

**BIOLOGICAL FUNCTIONS OF LSAMP, A GENE ASSOCIATED WITH
RAPID DISEASE PROGRESSION IN PROSTATE CANCER**

BY

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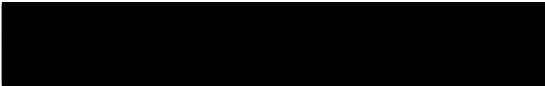
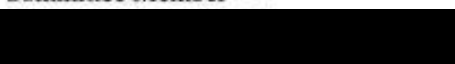
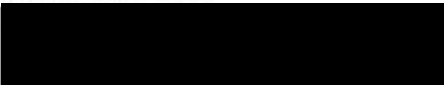
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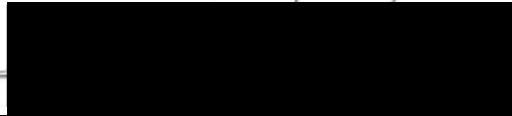
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ABSTRACT

BIOLOGICAL FUNCTIONS OF LSAMP: A GENE ASSOCIATED WITH RAPID DISEASE PROGRESSION IN PROSTATE CANCER

KEVIN BABCOCK, PHD, 2018

**THESIS DIRECTED BY: ALBERT DOBI, PHD, RESEARCH ASSISTANT PROFESSOR,
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INTRODUCTION: African American men have the highest prostate cancer incidence and mortality rates in the US. However, the biological basis for this disparity is not well understood. *LSAMP* inactivation has been implicated in various cancers, and recently identified in prostate cancer. A higher frequency of genomic inactivation was observed in AA prostate cancer, and this aberration significantly associated with rapid disease progression. However, the putative tumor suppressive function of *LSAMP* has not been characterized.

METHODS: The copy number and expression of *LSAMP* in prostate cancer cell lines was assessed. LNCaP, MDA PCa 2b, and DU 145 cell lines were stably transduced to express *LSAMP* in an inducible or constitutive manner. Proliferation and tumor growth rates were assessed by MTT assay, and subcutaneous xenograft model. *LSAMP* expressing and control DU 145 cells were used to assess extracellular matrix-cell adhesion, and cell-cell adhesion to fibroblast (NIH/3T3), normal human prostate stroma

derived (WPMY-1), and human endothelial (HUVEC) cell monolayers. LSAMP inhibition of specific signal transduction in prostate cancer cell lines was assessed by immunoblot and immunofluorescence assays.

RESULTS: Expression of *LSAMP* inhibited cell proliferation and tumor growth, and increased adhesion to extracellular matrix proteins. *LSAMP* expression also increased cell-adhesion to fibroblast and prostate stroma cell lines, and concomitantly decreased endothelial cell-adhesion. Furthermore, *LSAMP* decreased expression of specific receptor tyrosine kinases: FGFR2, FGFR4, and EPHA3, resulting in decreased phosphorylation of their downstream targets in the Akt and ERK1/2 signaling pathways, and leading to the dysregulation of β -catenin. Additionally, *LSAMP* alters the expression of several Integrins.

CONCLUSION: These studies provide the first evidence of *LSAMP*'s prostate tumor suppressive function, and a specific tumorigenic signal transduction pathway negatively regulated by LSAMP has been identified. The reversion to an indolent adhesive phenotype, growth attenuation, and signaling inhibition upon *LSAMP* expression in prostate cancer models provides novel insight to the biological mechanism underlying the aggressive prostate cancer phenotype prevalent among AA men.

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CHAPTER 1: Background and Significance

PROSTATE CANCER

Cancer is the second leading cause of death in Americans (264). Cancer arises as a continuum of sustained genetic alteration (200). This is manifested by oncogene activation and tumor suppressor inactivation in cells, leading to uncontrolled proliferation, evasion of growth suppression and cell death, angiogenesis, invasion and metastasis (103). Worldwide, the second most diagnosed cancer in men is prostate cancer (290). In 2018, prostate cancer is projected to be the cancer with the highest incidence and second highest mortality among American men, with 164,090 new cases and 29,430 deaths (264).

Prostate cancer is largely characterized by copy number aberrations and genomic structural variations, with point mutations occurring at a significantly lower frequency. In an advanced prostate cancer dataset, Barbieri *et al.* identified only four significantly mutated genes: Speckle-type POZ protein (*SPOP*), Tumor protein p53 (*TP53*), ATM serine threonine kinase (*ATM*) and Mediator of RNA polymerase II transcription, subunit 12 homolog (*MED12*) (17). A percentage of prostate cancer risk can be explained by heritability. A twin study revealed the percentage of heritability associated with prostate cancer risk to be higher than any other common cancer type, although environmental factors remain the principal contributor to the disease (112; 170). *BRCA2* (Breast Cancer 2) was the first germ line prostate cancer risk variant discovered, and was estimated to confer a five-fold increased risk for prostate cancer development (205). Genome wide association studies have expanded upon known prostate cancer risk variants. However, current discoveries only account for approximately one third of familial risk in prostate

cancer patients of European descent, and even less so in those of African descent, where prostate cancer incidence and mortality is the most prevalent (6).

In both normal prostate function and prostate cancer development Androgen receptor (*AR*) signaling plays a central role (259). The androgen pathway blockade remains the main post-surgical option in the treatment of prostate cancer. Androgen deprivation therapy (ADT) is primarily administered following the failure of radical prostatectomy, and subsequent biochemical recurrence (BCR). BCR is defined as a rise of Prostate Specific Antigen (PSA), following surgery or radiation, and indicates disease progression. ADT often only serves to postpone disease progression, resulting in the development of castration resistant prostate cancer (CRPC). Castration resistance is a process by which the tumor becomes unresponsive to standard hormone ablation therapy and is indicative of an aggressive phenotype.

Despite the involvement of common pathways, there is significant genomic heterogeneity in prostate cancer, even between each tumor focus within a single patient. Genomic comparisons have revealed independent tumor origins within distinct and inter-related regions (56; 171; 277). This was first defined through the discovery and characterization of the most common prostate cancer oncogene, *ERG* (ETS-related gene) (81; 217; 219; 289). Approximately half of Caucasian American (CA) prostate tumors contain an activated form of *ERG* oncogene as the result of a fusion with an androgen responsive promoter, most often *TMPRSS2* (Transmembrane protease, serine 2) (219; 289). This genetic rearrangement defines the main prostate cancer causal event, and is the major focus of research in the prostate cancer field. *PTEN* (Phosphatase and tensin

homolog), a tumor suppressor gene which negatively regulates PI3K/Akt signaling, is deleted in 40% of CA primary prostate tumors, and inactivated via mutation in another 5-10% (17; 93; 308). *PTEN* loss is associated with *ETS*-positive status, and both aberrations are prevalent in CA prostate cancer (274). The majority research focused on prostate cancer genomic alterations which emphasize the prevalence *ERG* and *PTEN* alterations have primarily been conducted using CA specimens, largely due to disparities in healthcare access (31; 110; 140). Emerging evidence from ethnicity based studies suggests the frequency of these alterations can be much less prevalent in African American (AA) prostate cancer, exhibiting less than half of the frequency of *ERG*-positive index tumors as observed in CA prostate cancers (59; 74; 140; 218; 254). Mutations and deletions in *SPOP*, and *CHD1* (Chromodomain-helicase-DNA-binding protein 1) correlate with *ETS*-fusion negative tumors, however, the molecular mechanisms governing these tumors remain largely unknown (17; 93).

TABLE 1: PROSTATE CANCER INCIDENCE AND MORTALITY PER 100,000.

	Non-Hispanic White	Non-Hispanic Black	Asian/Pacific Islander	American Indian/Alaska Native	Hispanic
Incidence	107.0	186.3	58.4	78.3	97.0
Mortality	18.2	40.8	8.7	19.7	16.1

Siegel et al., Cancer statistics, 2018. *CA* 68:7-30

PROSTATE CANCER DISPARITY

Worldwide, AA men are the single group with the highest prostate cancer incidence, and second highest prostate cancer mortality, behind only another population

with African descent, Trinidad (290). When compared to CA men, AA men are 1.7 times more likely to develop prostate cancer, and 2.2 times more likely to succumb to the disease (Table 1) (264). Socioeconomic factors are attributed to this disparity, however, after adjusting for these factors, significant disparities in incidence and mortality persist (51; 52; 252). This suggests genetic predisposition plays a significant role in the high rates of incidence and aggressive nature of prostate cancer in patients of African descent (45; 230). However, the key biological drivers of this disparity have been relatively poorly characterized.

Recent studies have highlighted some evidence of a biological basis for the AA prostate cancer disparity (138). Androgen and 5 α -reductase levels, an enzyme which reduces testosterone to dihydrotestosterone, were found to be higher in AA males when adjusted for age (141; 241; 242). Cytochrome p450 enzymes involved in steroid biosynthesis pathways, *CYP17* and *CYP3A4*, contain specific alleles which associate AA and increased prostate cancer risk (16; 147; 213; 225; 278; 304). AR expression is increased in epithelial prostate cancer tissue and decreased in the stromal tissue of AA patients (85; 143; 169). GermLine and somatic AR mutation patterns are significantly different in prostate cancers of AA and CA men (154). AA men have fewer CAG repeats, which is associated with prostate cancer risk (24; 89; 104). DNA damage excision repair proteins were uniquely found in AA patients to bind mutant AR (323). Further, expression of AR target genes, such as *RHOA* (Ras homolog gene family, member A), *ITGB5* (Integrin β 5), and *PIK3CB* (Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta isoform) associated with AA prostate cancer and increased invasion (300). In addition to androgens, estrogens also play a role in prostate cancer development

(30; 113). Increased estrogen levels, and polymorphisms in some estrogen related genes with a known prostate cancer risk, associate with AA prostate cancer (204; 238). Further, both benign and cancer samples from the prostates of AA men exhibited higher estrogen levels and Estrogen Receptor β tissue expression, when compared to CA specimens (1). Hormone signaling may contribute to the increased incidence and mortality observed in AA prostate cancer.

Factors other than hormone signaling may also play a role in the disparity. Epidermal Growth Factor Receptor (*EGFR*), a receptor tyrosine kinase (RTK) implicated in various cancers, is more commonly overexpressed in AA prostate cancer (262). A germLine polymorphism and several single nucleotide polymorphisms (SNPs) in *EphB2* (Ephrin type-B receptor 2), another RTK, associated with increased prostate cancer risk, and were found more frequently in AA prostate cancer (146; 236). Anti-apoptotic protein BCL2 (B-cell lymphoma 2) exhibited increased expression, and was observed alongside increased proliferation in AA prostate cancer (64; 98). Additionally, exosomal levels of anti-apoptotic protein, Survivin, are significantly increased in AA prostate cancer patients (139).

Distinct copy number variations and concomitant changes to gene expression also contribute to the differences between AA and CA prostate cancer (240). Amundadottir *et al.* and Freedman *et al.* found an allelic variant in 8q24 to be associated with prostate cancer, with an association twice as high for AA prostate cancer (10). Several other studies have corroborated the association of prostate cancer risk, 8q24, and AA ancestry (18; 28; 80; 99; 102; 235). In addition to copy number variations, other genomic and expression changes distinguish AA prostate cancer. The *HPC1* (hereditary prostate

cancer 1) locus is associated with early prostate cancer development, and is more common in AA families with multiple family members with prostate cancer (3; 55; 269). In addition, several microRNAs associated with aggressive prostate cancer show differential expression in AA versus CA (41; 260; 285; 286; 299). Other genes exhibit increased methylation in AA versus CA samples, including *RAR β* (Retinoic acid receptor β), and *SPARC* (secreted protein acidic and rich in cysteine) (63; 160). Additionally, increased methylation of *RAR β* and *CD44* (homing cell adhesion molecule) in AA prostate cancer is associated with increased risk and tumor grade, respectively (280; 313; 314). Specific changes observed in hormone signaling, genomic alteration and expression changes each may play some role in AA prostate cancer.

Although studies have contributed to the growing understanding of the ethnic disparity observed in prostate cancer, the key biological causes of the disparity have not been defined. Importantly, the translation of these studies to improvement in the ability to diagnose, prognose, or treat AA prostate cancer patients has been limited.

THE DISCOVERY OF THE FREQUENT DELETION OF *LSAMP* IN PROSTATE CANCER

The Center for Prostate Disease Research led a collaborative study of 7 AA and 7 CA prostate cancer whole genomes, and discovered a novel alteration at the *LSAMP* (Limbic System Associated Membrane Protein) locus, 3q13.31 (218). Further evaluation in tissue microarrays derived from a matched cohort of 59 CA and 42 AA tumor samples found the *LSAMP* deletion to be significantly more prevalent in AA specimens (26%) when compared to CA (7%). Deletions at the *LSAMP* locus were further observed by SNP array in 25% of AA (11/44) and 14% of CA (34/244) prostate tumors in an independent database (TCGA). The association of *LSAMP* with AA prostate cancer,

commonly *ERG*-negative and wild type-*PTEN*, contrasts with the frequent *PTEN* deletion and *TMPRSS2-ERG* fusion observed in CA prostate cancer. Patients with an *LSAMP* deletion had significantly reduced BCR free survival and a significant association with an advanced pathological T stage (218). Recently, the prevalence of *LSAMP* alteration in prostate cancer was corroborated in a Chinese prostate cancer genome study. Structural variations of the *LSAMP* locus which resulted in significant downregulation of *LSAMP* expression occurred at a frequency of 11%. Additionally, significant *LSAMP* downregulation was observed in 52% of prostate cancer specimens (234). *LSAMP* loss and downregulation has been recently observed in clinical samples, but does *LSAMP* truly exhibit significant biological function in prostate cancer?

LSAMP AND IGLON FAMILY MEMBERS: MOLECULAR CHARACTERISTICS

LSAMP is a 64-68 kD surface glycoprotein which acts as a selective cell adhesion molecule. The *LSAMP* gene maps to 3q13.31, contains 7 exons, and spans 654 kb (157; 221). The translated sequence contains 3 Ig domains, 8 putative N-linked glycosylation sites, a hydrophobic C-terminus typical for GPI-membrane anchors, a secretory signal sequence, and several putative phosphorylation sites (221). *LSAMP* is a member of the IgLON family, which also contains *OPCML* (Opioid-binding protein/cell adhesion molecule), *NEGR1* (Neuronal growth regulator 1), *NTM* (Neurotrimin), and *IGLON5* (IgLON Family Member 5) (76; 166; 221; 222; 322). IgLONs are cell adhesion molecules of the Ig superfamily, which play roles in development, regulation of cell proliferation, differentiation and migration (38). Within the nervous system, they play a role in axon growth and guidance, cell-cell recognition, synaptogenesis, and neural plasticity (72; 106; 108). IgLONs typically contain three Ig domains, facilitating

adhesion, and are anchored to the outer leaflet of the cell membrane via a GPI anchor. GPI anchors sequester IgLONs in lipid rafts, encouraging their lateral association, and potential facilitation of cell signaling through interaction with a transmembrane receptor (265). Despite the likelihood of association with transmembrane signaling molecules, LSAMP's specific role in signal transduction has not been previously revealed.

LSAMP exists in alpha and beta isoforms, with the latter containing 11-12 additional amino acids between the third Ig domain and the GPI anchor (39). In a recent study seeking to characterize LSAMP isoform distribution, Phillips *et al.* proposed the regulation of two alternative promoters play a role in isoform expression levels and behavior (220). Promoter-specific IgLON expression is differentially expressed in distinct areas of the brain (295). In neuronal precursors, *LSAMP* is induced in response to Collagen IV in combination with Tumor Necrosis Factor- α (TNF α) or Epidermal Growth Factor (EGF) (77). *LSAMP* knockout mice exhibit reduced Mineralocorticoid Receptor expression, as well as memory and emotional deficits, providing evidence of LSAMP's ability to affect transcriptional activity and phenotype (128; 129; 226).

IgLONs regulate neurite outgrowth through cell type specific mechanisms which may be attractive or repulsive, via homophilic and heterophilic interactions (87; 173). In the regulation of neurite outgrowth, individual Ig domains have been shown to mediate distinct functions in different neuronal cell subtypes (72; 333). In addition to their function at the membrane, IgLONs have also been shown to be shed from the cell surface in a metalloproteinase dependent manner, regulating neurite outgrowth (247). LSAMP serves as a ADAM10 (A Disintegrin and metalloproteinase domain-containing protein

10) substrate and negatively regulates outgrowth of neurons (248). However, the major role of IgLONs is cell adhesion and recognition, which occurs with or without regulation of neurite outgrowth (181). Early experiments suggest LSAMP as a homophilic adhesion molecule, but it has since been shown that all IgLONs interact with greater affinity heterophilically with the other family members, and two or more members are generally expressed in a given tissue at a time (173). IgLONs may function as independent cell adhesion molecules, or as heterodimeric proteins (DIgLONs). Depending on the expression of various IgLONs, DIgLONs may interact with identical or different DIgLONs on adjacent cells (232). Akeel *et al.* used pertussis toxin and a Rho-kinase inhibitor, to reverse the inhibition of LSAMP-NTM on neurite outgrowth, loosely implicating G-protein coupled receptor signaling and RhoA activation in a possible IgLON mechanism of action (5). However, despite suggestions of intracellular signaling, and observations of gene expression changes, no specific signal transduction mechanism mediated by *LSAMP* has been identified. What, if any, signaling pathways are involved?

LSAMP LOCATES IN A HIGHLY FRAGILE GENOMIC REGION

Genomic instability is a hallmark of cancer (197). Chromoplexy, a phenomenon first observed in prostate cancer, causes genetic material from one or more chromosomes to be scrambled and reassembled in a new configuration. In prostate cancer, this occurs through a proposed mechanism of AR-mediated DNA breaks and dysfunctional repair machinery (13). Similarly, chromothripsis is a phenomenon in which hundreds of chromosomal rearrangements occur in confined chromosomal regions (237; 273). Both are examples of chromoanagenesis, and can result in multiple tumor suppressor

inactivations and/or oncogene activations in a single event (114). Recent studies have implicated chromothripsis in many types of cancer, including 16.7% of high-risk prostate cancer (163; 237).

The *LSAMP* locus occurs within a double stranded break (DSB) cluster, making it more prone to rearrangement (307). The 3q13.31-q-terminal region containing *LSAMP* has been described as a jumping translocation in acute monocytic leukemia (148). Analysis of osteosarcoma samples revealed significant chromosomal breakage clustering within *LSAMP* (268). The prevalence of interchromosomal arrangements significantly distinguishes AA and CA prostate cancer (218). This suggests chromoanagenesis may be more prevalent in AA prostate cancer. An AA-associated SNP within the *LSAMP* locus has been associated with ulcerative colitis, showing there are ethnicity related SNPs within the locus which can contribute to a disease phenotype (33). AA-associated SNPs in the 3q13.31 region may contribute to an increased susceptibility to *LSAMP* deletion, interchromosomal rearrangement, or initiation of chromoanagenesis; however, this remains to be determined.

LSAMP AND IGLON FAMILY MEMBERS: DISEASE AND POTENTIAL SIGNALING

All members of the IgLON family have been implicated in various psychological and neuropsychiatric disorders, as well as cancer (22; 151; 152; 201; 297). In addition to these diseases, polymorphisms which result in *LSAMP* downregulation play a role in coronary artery disease through the enhanced proliferation of smooth muscle cells (303). *OPCML* is inactivated and displays tumor suppressor function in epithelial ovarian cancers, and various brain tumors (233; 255). *NEGR1* is commonly down regulated in many cancer types. *NEGR1* overexpression attenuates oncogenic phenotype, and its

depletion increases cell migration and invasion (142). The observations of IgLONs in cancer are suggestive of a tumor suppressive function.

Consistent with other IgLON family members, *LSAMP* inactivations via recurrent deletion, translocation, or methylation of the upstream CpG island, have also been reported in acute myeloid leukemia, ovarian carcinoma, and in clear cell renal carcinoma (48; 158; 201). Deletions in a 2.1 Mb region of 3q13.31 were identified in a high proportion of osteosarcoma tumors and cell lines, alongside reduced *LSAMP* expression levels (19; 157; 318). Additionally, Ntougkos *et al.* reported significant reduction of *LSAMP*, *OPCML*, and *NEGR1* expression in epithelial ovarian cancer (201). Ovarian oncogenesis is characterized by cell dedifferentiation which drives the epithelium to the various histological subtypes. Expression levels of IgLONs are associated with a histologic type, suggesting a role in the differentiation process of ovarian oncogenesis. *LSAMP* and *NEGR1* expression is positively correlated and most significantly reduced in serous, clear cell and endometrioid tumors. *LSAMP* expression was four times higher in moderately and well differentiated tumors than in the more aggressive poorly differentiated phenotype. Reduced *LSAMP* expression was found to be a negative prognostic indicator and significantly associated with reduced survival (201). Studies have observed loss of *LSAMP* and other IgLONs to be associated with cancer, however, the functional characterization of these molecules in cancer cell culture models is limited.

Previously, only a single study has modulated *LSAMP* expression in a cancer cell model to characterize its role in tumor progression. Barøy *et al.* reported a significant reduction in *LSAMP* copy number, mRNA expression and protein expression in osteosarcoma samples (19). *LSAMP* copy number loss and low expression have both

been associated with reduced survival in osteosarcoma. Copy number did not strictly correlate with mRNA or protein expression, with some samples of normal or increased copy number having undetectable or significantly reduced expression. This suggests copy number loss is not the only mechanism negatively regulating expression. Exogenous *LSAMP* expression in an osteosarcoma cell line with a *LSAMP* homozygous deletion significantly reduced proliferation and tumor growth. Gene expression analysis upon *LSAMP* re-expression in an osteosarcoma model revealed upregulation of hairy and enhancer of split 1 (*HES1*), cancer/testis antigen 2 (*CTAG2*), and kruppel-like factor 10 (*KLF10*). These genes are related to cancer, however, when samples with and without detectable levels of *LSAMP* protein were compared, no differences in the expression of these genes were observed. (19). Other than the observed attenuation of proliferation and tumor growth in an osteosarcoma model, no further function or mechanism has been reported for *LSAMP* in cancer. Does *LSAMP* mediate its function in prostate cancer by regulating cell-proliferation, or does cell-adhesion also play a role?

Characterization of the molecular signatures of AA prostate cancer models, where *LSAMP* aberrations are most prevalent, can give insight to the underlying mechanisms. Whole proteome comparison of isogenic AA derived tumor (RC-77T) and normal (RC-77N) immortalized cell lines revealed β -catenin upregulation, and Integrin downregulation in the tumor cell line (193; 284). Pathway analysis revealed RC-77N (normal) associated with cell-adhesion molecules and extracellular matrix (ECM)-Receptor interaction pathways. Whereas, RC-77T (tumor) associated with tight junctions, and adherens junctions pathways (193). This is consistent with other reports of elevated β -catenin, and downregulated Integrins in AA prostate cancer compared to CA prostate

cancer (145; 287). Can *LSAMP* inactivation be related to this signature observed in an AA prostate cancer model?

Studies of *LSAMP* in prostate cancer are quite limited. A single nucleotide polymorphism in an NFkB binding site found in an *LSAMP* intron has been shown to be a significant predictor of prostate cancer-specific mortality following androgen deprivation therapy, but prior to Petrovics *et al.*, *LSAMP* rearrangement or downregulation had not been reported in prostate cancer (123; 218). Although *LSAMP* has been implicated in various cancers as a putative tumor suppressor, no systematic functional or mechanistic studies have previously been performed to define the biological implications of the observed genomic aberrations. In order to define tumor suppressive functions and lead to improved prostate cancer treatment, further characterization of *LSAMP*'s function and molecular mechanism is needed.

Receptor Tyrosine Kinase Signaling

Previous studies of *LSAMP* have primarily been conducted in a neuronal context to study its adhesion mediated function and its affect on behavior. The role of *LSAMP* in any defined signaling pathway has not been previously identified. *LSAMP*, and the IgLON family, are anchored to the outer leaflet of the cell membrane via a GPI anchor, which contributes to an association with lipid rafts, and close proximity with intracellular signaling molecules. Any intracellular signaling affected by IgLONs would have to be mediated by a transmembrane protein. In a single study in an ovarian cancer model, *OPCML*, an IgLON family member, downregulated specific RTKs: EphA2 (Ephrin type-A receptor 2), FGFR1/3 (Fibroblast growth factor receptors 1 and 3), and HER2/4 (human epidermal growth factor receptors 2 and 4). Interaction with these RTKs induced

endocytosis and subsequent proteasomal degradation. RTK downregulation attenuated ERK (Extracellular signal-regulated kinase) and Akt (Protein kinase B) phosphorylation, and cell growth (180). *OPCML* has further been shown to potentiate Anti-EGFR and Anti-HER2 therapy in *HER2*-positive breast cancer (324). Given the molecular and functional similarities within the IgLON family, does *LSAMP* inhibit signaling through a similar mechanism in prostate cancer?

Fibroblast Growth Factor Receptors 2 and 4

If *LSAMP* regulates signaling through RTKs, it is likely to be through the interaction with a specific few. Fibroblast Growth Factor Receptors (FGFR) are RTKs involved in various cancers, and play a role in proliferation, invasion, angiogenesis, androgen independence, and metastasis (20; 46; 159). Angiogenesis is the formation of new blood vessels into an avascular region, facilitating the proliferation and metastasis of tumor cells (199). FGFR2 is expressed in basal epithelials, and 30% of localized prostate cancer, but not luminal epithelials, where prostate cancer often initiates (90; 209). FGFR2 increases proliferation *in vitro*, and is found in increasing quantities in a prostate cancer model through its progression (126; 239). FGFR2 signaling is central to preserving stemness and preventing differentiation of basal prostate stem cells (125). Additionally, FGFR2 was one of five antigens identified as a potential prostate cancer biomarker in a South African Cohort (2).

FGFR4 is expressed in both normal and malignant prostate tissue (302). However, specific activating ligands of FGFR4 are present exclusively in prostate cancer (203). A germLine polymorphism in *FGFR4* is associated with prostate cancer initiation, and *FGFR4* expression increased invasion (263). Inhibition of FGFR (1/4) signaling in a

prostate cancer model reduced ERK phosphorylation and invasion, and inhibited tumor growth and angiogenesis (75). FGFR4 was also found to be independently associated with prostate cancer specific survival, and higher expression levels associated with tumor grade (191). Further, FGF signaling has been shown to support androgen independence in CRPC, a hallmark of aggressive prostate cancer (27).

Ephrin A3

Another RTK, Ephrin Receptor A3 (*EPHA3*), is involved in cell-cell interaction, neural growth during development, prostate cancer bone metastasis, poor prostate cancer patient outcome, as well as metastasis in other cancers (132; 195; 207; 316). During development, Ephrin receptors and ligands display distinct tissue expression patterns and regulate the migration of cells and neuronal growth cones, via cell-adhesion and cytoskeletal regulation (37; 68; 182). Ephrins (Ephrin Receptor ligands) may serve as chemoattractants to Ephrin receptor expressing cells (211). Ephrins can be expressed on endothelial cells (115) and have been implicated in angiogenesis (212; 301)

EPHA3 is involved in angiogenesis, cancer stem cell (CSC) maintenance, and metastasis (132). *EPHA3* was first implicated in cancer when it was identified in a leukemic cell line, and is most well known in lung cancer, but has been implicated with aggressive disease in multiple cancers (65; 168; 175; 195; 309). In a multiple myeloma model, knockdown or blocking of *EPHA3* inhibited adhesion to fibronectin or stromal cells, and their ability to invade (162). Further, expression profiling following *EPHA3* knockdown showed modulation of several molecules involved in adhesion, migration, and invasion processes (162).

In prostate cancer, *EPHA3* was found to be upregulated in androgen independent cells (267). Ozdemir, *et al.*, implicates *EPHA3* as representing a core part of the bone specific stroma transcriptional response to prostate cancer induced osteoblastic metastasis (207). Wu *et al.* demonstrated *EPHA3* as an oncogene in prostate cancer (316). *EPHA3* expression enhanced proliferation and survival in LNCaP cells *in vitro*, and tumor development in nude mice. *EPHA3* was found to increase phosphorylation of Akt (Ser473, Thr308). They further found a positive correlation of *EPHA3* levels and Gleason grade. (316). Ephrin Receptor activation in PC3 induced inhibition of integrin mediated cell adhesion (183). Ephrin or Ephrin receptor expression has also been shown to disrupt E-cadherin mediated cell adhesion, mediated through FGF signaling (136; 311). Overexpression of Ephrin receptors may increase sensitivity of tumors to Ephrins, decreasing adhesion, increasing motility and invasiveness (67). *LSAMP* may play a role in the inhibition of these oncogenic receptors.

Integrins

Integrins are transmembrane receptors that facilitate cell adhesion to the ECM, and can facilitate intracellular signaling via Focal Adhesion Kinase (FAK) (91). Integrin dysregulation and focal adhesion abnormalities are implicated in various cancers (178). Expression of specific Integrins has been shown to be downregulated in localized prostate cancer (53). Downregulation of specific Integrins can increase prostate cancer cell motility (60). Integrins $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, αV , $\beta 1$, and $\beta 4$ are expressed in normal basal cells. $\alpha 3\beta 1$ and $\alpha 6\beta 1$ expression is associated with invasive prostate cancer, while expression of the others is often lost (58). Integrin $\alpha 4$ expression is significantly reduced in metastatic prostate cancer (153). Contrasting with its association with invasive

prostate cancer, in another study Integrin $\alpha 3\beta 1$ was shown to suppress prostate cancer migration, invasion and anchorage independent growth (296). In cases such as this, protein behavior can depend on its interactions. For example, Filamin-A may act as a metastatic potentiator with interaction with Integrin $\beta 1$ or Caveolin-1, but can act as a metastatic inhibitor in other contexts (250). Specific Integrin pairs have also been implicated in bone metastasis (161; 251). The Integrin $\alpha 6\beta 1$ -laminin interaction has been implicated in perineural invasion of prostate cancer (272). Others suggest Integrins play a role in clustering of circulating tumor cells (CTCs), facilitating a metastatic phenotype (105). However, adhesion of metastatic prostate cancer cells to bone marrow epithelial cells requires molecules other than Integrins (266). With each subunit and integrin pair mediating various cell specific functions through the interactions of complex signaling networks, the role of Integrins in cancer is complex (7; 127). In prostate cancer, Integrins are abnormally expressed. However, most α and β subunits are downregulated, and that downregulation is associated with an invasive phenotype (69; 91). Does LSAMP play a role in regulating Integrin expression?

ERK1/2 and Akt Signaling

RTKs and Integrins are transmembrane proteins which facilitate signaling through the Akt and ERK pathways. These signal transduction pathways play a central role in the cell cycle, survival, metabolism, and differentiation (109; 179). Hyperactivation of these pathways is prevalent in many epithelial cancers, including prostate cancer. Akt and ERK signaling is hyperactivated in the progression to CRPC, and is significantly associated with a poor outcome (53; 144; 156; 176; 334). PTEN, a phosphatase and major prostate cancer tumor suppressor, negatively regulates the Akt pathway through the

dephosphorylation of Akt (326). *PTEN* loss, which occurs in more than 2/3 of patients with advanced prostate cancer, and Akt phosphorylation predict prostate cancer BCR, and are associated with aggressive disease and poor outcome (47; 95). ERK signaling dysregulation has been strongly implicated in invasion and metastasis (281). ERK 1/2 hyperactivation is implicated in prostate cancer progression, and poor prognosis (88; 293).

ERK1/2 activation strongly correlated with Akt pathway dysregulation (144). In a mouse model, Akt and ERK pathways were activated in androgen independent lesions, and increased tumorigenicity and androgen independence (84). Akt regulates AR expression via crosstalk with the ERK pathway (116). Hormone ablation activates Akt and ERK1/2, mediating AR downregulation and neuroendocrine differentiation (117; 315). RTKs, and Integrins, via FAK, can activate both of these pathways through Ras and PI3K (Phosphatidylinositol-4,5-bisphosphate 3-kinase) (50). There is major crosstalk between these pathways, and their role in tumor progression is significant (43; 86). Does LSAMP inhibit signaling of these pathways?

Beta-Catenin Dysregulation: Cell-Cell Adhesion and Transcriptional Co-activation

ERK and Akt signaling have many downstream effects, including the activation of β -catenin. β -catenin pathway hyperactivation is implicated in many cancers (224). Glycogen Synthase Kinase-3 β (GSK3 β), also implicated in various cancers, phosphorylates cytoplasmic β -catenin, priming it for proteasomal degradation (194; 224). The Akt and ERK1/2 pathways converge at GSK3 β . Both Akt and ERK phosphorylate and inactivate GSK3 β , thus allowing cytoplasmic β -catenin to translocate to the nucleus and affect its transcriptional coactivation (194). Activated Akt inhibition of GSK3 β ,

resulting in stabilization and activation of β -catenin, has been confirmed in prostate cancer (257). Overexpression of β -catenin promotes prostate tumor progression *in vivo* (321). Additionally, β -catenin signaling is high in cancer stem cells, which are strongly implicated in recurrence (26; 164). β -catenin activity plays a significant role in prostate cancer and metastasis.

β -catenin has dual functions: membranous β -catenin regulates cell-cell adhesion through its association with cadherins, whereas, cytoplasmic β -catenin is active and free to translocate to the nucleus and affect transcriptional co-activation (270). Activated β -catenin contributes to prostate cancer invasion in part by the loss of cadherin-mediated cell adhesion, but also through transcriptional activity. These aberrant changes affect the gene expression of many molecules which may contribute to an aggressive phenotype, including the upregulation Matrix metalloproteinases (MMPs), which breakdown the ECM and are significantly associated with prostate cancer progression and metastasis (8; 32; 36; 57). Membranous β -catenin is reduced in prostate cancer compared to benign tissue, and its reduction associates with a higher Gleason grade (130). Additionally, membranous β -catenin and E-cadherin is reduced in prostate cancer development, and nuclear β -catenin is significantly higher in advanced prostate cancer (120; 130).

There is also interplay between β -catenin and AR signaling. AR can compete with TCF/LEF-1 (Lymphoid enhancer-binding factor 1), a transcription factor commonly associated with β -catenin, inhibiting transcription of TCF/LEF-1 sites (190). β -catenin binds to AR, and interactions can be modulated by other cofactors (317; 319). β -catenin coactivates mutant ARs and AR signaling in hormone ablated LNCaP (androgen dependent prostate cancer cell line (Supplementary Table 1), suggesting a role in

development of CRPC (270). Next generation sequencing of CRPC tumors showed alterations in multiple components of the β -catenin pathway which were not present in naïve, hormone ablation-responsive prostate cancer (93; 229). Cytoplasmic or nuclear staining of β -catenin was significantly higher in metastatic CRPC cases when compared to radical prostatectomy (62). β -catenin was also higher following ADT when compared to pretreatment samples (215). Meanwhile, other studies found conflicting results, with no significant β -catenin expression in CRPC (11). There is much evidence for the involvement of β -catenin nuclear localization in CRPC, but sample size, specimen processing, or staining methods may account for some of the observed variations(319).

In addition to its role in transcription, β -catenin regulates cellular adhesion. Cell-cell adhesion, critical for the maintenance of cell polarity, is mostly reduced in cancer, allowing cells to disrupt the normal structure and adopt a malignant phenotype (111). Additionally, cell-cell adhesion plays a role specifically in prostate cancer progression (186). GSK- β inhibition of β -catenin and cell-cell adhesion promotes the epithelial-mesenchymal transition (EMT) and metastatic characteristics in prostate cancer (83). A critical process in the progression to metastasis, the EMT occurs as epithelial cells lose polarity and cell-cell adhesion, and gain invasive characteristics as mesenchymal stem cells (103). E-cadherin and catenins interact with actin as a central component of adherens junctions, and are commonly dysregulated in the progression of prostate and other cancers (111; 216; 227). The E-cadherin-catenin association is responsible for maintenance of intercellular junctions and cytoskeletal stability, and its dissociation can result in β -catenin nuclear translocation and transcriptional co-activation (35; 100; 187).

Adhesion molecules play a significant role in prostate cancer progression. E-cadherin levels are reduced in prostate cancer progressively with increasing Gleason grade (9; 189). An adhesion molecule normally expressed on endothelial cells, such as VCAM-1 (Vascular cell adhesion protein 1), can be upregulated in tumor cells to mediate tumor-endothelial adhesion, and facilitate metastasis (49). Neuronal Cell Adhesion molecule (*NRCAM*), an Ig superfamily member involved in neural development and angiogenesis, is a β -catenin target frequently expressed in prostate cancer, and other cancers (4; 54; 244; 291). In an androgen-independent prostate cancer xenograft model, testosterone administration resulted in the downregulation of *NRCAM* and N-cadherin suggesting an association with CRPC and expression dependent on non-AR signaling (134). In a thyroid cancer model, *NRCAM* significantly increased growth and invasiveness by binding to EGFR and $\alpha 4\beta 1$ Integrins, activating ERK and Akt pathways. ERK and Akt pathway activation increased β -catenin activity, which further upregulates *NRCAM* (328). This models a mechanism by which activation of ERK and Akt pathways can contribute to β -catenin activity through the upregulation of a cell adhesion molecule. ERK, Akt, and β -catenin signaling play a central role in the development of distant metastasis (208). Does *LSAMP* inactivation contribute to an aggressive prostate cancer phenotype by the dysregulation of these signaling pathways, and what phenotypic changes occur as a result?

TABLE 2: FIVE YEAR RELATIVE SURVIVAL RATES OF PROSTATE CANCER BY DISEASE STAGE.

	All Stages	Local	Regional	Distant
5 Year Relative Survival Rate (%)	99	>99	>99	30

Local: confined to organ; Regional: extended to surrounding tissues and/or involves lymph nodes; Distant: spread to parts of body remote to primary tumor. Siegel et al., Cancer statistics, 2018. *CA* 68:7-30

SIGNIFICANCE

Prostate cancer is relatively common, affecting roughly 1 in 9 men in their lifetime (264). Most cases develop slowly and often do not cause symptoms or mortality (101; 264). Overtreatment of indolent disease may result in significant morbidity (61). Active surveillance, a term used to describe regular close monitoring with prostate specific antigen tests, digital rectal exams, ultrasounds or biopsies, is becoming more commonly prescribed. The majority of localized prostate cancer will remain indolent, and metastasis accounts for the large majority of prostate cancer related mortality (210; 264; 310). CRPC metastasis remains incurable despite intensive therapy, with the five year survival of distant disease at merely 30% (Table 2) (174; 264). Although there is significant genetic heterogeneity in prostate cancer, and patients often develop distinct tumor foci, recent data suggests metastases originate from a single tumor foci (97). Altered expression of metastatic driver genes results in the escape from the primary tumor site (intravasation), invasion through the vasculature (extravasation) establishment of a tumor site in a secondary tissue (105). The known genetic alterations which cause aggressive disease are limited, and studied mainly in the context of CA prostate cancer. Determining the specific molecular differences that distinguishes aggressive from

indolent disease is critical to improving the ability to make the critical decision of whether to monitor or treat.

The need to distinguish the molecular characteristics of aggressive disease is even more imperative in AA prostate cancer. AA prostate cancer occurs more often becomes aggressive more rapidly, and results in mortality more often (231; 264; 283). The ethnic disparity of prostate cancer suggests an increased frequency of distinct driver alterations in the AA prostate cancer genome. However, the key molecular mechanisms driving the disparity in AA prostate cancer, which predominantly exhibit *ERG*-fusion negative and wild-type *PTEN*, have not been identified.

Previous studies of *LSAMP* in other cancers, and the prevalence of genomic inactivation in prostate cancer, suggest a significant role in prostate tumor progression. The biology associated with *LSAMP* genomic aberrations has not been previously defined. Characterization of *LSAMP* is needed to confirm the significance of the genomic alteration, and to provide the first evidence of its function in prostate cancer. Further investigation could lead to the development of a crucial biomarker and the ability to molecularly stratify patient tumors with increased accuracy. Additionally, identifying a *LSAMP*-mediated signaling mechanism would provide new insights to the function of *LSAMP* and the molecular mechanisms underlying prostate tumor progression and the AA prostate cancer disparity. Furthermore, identification of a signaling pathway could provide potential therapeutic targets for prostate cancer patients with *LSAMP* inactivation. *LSAMP* loss may play a significant role in prostate cancer progression and the disproportionate incidence and mortality of AA prostate cancer. The investigation of

its function will contribute to the understanding of the biological mechanisms underlying this disparity and tumor progression, leading towards improved patient care.

CHAPTER 2: Materials and Methods

Cell Culture and Reagents

Neuroepithelioma cell line MC-IXC (CRL-2270), Osteosarcoma cell line Saos-2 (HTB-85), prostate derived cell line RWPE-1 (CRL-11609), and prostate cancer cell lines LNCaP (CRL-1740), MDA PCa 2b (CRL-2422), DU 145 (HTB-81), VCaP (CRL-2876), 22Rv1 (CRL-2505), PC3 (CRL-1435), NCI-H660 (CRL-5813), HUVEC (CRL-1730), NIH/3T3 (CRL-1658), and WPMY-1(CRL-2854) were previously obtained from American Type Culture Collection (ATCC; Manassas, VA), where they were regularly authenticated and tested for Mycoplasma contamination by the vendor (135-XV, 30-1012K). LAPC-4 was a kind gift from Dr. Charles Sawyer (UCLA), and cultured in IMDM (ATCC 20-2005) and 15% Fetal Bovine Serum (FBS) (30-2020); MDA PCa 2b cells were cultured in BRFF-HPC1 media (Athena Enzyme Systems, MD. Cat No. 0403) and 20% FBS; LNCaP cells were cultured in RPMI-1640 (ATCC 30-2001) and 10% FBS; DU 145 cells were cultured in EMEM (ATCC 30-2003) and 10% FBS; MC-IXC cells were cultured in a 1:1 mixture of EMEM and F12 (ATCC 30-2004) media and 10%FBS; Saos-2 cells were cultured in McCoy's 5A (ATCC 30-2007) and 15% FBS; RWPE-1 cells were cultured in K-SFM (Gibco, Cat No. 10724-011); VCaP cells were cultured in DMEM (ATCC 30-2002) and 10% FBS; 22Rv1 cells were cultured in RPMI-1640 and 10% FBS supplemented with 2 mM L-glutamine (Gibco, Cat No. 25030149), 10 mM sodium pyruvate (Gibco, Cat No. 11360070), 4.5 g/L glucose

(Gibco, Cat No. A2494001), 1.5 g/L sodium bicarbonate (Gibco, Cat No. 25080094); PC3 cells were cultured in F12 and 10% FBS supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate; NCI-H660 cells were cultured in RPMI-1640 and 5% FBS supplemented with 5 µg/mL insulin (Sigma Aldrich, Cat No. I6634), 10 µg/mL transferrin (ThermoFisher, Cat No. 11107018), 30 nM sodium selenite (Sigma Aldrich, Cat No. 214485), 10 nM β -estradiol (Sigma Aldrich, Cat No. E2758), and 10 nM hydrocortisone (Sigma Aldrich, Cat No. H6909); HUVEC cells were cultured in endothelial cell growth media (Sigma Aldrich, Cat No. 211-500); NIH/3T3 cells were cultured in DMEM and 10% FBS; WPMY-1 cell were cultured in DMEM and 5% FBS. Cells were trypsinized using 0.25% trypsin-EDTA (ThermoFisher Scientific, Cat No. 25200056), and washed with D-PBS for passaging. Cells were grown for less than six months following resuscitation. For doxycycline (Sigma Aldrich, Cat No. D9891) induction experiments, LNCaP and MDA PCa 2b cells were cultured in 10% Tetracycline Free FBS (Takara, Cat No. 631106). Further cell culture model characteristics available in Supplementary Table 1.

Constructs, Virus production, Stable Cell Line Generation

LSAMP cDNA was cloned into pENTR Directional TOPO cloning vector with TOP10 chemically competent *E. coli* (ThermoFisher Scientific, Cat No. K240020), followed by Not I (New England BioLabs, Cat No. R0189) and BamHI (New England BioLabs, Cat No. R0136) restriction enzyme digest and separation on an agarose gel to screen the colonies. Sanger sequencing was performed (USUHS BIC Genomics) to confirm the correct cloning of insert. The recombination reaction was performed

according to ViraPower HiPerform Lentiviral Expression System protocol (ThermoFischer Scientific, Cat No. K531000) using Gateway Clonase II Enzyme mix (ThermoFischer Scientific, Cat No. 11791020) to catalyze the recombination of *LSAMP* cDNA into pCW57.1 (a gift from David Root, Addgene plasmid # 41393) or pLenti6.3v5 (ThermoFischer Scientific, Cat No. K531520) in Stbl3 competent cells (ThermoFischer Scientific, Cat No. C737303). Plasmids were isolated from the TOP10 and Stbl3 colonies using Mini or Maxi plasmid purification kits (Qiagen, Cat No. 12125, 12163). BamHI digest and separation on an agarose gel was performed to screen the colonies, and Sanger sequencing was again performed to confirm the integrity of the insert. Virapower Lentiviral Packaging Mix (ThermoFischer Scientific, Cat No. K497500) and the empty or *LSAMP*-containing expression vector was co-transfected into HEK293FT cells (ThermoFischer Scientific, Cat No. R70007) to produce lentivirus. Lipofectamine 2000 transfection reagent (ThermoFischer Scientific, Cat No. 11558019), and the plasmids, were diluted into Opti-MEM (ThermoFischer Scientific, Cat No. 31985062), and administered to the cells for 24 hours, followed by a media change and subsequent 48-72 hour incubation. Harvested lentivirus supernatant was filtered through 0.45 µm PVDF filter, and were then used to transduce LNCaP, MDA PCa 2b, and DU 145 cells, along with Polybrene (Millipore, Cat No. TR-1003-G). The media was changed after 24 hours, and Puromycin (Sigma Aldrich, Cat No. P8833) conditioned selection media was added 72-96 hours following the transduction. Approximately ten colonies were subcultured, expanded, and validated for *LSAMP* expression by western blot and immunofluorescence. For *in vivo* imaging in future experiments, pGL4.50 (Promega, Cat No. E1310) was transfected into stably expressing LNCaP and MDA PCa 2b cells, and

selected with Hygromycin B (R & D Biosystems, Cat No. 4137). Prior to imaging luciferin was administered (GoldBio, Cat No. LUCK-500). LentiGuide-Puro (a gift from Feng Zhang, Addgene plasmid # 52963), and CRISPR lentiCRISPR v2 (a gift from Feng Zhang, Addgene plasmid # 52961) (246) containing a non-targeting or *LSAMP* specific sequence (constructs modified by Gene script), were used to assemble lentivirus, transduce and select PC3 cells, as described above.

Fluorescence *in situ* hybridization analysis

To assess *LSAMP* copy number by FISH analysis, MC-IXC, SAOS-2, LNCaP, MDA PCa 2b, VCaP, RWPE-1, LAPC-4, NCI-H660, and PC3 were grown in four well chamber slides (ThermoFisher Scientific, Cat No. 154917PK) and fixed in 10% neutral buffered formalin overnight. Slides were rinsed and dehydrated in graded ethanol concentrations before immersion in 100 mM Trizma Base and 50 mM EDTA (pH 7) at 94°C for 30 minutes. Slides were incubated in Digest All III (Invitrogen, Cat No. 00-3009), rinsed, and dehydrated again in ethanol. Probes (CytoTest LSP *LSAMP* CytoOrange, *CEP3* CytoGreen) were applied, and slides were placed on 94°C for 10 minutes before overnight incubation at 37°C. Slides were washed in SSC, and cover glasses were mounted with DAPI mount (Invitrogen, Cat No. P36935). Bacterial artificial chromosome clones (a kind gift from Dr. Joseph Cheng and Dr. Reinhard Ebner, CytoTest Inc.) were used to construct a 600kbp sequence which covered the length of the *LSAMP* locus. As a control, a CytoGreen fluorescently labeled probe specific to the chromosome 3 α -satellite centromeric sequence (*CEP3*). The performance of the probes in interphase nuclei was assessed in normal human lymphocytes. Centromere and *LSAMP* counts were

analyzed to determine whether there was an amplification or deletion. Cells with at least two centromeres were counted, and deletions were counted when greater than 75% of the cells showed a loss of *LSAMP*.

Quantitative Reverse Transcriptase PCR

Isolation of total RNA from cell lines was performed with RNA Bee (TelTest Inc., Cs-104B), chloroform-isoamyl alcohol (JT Baker, Cat No. 9180-22), cold isopropanol (Sigma Aldrich, Cat No. 19516), and centrifugation. The quality and concentration of the mRNA was assessed using a Nanodrop 3000. Synthesis of the cDNA was performed using Omniscript RT Kit (Qiagen, Cat No. 205111), 1 µg of cell line RNA and random primers at 37°C for 60 min followed by 93°C for 5 min, and then held at 10°C.

Quantitative-PCR was performed using 10ng of cDNA, Taqman gene expression master mix (ThermoFisher, Cat No. 4369016), and optimized forward and reverse primers and probes (Supplementary Table 2) for each of the expression assays on a Mx3000P qPCR System (Agilent Technologies). *GAPDH* and *B2M* genes were included as housekeeping genes for the normalization of *LSAMP* expression. Conditions for the PCR included 55°C for 2 min, followed by an initial denaturation at 95°C for 10 min, and 50 cycles of 95°C for 30 sec, 56°C for 1 min, and 72°C for 1 min. Expression was measured in cycle threshold (Ct) log 2 values. Expression was normalized to the geometric mean of *GAPDH* and *B2M* (endogenous controls) and analyzed according to the relative quantification method, as $\Delta Ct = Ct \text{ GAPDH} - Ct \text{ target}$. Fold difference between any two samples was calculated as $2^{(\Delta Ct1 - \Delta Ct2)}$.

Antibodies

LSAMP primary antibodies: mAb873 clone 110728 (223; 333)(R & D Systems, Cat No. MAB873) and 2G9 (Developmental Studies Hybridoma Bank, #AB213820, deposited by Levitt, P.R.) (166) were used. Specific antibodies against Na/K ATPase (Cat No. 3010), EphA1 (Cat No. 90673), phospho-EphA2 (ser897)(Cat No. 6347), EphA2 (Cat No. 6997), phospho-EPHA3 (Tyr779)(Cat No. 8862), EPHA3/4/5 (Cat No. 8793), EphA7 (Cat No. 64801), EphB4 (Cat No. 14960), EphB2 (Cat No. 83029), FGFR1 (Cat No. 9740), FGFR2 (Cat No. 11835), FGFR3 (Cat No. 4574), FGFR4 (Cat No. 2894), VEGFR2 (Cat No. 9698), VEGFR3 (Cat No. 2485), EGFR (Cat No. 2232), HER2 (Cat No. 2165), HER3 (Cat No. 12708), HER4 (Cat No. 4795), phospho-BRAF (Ser445)(Cat No. 2696), BRAF (Cat No. 9433), phospho-MEK1/2 (Ser217/221)(Cat No. 9154), MEK1/2 (Cat No. 9122), phospho-ERK1/2 (Thr202/Tyr204)(Cat No. 4370), ERK1/2 (Cat No. 9102), Phospho-Akt (Ser473)(Cat No. 4060), Akt (pan)(Cat No. 4685), phospho-GSK3 β (ser9)(Cat No. 5558), GSK3 α/β (Cat No. 5676), Nonphospho (active) β -catenin (ser45)(Cat No. 19807), phospho- β -catenin (Ser33/37/Thr41)(Cat No. 9561), β -catenin (Cat No. 8480), Axin1 (Cat No. 2087), phospho-FAK (Tyr397)(Cat No. 8556), FAK (Cat No. 3285), phospho- STAT3 (Tyr705)(Cat No. 9145), Integrins α 4 (Cat No. 8440), α 5 (Cat No. 4705), α V (Cat No. 4711), β 1 (D2E5) (Cat No. 9699), β 3 (Cat No. 13166), β 4 (Cat No. 14803), and β 5 (Cat No. 3629), Caspase 3 (Cat No. 9665), cleaved Caspase 3 (Asp175)(Cat No. 9664), Caspase 7 (Cat No. 12827), cleaved Caspase 7 (Asp198)(Cat No. 8438), Caspase 9 (Cat No. 9508), cleaved Caspase 9 (Asp330)(Cat No. 7237), PARP (Cat No. 9542), cleaved PARP (Asp214)(Cat No. 5625), as well as Anti-

rabbit (Cat No. 7074) and Anti-mouse IgG (Cat No. 7076) HRP linked secondary antibodies were all acquired from Cell Signaling Technologies. CD1 (Cat No. SC-718) and GAPDH (Cat No. SC-25778) antibodies were used from Santa Cruz Biotechnology. NRCAM (Cat No. ab24344) was acquired from Abcam. Activated β -catenin antibody (clone 8E7) (Cat No. 05-665) was purchased from Millipore, US. Alexa Fluor-488 goat anti-mouse secondary antibody (Cat No. A-11001) or Alexa Fluor-594 goat anti-rabbit (Cat No. R37117), for use in immunofluorescence assays, were obtained from Invitrogen. Biotinylated horse anti-mouse secondary antibody (Vector, Cat No. BA-2000) was used for immunohistochemistry.

Western Blot Analysis

Whole cell lysates were isolated using M-PER buffer (ThermoFischer Scientific, Cat No. 78501) and supplemented with a protease/phosphatase inhibitor cocktail (Cell Signaling Technologies, Cat No. 5872). Samples were diluted in PBS, with the addition of loading buffer (Invitrogen, Cat No. NP0007) and a reducing agent (50 mM DTT final concentration) (Invitrogen, Cat No. NP0004) and heated at 70°C for 5 min. Protein standards (Invitrogen, Cat No. LC5925) were run alongside the samples in a 4-12% BisTris gel (Invitrogen, Cat No. NP0321) with MES SDS running buffer (Invitrogen, Cat No. NP0002), and antioxidant (Invitrogen, Cat No. NP0005). Protein was transferred from the gel to a PVDF Filter (Invitrogen, Cat No. LC-2005) using a semi-dry transfer apparatus with transfer buffer (Invitrogen, Cat No. NP0006). TBST containing 5% bovine serum albumin (BSA) (Sigma Aldrich, Cat No. A7030) was used for blocking and antibody dilution. Primary antibodies were incubated over night at 4°C, following by

washing in TBST, and incubation in the secondary antibody for one hour. ECL blotting agents (GE, Cat No. GERPN2109) were used to develop the blots. When using mAb873 for the detection of LSAMP, the reducing agent and antioxidant were omitted. Relative quantification of western blots were performed using Image J. Values were normalized to the loading control.

Immunofluorescence Assay

Prostate cancer cells were cultured on poly-L-lysine-coated cover glass (BD Biosciences, Cat No. 354085). Cells were fixed in paraformaldehyde buffered with PHEM, and permeabilized with 0.1% Triton X-100. Cells were blocked with 1% normal mouse (Millipore, Cat No. NS03L) or normal horse serum (Vector laboratories, Cat No. 1074G), before incubation with the primary antibody overnight. PHEM buffer containing BSA, normal serum, and Tween-20 was used as a wash and antibody dilution buffer (mAb 873 1:400). Cells were rinsed prior to a 1-3 hour incubation with the secondary antibody, and a second primary antibody. Cells were again rinsed, prior to a 1-3 hour incubation with the second secondary antibody and DAPI (Invitrogen, Cat No. D21490) or Hoescht 33342 (Thermo Scientific, Cat No. 62249). For actin staining, AlexaFluor-594 Phalloidin (Invitrogen, Cat No. A12381) was used. Cover glasses were mounted on slides using Prolong Gold (ThermoFisher Scientific, Cat No. P36930). Images were captured using a 40x/0.65 N-Plan objective on a Leica DMIRE2 upright inverted light microscope, or a Plan Apochromat 63x/1.4 oil DICM27 objective on a Zeiss 700 laser confocal microscope.

Adhesion Assays.

DU 145 cells containing LSAMP or control vector, or MDA PCa 2b-LSAMP cells with and without pretreatment of doxycycline were trypsinized and seeded into a 48-well cell adhesion assay in duplicate wells of an ECM Array (Cell BioLabs, Cat No. CBA-070), or Fibrinogen coated plate (Cell BioLabs, Cat No. CBA-058). BSA coated wells served as a control. 1×10^6 DU 145 cells/mL suspended in serum free media were seeded for 60 minutes, or decreasing cell densities of MDA PCa 2b for 14 hours. Cells were removed, washed, and stained. The stain extracts were transferred onto a 96-well plate and absorbance was measured at 560 nm. Experiments were performed three times. For cell-cell adhesion assays, HUVEC, WPMY-1, or NIH3T3 cells were seeded and allowed to grow to monolayer in a gelatin (Sigma Aldrich, Cat No. G193) coated 48 well plates. DU 145 cells containing *LSAMP* or empty vector control were trypsinized at incubated with CellTraker CMFDA Dye (ThermoFischer Scientific, Cat No. C7025). The cells were washed and resuspended in serum free EMEM before being seeded in equal densities onto the various monolayers. $0.5\text{-}2 \times 10^5$ cells/well were allowed to attach for 5-90 minutes, before five rounds of washing to remove unattached cells. Fluorescently labeled DU 145 cells which remained attached were photographed with a 5x objective on a Leica DMIRE2 upright inverted light microscope. For quantification, attached cells from three separate fields per each of the quadruplicate wells were manually counted. Additional experiments combined the pretreatment of the monolayer with $\text{TNF}\alpha$, to upregulate adhesion molecules, or the pretreatment of DU 145 cells with anti-LSAMP monoclonal antibody mAb873 at 1:50. Cell-cell adhesion assays were performed in three independent experiments. Statistical analysis was conducted using a t-test.

MTT assay

2.5×10⁵ cells/mL DU 145, 5×10⁵ cells/mL MDA PCa 2b, or 5×10⁵ cells/mL LNCaP cells were seeded into 48 (200 µL) or 96 well (100 µL) plates, in quadruplicate or quintuplicate, with or without doxycycline for MDA PCa 2b and LNCaP, or *LSAMP* expressing DU 145 versus control. Additionally, for doxycycline optimization in LNCaP, LNCaP-*LSAMP* and LNCaP-control were treated with doxycycline in decreasing concentration. MTT assays were performed every 24-48 hrs after seeding using the CellTiter 96 Nonradioactive Cell Proliferation Assay (Promega, Cat No. G4100). MTT dye was added to the cells followed by the stop/solubilization solution 3 hours later, and incubated over night. Plates were read at 560 nm, with the background absorbance at 630 nm subtracted. Additionally, the absorbance read in wells containing the appropriate media, but no cells, was subtracted. Experiments were performed 3 times. Statistical analysis was conducted using a t-test.

Cancer Signaling Phosphoarray and pathway analysis

MDA PCa 2b-*LSAMP* cells were cultured with or without doxycycline for 72-96 hours prior to harvest. Protein was extracted using M-PER, and purified using polyacrylamide spin desalting columns (ThermoFisher Scientific, Cat No. 89849). Amersham CyDye mono-reactive dye (GE, Cat No. PA25001) was used to label the protein. Cancer Signaling Phospho Antibody Array (Cat No. PCS248) was obtained from FullMoon Biosystems, and performed according to manufacturer's protocol using an antibody array assay kit (Full Moon Biosystems, Cat No. KAS02). Genomatix Pathway Analysis was used to analyze the results (genomatix.de).

Invasion, Migration Transwell Assays

Invasion and migration assays were conducted using the Matrigel invasion kit (BD Biosciences, Cat No. #354480,) low concentration basement membrane extract invasion assay (Trevigen, Cat No. 3481-024K), basement membrane invasion assay (Cell BioLabs, Cat No. CBA-110), Collagen-based invasion assay (Sigma Aldrich, Cat No. ECM551), or migration assay (Cell BioLabs, Cat No. CBA-107). Boyden chambers were rehydrated with serum free medium. 10% FBS was added as a chemoattractant to the bottom of the wells. Following overnight starvation in serum free media, 1×10^4 - 4×10^5 LNCaP, MDA PCa 2b, or DU145 cells, without or without LSAMP expression, were resuspended in serum free media, and added to the upper chamber, and incubated for 12-72 hours. Non-invading cells were removed, and the chambers were transferred into methanol, and stained with trypan-blue (Gibco, Cat No. 15250-061) prior to analysis.

Soft Agar Assay

10^4 - 10^5 MDA PCa 2b cells, treated with or without doxycycline, were trypsinized and resuspended with the appropriate medium and plated in a six well plate containing 2 mL 0.3% agar base medium, with or without doxycycline, and 2mL 0.5% agar base. Cell colonies were evaluated at 2-4 weeks.

Analysis of *LSAMP* Expression in normal tissue and DNA Methylation of *LSAMP* promoter in Prostate Cancer

Existing databases were utilized to investigate tissue expression and methylation in prostate cancer samples. RNAseq (GTEx, HPA(292)), CAGE (279) (Fantom5)(172),

SAGE (CGAP), and microarray (BioGPS) (276) datasets were used to assess *LSAMP* expression in human prostate tissues. The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. Gene expression data for *LSAMP* (ENSG00000185565.7) used in this dissertation were obtained from the GTEx Portal on 02/23/18 (data source: GTEx Analysis Release V7 (dbGaP Accession phs000424.v7.p2)). The Human Protein Atlas <http://www.proteinatlas.org/ENSG00000185565-LSAMP/tissue> was accessed on 02/23/18 to view HPA, and Fantom5 datasets. The SAGE expression dataset of the Cancer Genome Anatomy Project (CGAP) at cgap.nci.nih.gov/, and the BioGPS dataset: (GeneAtlas U133A, gcrma) at <http://biogps.org/dataset/GSE1133/> was accessed as recently as 02/23/18.

LSAMP methylation in prostate cancer was determined using MethHC, a methylation database of human disease (124). Methylation data was acquired from Illumina Infinium HumanMethylation450 Beadchip, and expression data was obtained from RNA sequencing data, both from the TCGA portal. The database contains DNA methylation data from 387 prostate cancer samples, and expression data from 342. Beta values were calculated as $(\text{methylated probe intensity}) / (\text{unmethylated probe intensity} + \text{methylated probe intensity} + 100)$. Average beta values, ranging from 0 (least methylation) to 1 (highest methylation), were compared in tumor and matching normal samples. Promoter region was defined as 1500 bp upstream to 500 bp downstream, of the transcription start site as defined by UCSC RefSeq. For statistical analysis, a t-test was used between tumor and normal groups for the box plot, and the Pearson correlation

coefficient was calculated for the association between methylation and expression in the scatter plot.

Senescence-associated- β Galactoside staining

LNCaP cells with or without doxycycline and DU 145 cells, *LSAMP* or control, were plated in six well dishes, and grown for 48-72 hours to 50-70% confluency. Staining was performed using a senescence associated β -Galactoside staining kit (Cell Signaling Technologies, Cat No. 9860S). The plates were rinsed with PBS, followed by addition of the fixative, and rinsed again before addition of the staining solution. Stained cells were observed using the 5-10x objective of a Leica DMIRE2 upright inverted light microscope.

***In vivo* Tumorigenicity**

Male, athymic nude mice were acquired from Charles River Laboratories. Mice used were between 7 and 10 weeks of age. 3×10^6 DU 145 cells were injected subcutaneously into the lower flanks of 20 mice, 10 with cells expressing *LSAMP*, and 10 containing vector control. Tumor size was monitored weekly, and tumor volume was calculated ($[\text{length} \times \text{width}^2]/2$). Tumors will be harvested and fixed for further processing and slide preparations. Lung, liver, kidneys, bone, brain and lymph nodes were harvested to determine the presence of any secondary tumors. Studies were conducted according to the guidelines in *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, revised 1996), and were approved by the Uniformed Services University of the Health Sciences Institutional Animal Care and Use Committee.

Apoptosis Induction

The androgen ablation sensitive LNCaP cell line was treated with or without 25 μ M enzalutamide. Cells were additionally administered doxycycline at 0, 0.1, and 1 μ g/mL. Cells were cultured for 48-96 hours before harvesting for western blot analysis.

Immunohistochemistry

To assess the ability of monoclonal antibodies to detect LSAMP by IHC, LSAMP-expressing and control DU 145 cells were grown, fixed in formalin, embedded in paraffin and sectioned. Slides were deparaffinized in xylene at room temperature, and placed in decreasing concentrations of ethanol to hydrate and remove xylene. Slides were placed into 0.6% hydrogen peroxide in methanol for 20 minutes, and rinsed in PBS prior to antigen retrieval in a citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0) and microwaved for 15 minutes at 50% power. Following cooling, slides were rinsed and blocked with 1% normal horse serum in PBS for 30 minutes. LSAMP mAb (2G9) was diluted in PBS (1:80, 1:160, 1:320) and incubated at room temperature for one hour. Slides were rinsed before 30 minute incubation at room temperature with biotinylated horse anti-mouse antibody (1:200 in 5% nonfat dry milk in PBS). Slides were again washed before 30 minute incubation with ABC (Vector, Cat No. PK-6100). Slides were rinsed before 5 minute incubation with VIP Chromogen (Vector, Cat No. SK-4600). Slides were counterstained in Mayer's hematoxylin, rinsed, and dehydrated in ethanol and xylene before mounting the cover slip with Permount (Fisher Scientific, Cat No. SP15).

HEK293 lentiviral transient transduction and knockdown

HEK293 cells were transduced, as described above, with 4×10^3 transducing units (TU), or untransduced for control. 24 hours following transduction cells were transfected, as described above, with non targeting siRNA or pooled *LSAMP* siRNA (three oligonucleotides targeting *LSAMP* at nucleotides: 660-682, 1072-1094, and 1202-1224/1264-1288) at 25 or 50 nM, or untransfected as an additional control. 48 hours later, cells were harvested for immunoblot analysis. Additionally, HEK293 cells were transfected, as described above, with pCMV6 (Origene, Cat No. PS100001) containing *LSAMP* and a C-terminal Myc-DDK tag for immunofluorescence analysis.

CHAPTER 3: Results

Loss of heterozygosity often affects large genomic regions. It is necessary to show a functional role of *LSAMP* in prostate cancer progression, in order to bring clinical significance to the observed genomic aberration and distinguish *LSAMP* from adjacent genes, such as *ZBTB20* or *GAP43*. Although *LSAMP* aberrations have been observed in cancer, its function in prostate cancer has not been defined. Additionally, *LSAMP*'s role in signal transduction has not been identified. In order to elicit *LSAMP*'s functional role in prostate cancer progression, it is necessary to characterize the effect of *LSAMP* expression in prostate cancer models (Supplementary Table 1).

LSAMP COPY NUMBER IN PROSTATE CANCER CELL LINES IS VARIED

There has been limited research on the status or function of *LSAMP* in prostate cancer. Fluorescent *in situ* hybridization (FISH) was performed to assess *LSAMP* copy number to determine the prevalence of genetic aberration in commonly used prostate cancer cell culture models (Fig. 1). The copy number of chromosome 3 was determined using a *CEP3* (chromosome enumeration probed 3) probe which hybridizes to its centromere.

The copy number status of *LSAMP* in the prostate cancer cell lines was variable. *LSAMP* was amplified in PC3, with five copies and four copies of chromosome 3 (Fig. 1A). RWPE-1, LNCaP, LAPC-4, and NCI-H660 have a balanced copy number, with two, four, three, and three copies of both *LSAMP* and chromosome 3, respectively (Fig. 1B). RWPE-1 is a cell line of normal prostatic epithelial origin and was as expected to have two copies of each chromosome 3 and *LSAMP*. VCaP and MDA PCa 2b, a prostate

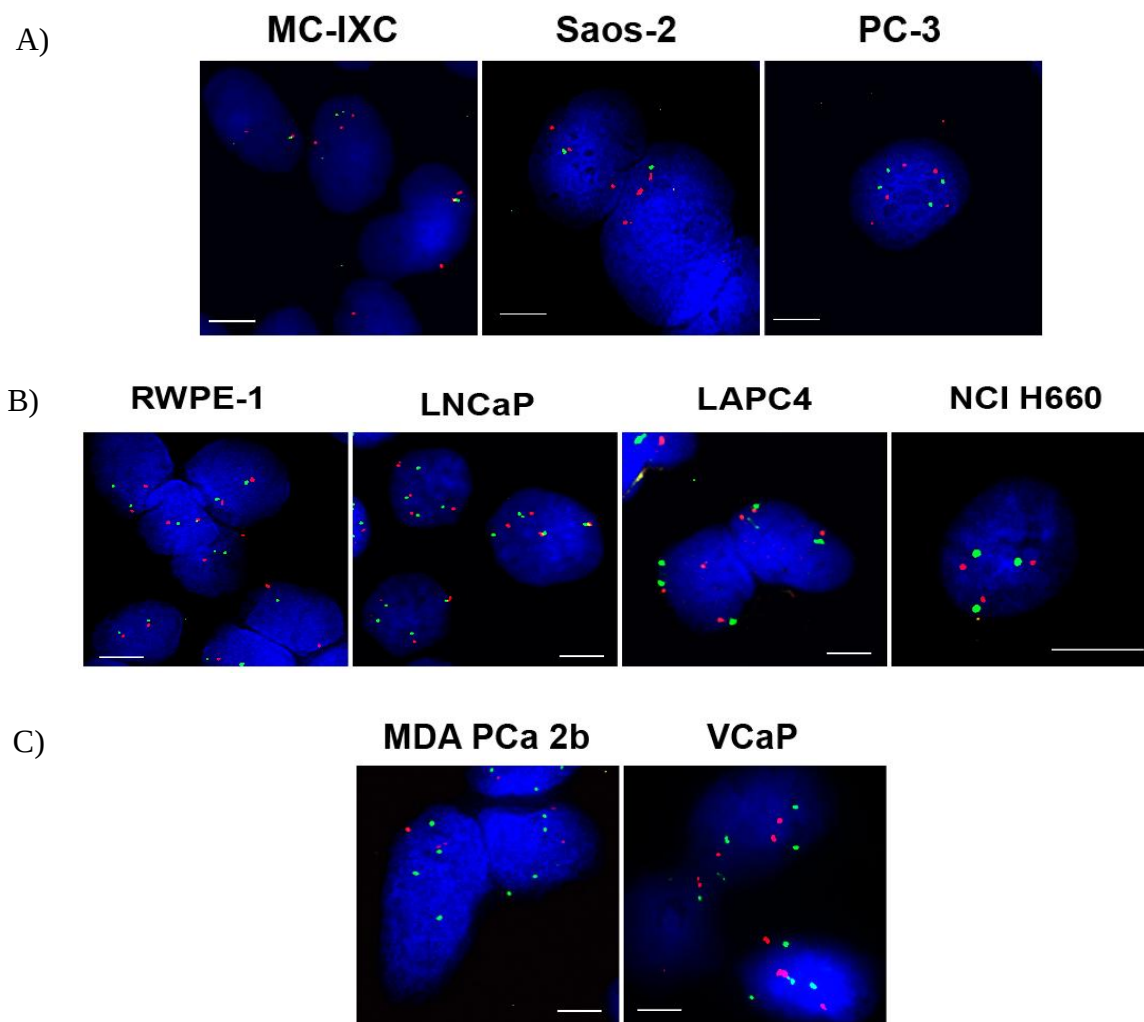


FIGURE 1. *LSAMP* COPY NUMBER IN PROSTATE CANCER.

Fluorescence *in situ* hybridization analysis of *LSAMP* copy number in interphase prostate cancer cells together with centromere of Chromosome 3, *CEP3*, as control. (A) Locus amplification: PC3 and positive control lines MC-IXC and Saos-2; (B) Balanced copy number: RWPE-1, LNCaP, LAPC-4, and NCI-H660. (C) *LSAMP* deletion: MDA PCa 2b and VCaP; Red=*LSAMP*, Green=*CEP3*(chromosome enumeration probed).

cancer cell line of AA origin, both showed a deletion of *LSAMP*, with three and two copies of *LSAMP*, respectively, and both with four copies of chromosome 3 (Fig. 1C). In MDA PCa 2b, this specific copy number aberration may have resulted from a haploid

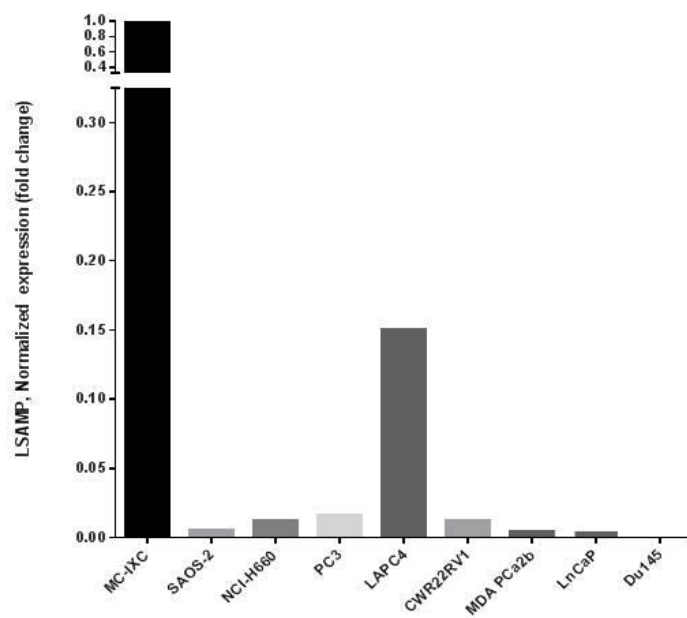
loss of *LSAMP* followed by a doubling of chromosome 3, as the genomic aberrations accumulated. Notably, the only AA prostate cancer line tested, MDA PCa 2b, exhibited the most significant relative copy number reduction, consistent with the increased frequency of *LSAMP* deletion observed in AA prostate cancer samples (196; 218).

Cell lines used as a positive control for *LSAMP* expression, MC-IXC and Saos-2, both harbor an amplification of *LSAMP*. MC-IXC, a neuroblastoma cell line, has four copies of *LSAMP* and two copies of chromosome 3 (Fig. 1A). Saos-2, an osteosarcoma cell line, has 3 copies of *LSAMP* and a single chromosome 3 (Fig. 1A). This amplification is consistent with the higher levels of expression observed in these lines (19).

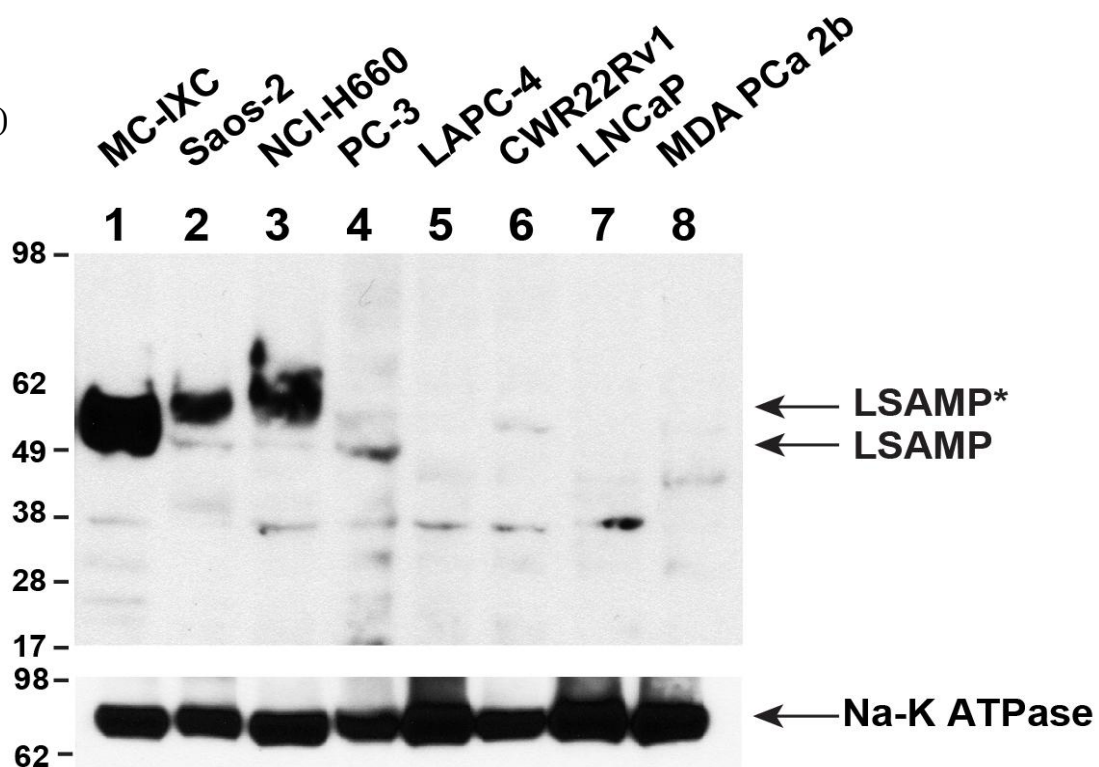
LSAMP EXPRESSION IS LOW OR UNDETECTABLE IN PROSTATE CANCER CELL LINES

LSAMP mRNA expression is detected in normal prostate tissue, as observed in multiple independent databases. Following the assessment of *LSAMP*'s genomic status in prostate cancer cell lines and confirming expression in normal tissue, expression levels were evaluated in prostate cancer cell lines. *LSAMP* transcript levels were the highest in LAPC-4; however protein expression was not detected (Fig. 2A, 2B). Very low levels of mRNA were detected in PC3, NCI-H660, and 22Rv1, and *LSAMP* expression was negligible in LNCaP, MDA PCa 2b, and DU 145 cell lines (Fig. 2A). Additional prostate cancer cell lines C42B, VCaP, RC58, RC92, RC165, and RC170 also exhibited negligible levels of *LSAMP* expression. *LSAMP* mRNA is rarely expressed at significant levels in prostate cancer cell lines.

A)



B)



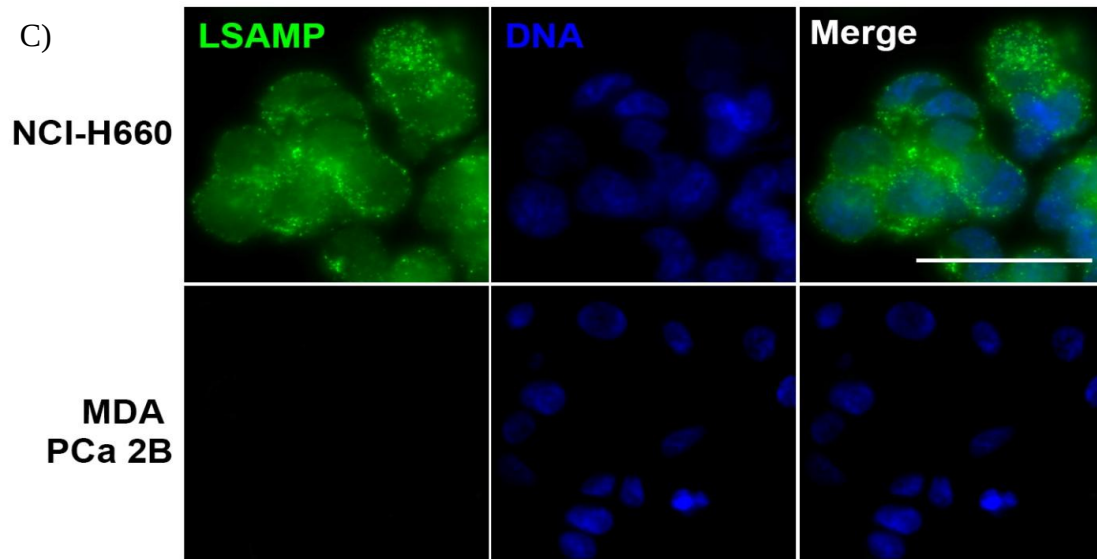


FIGURE 2. LSAMP EXPRESSION IS LOW OR UNDETECTABLE IN MOST PROSTATE CANCER CELL LINES A) *LSAMP* mRNA Expression is Negligible in most Prostate Cancer Cell lines. qRT-PCR analysis of prostate cancer Cell Lines detects endogenous *LSAMP* mRNA in LAPC-4, PC3, NCI-H660, 22Rv1, and positive controls MC-IXC and Saos-2. *LSAMP* protein expression is not detected in most prostate Cancer Cell lines, as assessed by B) immunoblot and C) immunofluorescence analysis. *LSAMP* protein is highly expressed in NCI-H660, and marginally detected in PC3 cells. *LSAMP* is not detected in MDA PCa 2b: African American prostate cancer cell line with haploid deletion for 3q13.31, and all other prostate cancer cell lines assessed. NCI-H660: epithelial neuroendocrine small cell carcinoma, prostate cancer line with *LSAMP* expression.

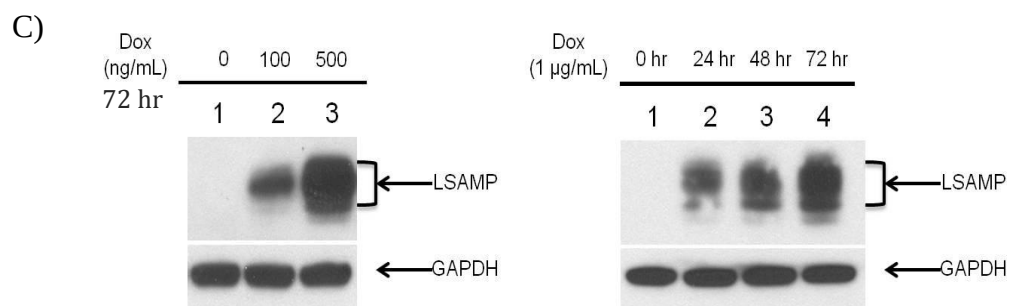
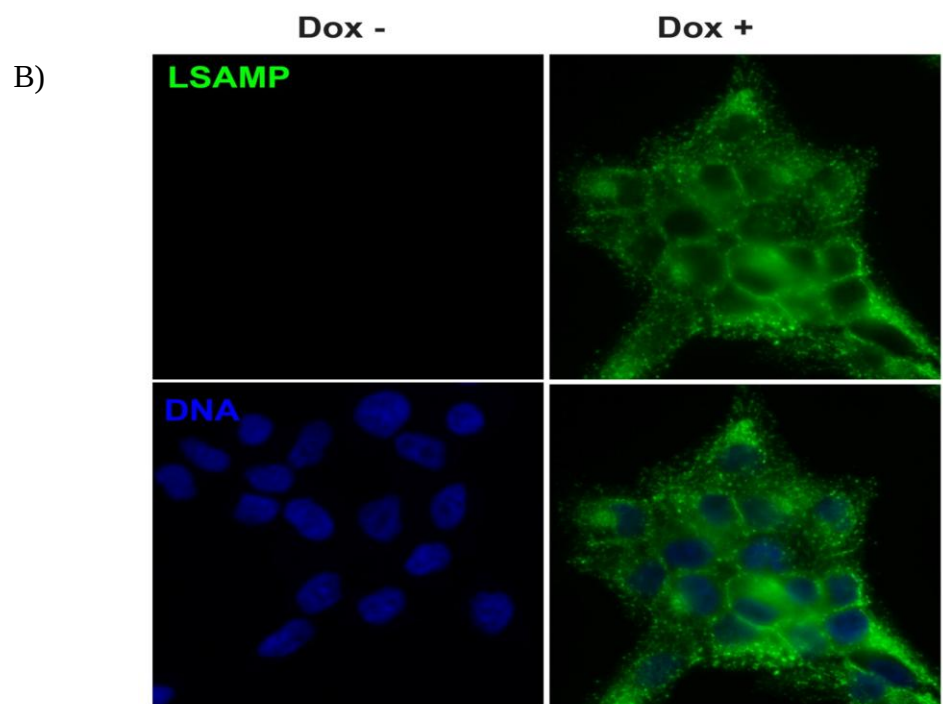
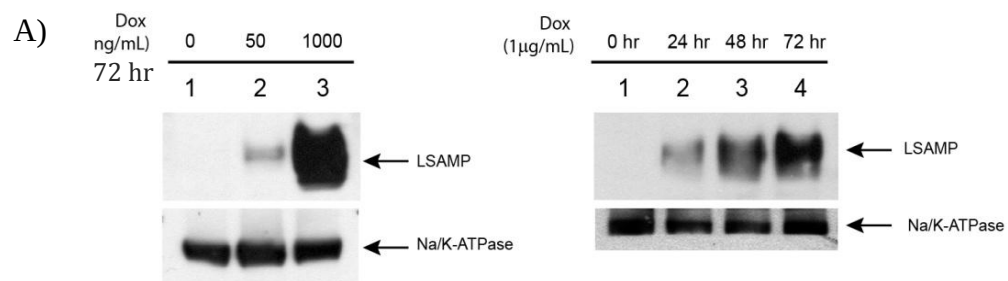
In addition to assessing *LSAMP* transcription, *LSAMP* protein was probed using monoclonal mouse antibody mAb873 (223; 333). Temperature and reducing agent conditions were optimized, and it was determined that the omission of reducing agents in the sample is necessary for consistent detection of *LSAMP* (Fig. S1). *LSAMP* expression was relatively high in NCI-H660, with a diffuse band on the immunoblot, consistent previous observations of glycosylated *LSAMP* protein (333) (Fig. 2B). Immunofluorescence analysis of NCI-H660 revealed strong punctate staining enhanced at the cell membrane (Fig. 2C). This is consistent with the pattern of staining observed in

MC-IXC, as well as previous studies (Fig. S2)(333). Positive control cell line MC-IXC showed the highest level of expression at both the mRNA and protein levels (Fig. 2A, 2B, S2). Positive control line Saos-2 exhibited low levels of mRNA expression of high levels of protein expression.

Other than LSAMP expression in NCI-H660, LSAMP was only faintly detected in PC3 by immunoblot when very high quantities of total protein were loaded (Fig. 2B). Additionally, the band seen on the immunoblot was a slightly smaller size and less diffuse than the other bands seen in cells with protein expression. This may reflect a lack of glycosylation, but is yet to be determined (333). Immunofluorescence staining of LSAMP in PC3 showed diffuse cytoplasmic staining. CRISPR-Cas9 lentiviral constructs were used to knockout *LSAMP* in PC3, and protein expression was undetectable (Fig. S3). Preliminary proliferation rate data showed no significant difference in growth. These observations suggest the endogenous protein may be functionally inactive, but this remains to be determined. Neuroendocrine prostate cancer cell line NCI-H660 exhibits endogenous membranous LSAMP, and expression in PC3 is marginal. All other prostate cancer cell lines assessed exhibit undetectable levels of LSAMP protein.

GENERATION AND VALIDATION OF PROSTATE CANCER MODELS TO ASSESS THE EFFECT OF LSAMP

LSAMP expression in prostate cancer cells with null expression can be used to investigate *LSAMP*'s function. Preliminary transient transfections exhibited low



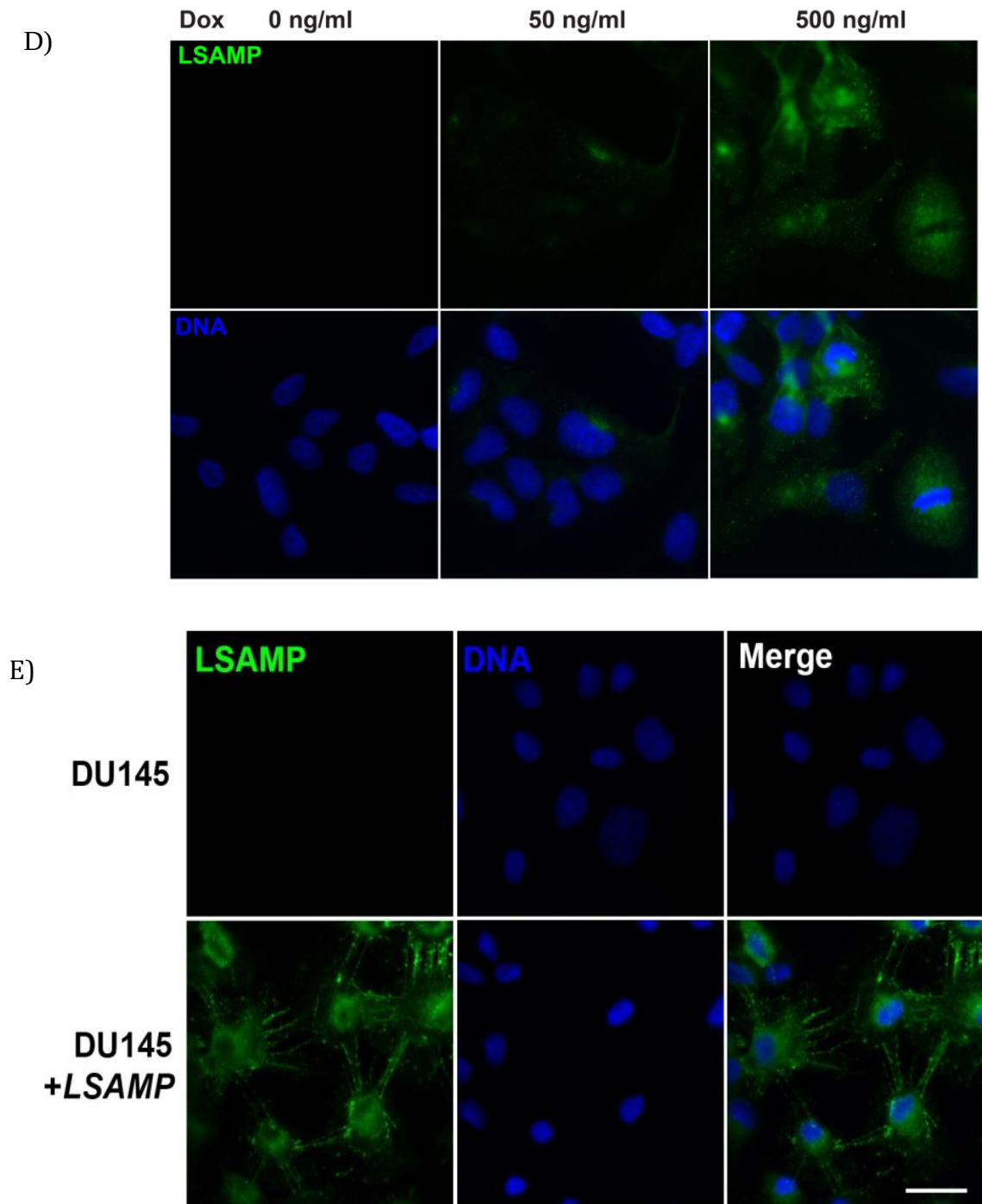


FIGURE 3. CONSTITUTIVE AND INDUCIBLE LENTIVIRAL EXPRESSION OF LSAMP IN PROSTATE CANCER CELL MODELS LSAMP expression was validated in stably transduced cell culture models via immunoblot and immunofluorescence in A-B) inducible MDA-PCa2B, C-D) inducible LNCaP, or constitutive expression in E) DU 145. Immunoblot analysis detects LSAMP induction in a doxycycline concentration and time dependent manner.

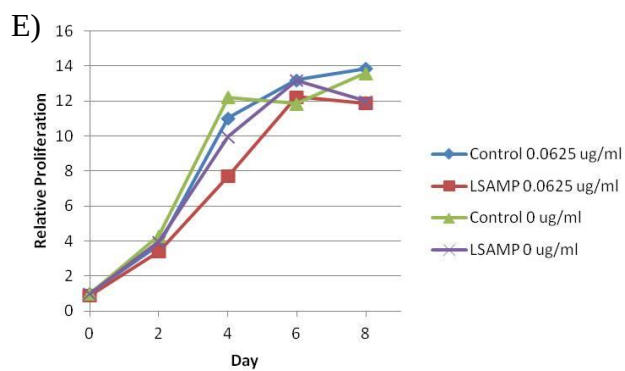
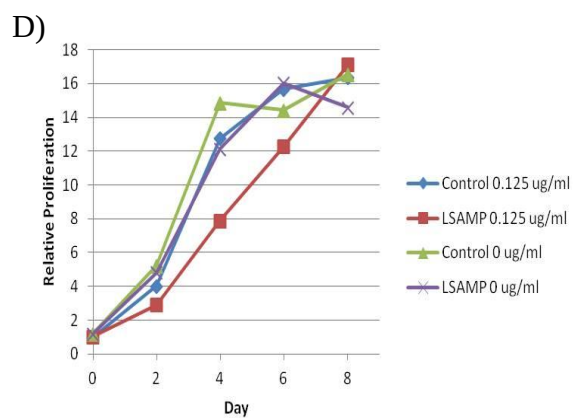
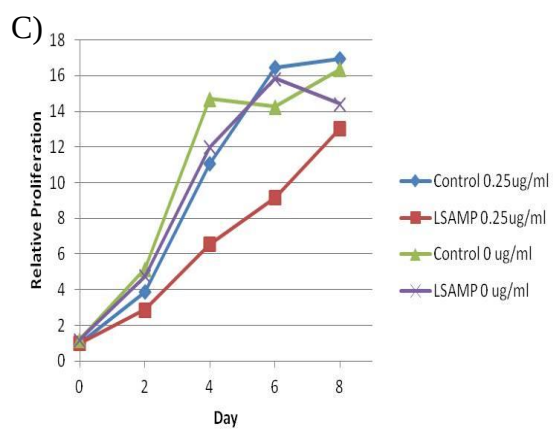
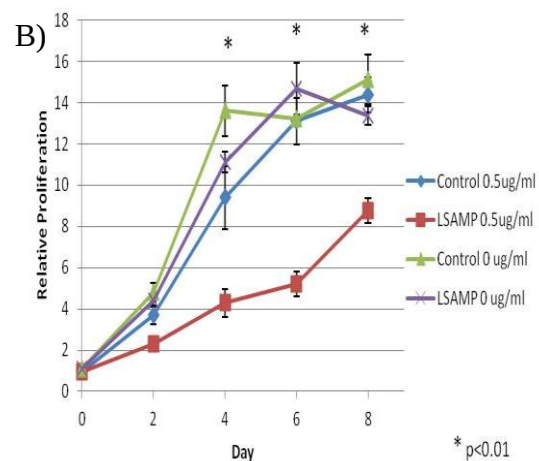
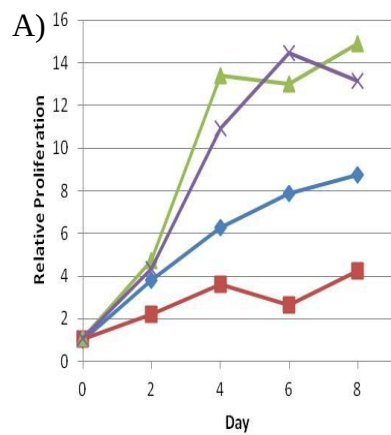
efficiency, so three prostate cancer cell lines with undetectable expression levels were selected to stably express *LSAMP* via lentiviral transduction. The LNCaP cell line was established from a lymph node of a CA patient, and is androgen dependent (Supplementary Table 1). MDA PCa 2b was established from the bone metastases of an AA patient, and is androgen independent but androgen responsive. DU 145 was established from the brain metastases of a CA patient, and is androgen independent (294). The progression from androgen dependence to independence is a hallmark of prostate cancer progression. These are suitable models to investigate *LSAMP*'s function in prostate cancer backgrounds of varied phenotype and ethnic origin.

LNCaP and MDA PCa 2b cell lines were transduced with doxycycline inducible lentivirus. DU 145 was transduced to constitutively express *LSAMP*, or with control lentivirus. *LSAMP* expression was validated in these cell lines by immunofluorescence assay (Fig. 3A, 3C, 3E). Protein expression was strong in DU 145 cells with constitutive expression, and upon doxycycline induction in LNCaP and MDA PCa 2b. *LSAMP* expression was not detected in the DU 145 transduced with control lentivirus, and without doxycycline induction in LNCaP and MDA PCa 2b. The pattern of staining was punctate, with strong membrane enrichment. In the doxycycline inducible cell lines, the expression of *LSAMP* was doxycycline concentration and time dependent (Fig. 3B, 3D). Lentiviral transduction to stably express *LSAMP*, in an inducible or constitutive manner, was validated, and these cell lines are appropriate for further functional studies.

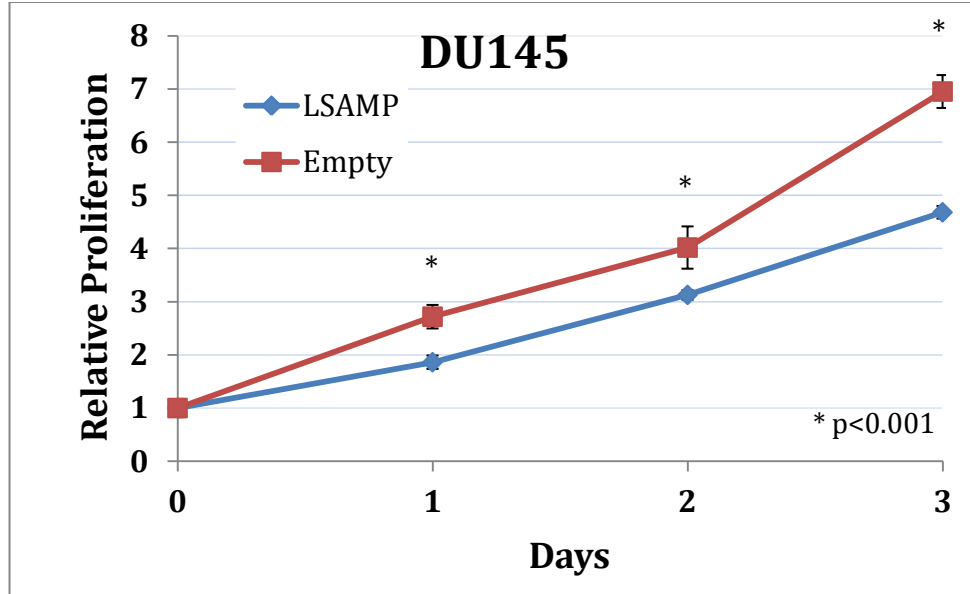
LSAMP EXPRESSION INHIBITS PROLIFERATION IN PROSTATE CANCER CELL LINES AND TUMOR GROWTH IN A SUBCUTANEOUS XENOGRAFT MODEL

Tumor suppressor inactivation often results in an increased proliferative capacity of tumor cells. MTT assays were performed to assess the proliferation rate in prostate cancer cell lines, with or without *LSAMP* expression. In LNCaP, MDA PCa 2b, and DU 145, *LSAMP* expression resulted in a significantly reduced proliferation rate, a new observation in the context of prostate cancer models (Fig. 4). Proliferation inhibition upon *LSAMP* expression is consistent with a previous study which expressed *LSAMP* in osteosarcoma cell lines and *LSAMP*'s proposed tumor suppressive role (19). In constitutively expressing DU 145, a significant decrease in proliferation rate observed at the first time point, 24 hours (Fig. 4F). Inducible *LSAMP* expression in LNCaP and MDA PCa 2b resulted in a significant reduction in proliferation at the second time point, 96 hours (Fig. 4B, 4G). One potential explanation for the delay is the increased time required for sufficient transcription, translation, and localization to the membrane following doxycycline administration, before *LSAMP* can exert its function, possibly including downstream signaling altered gene expression of secondary effectors.

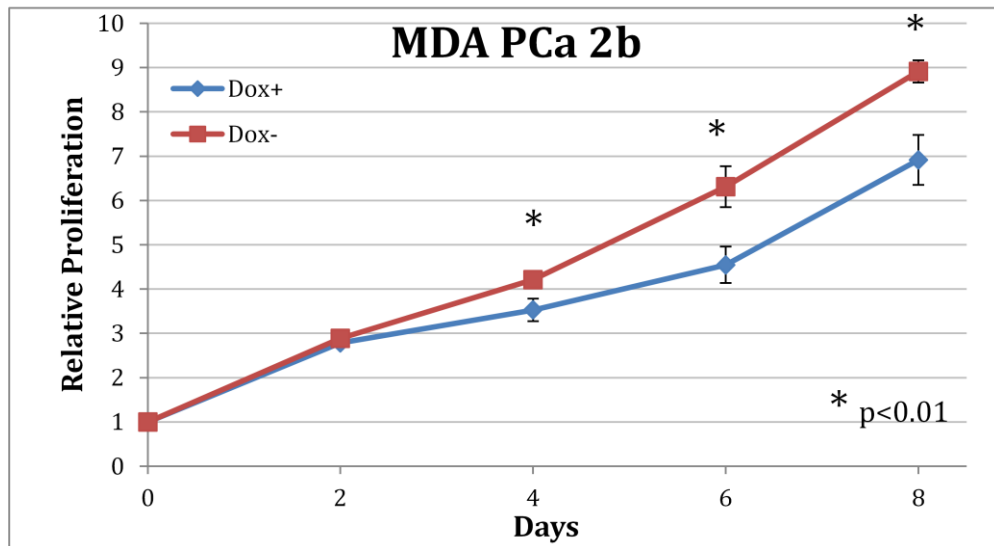
Previous studies have reported doxycycline-dependent growth inhibition of LNCaP cells at high concentrations (78). In order to discern *LSAMP*-dependent effects from doxycycline-dependent cell growth inhibition, the optimal doxycycline dose was assessed. Doxycycline was administered at various concentrations to the control and *LSAMP* inducible LNCaP cell lines (Fig. 4A-E). In the absence of doxycycline, *LSAMP* and control cell lines had matching proliferation rates. It was only at the highest concentration of doxycycline, that the proliferation rate of the control vector harboring



F)



G)



H)

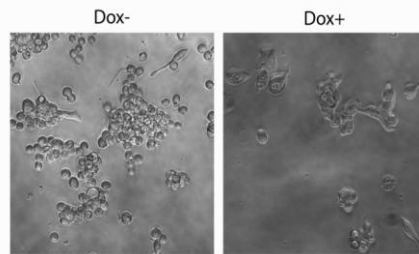


FIGURE 4. LSAMP EXPRESSION INHIBITS PROSTATE CELL PROLIFERATION. MTT assay analysis of A-E) LNCaP, F) DU 145 G) MDA PCA 2B. A-E) Doxycycline titration in inducible LSAMP and control stable cell lines reveals doxycycline dependent effects at 1 $\mu\text{g/mL}$, and an optimal concentration of 0.5 $\mu\text{g/mL}$. H) Representative image of MDA PCa 2b. P-value<0.01.

cell line was inhibited (Fig. 4A). This confirms that at high doses, doxycycline inhibits the growth of LNCaP. At the optimal concentration of 0.5 $\mu\text{g/mL}$, *LSAMP* expressing LNCaP had a significantly reduced proliferation rate, compared to all groups (Fig. 4B). With subsequent doxycycline dilutions, the inhibition of proliferation became less pronounced, consistent with the reduced *LSAMP* protein expression observed at lower concentrations (Fig. 4C-E). *LSAMP* inhibits proliferation in LNCaP, MDA PCa 2b, and DU 145 prostate cancer cell lines.

The role of *LSAMP* in prostate cancer was further characterized in a mouse xenograft model. DU 145 cells were selected for the subcutaneous xenograft to determine whether the effect of *LSAMP* expression was sufficient to attenuate tumor growth in an aggressive prostate cancer model. Subcutaneous xenografts of DU 145 in nude mice revealed a significant reduction in tumor growth with *LSAMP* expression ($p < 0.05$) (Fig. 5). Tumor volume (mm^3) of DU145 control xenografts increased an average of 75 mm^3 per week ($R^2 = 0.98$), with a median tumor volume of 490 mm^3 , and an average of 543 mm^3 (stdev=451) at the study endpoint. Tumor volume of DU145-*LSAMP* xenografts increased an average of 14 mm^3 per week ($R^2 = 0.95$), with a median tumor volume of 199 mm^3 , and an average of 184 mm^3 (stdev=155) at the study endpoint. Additionally, two *LSAMP*-negative tumors developed ulceration and the mice were euthanized at week six, and not included in subsequent measurements. Interestingly, two mice, previously with *LSAMP*-expressing tumors once as large as 114 mm^3 , exhibited no sign of a tumor nine weeks post-injection. Statistically significant differences were observed starting at week six. *LSAMP* expression did not interfere with DU 145 tumor formation with the cell number administered, as all mice in both groups developed tumors. However, *LSAMP*

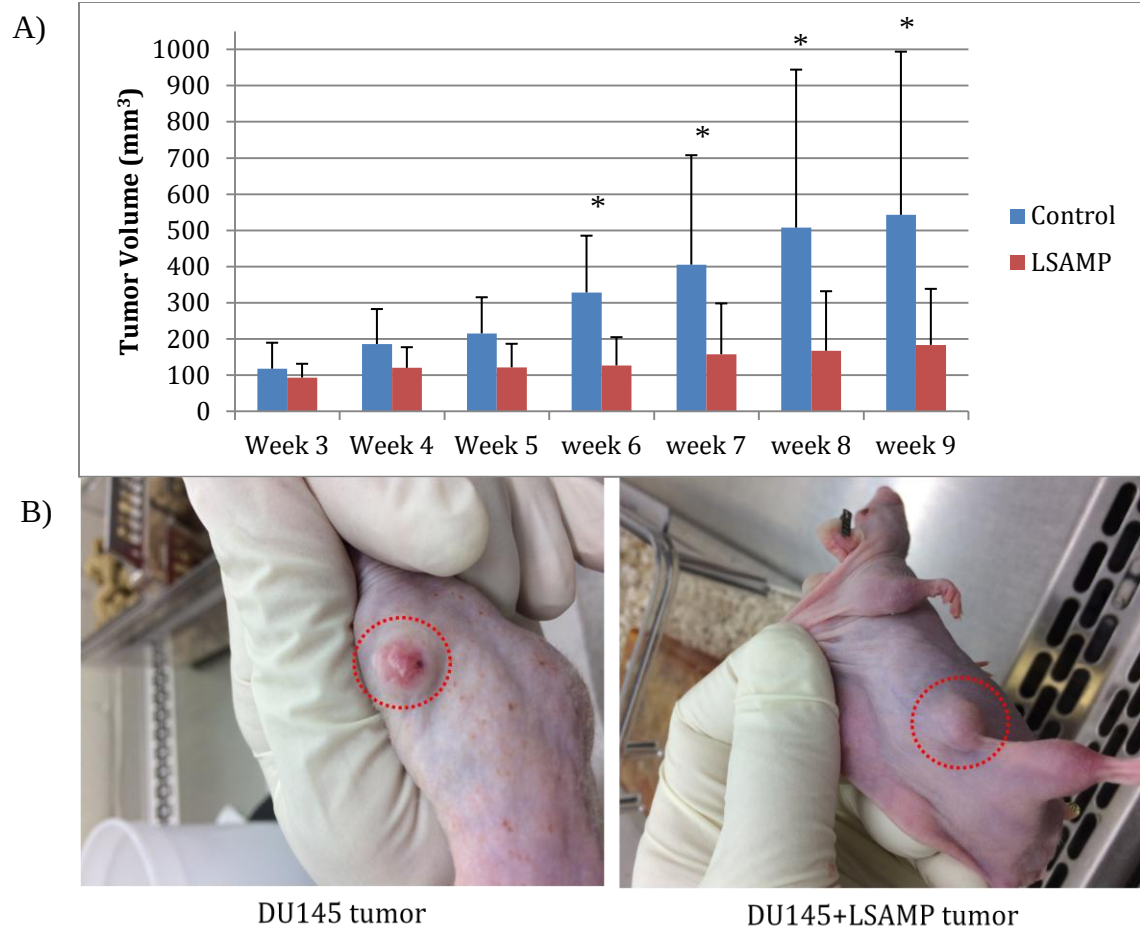


FIGURE 5: LSAMP EXPRESSION IN DU 145 SUBCUTANEOUS XENOGRAFTS INHIBIT TUMOR GROWTH A) Tumor volume of control (n=10) and LSAMP expressing DU145 (n=10). B) Image taken of xenograft tumors. P-value<0.05.

expression significantly reduced tumor growth. These observations and reduced tumor growth are consistent with the *in vitro* data, and with *LSAMP*'s tumor suppressive role in prostate cancer.

LSAMP EXPRESSION INCREASES ADHESION TO EXTRACELLULAR MATRIX PROTEINS

LSAMP and IgLON family members are cell adhesion molecules whose defects have been reported in various cancers (48; 142; 157; 201; 233). In addition to proliferation, *in vitro* investigation of apoptosis, senescence, migration, invasion, and anchorage independent growth were evaluated. These features were assessed under various conditions, and repeated, no measurable difference was observed upon *LSAMP* expression in the models tested (Fig. S4). In similar *LSAMP* expression experiments in osteosarcoma cell lines, Barøy *et al.* also observed no change in apoptosis, migration rate, or differentiation, although they did observe significantly decreased proliferation and tumor growth in cells expressing *LSAMP* (19). However, the association of *LSAMP* inactivation with BCR suggests a functional role beyond the inhibition of tumor cell proliferation (218).

Along the lines of reported IgLON involvement in cell-adhesion, the effect of *LSAMP* expression in prostate cancer cell lines on ECM-cell adhesion was assessed (173). *LSAMP* expressing DU 145 cells had significantly increased adhesion to Fibronectin (FN), Collagen I (Col I), with the most significant increase to Fibrinogen (FBG) (Fig. 6A, 6B). Fibrinogen, an ECM protein commonly associated with its role

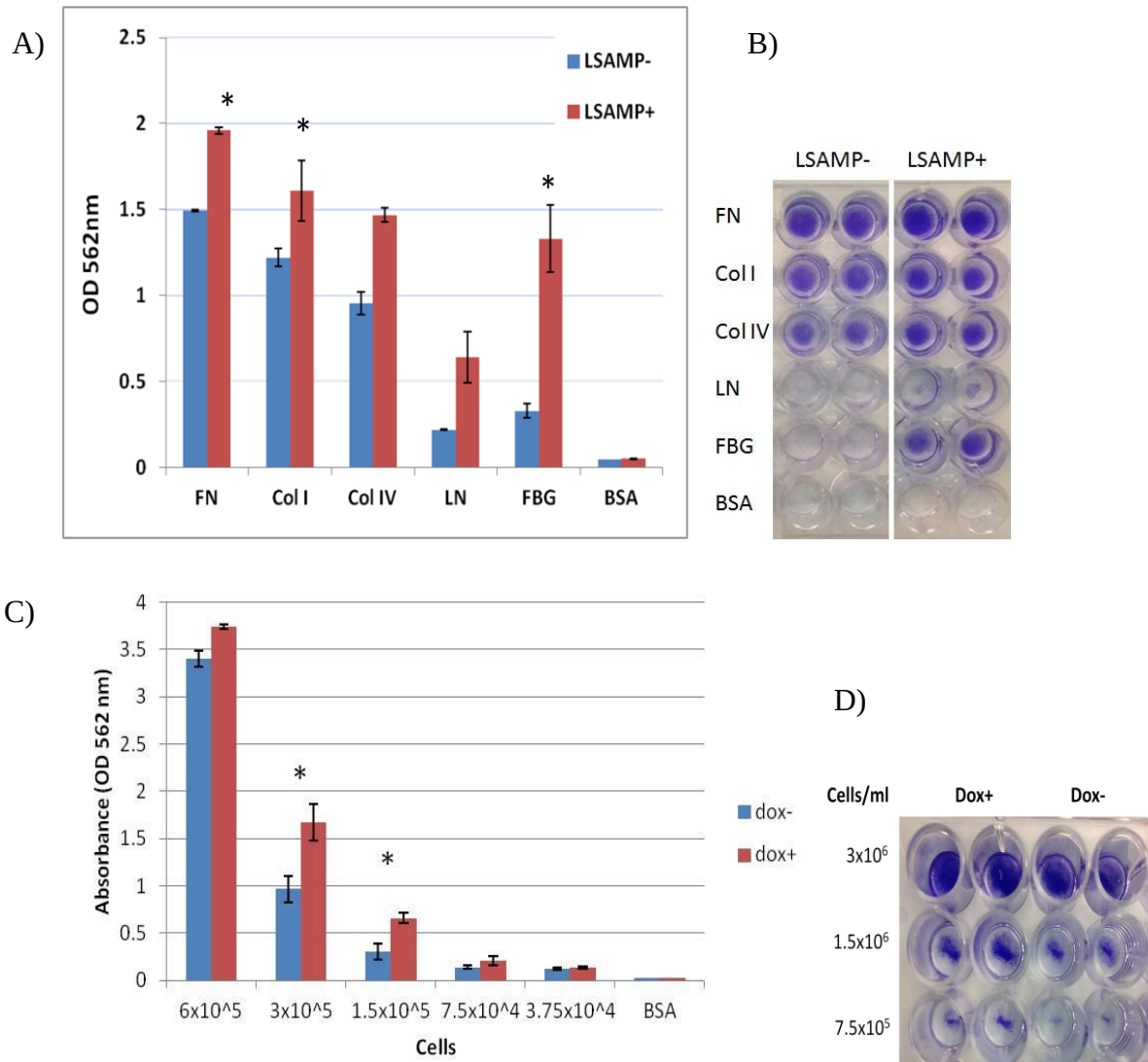


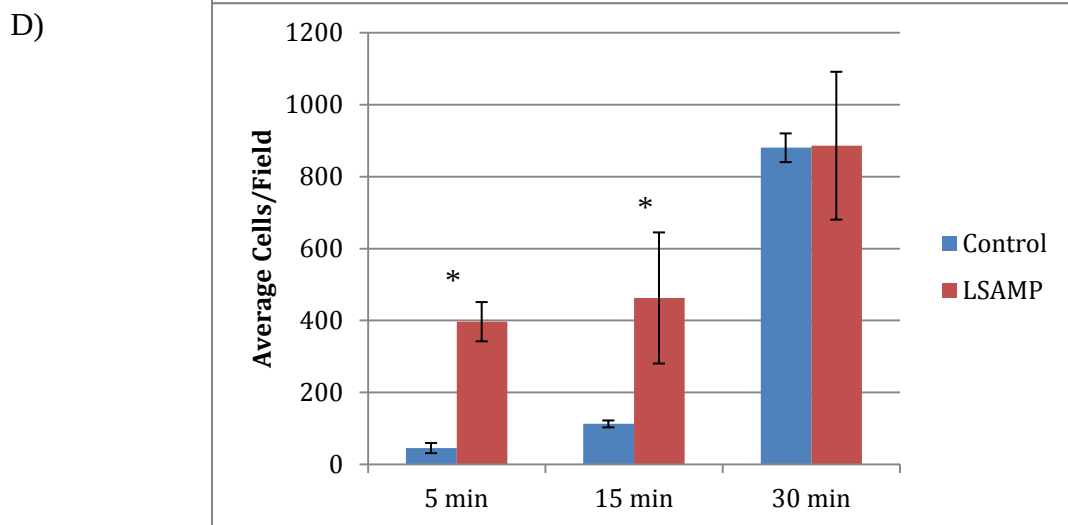
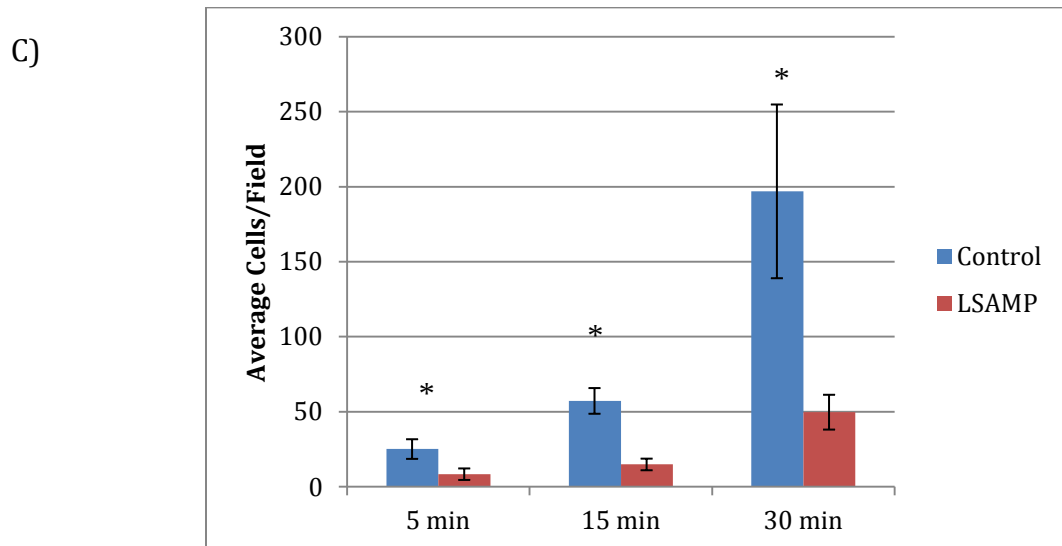
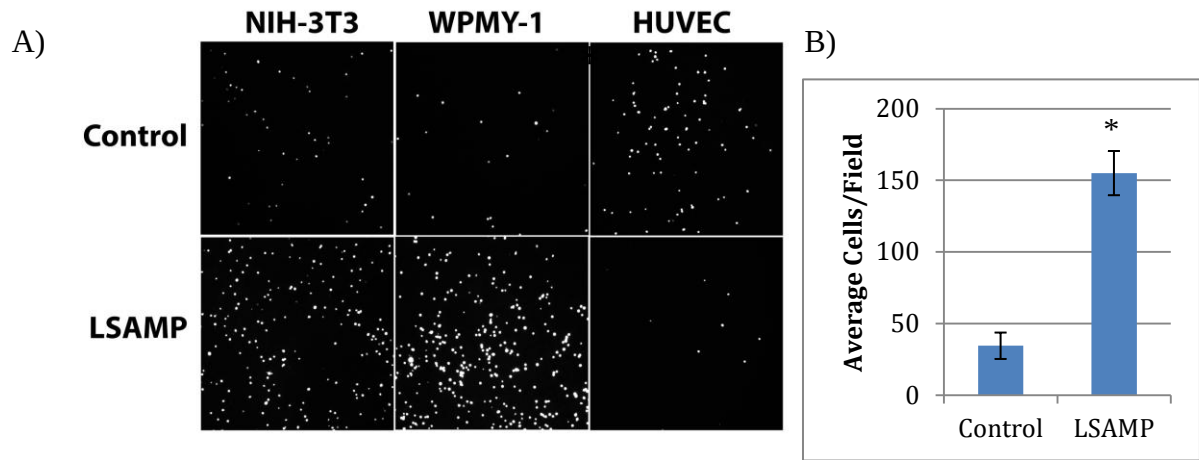
FIGURE 6. LSAMP EXPRESSION INCREASES ADHESION TO EXTRACELLULAR MATRIX PROTEINS A) Quantification, and B) Representative image of DU 145 adhesion to ECM array (Col I, Col IV, LN, FBG, FN), control versus LSAMP. C) Quantification and D) Representative image of MDA PCa 2b adhesion to Fibrinogen, doxycycline+ versus doxycycline-. LSAMP increases ECM-celladhesion. BSA coated well served as a control. P-Value<0.05. Fibronectin: FN; Collagen I: Col I; Collagen IV: Col IV; Laminin: LN; Fibrinogen: FBG.

in blood plasma, is found in the connective tissue of malignant and benign prostate, but not in the adjacent normal areas (312). Doxycycline-inducible MDA PCa 2b cells were further found to have a significant increase in adhesion to Fibrinogen at intermediate cell

densities (Fig. 6C, 6D). Other ECM proteins tested, Collagen IV (Col IV) and Laminin (LN) also exhibited increased adhesion to *LSAMP* expressing DU 145. However, statistically significant differences were not observed under the conditions tested, indicating a selectivity of adhesive interaction. Cell-adhesion to ECM proteins in this assay was seeding density and time dependent, as such, multiple seeding densities and incubation times were initially assessed. The adhesive characteristics of the prostate cancer lines were variable. DU 145 cells could adhere almost immediately, whereas MDA PCa 2b required over 12 hours, and LNCaP did not show adherence to the tested ECM proteins within the timeframes of observation. *LSAMP* expression in DU 145 and MDA PCa 2b cells increases adhesion to ECM proteins.

LSAMP EXPRESSION RESULTS IN INCREASED ADHESION TO FIBROBLAST AND PROSTATE STROMA CELL LINES, AND DECREASED ADHESION TO ENDOTHELIAL CELLS

To further investigate *LSAMP*'s role in prostate cancer cell-adhesion, the effect of *LSAMP* expression in DU 145 cell-adhesion to an endothelial cell (HUVEC), fibroblast (NIH/3T3), or prostate stromal derived (WPMY-1) monolayer was assessed. Fibroblast and prostate stroma cell lines produce ECM proteins, and in part model the native environment of prostate epithelial and tumor cells. Endothelial cell-adhesion assays assess tumor cell invasiveness. Increased tumor cell-adhesion to an endothelial monolayer suggests an increased ability of CTCs to initiate adhesion on the vascular endothelium and facilitate the establishment of a metastatic site. *LSAMP* expression was expected to increase cell-adhesion to fibroblast and stromal cells, but not endothelial cells, consistent with its role as an adhesion molecule and putative tumor suppressive function.



E)

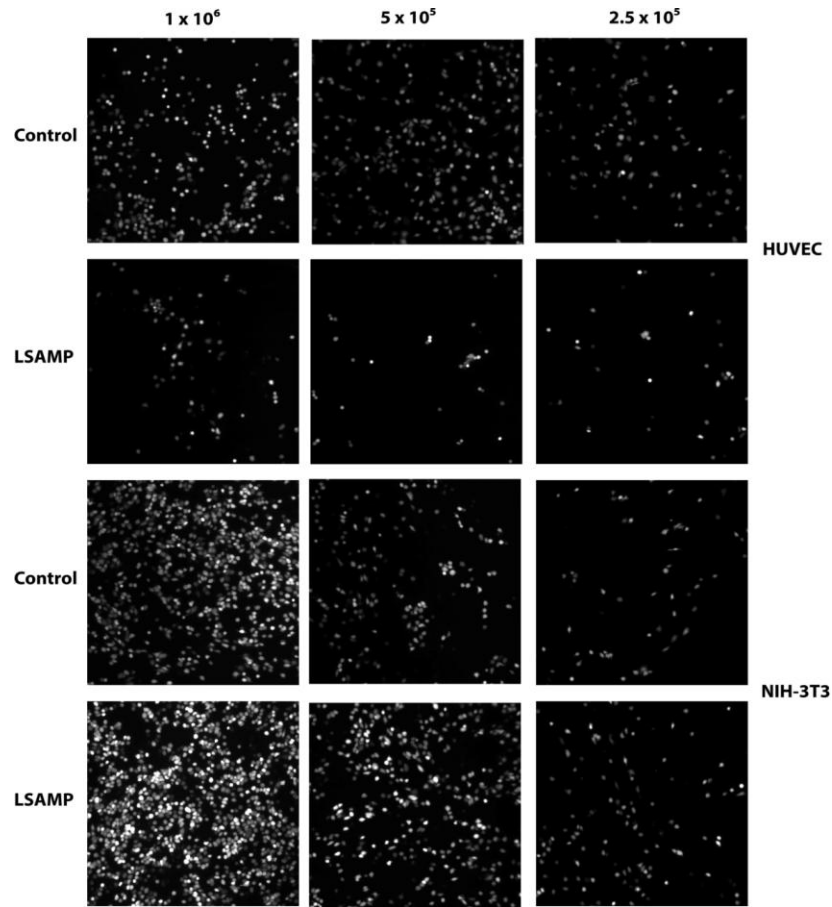


FIGURE 7. LSAMP EXPRESSION IN DU 145 INCREASES ADHESION TO FIBROBLAST AND PROSTATE STROMA CELL LINES AND DECREASES ADHESION TO ENDOTHELIAL CELL LINE A) Representative image of DU 145 cell-adhesion to endothelial (HUVEC), fibroblast (NIH/3T3), and prostate stroma derived (WPMY-1) monolayers. B) Quantification of cell-adhesion to NIH/3T3. C) Quantification of Adhesion to WPMY-1. D) Quantification of Adhesion to HUVEC. E) Additional representative image of DU 145 adhesion to HUVEC and NIH/3T3 at different cell densities. Numbers in cells/mL. P-value<0.05.

LSAMP expression in DU 145 resulted in significantly increased adhesion to both the mouse fibroblast line, NIH/3T3, and the human prostate stroma cell line, WPMY-1 (Fig. 7). *LSAMP* expression resulted in greater than a four-fold increase in adhesion to fibroblasts, which was evident after five minutes. *LSAMP* expressing DU 145 exhibited an approximate nine fold increase in adhesion to the normal prostate stromal monolayer at five minutes. The adhesion differences observed with the prostate stroma cell line

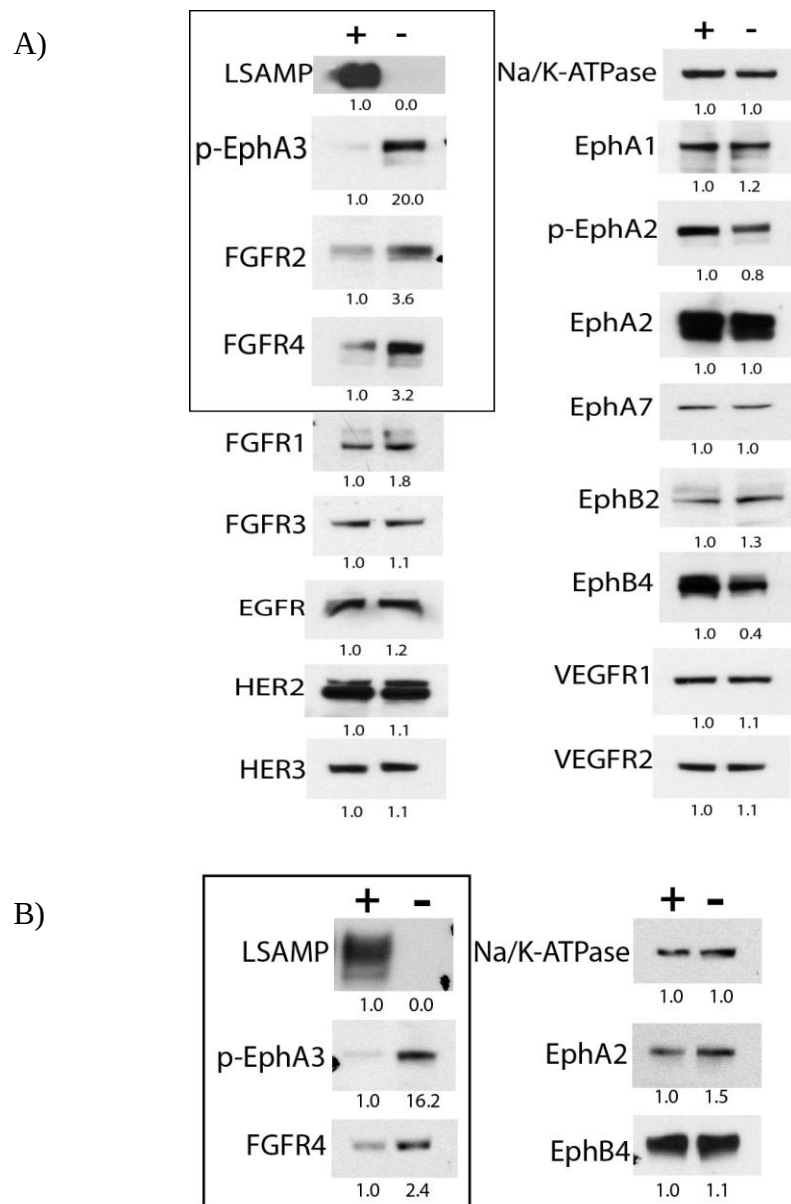


FIGURE 8. LSAMP DOWNREGULATES SPECIFIC RECEPTOR TYROSINE KINASES.

Immunoblot analysis of LSAMP expression in A) DU 145, and B) LNCaP shows downregulation of a specific set of RTKs: EPHA3, FGFR2, FGFR4. Values are relative normalized expression

diminished with time, and became insignificant after thirty minutes, possibly due to saturation. Surprisingly, *LSAMP* expressing DU 145 cells also exhibited a significant reduction in endothelial cell-adhesion. *LSAMP* expression resulted in a 3-4 fold decrease

in adhesion to the endothelial cells. This may be mediated by an indirect downregulation of molecules which mediate endothelial adhesion upon *LSAMP* expression, but this is yet to be determined. These alterations in adhesive affinities were consistently observed at multiple cell densities and time points, as well as with the pretreatment of the monolayer with TNF- α (Tumor Necrosis Factor α), which upregulates adhesion molecules (Fig. S5 A-C). Incubation of DU 145 with LSAMP antibody did not significantly inhibit adhesion, suggesting inability of the antibody to block LSAMP-mediated adhesion, or the involvement of other adhesion molecules which may be concomitantly upregulated with *LSAMP* expression (Fig. S5 D). The cell-adhesion characteristics mediated by *LSAMP* expression may inhibit key steps in metastatic development by increasing tumor cell-adhesion to normal stroma, and by decreasing tumor cell-adhesion to endothelial cells.

LSAMP EXPRESSION DOWNREGULATES SPECIFIC RECEPTOR TYROSINE KINASES AND INHIBITS DOWNSTREAM ACTIVATION OF THE ERK, AKT, BETA-CATENIN PATHWAYS

The decreased adhesion to endothelial cells following *LSAMP* expression suggests other adhesion molecules which may mediate this interaction may be downregulated. Crosstalk between adhesion molecules requires intracellular signaling, however, specific signaling transduction mediated by *LSAMP* has not been previously identified. To this end, potential *LSAMP* pathway mediators were investigated.

LSAMP expression resulted in significantly reduced expression of FGFR 2, FGFR4, and phospho-EPHA3 (Fig. 8). There are limited antibodies available to specifically detect unphosphorylated EPHA3, with high homology observed in the Ephrin Receptor Family. However, the reduction in phospho-EPHA3 suggests reduced activity, whether by inhibition of phosphorylation, or by endocytosis mediated degradation, as

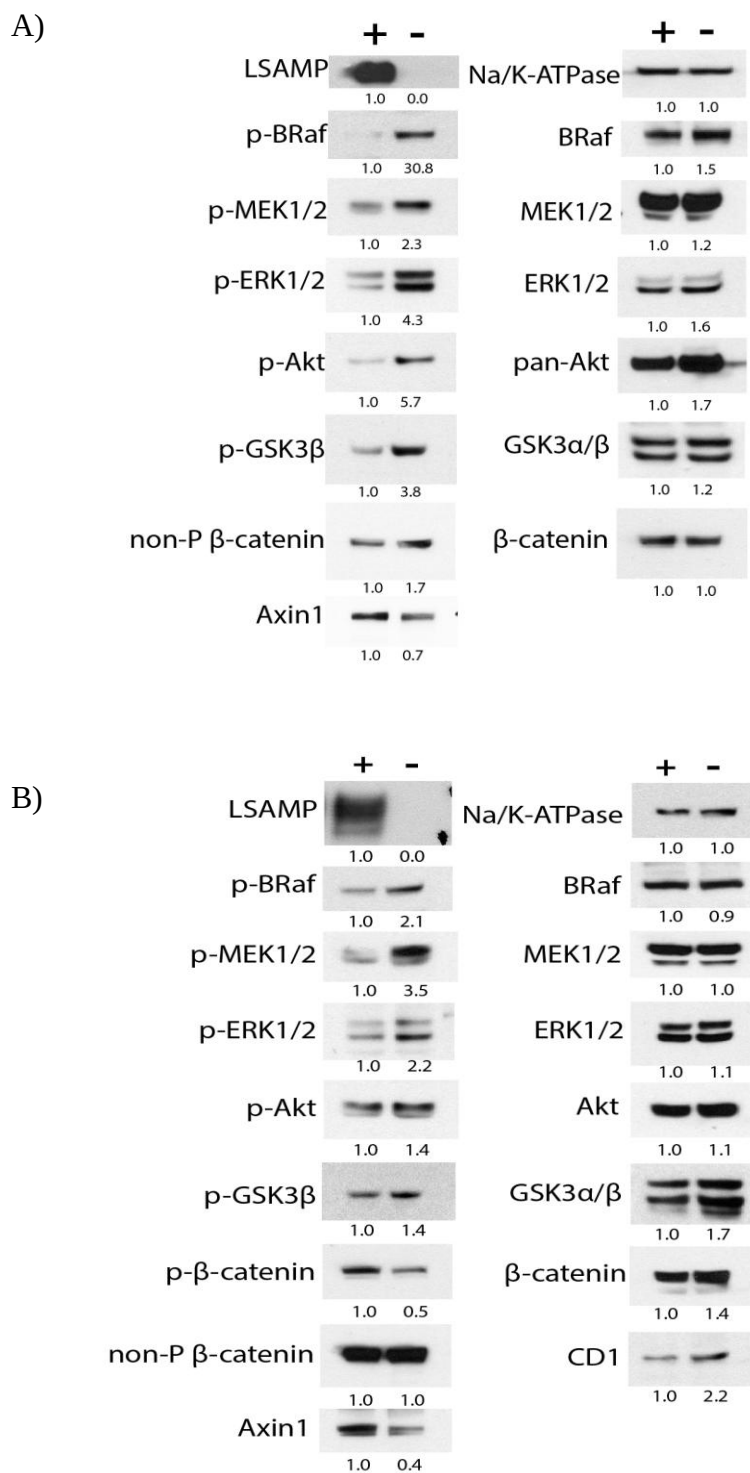


FIGURE 9. LSAMP INHIBITS DOWNSTREAM ERK, AKT, B-CATENIN PATHWAYS
 Immunoblot analysis following LSAMP expression in A) DU 145, and B) LNCaP reveals inhibition of ERK/Akt/ β -Catenin pathways. Values are relative normalized expression

suggested by the previous studies of an IgLON family member (180). LNCaP, however, did not express detectable levels of FGFR2. This is consistent with the absence of FGFR2 in normal prostate luminal cells, and the reported frequency in prostate cancer at only 30% (90). Although protein levels of these specific RTKs are reduced when LSAMP is expressed, it is unclear whether this is a direct downregulation by endocytosis and proteasomal degradation, or another mechanism yet to be clarified. Evaluation of several other RTKs shows no significant change in expression in the presence of LSAMP (Fig. 8). This suggests that LSAMP selectively regulates downstream ERK and Akt signaling by inhibiting specific set of RTKs.

In addition to RTK inhibition, DU 145 (Fig. 9A), and LNCaP (Fig. 9B), also exhibited a marked decrease in phosphorylated proteins downstream of RTKs. BRAF (v-Raf murine sarcoma viral oncogene homolog B), MEK1/2 (mitogen-activated protein kinase kinase 1 and 2), ERK1/2, Akt, and GSK3 β phosphorylation was reduced in the cells expressing LSAMP, without any significant effect on total protein levels. In *LSAMP* expressing DU 145 non-phosphorylated (active) β -catenin was reduced and expression of Axin, a negative regulator of β -catenin, was slightly higher, suggesting likely inhibition of β -catenin activity. Upon *LSAMP* induction in LNCaP, the phosphorylated (inactive) β -catenin and Axin expression was higher, and CD1 (Cyclin D1), a known β -catenin target, expression was lower (261). These observations, in both inducible and constitutively expressing prostate cancer cell lines, implicate *LSAMP* in the inhibition of β -catenin activity.

LSAMP expression also altered the expression levels of various Integrins (Fig. 10). Constitutively expressing DU 145-*LSAMP* cells expressed higher levels of Integrin

$\alpha 5$, αV , $\beta 1$, and $\beta 3$, and no significant change in $\beta 4$. Interestingly, the only Integrin found to be downregulated by LSAMP in DU 145, Integrin $\beta 5$, has been shown to be an AR target associated with high expression in AA prostate cancer cells (300). The increased Integrin expression was accompanied by an increase in the phosphorylation of Focal adhesion Kinase (FAK). Integrins and RTKs have extensive crosstalk (50). The increased Integrin expression may be the result a feedback mechanism to partially compensate for the attenuated ERK/Akt activity, but remains to be determined. *LSAMP* expression results in the upregulation of most Integrins in DU 145. This observation is consistent with the downregulation of most Integrins in prostate cancer, notably prostate cancers of AA men (91; 145; 193).

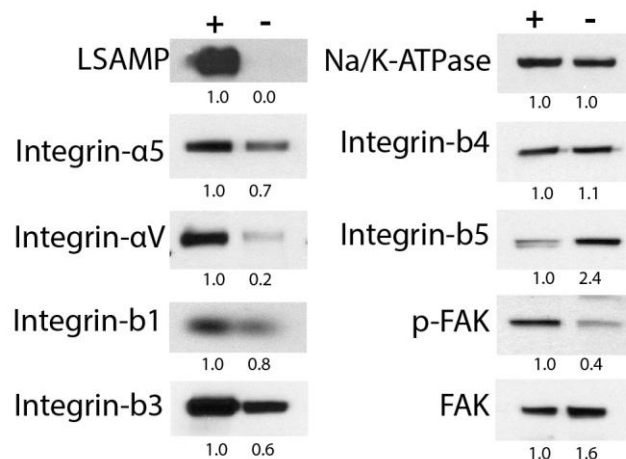
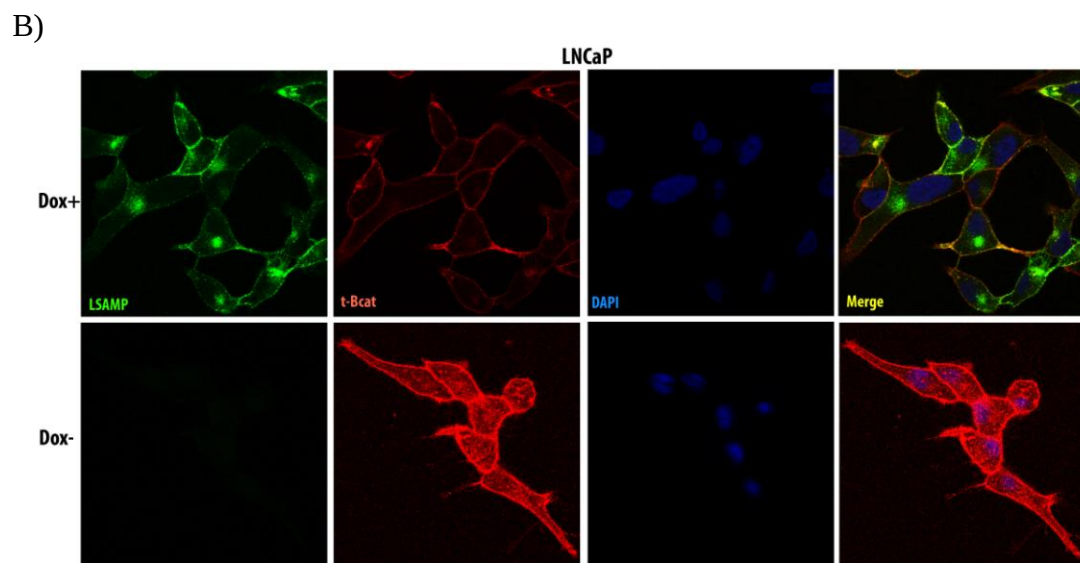
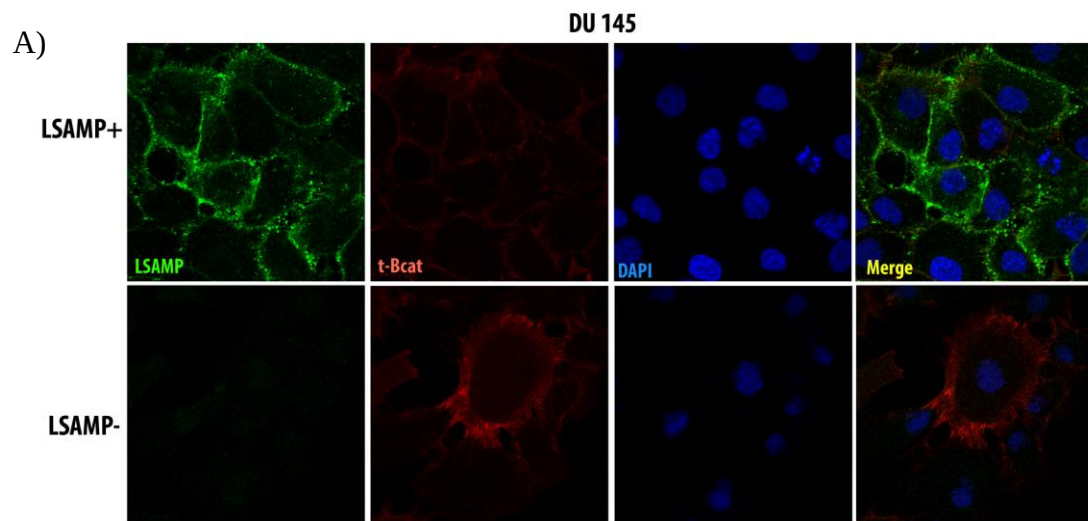
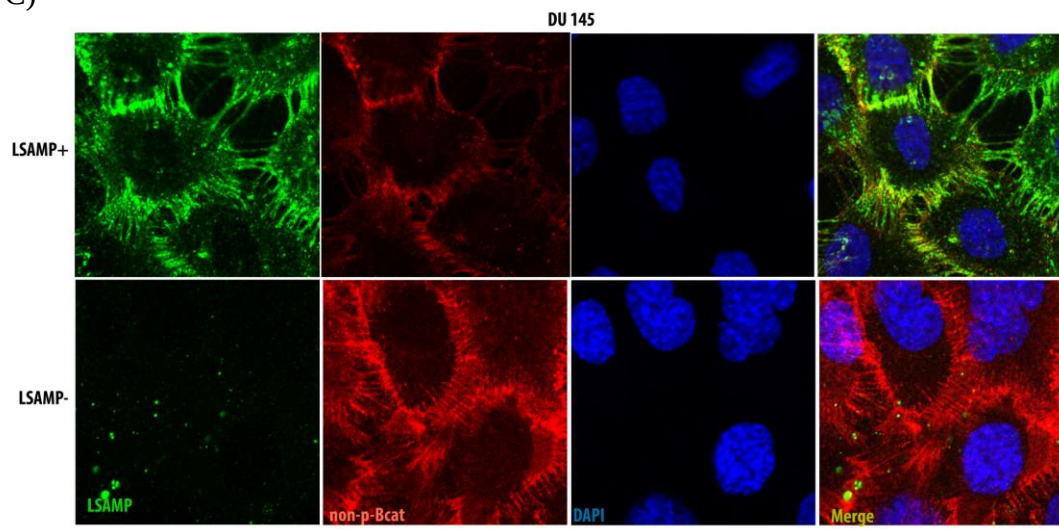


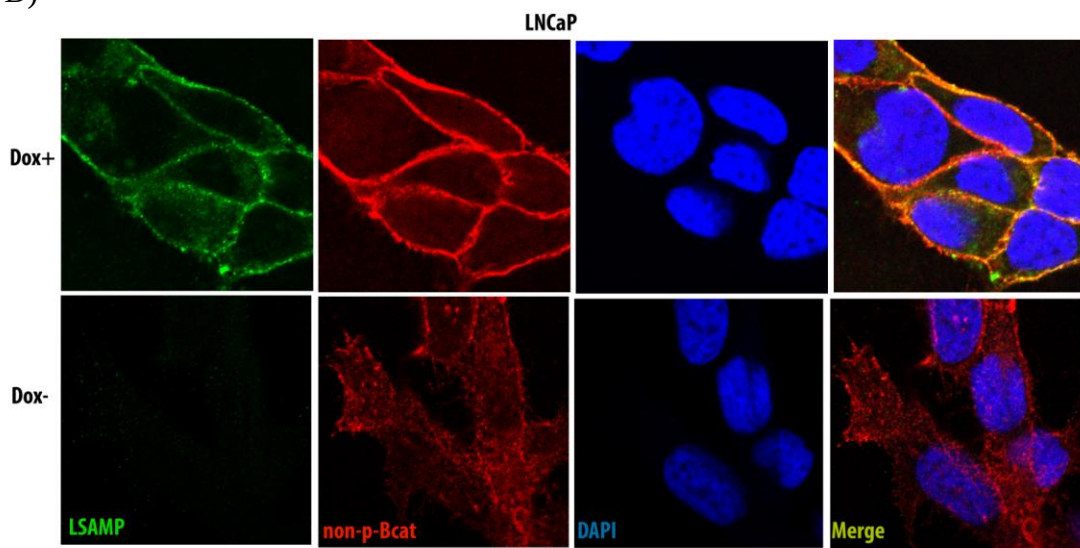
FIGURE 10. LSAMP ALTERS INTEGRIN EXPRESSION Immunoblot analysis following LSAMP expression in A) DU 145 reveals upregulation of several Integrins: $\alpha 5$, αV , $\beta 1$, $\beta 3$ accompanied by an increase in phosphorylated FAK. Integrin $\beta 5$, associated with high expression in AA prostate cancer, is downregulated with LSAMP expression. Values are relative normalized expression.



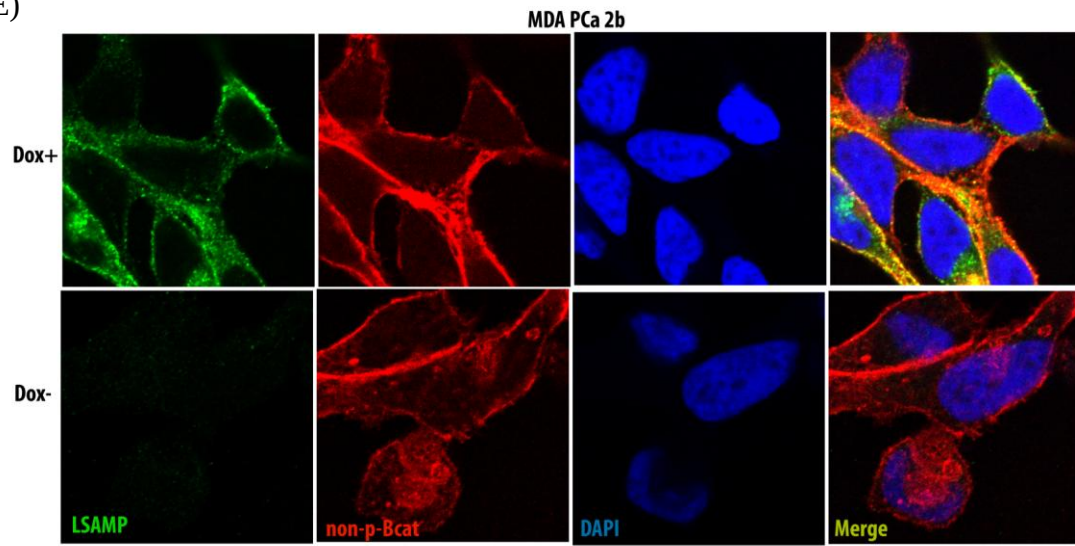
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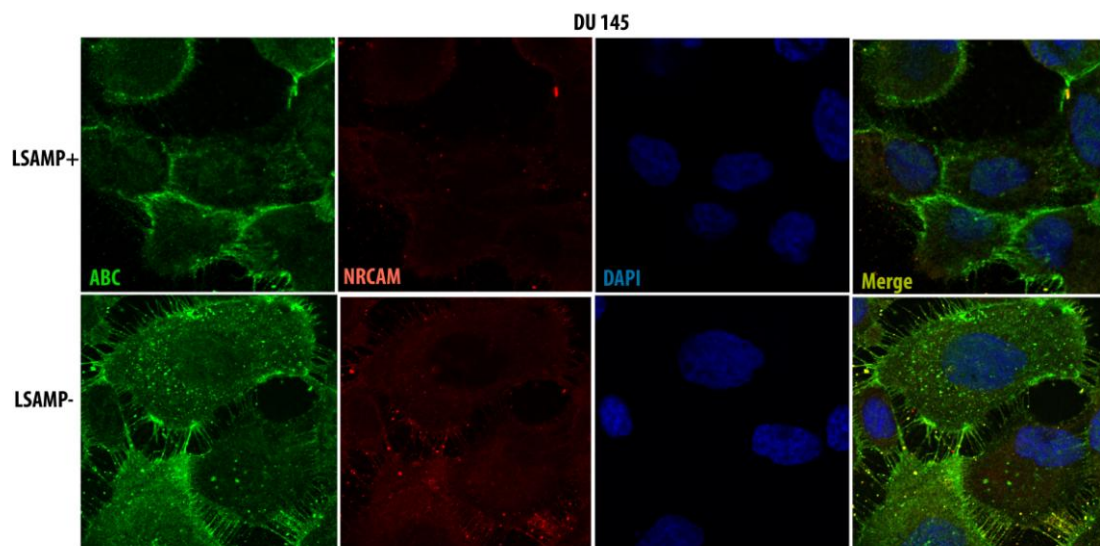
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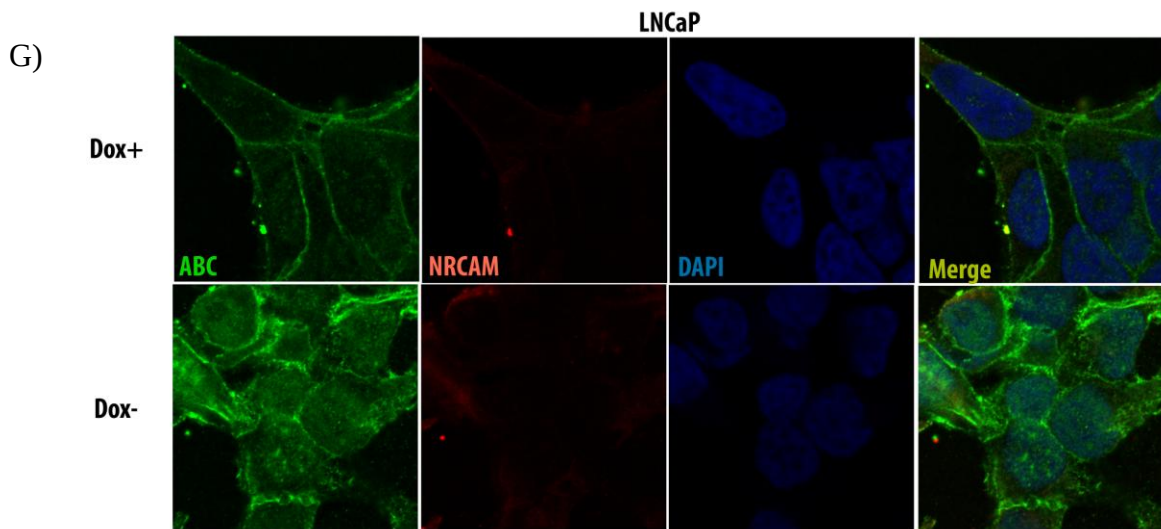


FIGURE 11. LSAMP EXPRESSION ALTERS LOCALIZATION OF BETA-CATENIN

Immunofluorescence confocal microscopy analysis of A) DU 145: LSAMP, total β -catenin. B) LNCaP: LSAMP, total β -Catenin. C) DU145: LSAMP, non-phospho- (active). D) LNCaP: LSAMP, non-phospho- β -catenin. E) MDA PCa 2b: LSAMP, non-phospho- β -catenin. F) DU 145: Activated β -catenin (ABC), NRCAM. G) LNCaP: Activated β -catenin, NRCAM. Green=LSAMP, Active β -catenin (ABC); Red= total β -catenin, NRCAM. Top row: LSAMP expressing prostate cancer cell. Bottom row: without LSAMP expression.

Cytoplasmic staining of β -catenin is reduced in LSAMP-expressing prostate cancer cell lines, while β -catenin remained mostly membranous (Fig. 11). The increase in cytoplasmic and nuclear staining observed in *LSAMP*-negative cells, coupled with the lack of significant downregulation of total β -catenin (Fig. 11A-B), is consistent with reduced cadherin mediated adhesion, and increased free β -catenin. This is observed in the decreased intensity of the active form of β -catenin in LSAMP expressing cells, as detected by two independent antibodies (Fig. 11C-E). The increase in free β -catenin, facilitated by ERK and Akt hyperactivation, indicates increased co-transcriptional activity of β -catenin. However, LSAMP's direct inhibition of β -catenin transcriptional

activity has yet to be assessed. Additionally, NRCAM, a cell adhesion molecule upregulated by β -catenin, exhibited reduced expression in *LSAMP* expressing cells, however, this requires additional investigation. The *LSAMP*-mediated downregulation of RTKs, decreased phosphorylation of ERK and Akt pathways, and β -catenin phosphorylation and localization changes are consistent with *LSAMP*'s tumor suppressive role, and identifies a novel signaling mechanism for *LSAMP*.

DYSREGULATION OF ADHESION MOLECULES AND BETA-CATENIN TARGETS IN AFRICAN AMERICAN CLINICAL SPECIMENS WITH *LSAMP* INACTIVATION

Datasets which give insight to the effect of *LSAMP* expression on transcription in prostate cancer is very limited. Analysis of existing unpublished RNA sequencing data from a small dataset of 3 AA prostate cancer patients with *LSAMP* aberration showed upregulation of various molecules involved in adhesion (Table 3), downregulation of several Integrins (Table 4), and differential regulation of known β -catenin target genes (Table 5).

The most significantly upregulated gene, *SPINK1* (serine protease inhibitor Kazal-type 1), was upregulated 385-fold, and has been shown to be a downstream effector of β -catenin and associated with tumorigenic characteristics via ERK pathway activation (258). *HOXC4* (Homeobox protein Hox-C4) expression has been associated with β -catenin activity in neural differentiation, and was found upregulated 92-fold (206). Additional known β -catenin targets such as *MMP9*, *MMP7*, and *TWIST1* (Twist-related protein 1) were also upregulated. Many genes involved in cell-adhesion were upregulated in *LSAMP* deleted tumors, consistent with the observation of increased

TABLE 3. RNASEQ ANALYSIS OF PATIENTS WITH *LSAMP* DELETION: UPREGULATED MOLECULES WHICH PLAY A ROLE IN ADHESION.

GENE	TUMOR/NORMAL (FOLD CHANGE)
PCDHA1	85.6
PCDH15	37.6
THBS4	25.8
PCDHGA1	16.1
PCDHGA4	12.9
ITGBL1	12.6
PCDHA11	11.5
PCDHA6	10.1
PCDHA13	9.50
PCDHB2	8.60
EPHA8	6.58
PDLIM5	4.94
PCDHGA5	3.90
PCDHB9	3.41
CTLA4	3.22
CDH8	3.12
PCDHGA2	3.01
EPCAM	2.99
PCDHAC1	2.98
PCDHA9	2.90
PCDHGB1	2.51
CDH13	2.48
EPHA6	2.39
PCDHA12	2.38
PCDH8	2.36
EPHB3	2.33
PCDH19	2.31
EPHA10	2.25
CDH11	2.23
PCDHB6	2.19
PCDHA3	2.16
CEACAM4	2.16
ALCAM	2.10

TABLE 4. RNASEQ ANALYSIS OF PATIENTS WITH *LSAMP* DELETION: DOWNREGULATION OF INTEGRINS.

GENE	TUMOR/NORMAL (FOLD CHANGE)
ITGA8	0.51
ITGA1	0.49
ITGA9	0.45
ITGA5	0.40
ITGB3	0.36

TABLE 5. RNASEQ ANALYSIS OF PATIENTS WITH *LSAMP* DELETION: MODULATION OF KNOWN BETA-CATENIN TARGETS

GENE	TUMOR/NORMAL (FOLD CHANGE)	REFERENCE
SPINK1	385.4	(258)
HOXC4	92.2	(206)
MMP9	37.7	(36)
FOXN1	11.3	(14)
MMP7	11.2	(32; 57)
NRCAM	10.3	(54)
TWIST1	9.88	(122)
RET	7.39	(330)
GREM1	5.29	(149)
LEF1	4.78	(79; 121)
BIRC5	4.28	(327)
VCAN	4.27	(228)
CTLA4	3.22	(256)
CLDN1	3.13	(185)
PTTG1	3.06	(331)
PCSK1	2.90	(198)
MYCN	2.61	(282)
MMP26	2.47	(177)
EPHB3	2.33	(21)
MYC	2.30	(107)
SALL4	0.42	(29)

endothelial cell adhesion. *NRCAM* (neuronal cell adhesion molecule) is involved in endothelial cell adhesion, was found to be significantly upregulated, 10 fold, in patients with *LSAMP* inactivation (4). *NRCAM* is also a β -catenin transcriptional target, and feeds forward into ERK1/2 and Akt signaling mediated activation of β -catenin, via Integrins and FAK (328).

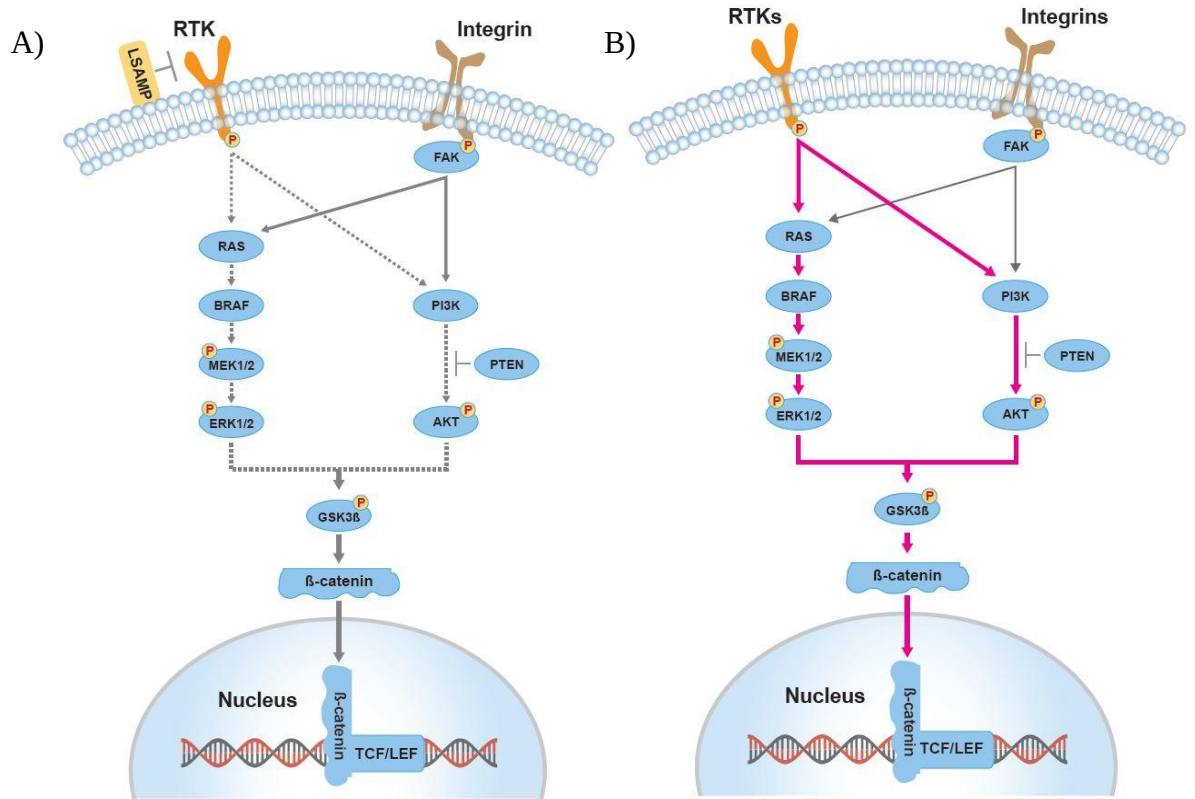
In addition to known β -catenin targets, adhesion molecules, and Integrins, other genes were found to be differentially regulated. Among them, *CXCL14* (Chemokine (C-X-C motif) ligand 14), an autocrine activator of fibroblast growth and invasion, has been found to increase prostate tumor growth and angiogenesis and positively correlate with Gleason score, was found to be upregulated 75-fold in cases with *LSAMP* deletion (12; 253). *CXCL9*, *10*, and *11*, were found to be downregulated, and have been previously been reported to have anti-angiogenic properties (288). This clinical dataset of *LSAMP* inactivated AA prostate cancer is very limited. However, observations of Integrin and β -catenin target expression modulation are consistent with the functional role of *LSAMP* identified in these studies. Additionally, the observed upregulation of several other adhesion molecules identifies potential targets responsible for mediating adhesion to endothelial cells.

TABLE 6: CANCER SIGNALING PHOSPHOARRAY: SIGNALING PATHWAYS MODULATED BY LSAMP EXPRESSION IN MDA PCa 2b

CANONICAL PATHWAY	P-VALUE
AKT(PKB)-Bad signaling (EPO signaling pathway (JAK2 STAT1 STAT3 STAT5))	6.26E-19
Signaling events activated by Hepatocyte Growth Factor Receptor (c-Met)	6.06E-18
Gene expression (VEGF signaling pathway)	1.16E-17
AKT(PKB)-Bad signaling (IL-7 signaling (JAK1 JAK3 STAT5))	6.79E-17
EGFR1	1.12E-16
ErbB2/ErbB3 signaling events	3.99E-15
Signaling events mediated by Stem cell factor receptor (c-Kit)	2.70E-14
trefoil factors initiate mucosal healing	4.70E-14
IL2-mediated signaling events	7.32E-14
Angiopoietin receptor Tie2-mediated signaling	2.88E-13

LSAMP INHIBITS RECEPTOR TYROSINE KINASE AND AKT SIGNALING PATHWAYS

Phosphoarray and pathway analysis of MDA PCa 2b, with and without *LSAMP* induction, revealed results centered on RTK and Akt signaling pathways (Table 6). Erythropoietin signaling was found to be the most significantly associated with *LSAMP* modulation. This pathway involves RTK signaling, via EpoR, through Akt and ERK, and has been found to promote angiogenesis, and is implicated in prostate cancer (133; 332). The second most significantly associated pathway, Hepatocyte Growth Factor signaling, is also implicated in prostate cancer, angiogenesis, and metastasis, as well as VEGF (vascular endothelial growth factor) and Angiopoietin receptor-Tie2 signaling (66; 73; 167; 188; 245; 298). Additionally, all of these pathways have been shown to be mediated by β -catenin dependent signaling (202).



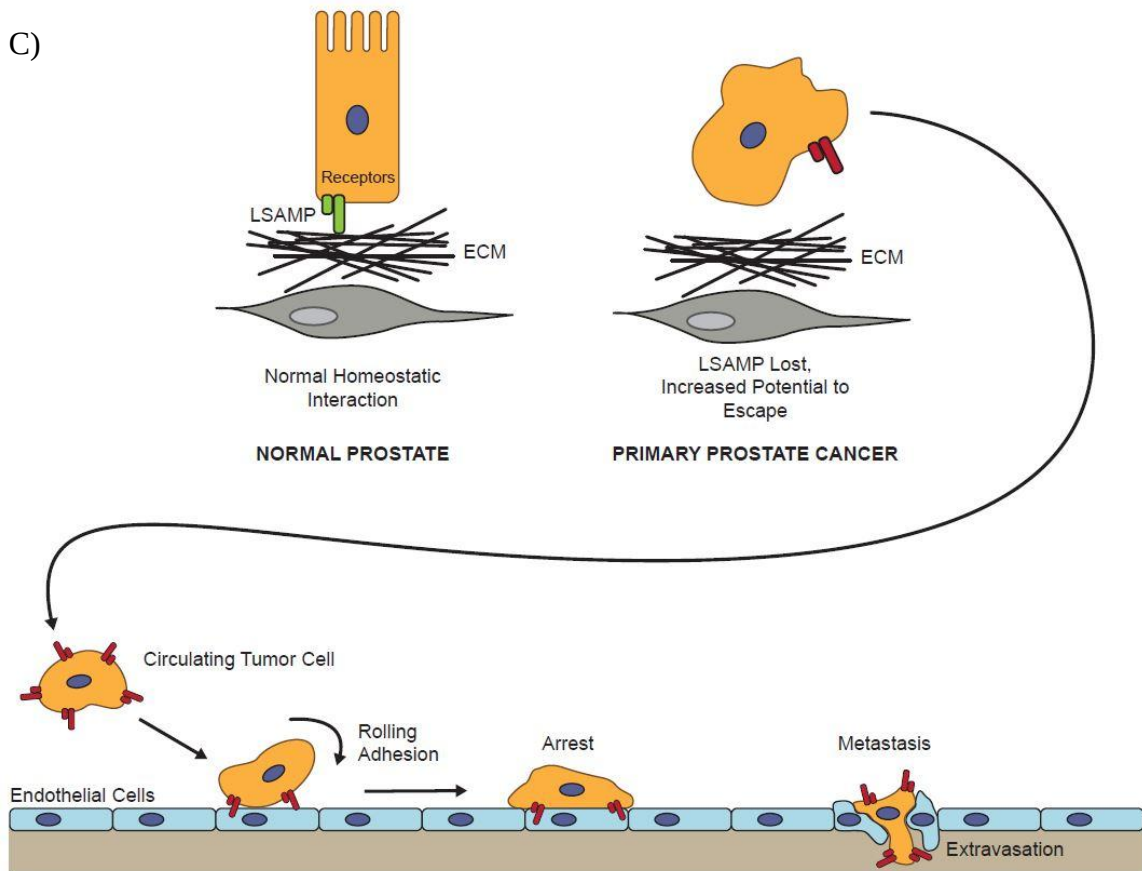


FIGURE 12. PROPOSED MODEL: LSAMP LOSS PLAYS A ROLE IN THE DYSREGULATION OF CELL-ADHESION, VIA THE LACK OF INHIBITION OF RTKS AND SUBSEQUENT HYPERACTIVATION OF ERK1/2, AKT, AND BETA-CATENIN . A) LSAMP inhibits specific RTKs, resulting in reduced downstream signaling and β -Catenin Activation, and higher Integrin expression. B) Loss of LSAMP results in lack of Inhibition of RTKs, and hyperactivation of ERK1/2 and Akt pathways, leading to increased activity of β -Catenin, and Integrin downregulation. C) *LSAMP* loss results in a malignant adhesive phenotype, with dysregulated interaction in its native environment through adhesion to ECM and prostate stroma, facilitating intravasation. Additionally, phenotypic changes as a result of dysregulated signaling, and possible aberrant upregulation of adhesion molecules, results in increased adhesion of CTCs to the vascular endothelium, facilitating extravasation and establishment of a metastatic site.

CHAPTER 4: Discussion

LSAMP inactivation is observed in various cancers, and has been recently identified in prostate cancer (48; 157; 218; 325). Signal transduction mediated by *LSAMP*, or its functional role in prostate cancer had not been previously identified. In the investigation of *LSAMP*'s role in prostate cancer, it was initially found that its expression did decrease the proliferative rate of prostate cancer cell lines, consistent with observations in osteosarcoma (19). Proliferation rate contributes to tumorigenesis, however, in prostate cancer where the majority of mortality arises from metastasis, increased proliferation is not likely to be the major factor contributing to the rapid disease progression observed with *LSAMP* loss (218).

LOSS OF LSAMP: ABERRANT ADHESION, DYSREGULATED SIGNALING, AND POTENTIAL IMPLICATIONS IN METASTASIS

The detachment of a tumor cell from its native environment is an initial step in progression to metastasis. The epithelial-stromal interaction in the normal prostate is mediated by ECM-receptor association and focal and cell adhesion pathways (92). A loss of this interaction can lead to prostate cancer development (329). Many biological investigations of potential oncogenes and tumor suppressors focus on proliferation rate, apoptosis, motility and invasion. However, cell-adhesion may play an underlying role in all of these features. Aberrations in cell-adhesion and its associated signaling can contribute to tumor progression and metastasis. Normal cells undergo apoptosis upon detachment from the ECM. Whereas, tumor cells can detach, facilitating migration and invasion as a part of the EMT process (96). Additionally, decreased cell-cell adhesion can promote enhanced growth with a lack of contact inhibition, as well as metastasis (44).

Furthermore, dysregulation of stromal microenvironment interaction can result in inflammation, which may contribute to further aberrations, secretions of growth factors and cytokines, and the aggressiveness of the tumor (15). Weakened adhesion to native stroma can facilitate increased motility and invasion, leading to intravasation. In addition to this contribution to the malignant cell's escape from its primary site, aberrant adhesion molecule expression mediates CTC adhesion on the vascular endothelium, facilitating extravasation and establishment of a metastatic site. *LSAMP* expression resulted in significantly increased affinity to fibroblast and native stroma cell lines. This phenotype conveys inhibited tumor progression. The lack of significant blocking with antibody pretreatment in the cell-adhesion assay and the upregulation of most Integrins, suggests the observed difference in adhesion to ECM proteins, and fibroblast and prostate stromal cell lines may in part be mediated by Integrins. *LSAMP* expression also significantly reduced adhesion to endothelial cells. This further conveys a decreased propensity to metastasize. The discovery of *LSAMP*'s effect on tumor cell-adhesion properties provides novel insight into its role in cancer progression.

In the proposed model, *LSAMP* is expressed in the normal prostate tissue, facilitating a normal interaction with neighboring cells and stroma (Fig. 12C). This interaction may in part be additionally mediated by Integrins and cadherin associated membranous β -catenin, which play a large role in ECM-cell and cell-cell adhesion, and are commonly downregulated in prostate cancer (69; 91; 120; 130). *LSAMP* inhibits FGFR2/4 and EPHA3 activation of the ERK and Akt pathways, reducing β -catenin activity (Fig. 12A). Upon *LSAMP* inactivation, FGFR2/4 and EPHA3 are no longer inhibited, and ERK and Akt pathways become hyperactivated, leading to increased β -

catenin activity (Fig. 12B). *LSAMP* inactivation, coupled with concomitant Integrin downregulation, and loss of membranous β -catenin weakens the interaction of the tumor cell to its native environment, facilitating an increased potential for intravasation and tumor progression. With β -catenin released from the membrane and GSK-3 β mediated degradation inhibited, this propensity to metastasize is further exacerbated by increased β -catenin co-transcriptional activity. β -catenin transcriptional coactivation can facilitate the EMT, increased motility, and invasion (229; 319). Additionally, β -catenin activity may be responsible for the upregulation of the molecules which mediate endothelial cell-adhesion, however this is yet to be determined. The loss of *LSAMP*, and the accompanying downregulation of Integrins, and RTK-Akt/ERK-mediated activation of β -catenin may significantly contribute to prostate tumor progression.

Previous studies of *LSAMP* and other members of the IgLON family have shown that in many cases during neuronal development and differentiation, expression of surface molecules on specific cell subtypes may be coordinated (34; 70; 71). This molecular patterning is critical to the interaction of these cells during development, differentiation, and cancer progression. The specific adhesion molecule repertoires of normal prostate tissue may undergo changes as a course of tumor progression, switching to a malignant adhesion molecule repertoire and facilitating metastasis. The adhesive affinity for its native environment is weakened, allowing escape, and the adhesive affinity for endothelial cells lining the blood vessels is increased, facilitating the adhesion of CTCs and allowing the opportunity for extravasation at a distant site. The switch in adhesive phenotype facilitated by the loss of *LSAMP* may be mediated by β -catenin transcriptional activity. In the presence of *LSAMP*, β -catenin is predominantly

membranous, mediating cell-cell adhesion, alongside Integrins. In the absence of *LSAMP*, Integrins are downregulated and ERK and Akt pathways are hyperactive, allowing an increase in β -catenin translocation into the nucleus to transcriptionally coactivate a variety of different genes, possibly including adhesion molecules such as *NRCAM*. *LSAMP* may be a determining factor in the adhesion molecule repertoires of the prostate tumor cell.

In addition to facilitating tumor progression, aberrantly expressed adhesion molecules may play a role in the homing of CTCs to a specific distant site and facilitating favorable interactions in the foreign stroma necessary for metastatic establishment. The interaction of CTCs with the endothelial cells of a specific tissue, may involve specific chemoattractant and adhesion molecule expression. Prostate cancer predominantly metastasizes to the lymph nodes, bone, lung, liver, pleura, and adrenals (40). The molecular basis for metastases from a specific origin targeting certain tissues is largely unknown. However, an example is *CXCR4*, which is highly expressed in breast cancer, and targets tissues with high *CXCL12* expression, such as the lung, liver, marrow, and brain (249). In addition to aberrant expression of adhesion molecules, β -catenin activity may also upregulate chemokines such as the *CXCL14*, observed to be upregulated 75-fold in *LSAMP* deleted clinical samples. Additionally, Ephrins, commonly expressed on endothelial cells, and Ephrin Receptors, such as *EPHA3*, can function in a manner similar to chemokines (115; 211) *LSAMP* loss may significantly contribute to the aberrant adhesive affinities of metastatic prostate cancer.

LSAMP loss contributes to the dysregulation of Integrins and RTK/ERK/Akt/ β -catenin signaling. The identification of *LSAMP*'s role in signal transduction is a novel

finding. This dysregulation may lead to a host of aberrant changes and an aggressive phenotype. *LSAMP* plays a role in inhibiting proliferation and tumor growth. However, *LSAMP* may play a more significant role in cell-adhesion mediated inhibition of metastatic progression. *LSAMP* contributes to the maintenance of normal adhesive interactions and inhibits prometastatic RTK/ERK/Akt/ β -catenin signaling and gene expression. The dysregulation of cell adhesion genes significantly distinguishing cancers of distinct ethnic origin is not completely novel; and has been found to significantly differentiate AA breast cancer from that of CA (94). Additionally, the dysregulation of β -catenin has previously been shown to be mediated by the downregulation of another Ig superfamily member in a metastatic AA prostate cancer model (214). Increased β -catenin activity and Integrin downregulation is associated with AA prostate cancer, and aggressive disease (130; 143; 193; 280). Furthermore, Integrin $\beta 5$, the only Integrin assessed to have notable downregulation with *LSAMP* expression, is highly expressed in AA prostate cancer (300). *LSAMP* loss may play a significant role in this phenotype. The reversion to a more indolent adhesive phenotype and inhibition of progression-associated signaling upon *LSAMP* expression in prostate cancer cell lines provides novel insight to the understanding of prostate tumor progression and the disproportionate incidence and mortality in AA prostate cancer.

FUNCTIONAL INACTIVATION OF *LSAMP* MAY BE MORE PREVALENT IN PROSTATE CANCER THAN OBSERVED IN CURRENT GENOMIC DATA

LSAMP deletion is detected in a significant proportion of prostate cancer, most notably in that of AA men (218). However, the prevalence of the *LSAMP* inactivation in

prostate cancer may be higher than currently reported. Prostate tumors are highly heterogeneous, and the tumor foci are often very small. Genomic data can have some degree of noise, due to sample impurities caused by a mixture of normal epithelia or another tumor clone with the sample being tested. Sample pre-amplification can facilitate the detection of a point mutation, but it may reduce the sensitivity necessary to detect the heterozygous deletion of a small tumor focus in a heterogeneous sample. Additionally, sequence depth and subsequent bioinformatic analysis may not be optimized to allow for the precision and sensitivity necessary. AA prostate cancer is more likely to harbor a *LSAMP* deletion, but currently the majority of data consists of CA specimens. Sequencing technologies have immensely expanded our understanding; however, remaining limitations may lead to an underrepresentation of *LSAMP* genomic aberrations.

Interchromosomal rearrangements are more prevalent in AA prostate cancer, and may result in an inactivating translocation. An inactivating translocation of *LSAMP* has been detected in AA prostate cancer genomic data (218). Genomic and RNA sequencing analysis of Chinese prostate cancer samples identified *LSAMP* deletions which corresponded to significant expression downregulation. In additions to deletions of *LSAMP*, intra- and interchromosomal translocations, as well as insertions corresponded with significant expression downregulation. Of the prostate cancer specimens with both structural variation of the *LSAMP* locus and significant downregulation, approximately 29% of the structural variants were not characterized as a deletion (234). Genomic aberrations which inactivate the gene may also leave both copies of *LSAMP* remaining. Partial sequences may also detected as intact, depending on the specific aberration and

the probes used. In cases such as these, SNP array and FISH analysis would be insufficient to detect the aberration.

In addition to chromosomal aberration, *LSAMP* expression can be reduced below endogenous levels by other mechanisms. *In silico* analysis of *LSAMP*'s promoter revealed significantly higher methylation in prostate cancer compared to matching normal samples (Fig. S6). This is consistent with significantly downregulated *LSAMP* expression in 52% of the Chinese prostate cancer samples, when genomic alteration occurred in only 20% (234). Also, *LSAMP* deletions are rarely homozygous, suggesting the remaining locus may be inactivated by other means, such as epigenetic modification (218). Many of the prostate cancer cell lines assessed had multiple copies of *LSAMP*, and negligible expression. Some prostate cancer cell lines, such as LAPC-4, had relatively high mRNA expression, and undetectable levels of protein, or in the case of PC3, possible expression of a nonfunctioning protein. In osteosarcoma samples, *LSAMP* copy number, transcript and protein levels also did not always strictly correlate (19). This suggests that the prostate tumor suppressive function of *LSAMP* may be inactivated by multiple mechanisms, whether by deletion or other genomic alteration, methylation, or aberrant mRNA and protein processing. The prevalence of deletion in AA, and its significant association with BCR has been shown (218). However, limitations in genomic datasets and multiple mechanisms of downregulation suggest the possibility of a higher prevalence of *LSAMP* inactivation in prostate cancer than current studies have identified.

CLINICAL RELEVANCE

Prostate cancer is common, and occurs in many men without causing any symptoms (101; 264). Prostate cancer grows relatively slow, and with current surgical and treatment options, the five-year survival for localized and regional disease is approaching 100%, while that of distant disease remains at merely 30% (264). AA men are disproportionately affected by this disease (264). What distinguishes indolent from aggressive, as well as what accounts for the biological basis of the AA prostate cancer disparity is largely unknown. New discoveries of the underlying mechanisms are critical to improving outcome. Genetic aberrations of *LSAMP* are observed in approximately 27% of AA prostate cancer patients, but with downregulation by other means, the number of patients which could potentially benefit from *LSAMP* studies may be substantial.

LSAMP loss contributes to increased proliferation and tumor growth. Further, *LSAMP* loss contributes to a malignant adhesive phenotype and increased pro-metastatic signaling. A single patient may have seven distinct large tumors, but it is the eighth tiny tumor with a loss of *LSAMP* which results in distant metastasis and mortality, and needs to be identified. With watchful waiting and active surveillance more commonly prescribed, diagnostic and prognostic biomarkers are crucial for determining the associated risk. *LSAMP* biomarker development may provide an essential tool in the diagnosis and prognosis of prostate cancer. This would be especially critical in the AA patient population, where the key drivers are largely unknown.

Defining the tumor suppressive function of *LSAMP* provides the first insight into its role in prostate cancer. *LSAMP* studies have contributed to the concept of tumor progression-associated adhesion molecule defects, and provide a model for the investigation of other adhesion molecules identified in cancer genomic studies. The novel

identification of the LSAMP-mediated inhibition of the RTK, ERK, Akt and β -catenin pathway provides the basis for the further investigation of a multitude of potential therapeutic targets for prostate cancer patients, or potentially other cancers, with *LSAMP* inactivation. Dysregulated cell membrane proteins provide additional targets for potential therapies, including immunotherapies (320). The *LSAMP* locus is fragile, consistent with higher interchromosomal rearrangements in AA prostate cancer, and its loss may serve in the identification of chromoanagenesis positive tumors (218; 268; 307). The large number of rearrangements in these tumors may make them susceptible to therapies similar to immune checkpoint blockade treatments used in tumors with mismatch repair deficiencies (165). In addition to implications for *LSAMP* and cancer, the adhesive phenotype aberration and identification of involvement in a specific pathway may give insight into the role of the IgLON family in cancer and neuropsychiatric disorders.

In the case of AA prostate cancer, there is a significant healthcare disparity. The rates of incidence and mortality in AA prostate cancer is disproportionately high, and the majority of studies are performed on tissues of CA origin. There is emerging evidence of the biological basis for the AA prostate cancer disparity, but little implementation in the clinic. The investigation of *LSAMP* loss, highlighting prometastatic signaling, and altered adhesion to specific cell types, provides insight into disease progression and the biological basis of the AA prostate cancer disparity. Additionally, they provide novel biological evidence of *LSAMP*'s tumor suppressive role in support of its use as a potential biomarker, and identified a novel pathway which provides potential therapeutic targets for a significant proportion of aggressive prostate cancer. These functional studies start to bridge the gap between genomic observations and personalized treatment, and provide

the groundwork for future investigation, potentially leading towards a more informed treatment decision and better treatment options.

FUTURE STUDIES

These studies have identified a novel *LSAMP*-mediated signaling mechanism and provided the first biological evidence of *LSAMP*'s function in prostate cancer. However, many details are yet to be defined. The use of clinical samples can validate *in vitro* and *in vivo* findings. Initial tests using paraffin embedded cell line controls to validate the use of *LSAMP* mAb 2G9 in immunohistochemistry has been performed (Fig. S7). RNA *in situ* hybridization or IHC to assess *LSAMP* expression in a large racially diverse cohort of prostate cancer patients is needed to confirm *LSAMP* downregulation in deleted tumors, and assess the frequency of expression downregulation. Clinical specimens can also be used to validate findings of *LSAMP*'s effect on specific RTKs, Integrins, and β -catenin localization, as well as to investigate a possible correlation between *LSAMP* loss and chromoanagenesis. Assessing *LSAMP* status in clinical metastatic samples can more definitively institute *LSAMP* as a biomarker. Additionally, further genomic studies could investigate whether any ethnicity associated SNPs convey prostate cancer risk.

EPHA3, FGFR2/4, ERK, Akt, and β -catenin are all implicated in angiogenesis. Pathways identified in MDA PCa 2b were also predominantly involved in angiogenesis. Increased endothelial cell affinity mediated by aberrant adhesion molecule expression may also facilitate angiogenesis. In addition to the significant difference in tumor growth, 8/10 *LSAMP*-negative tumors exhibited visible vascularization at the tumor site,

whereas 0/10 *LSAMP* expressing tumors exhibited this phenotype (Fig .7C). The vascularization observed was not dependent on tumor size, and suggests increased angiogenesis. The growth rates observed coupled with the visible vascularization at the tumor site only in those without *LSAMP*, suggests *LSAMP* expressing tumors may in part have limited growth due to lack of sufficient blood supply resulting from inhibition of angiogenesis, but this remains to be determined. To investigate *LSAMP*'s potential inhibition of angiogenesis, xenograft tumors will be sectioned and stained for vascular markers, such as CD31 and ERG. *In vitro* angiogenesis assays and identification of the extent of angiogenesis in clinical samples can validate *LSAMP*'s angiogenic inhibition. Additionally, *in vivo* investigations are ongoing to corroborate the findings.

FGFR2, FGFR4, and EPHA3 are observed to be downregulated with *LSAMP* expression. Co-immunoprecipitation experiments can be used to validate the interaction, and subsequent mass spectrometry can identify other potential interactions. Identifying the precise binding partners which are directly inhibited by *LSAMP* further defines the mechanism and may provide a specific therapeutic target which could be administered to all patients with *LSAMP* deletion or downregulation. Additionally, *LSAMP* protein function may be somewhat dependent on the availability of other member of the IgLON family it is known to interact with, OBCAM, and NTM. As prostate tumors are highly heterogeneous, determining the expression of other IgLONs may give further insight.

ERK/Akt pathway hyperactivation, GSK3 β phosphorylation, reduced Axin expression, increased CD1 expression, changes in phosphorylation, localization and intensity of β -catenin observed in immunofluorescence assays, and clinical expression data all point to increased β -catenin activation. However, *in vitro* assessment of β -catenin

transcriptional activity using a reporter plasmid in prostate cancer cell lines will confirm the extent of the *LSAMP*-mediated inhibition of transcriptional activity. RNA sequencing and proteome analysis can corroborate the findings of β -catenin activity, and will provide the initial data needed to identify additional molecules differentially regulated by *LSAMP* expression, specifically those that mediate endothelial cell-adhesion. Additionally, *in vitro* investigations can give insight into which aberrant proteins are best therapeutically targeted in an *LSAMP*-null context. Further definition of the *LSAMP* pathway in cancer could advance personalized medicine by providing therapeutic targets and potentially increase the quality of care for patients with *LSAMP* inactivation.

Several details underlying *LSAMP* expression-mediated adhesive affinity changes have not been defined. In addition to the investigation of molecules which mediate endothelial cell interaction, RGD peptides can be used to block Integrin function in adhesion assays to determine the extent of Integrin involvement in the adhesion to fibroblast and prostate stroma cell lines. A complex interaction exists between prostate cancer and its microenvironment, to include interaction with normal stroma and the potential development of cancer associated fibroblasts. Co-culture experiments with prostate cancer cells and fibroblasts or prostate stromal cell line would shed light on the capacity of *LSAMP* to maintain a normal interaction with stromal cells, limit the development of cancer-associated fibroblasts, and inhibit protumorigenic secretions. These proposed studies may define unknown details of *LSAMP*'s tumor suppressive function, leading towards both biomarker development, and personalized therapies for an aggressive disease which significantly contributes to the understudied AA prostate cancer disparity.

CONCLUDING REMARKS

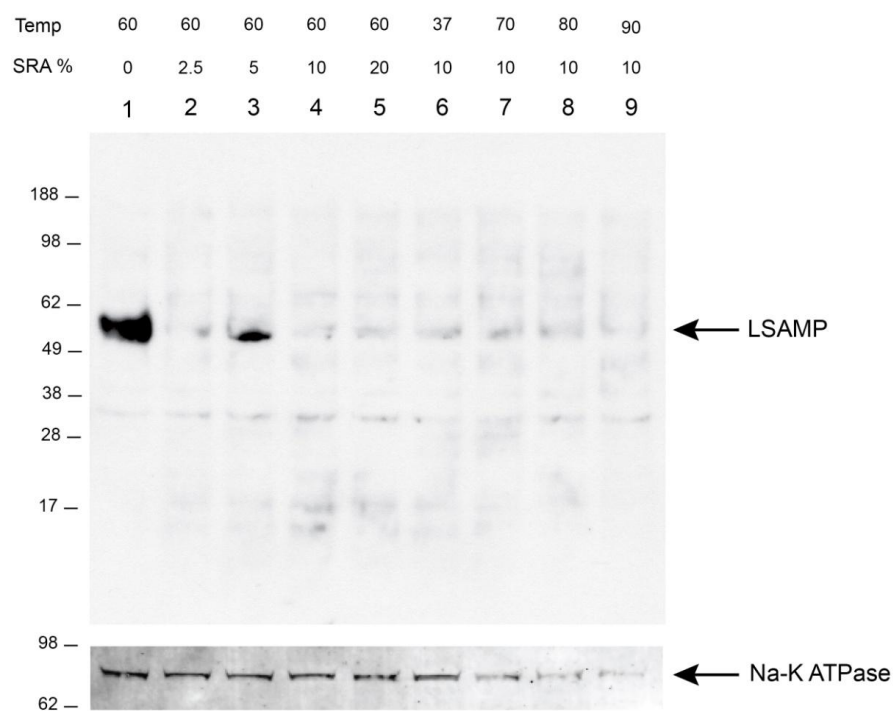
Lentiviral *LSAMP* expression and subsequent *in vitro* and *in vivo* characterization of its effects in prostate cancer cell lines presents evidence that *LSAMP* is the functional region in 3q13.31 commonly deleted, and provides the first functional evidence of *LSAMP*'s tumor suppressive activity in prostate cancer, or any cancer. The investigations also identified a novel mechanism by which *LSAMP* affects signal transduction, and connects *LSAMP* loss to the Integrin downregulation and β -catenin hyperactivation commonly observed in AA prostate cancer. Additionally, these studies, focused on tumor cell-adhesion, provide a model to validate the function of other IgLONs and adhesion molecules identified in cancer genomic studies. This functional characterization of *LSAMP* gives novel insight to the mechanisms underlying aggressive prostate cancer and the AA prostate cancer disparity.

Supplementary Data

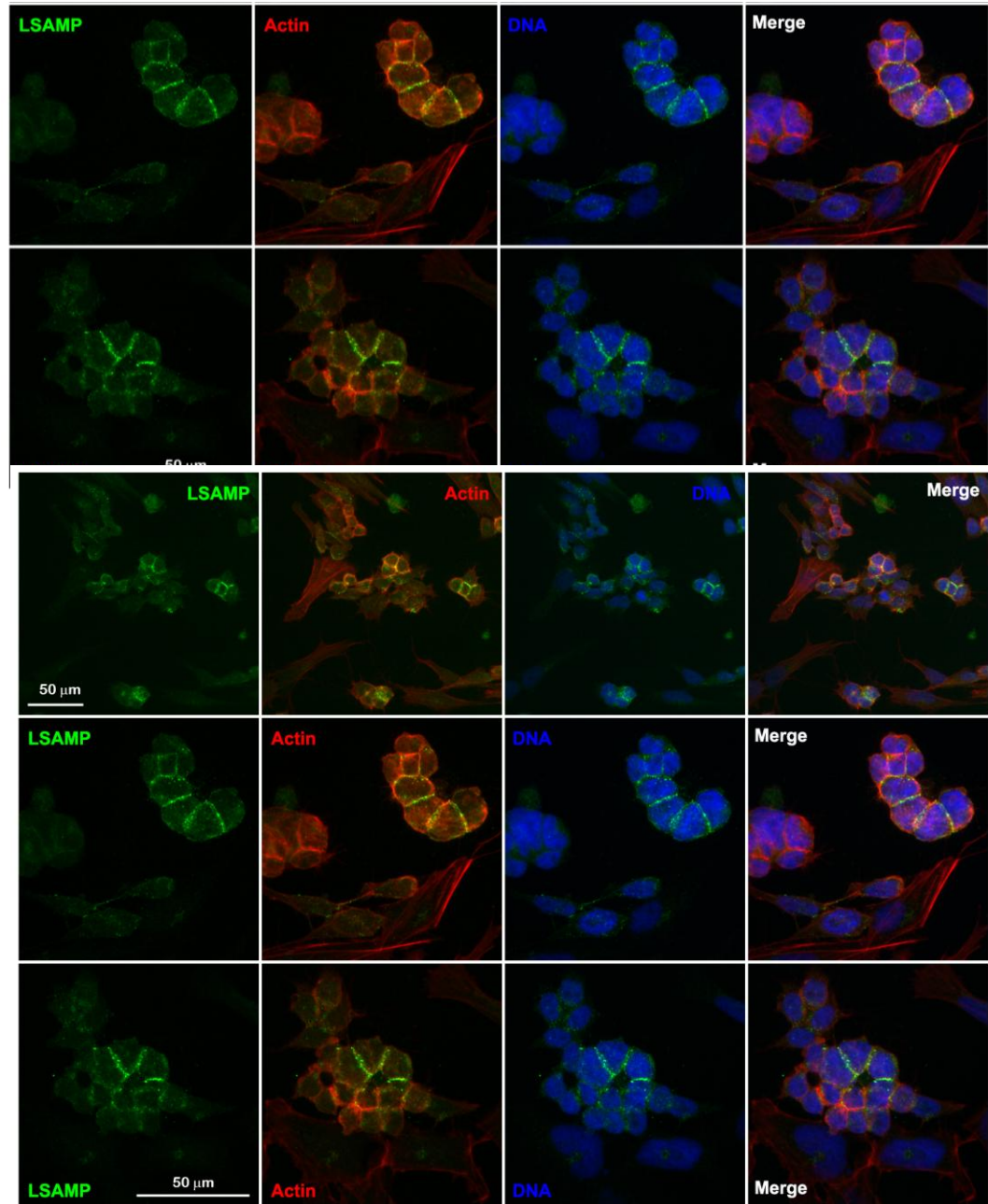
SUPPLEMENTARY TABLE 1. *IN VITRO* MODELS

Cell Line	Ethnic Origin	Site of Origin	Androgen Sensitivity	Reference
LNCaP	CA	Prostate Cancer, Supraclavicular Lymph Node Metastasis	AD/AS	(118; 119)
MDA PCa 2b	AA	Prostate Cancer, Bone Metastasis	AI/AS	(196)
DU 145	CA	Prostate Cancer, Brain Metastasis	AI	(184; 275)
PC3	CA	Prostate Cancer, Bone Metastasis	AI	(137)
22Rv1	CA	Prostate Cancer, Primary, Xenograft	AI/AS	(271)
VCaP	CA	Prostate Cancer, Vertebral Metastasis, Xenograft	AI/AS	(155; 243)
LAPC-4	CA	Prostate Cancer, Lymph Node Metastasis, Xenograft	AD	(150)
NCI-H660	CA	Neuroendocrine Prostate Cancer, Lymph Node Metastasis, SCC	AI	(42; 135)
RWPE-1	CA	Normal Prostate	N/A	(23; 305)
MC-IXC	CA	Neuroblastoma, Metastasis	N/A	(25)
Saos-2	CA	Osteosarcoma	N/A	(192)
NIH/3T3	N/A	Mouse Embryo, Fibroblast	N/A	(131)
WPMY-1	CA	Normal Prostate Stroma	N/A	(306)
HUVEC	CA	Umbilical Vein/Vascular Endothelium	N/A	(82)

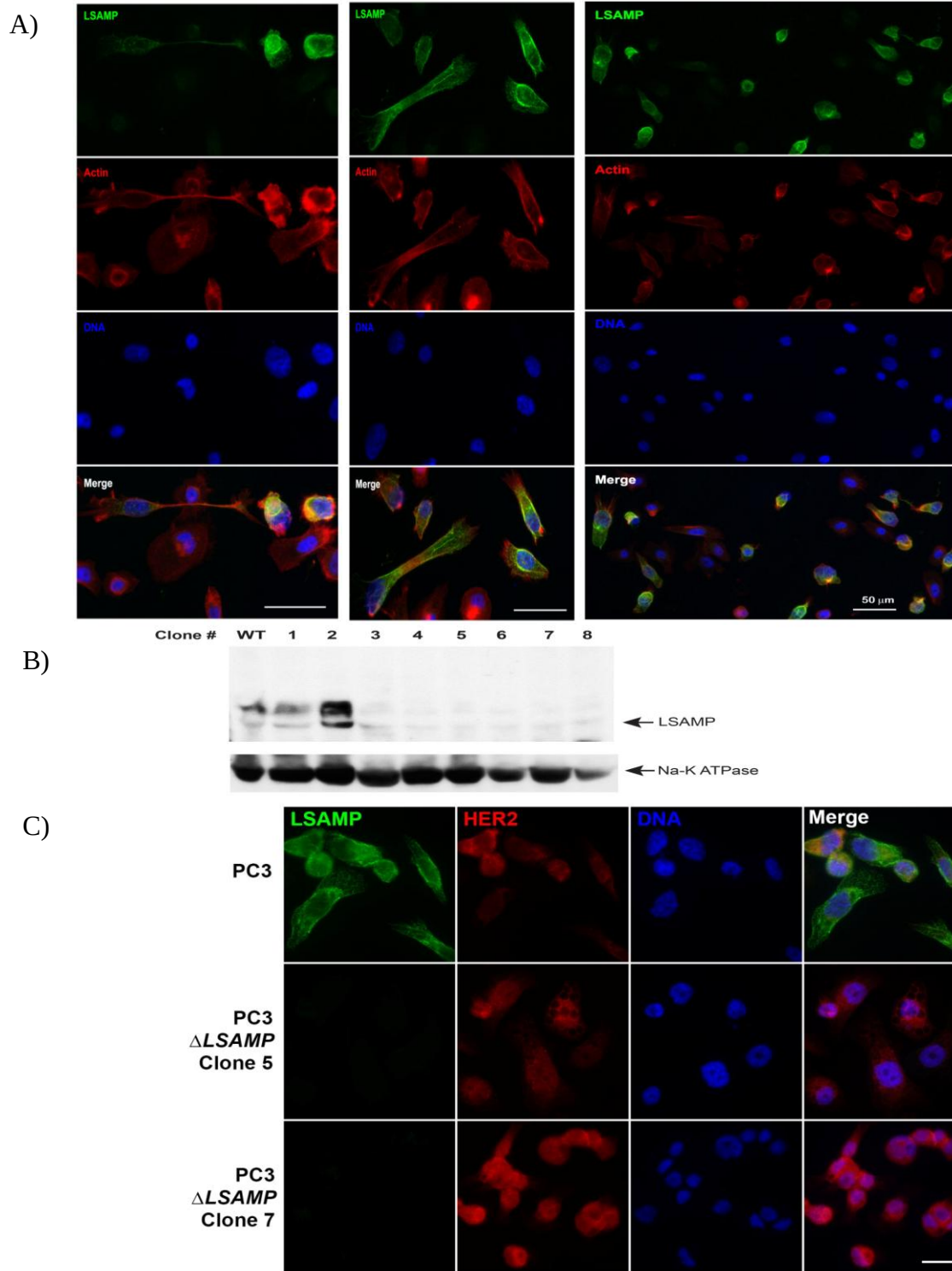
CA: Caucasian American. **AA:** African American. **AD:** Androgen Dependent. **AS:** Androgen Sensitive. **AI:** Androgen Insensitive. Van Bokhoven *et al.* The Prostate 57:205-225 (2003).



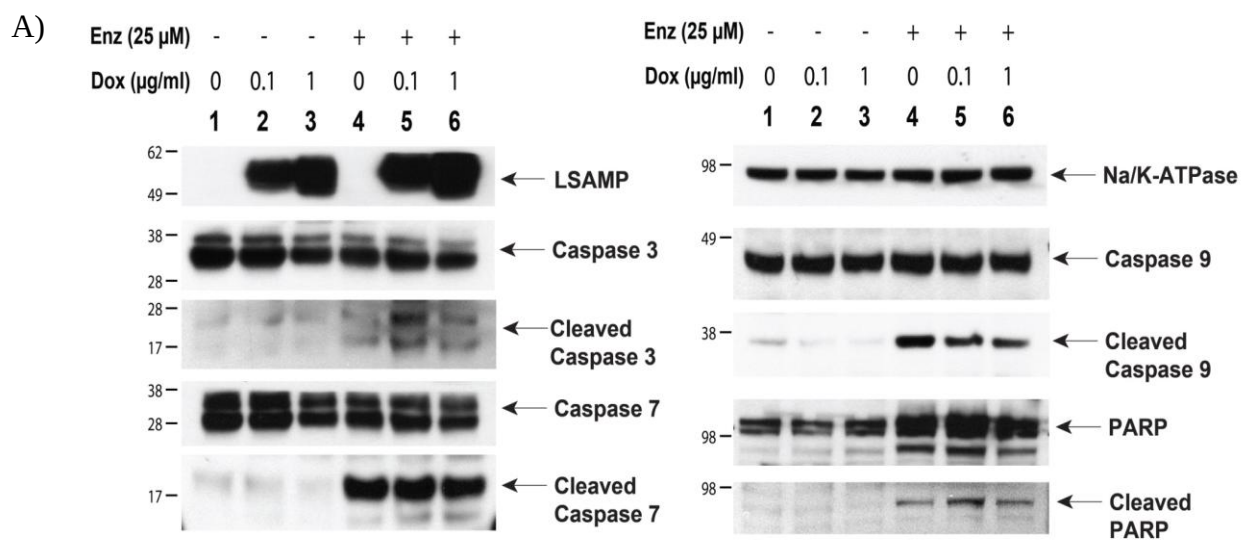
SUPPLEMENTARY FIGURE S1. EFFECT OF REDUCING AGENT CONCENTRATION AND TEMPERATURE ON DETECTION OF LSAMP. Immunoblot analysis of MC-IXC lysates following sample incubation at various temperatures, and the addition of various concentrations of sample reducing agent (SRA) reveals R&D mAb 873 detects conformational epitope, which is degraded with the addition of a reducing agent.



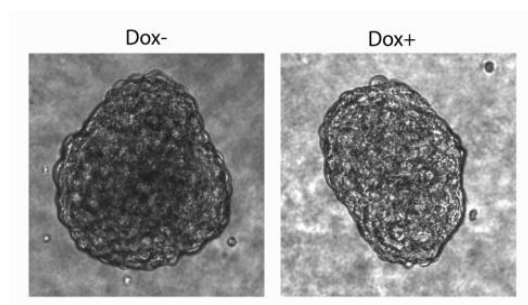
SUPPLEMENTARY FIGURE S2. IMMUNOFLUORESCENCE OF POSITIVE CONTROL
NEUROEPITHELIOMA CELL LINE MCI-XC shows punctate LSAMP staining
 enriched at intercellular junctions.



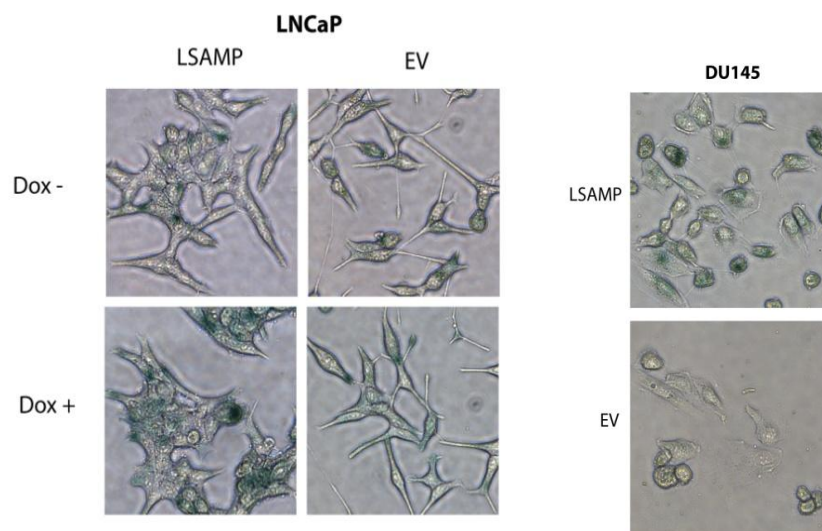
SUPPLEMENTARY FIGURE S3. PC3 EXPRESSES CYTOPLASMIC LSAMP AND PROTEIN EXPRESSION IS KNOCKED DOWN TO UNDETECTABLE LEVELS IN CRISPR-CAS9-PC3 CELL LINE shows A) Endogenous, mostly cytoplasmic LSAMP expression in a subset of cells. CRISPR-Cas9 knocks out expression as detected by B) Immunoblot, and C) Immunofluorescence assays.

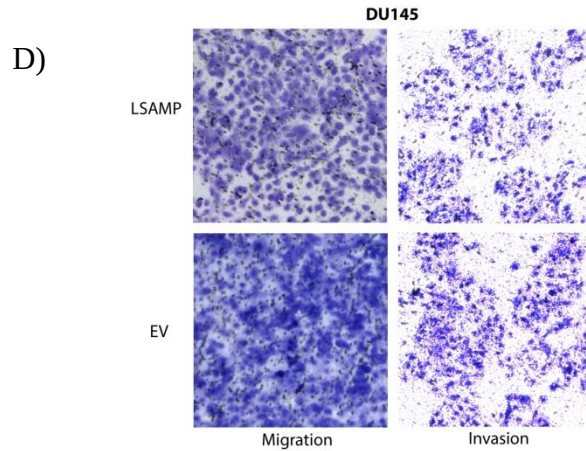


B)

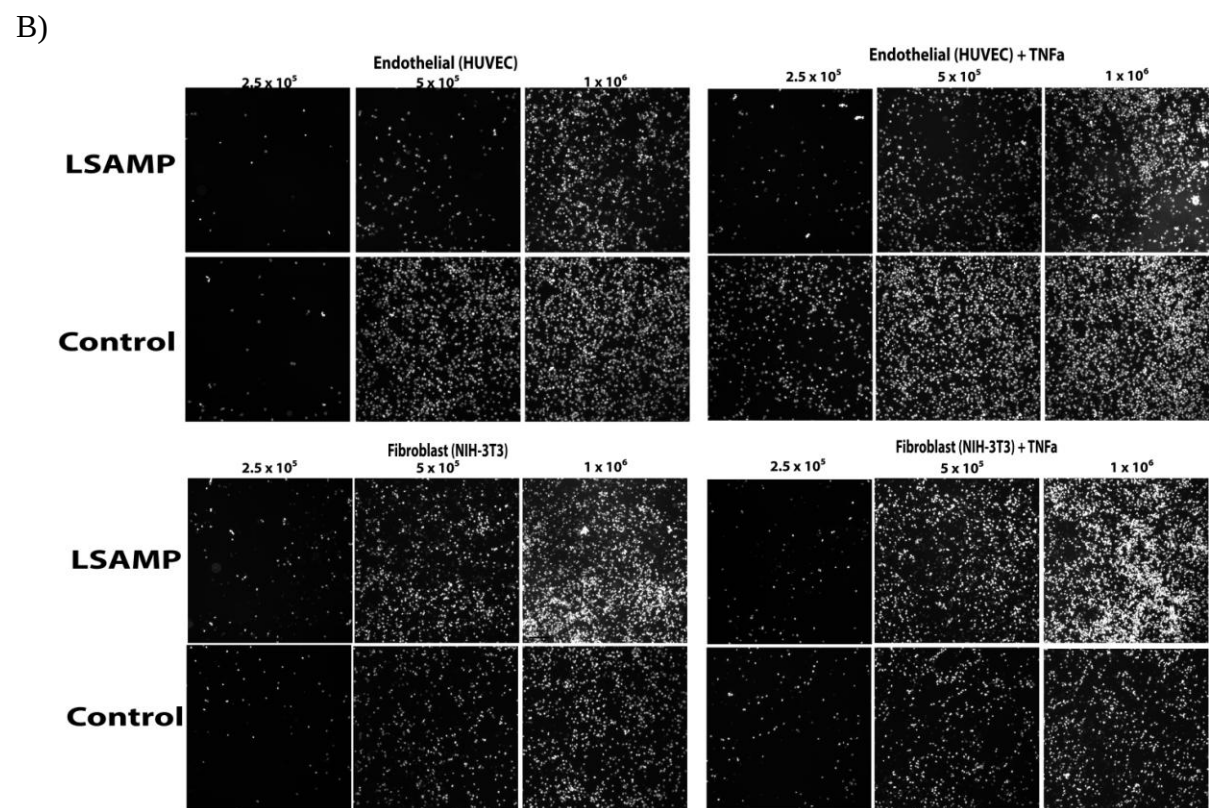
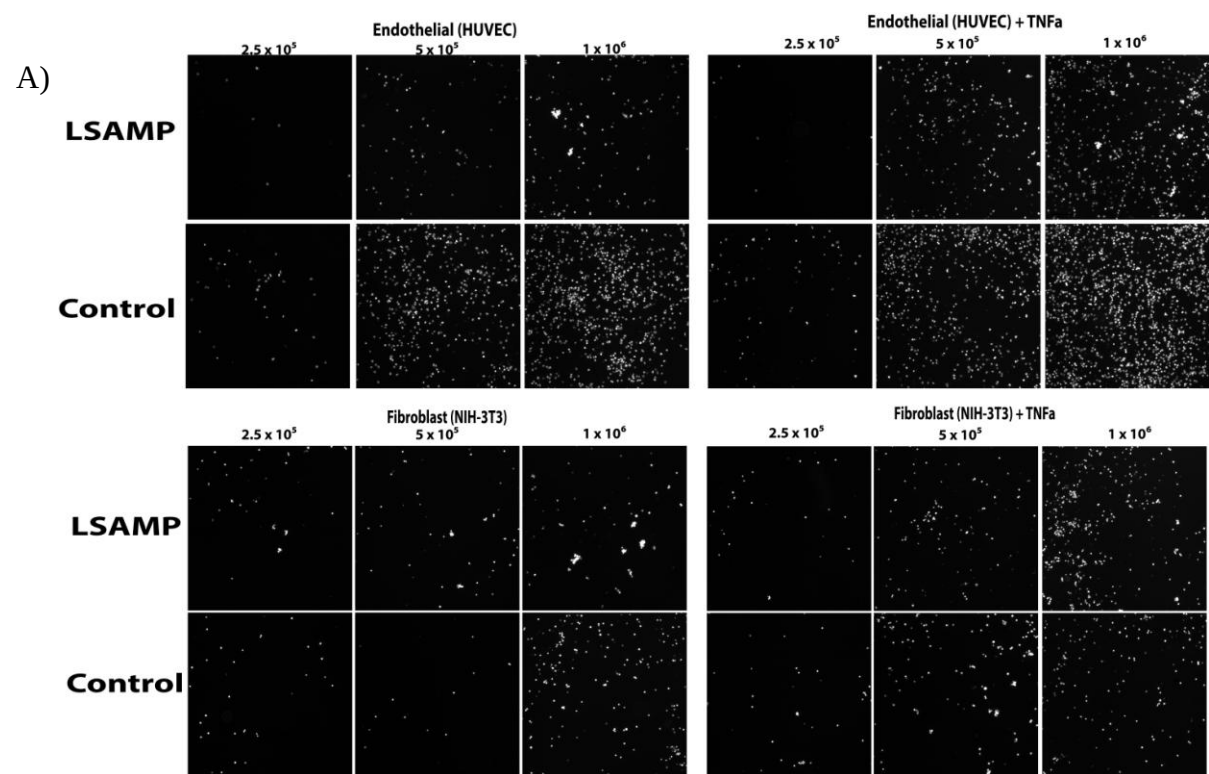


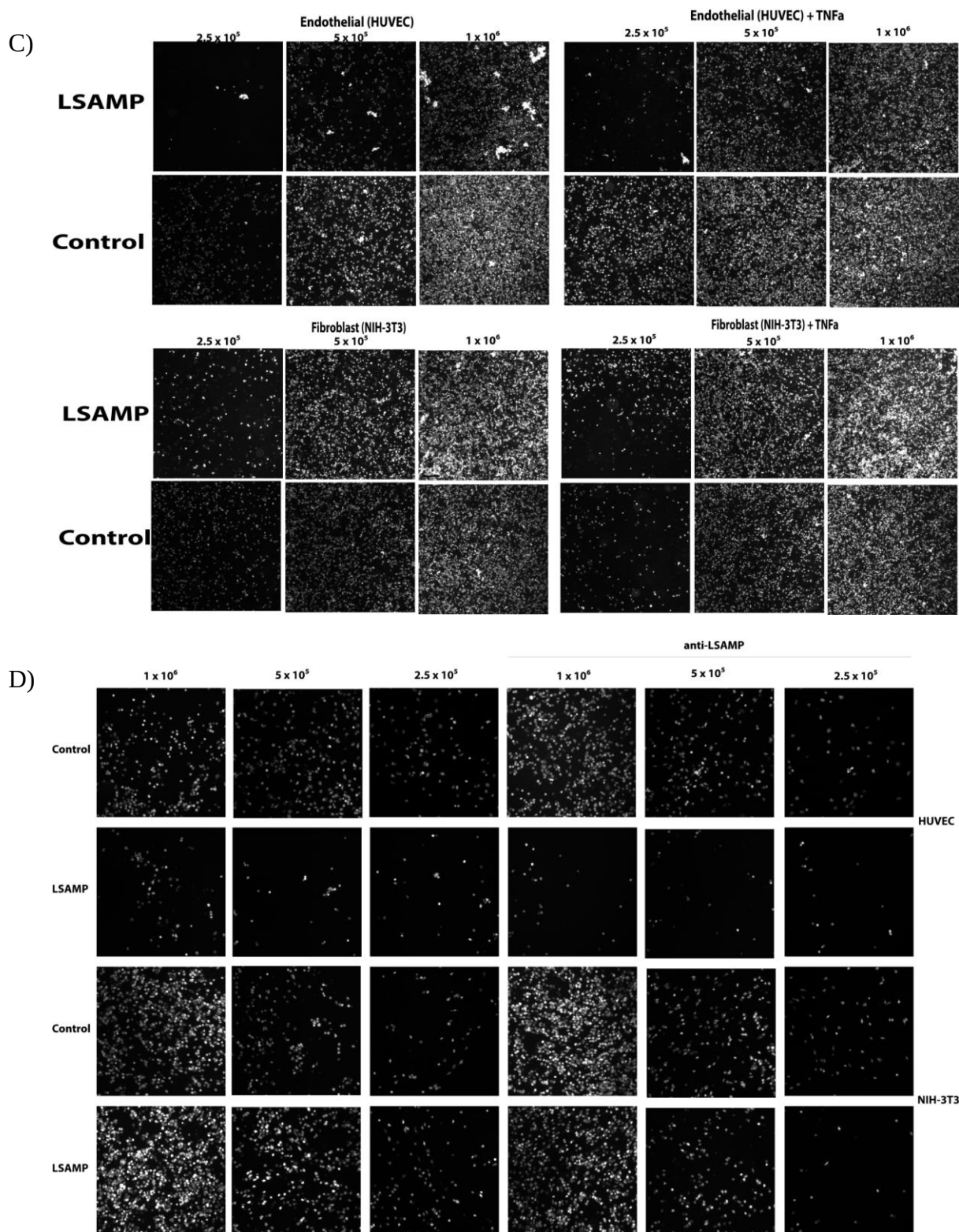
C)



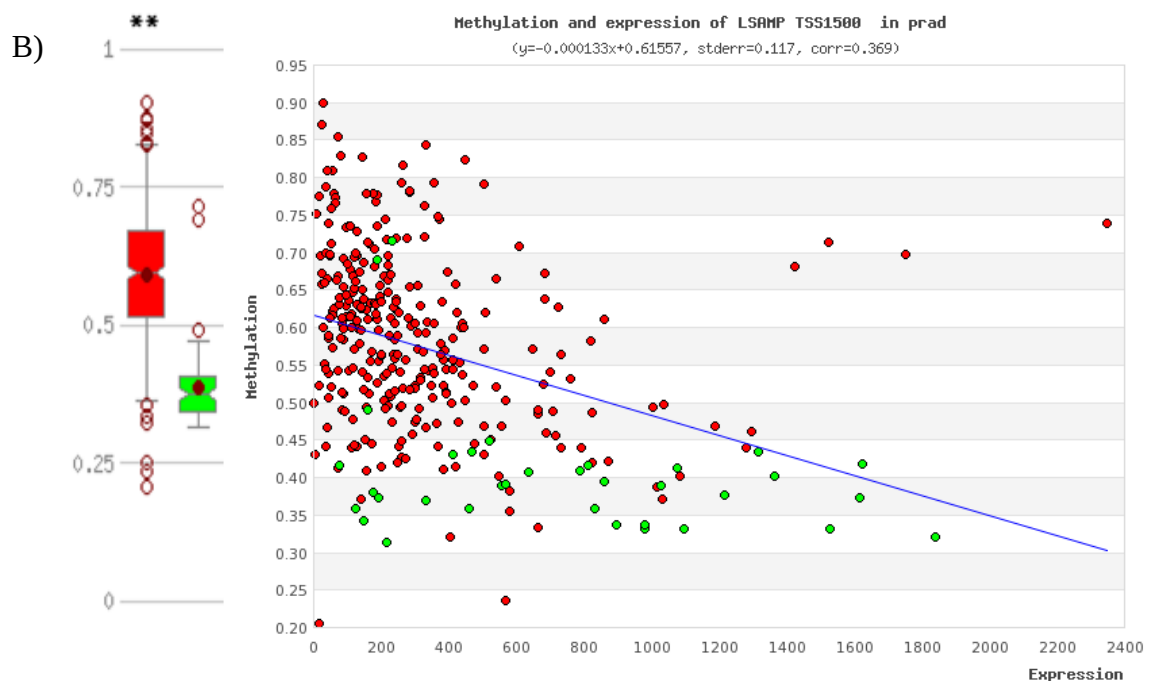
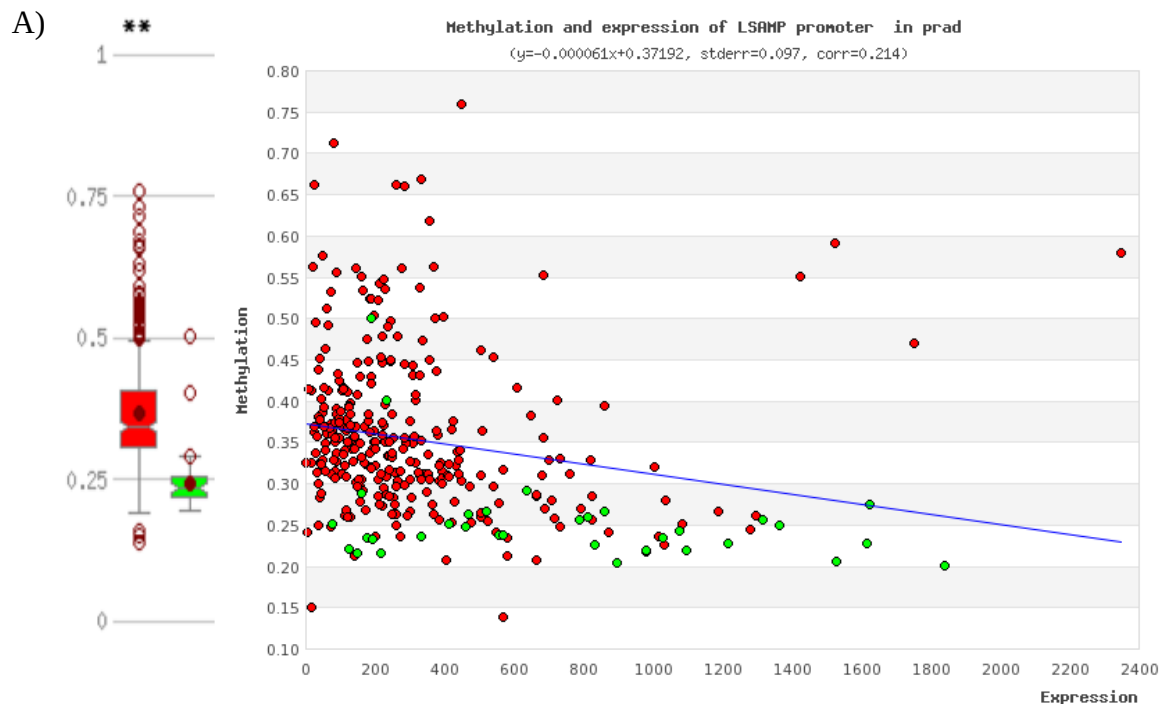


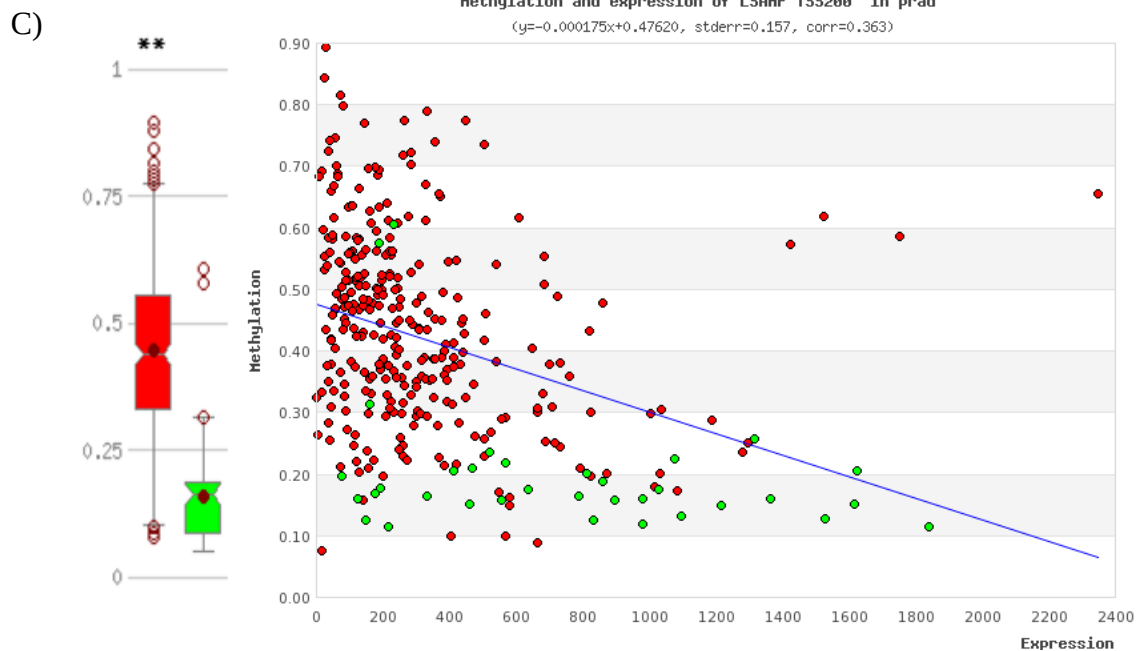
SUPPLEMENTARY FIGURE S4. APOPTOSIS, ANCHORAGE INDEPENDENT GROWTH, SENESCENCE, INVASION OR MIGRATION DIFFERENCES UPON LSAMP EXPRESSION NOT OBSERVED. Representative Images: A) LNCaP Enzalutamide induced Apoptosis showed no notable difference with LSAMP expression. B) Anchorage independent growth in MDA PCa 2b assessed by growth in soft agar showed no significant difference in colony number. B) Senescence not notably affected by LSAMP expression in LNCaP(control and LSAMP; left) and DU 145 (right) as detected by Senescence Associated β -Galactoside Staining. C) In vitro migration (left) or invasion (right) not notably affected upon LSAMP expression as assessed in boyden chamber assays.



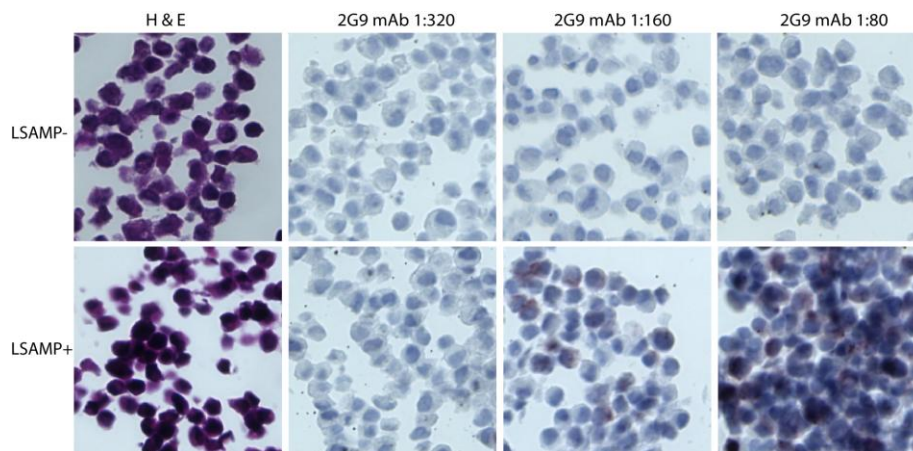


SUPPLEMENTARY FIGURE S5. DU 145 ADHESION TO HUVEC OR NIH/3T3 with TNF- α monolayer pretreatment, for A)15, B) 30, or C) 60 minute; or D) anti-LSAMP mAb pretreatment of DU 145.

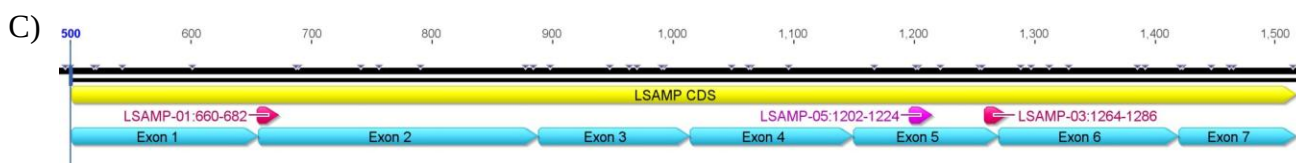
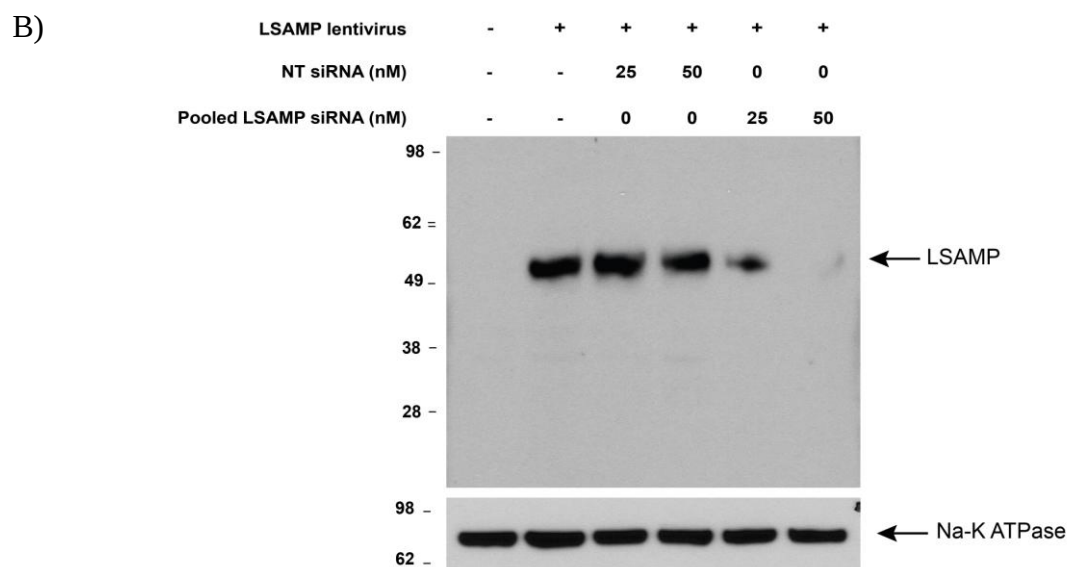
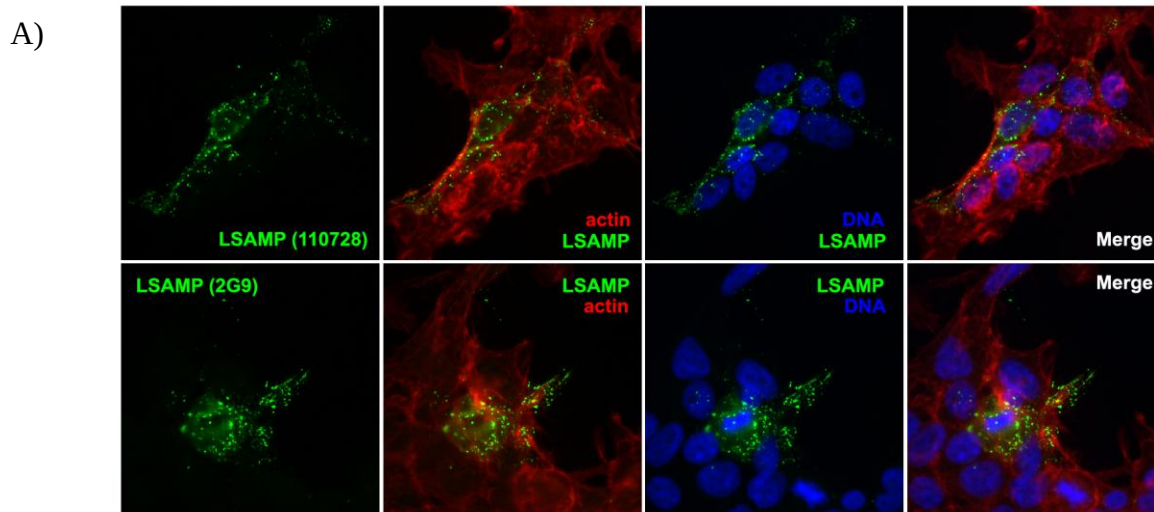




SUPPLEMENTARY FIGURE S6. LSAMP PROMOTER IS SIGNIFICANTLY METHYLATED IN PROSTATE CANCER. Comparing average methylation of prostate tumor samples (n=340) and matched normal samples (n=66) at the A) promoter (p-value= 1.7E-14), B) 1500 bp upstream of the transcription start site (TSS) (p-value= 3.2E-15), and C) 200 bp upstream of the TSS (p-value= 9.8E-15).

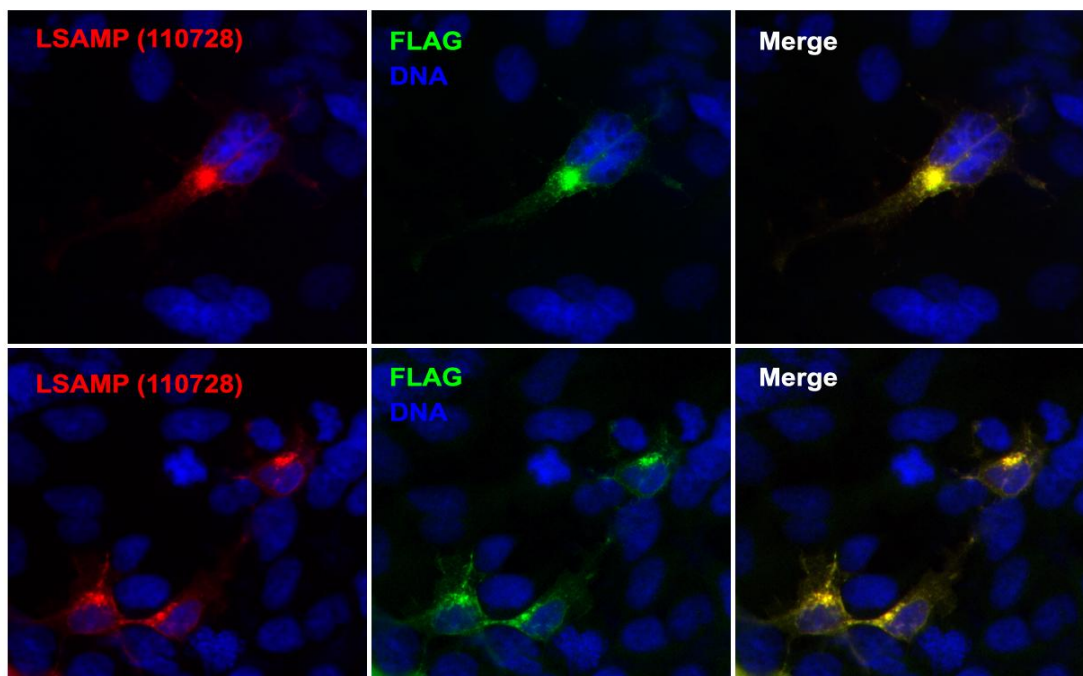


SUPPLEMENTARY FIGURE S7. 2G9 MAB DETECTS LSAMP IN FORMALIN-FIXED PARRAFIN-EMBEDDED DU 145



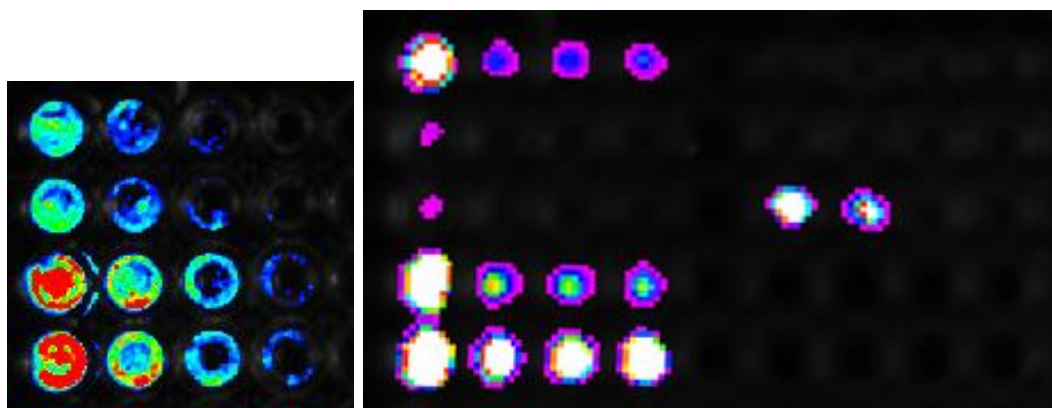
SUPPLEMENTARY FIGURE S8. HEK293 LENTIVIRAL TRANSDUCTION AND KNOCKDOWN VALIDATES CONSTRUCT AND ANTIBODY SPECIFICITY.

A) Immunofluorescence analysis of LSAMP and actin in HEK293 following transduction. B) Immunoblot analysis of LSAMP expression in HEK293 following lentiviral transient transduction and subsequent siRNA knockdown. C) Map of siRNA oligonucleotide targets on LSAMP mRNA. Transduced HEK293-FT Cells Show Fine, Punctuate LSAMP Staining (A). LSAMP Lentiviral Transduction & Subsequent siRNA Knockdown in HEK-293 Shows Specificity of Constructs and Antibody (B).



SUPPLEMENTARY FIGURE S9. N-TERMINAL LSAMP TAG INTERFERES WITH SIGNAL SEQUENCE DEPENDENT PROTEIN PROCESSING

Immunofluorescence of HEK-293 transfected with LSAMP-FLAG reveals N-terminal FLAG tag interferes with signal sequence dependent protein processing localization to the membrane.



SUPPLEMENTARY FIGURE S10. DOXYCYCLINE INDUCIBLE LSAMP- LUC2 DOUBLE STABLE LNCaP AND MDA PCa 2B CELL LINES

In vivo Extreme Bruker Imaging System was used to screen cells for bioluminescence prior to use *in vivo*. LNCaP (left): Duplicates of 2 of 13 clones with highest luminescence . MDA PCa 2b (right): 5 of 30 clones, compared with LNCaP (far right). Cells were diluted from left to right.

SUPPLEMENTARY TABLE 2. qPCR PRIMERS AND PROBES

P4266	LSAMP_Forward	CACTGGTCCTACTGAGATTGC
P4267	LSAMP_Reverse	CACTGGTCCTACTGAGATTGC
P4268	LSAMP_Probe	CACTGGTCCTACTGAGATTGC
P4564	B2M-Forward	CCGTGTGAACCATGTGACTT
P4565	B2M-Reverse	CAAACCTCCATGATGCTGCT
P4567	B2M-Probe	AGCCCAAGATAGTTAAGTGGGA
P4562	GAPDH-Forward	GAAGGTGAAGGTCGGAGTCA
P4563	GAPDH-Reverse	AGAGTTAAAAGCAGCCCTGG
P4566	GAPDH-Probe	GGATTTGGTCGTATTGGGCG

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