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## EPIDERMAL GROWTH FACTOR STIMULATES TRANSLOCATION OF THE CLASS II PHOSPHOINOSITIDE 3-KINASE PI3K-C2 $\beta$ TO THE NUCLEUS

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## SUMMARY

Although the class II phosphoinositide 3-kinase enzymes PI3K-C2 $\alpha$  and PI3K-C2 $\beta$  act acutely downstream of cell surface receptors they have also been localized to nuclei in mammalian cells. As with the class I PI3K enzymes, the relationship between the pool of enzyme present in cytoplasm and nuclei remains poorly understood. In this study we test the hypothesis that PI3K-C2 $\beta$  translocates to nuclei in response to growth factor stimulation. Fractionating homogenates of quiescent cells revealed that less than 5% of total PI3K-C2 $\beta$  resides in nuclei. Stimulation with epidermal growth factor sequentially increased levels of this enzyme in the cytosol and then in nuclei. Using detergent treated nuclei, PI3K-C2 $\beta$  co-localized with lamin A/C in the nuclear matrix. This was confirmed biochemically and phosphoinositide kinase assay showed a statistically significant increase in nuclear PI3K-C2 $\beta$  levels and lipid kinase activity following epidermal growth factor stimulation. C-terminal deletion and point mutation of PI3K-C2 $\beta$  demonstrated that epidermal growth factor driven translocation to the nucleus is dependent on a sequence of basic amino acid residues (KxKxK) that form a nuclear localization motif within the C-terminal C2 domain. Furthermore, when this sequence was expressed as an EGFP fusion protein, it translocated fluorescence into nuclei with an efficiency dependent upon copy number. These data demonstrate that epidermal growth factor stimulates the appearance of PI3K-C2 $\beta$  in nuclei. Further, this effect is dependent on a nuclear localization signal present within the C-terminal C2 domain indicating its bimodal function regulating phospholipid binding and shuttling PI3K-C2 $\beta$  into the nucleus.

## INTRODUCTION

The analysis of signal transduction events in mammalian nuclei has a significant history [1]. Phosphatidylinositol (PtdIns), diacylglycerol (DAG) and phosphoinositide kinase activity have all been demonstrated in cell nuclei stripped of their nuclear envelope using buffer containing detergents [2;3]. Phosphoinositide levels inside nuclei appear to alter independently of their content in the plasma membrane however stimulation of cells with cytokines and chemokines like insulin like growth factor (IGF-I) results in the translocation of phospholipase C $\beta$ , protein kinase C $\alpha$  and protein kinase C $\beta$  into nuclei [3-5]. IGF-I stimulation also decreased the nuclear content of PtdIns(4,5)P<sub>2</sub> accompanied by an increase in DAG suggesting increased PLC activity [6;7]. DAG, phosphoinositides, phosphoinositide kinases [8] and PLC $\beta$  and PKC all associate with the nuclear matrix [9;10]. The significance of phosphoinositide 3-kinase (PI3K) activity in nuclei has recently attracted significant interest [11].

The large family of PI3K enzymes is divided into three classes termed I, II and III based on sequence similarity and lipid products they generate *in vitro* [12]. Class I and II PI3K enzymes, 3-phosphoinositides, their downstream target Akt/PKB and the 3-phosphoinositide phosphatase PTEN have all been described in nuclei [13;14] in addition to other 3-phosphoinositide binding proteins such as a 43-kDa PtdIns(3,4,5)P<sub>3</sub>-binding protein containing a zinc finger motif and two pleckstrin homology domains [15]. Translocation of class I PI3K activity to nuclei was originally described in response to NGF stimulation [16].

In mammalian cells the class II PI3K isoforms PI3K-C2 $\alpha$ , PI3K-C2 $\beta$  and PI3K-C2 $\gamma$  [17–19] act as downstream targets of growth factor [20–22], chemokine [23] and integrin receptors [24]. In addition to their role in acute receptor mediated signaling, PI3K-C2 $\alpha$  and PI3K-C2 $\beta$  have also been described in nuclei [25–28]. Immunofluorescence analysis revealed PI3K-C2 $\alpha$  associated with nuclear speckles [25]. In contrast, analysis of rat liver nuclei revealed that PI3K-C2 $\beta$  is present in the nuclear matrix during compensatory liver growth [26]. However, this localization of PI3K-C2 $\beta$  depends on the cell type, since in HL-60 leukocyte cell line the majority of PI3K-C2 $\beta$  activity was detected in nuclear envelope [27;28]. This localization appears to be functionally relevant since the expression and catalytic activity of PI3K-C2 $\beta$  increases in nuclei following partial hepatectomy [26]. Despite the different intra-nuclear localization, activation of PI3K-C2 $\beta$  in hepatocytes and HL-60 cells appears to be dependent on a calpain-mediated hydrolysis [26;28–30]. Calpain cleavage of the PI3K-C2 $\beta$  C-terminus is related to cell-cycle progression. In contrast, activation of PI3K-C2 $\beta$  in the nuclei of HL-60 differentiated with ATRA correlates with its phosphorylation on tyrosine [27]

The localization of PI3K-C2 $\alpha$  in nuclei was previously shown to be dependent on a lysine rich motif within the C-terminal C2 domain [25]. Furthermore, this sequence of 11 amino acid residues was sufficient to translocate both DsRed and GFP into nuclei where the staining was enriched in nuclear speckles. In contrast, the localization of the PI3K-C2 $\beta$  isoform has not been imaged in nuclei. In this study we demonstrate that epidermal growth factor stimulates translocation of PI3K-C2 $\beta$  from cytoplasmic membranes to the nuclear matrix. In addition, we confirm that this response is dependent on a KxKxK nuclear localization motif present in its C-terminal C2 domain.

## EXPERIMENTAL PROCEDURES

### cDNA constructs

The N-terminal EE tagged C-terminal C2 domain deletion mutant of PI3K-C2 $\beta$  (1-1464) was prepared by PCR based on an equivalent PI3K-C2 $\alpha$  cDNA construct [31]. The N-terminal EE tagged isolated C-terminal C2 domain of PI3K-C2 $\beta$  (1488-1608) was produced by PCR. The PI3K-C2 $\beta$  NLS point mutant was produced using the QuickChangeXL site directed mutagenesis kit (Stratagene, Amsterdam, Netherlands) using PI3K-C2 $\beta$  -pBluescript cDNA as template. The C-terminal fragment was then subcloned into pcDNA 3.0-EE tagged PI3K-C2 $\beta$ . cDNA encoding PI3K-C2 $\beta$  amino acid residues 1519-1551 containing the sequence KRKTKVARKTC was amplified by PCR and subcloned into the pEGFP-C1 vector (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) either as a single copy pEGFP-NLS or in tandem (pEGFP-2xNLS).

### Cell Culture

HEK293 cells were routinely passaged in DMEM containing 10% FBS and antibiotic-antimycotic (Invitrogen, Paisley UK) in a humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C. Cells were transiently transfected. After 48 hours cell pools expressing recombinant enzymes were propagated in the presence of 700  $\mu$ g/ml G418 (Invitrogen) as “pooled” cultures. In this way N-terminal EE epitope tagged C-terminal deletion mutant lacking the C2 domain, C2 domain alone, and the point mutated NLS PI3K-C2 $\beta$  cDNA constructs were expressed. In all cases levels of recombinant enzyme was verified by Western blotting using either anti-EE or anti-PI3K-C2 $\beta$  antibody. Cells that expressed N-terminal EE tagged wild-type PI3K-C2 $\beta$  were selected again as “pools” in the presence of continuous culture in G418 (700  $\mu$ g/ml) [32]. 786-O cells were passaged every 3-4 days in RPMI media (Invitrogen) containing 10% FBS and antibiotic-antimycotic (Invitrogen).

### Enrichment of Nuclei

Cell cultures were serum starved overnight before incubation in the absence or presence of EGF then lysed in hypotonic cell lysis buffer containing 0.1% Na-DOC and 1% Triton X-100. Nuclei were purified by low speed centrifugation (500 x g) over a 1.2 M sucrose cushion [3]. Enrichment of nuclei was verified using marker enzymes (leucine arylamidase, Na<sup>+</sup>-K<sup>+</sup>-ATPase, succinate: cytochrome C oxidoreductase, KCN-resistant NADH oxidoreductase and 5-nucleotidase) and electron microscopy [26-29;33]. Nuclear matrix was isolated from nuclei using DNase I and RNase A, and chromatin-associated proteins were released upon incubation with 2 M ammonium sulphate. Chromatin was extracted from nuclei using progressively hypotonic Tris buffer (50, 10, 2 and 0.4 mM). Cytosol and postnuclear membranes were prepared as previously described [33].

### Western blotting

Proteins extracts were quantified using BCA assay (Pierce, Perbio UK Science Ltd, Northumberland, UK) then extracted in sample buffer. Electrophoresis performed using mini-Protean II apparatus (Bio-Rad Laboratories Ltd, Hertfordshire UK) and 12% polyacrylamide gels. Proteins were transferred onto PVDF membrane (GE Healthcare, Amersham UK) and Western blotted using either rabbit anti-PI3K-C2 $\beta$  or mouse anti-EE epitope tagged antibodies as previously described [20] or anti- $\alpha$  tubulin antibody (Abcam plc Cambridge UK). Proteins were visualized using horseradish peroxidase conjugated secondary antibody and ECL reagent (GE Healthcare).

### Transfection

HEK293 cells were transiently transfected using the calcium phosphate method as previously described [32]. 786-O cells were transfected using Fugene HD (Qiagen Ltd, Crawley West Sussex UK) according to manufacturers instruction with a Fugene:cDNA ratio of 3:1.

### Immunofluorescence staining and confocal analysis

Following 5-8 hours transfection, cells were trypsinised and plated onto poly L-lysine treated coverslips in media containing 10% FBS. Cultures were then fixed for 1 hour at RT with 3.7% paraformaldehyde in PBS and permeabilised for 5 minutes with PBS containing 0.1% Triton X-100. Cells were then mounted in Vectorshield containing DAPI (Vector Laboratories Ltd, Peterborough, UK).

For immunostaining of isolated nuclei,  $1 \times 10^6$  nuclei were fixed in 90% methanol overnight at  $-20^\circ\text{C}$ . Nuclei were incubated to block non specific binding with 2% BSA in PBS for 60 min at room temperature, and then reacted with the primary antibodies diluted 1 : 50 for 2 hours at room temperature. Anti-lamin A/C antibody was purchased from Abcam plc. After three washing steps, primary antibody binding was visualized by incubating the specimens with fluorescein or Cy5-conjugated affinity pure secondary antibodies diluted at 1 : 500 in 1% BSA/PBS for 1 h at room temperature. Fluorescence was analyzed by confocal laser scanning microscopy using either a LSM 510 Meta (Carl Zeiss, Germany) or a SP5 (Leica Microsystems (UK) Ltd, Milton Keynes UK) system.

### Lipid Kinase Assay

PI3K-C2 $\beta$  kinase assays were performed as previously described in the presence of  $\text{Ca}^{2+}$  (6 mM) and PtdIns as substrate [20].

## RESULTS

Nuclei isolated from HEK293 cells stably expressing recombinant PI3K-C2 $\beta$  were enriched from cell homogenates prepared with buffer containing 1% Triton and 0.1% deoxycholate. These preparations were free from  $\alpha$ -tubulin confirming separation from cytoskeletal proteins and outer nuclear membranes (Figure 1A). Purity from residual endoplasmic reticulum was supported by electron microscopy and the absence of marker enzymes (data not shown). Western blotting and lipid kinase activity of anti-PI3K-C2 $\beta$  immunoprecipitates (Table 1) confirmed the presence of PI3K-C2 $\beta$  enzyme in nuclei but indicated that this organelle contained < 5% of its total cellular content.

Stimulation of HEK293 cells expressing recombinant PI3K-C2 $\beta$  with EGF (100 ng/ml) revealed a time dependent increase in the level of PI3K-C2 $\beta$  enzyme and enzyme activity in the nuclei (Figure 1B). Maximal increase in PI3K-C2 $\beta$  levels were obtained between 90-120 minutes after EGF stimulation and then declined slowly until 180 minutes. Repeating this approach but then differentially centrifuging homogenates showed a time dependent increase of PI3K-C2 $\beta$  in the cytosol that was clearly evident after 30 minutes (Figure 2). This preceded an increase in the level of PI3K-C2 $\beta$  in nuclei increasing after 60 minutes becoming maximal at 90 minutes and corresponded to decreases in PI3K-C2 $\beta$  enzyme activity in post-nuclear membranes. Immunofluorescence analysis performed with affinity purified anti-PI3K-C2 $\beta$



antibody revealed that PI3K-C2 $\beta$  co-localized with lamin A/C in the nuclear matrix (Figure 3, upper plates). Chromatin and nuclear matrix were also purified from nuclei harvested from cells treated in the absence or presence of 100 ng/ml EGF for 90 minutes. Immunoprecipitation of PI3K-C2 $\beta$  enzyme from each sub-nuclear fraction revealed that the catalytic activity of this enzyme in quiescent cells was almost eight times higher in the nuclear matrix than in the chromatin extract (Figure 3 lower panel). EGF stimulation increased PI3K-C2 $\beta$  lipid kinase activity by 45% in the nuclear matrix with no significant change in the chromatin compartment. These data were confirmed by Western blotting. Similar experiments were also performed using non-transfected HEK293 cells (Figure 4). After immunoprecipitation, the catalytic activity of endogenous enzyme could only be detected in the nuclear matrix. In quiescent cultures the PI3K-C2 $\beta$  enzyme activity in the nuclear matrix was  $(203 \pm 27 \text{ dpm PtdIns(3)P/ mg of protein})$  versus  $334 \pm 31 \text{ dpm of PtdIns(3)P/ mg of protein}$  following EGF stimulation (mean  $\pm$  S. E.  $n=3$  experiments, performed in duplicate). These data were confirmed by Western blotting. It is important to highlight that the activity of endogenous enzyme is about 100 times lower when compared to that of recombinant PI3K-C2 $\beta$  (Figure 3 lower panel).

It was demonstrated previously that translocation of PI3K-C2 $\alpha$  to nuclei was dependent on its C-terminal C2 domain and that a sequence of 11 amino acid residues served as a nuclear localization signal [25]. Sequence alignment of all three class II PI3K enzymes revealed a conserved KRKTKxxxK motif (residues 1530-1538 of PI3K-C2 $\alpha$ ). To establish if translocation of PI3K-C2 $\beta$  to the nucleus is mediated via this putative NLS, a C-terminal deletion mutant ( $\Delta$ C2) was made together with a cDNA construct containing 3 point mutations altering residues K<sub>1530</sub>M, K<sub>1532</sub>Q and K<sub>1534</sub>I (Figure 5, upper plate). Each PI3K-C2 $\beta$  mutant was transiently expressed in HEK293 cells and these cultures were incubated in the absence or presence of EGF. Western blotting of corresponding cell lysates revealed that neither the  $\Delta$ C2 or point mutated PI3K mutant was detected in nuclei of either quiescent or EGF stimulated cells (Figure 5, middle plate). Furthermore, when the isolated C2 domain of PI3K-C2 $\beta$  was expressed in HEK 293 cells this also was present in nuclei, but EGF stimulation did not further increase its levels (Figure 5, lower plate).

786-O cells were transfected with cDNA plasmid to express EGFP, or EGFP fusion proteins containing a single copy of the PI3K-C2 $\beta$  nuclear localisation motif KRKTKVARKTC (residues 1530-1540) or two copies in tandem (2xNLS) (Figure 6). Whilst EGFP was distributed across the entire cell Figure 6 A-C, a single copy of the PI3K-C2 $\beta$  NLS sequence markedly enriched EGFP fluorescence in nuclei (Figure 6 D-F). This was further exaggerated when 2 copies of the NLS were expressed in tandem since in these cells fluorescence was almost entirely contained within nuclei (Figure 6 G-I).

A schematic representation of our data is shown in Figure 7. We propose that in quiescent cells PI3K-C2 $\beta$  is primarily localized either to endosomes and plasma membrane. Following EGF stimulation, the activated EGF receptor and PI3K-C2 $\beta$  travel along the endocytic pathway. Given that EGF stimulation increases the content of PI3K-C2 $\beta$  in the cytosol, it either dissociated from EGF receptor containing endosomes (A) or it is liberated directly from membranes (B). From the cytosol, PI3K-C2 $\beta$  is transported into the nucleus by an as yet unknown mechanism that may involve its association with an importin complex.

## DISCUSSION

In contrast to class I and III PI3K enzymes class II PI3K enzymes have a significantly higher molecular mass extended both at their N- and C-terminus. Despite sequence similarity with the class I enzymes, class II PI3Ks lack motifs that would allow their binding to either a p85 or p101-like adaptor [34] and an alternative dedicated regulatory binding protein has not been reported. Instead, class II PI3K enzymes possess an extended N-terminus that contains a coiled-coil domain, proline-rich sequences and a clathrin binding motif [21;35]. The proline-rich motifs of PI3K-C2 $\beta$  mediate its association with the activated EGF receptor via Grb-2 and it exists in complex with Shc and Eps8/Abi1/Sos1 [21;36]. Both PI3K-C2 $\alpha$  and PI3K-C2 $\beta$  associate with clathrin thus providing a link between receptor mediated intracellular signaling and clathrin mediated vesicle transport [35]. The C-terminus contains two regulatory domains termed Phox homology (PX) domain [37] and a C2 domain [38]. Both the PX and C2 domains are likely to contribute to phospholipid binding and although their deletion did not alter subcellular localization of PI3K-C2 $\alpha$  [31] the C2 domain contributes to regulating catalytic activity [39].

In contrast to the class I PI3K enzymes that have been extensively studied downstream of cell surface receptors and in nuclei, little is currently understood about the physiological relevance of class II PI3K enzymes [40]. However, class II PI3K enzymes have also been described in the nucleus although how they are imported or what role they serve in nuclei is unclear [26-29]. The best understood system for the transport of macromolecules between the cytoplasm and the nucleus is the classical nuclear import pathway, where proteins containing a negatively charged consensus NLS are imported by a heterodimeric import receptor [41]. This complex consists of the  $\beta$ -karyopherin importin  $\beta$ , which mediates interaction with nuclear pore complex and the adaptor protein importin  $\alpha$ , which directly binds the NLS. Our data demonstrate that EGF stimulation initially leads to the appearance of PI3K-C2 $\beta$  in the cytosol followed by its increased levels in the nucleus (Figure 2 and Table 1). Specifically in both naïve HEK293 cells and cells expressing recombinant PI3K-C2 $\beta$  enzyme, PI3K-C2 $\beta$  is localized in the nuclear matrix with minimal levels present in chromatin (Figure 3 and 4). This mitogen stimulated sequential translocation implicates the action of an import complex in the passage of class II PI3K enzymes from cytosol into nuclei. We demonstrate that the motif of basic amino acid residues within the C2 domain of class II PI3K enzymes proposed by Didichenko and Thelen [25] to serve as a NLS in PI3K-C2 $\alpha$  is required to localize PI3K-C2 $\beta$  in nuclei (Figure 5 and 6). Both deletion of the C2 domain or point mutation of three lysine residues (K1530M, K1532Q and K1534I) precluded constitutive and EGF driven localization of PI3K-C2 $\beta$  in nuclei. Furthermore, expression of the PI3K-C2 $\beta$  nuclear localization sequence as an EGFP fusion localized EGFP fluorescence to nuclei consistent with copy number.

Although, both PI3K-C2 $\alpha$  and PI3K-C2 $\beta$  have been identified in nuclei, PI3K-C2 $\alpha$  was localised by immunofluorescence to nuclear speckles [25], whilst PI3K-C2 $\beta$  was localized biochemically to the nuclear matrix [29] as confirmed by our immunofluorescence staining (Figure 3). We report less than 5% of cellular PI3K-C2 $\beta$  localized to the cell nucleus. This value is consistent with the small proportion of total class IA PI3K enzyme content that is recruited to plasma membrane following growth factor receptor stimulation [42].



The lysine rich motif that serves as a NLS is localized to the  $\beta 4$  front  $\beta$ -sheet of C-terminal C2 domain [38]. Liposome binding assays demonstrate that the C2 domain of PI3K-C2 $\alpha$  binds PtdIns(3,4)P<sub>2</sub> and PtdIns(4,5)P<sub>2</sub> with similar affinity. Furthermore, its phosphoinositide binding ability is dependent on three lysine residues (K1420, K1432, and K1434). Importantly, K1432, and K1434 contribute to the NLS. This infers that the C2 domain can either be used for phosphoinositide binding or nuclear transport. Alternatively, translocation of class II PI3K enzymes to the nucleus may involve a PtdIns(3,4)P<sub>2</sub> and/or a PtdIns(4,5)P<sub>2</sub> dependent mechanism. Metabolic labeling of inositol phospholipids and direct mass measurements has consistently failed to detect PtdIns(3,4)P<sub>2</sub> in cell nuclei favoring a role for PtdIns(4,5)P<sub>2</sub> [3;26-29;43]. Phosphoinositide dependent partitioning of phosphoinositide binding proteins between the nucleus and cytosol has been previously demonstrated. Phospholipase C- $\delta 1$  accumulates in the nucleus at the G(1)/S boundary and in G(0) phase of the cell cycle by a mechanism that is dependent on binding of its PH domain to PtdIns(4,5)P<sub>2</sub> [44].

Although in membrane compartments across the cytoplasm levels of PtdIns(4,5)P<sub>2</sub> greatly exceed PtdIns(3,4)P<sub>2</sub>, the timing of EGF stimulated increase in PI3K-C2 $\beta$  to nuclei cannot preclude a role for PtdIns(3,4)P<sub>2</sub> especially if presented by a nuclear import protein complex. Given the degree of sequence similarity (Figure 5), the NLS does not appear to determine the differential localization of PI3K-C2 $\alpha$  and PI3K-C2 $\beta$  to nuclear speckles and nuclear matrix respectively. Calpain activity on class II PI3K enzymes cleaves their C-terminal C2 domain [24]. Although this action increases catalytic activity of PI3K-C2 $\beta$  [39] potentially it could also serve to shuttle class II PI3K enzymes out of nuclei. However, calpain mediated hydrolysis of PI3K-C2 $\beta$  did not appear to alter its localization to nuclear matrix of rat hepatocytes [29] suggesting that another mechanism is also responsible for tethering this enzyme inside nuclei.

Our data demonstrate a sequential translocation of the class II PI3K-C2 $\beta$  enzyme from post-nuclear membranes to cytosol and nuclear matrix following EGF stimulation (Figure 2 and 7). A deletion mutant and expression of EGFP fusion proteins confirmed that both basal and EGF stimulated localization of PI3K-C2 $\beta$  in nuclei is dependent upon the C-terminal C2 domain and point mutation confirmed the role of the KxKxK lysine rich motif located within the phosphoinositide binding pocket. Whilst the relationship between nuclear import and phosphoinositide binding warrants further investigation we propose that the C-terminal C2 domain of class II PI3K enzymes plays a key regulatory role in enzyme localization to nuclei following growth factor stimulation.

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**Table 1.** PI3K-C2 $\beta$  enzyme levels increase in the nuclei of HEK293 cells following EGF stimulation.

	Control	EGF
Cytosol	199 $\pm$ 37	506 $\pm$ 72*
Nuclei	613 $\pm$ 77	1224 $\pm$ 91*
Postnuclear membranes	13336 $\pm$ 317	12038 $\pm$ 297*
Cell lysate	14417 $\pm$ 372	14235 $\pm$ 421

HEK 293 cells expressing recombinant PI3K-C2 $\beta$  were stimulated with EGF (100 ng/ml) for 90 minutes. After this time, PI3K-C2 $\beta$  was immunoprecipitated from the subcellular compartments and catalytic activity quantified using an in-vitro kinase assay with PtdIns as substrate. Data are expressed as dpm of PtdIns(3)P/ 100  $\mu$ g of protein and represent the mean  $\pm$  S. E. of three independent experiments, each performed in duplicate. \*,  $p < 0.05$  (Student's t-test) with respect to the control.

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## FIGURE LEGENDS

### FIGURE 1. EGF stimulates the translocation of PI3K-C2 $\beta$ to nuclei.

**Panel A** - Total cell lysate (TCL) and nuclei isolated in the presence of either 1% Triton X-100 (T) or 1% Triton X-100 and 0.1% deoxycholate (T+D) were prepared from HEK293 cells expressing recombinant PI3K-C2 $\beta$  enzyme. TLC (10  $\mu$ g protein) and nuclear extract (50  $\mu$ g protein) were then fractionated by SDS-PAGE, and Western blotted with anti-PI3K-C2 $\beta$  and  $\alpha$ -tubulin antibodies.

**Panel B** - HEK 293 cells expressing recombinant PI3K-C2 $\beta$  were incubated in the absence (squares) or presence (circles) of 100 ng/ml EGF for the times indicated. Cell nuclei were isolated and fractionated (50  $\mu$ g protein) by SDS-PAGE, then Western blotted with anti-PI3K-C2 $\beta$  antibody (upper plate) or PI3K-C2 $\beta$  immunoprecipitated and a lipid kinase assay performed using PtdIns as substrate (lower plate). Data are represented as mean  $\pm$  S. E. for three independent experiments, each performed in duplicate. \*,  $p < 0.05$  (Student's t-test) with respect to the control.

### FIGURE 2. EGF stimulates sequential translocation of PI3K-C2 $\beta$ from membrane to cytosol and nuclei.

HEK 293 cells expressing recombinant PI3K-C2 $\beta$  were stimulated with EGF (100 ng/ml) for the times shown. From harvested cells, either total cell lysates were prepared or fractionated by differential centrifugation to enrich cytosol, nuclei and postnuclear membranes. Proteins in lysate and post-nuclear membranes (10 $\mu$ g) and cytosol and nuclei (50 $\mu$ g) were fractionated by SDS-PAGE then Western blotted with anti-PI3K-C2 $\beta$  antibody.

### FIGURE 3. PI3K-C2 $\beta$ co-localizes with lamin A/C in the nuclear matrix of HEK293 cells.

**Upper panel** - Nuclei were isolated from HEK293 cells expressing recombinant PI3K-C2 $\beta$  using buffer containing 1% Triton and 0.1% deoxycholate. Enriched nuclei were fixed in methanol, and incubated with antibody to either PI3K-C2 $\beta$  (A) or lamin A/C (B). After incubation with anti rabbit-FITC (green) or anti-mouse-Cy5 (red) coupled secondary antibody respectively, staining was examined by confocal microscopy. Co-localization of both proteins is indicated as yellow in the overlaid image (C).

**Lower panel** - HEK293 cells expressing recombinant PI3K-C2 $\beta$  were incubated in the absence (solid bars) or presence (open bars) of 100 ng/ml EGF for 90 minutes. After this time, both chromatin (Chr) and nuclear matrix (NM) were enriched from isolated nuclei. Proteins (50  $\mu$ g) were fractionated by SDS-PAGE and Western blotted with anti-PI3K-C2 $\beta$  antibody (right plate). PI3K-C2 $\beta$  was immunoprecipitated from chromatin and nuclear matrix and its kinase activity quantified using PtdIns as substrate. Data are represented as mean  $\pm$  S. E. for three independent experiments, each performed in duplicate (left plate). \*  $p < 0.05$  (Student's t-test) with respect to the control.

### FIGURE 4. PI3K-C2 $\beta$ translocates to the nuclear matrix in naive HEK293 cells.

Serum starved HEK293 were incubated in the absence (solid bar) or presence (open bar) of EGF (100 ng/ml) for 90 minutes. After this time, chromatin (Chr) and nuclear matrix (NM) were enriched from isolated nuclei and aliquots of each fraction immunoprecipitated with anti-PI3K-C2 $\beta$  antibody. Lipid kinase activity was only detected in immunoprecipitates of the nuclear matrix (Figure 4, upper panel). Data represent mean  $\pm$  S. E. for three independent experiments, each performed in duplicate. PI3K-C2 $\beta$  enzyme was visualized by Western blotting after SDS-PAGE fractionation (Figure 4, lower panel).

**FIGURE 5. The C-terminal C2 domain of PI3K-C2 $\beta$  contains an EGF responsive nuclear localization signal.**

**Upper panel** – The putative nuclear localization signal identified in the C-terminus of PI3K-C2 $\alpha$  is aligned with the equivalent region in the other human class II PI3K enzymes. Stars denote sequence identity, dots sequence similarity. Details of point mutations introduced by site directed mutagenesis into cDNA sequence encoding the putative nuclear localization signal consensus (KxKxK).

**Middle panel** - HEK293 cells were transiently transfected to express either recombinant PI3K-C2 $\beta$  (WT), a PI3K-C2 $\beta$  C2 domain deletion mutant ( $\Delta$ C2), or a construct containing point mutation of the nuclear localization signal (NLS MUT). Cells were incubated in the absence or presence of 100 ng/ml EGF for 90 minutes. After this time, nuclei were isolated and cell lysates prepared. Proteins were fractionated by SDS-PAGE and Western blotted with anti-PI3K-C2 $\beta$  antibody. Cell lysates (10  $\mu$ g protein) and nuclei (50  $\mu$ g).

**Lower panel** - HEK293 cells were transiently transfected to express EE-tagged PI3K-C2 $\beta$ - C-terminal C2 domain. Cells were incubated in either the absence (-) or presence (+) of 100 ng/ml EGF for 90 minutes. After this time, lysate (10  $\mu$ g protein) or nuclei (50  $\mu$ g protein) were fractionated using 12 % SDS-PAGE and Western blotted with anti-EE epitope tagged antibody.

**FIGURE 6. The putative PI3K-C2 $\beta$  nuclear localization signal translocates EGFP to nuclei .** 786-O cells were transfected with either pEGFP-C1 vector (A-C), plasmid encoding pEGFP-PI3K-C2 $\beta$ <sub>(1519-155)</sub> fusion that contains the putative KRKTKVARKTC nuclear localization sequence (NLS) (D-F) or a pEGFP-2xNLS cDNA plasmid (G-I). After 5 hours, cells were plated onto glass coverslips. After 24 hours, cultures were fixed then permeabilised and cells mounted in media containing DAPI and visualized by confocal microscopy. EGFP fluorescence is shown in plates A, D and G, DAPI stained nuclei in plates B, E and H. The EGFP and DAPI staining was merged and the overlay shown in plates C, F and I.

**FIGURE 7. A schematic representation illustrating EGFR stimulated translocation from cell membranes into cytosol then nuclei.** In quiescent cultures PI3K-C2 $\beta$  is found highly associated with post-nuclear membranes, Following EGF stimulation PI3K-C2 $\beta$  can bind clathrin coated vesicles then dissociate into the cytosol (A) or it could dissociate directly from plasma membrane or endosomes (B). In the cytosol, PI3K-C2 $\beta$  could enter nuclei directly through nuclear pores or bound to an importin complex.

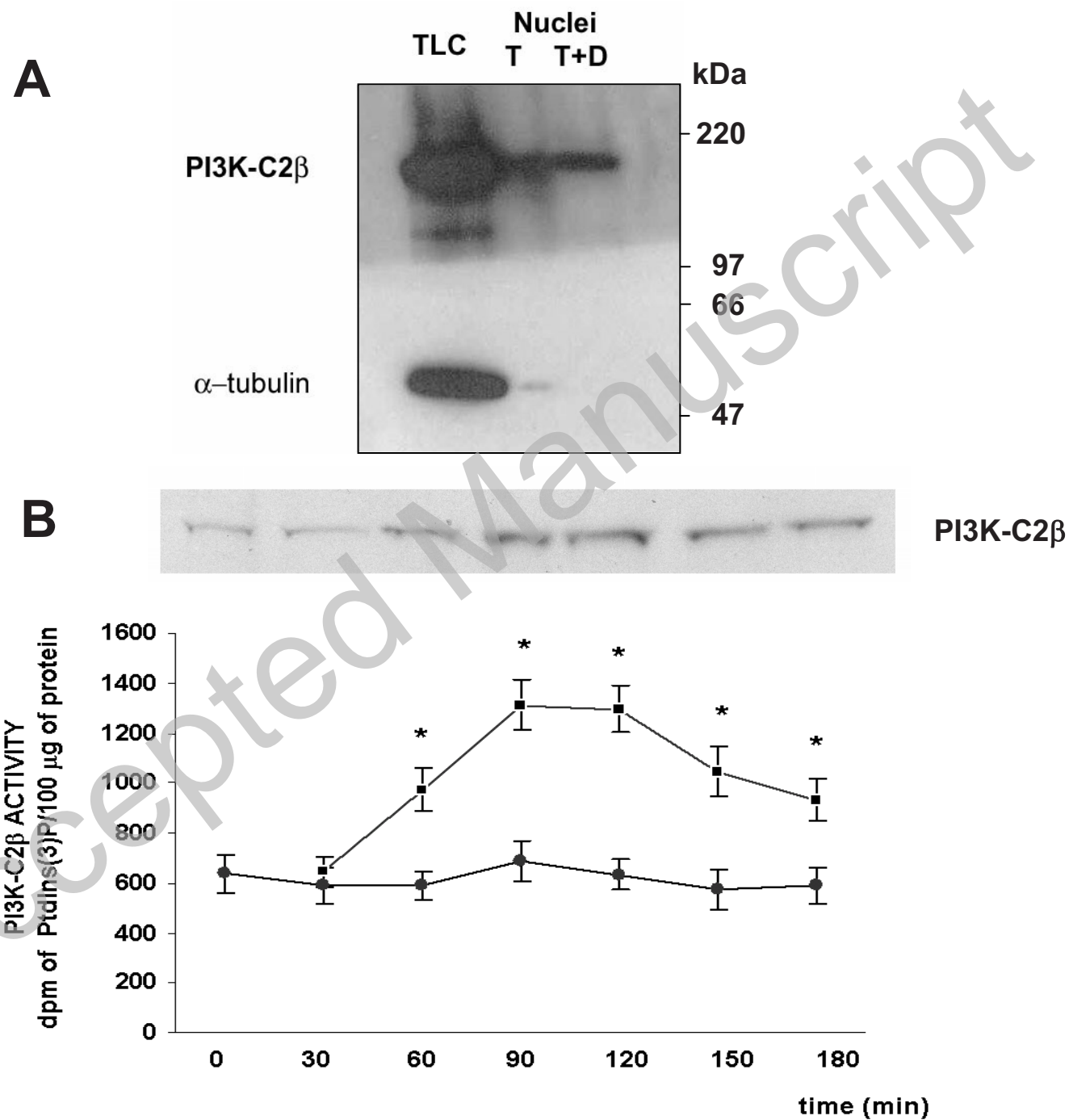
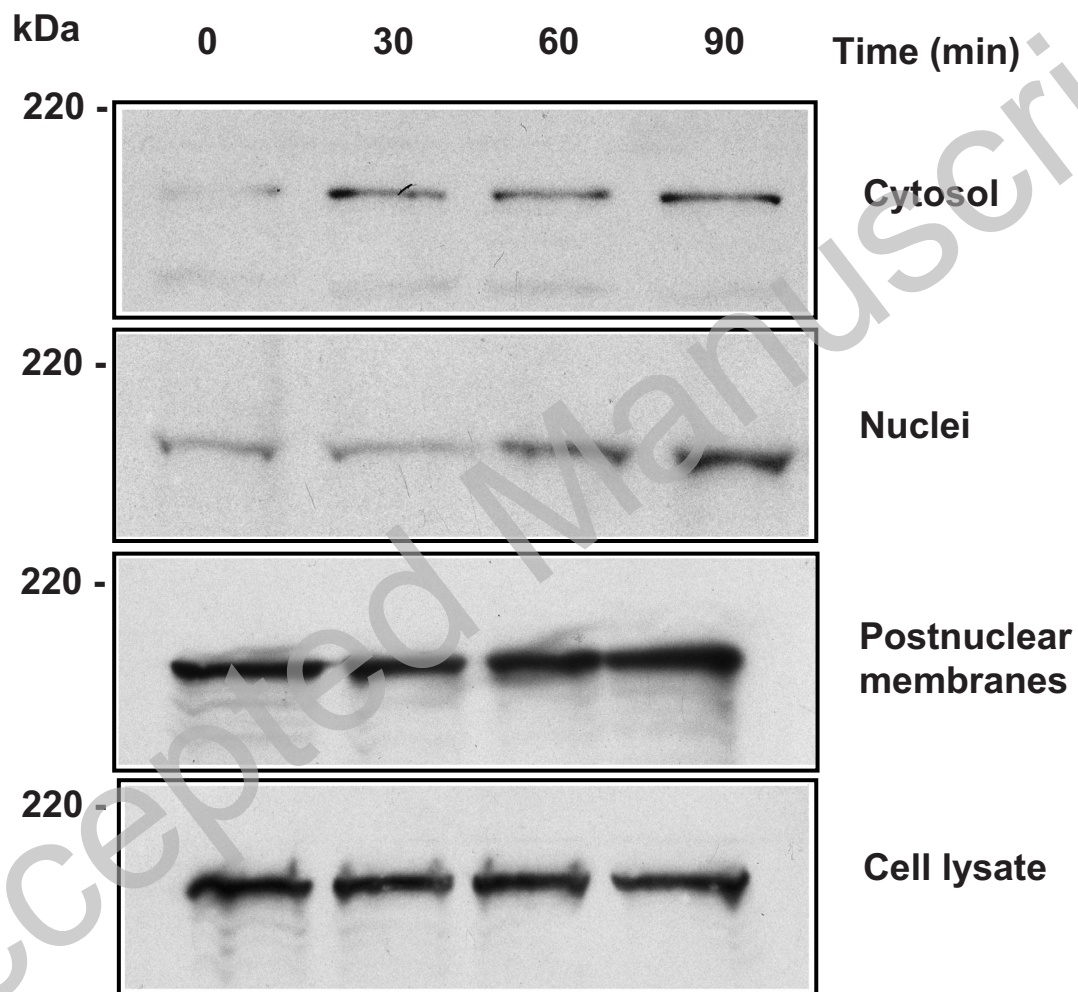


Figure 1

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**Figure 2**



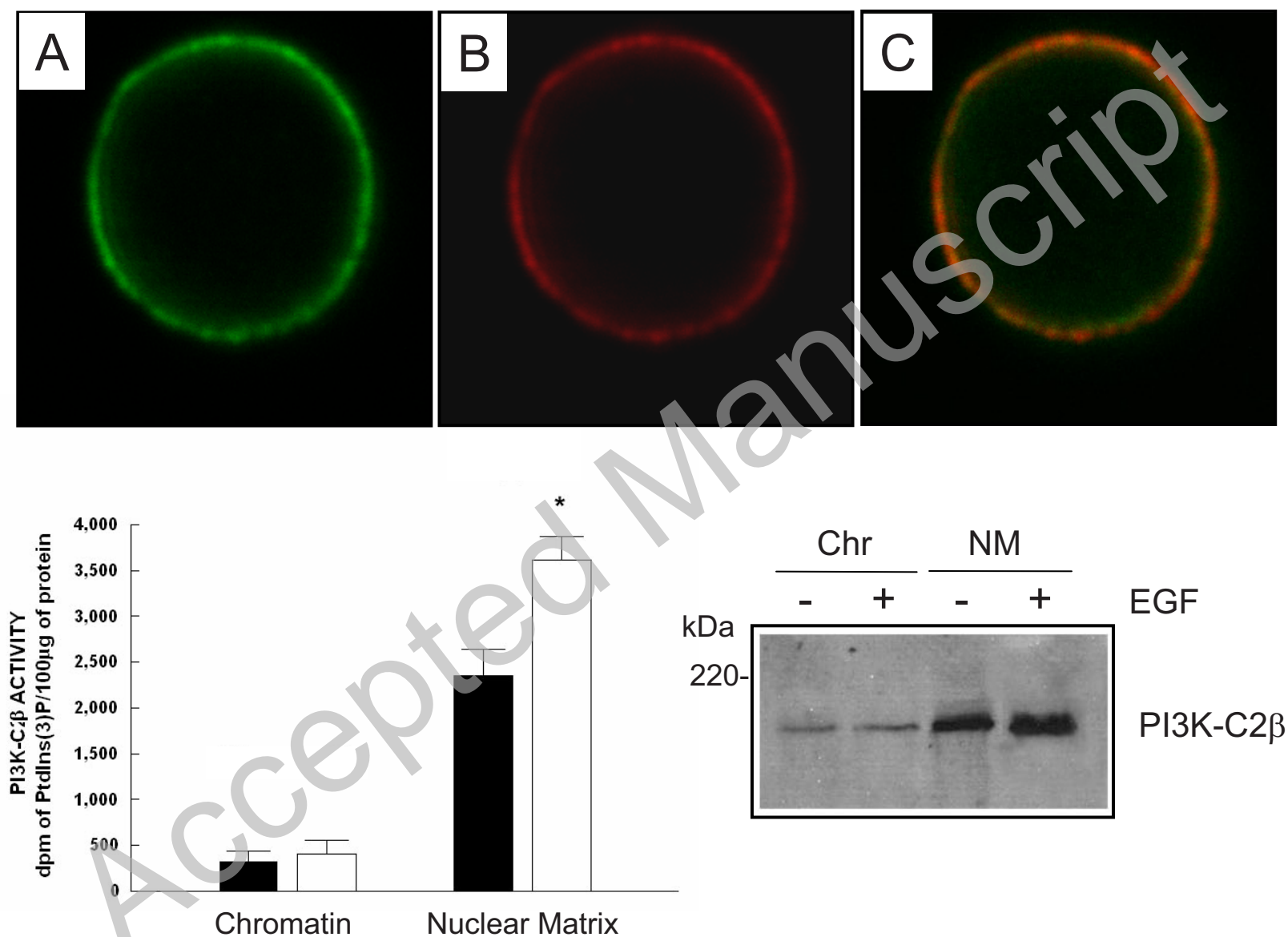


Figure 3

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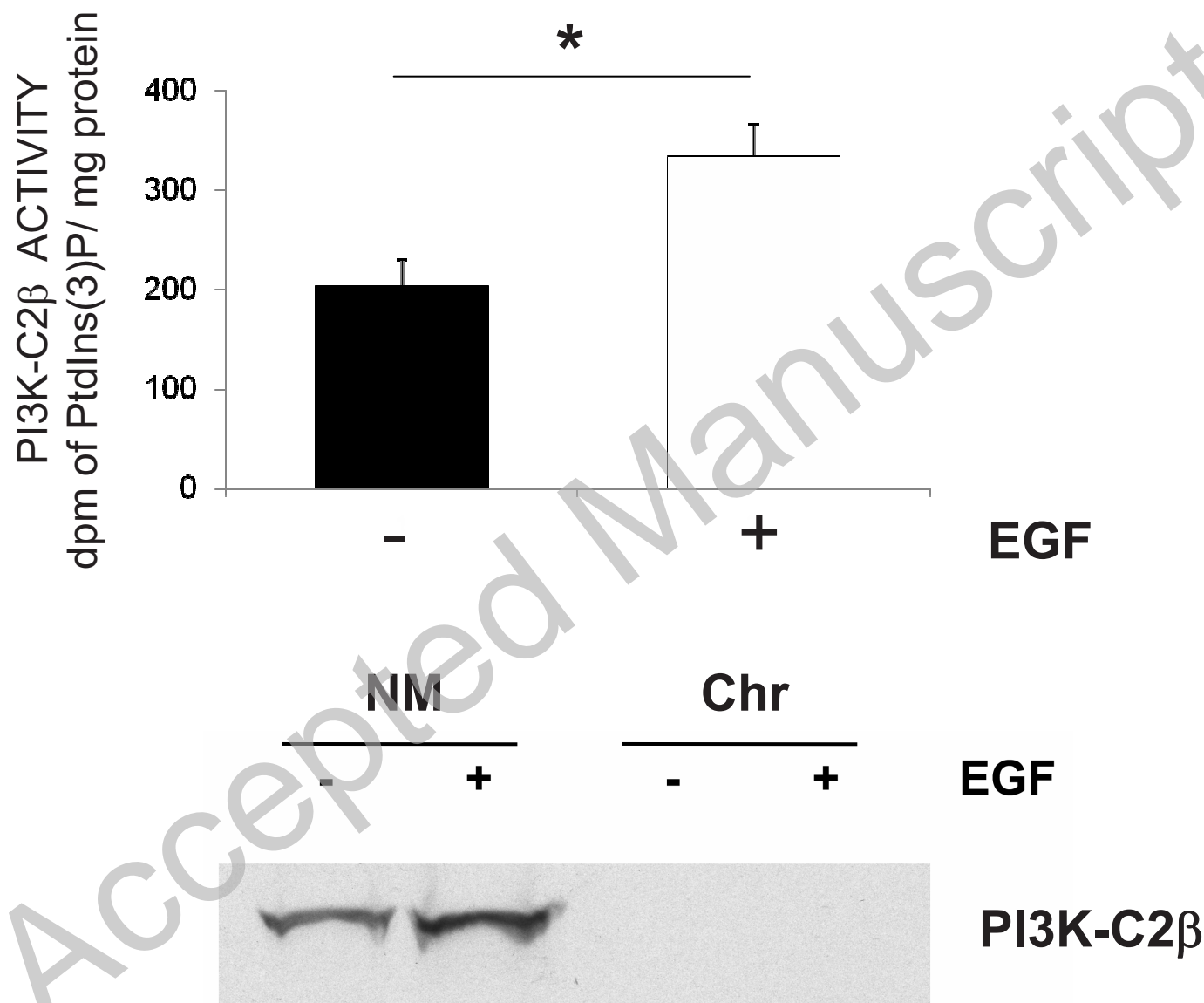


Figure 4

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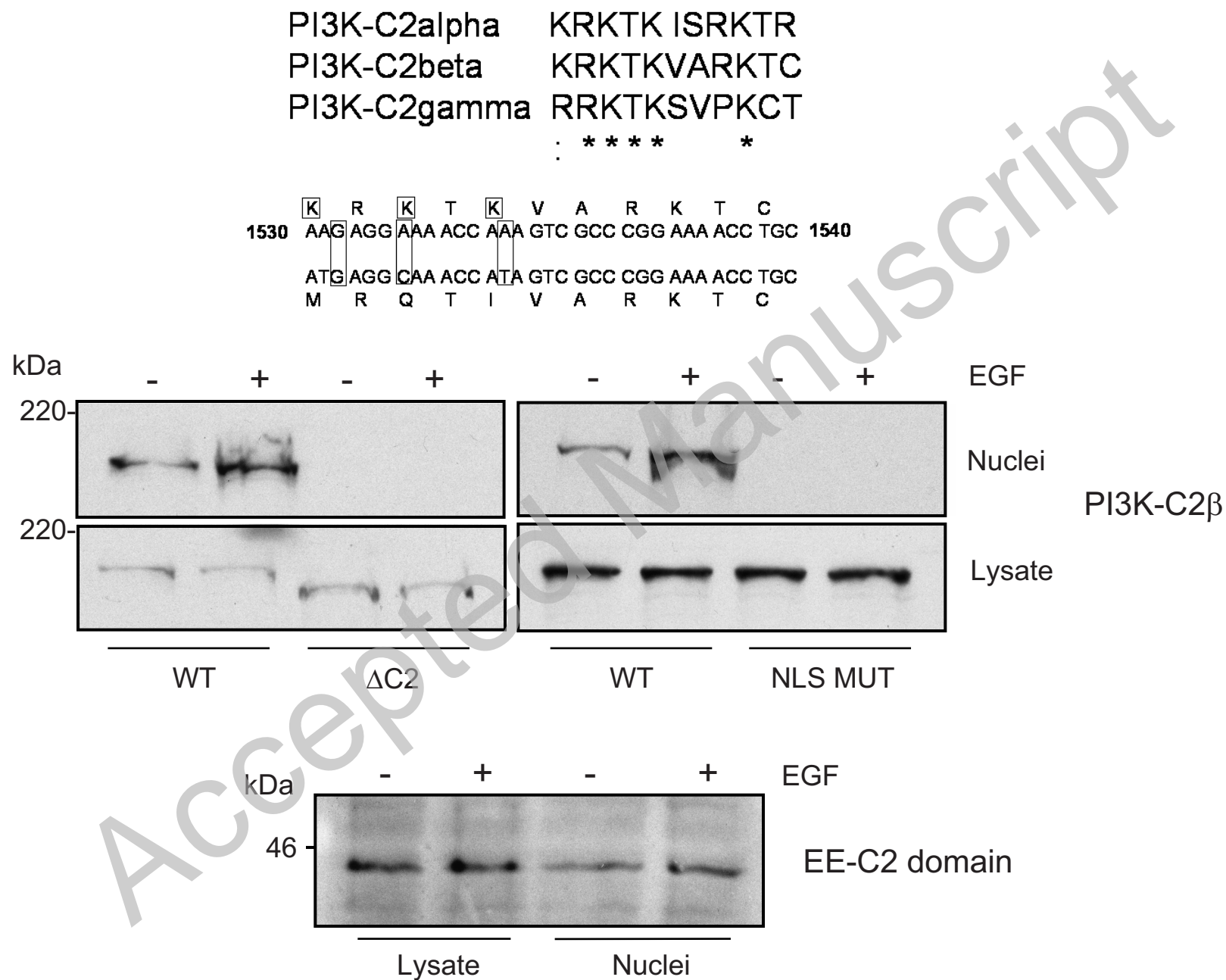
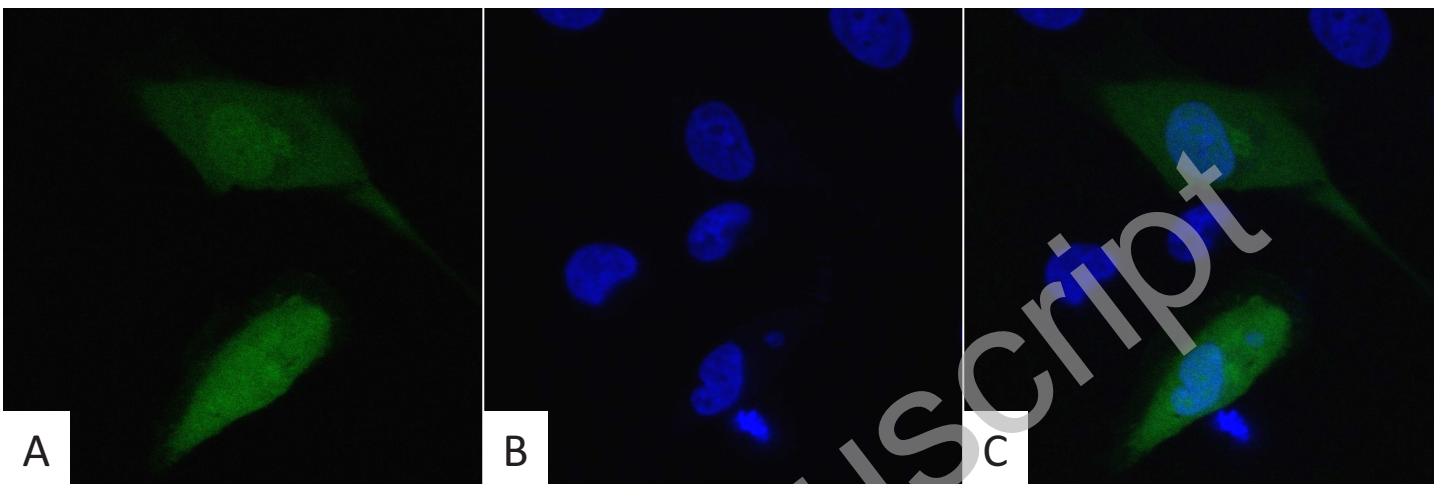


Figure 5

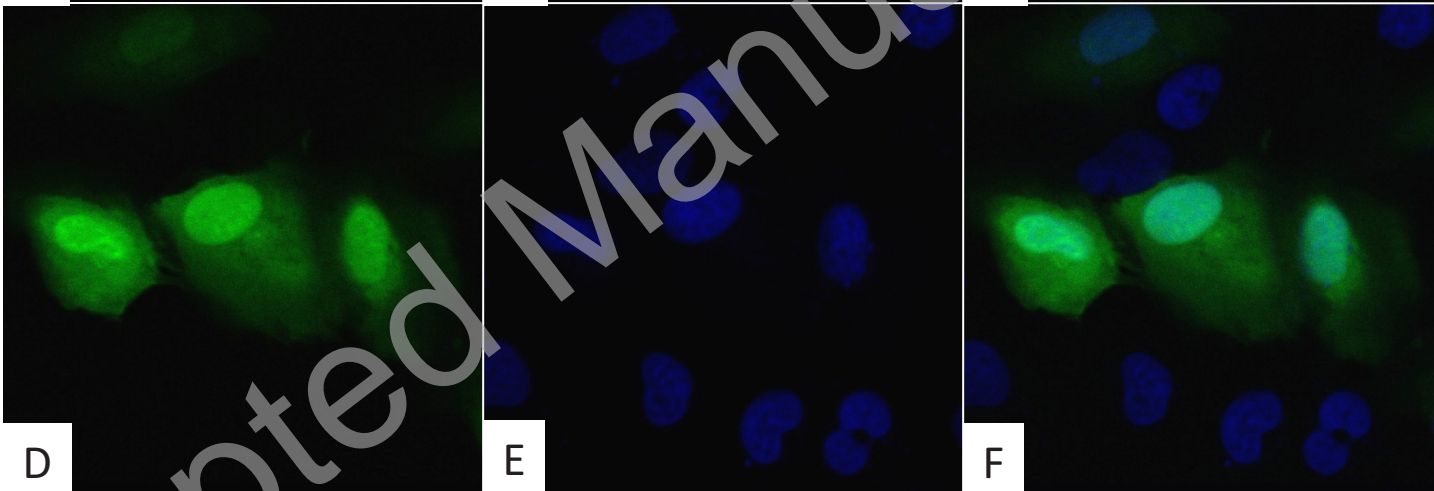
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EGFP



EGFP-NLS



EGFP-2xNLS

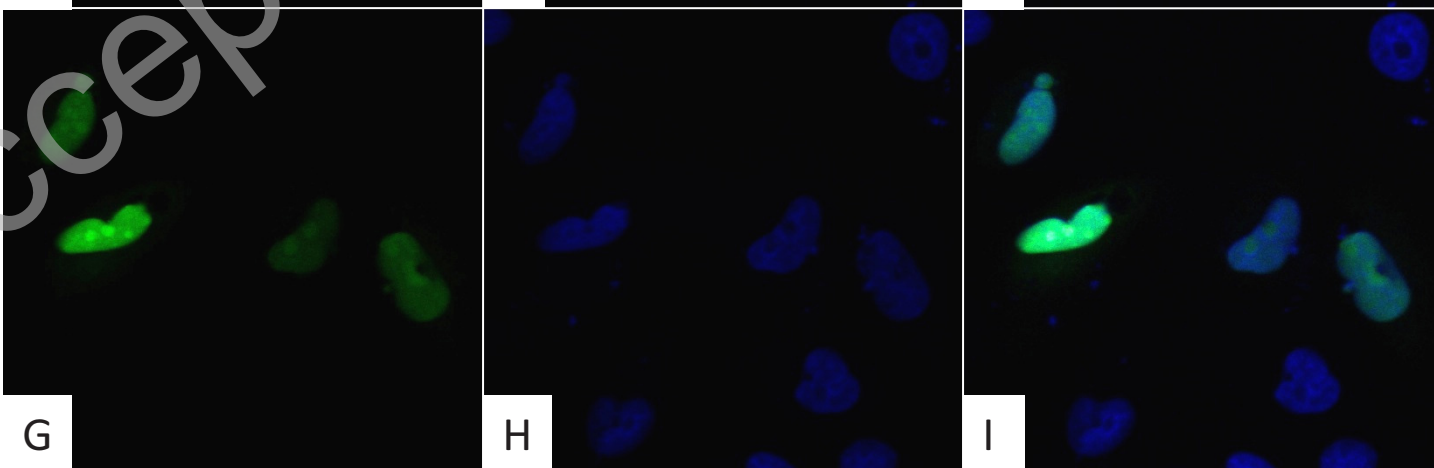


Figure 6

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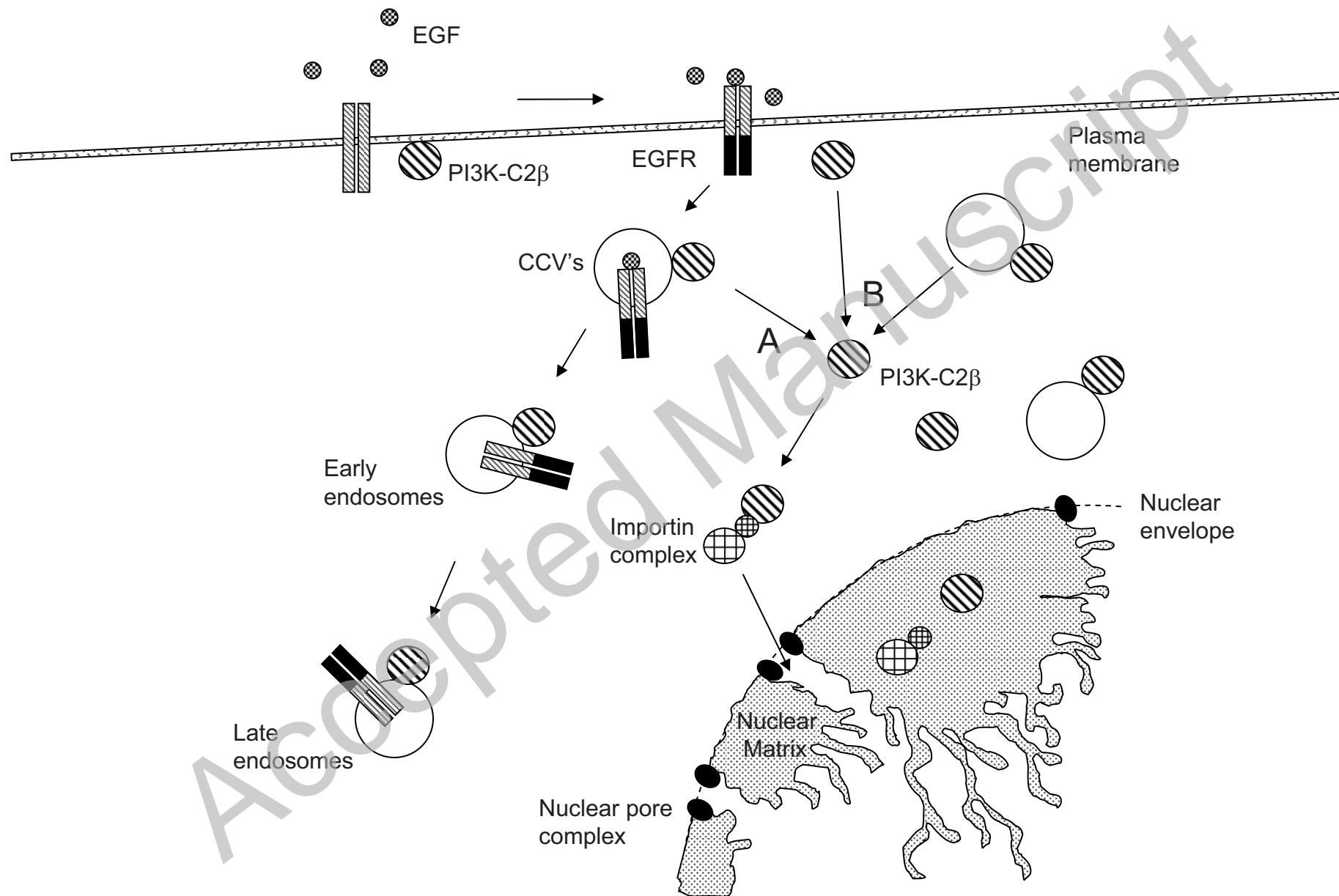


Figure 7

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