LYRIC/AEG-1 Is Targeted to Different Subcellular Compartments by Ubiquitylation and Intrinsic Nuclear Localization Signals

Hayley J. Thirkettle,1 Joanne Girling,1 Anne Y. Warren,2 Ian G. Mills,1 Kanagasabai Sahadevan,3 Hing Leung,4 Freddie Hamdy,5 Hayley C. Whitaker,1 and David E. Neal1

Abstract Purpose: LYRIC/AEG-1 has been reported to influence breast cancer survival and metastases, and its altered expression has been found in a number of cancers. The cellular function of LYRIC/AEG-1 has previously been related to its subcellular distribution in cell lines. LYRIC/AEG-1 contains three uncharacterized nuclear localization signals (NLS), which may regulate its distribution and, ultimately, function in cells. Experimental Design: Immunohistochemistry of a human prostate tissue microarray composed of 179 prostate cancer and 24 benign samples was used to assess LYRIC/AEG-1 distribution. Green fluorescent protein-NLS fusion proteins and deletion constructs were used to show the ability of LYRIC/AEG-1 NLS to target green fluorescent protein from the cytoplasm to the nucleus. Immunoprecipitation and Western blotting were used to show posttranslational modification of LYRIC/AEG-1 NLS regions. Results: Using a prostate tissue microarray, significant changes in the distribution of LYRIC/AEG-1 were observed in prostate cancer as an increased cytoplasmic distribution in tumors compared with benign tissue. These differences were most marked in high grade and aggressive prostate cancers and were associated with decreased survival. The COOH-terminal extended NLS-3 (amino acids 546-582) is the predominant regulator of nuclear localization, whereas extended NLS-1 (amino acids 78-130) regulates its nucleolar localization. Within the extended NLS-2 region (amino acids 415-486), LYRIC/AEG-1 can be modified by ubiquitin almost exclusively within the cytoplasm. Conclusions: Changes in LYRIC/AEG-1 subcellular distribution can predict Gleason grade and survival. Two lysine-rich regions (NLS-1 and NLS-3) can target LYRIC/AEG-1 to subcellular compartments whereas NLS-2 is modified by ubiquitin in the cytoplasm.

Lysine-rich CEACAM-1–associated protein (LYRIC; ref. 1), also known as metastasis adhesion protein (metadherin; ref. 2), and astrocyte elevated gene-1 (AEG-1; ref. 3) was originally identified as part of a serial analysis of gene expression, investigating proteins up-regulated in the androgen-treated LNCaP prostate cancer cell line (4). LYRIC/AEG-1 was subsequently found to be overexpressed in breast, brain, and prostate cancer (5–8) and localized to the cell membrane, endoplasmic reticulum, nucleolus, cytoplasm, and nucleus in various cell lines (1, 5, 6).

Various functions of LYRIC/AEG-1 have been suggested in a number of subcellular compartments; however, its exact function remains poorly understood. At the membrane of polarized cells, LYRIC/AEG-1 has been linked to cell adhesion through colocalization with ZO-1 at tight junctions (5). This may be linked to the loss of cell polarity that is known to occur with increased epithelial tumorigenicity (9). LYRIC/AEG-1 has been shown to suppress the activation of FOXO3a, a transcription factor regulated by AKT phosphorylation (7). The oncogenic potential of LYRIC/AEG-1 has been shown by its ability to transform melanocytes (3) and more recently shown by its ability to activate nuclear factor-κB through an interaction with CREB-binding protein/p300 (6, 10). Furthermore, LYRIC/AEG-1 can be induced by oncogenic Ha-ras through phosphatidylinositol 3-kinase/AKT (10, 11). Most recently, LYRIC/AEG-1 has recently been shown to interact with the cell cycle regulator BRCA2 and CDKN1A-interacting protein (BCCIPα) and down-regulate its expression by targeting it for degradation (12). Thus, LYRIC/AEG-1 has many functions in different
Translational Relevance

Previous studies noted the importance of LYRIC/AEG-1 in tumorigenesis, linking it to survival in breast tumors, and suggesting that it may function as a tumor suppressor. LYRIC/AEG-1 localization is thought to play a significant role in regulating its function, resulting in speculation about the importance and function of the lysine-rich regions as putative nuclear localization motifs. We have shown that LYRIC/AEG-1 is lost from the nucleus with increasing tumorigenesis and decrease survival in prostate tumors. This is consistent with a potential loss of tumor suppressor activity. A change in distribution could be used as a biomarker of tumor progression. By understanding how LYRIC/AEG-1 localization is regulated by its nuclear localization motifs, it may ultimately be possible to redistribute LYRIC/AEG-1 to the nucleus in tumors as a therapeutic tool to suppress tumorigenesis.

Materials and Methods

LYRIC/AEG-1 antibodies and plasmids. LYRIC/AEG-1 was detected using two sheep anti-LYRIC/AEG-1 antibodies kindly supplied with blocking peptides by Heidi Sutherland (MRC Human Genetics Unit, Edinburgh, United Kingdom) (1). The antibody that recognizes residues 197SHREKRQQRKRDV210 is called AK, whereas the COOH-terminal antibody recognizing residues 560SPRQQKKKKARRET662 is referred to as SS. Green fluorescent protein (GFP)-tagged NLS constructs were created using pQBI25-fC3 and pQBI25-fN2 plasmids (Q-BIOgene) and by designing primers (Sigma Genosys) to include lysine-rich regions and/or flanking secondary structure, as predicted by PredictProtein. Site-directed mutagenesis was done using QuickChange II Site-Directed Mutagenesis kit (Stratagene) following the manufacturer’s instructions. All primer sequences and plasmids are detailed in Supplementary Table S1. HA-tagged ubiquitin was a gift from Jochen Rink (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany) (16). The ASUMO-1 construct was a kind gift from Helen Hurst (Barts and the London School of Medicine and Dentistry, London, United Kingdom) (17).

Patient population. The patient population was identical to the one previously described (18). The tumor grade was classified by a uropathologist (A.W.) using the Gleason grading system and classified into low (Gleason 6 and below), moderate (Gleason 7), and high (Gleason 8-10). Following immunostaining, tissue core loss reduced the cohort to 179 cancer patients, 83 samples of coexisting benign prostate hyperplasia (BPH) and 24 control BPH patients. To confirm that staining in BPH samples from radical prostatectomy and TURP samples was identical, whole sections from two radical prostatectomy samples obtained from Addenbrookes Hospital, Cambridge, were probed for LYRIC/AEG-1. Staining was also done on 11 patient samples from the Royal Hallamshire Hospital, Sheffield, consisting of bone metastasis that had received no hormone manipulation therapy and were undergoing bilateral orchietomy at the time of biopsy. Informed consent and ethical approval was obtained for all procedures and studies.

Immunohistochemistry. Confirmation of tissue status (Gleason grades and BPH) was conducted by a uropathologist, who assessed and marked the blocks appropriately. Duplicate 0.6-mm tissue cores were cut and constructed according to predetermined tissue microarray (TMA) layout. Multiple 5-μm sections were cut from TMA for immunohistochemistry analysis. Full thickness bone biopsies (5-10 mm cores) taken from the iliac crest were fixed in formal saline (10%) for 48 h, decalcified for up to 3 wk in a solution containing EDTA before 4-μm sections cut, and mounted on slides as before (19). A normal bone TMA (US Biomax) was used as a control. A multinormal/tumor TMA (Zymed) was used to assess LYRIC/AEG-1 staining in a wide variety of tissues.

All staining was done under identical conditions to allow for comparison over different TMA slides. Tissue was deparaffinized, rehydrated, and antigen retrieval was done using the following breaths: 0.01 mol/L sodium citrate, 0.05% Tween for 15 min at 90°C. To reduce the possibility of epitope masking, LYRIC/AEG-1 was detected using a 1:1 ratio of AK and SS antibodies (1:50) overnight at 4°C. Negative controls were done using sheep IgG (Upstate Cell Signalling Solutions). Secondary biotinylated antibodies (Autogen Bioclear) were used at 1:100, followed by ABC complex (Vector Laboratories, Inc.). Staining was visualized with diaminobenzidine tetrahydrochloride (Vector Laboratories) and the nucleus was counterstained lightly with hematoxylin (Vector Laboratories). Tissues were mounted and visualized as described (18).

Immunostaining assessment. In those patients where duplicate cores remained intact, immunostaining was evaluated according to staining intensity and localization, independently of each other. Scoring was done independently by two observers (one an independent specialist uro-oncology pathologist) both blinded to the TMA plan. Staining was classified into absent, low, moderate, and high intensity. Localization was categorized according to the predominant staining region, i.e., nuclear, cytoplasmic, or membranous. If equal in two or more areas, this was classed as mixed cytoplasmic/nuclear. If staining was equal in all areas of the cell, it was classed as global. A consensus agreement was reached on intensity and localization on each core. Statistical analysis on immunohistochemistry data was done on the consensus score using
a using a two-tailed Fisher’s exact test. Results were considered significant if the P value was < 0.05.

For a subset of 50 patients, survival data was collected for up to 120 mo. Patients were grouped according to their LYRIC/AEG-1 staining pattern; those with any nuclear staining, including mixed cytoplasmic/nuclear, were placed in one group. Those patients with no nuclear staining were placed in a separate group. Data were analyzed using GraphPad Prism and statistical difference was determined using a two-tailed t test.

Cell culture. All cell lines were purchased from the Cancer Research UK cell bank except the benign prostatic PNT1a cells, which were a kind gift from Norman Maitland (Yorkshire Cancer Research, Harrogate, North Yorkshire, United Kingdom) and VCaP cells that were a kind gift from Marion Bussemakers (Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands). PNT1a, LNCaP, VCaP, PC3, and NIH3T3 cells were routinely cultured in RPMI medium (Life Technologies). COS-7 and MDBK cells were routinely cultured in DMEM (Life Technologies). All media were supplemented with 10% fetal bovine serum (Labtech). Androgen regulation experiments were done as previously described (20) and media were supplemented with either the dihydrotestosterone analogue R1881 (10 nmol/L; Sigma) or an equal volume of vehicle (ethanol) for 24 h before being harvested. For transfection, cells were grown to 40% confluency. DNA (1 μg per 6 well of a 24-well plate or 4 μg per 14-cm dish) was transfected using FuGENE

Fig. 1. A, a schematic representation of LYRIC/AEG-1 highlighting the leucine-rich region (black) and lysine-rich regions (light grey). Dotted lines, antibody epitopes for AK and SS. B, the specificity of the LYRIC/AEG-1 antibodies (AK and SS) was tested by separating 20 μg LNCaP (+/− R1881), COS, MDBK, and NIH3T3 whole cell lysate by SDS-PAGE, transferring to nitrocellulose, and probing with either AK or SS (1:2,000). Duplicate blots were incubated with antibody in the presence of 5 μg specific blocking peptide but otherwise treated in an identical manner. Proteins were visualized with ECL-Plus. C, a multitumor/normal TMA and bone sections were stained for LYRIC/AEG-1 (1:150; brown) and the nuclei were counterstained with hematoxylin (blue): benign lung (i), benign thyroid (ii), benign prostate (iii), prostate tumor (iv), normal bone (v), prostate bone metastases (vi), and thyroid tumor (vii, >60) and a zoomed image with arrows indicating the nucleolar staining (viii). All other images were captured using ×40 magnification except bone images that were collected at ×20 magnification.

www.aacrjournals.org ClinCancerRes2009;15(9)May1,20093005

Tumor and normal bone sections were stained for LYRIC/AEG-1 (1:150; brown) and the nuclei were counterstained with hematoxylin (blue).
6 (Roche) following the manufacturer’s protocol. Cells were grown for a further 48 h before confocal microscopy or Western analysis.

**Cellular fractionation.** All procedures were done on ice or at 4°C. Cells were harvested at 95% confluency by scraping into 3 mL PBS and centrifuging at 800 × g. Cells were resuspended in 500 µL modified RIPA buffer (as described in ref. 18) by “flicking” and incubated for 5 min before centrifuging at 800 × g for 5 min. The supernatant was retained as the cytoplasmic fraction. Nuclei were washed thrice in 500 µL RIPA buffer and resuspended in 100 µL RIPA buffer, and sonicated thrice for 10 s. The sonicated nuclei were centrifuged at 16,100 × g for 1 min and the supernatant was retained as the nuclear fraction. Protein concentrations were determined using the Bradford reagent (Bio-Rad).

**Cell lysates, Western blotting, and immunoprecipitation.** All procedures were done as described in ref. (20). Membranes were incubated with primary antibody—anti-RNA polymerase II (1:2,000), anti-GFP (1:5,000), anti-fibrillarin (1:1,000), anti-histone H3 (1:5,000), and anti-actin (1:5,000; all from Abcam); anti-γ-adaptin (1:2,000, BD Transduction Laboratories), anti-p65 (1:1,000, Abcam), or anti-LYRIC/AEG-1 SS or AK at 1:2,000. Secondary antibodies (1:1,000, DakoCytomation) were used for all antibodies. Protein bands were detected with ECL-Plus (GE Healthcare), and where they exceeded the dynamic range of film, diaminobenzidine (Vector Laboratories) was used. For peptide blocking experiments, duplicate Western blots were blocked in 5% bovine serum albumin–PBS either alone or with 5 µg of corresponding blocking peptide and incubated with membranes at 4°C overnight. Western blots were then completed as described. Densitometry was done using Image Scanner III and ImageQuant TL software from Amersham Biosciences. SUMOylated and ubiquitinylated LYRIC bands were normalized to GFP-LYRIC band intensity.

For immunoprecipitation, all of the procedure was done at 4°C. For each sample, 100 µL of protein G Sepharose beads (GE Healthcare) was washed in modified RIPA buffer. After preclearing, 1 mg of total protein was incubated with antibody, LYRIC/AEG-1 SS (5 µL) or GFP (1 µL). An equal concentration of sheep (Upstate Cell Signalling Solutions), mouse, or rabbit (Vector laboratories) immunoglobulin was used as controls. Following the addition of protein G, Sepharose beads were washed in modified RIPA buffer containing 2% NP40 and boiled for 10 min in 30 µL SDS-PAGE sample buffer before Western blot analysis.

---

Fig. 2. PNT1a (benign), PC3 (metastatic), and LNCaP and VCaP (tumorigenic) cell lines were treated with the synthetic dihydrotestosterone R1881 for 24 h and either whole-cell lysates were made (A) or cells were fractionated (B). Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Efficient fractionation and equal loading was confirmed by probing for γ-adaptin (cytoplasm) or histone H3 (nucleus). For whole-cell lysate, actin was used as a loading control. Blots were probed for LYRIC/AEG-1 using either AK or SS antibodies. All proteins were visualized with ECL-Plus except nuclear histone H3, which was visualized with diaminobenzidine. WB, Western blot.
Prostate TMAs were stained for LYRIC/AEG-1 and the intensity of LYRIC/AEG-1 staining was assessed as absent, low, moderate, or high. We compared the expression in BPH with all malignant prostate tissue (A, left) and then the tissue was subdivided into Gleason 4-6, Gleason 7, and Gleason 8-10 grade tumors (A, right). The localization of staining was assessed as nuclear, cytoplasmic, both nuclear and cytoplasmic, or global when it was also found at the membrane as well throughout the cell. LYRIC/AEG-1 localization was compared in BPH and all tumors (B, left) or after the tumors were subdivided into Gleason 4-6, Gleason 7, and Gleason 8-10 grade tumors (B, right). A subset of patients, for whom clinical outcome data were available, was analyzed for any correlation between nuclear LYRIC/AEG-1 staining and survival (C). Nuclear LYRIC/AEG-1 was classed as any nuclear staining and included mixed staining patterns. LYRIC/AEG-1 expression and distribution was also determined using identical criteria in serial hormone-naive, hormone-sensitive, and hormone-refractory tumors stained for LYRIC/AEG-1 (D). For clinical outcome (C), P values were calculated using a two-tailed t test. For all other data, P values were calculated using a two-tailed Fisher’s exact test. P values were considered significant when $P \leq 0.05$. 

Fig. 3. Prostate TMAs were stained for LYRIC/AEG-1 and the intensity of LYRIC/AEG-1 staining was assessed as absent, low, moderate, or high. We compared the expression in BPH with all malignant prostate tissue (A, left) and then the tissue was subdivided into Gleason 4-6, Gleason 7, and Gleason 8-10 grade tumors (A, right). The localization of staining was assessed as nuclear, cytoplasmic, both nuclear and cytoplasmic, or global when it was also found at the membrane as well throughout the cell. LYRIC/AEG-1 localization was compared in BPH and all tumors (B, left) or after the tumors were subdivided into Gleason 4-6, Gleason 7, and Gleason 8-10 grade tumors (B, right). A subset of patients, for whom clinical outcome data were available, was analyzed for any correlation between nuclear LYRIC/AEG-1 staining and survival (C). Nuclear LYRIC/AEG-1 was classed as any nuclear staining and included mixed staining patterns. LYRIC/AEG-1 expression and distribution was also determined using identical criteria in serial hormone-naive, hormone-sensitive, and hormone-refractory tumors stained for LYRIC/AEG-1 (D). For clinical outcome (C), P values were calculated using a two-tailed t test. For all other data, P values were calculated using a two-tailed Fisher’s exact test. P values were considered significant when $P \leq 0.05$. 

www.aacrjournals.org ClinCancerRes2009;15(9)May1,20093007
Research. 

on April 5, 2017. © 2009 American Association for Cancer Research.
**Results**

**LYRIC/AEG-1 localization alters with tumorigenesis and predicts longer survival.** Anti-LYRIC/AEG-1 AK or SS antibodies, which recognize amino acids 197-210 (AK) and 568-582 (SS; Fig. 1A), have been characterized previously in different subnuclear compartments (1). As multiple bands were seen by Western analysis, we examined their specificity using blocking peptides corresponding to the antigenic amino acids. We showed the loss of the majority of bands for both antibodies, suggesting that staining is specific for LYRIC/AEG-1 (Fig. 1B). We then used these antibodies to assess LYRIC/AEG-1 expression in human tissues by staining a TMA consisting of normal and cancerous tissues from a variety of organs.

All of the various tissues that we examined showed some LYRIC/AEG-1 staining (Fig. 1C). Predominantly nuclear staining was only seen in benign tissues, including the prostate, thyroid, and lung. Tumorigenic tissue had comparatively low levels of nuclear staining; however, nucleolar staining was noted (Fig. 1C, vii and viii), which was not seen in the benign tissues we examined. As prostate cancer is known to frequently metastasize to bone, we obtained 11 bone metastases and probed them for LYRIC/AEG-1 expression. When compared with normal bone controls, 81.8% (9 of 11) of prostate bone metastases showed an increased expression of LYRIC/AEG-1 (Fig. 1C, v). LYRIC/AEG-1 was almost exclusively distributed in the cytoplasm and membrane in the prostate bone metastases.

To identify if LYRIC/AEG-1 distribution was altered by tumorigenesis in the prostate, we examined its expression and localization in a range of cell lines: benign (PNT1a), tumorigenic LNCaP and VCaP, and highly metastatic (PC3) cells. All cells were treated with the dihydrotestosterone analogue R1881 for 24 hours to determine if androgens have any effect on LYRIC/AEG-1 distribution or expression. The cells were either lysed intact (Fig. 2A) or separated into nuclear and cytoplasmic fractions, which were confirmed by Western blotting for γ-adaptin (cytoplasm) and histone H3 (nucleus; Fig. 2B). In whole-cell lysates, we saw a singlet or doublet at ~75 kDa, consistent with previous reports using these and other anti-LYRIC/AEG-1 antibodies. The lower molecular weight band of the doublet was weaker with the SS antibody (amino acids 568-582) or lost with the AK antibody (amino acids 197-210) in the benign PNT1a cell line, suggesting that there are differences in LYRIC/AEG-1 distribution in benign and tumorigenic cells. Within the nucleus and cytoplasmic cell fractions, multiple LYRIC/AEG-1 bands were seen in all cell lines (Fig. 2B). Some nuclear translocation of LYRIC/AEG-1 was seen in response to R1881 treatment, particularly in VCaP cells. Loss of a 60-kDa nuclear LYRIC/AEG-1 band was particularly evident in the nuclear fractions of LNCaP and VCaP cells probed with AK, compared with PC3 and PNT1a cells. Some bands, such as those at 28 kDa, were only seen with the AK antibody, whereas a cytoplasmic band at 50 kDa was only seen with the SS antibody. Changes were also seen on lower molecular weight bands, such as the appearance of a nuclear 35 kDa band in LNCaP and VCaP cells, which suggests that differences in nuclear and cytoplasmic LYRIC/AEG-1 occur between prostate cells at different stages of tumorigenesis.

Our initial experiments (Fig. 2) and previously published data (4, 5) suggest that LYRIC/AEG-1 may be up-regulated in prostate cancer; thus, we used immunohistochemistry to stain a prostate TMA consisting of BPH (n = 63) and prostate cancer (n = 143) for LYRIC/AEG-1. The intensity of LYRIC/AEG-1 staining increased significantly in malignancy (P = 0.037; Fig. 3A, left). We then determined if these changes in LYRIC/AEG-1 expression were associated with Gleason score by dividing the cancers into low, moderate, and high grade (Fig. 3A, right). Although LYRIC/AEG-1 expression increases in low Gleason cancers before decreasing with higher Gleason grade, these changes were not statistically significant (P = 0.29).

As we have previously noted changes in LYRIC/AEG-1 distribution between benign and tumorigenic tissues (Fig. 1C) and prostate cell lines (Fig. 2B), we examined the localization of LYRIC/AEG-1 in the same prostate cancer TMA. LYRIC/AEG-1 was localized to the nucleus of luminal cells in 82.5% of benign cases (52 of 63; Fig. 3B, left). Some staining of basal cells was seen in benign tissue, which was not seen as basal cells were lost with tumorigenesis. In contrast, nuclear LYRIC/AEG-1 was only seen in 26.6% (38 of 143) of tumors. As a result, there was redistribution of LYRIC/AEG-1 to the cytoplasm alone (33.6%), the cytoplasm and nucleus (42.9%), or throughout the cell (8.8%). The decrease in nuclear LYRIC/AEG-1 was associated with increased Gleason grade (Fig. 3B, right; P < 0.001) and mirrored by reciprocal increased changes in cytoplasmic staining. Clinical follow-up was available for 50 of these patients and they were examined for any correlation between survival and the presence or absence of any LYRIC/AEG-1 in the nuclei of cells (Fig. 3C). Patients with nuclear LYRIC/AEG-1 had a mean survival of 70 months compared with 39 months for patients without any LYRIC/AEG-1 in the nucleus (P = 0.0023). This suggests that nuclear LYRIC/AEG-1 may have a function in the nucleus of normal tissue that is lost in tumorigenesis.

We also examined a small cohort of patients during hormone treatment to determine any effects hormone therapy may have on LYRIC/AEG-1 expression and localization. Patients not receiving hormone treatment (hormone naïve) showed greater high-intensity LYRIC/AEG-1 staining than patients receiving hormone treatment regardless of whether the patient was responding to hormone treatment (hormone sensitive) or had become hormone resistant (hormone insensitive; Fig. 3D, left; P = 0.009). There was no significant change in LYRIC/AEG-1 localization (P = 0.441; Fig. 3D, right) with hormone ablation therapy although examination of patients with serial biopsies taken throughout treatment revealed a more nuclear LYRIC/AEG-1 distribution in patients that remained hormone sensitive (Supplementary Table S2).

**Characterization of LYRIC/AEG-1 nuclear localization signals.** As LYRIC/AEG-1 is capable of localizing to both the cytoplasm and nucleus in cells, we have examined the regulation of LYRIC/AEG-1 localization by its NLS motifs. Putative LYRIC/AEG-1 NLS motifs as suggested by Sutherland et al. (1) were designated NLS-1, NLS-2, and NLS-3 and tagged with GFP (Fig. 4A). Using PSORTII, we predicted that all of the NLS peptides contained either an NLS or NoLS shown as bold (NLS) or underlined (NoLS; Fig. 4A). The localization of the GFP-NLS fusion constructs was determined by confocal microscopy using 4′,6-diamidino-2-phenylindole (DAPI) to colocalize with the...
Fig. 4. A schematic representation of LYRIC/AEG-1 highlighting the NLS and exNLS peptides (A). Gray boxes, the predicted α-helical secondary structure. Predicted NLS and exNLS regions are indicated with amino acid numbering; NLS are indicated in bold and NoLS are underlined. COS-7 cells were transfected with GFP-tagged NLS or exNLS peptide fusion constructs (B; green) in 24-well plates. Forty-eight hours posttransfection, cells were fixed and stained for the nucleolar marker fibrillarin (red) and DNA was stained with DAPI (blue). Schematic representations of each construct consistent with A are given above each picture. Scale bars, 10 μm.
**Fig. 5.**

**A.** GFP-tagged ΔexNLS LYRIC/AEG-1 constructs were immunoprecipitated using an anti-GFP antibody from 1 mg whole-cell lysates made from COS-7 cells transfected with the ΔexNLS constructs. A proportion of protein input as well as the precipitated proteins were separated by SDS-PAGE and analyzed by Western blotting for the known LYRIC/AEG-1 interacting protein, p65. Western blots were reprobed for GFP as a control. **B.** COS-7 cells were transfected with GFP-tagged ΔexNLS constructs (shown in green) in a 24-well plate. Forty-eight hours posttransfection, cells were fixed and stained for the nuclear marker fibrillarin (red) and DNA was stained with DAPI (blue). DAPI image shows GFP, fibrillarin, and DAPI merged image. Schematic representations of each construct consistent with Fig. 4A are given above each picture. Scale bars, 10 μm.

<table>
<thead>
<tr>
<th>1% Input</th>
<th>IP: GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>LYRIC</td>
</tr>
<tr>
<td>WB: p65</td>
<td>WB: GFP-LYRIC</td>
</tr>
</tbody>
</table>

---

**Fig. 5.** 4. GFP-tagged ΔexNLS LYRIC/AEG-1 constructs were immunoprecipitated using an anti-GFP antibody from 1 mg whole-cell lysates made from COS-7 cells transfected with the ΔexNLS constructs. A proportion of protein input as well as the precipitated proteins were separated by SDS-PAGE and analyzed by Western blotting for the known LYRIC/AEG-1 interacting protein, p65. Western blots were reprobed for GFP as a control. 8. COS-7 cells were transfected with GFP-tagged ΔexNLS constructs (shown in green) in a 24-well plate. Forty-eight hours posttransfection, cells were fixed and stained for the nuclear marker fibrillarin (red) and DNA was stained with DAPI (blue). DAPI image shows GFP, fibrillarin, and DAPI merged image. Schematic representations of each construct consistent with Fig. 4A are given above each picture. Scale bars, 10 μm.
nucleus and fibrillarin as a nucleolar marker. GFP alone localized throughout the cell, suggesting that any targeting of the fusion protein was a result of the associated LYRIC/AEG-1 NLS peptide. All of the NLS constructs showed some degree of nuclear or nucleolar distribution (Fig. 4B; Supplementary Table S3); however, there were no distinctive differences between the different lysine-rich regions and none of the peptides were capable of completely translocating GFP to the nucleus. We then extended the NLS regions to include predicted flanking secondary structures (Fig. 4A). The resulting peptides contained additional NLS and NoLS motifs compared with the shorter NLS peptides. These peptides were designated exNLS-1, exNLS-2, and exNLS-3 (Fig. 4A). The exNLS peptides were tagged with GFP to make a GFP fusion protein and examined by confocal microscopy as before. The extended constructs targeted GFP to nuclear compartments much more effectively (Fig. 4B; Supplementary Table S3). The additional residues in exNLS-3 resulted in an almost exclusively nuclear and nucleolar localization. ExNLS-1, which contained an additional NoLS sequence compared with the shorter NLS fusion, was targeted much more strongly to the nucleolus. ExNLS-2 still retained a largely cytoplasmic localization.

To confirm that the regions identified as exNLS-1 and exNLS-3 are required for the translocation of LYRIC/AEG-1 into the nucleus and nucleolus, key lysine residues (as defined by the sequence alignment shown in Supplementary Fig. S4) from each exNLS region were mutated to alanines in the wild-type GFP-LYRIC/AEG-1 fusion protein using site-directed mutagenesis. The resulting constructs still retained nuclear translocating ability, suggesting that the multiple lysines within the LYRIC/AEG-1 NLS regions are able to compensate for one another (Supplementary Fig. S4). To overcome this redundancy, all of the exNLS regions were deleted either alone or in combination (indicated by Δ) from the wild-type GFP-LYRIC/AEG-1 fusion protein. To show that these constructs were all folded correctly, they were used to immunoprecipitate p65, a known LYRIC/EAG-1 interacting protein (Fig. 5A; ref. 6). All constructs retained p65 binding ability. All of the ΔexNLS deletion constructs showed a cytoplasmic and perinuclear distribution and defective nuclear translocation (Fig. 5B; Supplementary Table S3). The LYRIC/AEG-1 deletion mutant lacking all NLS regions (ΔexNLS-1/2/3) was completely cytoplasmic, indicating that the signal peptide mediating nuclear import resides within the extended lysine-rich regions. The ΔexNLS-2/3, which contains only exNLS-1, could not translocate LYRIC/AEG-1 into the nucleus although the exNLS-1 peptide was capable of targeting GFP to the nucleolus (Fig. 4B), suggesting that the NLS regions may work cooperatively in regulating LYRIC/AEG-1 nuclear translocation. Nucleolar localization was retained in cells transfected with mutants containing exNLS-3 but lost when exNLS-3 was deleted. This is consistent with exNLS-3 being essential for LYRIC/AEG-1 nucleolar localization.

**LYRIC/AEG-1 is posttranslationally modified within exNLS-2.** Despite its predicted molecular weight of 64 kDa, LYRIC/AEG-1 is detected by Western blotting as a 75-kDa band (Fig. 2) by multiple anti–LYRIC/AEG-1 antibodies. As lysine residues are also known to be modified by ubiquitin and/or SUMO, we investigated if the discrepancy in the molecular weight of LYRIC/AEG-1 was due to its posttranslational modification. LYRIC/AEG-1 was immunoprecipitated from COS-7 cells expressing endogenous protein. Other cells were transiently transfected with wtLYRIC/AEG-1 plus either SUMO-1 or ubiquitin. Using endogenous protein, a single 75-kDa band consistent with LYRIC/AEG-1 was detected when Western blots were probed for ubiquitin, but almost undetectable when blots were probed for SUMO-1 (Fig. 6A, left). Ubiquitin modification of LYRIC/AEG-1 was even more evident when LYRIC/AEG-1 and ubiquitin were overexpressed together (Fig. 6A, central panel). Overexpression of SUMO-1 with wtLYRIC/AEG-1 also resulted in detection of SUMOylated LYRIC/AEG-1 (Fig. 6A, right). Once again, SUMOylated LYRIC/AEG-1 was present at a much lower degree than ubiquitinated LYRIC/AEG-1, suggesting it is a much less abundant protein modification, especially under physiological conditions. To identify if LYRIC/AEG-1 modification occurs in a specific subcellular compartment, cells were fractionated into nuclear and cytoplasmic fractions (Fig. 6B, left). Ubiquitylated LYRIC/AEG-1 could only be detected in the cytoplasm, suggesting that it may influence LYRIC/AEG-1 localization (Fig. 6B, middle), possibly by helping retain LYRIC in the cytoplasm. SUMOylated LYRIC/AEG-1 was barely detectable in both subcellular compartments (Fig. 6B, right).

To define which residues were modified by ubiquitin and SUMO, we used the ΔexNLS-LYRIC/AEG-1 constructs that lacked specific exNLS regions (Fig. 5). LYRIC/AEG-1 ubiquitylation and any low-level SUMOylation were both lost when exNLS-2 was deleted (ΔexNLS-2; Fig. 6C, left and middle). Modification was restored to almost wild-type levels when a construct containing only exNLS-2 was used (ΔexNLS-1/3). This confirms that ubiquitylation and possibly SUMOylation of LYRIC/AEG-1 occurs in the exNLS-2 region. To determine if ubiquitylated LYRIC/AEG-1 was targeted for degradation by polyubiquitinylation, cells were treated with the proteasome inhibitor MG132 for 16 hours before lysing. Probing for ubiquitin resulted in a single band at 75 kDa and not a ladder of polyubiquitinated bands often seen with proteins destined for proteosomal degradation (Fig. 6D). We also noted no obvious stabilization of LYRIC/AEG-1 following MG132 treatment. These results suggest that ubiquitination may not target LYRIC/AEG-1 for degradation and that the ubiquitin modification of LYRIC/AEG-1 is more likely to be monoubiquitination.

**Discussion**

LYRIC/AEG-1 has previously been localized to numerous subcellular compartments in a number of studies in cell lines and tissues (1, 2, 5, 7). In addition, studies on small cohorts of breast and prostate cancer patients have suggested it may be overexpressed in tumors compared with benign tissue (2, 7). Using a large cohort of 206 patients, we have confirmed that LYRIC/AEG-1 is overexpressed in tumorigenic prostate tissue (Fig. 3A). However, the most significant change is in LYRIC/AEG-1 distribution, which changes from nuclear in benign tissue to a predominately cytoplasmic distribution in tumors, a trend that differs significantly across prostate increasing tumor grades (Fig. 3B). Localization of LYRIC/AEG-1 to the nucleus has previously been shown in prostate (7) but not in breast (8). By examining patient survival, we have linked nuclear LYRIC/AEG-1 with an increase in mean survival of 31 months consistent with an increase in survival seen in breast tumors when LYRIC/AEG-1 is overexpressed in breast cancer (8). Together, these data strongly suggest a tumor suppressor function for LYRIC/AEG-1 as has been previously suggested.
Nuclear LYRIC/AEG-1 is also responsible for promoting FOXO-3a–induced apoptosis (7), a mechanism that is more likely to be lost in tumorigenesis. Characterizing the regulation of LYRIC/AEG-1 distribution to different subcellular compartments may help us to understand how its function is regulated within the cell in both normal and tumorigenic tissues. LYRIC/AEG-1 has an unusually large proportion of lysine residues, which has been suggested to act as NLS (1, 6). We termed the three clusters of lysine residues NLS-1, NLS-2, and NLS-3 (Fig. 4A) and by tagging them to GFP.

Fig. 6. LYRIC/AEG-1 was immunoprecipitated (IP) from 1 mg whole-cell lysate (WCL) from untransfected COS-7 cells (endogenous) or COS-7 cells transfected with LYRIC/AEG-1 and either ubiquitin or SUMO-1 (A) using SS antibody and analyzed by Western blotting for SUMO, ubiquitin, and LYRIC/AEG-1 (AK). COS-7 cells were transfected with LYRIC/AEG-1 and either ubiquitin or SUMO and 48 h later were fractionated into cytoplasmic or nuclear fractions (B). Efficient fractionation was confirmed by Western blotting for γ-adaptin (cytoplasm) and RNA polymerase II (nucleus). After immunoprecipitating LYRIC/AEG-1 from each fraction, Western blots were probed for ubiquitin, SUMO-1, and LYRIC/AEG-1 (AK). Cells were also transfected with ΔexNLS LYRIC/AEG-1 constructs and ubiquitin or SUMO-1 and lysates were made. LYRIC/AEG-1 was immunoprecipitated from 1 mg whole-cell lysate using anti-GFP antibody and analyzed by Western blotting for ubiquitin, SUMO, and GFP. ECL-plus was used to detect all bands, except where they exceeded the dynamic range of film (RNA pol II and cytoplasmic LYRIC AK blots) where diaminobenzidine was used. SUMOylated/ubiquitinated LYRIC band intensities were compared after normalizing with GFP (C). COS-7 cells transfected with LYRIC/AEG-1 and ubiquitin were also treated with the proteasome inhibitor MG132 or vehicle (DMSO) 16 h before harvest. Immunoprecipitated LYRIC/AEG-1 from whole-cell lysate was analyzed using Western analysis for ubiquitin and LYRIC/AEG-1 (D).
to create fusion proteins, we have shown that they possess only a limited ability to target proteins to the nuclear compartment (Fig. 4B). When the NLS peptides were extended to include flanking regions (exNLS; Fig. 4A), additional NLS and NoLS residues were included and, as a result, their ability to target GFP to the nucleus and nucleolus was dramatically increased. This shows that the short lysine-rich regions alone are not sufficient to confer nuclear trafficking ability and that other lysine and charged residues found in the flanking regions are essential for this function, as reported for other proteins (21). Deleting all three exNLS regions seemingly does not alter LYRIC/AEG-1 folding as ΔexNLS-1/2/3 still retained p65 binding capacity while resulting in a completely cytoplasmic form of LYRIC/AEG-1 (Fig. 5; ref. 6).

To some extent, almost all NLS constructs targeted LYRIC/AEG-1 to the nucleolus (Figs. 4 and 5; Supplementary Table S3), consistent with our observations of nuclear LYRIC/AEG-1 in tumorigenic tissue (Fig. 1C, vii and viii) and existing reports that LYRIC/AEG-1 localizes to the dense fibrillar compartment of the nucleolus in a mouse embryonic cell line (1). When exNLS-3 was deleted, the remaining NLS could not target LYRIC/AEG-1 to the nucleolus (Fig. 5), suggesting that exNLS-3 is absolutely required for LYRIC/AEG-1 redistribution to the nucleolus. When the exNLS-1 region was deleted, all nuclear localization was abolished and LYRIC/AEG-1 was distributed throughout the cytoplasm. Completely nuclear LYRIC/AEG-1 was rarely seen in cultured cells, possibly due to the leucine-rich putative nuclear export signal (amino acids 61–68; Fig. 1A), which was retained in the LYRIC/AEG-1 deletion mutants. The extension of NLS-3 resulted in the inclusion of a bipartite NLS (Fig. 4A), giving rise to a more nuclear rather than nucleolar localization (Fig. 4B). The most potent NLS is exNLS-1, which targeted GFP exclusively to subnuclear compartments. We hypothesize that the different LYRIC/AEG-1 NLS regions may work cooperatively to regulate nuclear and nucleolar localization, with exNLS-1 and exNLS-3 being required for nuclear translocation and exNLS-3 for redistribution within the nucleus to the nucleolar subcompartment. Several publications, using a variety of anti-LYRIC/AEG-1 antibodies, have detected wtLYRIC/AEG-1 by Western blotting at 75 kDa. We have shown that this band represents LYRIC/AEG-1 modified by ubiquitin and, to a lesser extent, SUMO-1 on lysines within the exNLS-2 region (Fig. 6). Although SUMOylation of LYRIC/AEG-1 can be seen, it occurs at extremely low levels, particularly using endogenous proteins, indicating that ubiquitin modification, rather than SUMOylation, is more likely to be significant in physiologic systems. The exNLS-2 region that is modified by ubiquitin is the least effective as an NLS or NoLS. However, exNLS-2 modification may cooperate with exNLS-1 and exNLS-3 to act as a regulator to control nuclear translocation. Treatment of cells with a proteasome inhibitor failed to stabilize LYRIC/AEG-1 or generate multiple ubiquitinylated bands, leading to the suggestion that LYRIC/AEG-1 is modified by monoubiquitin, a known method of protein targeting (13, 14). Cell fractionation showed that ubiquitinylated LYRIC/AEG-1 is almost exclusively cytoplasmic (Fig. 6B). Such strict ubiquitination of LYRIC/AEG-1 in the cytoplasmic compartment suggests that regulating LYRIC/AEG-1 cellular distribution may be essential for cell maintenance or even survival within a tumorigenic microenvironment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Professor N. Maitland (Yorkshire Cancer Research) for the PNT1a cells. J. Lippitt (Sheffield) for the preparation of bone metastases, and J. Marioni (Cambridge) for his advice and assistance with statistical analysis; Cancer Research UK Genomics Core Facility, in particular Nick Mathews (Cambridge Research Institute), for help with DNA sequencing; all the members of the EU-FP6 PRIMA project (PRostate cancer Integral Management Approach) and The European Union for financial support (PRIMA: LSHC-CT-2004-504587); The University of Cambridge, Cancer Research UK, and Hutchison Whampoa Limited; and the National Institute for Health Research, which funds the Cambridge Bio-medical Research Centre, Cambridge, United Kingdom.

References

LYRIC/AEG-1 Is Targeted to Different Subcellular Compartments by Ubiquitinylation and Intrinsic Nuclear Localization Signals


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/15/9/3003

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2009/05/20/1078-0432.CCR-08-2046.DC1

Cited articles
This article cites 21 articles, 5 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/15/9/3003.full.html#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
/content/15/9/3003.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.