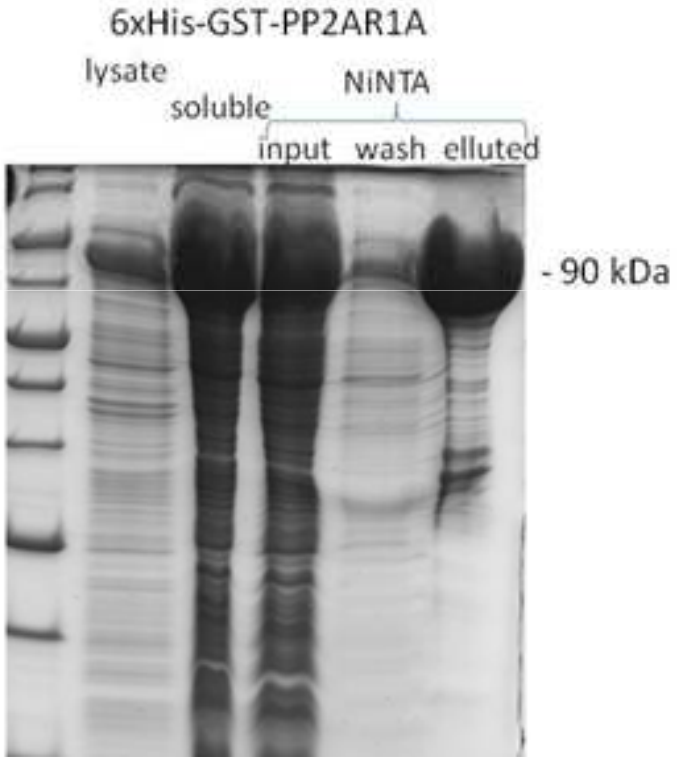
Spherulites appeared in some conditions. So far we've been unable to optimize these preliminar crystals.

PP2R1A expression in E.coli

Expression of His-GST tagged PP2R1A

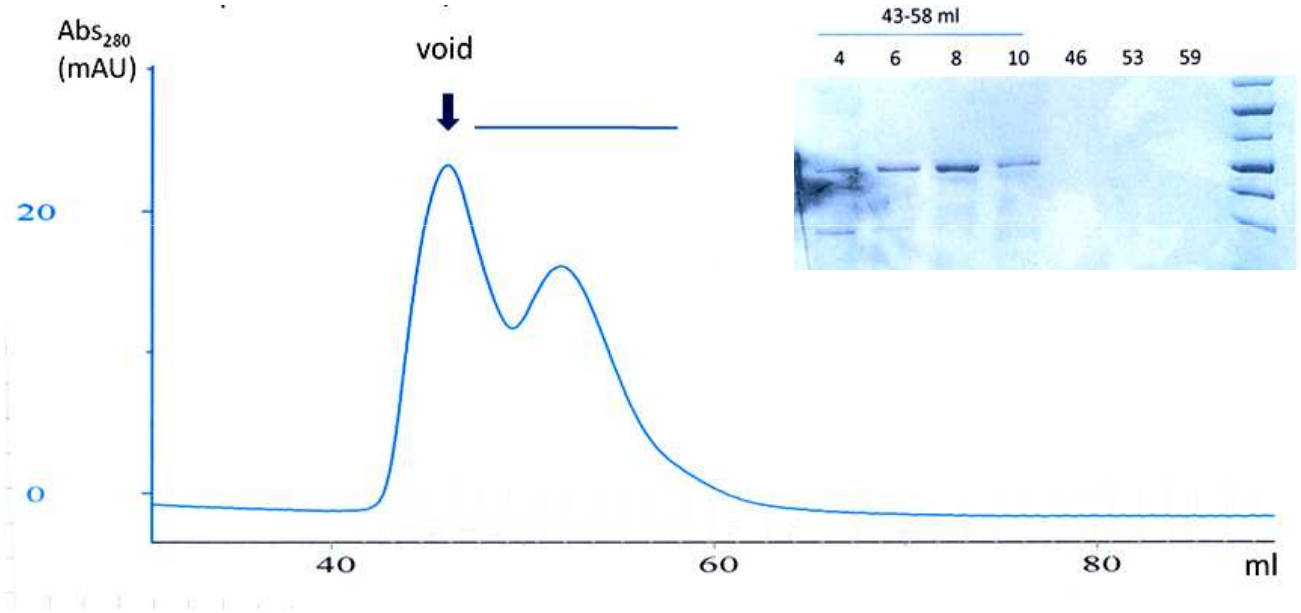


Groves et al. Cell (1999)

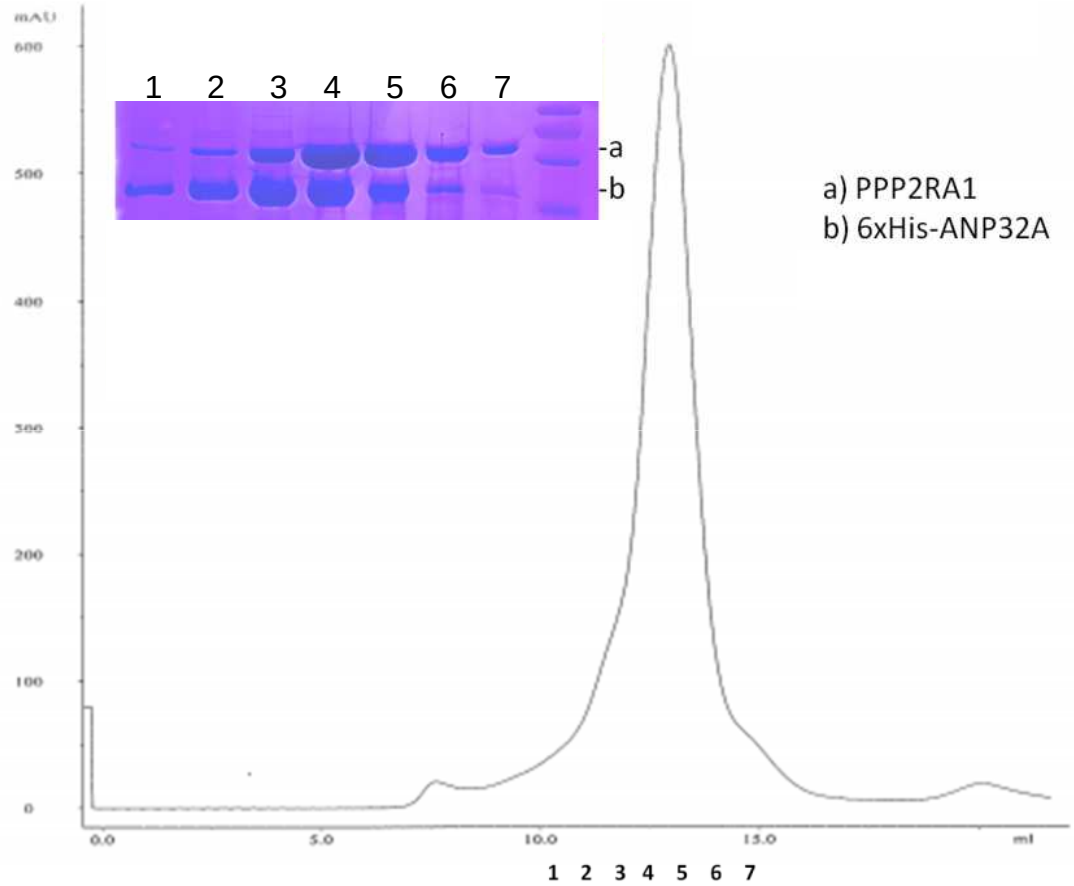


PPP2R1A expression in E.coli

GST tag can be efficiently removed from the A subunit.
Pure protein was obtained after size exclusion chromatography.



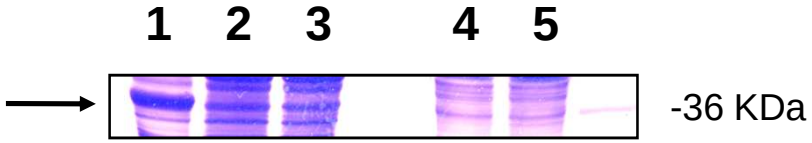
A (scaffold) subunit and ANP32A (full length) does not form a complex



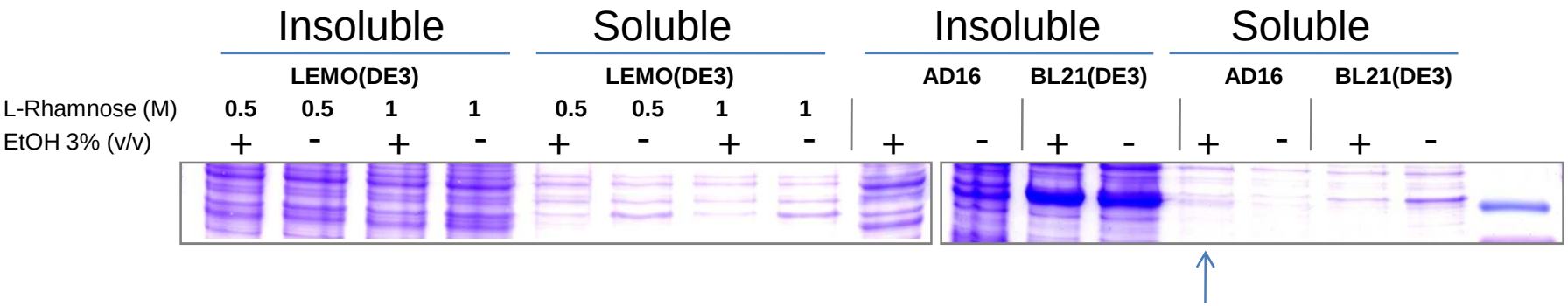
On the basis of previous data ANP32A should not directly bind to the scaffolding subunit A, but indirectly through the C subunit.

Expression PP2AC-6xHis in E.coli

Origami strain



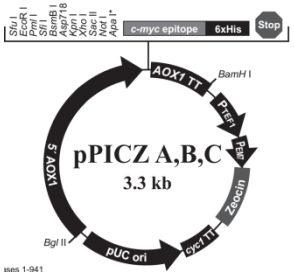
1 - Insoluble fraction
2,3 - Soluble fraction
4,5 - Elution NiNTA



Protein is insoluble in E.coli. Very small amounts of soluble protein could be seen in AD16 strain.

6xHis-PP2AC expression in yeast KM71H Pichia pastoris

-Cloning into pPICZ-A vector for intracellular expression.

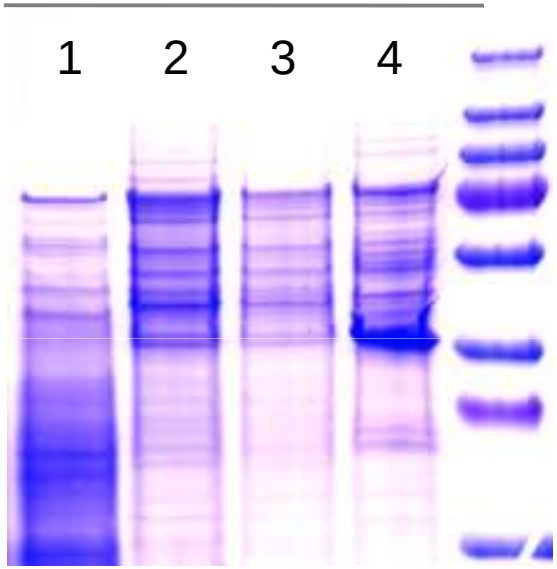


-Linearize construct and transform KM71H (Mut^S).

-Select transformants with Zeocin.

-Grow and induce with MetOH.

NiNTA affinity

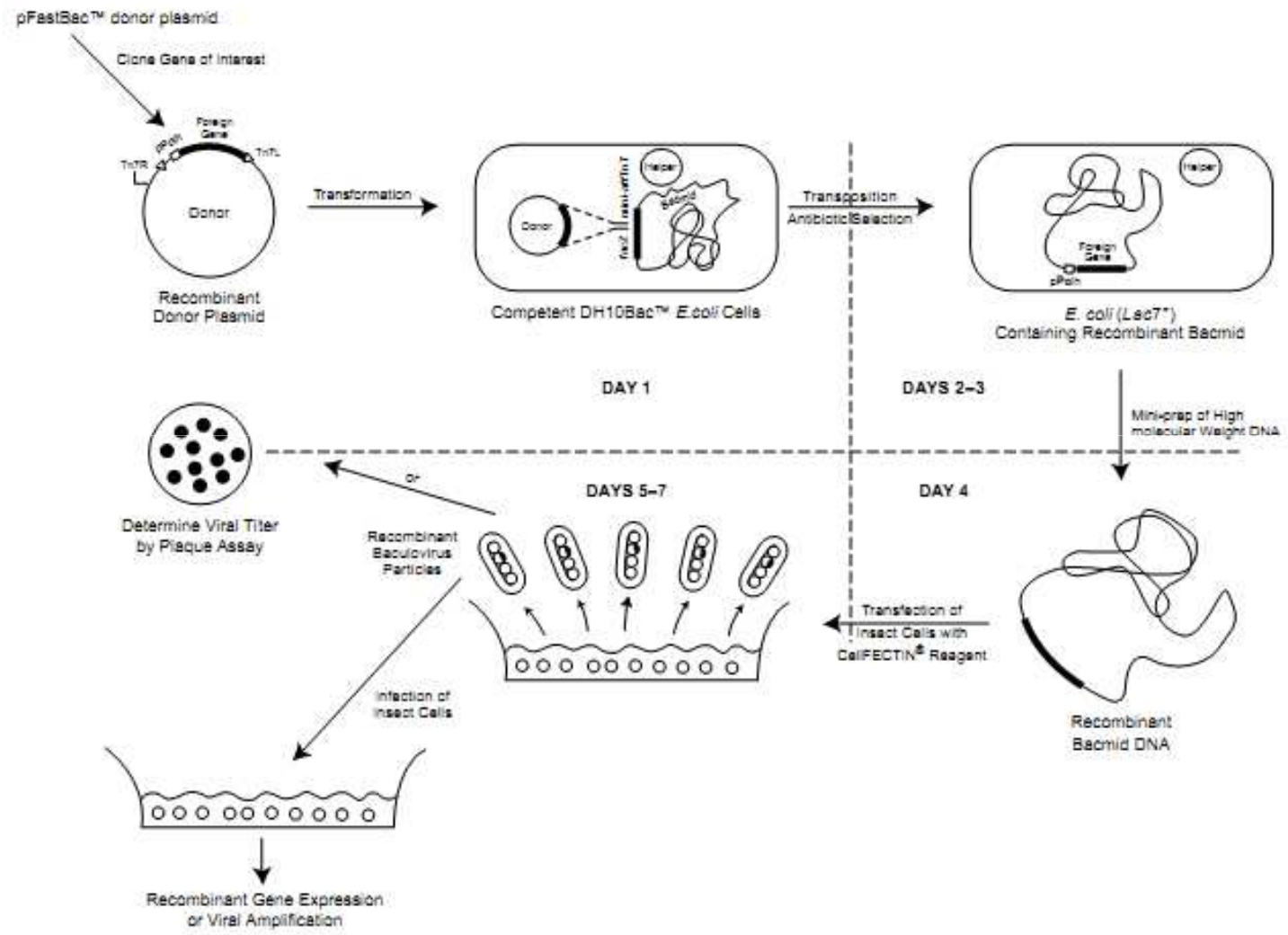


1- Soluble fraction
2- Wash1
3- Wash2
4- Elution

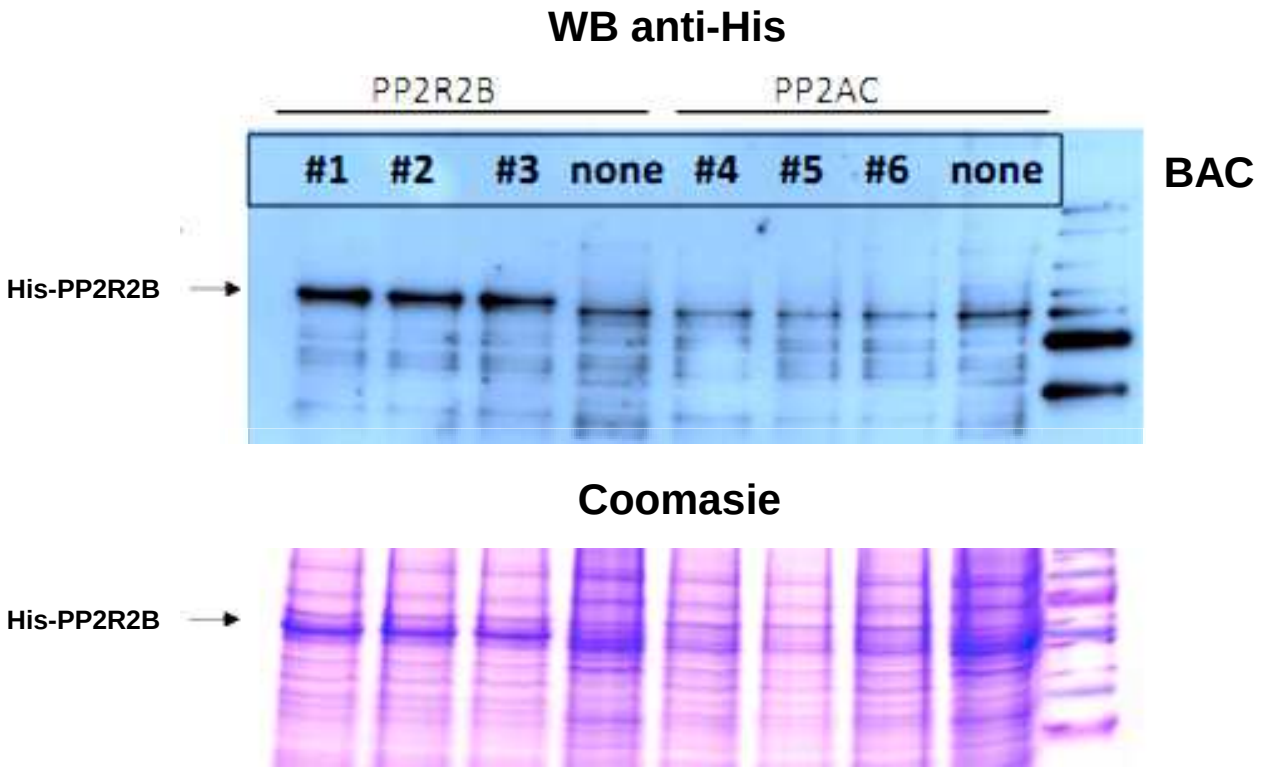
Protein is a contaminant from Pichia pastoris (a methanol inducible protein).
Similar results obtained when trying to express PP2R2B.

gi|254568544 **Mass:** 37318 **Score:** 416 **Matches:** 3(3) **Sequences:** 3(3)
Mitochondrial alcohol dehydrogenase isozyme III [Komagataella pastoris GS115]

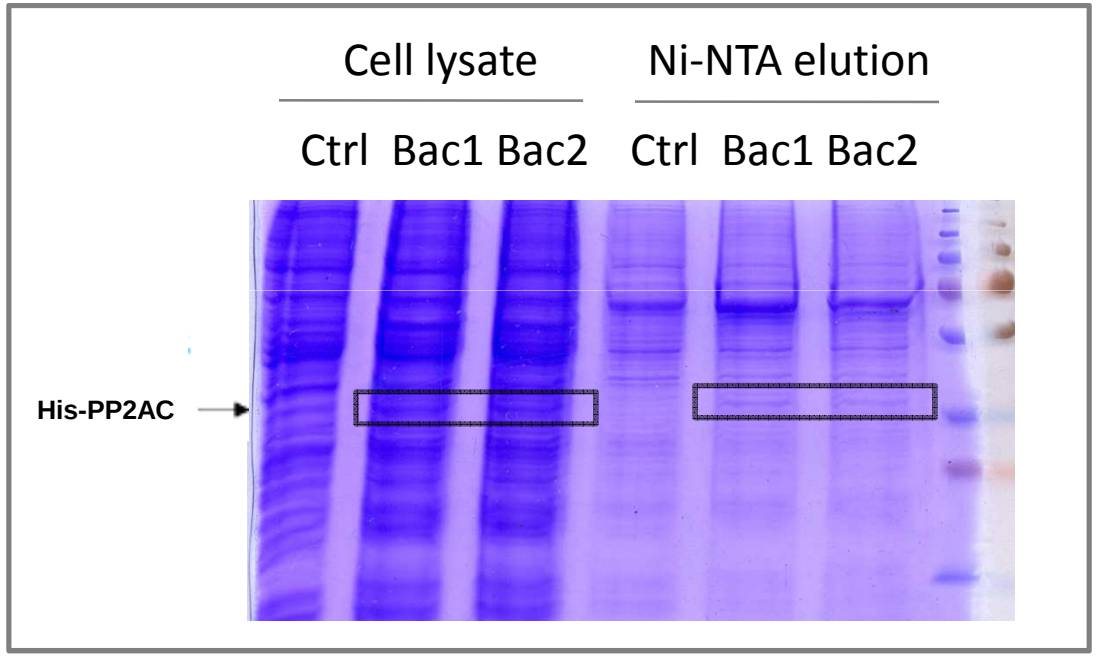
Baculovirus-mediated expression in Hi5 (insect) cells



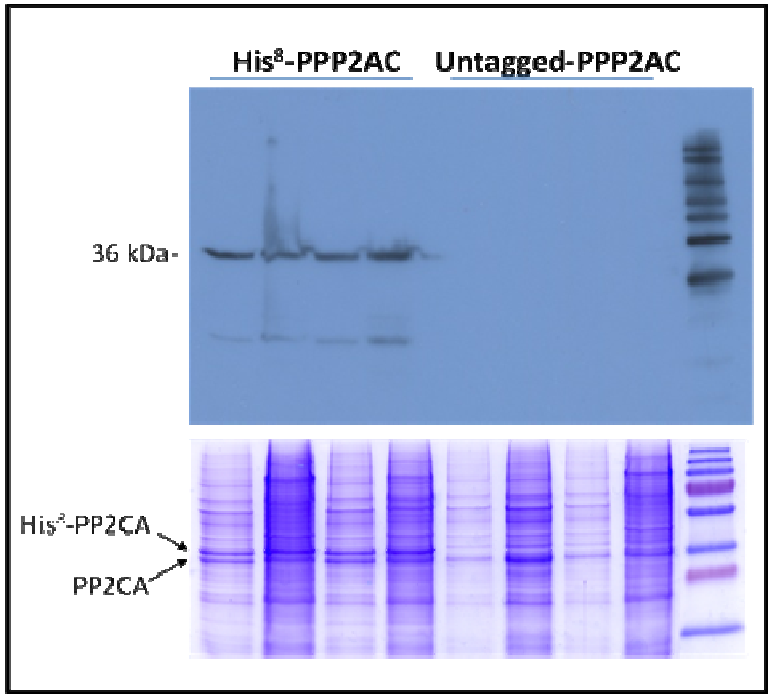
Expression of of PP2AC and PP2R2B in Hi-5 cells

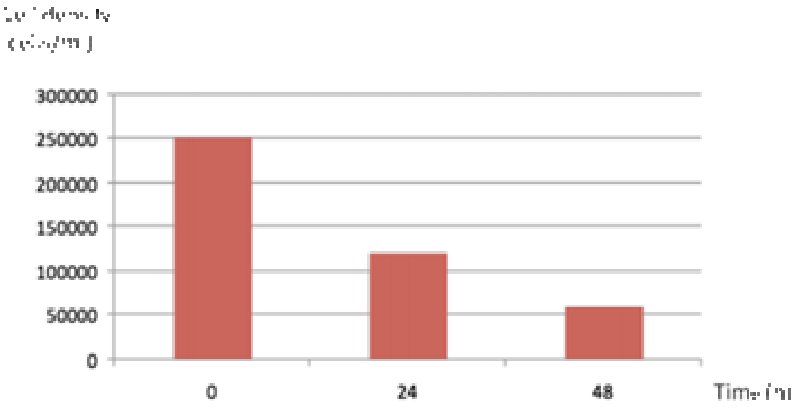


Expression of of PP2AC in Hi-5 cells

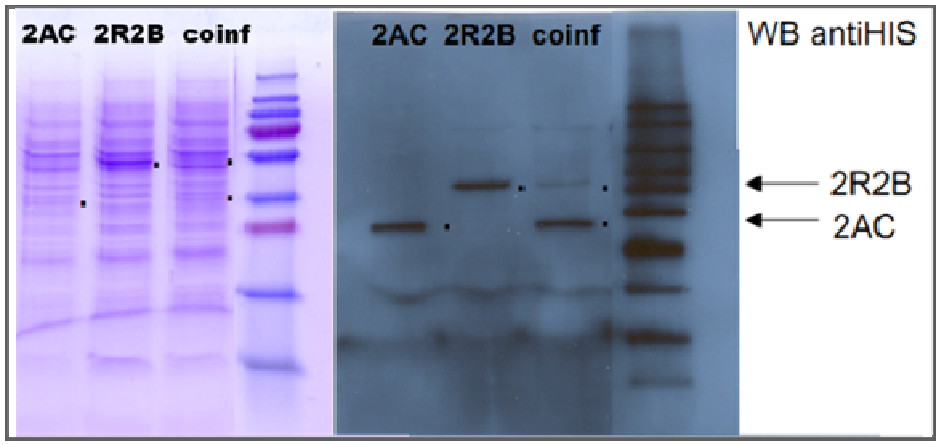


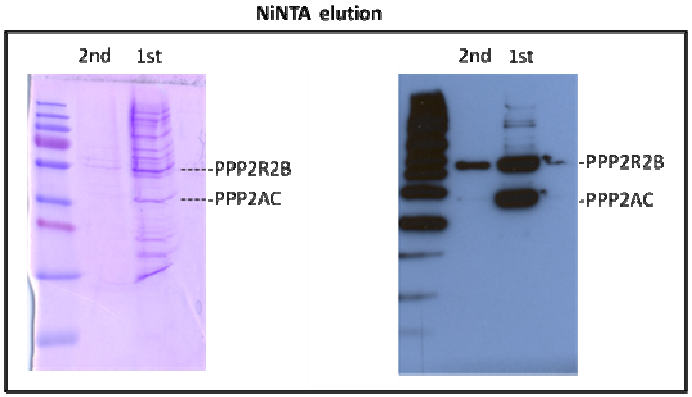
Expression of D88N mutants and untagged PP2AC in Hi-5 cells



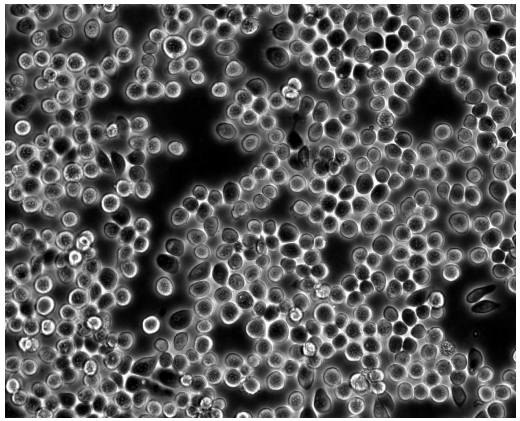


Co-expression of PP2AC and PP2R2B

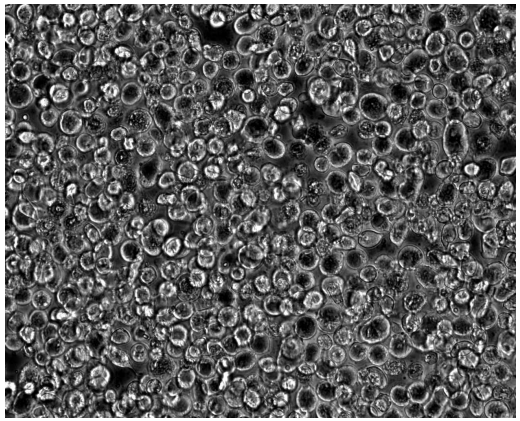




Expression of PP2AC in Sf-9 insect cells

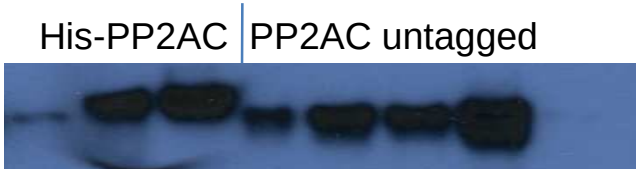


96h control not infected



96h infection 9His-2AC-D88N

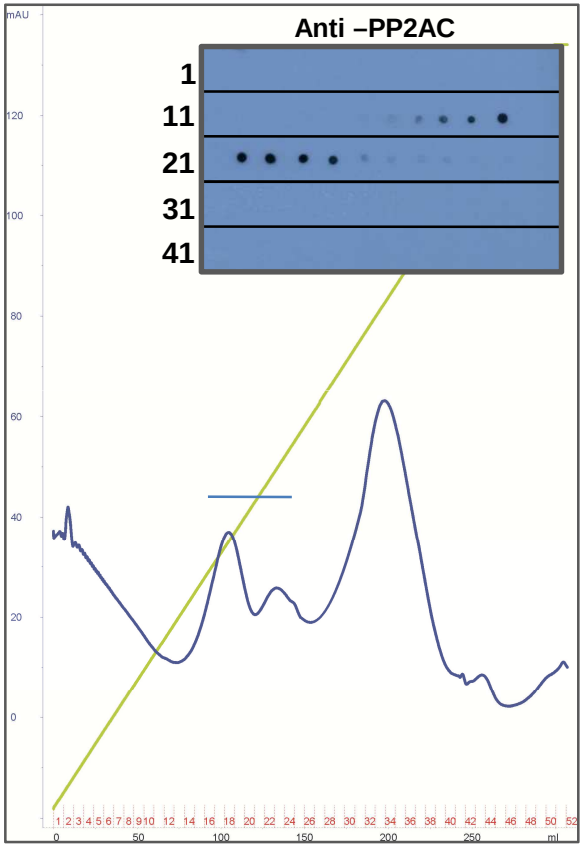
WB anti-PP2AC

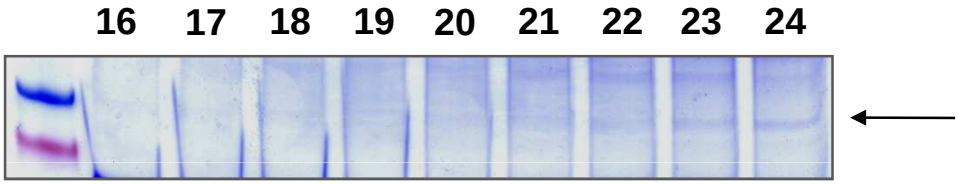


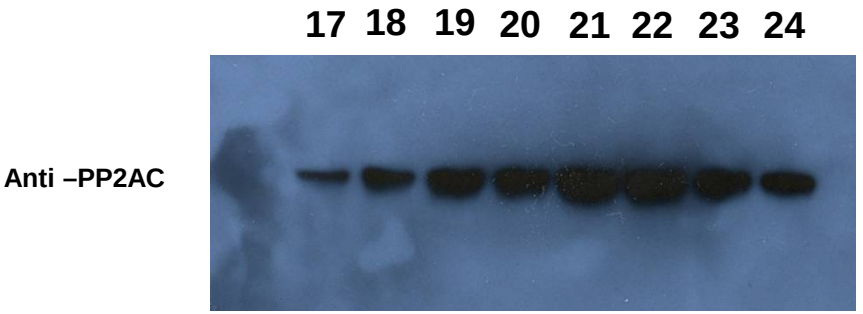
Yields were not improved with respect to Hi-5 cells.

New approach from endogenous source (bovine heart)

Ion Exchange Chromatography







Moderate amounts of endogenous PP2AC are obtained.

Target expression summary

	Expressed	Soluble	Available	Crystallized
ANP32A	**Yes (E.coli)**	**Yes**	**His-ANP $_{1-149}$** **His-ANP $_{1-164}$** **His-ANP $_{1-249}$** **ANP $_{1-149}$** **ANP $_{1-249}$**	**Spherulites** **ANP $_{1-249}$**
PP2R1A	Yes (E.coli)	Yes	His-GST-PP2R1A PP2R1A	
PP2R2B	Yes (E.coli) No (Pichia) Low (Insect)	No --- Yes	--- --- <300 ug	
PP2AC	Yes (E.coli) No (Pichia) Low (Insect) Low (endogenous)	?? --- Yes Yes	--- --- <100 ug <300 ug	
HEK293	...			
Schneider cells	...			

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<u>Collaborators</u>

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Dr. Ivelisse Sanchez

Proteolysis Lab