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Considerable progress has been made in isolating complexes of SNAP-associated polypeptides critical to understanding the determinants of the SNAIL-SNAG domain/SNAP interaction in vitro and in vivo. We have analyzed our unique panel of HEK293 stable cell lines expressing affinity tagged Ajuba and the LIMD1 proteins and examined differences in the cytoplasmic and nuclear complexes. We have developed useful polyclonal antibodies that sensitively detect and discriminate between Ajuba and LIMD1. We are currently mapping the SNAG/SNAP interaction surfaces. Our future goal is to establish dominant negative proteins able to disrupt the SNAG/SNAP interaction and reactive E-Cadherin to control the metastatic phenotype.

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Table of Contents

Introduction: This research project is based on the finding that an elevated level of SNAIL, a SNAG repression domain-containing zinc finger protein, in a spectrum of human tumors is correlated with the loss of E-Cadherin expression. SNAIL acts as a potent transcriptional repressor of E-Cadherin expression via direct binding to E-box elements (GCAGGTG) in the E-Cadherin promoter [1], and this repression is abolished by mutation of the SNAG domain. The E-Cadherin glycoprotein regulates the cellular adhesive properties required to establish and maintain the tissue architecture of many epithelial cell types, including normal breast epithelia. Loss of E-Cadherin expression in breast carcinoma is implicated in the loss of tumor cell adhesion and the acquisition of the metastatic phenotype. This progression of benign tumors to invasive carcinoma is the single most important obstacle that must be confronted in order to eliminate the morbidity and mortality from this disease. The proposed strategies for reactivating E-Cadherin expression, have the long-term goal of halting breast tumor progression and metastatic spread. We have propose that targeting the SNAG repression function of SNAIL should lead to reactivation of E-Cadherin and have made significant progress in establishing reagents and isolating protein complexes that will be relevant for targeting the SNAG domain and testing this hypothesis in model systems.

Body: Our preliminary studies were focused on the following characterization of the SNAG domain: 1. the SNAG of Gfi-1domain was demonstrated using reporter assays to be a dominant, transferable, DNA binding-dependent repression module [2, 3], 2. targeted mutagenesis was applied to define the amino acid residues critical for SNAG-mediated repression, 3. a yeast two-hybrid screen was employed to isolate two SNAG domain Interacting Proteins (SNAPS). The first, SNAP13, had been previously cloned as the LIM domain protein [4, 5] designated LIMD1 [6], and the second, SNAP20, was also an independently isolated LIM domain protein designated, AJUBA [7,8]. 4. We found that in the presence of a wild-type SNAG protein that can interact with AJUBA, AJUBA displays a distinctly nuclear localization; however, in the absence of a SNAG protein or in the presence of a mutant SNAG protein, AJUBA is cytoplasmic. Our finding that mutations in the SNAG domain that abolish repression, also prevent nuclear localization of AJUBA suggest that we have discovered a novel co-repressor that mediates SNAG domain repression via nuclear-cytoplasmic shuttling.

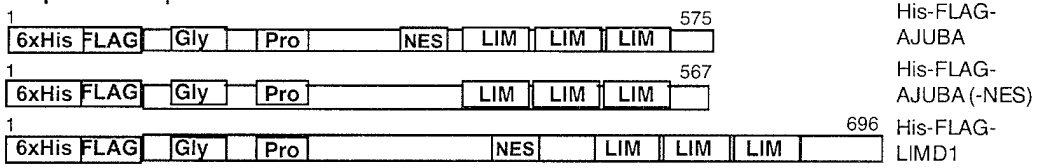
Because of its significant role in metastatic breast cancer, our goal is to extend these preliminary studies of model SNAG:SNAP interactions to the context of E-Cadherin, the target gene of the SNAG zinc finger protein SNAIL[1]. The proposed strategies are aimed at disrupting the interactions between the SNAG domain of SNAIL with its endogenous SNAP in order to abolish SNAIL-mediated repression of the E-Cadherin gene and lead to a reversal of the metastatic phenotype. The Specific Aims defined in our original proposal included the following: (1) Reconstitute, map and determine the specificity of the SNAIL-SNAG domain-SNAP interaction *in vitro* and in vivo. (2) Identify dominant negative and peptide-based inhibitors of the SNAG-SNAP interaction and use them as tools to manipulate SNAIL-mediated repression in vivo. (3) Define the set of genes that are under control of the SNAIL in breast cancer cells using cDNA microarray analysis. Last year we reported our accomplishments toward satisfying tasks associated with Specific Aim 1 which involved subcloning full-length cDNAs for the SNAP proteins AJUBA and LIMD1 to NH2-terminal 6xHIS and a FLAG tagged pcDNA3 mammalian expression vectors (Figure 1A). For AJUBA, a construct containing a specific deletion of the AJUBA nuclear exclusion signal (NES) was also constructed. We showed that all of these mammalian expression constructs permit stable protein expression when transfected in mammalian cell lines such as COS-1 cells, 293-HEK cells and Hep2 cells. The expressed proteins are readily detectable biochemically (Figure 1B) and by immunofluorescence

microscopy using the anti-FLAG M2 antibody (Figure 1C). Both the FLAG-LIMD1 protein, and His-FLAG-Ajuba protein containing the NES domain exhibit a predominantly cytoplasmic localization, however the Ajuba protein (-NES) lacking the nuclear exclusion signals is abundantly distributed in both cytoplasm and nucleus. We also purified unique Ajuba and LIMD1 antigens (1D) and used them to generate rabbit polyclonal antiserum specific for LIMD1 and the Ajuba proteins. Figure 1E depicts the autoradiogram demonstrating that the polyclonal sera are capable of detecting the full length Ajuba (~58 kDa) and the LIMD1 (~71 kDa) proteins. Hence we have successfully generated antisera that should be useful in detecting and discriminating between these proteins when expressed endogenously.

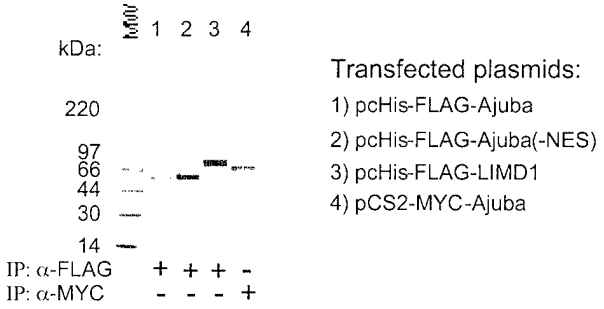
This year we have focused considerable attention towards establishing reliable epitope-tagged affinity chromatography methodology for the reproducible isolation of biochemical complexes of SNAP associated proteins. These studies used our panel of HEK293 cell lines that stably express the Ajuba protein, the Ajuba (-NES) protein, and the LIMD1 protein as shown in the anti-FLAG M2 antibody immunoblot analysis in Figure 2A. Both nuclear and cytoplasmic fractions were subjected to purification and the progress was followed using the anti-FLAG antibody. The silver-stained gel in Figure 2B reveals that there are LIMD1 associated proteins in the cytoplasm, that do not associate with Ajuba. In the nuclear fraction both Ajuba (-NES) and LIMD1 appear to share some common associated proteins. Figure 3 illustrates an intensive study of His-FLAG Ajuba protein and its associated polypeptides from both the S100 (cytoplasmic) and the nuclear extracts. We are currently subjecting the eluted polypeptides to MALDI-mass spectrometry for identification. These studies will identify both the nuclear and cytoplasmic components of a novel signaling system involving GFI1-SNAG domain and AJUBA. These purifications will be repeated using the antibodies to Ajuba and LIMD1. Identification of these associated polypeptides will very be informative with respect to the role in both SNAG-mediated transcriptional repression and in the biological function of GFI1 and AJUBA and will focus our future attempts at development of peptide inhibitors targeting protein-protein interactions within the complexes described in specific aim 2.

Our current efforts are directed at finishing tasks within Specific aim 2 by mapping SNAG-SNAP interaction and domain boundaries and establishing these constructs in epitope-tagged, bacterial and mammalian expression vectors Our initial mutagenesis efforts will target the SNAP LIM domains. We will continue to perform *in vitro* interaction assays using a combination of GST chromatography, and co-immunoprecipitation using transient co-transfection of tagged SNAP and SNAIL expression constructs and co-immunoprecipitation of cell extracts. We will also engineer a set of point mutations focusing on the potential protein-protein interaction surfaces in each SNAP. Finally, to determine the specificity of the interaction we will utilize the other LIM domain family members of which SNAPs are a member HIC5 [9, 10], TRIP6 [11], Zyxin [12] and LPP [13]. We will utilize epitope tagged versions and we will determine whether these proteins can interact directly with the SNAIL-SNAG domain via the assays described above. The outcome of these studies will enable us to identify dominant negative and peptide-based inhibitors of the SNAG-SNAP interaction and use them as tools to manipulate SNAIL-mediated repression *in vivo* as described in Specific Aim 2. The goal of this aim is to utilize truncated versions of the SNAIL and SNAP proteins as dominant negatives in vivo in order to manipulate E-Cadherin expression in the MDA-MB 435 cell lines that are highly malignant and have low E-Cadherin expression to evaluate the metastases potential in SCID mice based models.

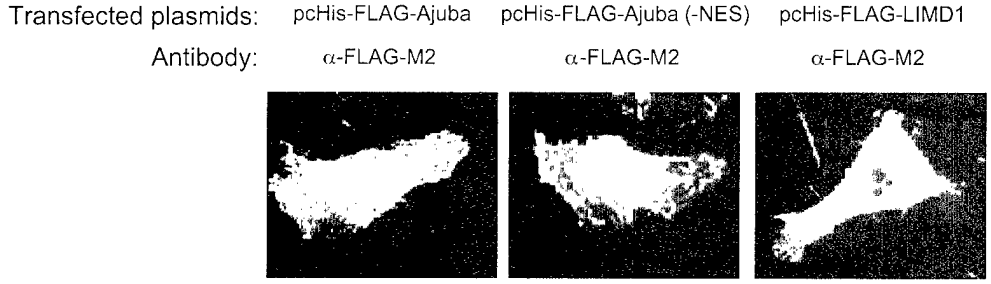
A. Expression plasmids:



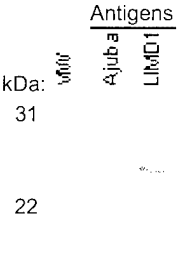
B. COS-1 Transfection and Immunoprecipitation



C. Immunolocalization of His-FLAG-Ajuba, His-FLAG-Ajuba (-NES) and His-FLAG-LIMD1 proteins



D. Ajuba and LIMD1 antigens



E. Characterization of Ajuba and LIMD1 antibodies

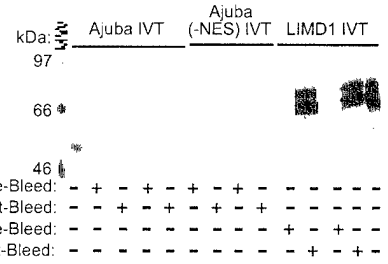
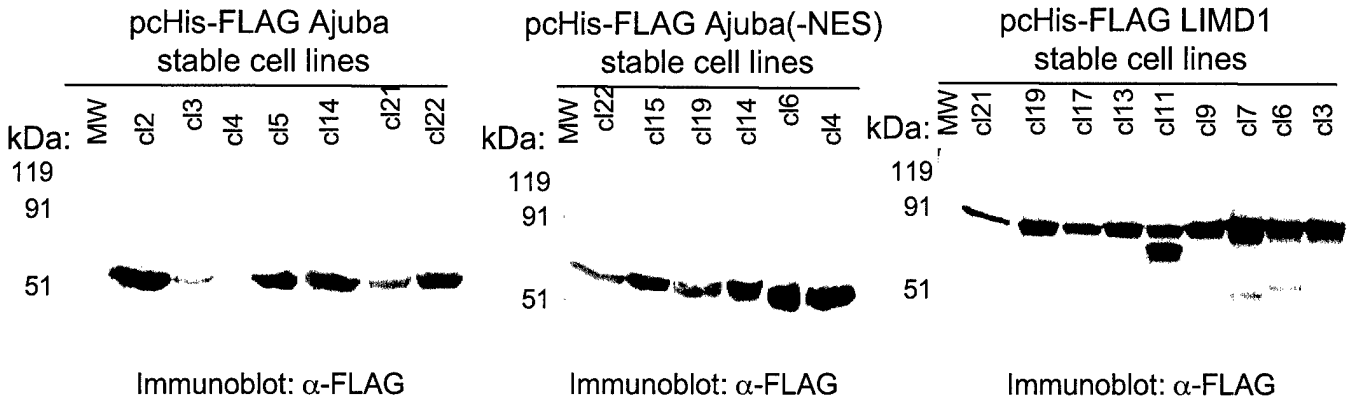


Figure 1: A. The mammalian expression plasmids were constructed by sub-cloning the cDNAs encoding His-FLAG-Ajuba, His-FLAG-Ajuba(-NES) and His-FLAG-LIMD1 genes into pcDNA3 vector. The pcHis-FLAG-Ajuba(-NES) construct lacks amino acids 289-297 of Ajuba, which harbors a functional leucine-rich nuclear export signal. **B**. Indicated expression plasmids were transfected into COS-1 cells, the metabolically labeled extracts were immunoprecipitated with α-FLAG antibody and analyzed. **C**. HEp2 cells that were transiently transfected with the expression plasmids, immunostaining was carried out with α-FLAG-M2 antibody and single cell images are presented. **D**. BL21(DE3) recombinants of pQE30-Ajuba or pQE30-LIMD1 were induced with IPTG and the crude cell lysates were bound to Ni^{2+}-NTA beads. After washing the beads, the bound proteins were serially eluted using buffer containing 400 mM imidazole and portions were analyzed on 12% SDS-PAGE. **E**. The ^{35}S-labeled Ajuba, Ajuba (-NES), and LIMD1 IVT proteins were immunoprecipitated with either pre- or test-bleeds and analyzed by fluorography. Note that the Ajuba antiserum detects both Ajuba and Ajuba (-NES) proteins.

A. Stable clones expressing His-FLAG-Ajuba, His-FLAG-Ajuba (-NES) & His-FLAG-LIMD1 proteins



B.

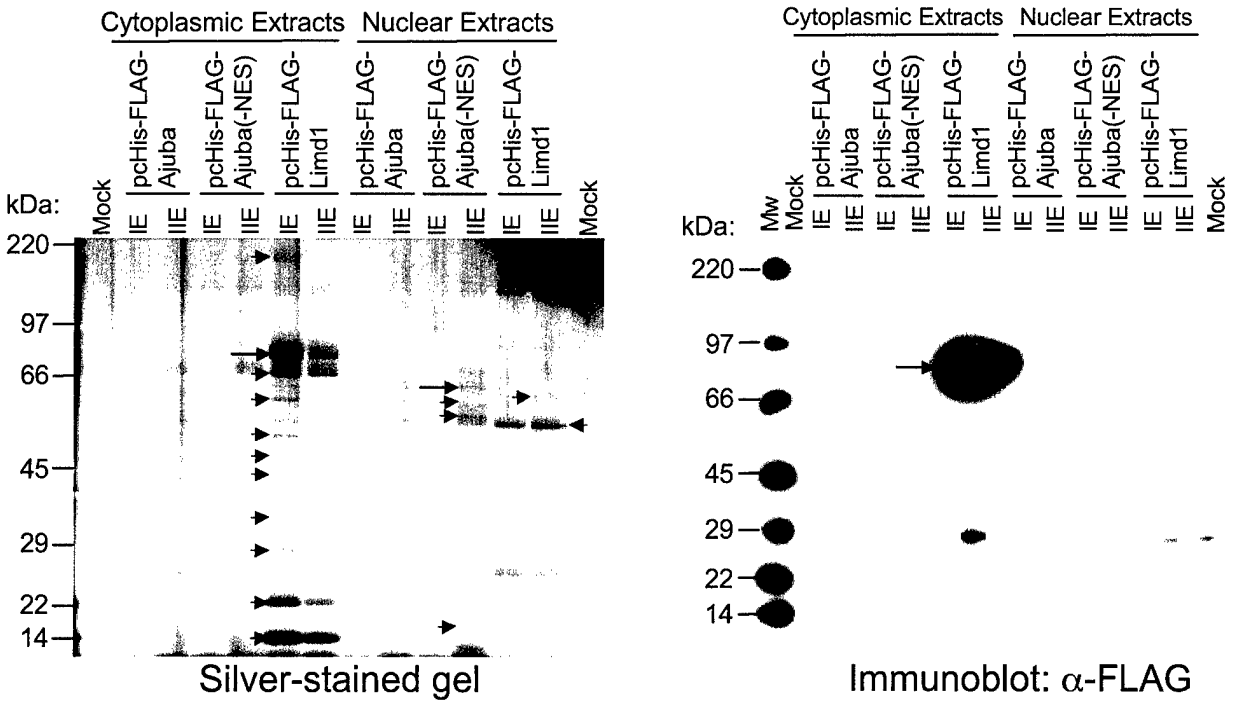


Figure 2: **A.** HEK 293 cells were transfected with the pcHis-FLAG-Ajuba, pcHis-FLAG-Ajuba (-NES) and pcHis-FLAG-LIMD1expression plasmids and selected for stable integrands using G418. Cell lysates were prepared from twenty-four single cell clones and analyzed by immunoblotting with α-FLAG antibody as primary and alkaline phosphatase labeled α-mouse IgG as secondary antibodies. Presence of immunoreactivity was tested by developing the color using BCIP and NBTsubstrates. Each panel illustrates mainly the good expressors. **B.** Nuclear and cytoplasmic fractions obtained from the transfected cells were incubated with the a-FLAG affinity resin. After washings, the bound proteins were eluted using FLAG peptide and electrophoresed on two 4-12% Nu-PAGE gels. One gel was processed for silver staining while the other gel was immunoblotted with α-FLAG antibody. Mock represents the extracts prepared from cells transfected with pCMV2-FLAG vector. IE and IIE stand for first and second elutions respectively. The filled block arrow represents the His-FLAG-LIMD1 protein and the open block arrow depicts the His-FLAG-Ajuba (-NES) protein. The arrowheads represent the associated polypeptides that are unique.

AJUBA Complex Purification

His-FLAG Ajuba and associated polypeptide elution fractions:

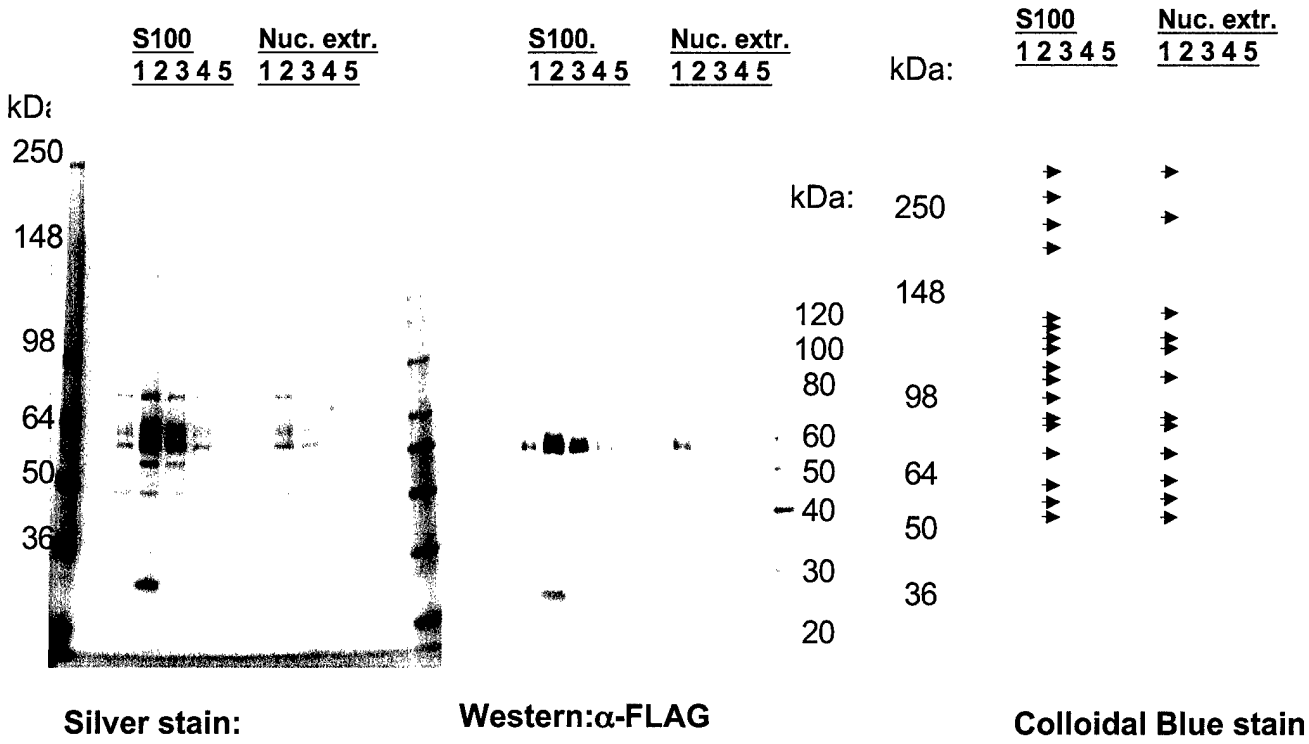


Figure 3: The stable 293-HEK clone (cl2) expressing HIS-FLAG-Ajuba fusion protein was cultured in 40 x 150mm dishes to 90% confluency and S100 (cytoplasmic) and nuclear extract fractions were prepared by a modification of the Dignam procedure. The associated polypeptides were eluted using FLAG peptide in 5 fractions. 15μl aliquots of each fraction were electrophoresed on two 4-20% Tris-glycine gels, one for silver staining and the other for immunoblotting and probing with the anti-FLAG M2 monoclonal antibody. 450μl of S100 and NE peak fractions (#2) were TCA precipitated and resolved in a single well of a 1.5 mm thick, 4-12% acrylamide Tris-glycine gel and stained with collodial blue. The sequences of individual poypeptides are currently being determined by LC- MS/MS analysis (indicated by arrowheads).

Key Research Accomplishments:

- We have obtained epitope-tagged full length cDNA clones of the SNAG domain zinc finger protein, SNAIL, and the SNAPs, Ajuba and LIMD1 and verified stable protein expression in mammalian cells.
- We have demonstrated that Ajuba can interact with SNAIL by co-immunoprecipitation and have shown that wild type but not SNAG-domain-deleted SNAIL can augment SNAIL-mediated repression of transiently transfected E-Cadherin promoter Luciferase reporter plasmids.
- We have generated and characterized extremely useful polyclonal antibodies capable of detecting and discriminating between Ajuba and LIMD1.
- We have generated a panel of HEK293 stable cell lines expressing Ajuba and the LIMD1 proteins.
- We have examined differences in the biochemical complexes associated with Ajuba and LIMD1 in cytoplasmic compared to nuclear fractions and identified associated polypeptides using MALDI-mass spectrometry.

Key Reportable Outcomes:

Manuscripts:

1. Kasirajan Ayyanathan, Rakesh Goyal, Greg Longmore, and Frank J. Rauscher III. Functional analysis of the SNAG repression domain from the GFI-1 proto-oncogene: Identification of a novel LIM domain protein that functions in the repression pathway (Manuscript in preparation for PNAS).

Presentations: **None**

Cell Lines developed:

HEK293 stable cell lines expressing Ajuba and the LIMD1 proteins.

Antibodies developed:

Rabbit sera #3201 and #3149 highly reactive for the LIMD1 antigen (amino acids 1-158)

Rabbit sera #3215 and #3198 reactive for the Ajuba antigen (amino acids 1-216)

Funding applied for based on work supported by this award:

NIH RO1 CA 095561 Functions of the SNAG Repression domain in oncogenesis

Research opportunities based on training provided by this approval:

Hongzhuang Peng Ph.D.

Conclusions: We have specifically proposed that targeting the SNAG repression function of SNAIL should lead to reactivation of E-Cadherin and have made significant progress in establishing reagents and defining relevant complexes for testing this hypothesis in model systems. We have obtained epitope-tagged full length cDNA clones of the SNAG domain zinc finger protein, SNAIL, and the SNAPs, Ajuba and LIMD1 and verified stable protein expression in mammalian cells. We have demonstrated that Ajuba can interact with SNAIL by co-immunoprecipitation and have shown that wild type but not SNAG-domain-deleted SNAIL can augment SNAIL-mediated repression of transiently transfected E-Cadherin promoter Luciferase reporter plasmids. We have generated a panel of HEK293 stable cell lines expressing Ajuba and the LIMD1 proteins and examined differences in the biochemical complexes associated with Ajuba and LIMD1 in cytoplasmic compared to nuclear fractions. We have generated and characterized extremely useful polyclonal antibodies capable of detecting and discriminating between Ajuba and LIMD1.

Our current efforts are directed at creating a panel of deletion and point mutations of Ajuba and LIMD1 proteins to map the protein-protein interaction surfaces with the SNAIL SNAG domain, and test the interaction specificity against other SNAPs (HIC5 [9, 10], TRIP6 [11], Zyxin [12] and LPP [13]). The goal of these studies is to identify dominant negative and peptide-based inhibitors of the SNAG-SNAP interaction. These tools will be used to manipulate SNAIL-mediated repression *in vivo* and to test whether reactivation of E-Cadherin expression will significantly inhibit the metastatic potential of MDA-MB 435 cell lines in SCID mice based models.

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