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N-terminus of pro-EMAP II regulates its binding with C-terminus, Arginyl-tRNA Synthetase, and Neurofilament light protein*

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*Running Title: Putative *Leucine-Zipper of EMAP II*

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Background: Functional domains of pro-EMAP II play an important role in Multi-Aminoacyl tRNA Synthetase (MSC) Complexes.
Results: Pro-EMAP II's N-terminus binds to its C-terminus, arginyl-tRNA synthetase and neurofilament light subunit, and contains a putative leucine-zipper.
Conclusion: N-terminus of pro-EMAP II facilitates its interaction with MSC complexes.
Significance: Understanding these binding domains may provide important insight into transcriptional regulation.

ABSTRACT

Pro-EMAP II, one component of the Multi-Aminoacyl tRNA Synthetase (MSC) Complex, plays multiple roles in physiological and pathological processes of protein translation, signal transduction, immunity, lung development and tumor growth. Recent studies determined that pro-EMAP II has an essential role in maintaining axon integrity in central and peripheral neural systems where deletion of pro-EMAP II's C-terminus was reported in a consanguineous Israeli Bedouin kindred suffering from Pelizaeus-Merzbacher-like disease. We hypothesized that pro-EMAP II's N-terminus had an important role in the regulation of protein-protein interactions. Using a GFP reporter system, we defined a putative leucine-zipper in the N-terminus of human pro-EMAP II protein (amino acid residues 1-70), which can form specific strip-like punctate structures. Through GFP punctate analysis, we uncovered that pro-EMAP II's C-terminus (147-312 amino acid residues) can repress the GFP punctate formation. Pull-down assays confirmed the binding between pro-EMAP II N-terminus and its C-terminus is mediated by a putative leucine-zipper. Furthermore, the pro-EMAP II 1-70 aa region was identified as the binding partner of the arginyl-tRNA synthetase (RARS), a polypeptide of MSC complex. We also determined that the punctate GFP pro-EMAP II 1-70aa aggregate co-localizes and binds to the neurofilament light (NFL) subunit protein that is associated with pathologic neurofilament network disorganization and degeneration of motor neurons. These findings indicate the structure and binding interaction of Pro-EMAP II protein and suggest a role of this protein in the pathological neurodegenerative diseases.

Endothelial Monocyte-Activating Peptide II (EMAP II) was initially isolated and identified as a 22kD pro-inflammatory cytokine secreted from a murine methylcholanthrene A-induced fibrosarcoma (1-4). Later studies confirmed that EMAP II was the C-terminus (147-312aa) of pro-EMAP II cleaved under apoptosis and by protease inhibitors (5-7). Pro-EMAP II, also known as p43, AIMP1 and Scye I, encodes one component of Multi-Aminoacyl tRNA Synthetase Complex (MSC) and is involved in protein translation. Mechanistically, the C-terminus of EMAP II has been determined to induce apoptosis in endothelial cells, inhibit angiogenesis and tumor growth through inhibition of vascular endothelial growth factor (VEGF) mediated signaling and

alpha5beta1 integrin mediated deposition of the extracellular matrix protein fibronectin (8-10). Expression and secretion of EMAP II is relevant to prognosis of some cancer patients and found to be involved in diverse biological process such as lung development, immune responses and glucose metabolism in pancreas (11-15). These imply the importance of EMAP II in the diagnosis and treatment of cancer and other diseases (16-20).

Intracellularly, pro-EMAP II directly interacts with other components of MSC such as p38, arginyl-tRNA synthetase (RARS) and glutaminyl-tRNA synthetase (QARS) (21-24). Such interaction plays an important role in the assembly and function of MSC. In addition, through C-terminus binding with E3 ubiquitin ligase Smurf2, pro-EMAP II stabilizes Smurf2 and down regulates the TGF-beta signaling pathway (25). Additional studies unveiled functional mechanisms and domains of pro-EMAP II protein (26,27). Park SG et al identified pro-EMAP II 's N-terminus 4-46aa region to be important in promoting fibroblast cell proliferation and wound repair (28). Pro-EMAP II was also determined to bind with heat shock protein HSP90B1/gp96, which plays critical role in innate and adaptive immunity (16,29). Based on sequence analysis, Guo M et al (2010) (30) introduced a novel concept that during evolution, the N-terminus of pro-EMAP II was preserved due to evolutionary pressure and the C-terminus gained cytokine functions through the accumulation of mutations and this may be the reason for the diverse functions of EMAP II.

Recently studies have determined some biological roles for pro-EMAP II. Zhu X et al (2009) (31) found that pro-EMAP II directly interacts with neurofilament light (NFL) chain and down-regulates neurofilament phosphorylation. Depletion of pro-EMAP II resulted in neurofilament network disorganization and degeneration of motor neurons. In 2010, pro-EMAP II truncation at the C-terminus was identified in consanguineous Israeli Bedouin kindred who suffered from Pelizaeus-Merzbacher-like disease (PMLD) (32,33) (34), indicating the requirement of further evaluation of the structure and functionality of pro-EMAP II in different biological processes. The role of pro-EMAP II in MSC, regulation of gene expression and modification, the interaction with other proteins and the functionality in different cell types requires further and detailed exploration. Here we established an approach to study the structure and function of pro-EMAP II using GFP punctate analysis and pull-down assay. We found that the N-terminus of pro-EMAP II can form specific strip like punctate structure. We characterized this structure as a putative leucine-zipper of pro-EMAP II. We also determined that the C-terminus of pro-EMAP II could bind with its N-terminus to repress punctate structure formation, and co-localizes to the neurofilament light subunit protein. Furthermore, our studies indicate that the 1-70aa region in the N-terminus of pro-EMAP II is responsible for its binding to the MSC subunits.

EXPERIMENTAL PROCEDURES

Cells, reagents and antibodies:

Human lung adenocarcinoma A549, Human Embryonic Kidney 293 and human neuroblastoma SH-SY5Y cells utilized in studies for neuronal function and differentiation. Cells were maintained in DMEM containing 10% fetal bovine serum and 5 mg/ml L-glutamine at 37°C in 10% CO_2. Human fetal kidney HEK293 cells were maintained in DMEM containing 5% fetal bovine serum at 37°C in 5% CO_2.

GeneExpresso™ 8000 In Vitro DNA Transfection Reagent was purchased from InnoVita Incoporation, Gaithersburg, MD; Lipofectamine 2000 and Hoechst 33342 from Invitrogen, Carlsbad, CA; TransIT LT 1 from Mirus Bio, LLC, Madison, WI, PS-341 from Millenium Pharmaceuticals, Cambridge, MA; Bredfeldin A, B-1080 Bafilomycin A1 (Baf A1) and Chloroquine were from Sigma-Aldrich St. Louis, MO and Bio-Rad Protein Assay Dye Reagent Concentrate was from Bio-Rad Laboratories, Inc., Hercules, CA. Disuccinyl suberate (DSS) was purchased from Thermo Fisher Scientific Rockford, IL and BL21 DE3 strain was purchased from Stratagene (Cat.230255). Antibodies against GFP were purchased from Invitrogen (cat#A11122) and Santa Cruz Biotecnologies (Santa Cruz, CA) (cat#sc-9996). Antibody against EMAP II was raised from rabbit in our lab (ref). Antibodies against GM130 (sc-55590) and GST (sc-33613) were purchased from Santa Cruz.

PCR cloning and detection:

For cloning, PCR reactions were carried out using high fidelity DNA amplification kit (Sigma-Aldrich) containing 10 pmol of each primer and 10 ng of DNA template. For RT-PCR and real-time PCR, total RNA was isolated from cells using Trizol reagent. Reverse Transcription was carried out using Superscipt III RT-PCR System according to manufacture's instruction (Invitrogen Corp). Oligonucleotides were

synthesized by Invitrogen and Sigma-Aldrich. N-terminus 1-102aa of RARS gene was amplified using oligos 5'-AACGAATTCAACCATGGACGTACTGGTGTCTGAGT-3' and 5'-CTTGTCGACCACTAGCAGAGGAGGATTTTCCA-3'. N-terminus 1-108aa of human QARS gene was amplified using oligos 5'-AACGAATTCAACCATGGCGGCTCTAGACTCCCTGT-3' and 5'-CTTGTCGACCTCGAAGTCCACAGTGTCGAT-3'.

Plasmid Construction:
The EMAP II plasmid was used as a template for amplification of N-terminus, C-terminus and truncation of human pro-EMAP II using oligonucleotides (Supplemental Table 1). PCR fragments were digested and cloned into vector pEGFP-N3 for overexpression of GFP fusion protein. Site-directed mutation was performed using overlapped oligonucleotides for PCR. In brief, N-terminus and C-terminus fragments containing mutations were amplified respectively. The PCR fragments were combined and used for PCR template. Site-directed mutated fragments were inserted into pEGFP-N3 vector. PCR cloned fragments were also cloned into vector pET28a(+) and pGEX-4T-3 for overexpression of His- or GST-tagged protein in *E. coli*. DNA sequencing and/or restriction digestion analysis was used to confirm plasmid sequence. Reporter plasmid EYFP-GalT, Lamp1-YFP, pEGFP-C1-wtVHL, EGFP-supervillin and DsRed-rab7 WT were purchased from Addgene (Cambridge, MA).

Transfection and stable clones:
Cells were seeded in 24-well plates or in chamber slides 24 hours prior to transfection. A549 cells were transfected using reagent TransIT LT1 or GeneExpresso^{TM} 8000. HEK-293 cells were transfected using Lipofectamine 2000 according to manufacture's protocol. Analysis for GFP punctate formation was performed on live cells. Cells were observed and photographed after 20 hours under fluorescence microscopy. 100-200 GFP positive cells were analyzed per experiment and these were confirmed with using 3-6 replicates from independent experiments performed on different occasions. For drug treatment experiments, final concentration of 5 mg/ml BFA, 50 nM Baf A1, 1 mM DTT, 10 nM PS-341, 50 mM chloroquin or PBS was applied to cells 1 hour after transfection. For immunofluorescence analysis, cells were treated with 10 mg/ml of BFA or PBS for 4h, or treated with 10 nM of PS-341 or PBS for 8h prior to further analysis. For stable clones of A549 or SH-SY5Y, cells were selected in complete medium containing 800 μg/ml or 600 μg/ml of G418 for 2 weeks. Single colonies were picked up for expanding cultures. Expression of GFP-fusion protein was confirmed using fluorescence microscopy and Western blotting analysis.

Cross-linking using DSS:
Transfected cells were harvested and washed three times with PBS. Cells were then suspended in PBS at 1 X 10^7 cells/ml and incubated with 1 mM DSS for 20 min. Reactions were quenched by adding Tris-HCl buffer (pH 7.5) to a concentration of 20 mM and incubated for 15 min. Cells were then pelleted, rinsed, suspended in 1 X SDS loading buffer, and sonicated to disrupt sticky genomic DNA prior to being subjected to SDS-PAGE analysis.

Immunofluorescence:
Cells were rinsed with PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. After blocking in CAS solution, cells were incubated with the specific primary antibody, followed by specific secondary FITC or Cy-3 conjugated secondary antibodies. Cells were washed with PBS for 4 times and mounted with SlowFade Gold Antifade Reagent with DAPI (Invitrogen). Fluorescent Microscope was used for imaging analysis.

Immunoblot analysis:
Cells were lysed at 4°C in RIPA buffer or 1 X lysis buffer (50 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM DTT, and 1X protease inhibitor cocktail). Lysate was cleared by centrifugation at 4°C at 13,200rpm for 20 minutes. The protein concentration was measured using BioRad Protein Assay reagents according to manufacture's manual. Equal amounts of protein were subjected to SDS-polyacrylamide gel and blotted to PVDF membrane. The blots were blocked in 5% non-fat milk, incubated with primary antibody, followed by the appropriate secondary antibody. Specific signals were measured using a detection kit from Amersham Biosciences.

Pull-down assay:
Pull-down assays were performed using ProFound^{TM} Pull-Down PolyHis Protein:Protein Interaction Kit

(Pierce). Briefly, BL21 DE3 competent *E. coli* were transformed with plasmids constructed in vector pET28a(+) and pGEX-4T-3 and fusion proteins were induced with 2 μg/ml IPTG. Following rinsing with TBS at 4°C, pellets were suspended in Profound lysis buffer and incubated for 30 min on ice. The crude *E. coli* lysate was clarified through 12,000xg centrifugation, the supernatant was collected and used as bait or prey protein for pull-down assay. When PolyHis-tagged proteins were used as bait protein, 40 mM final concentration of Imidazole was used in binding and washing buffers and proteins were eluted with buffer containing 290 mM of Imidazole.

Flow cytometry sorting and mass spectrometry:
SH-SY5Y stable clone cells overexpressing 1-70aa of pro-EMAPII fused with GFP were trypsinized for harvesting. After washing with PBS, cells were suspended in lysis buffer containing 1% Triton X-100 and rotated at 4°C for 30 min, followed by centrifugation at 1,000 rpm for 10 min. Pellets were suspended in lysis buffer and washed again. Pellets were then re-suspended in lysis buffer and sonicated. Supernatant was transferred to new tubes after centrifugation at 1,500 rpm for 10 min and sorted by flow cytometry as previously described (35). Enrichment of GFP punctates were confirmed under microscopic observation. GFP punctates were pelleted down after centrifugation at 5,000 rpm for 10 min. Pellet was suspended in 1X SDS loading buffer, boiled, and subjected to 12 % SDS-PAGE and run into separation gel about 1 cm. The gel was stained and destained according to Coomassie blue staining protocol. The gel was cut out and analyzed by mass spectrometry.

Statistical Analysis:
Prism software was used to perform all statistical analyses. All results are expressed as mean ± SEM. The significance of differences between two sample means was determined by unpaired two-tailed Student's *t* tests using 95% confidence intervals. A p value less than 0.05 were considered significant. Water solubility was determined using software at http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp. Coiled-coil structures were identified using Lupas software at http://www.ch.embnet.org/software/COILS_form.html.

RESULTS

Overexpression of truncated isoforms of the pro-EMAP II protein uncovered an N-terminus 1-70aa region responsible for the aggregation of EMAP II punctate formation

Although much is known regarding the activities and functions of mature C-terminus EMAP II, little is understood about the functions of its intracellular precursor form pro-EMAP II protein. A549 cells overexpressing the full-length pro-EMAP II (1-312aa) fused with GFP were noted to have dotted-like GFP punctate structure in <1% of GFP-positive cells (Figure 1B). Multiple truncations of the C and N terminus regions of EMAP II were utilized to identify the region of pro-EMAP II responsible for the punctate formation. We determined that the 1-70 amino acid region in the N-terminus promoted the GFP punctate formation as 45.2% of GFP positive cells contained GFP punctate formation ($p<0.01$ compared to full-length pro-EMAP II, Figure 1D, 1J) as compared to 0% GFP punctate formation in truncated 99-312aa (Figure 1I, 1J) and 13% GFP punctate lesions in pro-EMAP II 1-192aa (Figure 1C, 1J). Furthermore, truncation of the pro-EMAP II protein to 1-60aa abolished the GFP punctate formation (Figure 1E, 1J). When 21aa (22-70aa) (Figure 1F, 1J), 38aa (39-70aa) (Figure 1G, 1J) or 58aa (59-70aa) (Figure 1H, 1J) of the N-terminus of pro-EMAP II were truncated, GFP punctate ratio dramatically decreased ($p<0.01$ comparing to 1-70aa truncation). These results suggested that the entire 1-70aa region of pro-EMAP II is essential for GFP punctate formation.
In addition to the quantitative amount of GFP punctation, profound morphological change of GFP punctate was also observed in transfection with 1-70aa truncations. In transfection with full-length pro-EMAP II, GFP punctate was dotted-like in structure and peri-nuclei as shown in the left panel of Figure 1K. In contrast, the pro-EMAP II 1-70aa truncation expressed marked variation of punctate formation that was much stronger and strip-like shaped as shown in Figure 1L-S, however no branches in the strip-like GFP punctate pattern were observed. Similar results were observed in transfection of HEK293 (Figure 2A-C, G) and SH-SY5Y neuroblastoma cells (Figure 2D-G) using different transfection reagent such as Lipofectamine 2000, TransIT LT1, Fugene 6, GeneExpresso™ 8000 (data not shown). No punctate lesions were noted in the C-terminus EMAP II 147-312aa cells (data not shown).

Analysis of GFP-punctate formation uncovered the role of leucine-like residues in 1-70aa region of pro-EMAP II.

Across species, the 1-70aa region of pro-EMAP II (AIMP1) is highly conserved during evolution (Figure 3A), implying the importance of N-terminus of pro-EMAP II. To investigate the function of the putative acetylation sites of pro-EMAP II's 1-70aa region, ten lysine residues were identified and stripe-replacement was performed to replace lysine residues with alanine residues found between amino acid 8 to 11 (Figure 4D) and 46 to 63 (Figure 4C). Transfection analysis showed no significant difference in GFP punctate formation between alanine replacements (Figure 4C and 4D) and wild type (Figure 4B) (Figure 4Q).

We next turned our attention to the hydrophobic amino acids leucine and isoleucine residues within this region that have frequently been identified in protein–protein interactions and binding recognition sites for hydrophobic ligands respectively. Site-directed replacement of leucine or isoleucine residues with alanine residue at amino acid I21→A, L25→A, L31→A, L32→A, I37→A, L38→A, L42→A, L55→A, I59→A, and L62→A abolished GFP punctate formation (Figure 4E,G-O,Q). However, while replacement of isoleucine residues with an alanine residue at amino acid I22→A showed no significant difference (Figure 4F), exchange of a leucine for an alanine at amino acid L66→A showed a significant decrease in GFP punctate formation comparing with wild type (Figure 4P,Q). These results suggested leucine and isoleucine residues are important in the GFP punctate formation of 1-70aa pro-EMAP II.

Regions within the C-terminus of pro-EMAP II were determined to induce repression of the GFP punctate formation and induce morphologic GFP punctate alterations.

Overexpression of full-length pro-EMAP II markedly decreased the GFP punctate formation as compared with the 1-70aa isoform. To define the region within the pro-EMAP II protein that repressed its GFP puncate formation, serial internal truncation were performed. Internal truncation of pro-EMAP II between amino acids 71 - 253 dramatically increased the amount of GFP punctate formation while truncation of pro-EMAP II between amino acids 71-157, 71-203 and 71-94 did not significantly impact GFP punctate formation (Figure 5A, B). Further internal truncations between amino acids 204-253 were performed to identify the region within the amino acid span of 204-253 that impacted the GFP punctate formation of pro-EMAP II. We determined that the internal truncation of 71-233aa resulted in a 1.8 fold increase in GFP punctate formation ($p<0.01$), as compared 71-223aa truncation, indicating that the amino acid region between 203-233 of pro-EMAP II represses GFP punctate formation (Figure 5C, D).

To further narrow the repressing region, fragments of C-terminus of pro-EMAP II were linked with 1-70aa region through a 15aa flexible peptide linker [$(GGGGS)_3$] (Figure 5F) (36). Optimization of codon pair use within the (GGGGS)3 linker sequence is known to enhance protein expression. Transfection experiments containing C-terminus pro-EMAP II fragments 147-170aa, 181-210aa or 200-230aa resulted in a respective marked 3.2-, 4.5- or 7.0-fold decrease of GFP punctate formation as compared with 1-70aa alone, implying that multiple regions within the C-terminus have repressive effects on GFP punctate formation (Figure 5F). In addition to the amount of punctate protein formation, a change in morphology of GFP punctate was also observed in A549 cells transfected with full-length pro-EMAP II, while internal truncation at 71-203aa resulted in a dot-like GFP punctation (Figure 5E). However, internal deletion of the 71-212aa span induced a small strip-like change to the GFP punctate structure while further internal deletions of 71-223aa, 71-233aa, 71-253aa resulted in strong and strip-like GFP punctate structure. Analysis of pro-EMAP II identified three potential coiled-coil structures, 1M-80FI, 108G-148K, and and 199-219R with these sequences extending more than 35 residues suggesting a probability of >80-90% that they will assume a coiled-coil structure (Figure 3B).

The 1-70aa region of pro-EMAP II binds with its C-terminus.

To examine if 1-70aa region of pro-EMAP II binds with itself, HEK293 cells were transfected with GFP control and 1-70aa fused with GFP. Cells were cross-linked using DSS. Western blotting analysis determined that multiple bands of 35kD increment were observed in 1-70aa transfection treated with DSS, but not in GFP control and untreated cells (Figure 6A). This suggested that the 1-70aa region of pro-EMAP II fused with GFP might form a polymer structure within cells. Using his-tagged 1-70aa as bait and GST-tagged pro-EMAP II fragments recombinant protein as prey (Figure 6B), a his-tag pull-down assay was performed to determine binding between the N and C terminus regions of pro-EMAP II. As shown in Figure 6C, his-tagged 1-70aa

strongly pulled down GST-tagged 1-70aa and GST-tagged C-terminus of pro-EMAP II (146-312aa), but not GST control, GST tagged 74-146aa or 1-70aa mutant. These experiments revealed the binding of 1-70aa N-terminus region to the C-terminus of pro-EMAP II.

EMAP II's N-terminus region 1-70aa is a binding partner of the MSC complex RARS but not QARS:
Previous reports support the N-terminus region of pro-EMAP II as a component of the MSC where it has been identified as binding to itself as well as to p38, RARS, QARS and the leucine zipper of AIMP2. To establish the binding domain of two of the key MSC proteins, RARS and QARS, we examined their ability to bind to the pro-EMAP II N-terminus region extending from 1-70aa. Using his-tagged 1-70aa of pro-EMAP II as bait and recombinant GST-tagged RARS and QARS N-terminus as prey, we determined that pro-EMAP II pulled down GST-tagged RARS (1-102aa) but not GST control or GST-QARS (1-108aa) (Figure 7A). Point-mutations of pro-EMAP II at I21->A, I37->A, L38->A, I21->A I22->A L25->A (Figure 7B and 7C) defined the RARS binding region to be dependent on the leucine at amino acid 42 and 55 as pro-EMAP II mutant L42->A L55->A did not immunoprecipitate with RARS (Figure 7B, C). Furthermore, co-transfection of 1-70aa of pro-EMAP II fused with GFP or DsRed reporter resulted in two-colored co-localized punctate structure (Figure 7E), whereas of GFP empty vector control and DsRed fused with 1-70aa did not co-localize (Figure 7D). Punctate RARS protein expression was dependent on the presence of EMAP II 1-70aa (Figure 7G) as overexpression of RARS (1-74aa) fused with GFP alone showed no punctate (Figure 7F).

GFP Punctate pro-EMAP II 1-70aa are cytoplasmic.
In order to better understand the role of the 1-70aa pro-EMAP II punctate formations, we examined its pattern of intracellular distribution in conjunction with known organelle markers. Live staining of transfected cells with nuclei stain Hoechst 33343 indicated that 1-70aa GFP punctate was not nuclear (Figure 8A,B,C). Although, the addition of a nuclear localizing signal (NLS) fused to the 1-70aa and GFP partitioned the 1-70aa GFP fusion protein to the nucleus, no GFP punctate lesions were observed in nuclei (Figure 8D). On account of the cytoplasmic partitioning of the 1-70aa pro-EMAP II GFP punctate lesions, we examined whether the Golgi apparatus, lysosomes, aggresomes or endosomes markers co-localized with these punctate lesions. Due to a significant lack of effective organelle antibodies, co-transfection experiments were performed. Co-transfection of 1-70aa GFP with the late endosome marker Rab7 fused with DsRed (Figure 8E across upper panels), with Golgi marker GalT (Figure 8F across middle panels) or lysosomal marker lamp1 fused with YFP showed no punctate co-localization (Figure 8G across lower panels). These studies were validated by examination of purified punctate lesions and subsequent mass spectrometry analysis of binding partners isolated and bound to the punctate lesions. Cross-linking assays revealed no interaction between the GFP punctate pro-EMAP II 1-70aa formations and known organelle markers (data not shown). While treatment of cells with BFA to induce the disassembly of Golgi apparatus does not alter the 1-70aa GFP punctate formation (Figure 8H-M), consistent with the above observation that 1-70aa GFP punctate does not co-localized with Golgi apparatus or the nuclei**.** Similarly, the 1-70aa GFP punctate did not co-localize with the aggresome markers Vimentin and Ubiquitin even in the presence of the proteasome inhibitor PS-341 (data not shown). Furthermore, treatment of cells transfected with the mutant L66->A that demonstrates reduced punctate formation using PS-341, did not increase GFP punctate formation (data not shown).

Insoluble 1-70aa pro-EMAP II GFP punctate formation binds to NFL.
To determine binding partners associated with the 1-70aa GFP punctuated formation, GFP punctate lesions were isolated following transfection of 1-70aa pro-EMAP II into neuroblastoma SH-SY5Y cells. Our initial observations determined that these punctate formations were predominately insoluble as the 1-70aa GFP was detected in insoluble fraction while GFP control was detected only in supernatant by Western blot analysis (Figure 9A). This was further supported by microscopic fluorescent analysis that the 1-70aa GFP punctate formations were observed in cell debris after lysis in buffer containing 1% Triton X-100 and intensive wash (Figure 9B). Following a brief sonication, 1-70aa GFP punctate was released from debris into supernatant, pelleted and underwent flow cytometry enrichment. Mass spectrometry analysis of the enriched 1-70aa GFP punctate formations and their binding partners determined that they bound NFL polypeptide with 11 peptide spectrum matches. Immunoprecipitation from cells transiently transfected with 1-70aa GFP EMAP II N-terminus confirms binding of NFL with the pro-EMAP II N-terminus (Figure 10E). As mass

spectrometry analysis and previous reports indicate co-localization of pro-EMAPII and the neurofilaments found in neurons and neuroblastoma cells SH-SY5Y, co-transfection with 1-70aa GFP and DsRed-NFL were undertaken. Human NFL subunit gene was cloned and fused with DsRed. Transfection of the DsRed-NFL in A549 cells and SH-SY5Y cells induced a punctate formation (Figure 10A-D). Furthermore, co-transfection of DsRed-NFL and with either the 1-70aa (Figure 10A,C) or full-length pro-EMAP II fused (Figure 10B,D) with GFP resulted in co-localization and co-punctate lesions supporting an interaction between 1-70aa region of pro-EMAP II and NFL fibers (Figure 10A-D). C-terminus 146-312aa EMAP II C-terminus does not immunoprecipitate with NFL (Figure 10E).

DISCUSSION

Pro-EMAP II is abundantly expressed in all cell types and is a component of the multi-enzyme assemblage of eleven polypeptides that form the essential link to the structural organization of the mammalian cells cytoplasmic translation apparatus, the MSC complex. Based on bioinformatical analysis, pro-EMAP II protein was predicted to have of an N-terminus and C-terminus (37). The N-terminus contains a coiled-coil structure, which may play an important role in protein-protein interaction. Furthermore, the N-terminus of pro-EMAP II has been reported to interact with its own N-terminus, p38, RARS, QARS and leucine zipper of AIMP2 (21,23,24,38) suggesting that the N-terminus may mediate the association of pro-EMAP II to MSC. However, the structure and function of N-terminus of pro-EMAP II remains obscure. Through GFP punctate analysis and pull-down assay, we showed that the truncation of pro-EMAP II to 1 to 70aa resulted in strong and specific strip like punctate structure that was mediated by the leucine-like residues within the 1-70aa region of pro-EMAP II. In addition to the self-binding of the 1-70aa region, we determined that it also binds the C-terminus of pro-EMAP II through a putative leucine zipper motif and is the region that is the binding partner of the MSC complex polypeptide RARS but not the QARS. We also determined that the punctate GFP pro-EMAP II 1-70aa aggregate co-localizes and binds to the NFL subunit protein. These findings show that the 1-70aa region of the pro-EMAP II N-terminus is responsible for the binding of MSC subunits and may be the region of the N-terminus of pro-EMAP II that facilitates the interaction of the MSC complex with RNA-binding.

Transcriptional regulation is dependent on the flexibility and interaction of the participating protein complexes. Leucine zippers play an important role in this process by mediating protein-protein interactions and transcriptional regulation where they serve as folding triggering sequences in coiled-coil structures (39) (40). Pro-EMAP II, a component of the MSC complex, functions as a cofactor of aminoacyl-tRNA synthetases whose involvement is as a general RNA binding domain (41). We hypothesized that pro-EMAP II contained a putative leucine zipper that would allow it greater flexibility in the mediation of protein-protein interactions. Using mutation analysis, we showed that specific leucine or isoleucine residues are critical for GFP punctate formation, but abundant lysine residues have little effect. Our cross-linking and pull-down assay results showed that the 1-70aa region of pro-EMAP II interacts with itself, the C-terminus of pro-EMAP II and N-terminus RARS. Importantly, some leucine mutants abolished the binding with RARS suggesting the relevance of the leucine binding regions. Thus, our data provide detailed information to support that N-terminus of pro-EMAP II functions as a putative leucine zipper, which is consistent with previous prediction and other reports (38).

In contrast to the GFP punctate lesions seen following overexpression of the 1-70aa pro-EMAP II, not all leucine-zipper-like domains of the MSC proteins have a similar expression pattern. For example, although RARS is one component of MSC and contains a leucine-zipper-like domain at the N-terminus, overexpression of RARS leucine zipper domain showed no GFP punctate structure. This implies the specific characteristics of the putative leucine zipper of pro-EMAP II. The underlying mechanism of GFP punctate formation remains unclear. Cross-linking analysis and pull-down analysis suggest protein-protein interaction and protein polymerization may play some role in forming strip like structure. Co-transfection experiments suggest the punctate structure is not co-localized with the markers of aggresome, Golgi apparatus, lysosome or endosome. BFA treatment experiments also suggest GFP punctate formation is not relevant to ER pathway. Our data showed 1-70aa GFP colocalizes with NFL. This is consistent with the finding of colocalization and interaction of pro-EMAP II with NFL (31), which implies GFP punctate formation may be related to neurofilament structures.

Internal interaction of proteins is critical for its proper and effective folding and maintenance of a proteins functional structure (42). Protein misfolding and aggregation may play an important role in the pathological process of disease processes such as neurodegenerative diseases. Fore example, in transfection experiments with full-length of pro-EMAP II, only rare GFP punctate was observed compared with the transfection with the N-terminus 1-70aa GFP. However, presence of the C-terminus of pro-EMAP II induced a repressive effect on GFP punctate formation. These findings suggested that the C-terminus of pro-EMAP II might fold back and bind with its N-terminus region to cover up the active moiety of putative leucine zipper. Through truncation of C-terminus, we were able to expose the putative leucine zipper resulting in the GFP punctate formation. On the other hand, folding up between N-terminus and C-terminus may also prevent the exposure of an active C-terminus moiety. In our experiments to establish stable clones, we could easily obtain clones overexpressing 1-70aa GFP but it was difficult to obtain stable clones overexpressing C-terminus EMAP II fused with GFP (data not shown). Previous studies in our laboratory also showed that the overexpression of C-terminus of pro-EMAP II inhibits cell proliferation (43). These data suggest that the N-terminus putative leucine zipper plays an important role in maintenance of proper soluble structure status and function of full-length pro-EMAP II, which are the results of natural selection during evolution.

Although the mechanism of GFP punctate formation is complicated, it is the results of specific or nonspecific protein internal interaction or protein-protein interactions. As we know, most native proteins are soluble in vivo. When proteins are truncated or mutated, spatial hindrance will be removed and aggregates and other specific punctate structures may form. As shown in our experiments, overexpression of pro-EMAP II resulted in small dotted like punctate structures in a few cells. Gradual truncation of C-terminus of pro-EMAP II not only increases the ratio of GFP punctate formation, but also resulted in strong and long strip like punctate structure, mimicking the process of gradual exposure of a putative leucine zipper of pro-EMAP II to environment following gradual removal of its C-terminus. Our experiments suggest that GFP punctate analysis can be used as one of the powerful tool to dissect protein structural information.

Feinstein M et al (33) found truncation of C-terminus of pro-EMAP II in consanguineous Israeli Bedouin kindred, who suffer from a severe axonal disease. Loss of C terminus may be the cause of the disease. To our notice, the 1-97aa of N-terminus of pro-EMAP II remains intact in these patients with C-terminus truncation. In brain, pro-EMAP II is highly expressed. In our experiments, truncation of C-terminus resulted in strong strip like punctate formation. It is possible that such kind of punctate structure could be found in the neurons of these patients. Our data in SH-SY5Y human neuronal cells that have been widely studied as *in vitro* models of neuronal function, differentiation, (44-46) and in Parkinson's disease (47-49), we show co-localization of 1-70aa GFP with NFL. This is consistent with the report that pro-EMAP II co-localizes and binds with neurofilament. Knock-out mice of pro-EMAP II show axon development defect in motor neurons (31). These findings suggest that pro-EMAP II may play an important role in axon assembly. It is well known that protein aggregation plays an important role in many neurodegenerative diseases such as Alzheimer disease. If punctate formation or exposure of the putative leucine zipper occurs within these disease process, one may consider their potential role in disease progression as our experiments show that even one amino acid mutation abolishes GFP punctate formation. This implies the possibilities to alter protein structure through targeting the key amino acid residues leading to prevention and treatment of neurodegenerative diseases.

In conclusion, we have established an approach to study the structure and function of pro-EMAP II using GFP punctate analysis and pull-down assay. Utilizing this technique, we determined that the N-terminus of pro-EMAP II can bind with its C-terminus to repress punctate structure formation, has a putative leucine zipper, is the region responsible for the binding of MSC subunits, and co-localizes to the NFL subunit protein These findings indicate the structure and binding interaction of pro-EMAP II protein and suggest a role of this protein in the pathological neurodegenerative diseases.

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FOOTNOTES

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FIGURE LEGENDS:

FIGURE 1: Regulation of GFP punctate structure formation EMAP II aggregation by the 1-70aa N-terminus region of pro-EMAP II in A549 cells. Compared to empty vector alone, transfection of full-length pro-EMAP II (1-312aa) (B) fused to a GFP expression vector demonstrated a <1% of dotted-like GFP punctate structures as compared to empty vector alone (A). Analysis of GFP punctate formation in multiple truncated forms of pro-EMAP II (B-I, arrows) was performed in live cells. Calculation of percentage of GFP punctuated cells revealed that the presence of the 1-70aa region (D- arrows, J) was critical for this formation (J) as most constructs resulted in minimal to no punctate formation (J, construct aligned with graph representing percentage of GFP punctate cells found in transfected GFP positive cells with each construct). Morphologic variation of GFP punctate structures with strip-like regions of variable lengths (arrows) were identified in cells transfected with 1-70aa truncation (L-S) as compared to the consistent perinuclear punctate lesions seen in the full-length pro-EMAP II transfected cells (K, arrow). Representative image shown and analysis were performed on an n=100-200 cells/experiment and each experiment were repeated 3-6 times on different occasions. Magnification: A-I: 100x K-S: 200X

FIGURE 2: HEK293 and SH-SY5Y neuroblastoma cells exhibit GFP aggregates when transfected with 1-70aa N-terminus region of pro-EMAP II. Following transfection with 1-70aa pro-EMAP II, GFP punctation was visualized in HEK293 cells and SH-SY5Y cells (G, arrows C and F), respectively) as compared to empty vector alone (A, D), or the C-terminusterminus pro-EMAP II (no punctate lesions seen, data not shown) in living cells. Representative image shown and analysis were performed on an n=100-200 cells/experiment and each experiment was repeated 3-6 times on different occasions. Magnification: 200X. (B) Calculation of percentage of GFP punctated cells in SH-SY5Y cells.

FIGURE 3: Region 1-70aa of pro-EMAP II (AIMP1) is highly conserved across species and is predicted to have a Coiled-coil Structure. Evaluation of the N-terminus of pro-EMAP II determined that the region is highly conserved in evolution (A). Using the Lupas Software, three potential coiled-coil structure in pro-EMAP II protein were identified, 1M-80FI, 108G-148K, and 199L-219R with these sequences extending more than 35 residues suggesting a probability of >80-90% that they will assume a coiled –coil structure (B).

FIGURE 4: Leucine-like residues in the 1-70aa region of pro-EMAP II have a role in GFP-punctate formation. Stripe replacement of lysine residues with alanine residues (lysine mut in C and 8-11aa mut in D) had no impact on GFP punctate lesions in A549 cells transfected with these constructs as compared to wild type 1-70aa GFP (B) control. In contrast, replacement of the hydrophobic leucine or isoleucine residues with alanine resides at I21 →A (E), L25→A (G), L31→A (H), L32→A (I), I37→A (J), L38→A (K,Q), L42→A (L), L55→A (M,Q), I59→A (N), and L62→A (O) abolished GFP punctate formation. While exchange of a leucine for an alanine at L66→A reduced GFP punctate formation (P,Q). Site-directed mutagenesis of I22→A showed no significant difference in GFP punctation (F). Magnification: 100X.

FIGURE 5: C-terminus region of pro-EMAP II suppresses the GFP punctate formation and alters GFP punctate morphology. A549 cells transfected with constructs of serial internal truncations between amino acids 71-253 of pro-EMAP II markedly increased GFP punctate formation (A, B). Additional internal truncations between 204-253 increased GFP punctate formation (C, D) with the elimination of the 71-233 having a significant impact over 71-203 suggesting the 203-233 aa region of pro-EMAP II represses GFP punctate formation (C,D). Decreases in GFP punctate formation in the truncated forms of pro-EMAP II was associated with marked morphologic changes where internal deletion of the 71-212aa span induced a small strip-like change, while further internal deletions of 71-223aa, 71-233aa, 71-253aa resulted in strong and strip-like GFP punctate structure (E). Using a flexible linker, transfection with c-terminus pro-EMAP II fragments 147-170aa, 181-210aa or 200-230aa decreased GFP punctate formation by 3.2, 4.5, and 7 fold as compared to 1-70aa alone (F). Magnification: 100X- A,C, 200X- E.

FIGURE 6: N-terminus region of pro-EMAP II binds with its C-terminus. (A) Cross-linking of HEK293 cells transfected with 1-70aa fused with GFP resulted in multiple bands at 35kD increments in Western blot analysis as compared to non-crosslinked and empty GFP vector controls. (B) Multiple fragments of GST-tagged pro-EMAP II recombinant protein were designed and used as prey, with his-tagged 1-70aa pro-EMAP II recombinant protein as bait. Following isolation, GST tagged 1-70aa and 146-312aa pro-EMAP II were found to bind strongly to his-tagged 1-70aa pro-EMAP II, but not with GST control, GST tagged 74-146aa or 1-70aa mutant (B). (C) Input recombinant pro-EMAP II protein isolates are shown by Comassie Blue stain.

FIGURE 7: Pro-EMAP II N-terminus region 1-70aa is a binding partner of the MSC complex RARS. (A) Recombinant His tagged 1-70aa pro-EMAP II protein pulled down GST-RARS but not GST-QARS or GST alone. (B) Using His-RARS as bait, point mutations of pro-EMAP II determined that the RARs binding was dependent on the leucine at amino acid position 42 as mutation of this aa (L42->A) inhibited pull down of GST-L42->A 1-70aa pro-EMAP II. Recombinant input shown below. (C) Similarly, mutation of the leucine at amino acid position 55 (L55->A) of 1-70aa pro-EMAP II inhibited its pull down with RARS. Recombinant input shown below. Co-transfection of pro-EMAP II 1-70aa-GFP with DsRed-1-70aa pro-EMAP II resulted in co-localization of the intracellular punctate regions (E). Similarly, RARS (1-74aa)-GFP forms co-localized punctate intracellular regions when co-transfected with DsRed-1-70aa pro-EMAP II (G). In contrast, transfection with RARS(1-74aa)-GFP alone had no punctate lesions (F) and GFP empty vector with DsRed -1-70aa pro-EMAP II resulted only DsRed-1-70aa pro-EMAP II punctation without co-localization (D). Magnification: 100X (D-G).

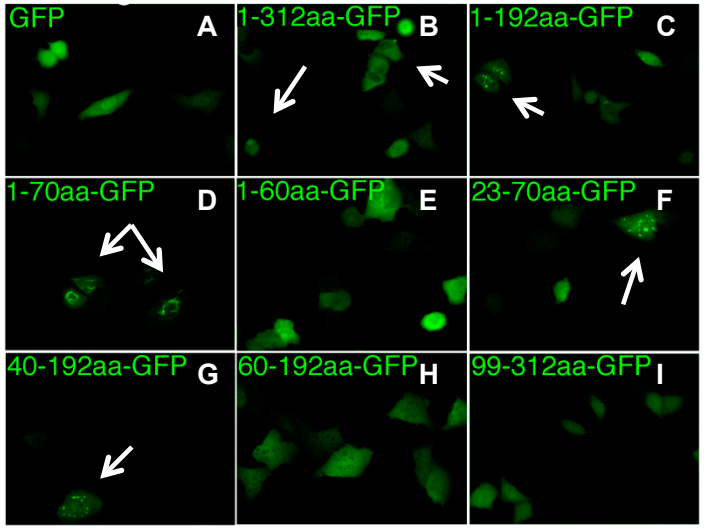
FIGURE 8: The N-terminus 1-70aa pro-EMAP II punctate lesions are cytoplasmic. The GFP punctate lesions in A549 cells transfected with 1-70aa GFP pro-EMAP II did not co-localize with DAPI staining and were noted to be cytoplasmic (A,B,C). Addition of a nuclear localizing sequence (NLS) to the 1-70aa GFP pro-EMAP II construct resulted in nuclear localization but lacked punctate formation in nuclei (D). 1-70aa GFP pro-EMAP II cytoplasmic punctate lesions were not associated with the endosome marker Rab7 (E), Golgi marker GalT (F), or the lysosomal marker lamp1 (G). BFA treatment disassembled the Golgi apparatus (K,M) as compared to untreated control (J,L), but didn't alter 1-70aa GFP pro-EMAP II punctate formation (H,I,L,M) or promote interaction between these formations and the disassembled Golgi (M). Magnification: 100X E-G, 200X C,D, 400X A,B,H-M.

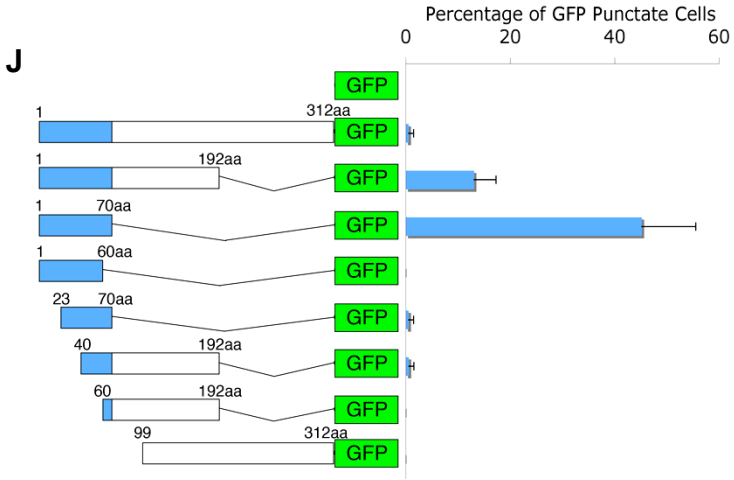
FIGURE 9: Isolation of insoluble 1-70aa pro-EMAP IIGFP punctate formation using flow cytometry enrichment. GFP punctate lesions were isolated from neuroblastoma SH-SY5Y cells transfected with 1-70aa pro-EMAP II. (A) Western analysis of the supernatant and pellet determined that the 1-70aa GFP was predominately found in the insoluble fraction (pellet) in contrast to the GFP vector control that was found only in the supernatant. (B) Systematic lysis with 1% triton X-100, sonication, pelleting and flow cytometry enrichment of the GFP punctate formations was performed and evaluated by light microscopy. Magnification: 100X

FIGURE 10: Punctate formations of 1-70aa and 1-312aa GFP pro-EMAP II co-localize and immunoprecipitates with NFL. Cells were co-transfected with either 1-70aa or 1-312aa GFP pro-EMAP II and DsRed- human NFL gene. Co-localization and co-punctate lesions were identified in cells co-transfected with DsRed-NFL and either the 1-70aa GFP (A,B) and 1-312aa GFP pro-EMAP II (B,D). Cell lysate from using transient transfected with 1-70aa GFP EMAP II, 1-312aa GFP pro-EMAP II, 146-312aa GFP EMAP II, or empty

vector were immunoprecipitated with GFP antibody. Western blot analysis using an NFL antibody confirms binding of the NFL with the EMAP II N-terminus domain and pro-EMAP II, but not the C-terminus (E) (IP with empty vector and control cell lysate was negative, data not shown). Magnification: 100X

Figure 1





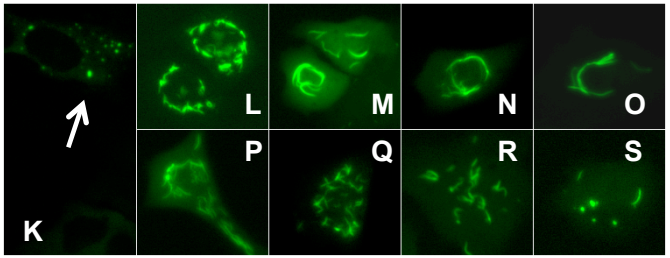


Figure 2

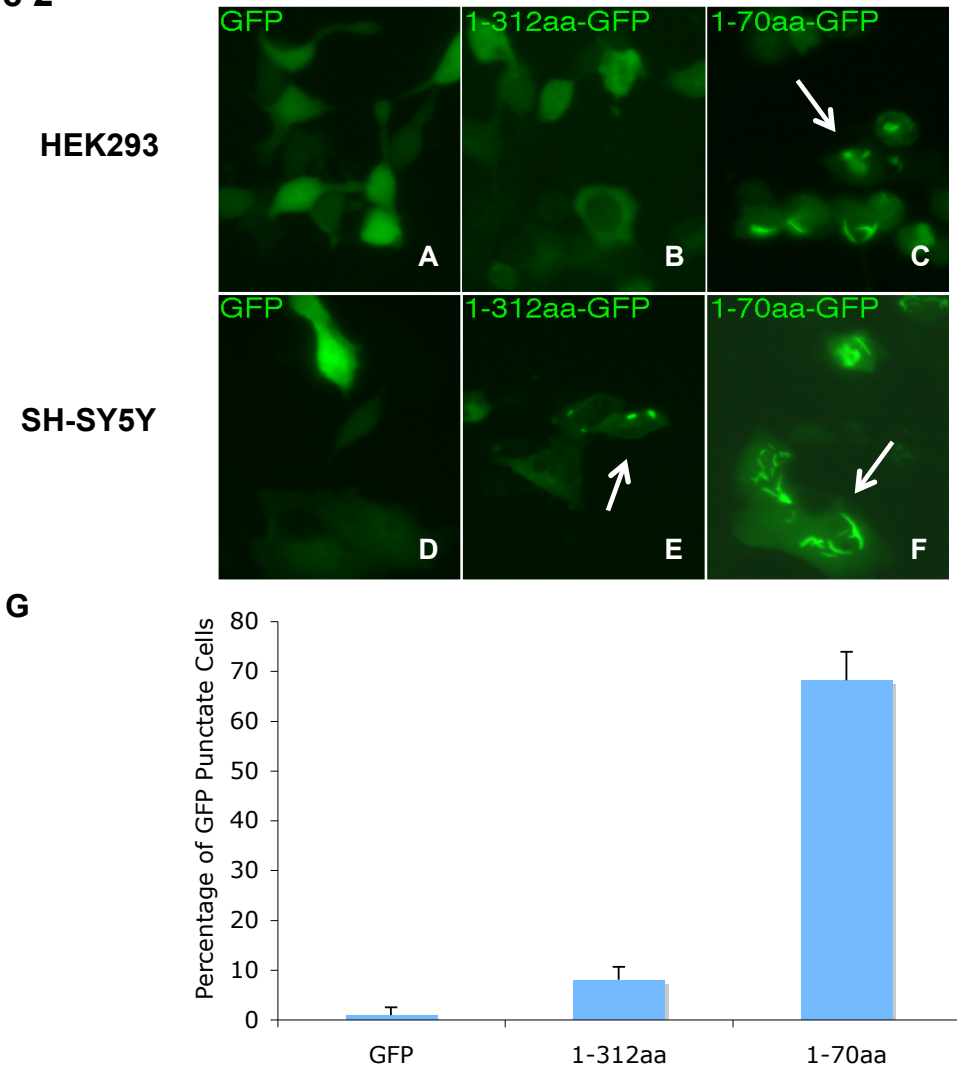
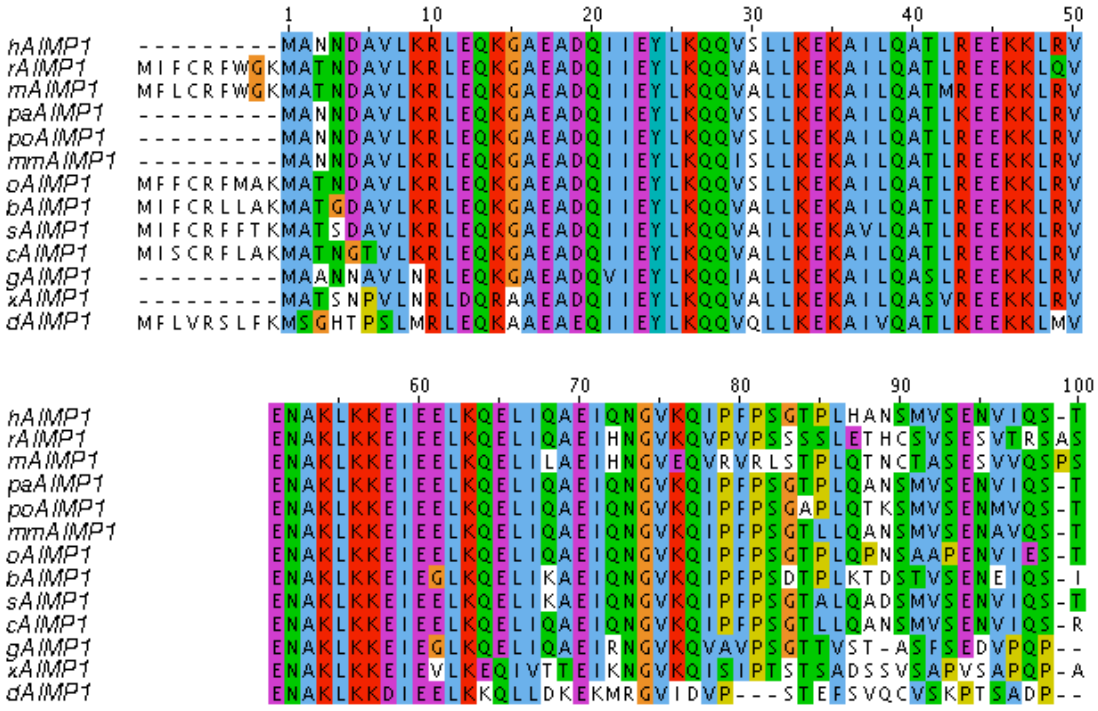


Figure 3

A



B

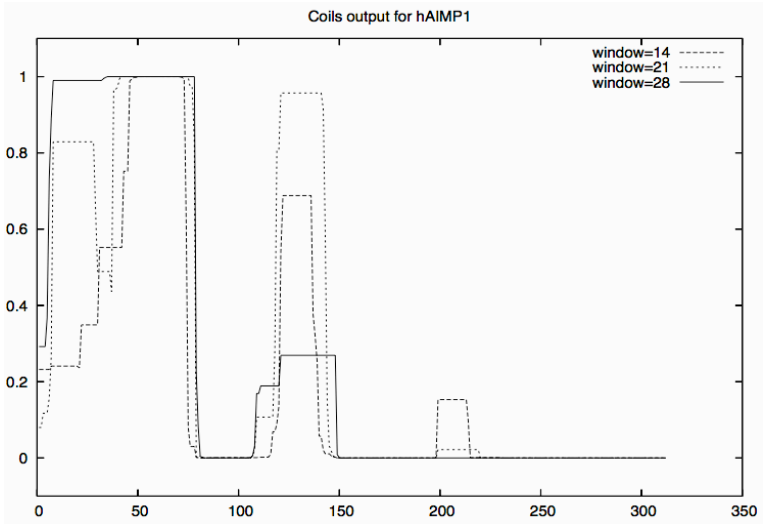
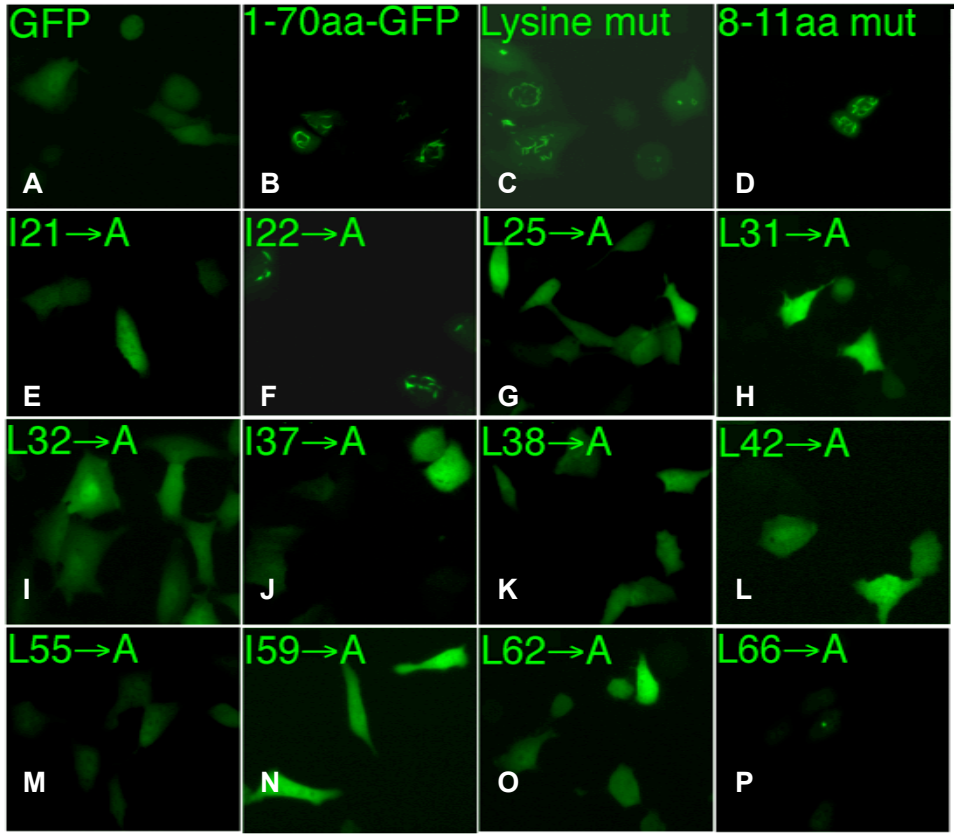


Figure 4



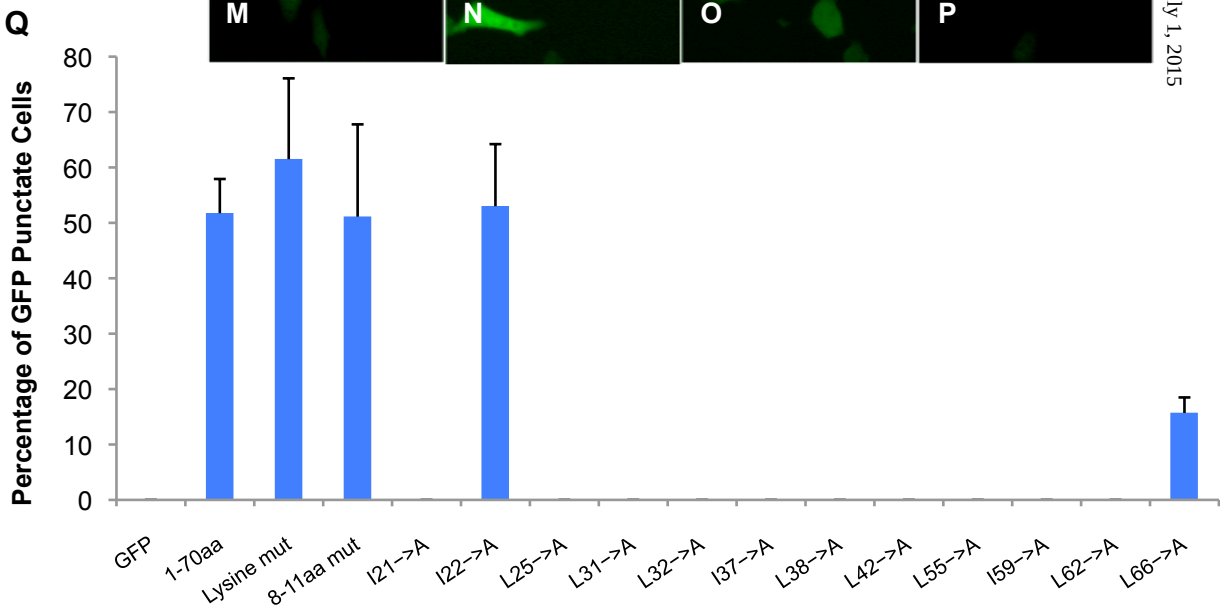


Figure 5

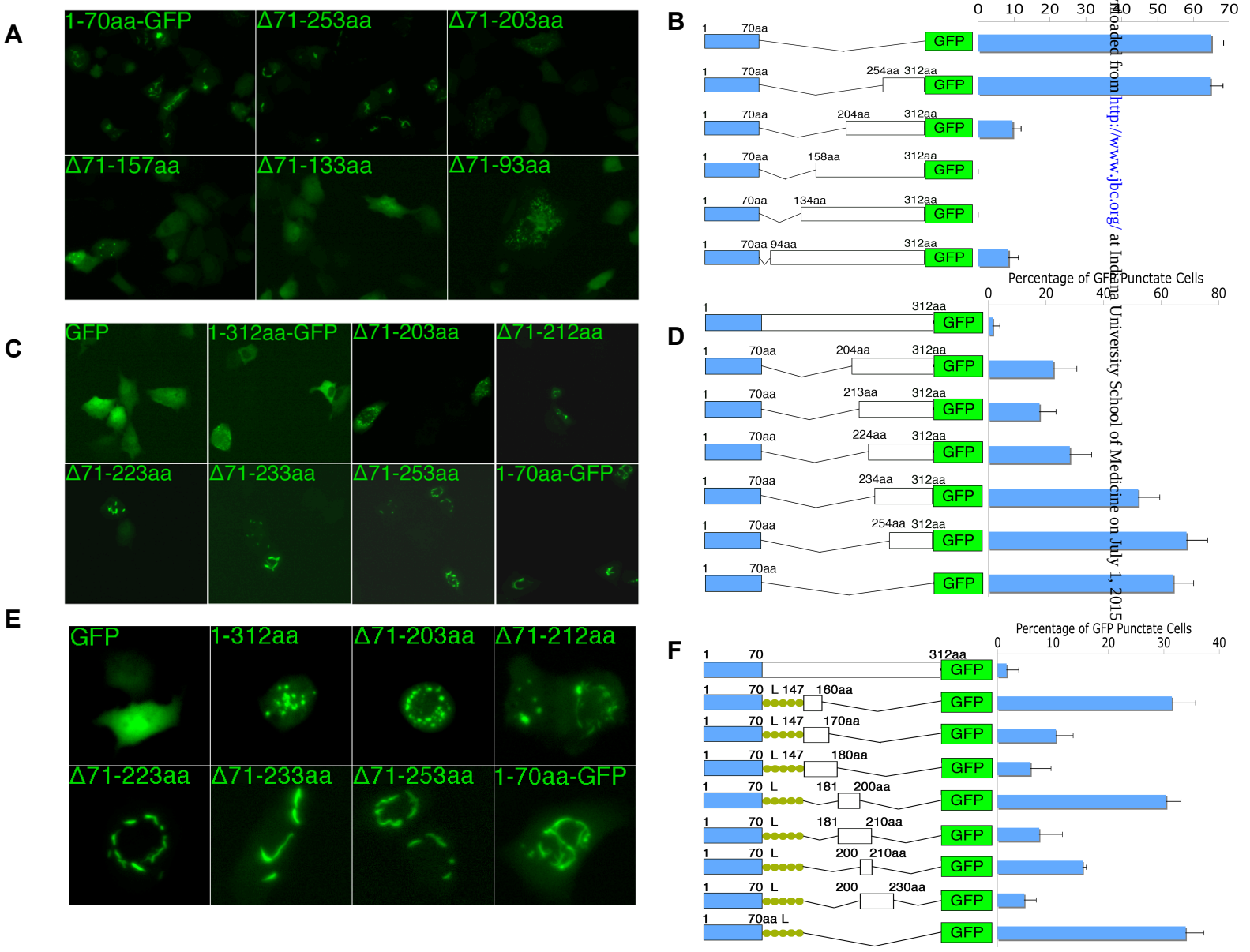


Figure 6

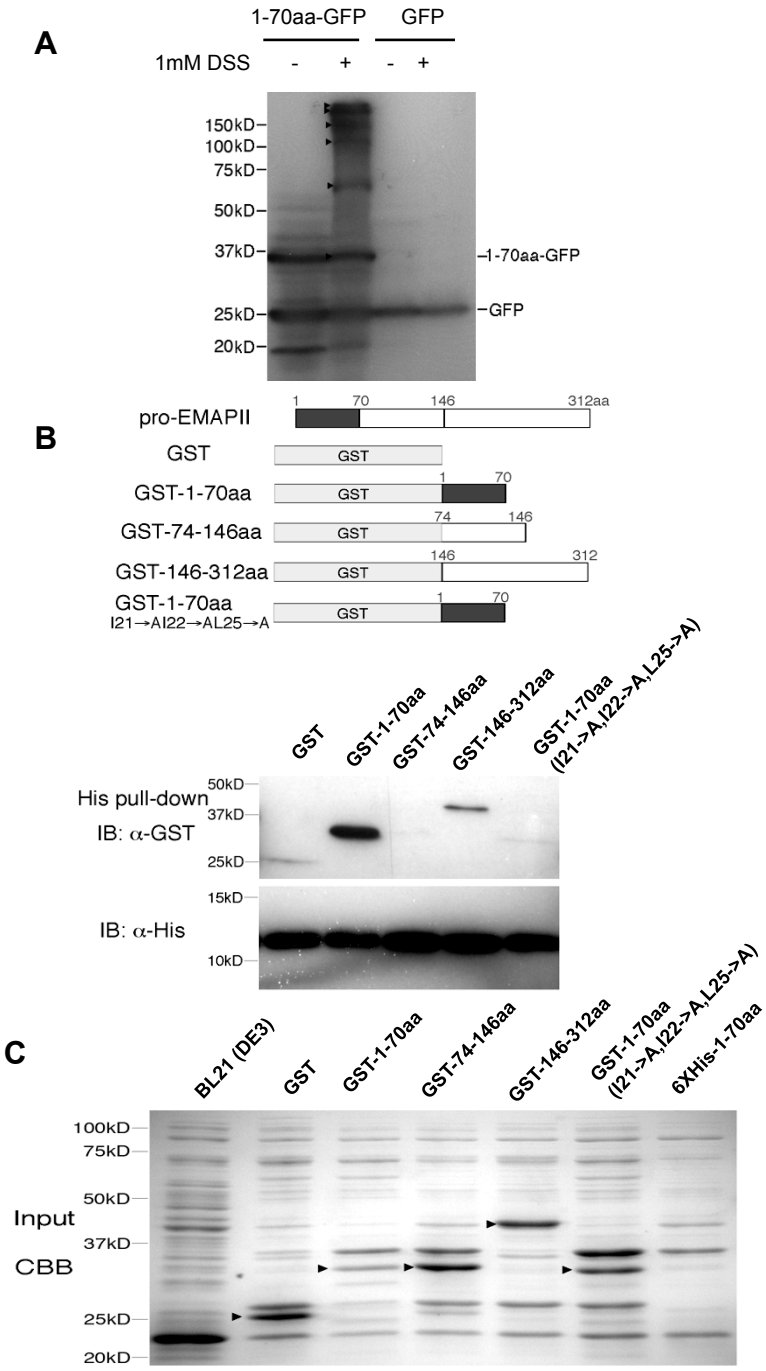


Figure 7

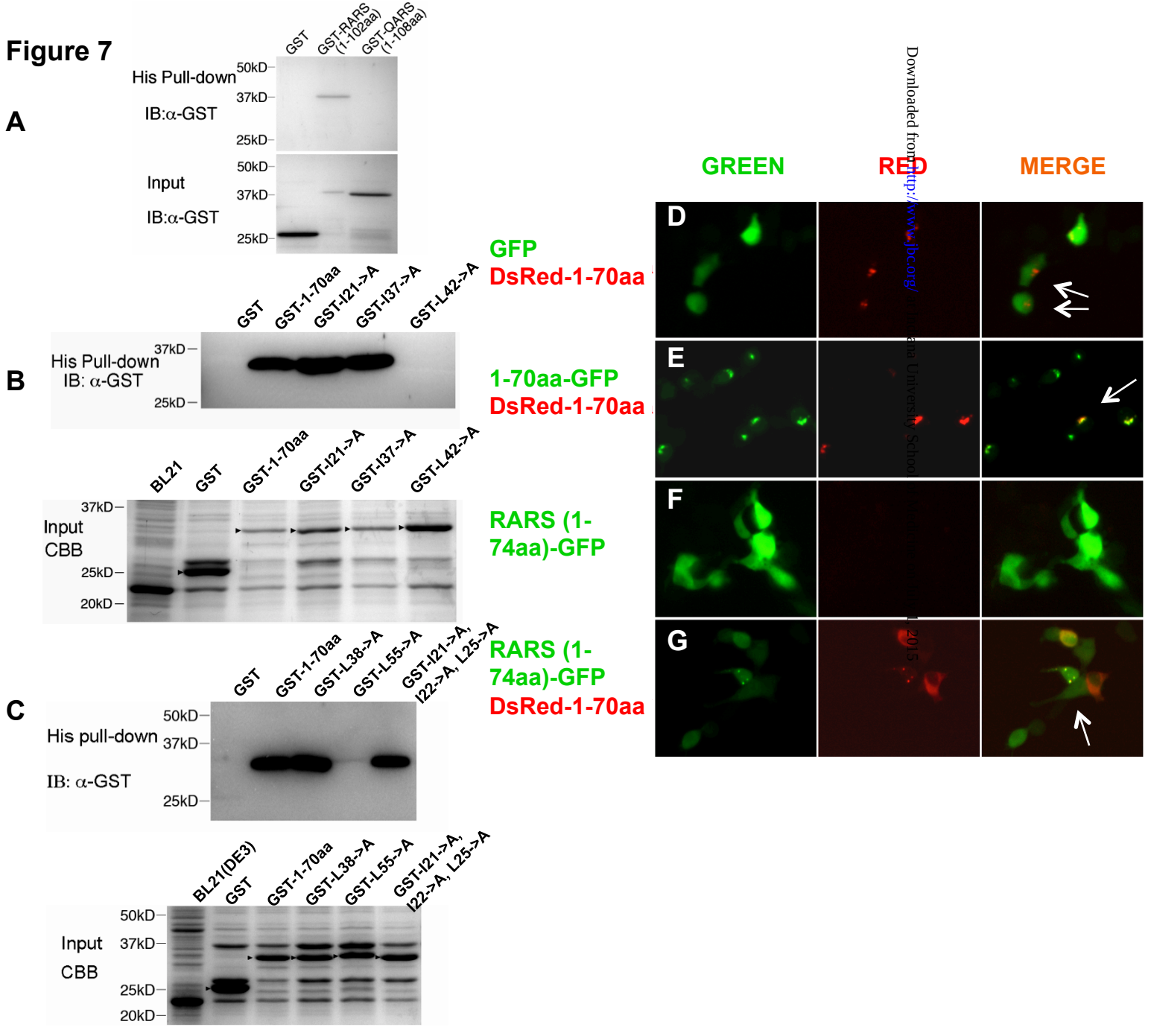
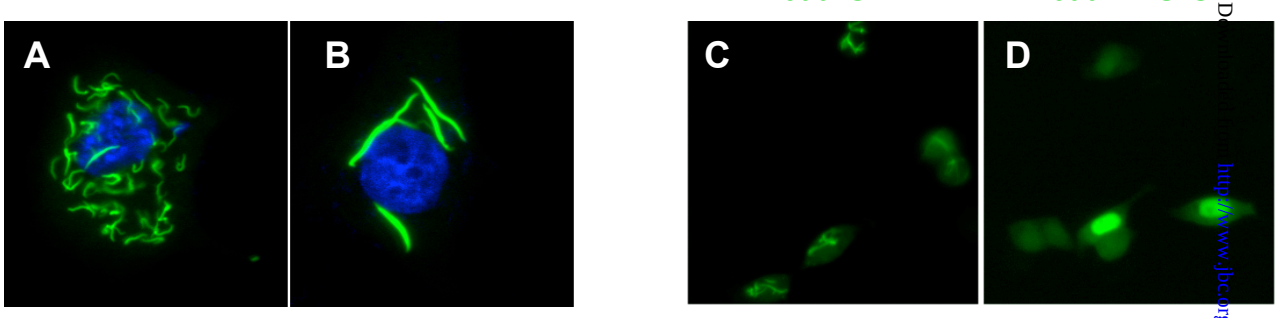
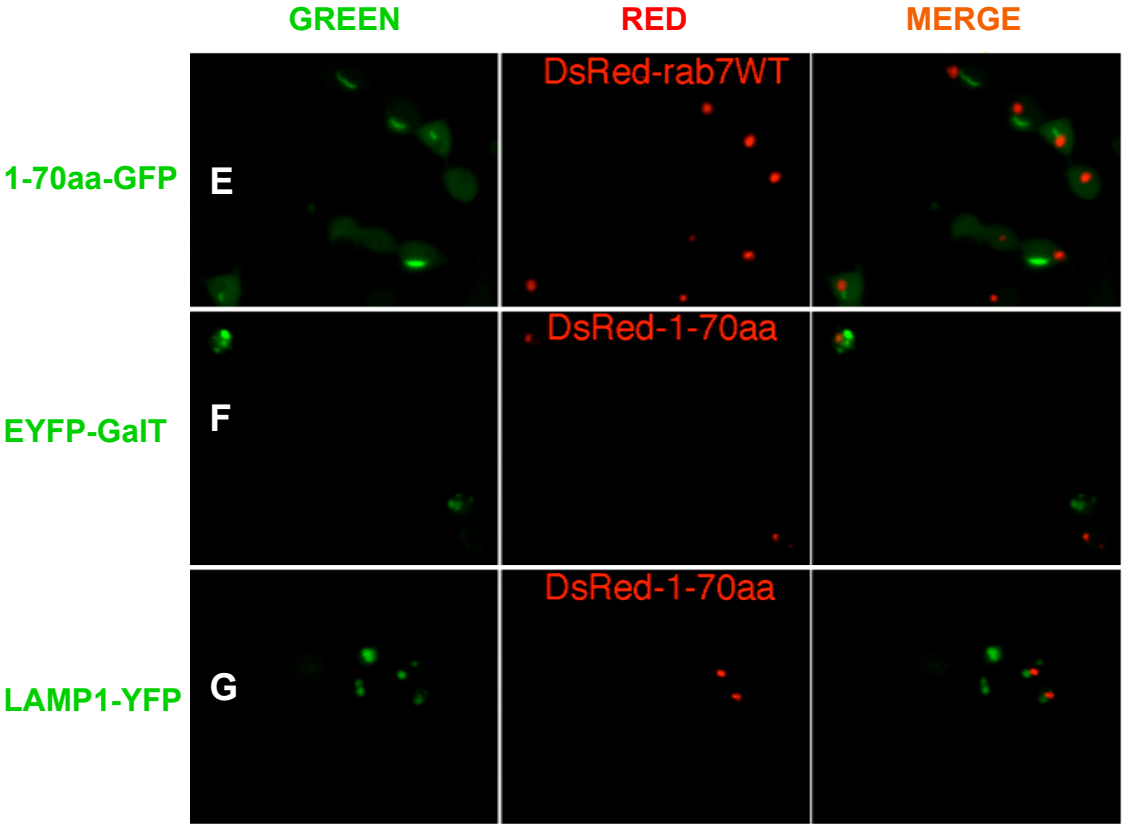


Figure 8





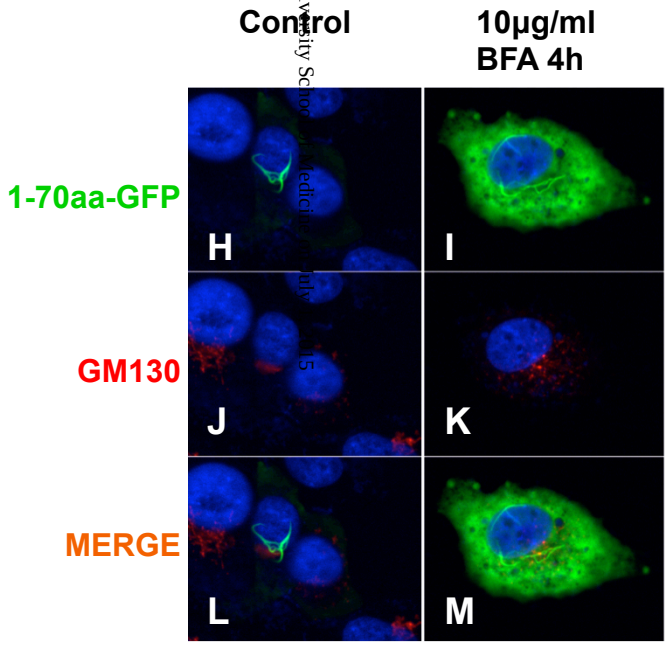


Figure 9

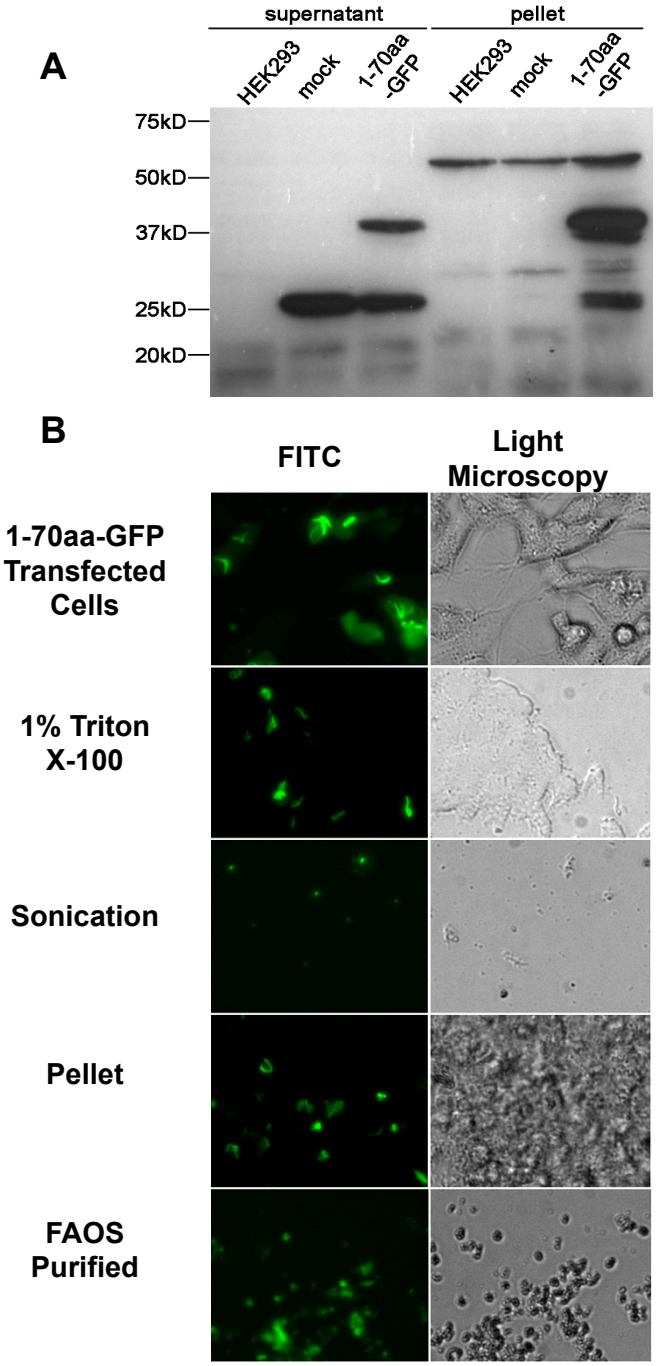
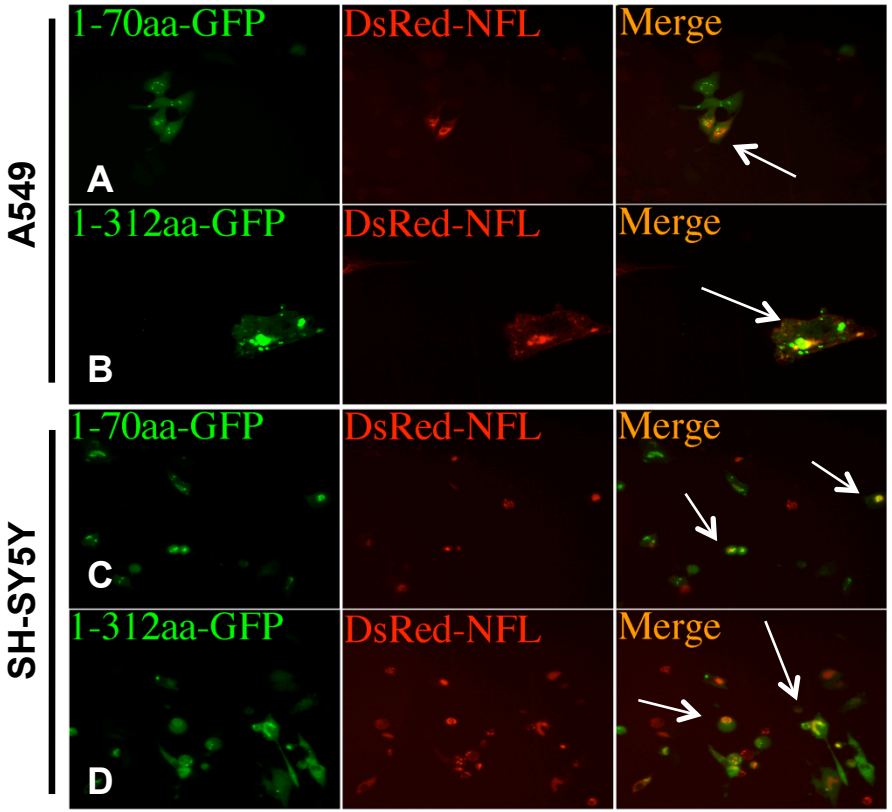
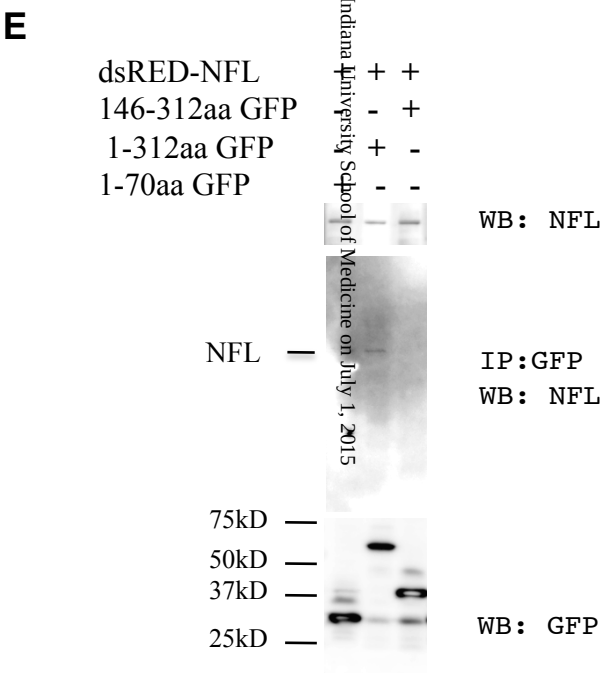


Figure 10





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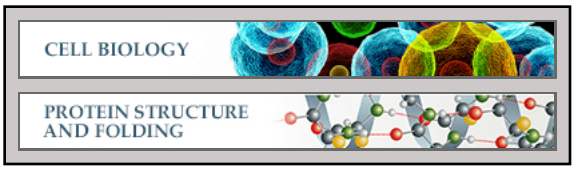

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