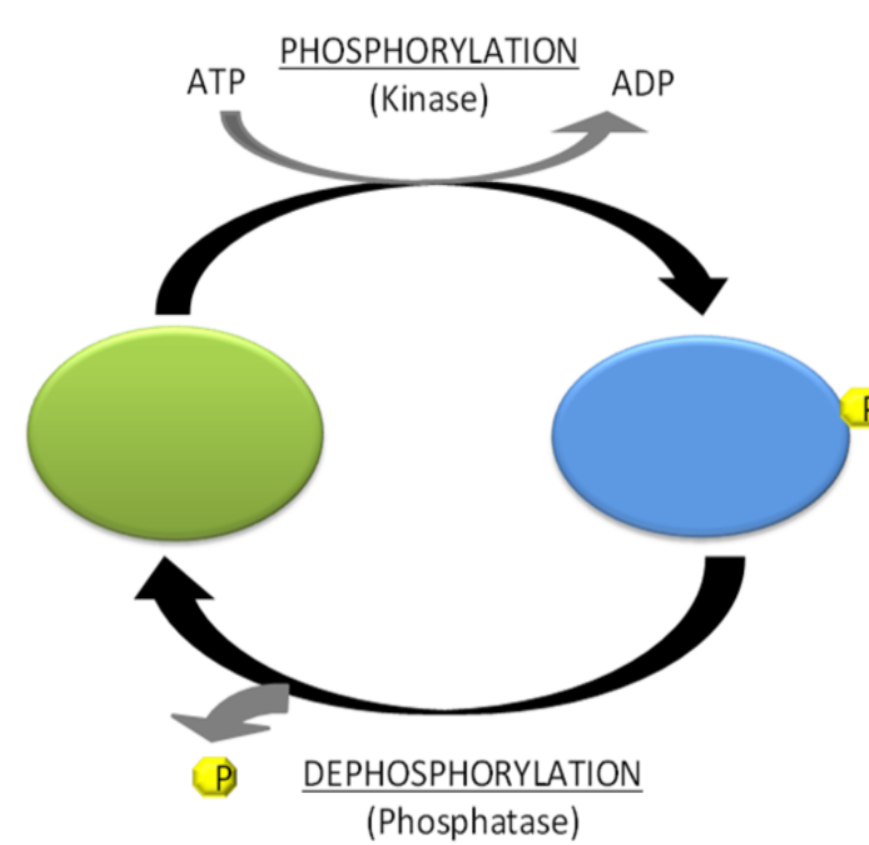


Introduction

Reversible phosphorylation by protein kinases and phosphatases has been established as a key regulator of many pathways in the cell. Over 400 protein kinases phosphorylate Ser/Thr residues in proteins, while only ~40 protein phosphatases dephosphorylate these same residues. How is it then, that so few phosphatases can counter the activity of a much larger number of kinases?

The answer lies in the mechanisms of regulation of the kinases and phosphatases themselves. While most Ser/Thr kinases have built-in substrate specificity (only phosphorylate a few substrates), the counteracting Ser/Thr phosphatases are non-specific on their own. Their specificity instead comes from regulatory proteins with which they associate in holoenzyme complexes. These "targeting subunits" direct the phosphatases to specific substrates in specific locations, at specific times.



PP1 Interactome

Our lab focuses on one such Ser/Thr phosphatase, protein phosphatase 1 (PP1). PP1 regulates various pathways of the cell and its activity is controlled by formation of regulated enzyme complexes with >200 different targeting subunits. Human cells express 3 isoforms of PP1, PP1 α , PP1 β and PP1 γ , which show distinct localization patterns throughout the entire cell (Fig. 1). Our lab studies these isoforms using a combination of live cell fluorescence imaging and quantitative mass spectrometry-based proteomics¹. Each PP1 isoform is tagged with a fluorescent reporter molecule and stably expressed at low levels in cultured cells. The dynamic localization of each isoform can be followed in live cells, and the proteins then pulled down so that we can identify proteins with which they interact². We are currently mapping the interactome of each PP1 isoform in specific cellular compartments.

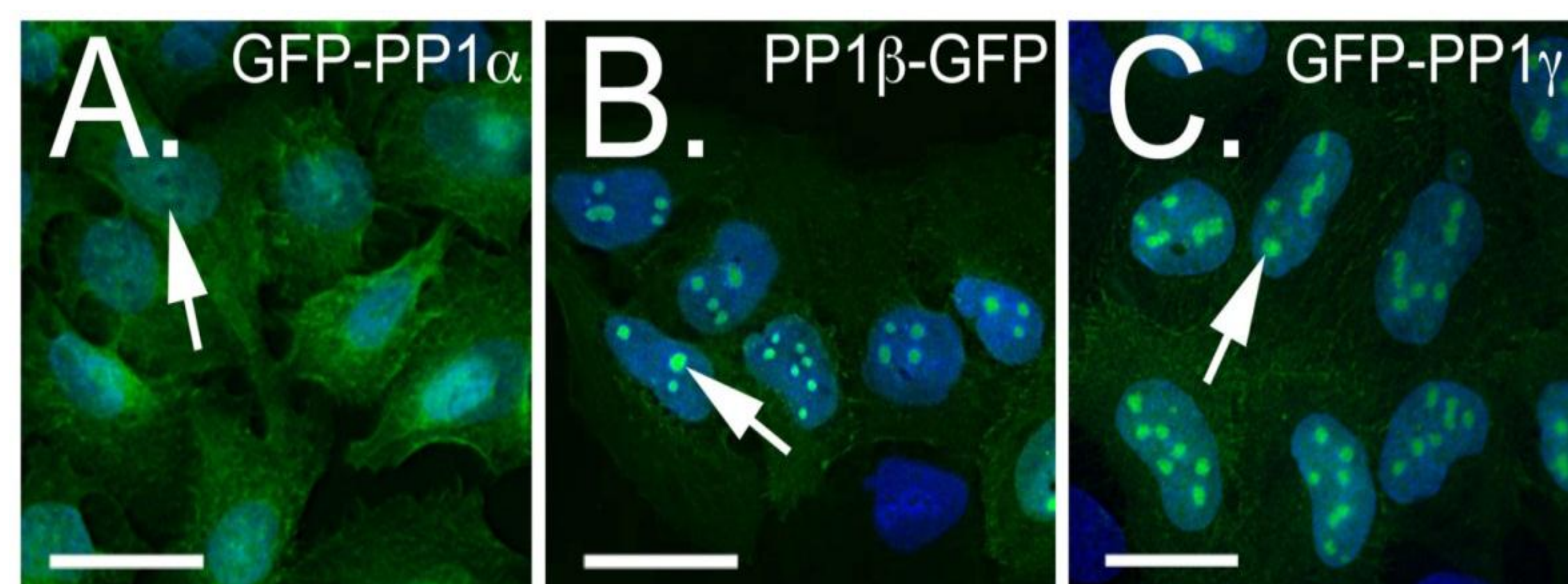


Figure 1. GFP-tagged PP1 isoforms stably expressed in U2OS cells. The arrows point to nucleoli (sites of ribosome synthesis). A. GFP-PP1 α , B. PP1 β -GFP, C. GFP-PP1 γ .

Consortin (c1orf71)

Using our quantitative proteomic approach, the protein consortin was found to associate with all three isoforms of PP1, primarily in the cytoplasm (although there is some evidence for a potential interaction in the nucleus). Little is known about this protein, with 3 isoforms (splice variants) predicted to be expressed in cells. Figure 2 shows a sequence alignment of the 3 isoforms, with the peptides detected in our PP1 interactome experiments highlighted in blue. Although most of the peptides we detected are found in more than one isoform, we did find two peptides specific to isoform 1 (which is the only isoform that contains the PP1 binding domain⁴).

One study in the literature has linked consortin to connexin recycling at the Golgi apparatus⁵. The authors observed that consortin binds to connexin 26 and 30 as well as adaptors on the Golgi apparatus, and that disrupting targeting of consortin to Golgi results in an intracellular accumulation of connexin. They did not, however, make the connection to PP1 binding. Our hypothesis is that consortin's role(s) within the cell involves recruitment of PP1 phosphatase activity.

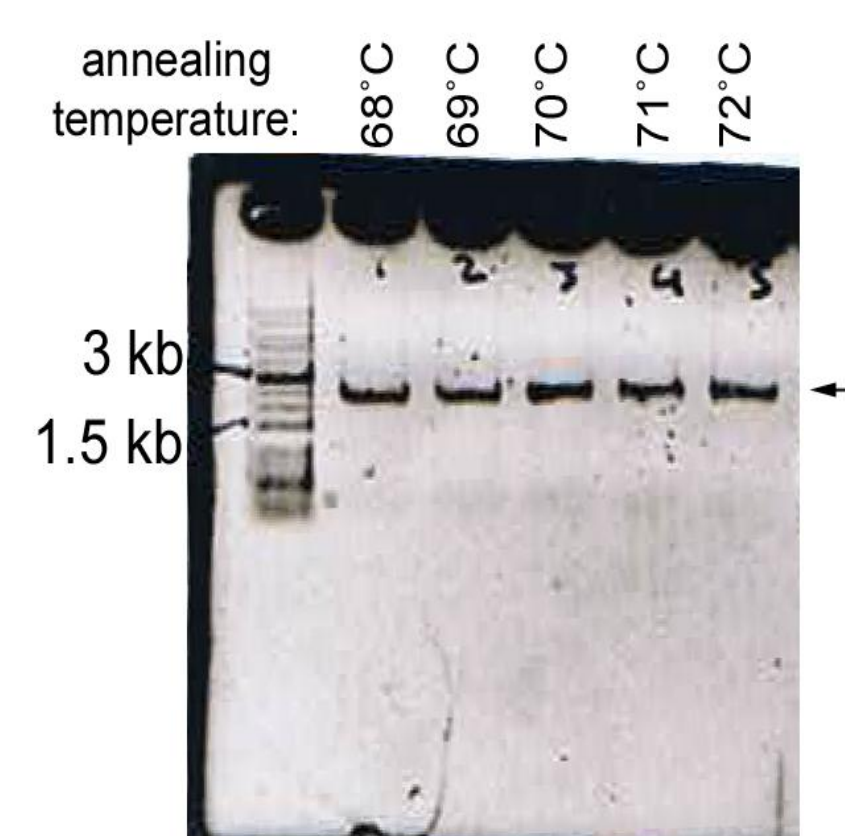
Approaches and Methods

Aim

The aim of my project is to validate Consortin as a PP1 regulatory subunit and compare two of its splice variants (isoforms 1 and 2). This will be done by cloning the genes and expressing the proteins in live cells with a fluorescent tag (GFP) to observe their localization and to demonstrate pulldown of endogenous PP1 specifically with tagged Consortin1.

PCR

To amplify the coding sequences of Consortin isoforms 1 and 2, we designed primers that would anneal specifically to the 5' and 3' ends of each sequence. The primers also contain the restriction sites Asp 718 (forward) and Bam H1 (reverse) for ligation into the multiple cloning site in the pEGFP-C1 vector.



A range of annealing temperatures were tested to optimize the PCR reaction. PCR products were run on agarose gels and the appropriate bands cut out and purified (Qiagen Gel Purification Kit). While we readily obtained a PCR product for isoform 2 (amplifying it from a full-length I.M.A.G.E. clone obtained from Open Biosystems), we have not yet been able to isolate isoform 1 by amplification from a cDNA library (no I.M.A.G.E. clone available). We are continuing to try to clone isoform 1.

Digestion and Ligation

For isoform 2, purified PCR product and pEGFP-C1 vector were digested with Asp 718 and Bam H1 restriction enzymes. To prevent the re-ligation of pEGFP-C1, digested ends were dephosphorylated using CIP (Alkaline Phosphatase, Calf Intestinal). Both vector and insert were purified using the Qiagen Gel Purification Kit and quantified using a Nanodrop spectrophotometer. Ligation of the insert and vector was carried out overnight at 16°C. A control reaction was also set up, containing only digested vector and no insert.

Consortin/Isoform	Sequence	Position
Consortin/Isoform1	MDDSDPTTYLQIEPQDCCHFGDSVRSVTCPLPSASDENQLDGDGHEHLTSSDSAMGX	60
Consortin/Isoform2	MDDSDPTTYLQIEPQDCCHFGDSVRSVTCPLPSASDENQLDGDGHEHLTSSDSAMGX	60
Consortin/Isoform3	MDDSDPTTYLQIEPQDCCHFGDSVRSVTCPLPSASDENQLDGDGHEHLTSSDSAMGX	60
Consortin/Isoform1	PQVSEQDNLNNSCTLCEVAAGENLQNTLCEASRDEQAFQKDKKIPKRSRPSKKGOT	120
Consortin/Isoform2	PQVSEQDNLNNSCTLCEVAAGENLQNTLCEASRDEQAFQKDKKIPKRSRPSKKGOT	120
Consortin/Isoform3	PQVSEQDNLNNSCTLCEVAAGENLQNTLCEASRDEQAFQKDKKIPKRSRPSKKGOT	120
Consortin/Isoform1	AKKIPPLPFGDIAPLMQKVLAVYAVDDEEAABVNAEQEPAKVLQSLFSLIRGE	180
Consortin/Isoform2	AKKIPPLPFGDIAPLMQKVLAVYAVDDEEAABVNAEQEPAKVLQSLFSLIRGE	180
Consortin/Isoform3	AKKIPPLPFGDIAPLMQKVLAVYAVDDEEAABVNAEQEPAKVLQSLFSLIRGE	180
Consortin/Isoform1	VEQLDGRALPLCLHQIASEYFQEDYERKMKFTQLERLVEHQLLANLSAIQBNQKWT	240
Consortin/Isoform2	VEQLDGRALPLCLHQIASEYFQEDYERKMKFTQLERLVEHQLLANLSAIQBNQKWT	240
Consortin/Isoform3	VEQLDGRALPLCLHQIASEYFQEDYERKMKFTQLERLVEHQLLANLSAIQBNQKWT	240
Consortin/Isoform1	VQHTVVALRNSKGFNGEDFERLTKICATHQDPLLSKHKIAAVEKSKERCSTQLLVE	300
Consortin/Isoform2	VQHTVVALRNSKGFNGEDFERLTKICATHQDPLLSKHKIAAVEKSKERCSTQLLVE	300
Consortin/Isoform3	VQHTVVALRNSKGFNGEDFERLTKICATHQDPLLSKHKIAAVEKSKERCSTQLLVE	300
Consortin/Isoform1	DPKEGATTKESSEKTKLCTESSEKSHQTVPLGSSPCCHQMDVQDSDPSLSTAGKDM	360
Consortin/Isoform2	DPKEGATTKESSEKTKLCTESSEKSHQTVPLGSSPCCHQMDVQDSDPSLSTAGKDM	360
Consortin/Isoform3	DPKEGATTKESSEKTKLCTESSEKSHQTVPLGSSPCCHQMDVQDSDPSLSTAGKDM	360
Consortin/Isoform1	EELLCSAEATLALHTQSSFTAGSPGDPSSDSEDSDSLQLAQTEACQVARIQIAED	420
Consortin/Isoform2	EELLCSAEATLALHTQSSFTAGSPGDPSSDSEDSDSLQLAQTEACQVARIQIAED	420
Consortin/Isoform3	EELLCSAEATLALHTQSSFTAGSPGDPSSDSEDSDSLQLAQTEACQVARIQIAED	420
Consortin/Isoform1	PKVFLSSKSTPELISFGCDRIIPALISEGKYQAQRKELRPLRDSALPTDQLENNE	480
Consortin/Isoform2	PKVFLSSKSTPELISFGCDRIIPALISEGKYQAQRKELRPLRDSALPTDQLENNE	480
Consortin/Isoform3	PKVFLSSKSTPELISFGCDRIIPALISEGKYQAQRKELRPLRDSALPTDQLENNE	480
Consortin/Isoform1	LNELQDPDLTSDGKSPQAQSDSDGSENVLCNNOISDLGILLPEVCMAPKEDKDDQGL	540
Consortin/Isoform2	LNELQDPDLTSDGKSPQAQSDSDGSENVLCNNOISDLGILLPEVCMAPKEDKDDQGL	540
Consortin/Isoform3	LNELQDPDLTSDGKSPQAQSDSDGSENVLCNNOISDLGILLPEVCMAPKEDKDDQGL	540
Consortin/Isoform1	NKETDYVNSLLECKLQTEDESLVENDQDDSDLLQDLSPFEASVLEQLMPSDESCLIS	600
Consortin/Isoform2	NKETDYVNSLLECKLQTEDESLVENDQDDSDLLQDLSPFEASVLEQLMPSDESCLIS	600
Consortin/Isoform3	NKETDYVNSLLECKLQTEDESLVENDQDDSDLLQDLSPFEASVLEQLMPSDESCLIS	600
Consortin/Isoform1	LDLAKRRIEAVNQR	660
Consortin/Isoform2	LDLAKRRIEAVNQR	660
Consortin/Isoform3	LDLAKRRIEAVNQR	660
Consortin/Isoform1	GGSCILLVLCIAVTVFLSVGQALYCFQGMDSVPVCTDFADNMDFVYKLGQVAREKH	720
Consortin/Isoform2	GGSCILLVLCIAVTVFLSVGQALYCFQGMDSVPVCTDFADNMDFVYKLGQVAREKH	720
Consortin/Isoform3	GGSCILLVLCIAVTVFLSVGQALYCFQGMDSVPVCTDFADNMDFVYKLGQVAREKH	720
Consortin/Isoform1	WYLS 725	
Consortin/Isoform2	----	
Consortin/Isoform3	----	

Figure 2. Alignment of the three predicted human consortin isoforms (splice variants).

Isoform 1 is 725 amino acids (2.175 kb) and MW 79.59 kDa. It contains the PP1 binding motif (amino acids 645-648, red box), a nuclear localization signal (amino acids 625-647) and a trans-membrane domain (amino acids 660-682).

Isoform 2 is 616 amino acids (1.848 kb) and MW 67.54 kDa.

Isoform 3 is 263 amino acids (0.789 kb) and MW 29.30 kDa.

Peptides detected in our PP1 interactome screens are highlighted in turquoise (found in all 3 isoforms), pale blue (found in isoforms 1 and 2) or dark blue (found only in isoform 1).

Transformation

Ligation reactions were transformed into DH5 α bacteria and plated on agar plates containing kanamycin. The pEGFP-C1 vector contains a kanamycin resistance gene, and therefore only bacteria that contain the circular plasmid should grow. Two control plates were run, one with the control vector alone ligation (no insert) and one with non-transformed bacteria. As expected, both showed very little growth.

To verify that the bacterial colonies did contain the construct rather than re-ligated empty pEGFP vector or contaminant bacteria, five colonies were chosen from plate containing the pEGFP/Consortin2 ligation reaction and grown for purification of plasmid DNA using the QIAGEN Miniprep kit. The isolated plasmids were digested with Asp 718 and BamH1 and migrated on an agarose gel. One of the colonies was found to contain the insert.

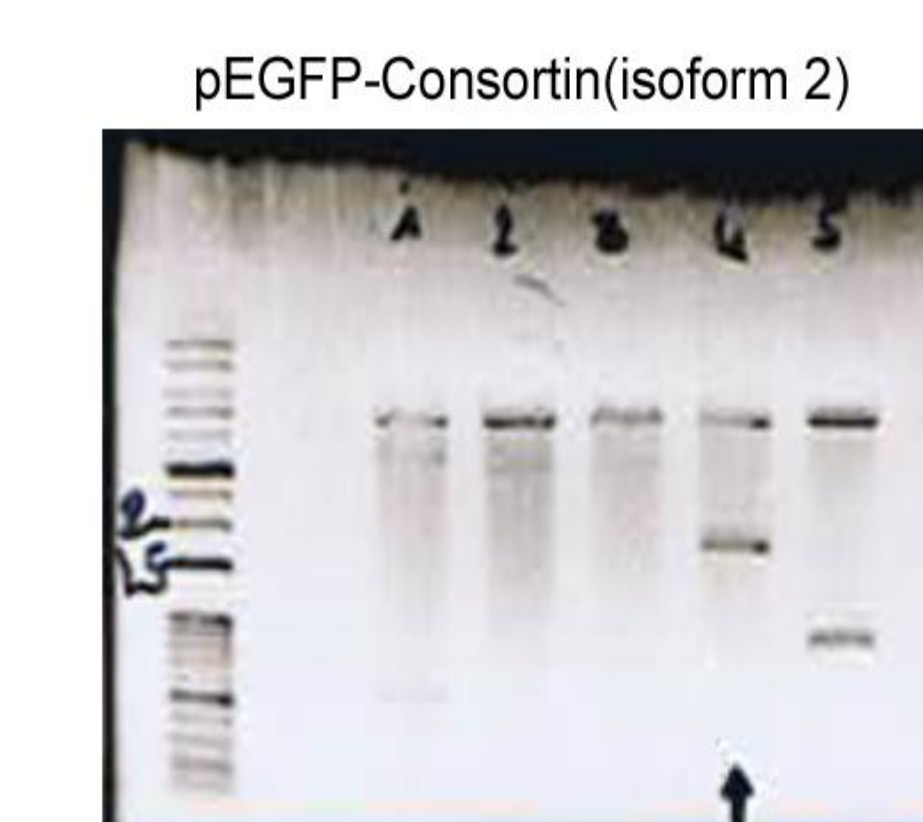


Figure 3. Image of agarose gel demonstrating the screening of pEGFP-consortin2 clones by restriction enzyme digest. The first column is the DNA ladder, there is a blank lane and then the next five lanes contain potential GFP-consortin2 clones. Of the chosen colonies, only colony 4 released an insert of the expected size (~1.8 kb), indicating that it is indeed pEGFP-consortin2 (which will be validated by sequencing). Colony 5 released a smaller insert, while colonies 1-3 contain re-ligated empty pEGFP-C1 vector.

Results and Future Research

Although we have so far been unable to clone isoform 1, we successfully cloned isoform 2 and expressed it transiently as a GFP-tagged protein by transfecting our pEGFP-Consortin2 plasmid into cultured HeLa cells (Fig. 4). Interestingly, the fusion protein is primarily located in the nucleus, with a weaker cytoplasmic signal. This isoform does not contain the nuclear localization signal present in isoform 1 (see Fig. 2), and therefore its localization to the nucleus may be due to interaction with other proteins.

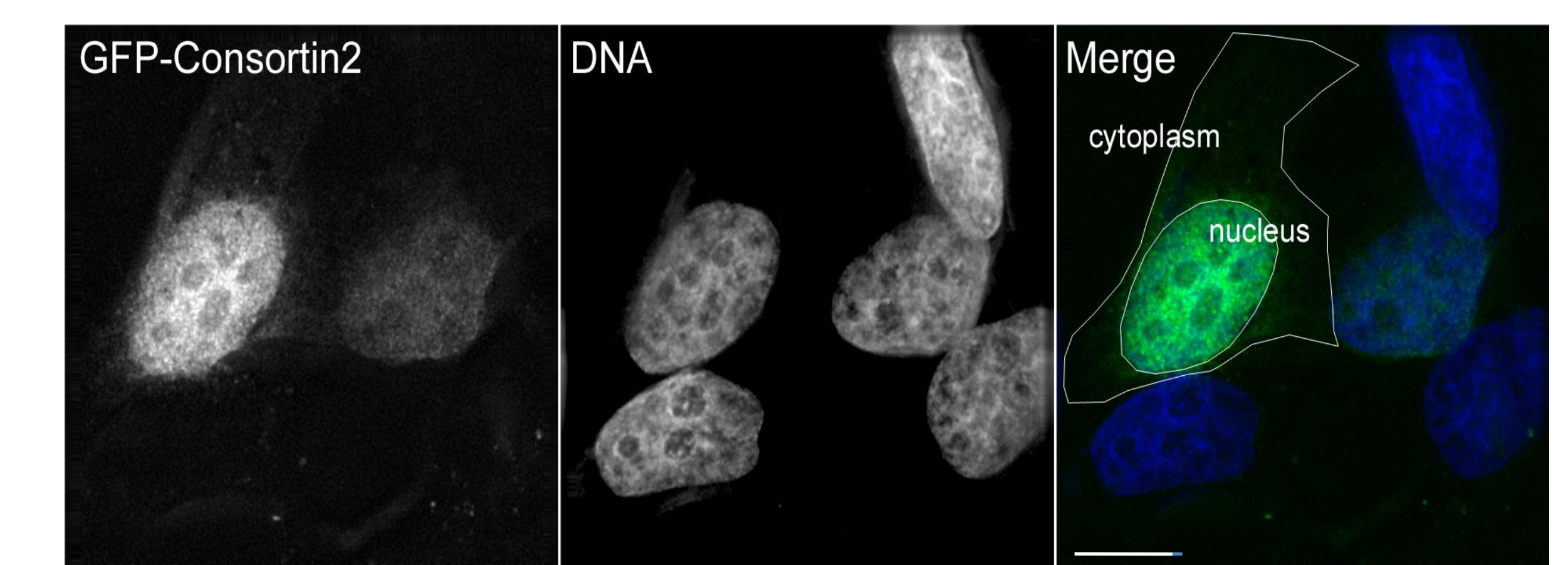


Figure 4. GFP-Consortin2 transiently expressed in HeLa cells. GFP-Consortin2 (green in the merged image panel) was imaged in paraformaldehyde-fixed cells using a DeltaVision CORE (Applied Precision) restoration fluorescence imaging system. As a marker for nuclei, DNA was stained using the dye Hoechst 33342 (blue in the merged image panel). Scale is 5 μ m.

Although frustrated by our problems in cloning isoform 1 from a cDNA library in the time available, we are intrigued by our initial results with isoform 2, which suggest that consortin's role within the cell may be more complicated than previously realized. We will continue to study both isoforms in parallel once we have cloned isoform 1.

Future research

We will continue our attempts to clone isoform 1, both from cDNA libraries and from nested expressed sequence tags (ESTs). Once we have cloned it we will:

1. Establish that it binds to PP1 via its RVxF motif (comparing it to isoform 2, which should not bind PP1)
2. Use site directed mutagenesis to alter the RVxF motif to RAXA and thereby create a non PP1-binding mutant of Consortin1
3. Compare the effect of wild type vs. mutant Consortin1 on Golgi-related activities, including connexin recycling
4. Compare the intracellular roles of Consortin1 and Consortin2

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