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Regulation of the localisation and function of the oncogene LYRIC/AEG-1 by ubiquitination at K486 and K491

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ABSTRACT

The pivotal role of LYRIC/AEG-1 in malignant transformation, tumourigenesis and chemo-resistance has previously been demonstrated in different cell types and sub-cellular compartments. The localisation of LYRIC/AEG-1 appears crucial to its function and is regulated by three lysine-rich nuclear localisation signal regions, one of which was previously demonstrated to be modified by ubiquitin. Here we show that mutation of LYRIC/AEG-1 at K486 and K491 results in a loss of ubiquitination. A K486/491R double mutant that is incapable of ubiquitination shows reduced binding to the NFκB subunit p65 or importin-β resulting in a distinctive peri-nuclear localisation of LYRIC/AEG-1. We also provide evidence to suggest that TOPORS, an E3 ligase that also regulates p53 modification may be responsible for LYRIC/AEG-1 ubiquitin modification. Overall we demonstrate that specific sites of LYRIC/AEG-1 ubiquitination are essential for regulating LYRIC/AEG-1 localisation and functionally interacting proteins.

1. Introduction

Many studies have shown that LYRIC/AEG-1 has a pivotal role in the development and progression of tumourigenesis (Brown and Ruoslahti, 2004; Chen et al.; Emdad et al., 2009; Hu et al., 2009; Jiang et al., 2012; Lee et al., 2009; Li et al., 2008; Liao et al., 2011; Song et al., 2009; Wang et al., 2011; Xia et al.; Yoo et al., 2009; Yu et al., 2009). Differential LYRIC/AEG-1 expression and altered localisation has been demonstrated in a variety of cancers, with increased expression frequently linked to a poor prognosis (Chen et al.; Jiang et al., 2012; Lee et al., 2009; Li et al., 2008; Liao et al., 2011; Song et al., 2009; Thirkettle et al., 2009a; Wang et al., 2011; Xia et al.; Yoo et al., 2009; Yu et al., 2009). Previous studies have shown that LYRIC/AEG-1 increases chemo-resistance and cell survival in response to hypoxia and glucose starvation, possibly by initiating protective autophagy (Bhutia et al.; Hu et al., 2009; Li et al., 2012). LYRIC/AEG-1 is regulated

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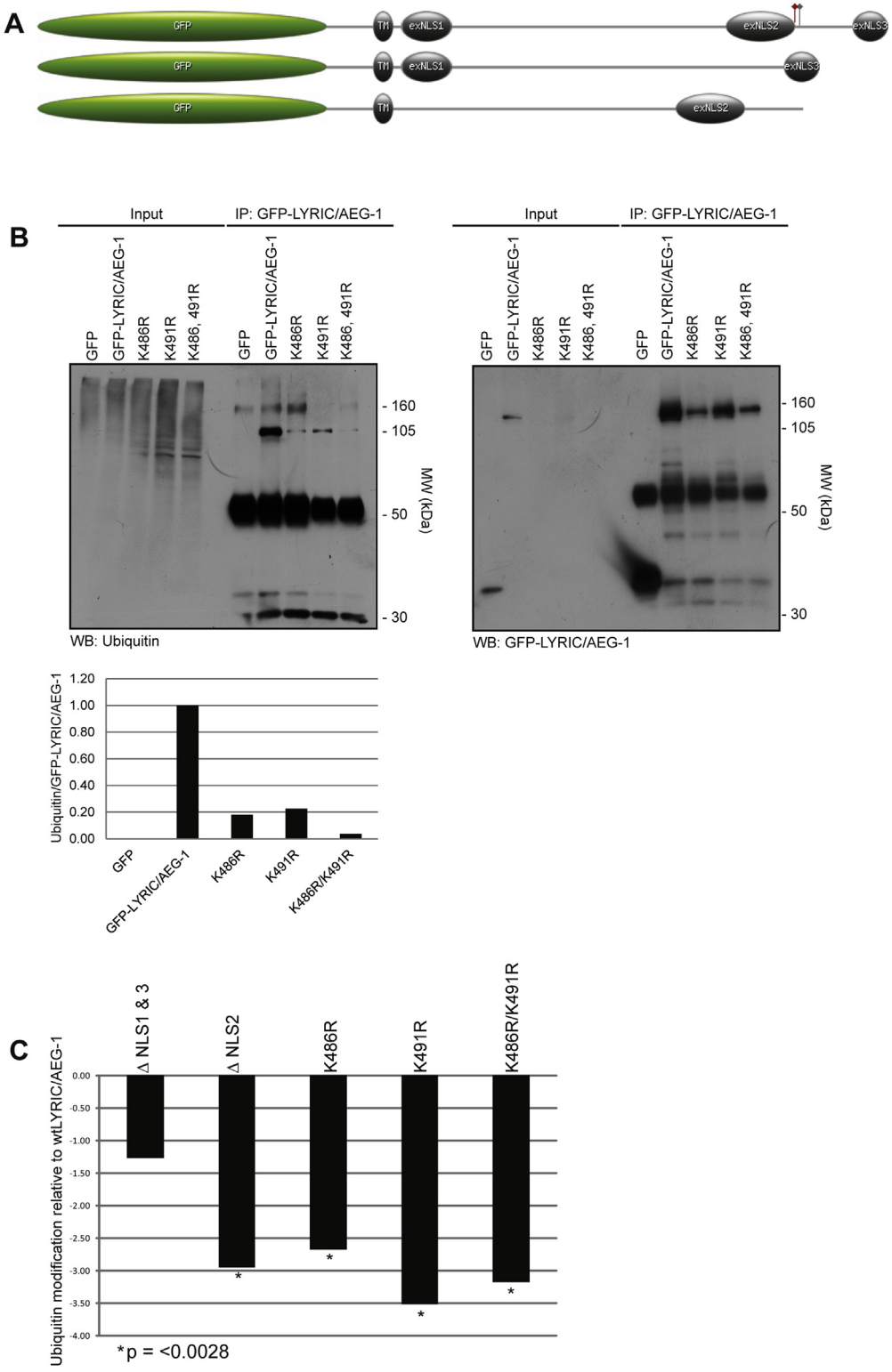

Figure 1 – LYRIC/AEG-1 is modified by ubiquitin on residues K486 and K491. (A) LYRIC/AEG-1 has a putative transmembrane domain, lysine residues clustered in *ex*tended *n*uclear *l*ocalisation *s*ignal regions (exNLS-1, -2 and -3). A series of GFP-tagged constructs were made deleting the exNLS regions or containing K486R, K491R or both K486/491R mutants (highlighted as diamonds). (B) COS-7 cells were transfected with GFP-LYRIC/AEG-1 constructs. All experiments were conducted with ubiquitin overexpression. GFP antibodies were used to immunoprecipitate LYRIC/AEG-1, separated by SDS-PAGE and analysed by Western blotting for ubiquitin. Blots were visualized using ECL or, where the signal exceeded the dynamic range of film, diaminobenzidene. The band at 160 kDa is considered non-specific. This band at 50 kDa is IgG. Densitometry showing the amount of ubiquitin normalised to GFP-LYRIC/AEG-1 construct is shown in the lower panel. (C) For mass spectrometry immunoprecipitated samples were separated by SDS-PAGE and bands corresponding to ubiquitinated LYRIC/AEG-1 excised,

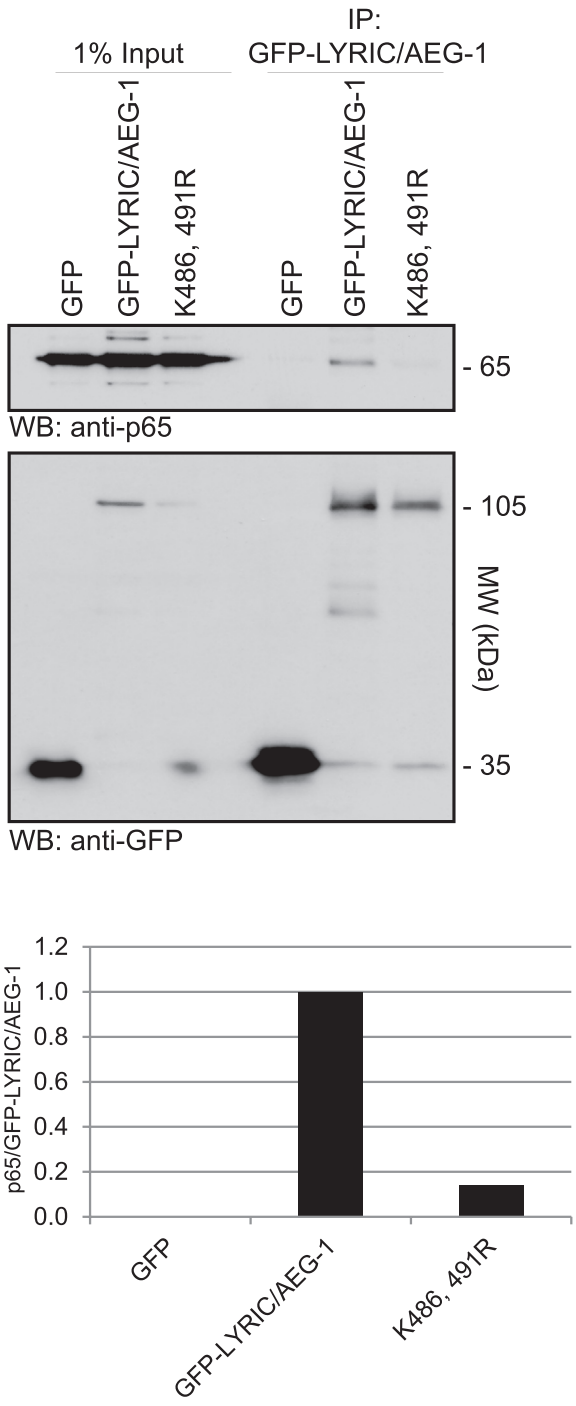

Figure 2 – Mutation of ubiquitination sites K486 and K491abrogates the interactions between LYRIC/AEG-1 and nuclear shuttling proteins. COS-7 cells were transfected with GFP-LYRIC/AEG-1 constructs and GFP-LYRIC immunoprecipitated from whole cell lysate, samples analysed using Western blotting and probed for endogenous p65 and GFP-LYRIC/AEG-1 as control. Densitometry showing the amount of p65 normalised to GFP-LYRIC/AEG-1 construct is shown in the lower panel.

by H-Ras and PI3K and there is mounting evidence for its role in regulating ubiquitination (Ash et al., 2008; Emdad et al., 2006; Yoo et al.).

Ubiquitin modifications are known to result in a broad range of responses, dependent upon the number of ubiquitin molecules and their linkage (Denuc and Marfany, 2010; Ikeda and Dikic, 2008). The addition of K48-linked ubiquitin moieties is associated with proteasomal dependent degradation of the substrate protein, whereas K63-linked ubiquitin moieties are associated with endocytosis and DNA repair {Aguilar and Wendland, 2003 #48}. Signalling activation and protein trafficking are more closely associated with monoubiquitination. Ubiquitin monomers can also elicit a response analogous to phosphorylation (Ikeda and Dikic, 2008) that regulates signalling or sub-cellular localisation of proteins such as PTEN, p53 and FOXO3a (Salmena and Pandolfi, 2007).

Ubiquitination is rate limited by the E3 ligases responsible for recognising specific ubiquitin targets. TOPORS is a unique E3 ligase which can ligate both ubiquitin and small ubiquitin-like modifier (SUMO) to substrate proteins (Denuc and Marfany, 2010). It contains an N-terminal RING-domain which ligates ubiquitin to its target proteins including DNA topoisomerase I, p53 and NKX3.1 (Guan et al., 2008; Weger et al., 2005). Depending on cellular context, TOPORS has been documented as a proto-oncogene or tumour suppressor (Guan et al., 2008; Saleem et al., 2004).

LYRIC/AEG-1 contains an usually large number of lysine residues (>12%) with the majority clustered within *e*xtended *n*uclear *l*ocalisation signal regions (exNLS). These regions have previously been defined as exNLS-1 (aa78–130), exNLS-2 (aa415–486) and exNLS-3 (aa546–582) and shown to act as nuclear and nucleolar localisation signals (Thirkettle et al., 2009a). However, only exNLS-2, which contains 18 lysine residues, was shown to be modified by mono-ubiquitin (Thirkettle et al., 2009a) causing almost all of LYRIC/AEG-1 to run at a higher MW of 105 kDa.

Here we identify the specific sites of LYRIC/AEG-1 ubiquitination by TOPORS in the exNLS-2 region. Mutagenesis of these residues leads to a highly significant reduction in LYRIC/AEG-1 ubiquitination which results in altered sub-cellular distribution and impairs the interaction between p65, importin-β and LYRIC/AEG-1.

2. Materials and methods

2.1. *Plasmids*

GFP-tagged LYRIC/AEG-1 constructs were created using pQBI25-fC3 plasmid (Q-BIOgene) as described (Thirkettle et al., 2009a). Site directed mutagenesis was performed using QuickChange II Site-Directed Mutagenesis kit (Stratagene) following the manufacturer's instructions to create GFP-K486R-LYRIC/AEG-1 alone, GFP-K491R-LYRIC/AEG-1

digested and subjected to mass spectrometry. Defined ubiquitin peptides (Supplementary Figure S2) were used to quantify the amount of ubiquitin present. Data was normalised to LYRIC/AEG-1 levels using defined GFP peptides (Supplementary Figure S3) and a mixed effects model. Values are given relative to wtLYRIC/AEG-1. * indicates significant *p*-values.

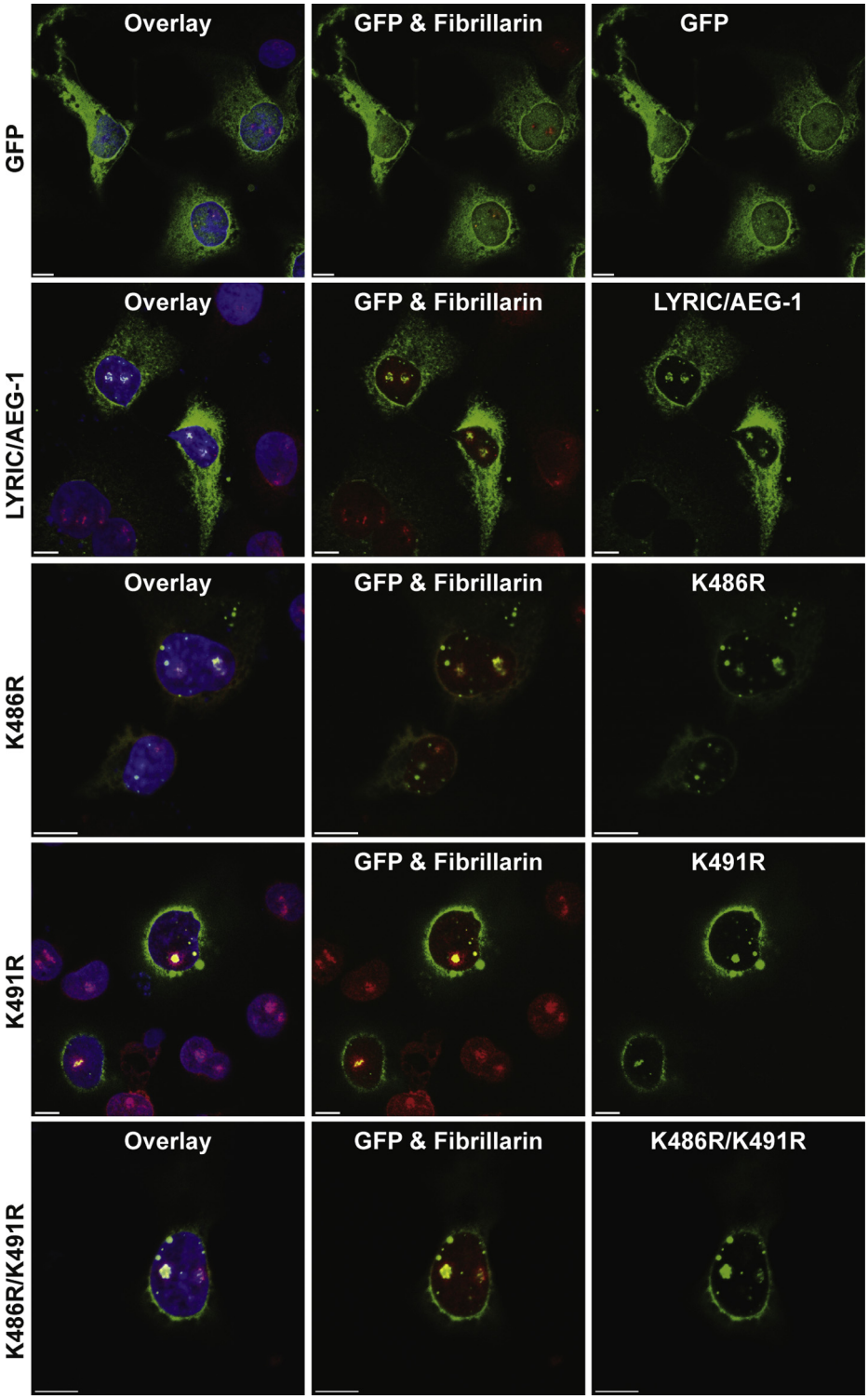

Figure 3 – Mutating K486 and K491 alters LYRIC/AEG-1 sub-cellular localisation. COS-7 cells were transfected with GFP or GFP-LYRIC/AEG-1 constructs (green) and ubiquitin. The nucleolus was labelled by staining for the nucleolar marker fibrillarin (red), the nuclei were visualised with DAPI (blue). Images captured using a Nikon confocal microscope, scale bars represent 10 um.

alone or a GFP-K486R/K491R-LYRIC/AEG-1 double mutant. Primer sequences are detailed in Supplementary Figure S1. TOPORS constructs were supplied by Dr. Stefan Weger (The Free University of Berlin, Germany) (Weger et al., 2005).

2.2. *Cell culture and transfection*

COS-7 cells purchased from the Cancer Research UK cell bank were routinely cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Labtech). For transfection cells were

grown to 40% confluency. DNA (1 μg/6 wells of a 24 well plate or 6 μg/per 14 cm dish) was transfected using FuGENE 6 (Roche) following the manufacturer's protocol. Cells were grown for a further 48 h prior to confocal microscopy or Western analysis.

2.3. Western blotting, immunoprecipitation and densitometry

All Western blotting procedures were performed as described (Whitaker et al., 2007) including the addition of 0.5% (w/v) sodium deoxycholate. Membranes were incubated with primary antibody; anti-GFP (1:5000), anti-actin (1:5000), anti-p65 (1:1000), anti-importin-β (1:5000) (all from Abcam), anti-ubiquitin (1:1000, Santa Cruz Biotechnology) and anti-TOPORS (1:1000, Sigma). Secondary antibodies (1:1000, Dako-Cytomation) were used against 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. Immunoprecipitation was performed at 4 °C using the method previously described (Thirkettle et al., 2009a). For densitometry all available Western blots ($n > 3$) were scanned using a Typhoon scanner (GE Healthcare) and analysed using ImageQuant TL (GE Healthcare). To overcome any variation in transfection/translation efficiency the amount of ubiquitination was normalised using GFP-LYRIC/AEG-1 values (ubiquitination/GFP-LYRIC/AEG-1).

2.4. Mass spectrometry

Proteins were separated using pre-cast 8% gels (Pierce) and stained using Biosafe Coomassie (Molecular Probes). Protein bands were excised and incubated overnight with destain (1:1 ratio of acetonitrile and 10 mM triethylammonium bicarbonate). Bands were washed in destain, dehydrated and reduced using 10 mM DTT, alkylated with 50 mM iodoacetamide then washed and dehydrated once more. Proteins were digested at 37 °C overnight using porcine trypsin (Promega) (10 ng/μl). Digestion was stopped by flash freezing before the addition of 5% formic acid (Fluka, UK) and sonication for 15 min, and repeated prior to sample dehydration.

Digested samples were resuspended in 8 μl 1% formic acid for 20 min prior to analysis using a nano-reverse phase LC- MS using a QTOF 6510 mass spectrometer with Chip Cube™ source interface and 1200 series HPLC running MassHunter B.01.03 (Agilent, USA). Samples were loaded onto a chip containing integrated 40 nl enrichment and 150 mm analytical columns and electrospray needle using 3 μl/min 0.1% formic acid. Peptides were eluted at 300 nl/min using a linear gradient from 10 to 70% elution buffer (80% acetonitrile/20% 0.1% formic acid (v/v)) for 10 min. After 1 min the gradient was increased to 100% elution buffer and 100% buffer was continued for a further 6 min. MS data was recorded in the 290–2500 m/z range at 6 spectra per second. MS/MS data was acquired in the 57–3000 m/z range at 4 spectra per second. Data was searched against UniProt (KB15.5j) and the known LYRIC/AEG-1 constructs using Mascot Daemon version 2.2.2 (Matrix Science, UK) using a peptide tolerance of 10 ppm and a fragment tolerance of 0.05 Da In addition to statistical scoring, the validity of the data was confirmed by manual assessment of the data using Scaffold software 2.1 (Proteome Science, USA).

2.5. Mass spectrometric label-free quantitation and statistics

Three biological replicates and two technical replicates were analysed per condition. Data were acquired using the Agilent's LC-Chip Cube™- 6510 QTOF data-dependent mode with the settings described above with the exception of collecting profile data. Data was acquired randomly as determined by list randomizer (http://www.random.org/lists/). Using Trapper version 4.2.0 (Natalie Tasman, Institute for Systems Biology, USA) files were converted to mzXML format and imported into Progenesis LC-MS version 2.5.3478.16299 (Nonlinear Dynamics, UK) for LC-MS run alignment and MS peak extraction for label-free quantitation after ion peak identification by MASCOT. Peaks of interest were analysed and validated using the statistical software R (version 2.10.1) (Team, 2010).

To normalize for variability in protein input the data was analysed using a mixed effects model, where the two fixed effects were GFP-LYRIC/AEG-1 constructs and ubiquitin. To reflect the pairing of measurements within these fixed effects (induced by the fact that each replicate provides two measurements) we also had a single mixed effects term indicating which replicate ion abundance mass spectrometric measurement it came from (analogous to the paired t-test that would have been carried out for a single fixed effect). Analysed data was the log 2 median of the unmodified ubiquitin normalized abundances obtained from Progenesis LC-MS label-free quantitation software.

2.6. Confocal microscopy

Cells were grown on glass coverslips in 24-well plates, transfected as described. Cells were fixed stained and mounted as previously described (Thirkettle et al., 2009a). Images were taken using a Nikon eclipse 90i confocal microscope with 60× objective. All scale bars represent 10 μM.

2.7. Yeast two-hybrid assay

A yeast two-hybrid assay was performed by Dualsystems Biotech AG, Zurich, Switzerland using pLexA-DIR-LYRIC/AEG-1 aa73-582 as bait and a human placental cDNA library as described (Thirkettle et al., 2009b).

3. Results

Previously, we have demonstrated that cytoplasmic LYRIC/AEG-1 is modified within the exNLS-2 region by mono-ubiquitin, leading to a 105 kDa MW band and no increase in LYRIC/AEG-1 degradation (Thirkettle et al., 2009a). Using immunoprecipitation of GFP-tagged wtLYRIC/AEG-1 and ΔexNLS constructs (shown in Figure 1A) we have previously demonstrated that deletion of the entire exNLS-2 region (aa415–486), and not exNLS-1 (aa78–130) or exNLS-3

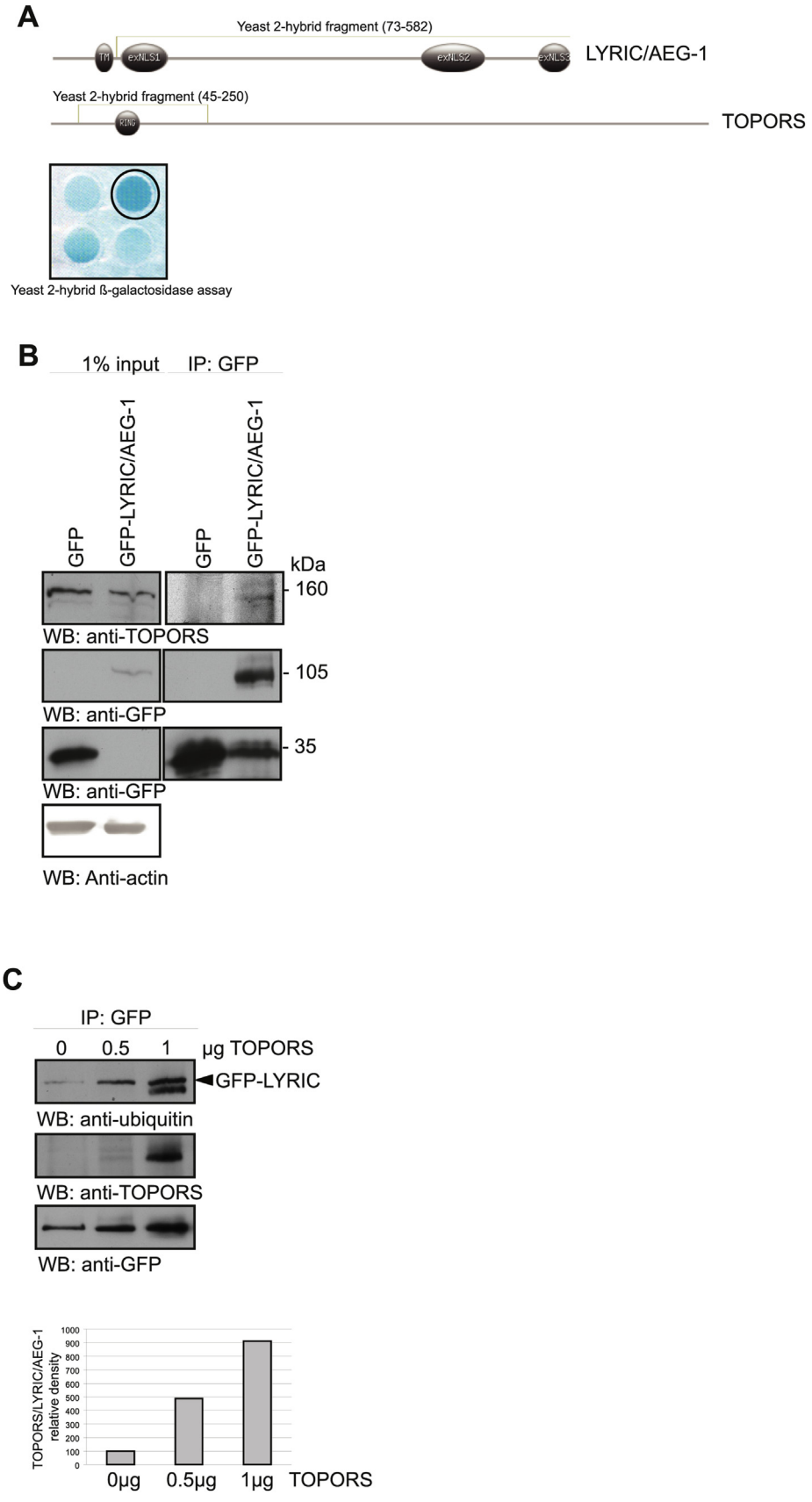

Figure 4 – LYRIC/AEG-1 potentially interacts with the E3 ligase TOPORS. A) A yeast two-hybrid assay was using pLexA-DIR-LYRIC/AEG-1 aa73-582 as bait and a human placental cDNA library. A β-galactosidase assay confirmed interaction between LYRIC/AEG-1 and aa 45–250 of TOPORS (circled in black), which included the RING domain. B) LYRIC/AEG-1 was immunoprecipitated from COS-7 cells using a GFP

(aa546–582), leads to an almost complete loss of LYRIC/AEG-1 mono-ubiquitination (Thirkettle et al., 2009a).

We identified two potential specific mono-ubiquitination sites by mass spectrometry at lysine residues K486 and K491. K486 lies within the exNLS-2 region while K491 lies 5 residues upstream of exNLS-2 (Figure 1B, top panel). To determine if K486 and K491 are the primary sites of ubiquitin modification single and double point mutations were made to ablate mono-ubiquitination without disrupting the protein charge or the potential to act as an NLS (K486R, K491R and K486/491R). GFP-tagged wild-type and mutant constructs and ubiquitin were over-expressed in mammalian cells and immunoprecipitated using the GFP-tag. Mutation of either K486 or K491 resulted in a significant reduction in LYRIC/AEG-1 mono-ubiquitination. When both K486 and K491 were mutated mono-ubiquitination was almost entirely ablated (Figure 1B).

To validate these findings we employed a mass spectrometry quantification method. Ubiquitination was normalised to the amount of GFP-tagged construct using the number of defined GFP peptides per construct as a control for GFP-LYRIC/AEG-1 input (Figure 1C). Due to the lysine-rich nature of LYRIC/AEG-1 the standard mass spectrometry methods were unsuitable and ubiquitination of each construct was quantified using a label-free mass spectrometry and a mixed effects model. Using this method the most consistent and robust ubiquitin and GFP peptides that could be detected were defined and measured for each construct (shown in Supplementary Figures S2 and S3).

Using mass spectrometry we confirmed that deletion of exNLS-2 resulted in a significant loss of ubiquitination compared to wtLYRIC/AEG-1 ($p = 0.0012$) (Figures 1A and 1C). When K486 and K491 were modified a highly significant loss of ubiquitination was seen; K486R mutant ($p = 0.0028$), K491R mutant ($p = 0.0004$) and the K486/491R double point mutant ($p = 0.0009$) (Figures 1B and 1C). There was also a small, but not significant, loss of ubiquitination when exNLS1/3 was deleted ($p = 0.43$).

We have previously demonstrated that deletion of the entire exNLS-2 region had no effect on LYRIC/AEG-1 function or stability and that treatment with MG132 failed to promote LYRIC/AEG-1 poly-ubiquitination, both of which are consistent with mono-ubiquitination (Thirkettle et al., 2009a). To establish if ubiquitination of LYRIC/AEG-1 had any effect on its function we examined the interaction between LYRIC/AEG-1 and p65, a known interacting protein proposed to shuttle into the nucleus with LYRIC/AEG-1 (Emdad et al., 2006; Sarkar et al., 2008). WtLYRIC/AEG-1 interacts with p65 but this interaction was lost with the non-ubiquitinated K486R/K491R mutant (Figure 2A). Mass spectrometry data identified a number of peptides corresponding to importin-β, a nuclear import chaperone, suggesting that it may interact with LYRIC/AEG-1 (Supplementary Figure S4) (Harel and Forbes, 2004). This interaction was confirmed by immunoprecipitating GFP-LYRIC/AEG-1 or the GFP-LYRIC/AEG-1 K486/491R mutant from COS-7 cells overexpressing the constructs. Importin-β was shown to interact with the wtLYRIC/AEG-1 but only at very low levels in the K486/491R mutant suggesting the ubiquitination of LYRIC/AEG-1 might regulate its interaction with importin-β.

Using confocal microscopy we examined the localisation of the GFP-tagged LYRIC/AEG-1 constructs. Previously we have reported that wtLYRIC/AEG-1 localises throughout the cytoplasm, nucleolus and to a lesser extent the nucleoplasm, as shown by co-localisation with fibrillarin (Figure 3) (Thirkettle et al., 2009a). All of the LYRIC/AEG-1 ubiquitin mutants are expressed at lower levels than wtLYRIC/AEG-1 despite being transfected with identical amounts of DNA. All of these mutants exhibit loss of the wild-type diffuse cytoplasmic and nucleoplasmic localisation. K491R and K486/491R display a striking peri-nuclear localisation. All nuclear LYRIC/AEG-1 in the K486R, K491R and K486R/K491R double mutants resides within the nucleolus and nuclear bodies and is lost from the nucleoplasm.

To identify potential E3 ligases for the ubiquitination of LYRIC/AEG-1 we used a yeast two-hybrid assay with C-terminal LYRIC/AEG-1, (aa 73–582), as bait (Thirkettle et al., 2009b) (Figure 4A). The screen identified only one E3 ligase, the N-terminal fragment of TOPORS (aa 45–250), containing the RING finger domain, as an LYRIC/AEG-1 interacting partner (Figure 4A) (Rajendra et al., 2004). The interaction between wtLYRIC/AEG-1 was supported using immunoprecipitation of GFP-tagged wtLYRIC/AEG-1 from COS-7 cells co-transfected with wtGFP-LYRIC/AEG-1 and TOPORS (Figure 4B). To determine if LYRIC/AEG-1 is a potential substrate for the TOPORS ubiquitin E3 ligase activity we immunoprecipitated wtLYRIC/AEG-1 from COS-7 cells transfected with an increasing concentration of TOPORS (Figure 4C). Western blot analysis showed that the ubiquitination of LYRIC/AEG-1 increased with increasing TOPORS expression. We have shown in previous studies using MG132 that ubiquitination of LYRIC/AEG-1 does not regulate its degradation by the proteasome (Thirkettle et al., 2009a).

4. Discussion

Mono-biquitination is known to regulate the localisation and function of a number of proteins including PTEN, p53 and FOXO3a (Denuc and Marfany, 2010; Salmena and Pandolfi, 2007). The large number of functions attributed to LYRIC/AEG-1 suggests it plays a pivotal role in tumourigenesis (Ash

antibody after cells were transfected with either GFP-LYRIC/AEG-1 or GFP alone. All cells were also co-transfected with ubiquitin and TOPORS Western blots were probed for GFP, ubiquitin and TOPORS. Blots were visualised using ECL or, where the signal exceeded the dynamic range of film in the case of actin, diaminobenzidene C) COS-7 cells were transfected with 0–1 ug of TOPORS as well as 2 ug LYRIC/AEG-1 and 2 ug ubiquitin and GFP-wt LYRIC/AEG-1 immunoprecipitated from 1 mg whole protein lysate. Samples were analysed using Western analysis probing for ubiquitin, TOPORS and GFP and densitometry performed to quantify the increase in ubiquitinated LYRIC/AEG-1 relative to the amount of precipitated GFP-LYRIC/AEG-1. The arrow indicates GFP-LYRIC/AEG-1 after reprobing following the TOPORS Western blot.

et al., 2008; Bhutia et al.; Brown and Ruoslahti, 2004; Emdad et al., 2009; Hu et al., 2009; Li et al., 2012; Yoo et al.). Previously we have shown that the exNLS-2 region of LYRIC/AEG-1 is modified by mono-ubiquitin, rather than poly-ubiquitin and that this modification does not alter LYRIC protein stability (Thirkettle et al., 2009a). We also saw modification by SUMO-1, but at much lower levels than mono-ubiquitin suggesting that mono-ubiquitin may play a more important role in post-translational modification of LYRIC/AEG-1. Deletion of exNLS-2, but not exNLS-1 or exNLS-3, results in an abrogation of LYRIC/AEG-1 modification by mono-ubiquitin (Figure 1A). We have previously reported that LYRIC/AEG-1 localisation and expression is up-regulated in prostate tumourigenesis supported by reports of overexpression multiple other cancers (Brown and Ruoslahti, 2004; Chen et al.; Jiang et al., 2012; Lee et al., 2009; Li et al., 2011, 2009, 2008; Liao et al., 2011; Song et al.; Song et al., 2009; Thirkettle et al., 2009a; Xia et al.; Yoo et al., 2009; Yu et al., 2009). We and others have previously shown that LYRIC/AEG-1 can move between the cytoplasm and nucleus (Figure 3) (Emdad et al., 2006; Sarkar et al., 2008; Thirkettle et al., 2009a). We have demonstrated that shuttling into the nucleus and interaction with proteins known to shuttle into the nucleus is attenuated by mono-ubiquitination of two key lysine regions at K486 and K491 (Figure 3). Mutating K486 and K491 of LYRIC/AEG-1 resulted in a dramatic effect on its localisation to peri- and sub-nuclear compartments and complete loss of cytoplasmic and nucleoplasmic LYRIC/AEG-1 (Figure 3). This suggests the retention of non-ubiquitinated LYRIC/AEG-1 within inactive sub-cellular compartments where it is unable to bind with known interacting protein p65 and the nuclear import protein importin-β (Figure 2 and Supplementary Figure S4) (Emdad et al., 2006; Sarkar et al., 2008).

Using quantitative mass spectrometry supported by Western blotting we have shown that mutation of K486 and K491 results in a dramatic loss of LYRIC/AEG-1 mono-ubiquitination (Figure 1B and C). This suggests that K486 and K491 are the major sites of mono-ubiquitination and that multiple other neighbouring lysine residues do not act co-operatively to compensate for the loss of a single lysine at either K486 or K491. Although K486 lies with the reported exNLS-2 region, K491 lies just outside the exNLS-2 region previously identified as the mono-ubiquitinated region of LYRIC-AEG-1. This is consistent either with co-operativity between the K486 and K491 mono-ubiquitination sites or binding of the E3 ligase needed to modify K491 to a site within exNLS-2.

E3 ligases are the rate limiting step and key regulators in ubiquitin modification. LYRIC/AEG-1 has previously been demonstrated to interact with BRCA2 and CDKN1A-interacting protein and indirectly alter its stability (Ash et al., 2008). We identified TOPORS, an oncogene and E3 ligase known to ubiquitinate proteins including p53, topoisomerase I and IκB kinase epsilon (Renner et al.), as an E3 ligase that potentially interacts with LYRIC/AEG-1. TOPORS was shown to interact with LYRIC/AEG-1 by yeast two-hybrid assay and co-immunoprecipitation Figure 4A and B. In addition, increasing amounts of TOPORS alters LYRIC/AEG-1 ubiquitination in a dose-dependent manner (Figure 4C).

LYRIC/AEG-1 has been implicated in tumour development, progression and treatment resistance (Bhutia et al.; Chen et al.; Hu et al., 2009; Jiang et al., 2012; Lee et al., 2009; Li et al., 2012, 2008; Liao et al., 2011; Song et al., 2009; Thirkettle et al., 2009a; Wang et al., 2011; Xia et al.; Yoo et al., 2009; Yu et al., 2009). Understanding the mechanism of LYRIC/AEG-1 modification by ubiquitin and its effects on LYRIC/AEG-1 sub-cellular trafficking provides new information on the how LYRIC/AEG-1 function is regulated in cells and offers potential new avenues for therapeutic intervention.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2014.01.009.

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