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RESEARCH ARTICLE

Nuclear upregulation of class I phosphoinositide 3-kinase p110β correlates with high 47S rRNA levels in cancer cells

Fatemeh Mazloumi Gavgani[1,§,*], Thomas Karlsson[1,§], Ingvild L. Tangen[2,3], Andrea Papdiné Morovicz[1], Victoria Smith Arnesen[1,‡], Diana C. Turcu[1], Sandra Ninzima[1], Katharina Spang[1], Camilla Krakstad[2,3], Julie Guillermet-Guibert[4] and Aurélia E. Lewis[1,¶]

ABSTRACT

The class I phosphoinositide 3-kinase (PI3K) catalytic subunits p110α and p110β are ubiquitously expressed but differently targeted in tumours. In cancer, *PIK3CB* (encoding p110β) is seldom mutated compared with *PIK3CA* (encoding p110α) but can contribute to tumorigenesis in certain PTEN-deficient tumours. The underlying molecular mechanisms are, however, unclear. We have previously reported that p110β is highly expressed in endometrial cancer (EC) cell lines and at the mRNA level in primary patient tumours. Here, we show that p110β protein levels are high in both the cytoplasmic and nuclear compartments in EC cells. Moreover, high nuclear:cytoplasmic staining ratios were detected in high-grade primary tumours. High levels of phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] were measured in the nucleus of EC cells, and pharmacological and genetic approaches showed that its production was partly dependent upon p110β activity. Using immunofluorescence staining, p110β and PtdIns(3,4,5)P_3 were localised in the nucleolus, which correlated with high levels of 47S pre-rRNA. p110β inhibition led to a decrease in both 47S rRNA levels and cell proliferation. In conclusion, these results present a nucleolar role for p110β that may contribute to tumorigenesis in EC.

This article has an associated First Person interview with Fatemeh Mazloumi Gavgani, joint first author of the paper.

KEY WORDS: Phosphoinositide 3-kinase, *PIK3CB*, p110β, Nucleolus, rDNA transcription, Endometrial cancer

INTRODUCTION

The phosphoinositide 3-kinase (PI3K) signalling pathway orchestrates a myriad of cellular processes regulating cell survival, proliferation and growth as well as metabolism. Class I PI3Ks consist of heterodimers of catalytic (p110α, β, δ or γ; encoded by *PIK3CA*, *PIK3CB*, *PIK3CD* and *PIK3CG*, respectively) and regulatory (p85α and its variants, p85β or p55γ; encoded by *PIK3R1*, *PIK3R2* and *PIK3R3*, respectively) subunits and phosphorylate the 3′-hydroxyl group of the glycerophospholipid phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] to generate phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] (Vanhaesebroeck et al., 2012). PtdIns(3,4,5)P_3 binds to effector proteins including the serine/threonine kinases AKT (also known as protein kinase B), 3-phosphoinositide-dependent protein kinase 1 (PDK1) and SIN1 (also known as MAPKAP1) via their phosphoinositide-binding pleckstrin homology (PH) domain (Manning and Toker, 2017). AKT is activated by phosphorylation on Thr308 and Ser473 by PDK1 and mammalian target of rapamycin complex 2 (mTORC2), respectively (Salamon and Backer, 2013). Activated AKT phosphorylates numerous substrates localised at different subcellular sites including the nucleus (Hers et al., 2011; Manning and Toker, 2017). The production of PtdIns(3,4,5)P_3 is regulated by phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a lipid phosphatase that dephosphorylates PtdIns(3,4,5)P_3 back to PtdIns(4,5)P_2, thereby opposing PI3K-mediated signalling and hence limiting the potential cancer-promoting effects of class I PI3K activity (Chalhoub and Baker, 2009).

The class I p110α and p110β subunits are ubiquitously expressed, share the same enzymatic properties, generate the same lipid product and initiate the same signalling cascade. Despite these shared features, the two isoforms are both essential in development as individual-knockout mice are embryonically lethal, suggesting non-redundant functions (Bi et al., 2002, 1999). Moreover, their mode of activation can be distinct. Whereas p110α carries out most of receptor tyrosine kinase (RTK)-mediated PI3K signalling, p110β can be activated by G-protein-coupled receptors as well as RTKs (Ciraolo et al., 2008; Guillermet-Guibert et al., 2008; Jia et al., 2008) but through different adaptor proteins (Fritsch et al., 2013). In cancer, the oncogenic properties of p110α are due to activating mutations of its gene *PIK3CA* (Samuels et al., 2004). In contrast, *PIK3CB*, the gene encoding p110β, is rarely mutated in cancer, with only three reports so far describing activating mutations (Dbouk et al., 2013; Pazarentzos et al., 2016; Whale et al., 2017). *PIK3CB* was, however, shown to be the key isoform mediating tumour growth in PTEN-deficient tumours, in particular in breast, prostate and ovarian cancer cells (Jia et al., 2008; Ni et al., 2012; Schmit et al., 2014; Torbett et al., 2008; Wee et al., 2008). This property may be due to its ability to promote oncogenic transformation in its wild-type state (Kang et al., 2006). Furthermore, the importance of p110β in tumorigenesis was highlighted in a study by Juric et al. (2015). This study showed that *PIK3CA*-mutant breast cancer cells, which were initially sensitive to p110α-specific inhibition, eventually developed resistance with acquired loss of PTEN in

[1]Department of Biological Sciences, University of Bergen, Bergen 5008, Norway. [2]Centre for Cancer Biomarkers, Department of Clinical Science, University of Bergen, Bergen 5021, Norway. [3]Department of Gynaecology and Obstetrics, Haukeland University Hospital, Bergen 5021, Norway. [4] Inserm U1037, Centre de Recherches en Cancérologie de Toulouse (CRCT), Institut National de la Santé et de la Recherche Médicale (INSERM), Université Toulouse III Paul Sabatier, 31037 Toulouse, France.
*Present address: The Sars International Centre for Marine Molecular Biology, Bergen 5008, Norway. ‡Present address: Department of Biomedicine, University of Bergen, Bergen, Norway.
§These authors contributed equally to this work

¶Author for correspondence (aurelia.lewis@uib.no)

I.L.T., 0000-0002-1760-0980; V.S.A., 0000-0002-2160-3404; K.S., 0000-0003-3746-5154; C.K., 0000-0002-0174-8139; J.G.-G., 0000-0003-3173-4907; A.E.L., 0000-0002-3613-5770

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metastatic lesions. Resistance could, however, be reversed when p110β inhibition was combined. Regarding their functions, a few studies have reported that the two isoforms can contribute differently to cell survival and proliferation, with p110α playing more of a role in cell survival and p110β in cell cycle progression and DNA replication (Benistant et al., 2000; Marqués et al., 2008, 2009). Another distinguishing feature of these two isoforms is their subcellular localisation. Although p110α and β are both found in the cytoplasm and share and/or compete for similar upstream receptor activation and downstream signalling cascades, p110β harbours a nuclear localisation signal and is found in the nucleoplasm, the chromatin fraction (Kumar et al., 2011; Marqués et al., 2009) as well as in the nucleolus together with its product PtdIns(3,4,5)P_3 (Karlsson et al., 2016). This would suggest that p110β can orchestrate different processes emanating from the nucleus and explain, at least partly, the pleiotropic aspects of the PI3K pathway.

The PI3K pathway is the signalling pathway most frequently altered in endometrial cancer (EC), with more than 80% of tumours harbouring somatic alteration in at least one gene component of the pathway (Mazloumi Gavgani et al., 2018). This includes high frequency mutations in *PTEN*, *PIK3CA* and *PIK3R1* and low frequency mutations in *AKT1* and *PIK3R2* (Cheung et al., 2011; O'Hara and Bell, 2012). Loss of function of the tumour suppressor gene *PTEN*, due to loss of heterozygosity or somatic mutations, is the most common event in type I endometrioid EC and occurs early in 18–48% of lesions with atypical hyperplasia (Hayes et al., 2006; Mutter et al., 2000). *PIK3CA* is the second most frequently mutated gene in EC, with mutations occurring in type I endometrioid EC and type II non-endometrioid EC serous lesions (Cheung et al., 2011; Hayes et al., 2006; Kandoth et al., 2013; Konopka et al., 2011; Kuhn et al., 2012; Le Gallo et al., 2012; Oda et al., 2005; Rudd et al., 2011). In addition, mutations in *PTEN* were found to co-exist with those of *PIK3CA* or *PIK3R1*, thereby leading to enhanced activation of the PI3K pathway (Konopka et al., 2011; Oda et al., 2008, 2005; Urick et al., 2011). *PIK3CA* gene amplification can also account for other mechanisms of PI3K pathway activation and tend to segregate more frequently to aggressive and invasive type II tumours (Kandoth et al., 2013; Konopka et al., 2011; Kuhn et al., 2012; Salvesen et al., 2009). In contrast to *PIK3CA*, mutation events are infrequent in *PIK3CB* and account for 2– 10% in EC, according to public data from the Catalogue of Somatic Mutations In Cancer (COSMIC, v90) or The Cancer Genome Atlas (Forbes et al., 2011; Kandoth et al., 2013; Kuhn et al., 2012). Of those, only two mutations located in its catalytic domain have been characterised recently and found to have oncogenic properties (Kim et al., 2016; Pazarentzos et al., 2016). *PIK3CB* mRNA levels were found to be elevated in endometrial tumours when compared to the levels found in normal tissue in a few patient samples (An et al., 2007). In a recent study, we have shown that p110β protein levels are elevated in EC cell lines and that mRNA levels are increased in grade 1 endometrioid endometrial lesions compared to complex hyperplasia (Karlsson et al., 2017). The cancer-promoting molecular mechanisms of p110β remain, however, largely unknown. We have recently reported the presence of p110β and of its product PtdIns(3,4,5)P_3 in the nucleolus of the breast cancer cell line AU565 (Karlsson et al., 2016). This would suggest that p110β could regulate different functions from the nucleolus and, in particular, that its increased levels may contribute to EC development. In this study, we showed an increase in the nuclear levels of both p110β and its lipid product PtdIns(3,4,5)P_3 in EC cells. We further showed that high p110β levels correlated with high 47S pre-rRNA levels, which was partly dependent on p110β activity. These results therefore suggest the involvement of this kinase and PtdIns(3,4,5)P_3 in increased nucleolar activity in EC cells.

RESULTS

p110β is cytoplasmic and nuclear in endometrial cancer cell lines and patient tumours

Previous studies have shown that the PI3K catalytic isoforms p110α and p110β are differently localised and that this may contribute to their different cellular functions (Karlsson et al., 2016; Kumar et al., 2011; Marqués et al., 2009). Using cell fractionation and western blotting, we determined the subcellular localisation of these two isoforms as well as the two regulatory subunits p85α and p85β in EC cells, and compared this with their localisation in a non-tumour immortalised endometrial cell line (EM). As shown in Fig. 1A, p110α was restricted to the cytoplasmic fraction in all cell lines. p110β was detected in both the cytoplasmic and nuclear fractions at low levels in EM cells but was found at higher levels in cancer cell lines. In the majority of cell lines, p85α was restricted to the cytoplasmic fraction. In contrast, p85β was mostly concentrated in the nuclear fraction, with high levels in EM, KLE, EFE-184 and MFE-280 cells and lower levels in the remaining cell lines (Fig. 1A). To determine whether the expression and localisation pattern of p110β could also be observed in human tissues, we examined a patient cohort of 714 primary endometrial tumours by immunohistochemistry of a tissue microarray (TMA). The level and intensity patterns of p110β were scored separately in the cytoplasm and nucleus. Whereas most patients showed p110β cytoplasmic detection with various degrees of intensity (Fig. 1B, left panel), 23% of all cases showed nuclear staining (Fig. 1B, right panel). In addition, a significant correlation was observed between high nuclear to cytoplasmic score ratio for p110β and higher grades or histological type II (non-endometrioid) endometrial tumour (Fig. 1C).

The levels of PtdIns(3,4,5)P_3 are increased in nuclei of EC cells in a p110β activity-dependent manner

We next determined whether the presence of p110β in the nucleus correlated with nuclear PI3K pathway activity by first assessing the presence of active AKT (as indicated by phosphorylation at Ser473 of AKT1, p-S473-AKT; of note, the antibody used can also detect AKT2 and AKT3 when phosphorylated on the corresponding serine residues Ser474 and Ser472, respectively). As shown in Fig. 2A, the cytoplasmic and nuclear levels of p-S473-AKT were low in EM, KLE, EFE-184 and MFE-280 cells, while high levels were observed in PTEN-deficient cells, MFE-296, MFE-319, RL95-2 and Ishikawa cells, consistent with our previous study using total cell extracts in the same cells (Karlsson et al., 2017). Interestingly, high nuclear p-S473-AKT levels were correlated with low levels of nuclear p85β (Fig. 1A). Furthermore, we determined the nuclear level of PtdIns(3,4,5)P_3 of all cells examined following nuclear isolation, lipid extraction and detection with GST–GRP1-PH, a PtdIns(3,4,5)P_3-specific probe (Guillou et al., 2007; Fig. 2B and Fig. S1 showing the specificity of the probe). The purity of the fractionation was verified by western blotting using markers for the cytoplasm, nucleus and endoplasmic reticulum (Fig. S2). Nuclear PtdIns(3,4,5)P_3 levels were high in most cancer cells and highest in RL95-2 cells, compared to levels in EM cells (Fig. 2C). To test whether p110β is responsible for the synthesis of nuclear PtdIns(3,4,5)P_3, we treated the PTEN-deficient cell line RL95-2 with TGX-221, a p110β selective inhibitor (Fig. 2D) or induced its knockdown by siRNA (Fig. 2E). Both the inhibition and partial knockdown of p110β (31%±14; mean±s.d.) reduced the levels of nuclear PtdIns(3,4,5)P_3. In addition, both treatments led to a decrease in the level of both total AKT and p-S473-AKT in the

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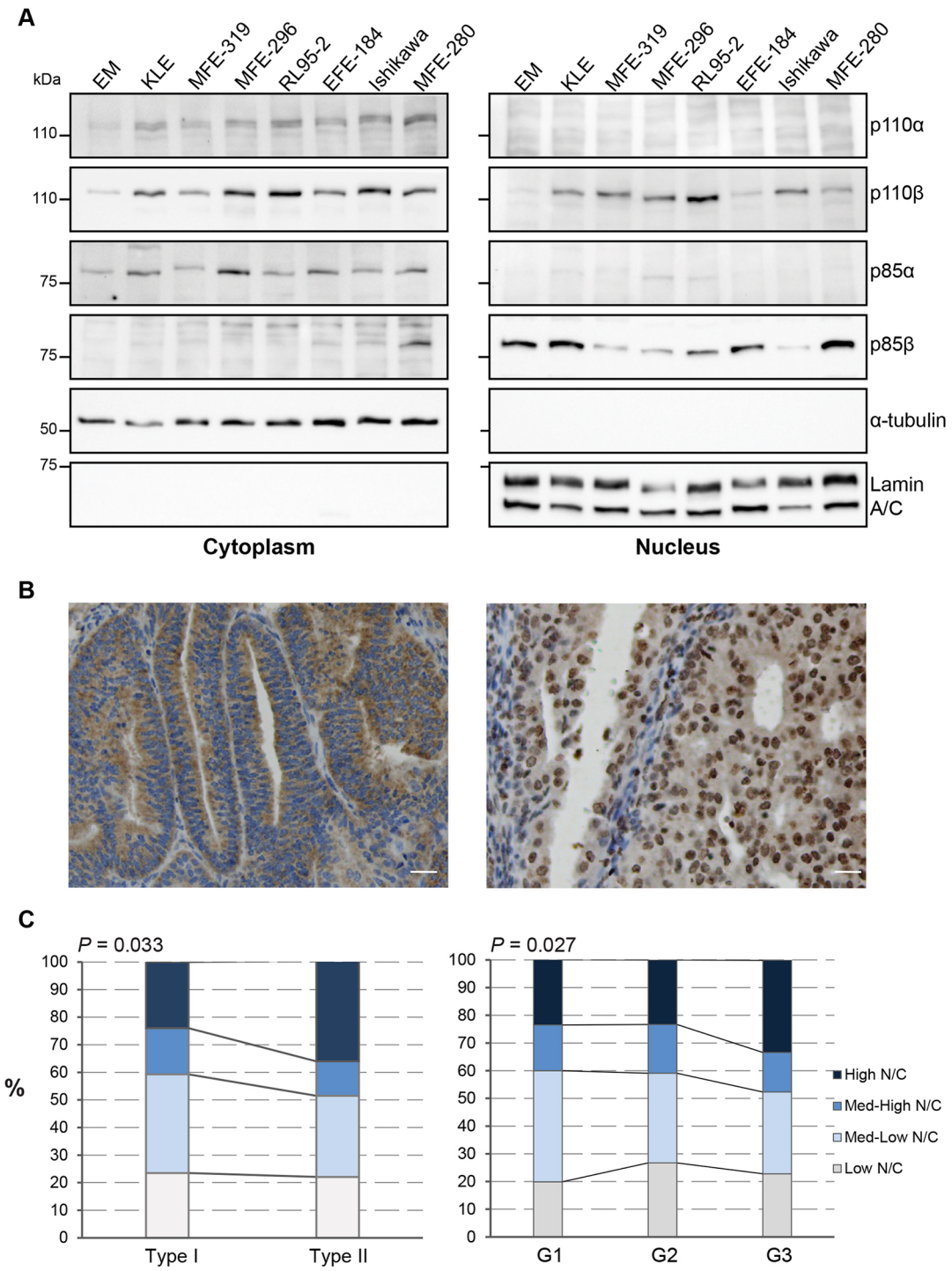

Fig. 1. p110β selectively localises to the nucleus in endometrial cancer cells. (A) Actively growing cells of the indicated cell lines were fractionated into cytoplasmic and nuclear fractions. Equal protein amounts (30 µg) were resolved using SDS–PAGE and analysed by western blotting using antibodies against the indicated proteins. Blots are representative of at least three replicates. (B) Representative immunohistochemistry images of cytoplasmic (left) and nuclear (right) p110β staining in primary endometrial tumours, detected using anti-p110β antibody. Scale bars: 20 µm. (C) Quantification of nuclear (N) to cytoplasmic (C) score ratios measured following p110β immunohistochemistry of 714 patient histology samples. The ratios were divided into quartiles and named low, medium/low (Med-Low), medium/high (Med-High) and high, and the percentage of the samples in each quartile for each group is shown. G represents the grade of the tumour. G1, *n*=267; G2, *n*=210; G3, *n*=237. *P*-values were calculated using a Chi-square test.

nuclear fraction, which resulted in a lack of change in p-S473-AKT:AKT ratio in these fractions (Fig. 2D,E). This would suggest that the decrease in nuclear p-S473-AKT is due to loss of its translocation from the cytoplasm and that the lower AKT activity observed in the nucleus is not linked to a decrease in nuclear PtdIns(3,4,5)P_3.

p110β and PtdIns(3,4,5)P_3 are nucleolar in EC cells

Using immunofluorescence staining, we found that p110β was localised in the cytoplasm, the nucleoplasm and strongly in nucleoli together with the nucleolar protein nucleophosmin in three cell lines with high nuclear p110β (Fig. 3A), which is consistent with our

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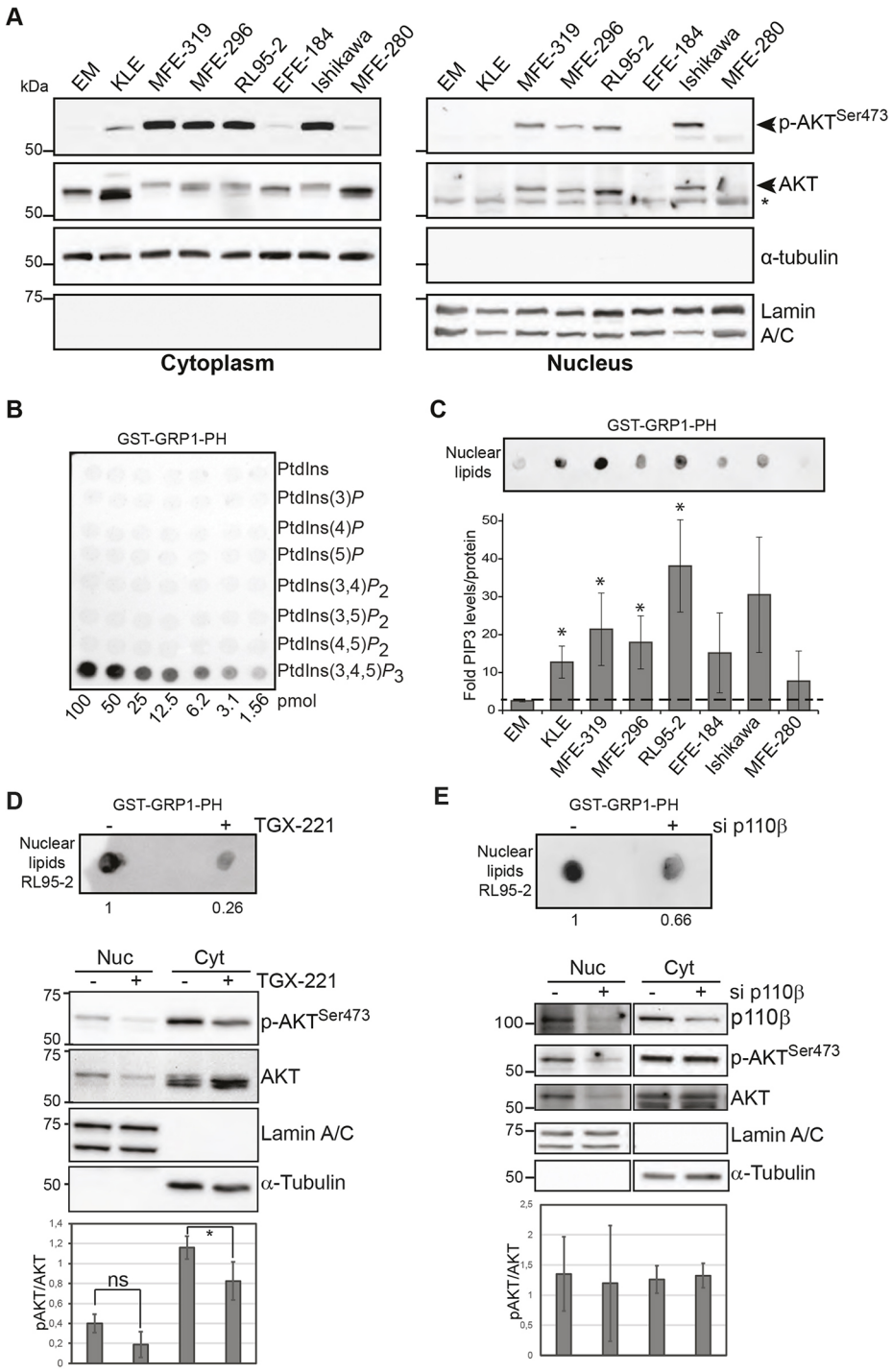

Fig. 2. Nuclear PtdIns(3,4,5)P_3 levels are elevated in endometrial cancer cells. (A) Actively growing cells of the indicated cell lines were fractionated into cytoplasmic and nuclear fractions. Equal protein concentrations were resolved using SDS–PAGE and analysed by western blotting using the antibodies against the indicated proteins.* indicates an unspecific band. Blots are representative of at least three replicates. (B) PIP array spotted with 1.56–100 pM of each of the seven indicated polyphosphoinositide species and phosphatidylinositol (PtdIns), incubated with GST–GRP1-PH and an anti-GST HRP-conjugated antibody. (C) Upper panel: PtdIns(3,4,5)P_3 (PIP3) detection in nuclear acidic lipids extracted from actively growing cells, as determined by overlay assay with GST–GRP1-PH domain and anti-GST HRP-conjugated antibody. Lower panel: PIP3 signal/mg nuclear protein ratio quantification for each cell line. The dashed line represents the value from EM cells. Data are presented as mean±s.d. n=3. *P<0.05 (two-way, unpaired Student's t-test for each cell line compared to EM cells). (D) RL95-2 cells treated with (+) or without (–) 10 µM TGX-221 for 3 d. Upper panel: lipid overlay detection of PIP3 in acidic lipids extracted from isolated nuclei using GST–GRP1-PH and anti-GST HRP-conjugated antibody. Numbers indicate the TGX-221:DMSO PIP3 signal ratio quantification. Middle panel: western blotting of cytoplasmic (Cyt) and nuclear (Nuc) fractions using antibodies against the indicated proteins. Lower panel: p-S473-AKT:AKT signal ratio quantification for each fraction. Data are presented as mean±s.d. n=3. *P<0.05; ns, not significant (two-way, unpaired Student's t-test). Lipid and western blots are representative of three matching replicates. (E) RL95-2 cells transfected with 200 nM control non-targeting siRNA or p110β-targeting siRNA (si p110β) for 2 d . Upper panel: lipid overlay detection of PIP3 in acidic lipids extracted from isolated nuclei using GST–GRP1-PH and anti-GST HRP-conjugated antibody. Numbers indicate the si p110β:si control PIP3 signal ratio quantification. Middle panel: western blotting of cytoplasmic and nuclear fractions using antibodies against the indicated proteins. Lower panel: p-S473-AKT:AKT signal ratio quantification for each fraction. Data are presented as mean±s.d. n=3. Lipid and western blots are representative of three matching replicates.

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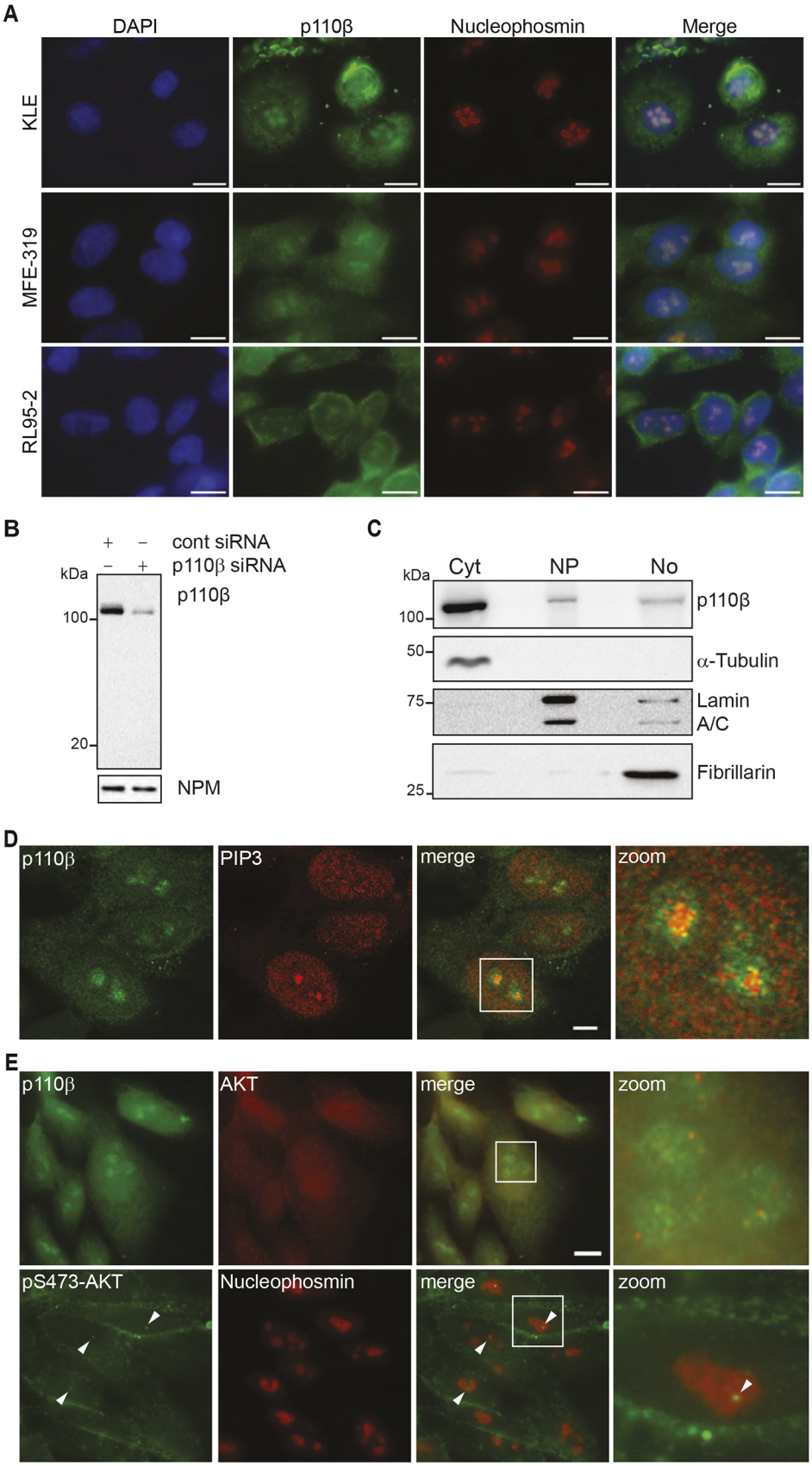

Fig. 3. p110β and PtdIns(3,4,5)P_3 are nucleolar. (A) Co-immunostaining of p110β using the Abcam 151549 anti-p110β antibody and anti-nucleophosmin in actively growing KLE, MFE-319 and RL95-2 cells, imaged by epifluorescence microscopy. DNA is stained with DAPI. Images are representative of at least three replicates. Scale bars: 10 µm. (B) Western blotting of KLE whole-cell extracts obtained following treatment with 100 nM control siRNA (cont siRNA) or p110β-targeting siRNA for 40 h, using the Abcam 151549 anti-p110β antibody and anti-nucleophosmin (NPM). Blots are representative of two experiments. (C) Subcellular fractionation (Cyt, cytoplasm; NP, nucleoplasm; No, nucleolus) of RL95-2 cells and western blotting of equal protein amounts from each fraction using antibodies against the indicated proteins. Blots are representative of three biological replicates. (D,E) Confocal (D) and epifluorescence (E) microscopy of actively growing RL95-2 cells co-stained with antibodies against the indicated proteins and PtdIns(3,4,5)P_3 (PIP3). Images are representative of three replicates. Boxes indicate regions shown in zoom images. Arrowheads indicate nucleolar p-S473-AKT signal. Scale bars: 5 µm.

previous study of AU565 cells (Karlsson et al., 2016). The nucleolar localisation of p110β was validated using a different antibody (Fig. S3). The specificity of the anti-p110β antibody was validated by knockdown using siRNA and western blotting (Fig. 3B). Nucleolar fractionation of RL95-2 cells confirmed the presence of p110β in the same compartments by western blotting (Fig. 3C). α-

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tubulin, lamin A/C and fibrillarin were used as cytoplasmic, nuclear and nucleolar markers, respectively, to validate the fractionation procedure. Lamin A/C was, however, found in both the nucleoplasmic and nucleolar compartments, as previously reported (Martin et al., 2009). p110β was detected with PtdIns(3,4,5)P_3 both in the nucleoplasmic and nucleolar compartments of RL95-2 (Fig. 3D) and MFE-319 cells (Fig. S4). The specificity of the anti-PtdIns(3,4,5)P_3 antibody was validated by lipid overlay assays using PIP arrays and by competition with free lipids observed using immunofluorescence staining (Fig. S5). Hence, pre-incubation of the antibody with different polyphosphoinositides showed that nucleoplasmic and nucleolar staining was abolished by the presence of PtdIns(3,4,5)P_3 but not by PtdIns3P and PtdIns(3,4)P_2. Furthermore, AKT was found to colocalise with the nucleolar pool of p110β, and its active p-S473 form with the nucleolar protein nucleophosmin as discrete foci (Fig. 3E, arrowheads). In addition, both AKT and its active form were detected on apparent plasma membranes.

The nucleolar pool of PtdIns(3,4,5)P_3 is partly dependent upon the activity of p110β

The main function of the nucleolus is to synthesise ribosomes, requiring rDNA transcription, processing and assembly with ribosomal proteins (Boisvert et al., 2007). rRNA synthesis oscillates during the cell cycle; it is abolished during mitosis and re-activated in G1 phase, with highest activity thereafter in S and G2 phases (Gebrane-Younes et al., 1997; Grummt, 2003; Roussel et al., 1996). Similarly, p110β is activated during G1 in the nucleus and contributes to G1 to S phase transition (Marqués et al., 2008, 2009). We therefore examined the appearance of p110β and PtdIns(3,4,5)P_3 during the reformation of nucleoli as cells exited mitosis. We combined nocodazol treatment and mitotic shake-off to synchronise and enrich for mitotic HeLa cells. After plating the collected mitotic cells on coverslips, the cells were left to recover for different time points before they were fixed and labelled with a GFP–GRP1-PH probe and immunostained with anti-nucleophosmin. After 1 h, the cells were still in mitosis, and both p110β and PtdIns(3,4,5)P_3 were present mostly in non-DNA regions. We found that p110β colocalised with RPA194 (also known as POLR1A), a subunit of the RNA polymerase I, 3 h after re-plating, as the cells exited mitosis and the nucleoli started to re-form. With a lag of up to 2–5 h, PtdIns(3,4,5)P_3 started to reappear in nucleoli (Fig. 4A). To determine whether the pool of PtdIns(3,4,5)P_3 present in nucleoli is produced due to the kinase activity of p110β, we compared the nucleolar appearance of PtdIns(3,4,5)P_3 in wild-type (WT) and p110β$^{D931A/D931A}$ kinase-inactive mouse embryonic fibroblast (MEF) cells during the reformation of nucleoli as cells exited mitosis (Fig. 4B). In line with our results in HeLa cells (Fig. 4A), PtdIns(3,4,5)P_3 was detected together with nucleophosmin in the p110β WT MEFs. In contrast, the p110β kinase-inactive MEFs demonstrated a decrease in PtdIns(3,4,5)P_3 nucleolar staining.

High nucleolar p110β levels correlate with high 47S rDNA transcription

Elevated levels of nucleolar activity have been correlated to an increased risk of cancer development (Montanaro et al., 2012). We next examined whether EC cells with high levels of p110β and PtdIns(3,4,5)P_3 in the nucleus had higher levels of 47S rDNA transcription compared to those in EM cells using quantitative RT-PCR. Consistently, all EC cell lines examined demonstrated higher levels than those in EM cells, with KLE cells showing the highest levels (Fig. 5A). Using a selective inhibitor of p110β, KIN-193, we next tested whether p110β is implicated in 47S rDNA transcription. A 30% decrease in 47S rDNA transcription was observed following 22 h of treatment, whereas RNA Pol I inhibition almost completely blocked transcription (Fig. 5B). Inhibition of p110β also led to a gradual decrease in cell numbers (Fig. 5C), in agreement with the findings of our previous study using TGX-221 (Karlsson et al., 2017). This would be consistent with the fact that rDNA transcription is a rate-limiting factor for ribosome biogenesis and, hence, of cell division (Grummt, 2010).

DISCUSSION

Cellular compartmentalisation provides an important mode of regulation for signalling cascades to achieve specificity and to precisely coordinate cellular outputs. The PI3K pathway has been extensively studied from a cytoplasmic perspective. However, few studies have detailed the distinct intracellular localisation of PI3K enzymes. The PI3K p110α is restricted to the cytoplasm, whereas p110β is present both in the cytoplasm and the nucleus, and in particular in the nucleoplasm, the chromatin fraction and the nucleolus (Karlsson et al., 2016; Kumar et al., 2011; Marqués et al., 2009). The compartmentalisation of these enzymes is likely to impact signalling networks and to mediate different cell functions, hence accounting for the pleiotropic effects attributed to PI3K signalling. Although the PI3K signalling pathway is pivotal in cancer, the impact of the subcellular localisation of PI3K on processes attributed to tumorigenesis is still poorly understood. Our findings demonstrate for the first time that p110α and p110β are differently compartmentalised in EC cells. Consistent with previous studies in other cell types (Kumar et al., 2011; Marqués et al., 2009), p110α is cytoplasmic while p110β is both cytoplasmic and nuclear. This would suggest that, in the cytoplasm, p110α and p110β isoforms can share some of the functions attributed to PI3K signalling, perhaps due to their reported cross-activation (Perez-Garcia et al., 2014). In cancer cells, the presence of genetic mutations affecting *PIK3CA* or *PTEN* would influence PtdIns(3,4,5)P_3-mediated downstream functions induced by p110α and p110β, respectively, in this compartment. Concerning the nucleus, we found that the levels of p110β are high in the nucleus of EC cell lines compared to levels in the nucleus of EM cells. In clinically annotated tumour samples, we showed a correlation between the nuclear p110β levels and endometrial cancer progression; tumours with higher grade presented high p110β nuclear to cytoplasmic ratio. These results indicate the importance of the levels of this isoform combined with its compartmentalisation status. At least, this would be consistent with the fact that the overexpression of p110β has previously been shown to lead to cell transformation in its wild-type state (Kang et al., 2006). However, this is the first study showing the potential importance of an increase in the nuclear compartmentalisation of p110β during disease progression in clinical samples.

Furthermore, our studies demonstrate that EC cells not only have high nuclear levels of p110β, but also have elevated levels of PtdIns(3,4,5)P_3, its lipid product, as well as the active form of the oncoprotein AKT, p-S473-AKT, the key signalling effector of PtdIns(3,4,5)P_3. Here, we demonstrated that upon p110β inhibition, the levels of p-S473-AKT are decreased in EC nuclei. The nuclear PtdIns(3,4,5)P_3 levels were also reduced in these cells following p110β inhibition, which suggests that the nuclear pool of PtdIns(3,4,5)P_3 is, at least partly, the product of the kinase activity of p110β. Another pool could be dependent upon the activity of inositol polyphosphate kinase (IPMK). IPMK was initially discovered as an inositol (1,4,5) trisphosphate kinase, but was thereafter reported

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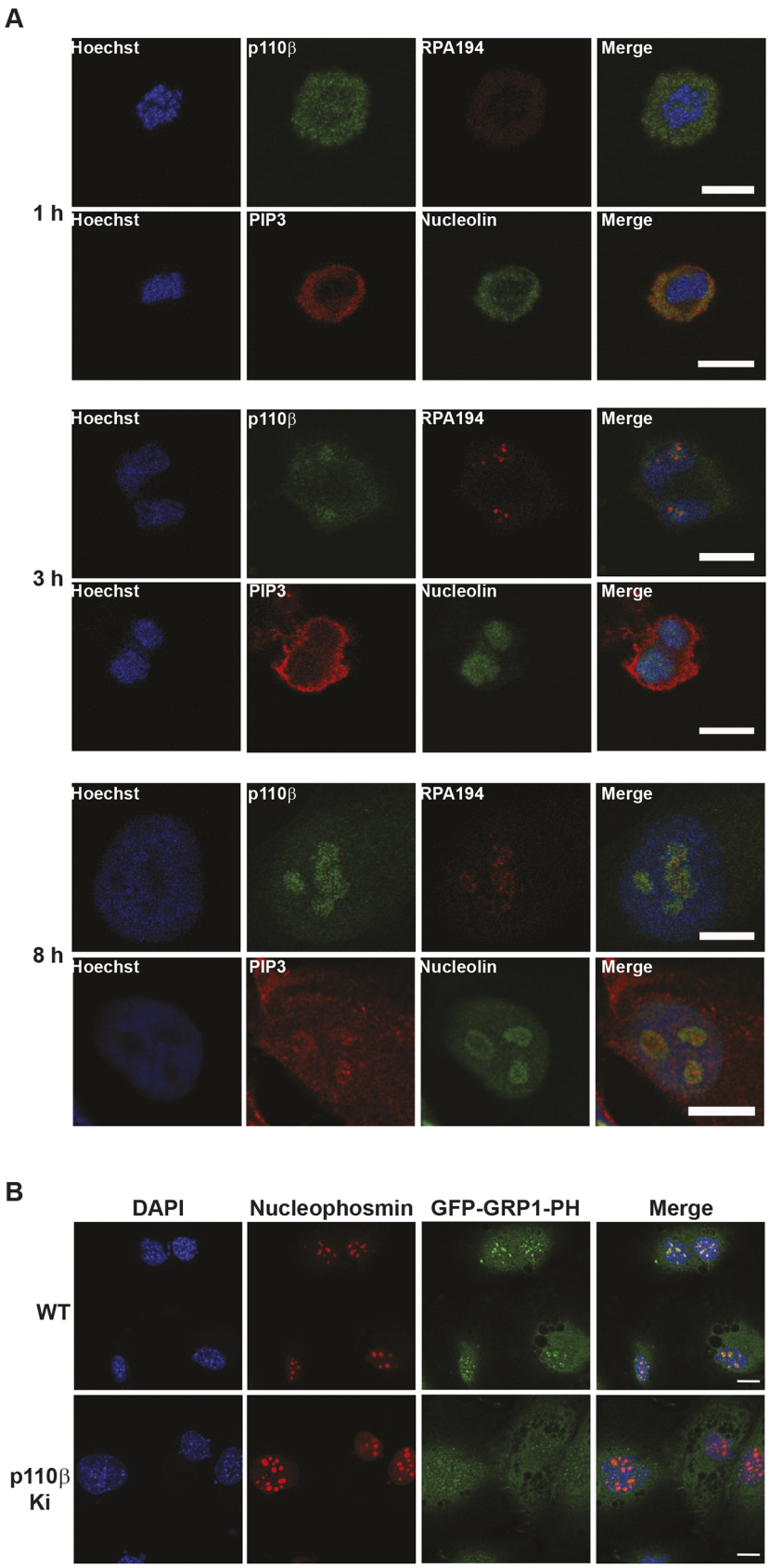

Fig. 4. p110β contributes to the synthesis of PtdIns(3,4,5)P_3 in the nucleolus. (A) HeLa and (B) MEF p110β WT and kinase-inactive (p110β Ki) cells were treated for 16 h with 50 ng/ml nocodazole, collected after mitotic shake-off and re-plated on poly-L-lysine coverslips. Cells were fixed after 1, 3 and 8 h (A) or 4 h (B). Immunofluorescence staining was performed as indicated, followed by confocal microscopy. PIP3, PtdIns(3,4,5)P_3; RPA194, RNA polymerase I 194 kDa. Images are representative of three biological replicates. Scale bars: 10 µm.

to also act on PtdIns(4,5)P_2 to generate PtdIns(3,4,5)P_3 (Maag et al., 2011; Resnick et al., 2005). The existence of a molecular link within the nucleus between PtdIns(3,4,5)P_3 and AKT is, however, not clear from this study. Additional mechanisms of regulation required for the activation of AKT were not explored. These may include the PtdIns(3,4,5)P_3-dependent activation of PDK1 and mTORC2, known

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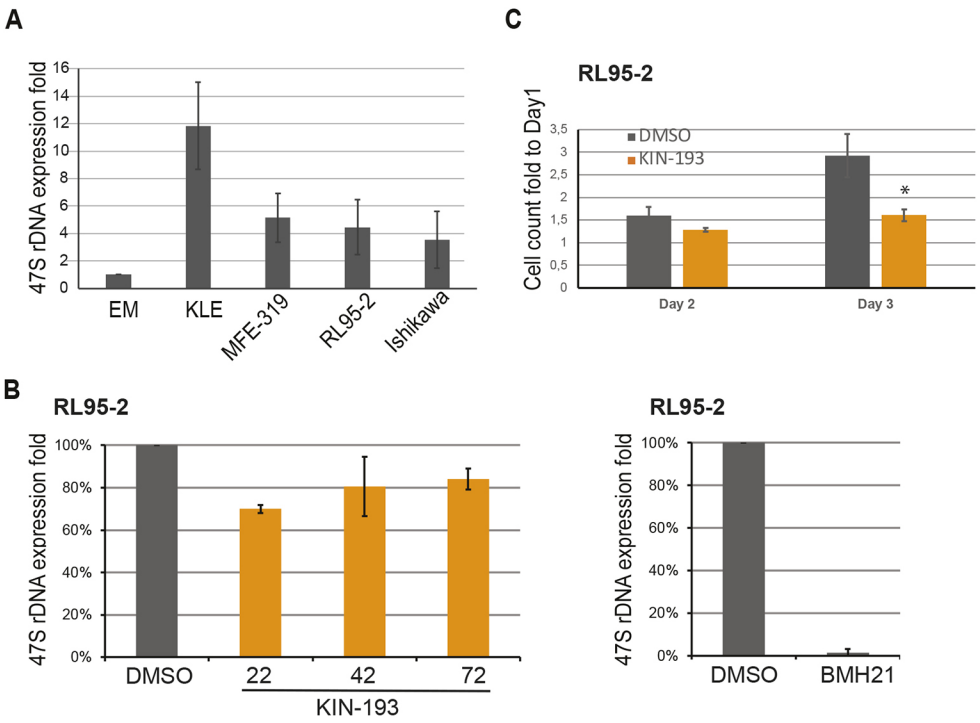

Fig. 5. p110β contributes to 47S pre-rRNA transcription. (A) 47S pre-rRNA expression relative to β-actin (*ACTB*) mRNA expression in KLE, MFE 319, RL95-2, Ishikawa and EM cells. The expression of all samples is normalised to that in EM cells. (B) 47S pre-rRNA expression relative to β-actin (*ACTB*) mRNA expression in RL95-2 cells treated with DMSO or the p110β inhibitor KIN-193 (10 µM) for 22, 42 and 72 h (left), or with the RNA polymerase I inhibitor BMH21 (1 µM) for 3 h (right). Expression is normalized to levels in DMSO-treated samples. (C) Effect of KIN-193 (10 µM) on RL95-2 cell counts, shown as cell count ratios of day 2:day 1 and day 3:day1. *P<0.05 (two-way, unpaired Student's *t*-test). In A–C, data are presented as mean±s.d. n=3.

to be critical for the phosphorylation and full activation of AKT. This may be plausible, as they have been detected in the nucleus in previous studies (Lim et al., 2003; Rosner and Hengstschlager, 2008; Scheid et al., 2005; Sephton et al., 2009).

A clear relationship between elevated nucleolar activity and increased risk of cancer has been previously shown (Montanaro et al., 2012; Pelletier et al., 2018). In this study, the panel of EC cells examined indeed showed high levels of rDNA transcription compared to levels in a non-tumour cell line. Nucleolar processes need to be tightly regulated with high fidelity to ensure appropriate cell growth and proliferation in response to external signals. One potential molecular link regulating these processes is the PI3K pathway. Previous studies have shown that the transcription and processing of the pre rRNA is stimulated in a PI3K and mTOR-dependent manner (Hannan et al., 2003; Iadevaia et al., 2012; James and Zomerdijk, 2004). However, the responsible PI3K isoform was not identified in those studies. This study identified p110β as the isoform contributing, at least partially, to 47S rRNA synthesis. The decrease in 47S rRNA levels observed following the inhibition of p110β could also indicate a modification of 47S rRNA processing rate. Although the response to p110β inhibition exhibited by this process was quite low, it was mirrored by a slow decrease in cell proliferation. This suggests that a 30% decrease in rRNA synthesis might be sufficient to induce a progressive decrease in cell division. On the other hand, it could also suggest that RL95-2 cells are to a certain extent resistant to the treatment and that other factors may contribute. In a systematic study investigating the effects of KIN-193 on cell proliferation in a panel of 422 cancer cell lines, although a larger proportion of PTEN-deficient cell lines were responsive to this inhibitor compared to WT cell lines, an important subset of cell lines was resistant despite being PTEN negative (Ni et al., 2012). RL95-2 cells express high p110β protein levels, and this may influence the efficacy of the inhibitor. However, other factors may influence how this inhibitor acts on p110β in the nucleus.

Several key members of the PI3K pathway are found in the nucleolus. For example, mTORC1 is known to localise to the nucleolus and has been shown to regulate nucleolar function (Iadevaia et al., 2012). Nuclear AKT has also been shown to regulate rDNA transcription by activating the TIF-1 transcription factor (Nguyen and Mitchell, 2013). This would correlate well with our findings detecting the active form of AKT in the nucleolus. How the PI3K p110β and AKT are active in a nucleolar context (i.e. in a membraneless environment) is, however, unclear. That said, p110β is thought to have basal activity, which can be greatly increased in PTEN-deficient tumour cells (Jia et al., 2008). p110β, p85β and PTEN have been reported to form a trimeric complex, which lowers the activity of p110β (Rabinovsky et al., 2009). Loss of PTEN would disrupt this complex, which could be exacerbated by lower levels of p85β in certain cancer cells (see Fig. 1A). Whether this complex exists in the nucleolus is so far unknown. PTEN, as well as a long PTEN isoform (PTENβ), were detected in the nucleolus of HeLa cells (Li et al., 2014; Liang et al., 2017), but p85β has so far only been detected in the nucleoplasm (Kumar et al., 2011). In this study, we showed that both PtdIns(3,4,5)P_3 and p110β were localised in the nucleolar compartment, raising the possibility of p110β acting as a regulator of nucleolar functions in a kinase-dependent manner. Preliminary data showed the detection of the PtdIns(3,4,5)P_3 C38:4 molecular species from isolated nucleoli of RL95-2 cells by LC-MS/MS analyses (data not shown), which is consistent with the reported most common chemical form of fatty acyl chains for polyphosphoinositides (Anderson et al., 2016; Clark et al., 2011; Traynor-Kaplan et al., 2017). We showed that p110β contributed to the appearance of PtdIns(3,4,5)P_3 in the nucleolus in MEF cells. Indeed, the p110β kinase-inactive MEFs demonstrated a decrease in the level of PtdIns(3,4,5)P_3 nucleolar staining compared to that in WT MEFs. The kinase-inactive cells are PTEN positive, and the decrease in PtdIns(3,4,5)P_3 levels could be partly be due to the presence of PTEN. The PI3K substrate PtdIns(4,5)P_2 has also previously been detected in the nucleolus (Sobol et al., 2013; Yildirim et al., 2013), albeit in a small proportion (4%) compared to its major localisation in nuclear speckles or nuclear islets (Boronenkov et al., 1998; Osborne et al., 2001; Sobol et al.,

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2018), and has been shown to play a role in RNA polymerase I activity (Yildirim et al., 2013). PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 may positively contribute to different rRNA processes via their interaction with different nucleolar effector proteins. Finally, immunofluorescence staining indicated the presence of both total AKT and phosphorylated AKT in nucleoli. However, AKT may not be locally activated in the nucleolus by PtdIns(3,4,5)P_3. The presence of active AKT in the nucleolus may rather be dependent upon its cytoplasmic activation followed by its translocation to the nucleus, consistent with previous studies (Martelli et al., 2012). Nucleolar PtdIns(3,4,5)P_3 may indeed have other effector proteins. In line with this, we have recently mapped the nuclear PtdIns(3,4,5)P_3 interactome and identified a majority of proteins annotated to the nucleolus (Gavgani et al., 2020 preprint).

In conclusion, our findings demonstrate that RL95-2 endometrial cancer cells, high in nuclear PtdIns(3,4,5)P_3 and p110β levels, have high pre-rRNA levels. The proliferation of these cells was shown to be at least partly dependent upon the activity of p110β (Karlsson et al., 2017). This suggests that an increase in ribosome production can contribute to sustained cell proliferation and, subsequently, cancer progression. Nucleolar p110β would hence provide a mode of regulation of ribosome synthesis necessary for protein synthesis and, ultimately, cell division. p110β can therefore potentially increase tumour progression in EC cells by producing the nucleolar pool of PtdIns(3,4,5)P_3 and thereby increasing the biogenesis of ribosomes required for tumour growth. However, the exact molecular mechanisms by which PtdIns(3,4,5)P_3 or p110β can influence nucleolar function remains to be further explored.

MATERIALS AND METHODS

Reagents

Antibodies used in western blotting and immunostaining are listed in Table S1. The selective p110β inhibitors TGX-221 (Cayman Chemical) (Jackson et al., 2005) and KIN-193 (Ni et al., 2012) (AZD6482, Selleckchem) were dissolved in DMSO at 10 mM and stored at −80°C. The final concentration used for these inhibitors was 10 µM. According to previous reports, the surviving fraction of 50% for both inhibitors (Weigelt et al., 2013) and the IC_{50} of KIN-193 in cell proliferation assays (Ni et al., 2012) is >10 µM in RL95-2 cells. The RNA polymerase I inhibitor, BMH21 (Selleckchem) was dissolved in DMSO at 1 mM and used at 1 µM (Peltonen et al., 2014). *PIK3CB*-targeting (sc-37269) siRNA, control non-targeting (sc-37269C) siRNA and transfection reagents were from Santa Cruz. The pGEX-4T1-EGFP–GRP1-PH plasmid was from Dr Julien Viaud (INSERM U1048, Toulouse, France).

Protein expression and purification

The pGEX-4T1-EGFP–GRP1-PH plasmid was transformed into *E. coli* BL21-RIL DE3. The bacteria were grown at 37°C and further induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside overnight at 18°C. Bacterial pellets were lysed in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton-X100, 5 mM DTT and 10% glycerol supplemented with protease inhibitor cocktail (Sigma) and 0.5 mg/ml lysozyme, for 30 min on ice. Following sonication and centrifugation (13,000 ***g***, 15 min at 4°C) of the lysates, GST–EGFP–GRP1-PH was purified from the supernatant using glutathione-agarose 4B beads, analysed by SDS-PAGE and Coomassie staining.

Cell lines, cell culture conditions and cell counts

Cancer cell lines were obtained from ATCC (KLE, RL95-2), DSMZ Germany (MFE-296, MFE-319, EFE-184 and MFE-280) and Sigma-Aldrich (Ishikawa). EM-E6/E7-hTERT (EM), a non-transformed endometrial cell line isolated from glandular endometrial tissue and immortalised with E6/E7 and human TERT (Kyo et al., 2003; Mizumoto et al., 2006), was a gift from Professor Pamela M. Pollock (Queensland University of Technology, Brisbane, Australia). All cells were authenticated by short tandem repeat DNA profiling (IdentiCell Service, Dept. Molecular Medicine, Aarhus University Hospital, Denmark for all cancer cell lines and MD Anderson Cancer Center, USA for EM cells), as previously described (Karlsson et al., 2017). p110β$^{D931A/D931A}$ kinase-inactive and p110β$^{WT/WT}$ MEFs immortalised by Shp53 were from Dr Julie Guillermet-Guibert (Université Toulouse III-Paul Sabatier, Toulouse, France; Guillermet-Guibert et al., 2015). All EC cells, HeLa and MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% foetal bovine serum (FBS; Sigma) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). EM cells were cultured in DMEM/Ham's F12 (Sigma) supplemented with Insulin-Transferrin-Selenium, 10% FBS and antibiotics and changed to DMEM containing 10% FBS and antibiotics 24 h before harvest. Actively growing cells were harvested when they reached a maximum of 80% confluence and received fresh growth medium the day before harvest. Cell counts were performed as previously described (Karlsson et al., 2017).

Cell synchronisation

Cells grown to 70% confluence were treated with 50 ng/ml of nocodazole (Sigma) for 16 h. The mitotic cells were collected by mechanical shake-off (repeated twice). The cells were pelleted by centrifugation at 70 ***g*** for 5 min. After washing the pellet twice with 10 ml of growth medium, the cell pellet was plated on coverslips covered with poly-L-lysine. The cells were collected at different time points after re-plating.

Whole-cell extract preparation and subcellular fractionation

Whole-cell extracts were prepared in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl pH 8.0, 0.5% deoxycholic acid, 150 mM NaCl, 1% NP-40 and 0.1% SDS) supplemented with 5 mM NaF, 2 mM Na_3VO_4 and 1× protease inhibitor cocktail (Sigma). Nuclear fractionation was carried out according to O'Carroll et al. (2009), and nuclear pellets were lysed in RIPA buffer. RL95-2 cells required an additional syringing step of the nuclear pellet resuspended in wash buffer (10 mM Tris-HCl pH 7.5 and 2 mM $MgCl_2$) to avoid cytoplasmic contamination. The nucleolar fractionation was performed according to the protocol described by Lam and Lamond (2006) with minor changes. In brief, cells were grown in 10×15 cm dishes up to 70% confluence. Fresh medium was added to the cells 1 h prior to fractionation. Cells were trypsinised and washed three times with cold phosphate-buffered saline (PBS). The cell pellet was collected by centrifugation and resuspended in 5 ml of buffer A containing 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1% Igepal and protease inhibitor cocktail. After 5 min of incubation on ice, the cells were syringed 16 times using a 23-gauge needle. After centrifugation at 200 ***g*** for 5 min at 4°C, the supernatant was collected as the cytosolic fraction and the nuclear pellet was resuspended in 3 ml of buffer S1 (0.25 M sucrose, 10 mM $MgCl_2$ and protease inhibitor cocktail). The suspension was layered over 3 ml of buffer S2 (0.35 M sucrose, 0.5 mM $MgCl_2$ and protease inhibitor cocktail) and centrifugation was performed at 1400 ***g*** for 5 min at 4°C. The pellet was then resuspended in 3 ml of buffer S2 and sonicated (seven times: 10 s on, 10 s off) on ice. The lysate was then layered over 3 ml of buffer S3 (0.88 M sucrose, 0.5 mM $MgCl_2$ and protease inhibitor cocktail), and centrifugation was performed at 3000 ***g*** for 10 min at 4°C. The top layer was collected as the nucleoplasmic fraction, and the pellet, which contained the nucleoli, was washed once with 500 µl of buffer S2 and centrifuged (1430 ***g***, 5 min at 4°C). Nucleolar pellets were resuspended in RIPA buffer.

Western blotting

Protein concentrations were measured using the bicinchonic acid protein assay kit (Thermo Fisher). Equal amounts of proteins (30–50 µg) were resolved on denaturing SDS-polyacrylamide gels, immunoblotted on to 0.45-µm-thick nitrocellulose membranes and detected by enhanced chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher) and visualised using a BioRad ChemiDocTM Xrs+.

RNA extraction and quantitative RT-PCR

Cells were pelleted, washed two times with PBS and resuspended in 1 ml TriReagent (Sigma) then incubated at room temperature for 5 min. 200 µl of

chloroform was added, mixed vigorously, incubated at room temperature for 1 min and centrifuged at 12,000 ***g*** and 4°C for 15 min. Phenol-chloroform-isoamyl alcohol mixture (Sigma) was added (500 µl) to the upper phase, mixed, incubated at room temperature for 2 min and centrifuged at 12,000 ***g*** and 4°C for 10 min. Chloroform (500 µl) was added to the upper phase, mixed, incubated at room temperature for 1 min and centrifuged at 12,000 ***g*** and 4°C for 10 min. 20 µg of RNA-grade glycogen (Thermo Fisher Scientific) and 500 µl isopropanol were added to the upper phase, mixed and incubated at room temperature for 20 min before centrifuging at 13,000 ***g*** and 4°C for 20 min. The pellet was resuspended in 1 ml of ice-cold ethanol (70%) and centrifuged (at 8000 ***g*** and 4°C) for 5 min. The extracted RNA was dissolved in RNase-free water for RT-qPCR analysis. cDNA was generated from 1 µg of RNA using random primers, according to the protocol from the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), with RNase Inhibitor.

Real-time qPCR

Real-time qPCR was performed from three biological replicates in triplicates on a Roche Light Cycler 480 using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific or Life Sciences). The reaction mix contained 10 µM of each primer and 1 ng of cDNA. The primers used for the target human 47S rRNA allow the amplification of the sequence (between +302 and +548) spanning the first cleavage site positioned at +414 within the 5′ external transcribed spacer region: 5′-GTGCGTGTCAGGCGTTCT-3′ and 5′-GGGAGAGGAGCAGACGAG-3′ (Popov et al., 2013). The human β-actin (*ACTB*) was used as a reference gene and the following primers were used for its amplification: 5′-TGCGTCTGGACCTGGCTG-GC-3′ and 5′-GCCTCAGGGCAGCGGAACC-3′ (Gabriel et al., 1998). The cycling parameters used were those from the manufacturer's instructions. 58°C was used for the annealing step, and 42 cycles were performed. Calibration curves based on five serial cDNA amounts (0.016, 0.08, 0.4, 2 and 10 ng/µl) were used for calculation of the reaction efficiencies. The 47S rRNA expression was normalised to that of *β-ACTIN* using to the $C_T(N^{-\Delta\Delta Ct})$ method, where N represents the primer efficiency, which was measured in each experiment.

Lipid extraction from nuclear fractions

Following cell fractionation, the nuclear pellets were resuspended in nuclear resuspension buffer (10 mM Tris-HCl pH 7,4, 1 mM EGTA, 1,5 mM KCL, 5 mM $MgCl_2$ and 320 mM sucrose), and the number of nuclei was counted. Lipids were extracted from each nuclear fraction using a method adapted from Gray et al. (2003). Nuclei were incubated in 1 ml MeOH:$CHCl_3$ (2:1) to extract neutral lipids for 10 min at room temperature with shaking at 1200 rpm and vortexing three or four times. The samples were centrifuged at 3000 ***g*** for 5 min at 4°C, supernatants were discarded and the same procedure was repeated. The acidic lipids were then extracted with 750 µl MeOH:$CHCl_3$:0.1 M HCl (80:40:1) and incubated for 15 min at room temperature and vortexed four times during the incubation followed by centrifugation at 3000 ***g*** for 5 min at 4°C. The pellets were resuspended with 250 µl $CHCl_3$ and 450 µl 0.1 M HCl and centrifuged at 3000 ***g*** for 5 min at 4°C. After centrifugation, a phase split between the organic and aqueous phases was apparent. The organic phase (bottom phase) was collected in conical glass tubes and dried at 60°C under N_2) gas. Lipids were resuspended with 4–6 µl of MeOH:$CHCl_3$:H_2O (2:1:0.8) and vortexed for 30 s before being sonicated in an ice bath for 5 min and vortexed again for 30 s. Proteins were recovered from lipid extraction, and the protein concentration was estimated for validation of the fractionation by western blotting.

Lipid overlay assay

Lipids obtained from lipid extraction were spotted on Hybond-CExtra membranes, 2 µl at a time. The membranes were left to dry for 1 h at room temperature protected from light. The membranes were next blocked for 1 h at room temperature with the appropriate blocking buffer (1% fat-free milk in PBS pH 7.4) and further incubated with 0.5 µg/ml GST–GRP1-PH in the same buffer overnight at 4°C and protected from light. GST–GRP1-PH was expressed and purified as described previously (Lewis et al., 2011). The membranes were washed 6×5 min in PBS-T (PBS containing 0.05% Tween 20) and then incubated with anti-GST conjugated to HRP (1:30,000) in blocking buffer for 1 h at room temperature. The blots were washed 6×5 min with PBS-T. The signal was detected by ECL using the SuperSignal West Pico Chemiluminescent Substrate, or with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific), and detected with a BioRad ChemiDoc Xrs+. Lipid spot densitometry was quantified using ImageJ (NIH, Bethesda, MD).

Immunostaining and microscopy

Cells grown on coverslips were fixed with 3.7% paraformaldehyde in PBS for 10 min, washed thrice with PBS, permeabilised with 0.25% Triton X-100 in PBS for 10 min, blocked for 1 h with blocking buffer (3% BSA in PBS containing 0.05% Triton X-100) and incubated with primary antibodies diluted in blocking buffer overnight at 4°C. Samples were subsequently incubated with fluorescently-labelled secondary antibodies diluted in blocking buffer for 1 h at room temperature. For labelling with GFP–GRP1-PH, cells were blocked in 3% fatty-acid-free BSA and 0.05% Triton-X100 in PBS for 1 h at room temperature followed by incubation with 40 µg/ml of the probe in 1% fatty-acid-free BSA and 0.05% Triton-X100 in PBS for 2 h at room temperature. Washes were performed with 0.05% Tween-20 in PBS after antibody incubation. The coverslips were mounted in ProLong Gold Antifade reagent containing 4′,6-diamidino-2-phenylindole (DAPI) or without DAPI (in which case DNA labelling was performed using Hoechst 33342). Images were acquired with a Leica DMI6000B fluorescence microscope using 40× or 100× objectives, or a Leica TCS SP5 confocal laser-scanning microscope using a 63×/1.4 oil immersion lens. Images were processed with Leica application suite V 4.0 and Adobe Photoshop CC 2018.

Patient series and immunohistochemistry

Tissue was collected from patients diagnosed with endometrial cancer at Haukeland University hospital during the period 2001–2013 and included a total of 714 primary tumours (G1=267, G2=210, G3=237). Clinical data were collected as described previously (Berg et al., 2015; Tangen et al., 2014). The patient cohort used for p110β immunohistochemistry is described in detail in Tangen et al. (2014). This study was conducted in line with Norwegian legislation and international demands for ethical review, and was approved by the Norwegian Data Inspectorate, Norwegian Social Sciences Data Services and the Western Regional Committee for Medical and Health Research Ethics (REK 2009/2315, REK 2014/1907). Patients provided informed consent. TMA sections were stained and scored for p110β expression following a protocol previously described (Tangen et al., 2014). Briefly, three cylinders of 0.6 mm were retrieved from high tumour purity areas using a custom-made precision instrument (Beecher Instruments, Silver Spring, MD, USA) and mounted in a paraffin block. TMA sections (5 µm) were stained for p110β expression (ab151549) and scored visually by light microscopy using a 20× objective by two independent observers (C.K. and I.L.T.). Scoring was performed blinded for information regarding clinical characteristics and outcome for the cytoplasmic and the nuclear areas. A semi-quantitative and subjective scoring method was used, and a staining index was calculated as a product of the staining intensity score (0, no staining; 1, weak; 2, moderate; and 3, strong) and the area of positive tumour cells score (1≤10%, 2=10–50% and 3≥50%), leading to scores ranging from 0 to 9 independently for the cytoplasm and nuclei. The ratio between the nuclear and the cytoplasmic scores was then calculated, and the resulting values were thereafter divided in quartiles for analyses. The quartiles were named low, med/low, med/high and high.

Statistical analysis

For clinical samples, statistical analyses were performed using the software package SPSS 22 (SPSS Inc, Chicago, IL) and the values of $P<0.05$ were considered statistically significant. Correlations between groups were evaluated using the Mann–Whitney U test for continuous variables. For cell lines, Student's *t*-test was used.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.E.L.; Methodology: T.K., A.E.L.; Validation: F.M.G., A.P.M., V.S.A., D.C.T., S.N., K.S.; Investigation: F.M.G., T.K., I.L.T., A.P.M., V.S.A., S.N., K.S., C.K.; Resources: J.G.-G.; Writing - original draft: A.E.L.; Writing - review & editing: F.M.G., A.P.M., V.S.A., D.C.T., C.K., J.G.-G., A.E.L.; Visualization: F.M.G., T.K., C.K., A.E.L.; Supervision: A.E.L.; Project administration: A.E.L.; Funding acquisition: A.E.L.

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