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Phosphorylation of annexin A2 at serine 25 is required for endothelin-1 stimulated cell proliferation and AKT activation in melanoma cells

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Abstract

Endothelin (ET)-1 contributes to melanoma progression via cell proliferation, invasion, and migration. We previously reported that annexin A2 (AnxA2) binds to ET receptors. In this study, we aimed to further investigate role of AnxA2 in melanoma cell proliferation after ET-1 stimulation. *AnxA2* knockdown inhibited ET-1-stimulated cell proliferation and AKT activation in SK-MEL28 melanoma cells. ET-1 stimulation phosphorylated serine on AnxA2, and AnxA2 Ser25 phosphorylation-deficient mutant (AnxA2 S25A) cells showed lower ET-1-stimulated cell proliferation and AKT activation than the rescue *AnxA2* knockdown (AnxA2 res) and AnxA2 Ser11 phosphorylation-deficient mutant (AnxA2 S11A) cells. Although AnxA2 S25A was localized to the plasma membrane, it exhibited lower colocalization with ET receptors than AnxA2 res and AnxA2 S11A on the plasma membrane. These results suggest that phosphorylation of AnxA2 Ser25 affects the colocalization of AnxA2 and ETRs and plays an important role in cell proliferation and AKT activation in ET-1 stimulated melanoma cells.

Keywords

annexin A2, endothelin, melanoma, cell proliferation

Introduction

Annexin A2 (AnxA2) is a calcium-dependent lipid-binding protein that participates in various cellular processes, including endocytosis, exocytosis, actin cytoskeletal remodeling, and signal transduction [1, 2]. AnxA2 also contributes to cancer progression via cell proliferation, invasion, migration and endothelial−mesenchymal transition [1, 3]. Phosphorylation of AnxA2 alters its structure [4]. AnxA2 is primarily phosphorylated at Ser11, Ser25, and Tyr23 (with Ser1 being the first amino acid) by various stimuli such as selenomethionine [5] and insulin [6]. Phosphorylation of Ser11 and Ser25 is mainly mediated by protein kinase C (PKC) [1, 2, 7]. Whereas PKC activity is regulated by AnxA2, Ser11 phosphorylation-deficient (S11A) and/or Ser25 phosphorylation-deficient (S25A) mutations in AnxA2 inhibit PKC activity [7], indicating that these two proteins form a feedback loop. Serine phosphorylation of AnxA2 is associated with the regulation of nuclear-cytoplasmic shuttling, mRNA binding, and binding to the S100 family [1, 8]. Tyrosine phosphorylation of AnxA2 is performed by Src-family and receptor tyrosine kinases and regulates actin rearrangement, cell adhesion, and membrane trafficking [6, 9, 10].

Endothelin (ET) was originally identified as a vasoconstrictor peptide produced by endothelial cells [11]. To date, three ET peptides have been identified: ET-1, ET-2 and ET-3 [12-14]. ET receptors are of two types: ET type A receptor (ET_AR) and ET type B receptor (ET_BR). ETs activate various signaling pathways, including the mitogen-activated protein kinase,

phosphatidylinositol 3-kinase (PI3K)/AKT, and PKC pathways, through ET receptors (ETRs). In addition to vasoconstriction, ETs are involved in cell proliferation, migration, and epithelial–mesenchymal transition, and progression in various tumors [12, 13]. In melanoma cells, ETs promote cell proliferation, migration, and release of cytokines, contributing to melanoma progression in vitro and in vivo [15, 16]. However, the mechanisms by which ET affects these processes remain largely unknown.

We previously reported that ET_AR and ET_BR bind to AnxA2, and that ET-1 stimulation causes serine phosphorylation of AnxA2 in EA.hy926 human umbilical vein endothelial cells. Additionally, we revealed that ET-1 activates the extracellular signal-regulated kinase and that *AnxA2* knockdown inhibits ET-1-stimulated extracellular signal-regulated kinase activation in EA.hy926 cells [17]. Melanoma and human umbilical vein endothelial cells are both affected by ET-1 [15, 16]. In this study, we aimed to investigate the mechanism of ET-1-stimulated melanoma cells proliferation via *AnxA2* knockdown and AnxA2 phosphorylation site mutations.

Materials and Methods

Cell lines

SK-MEL28 and 293T cells were purchased from the American Type Culture Collection. All cells were cultured in the Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque)

supplemented with 10% fetal bovine serum (FBS; Biowest) at 37 °C in a humidified atmosphere containing 5% CO_2.

Antibodies and chemicals

The following primary antibodies were used in this study: mouse monoclonal antibodies against AnxA2 (Proteintech), Myc tag (Cell Signaling Technology), phosphoserine (Merck), phosphotyrosine (Cell Signaling Technology), and β-actin (Sigma), rabbit monoclonal antibodies against AKT pS473 (Cell Signaling Technology), ET_AR (Abcam), and ET_BR (Abcam), and rabbit polyclonal antibodies against AKT (Cell Signaling Technology). Additionally, horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies were purchased from Jackson ImmunoResearch Laboratories. Alexa Fluor 488- and Alexa Fluor 555-conjugated anti-mouse and anti-rabbit goat IgG antibodies were purchased from Thermo Fisher Scientific. ET-1 was purchased from the Peptide Institute.

Gene silencing

Doxycycline (Dox)-induced *AnxA2* knockdown was performed using lentiviruses constructed by inserting the *AnxA2* short hairpin RNA (shRNA) sequence (sh1: TRCN0000056145 and sh2: TRCN0000296322; Merck) into the Tet-pLKO-puro vector (Addgene). The previously reported

irrelevant (*Irr*) shRNA target sequence was also used [18]. Briefly, Tet-pLKO-puro/*AnxA2* shRNA and Tet-pLKO-puro/*Irr* were transfected into 293T cells along with the VSV-G envelope-expressing plasmid pMD2.G (Addgene) and packaging plasmid psPAX (Addgene) using PEI MAX (Polysciences). After 48 h, the cultured supernatants were harvested and centrifuged at 450 × *g* for 10 min at 4 °C. The supernatants were added to SK-MEL28 cells in the presence of 7.5 μg/mL polybrene (Nacalai Tesque) as lentivirus preparations. The infected SK-MEL28 cells were subsequently selected using 0.5 μg/mL puromycin (Merck). For gene silencing, SK-MEL28 cells were cultured in a medium supplemented with 50 ng/mL Dox (FUJIFILM Wako Pure Chemical Corporation) for 48 h.

Plasmid construction

For *AnxA2* knockdown rescue experiment, *AnxA2* rescue cDNA (*AnxA2 res*) was constructed by substituting the nucleotides in the shRNA target sh1 sequence of *AnxA2* to 5′-AGGGAC-GCCTTAAATATCGAG-3′ without changing the coding amino acids via polymerase chain reaction-based mutagenesis. Phosphorylation-deficient mutations at S11A and S25A of *AnxA2 res* (*AnxA2 S11A* and *AnxA2 S25A*) were created by adding further mutations to *AnxA2 res* using the polymerase chain reaction-based mutagenesis method. These fragments were inserted into pcDNA6/Myc-HisA and amplified using the BamHI-Annexin primer (5′-

GAGCTCGGATCCACCATGTCTACTGTTCACGAAA-3′) and NotI-pcDNA6-BGH primer (5’-TTTATTGCGGCCGCACTAGAAGGCACAGTCGAGG-3’). To construct the Dox-inducible expression vector, the amplified fragments were digested with BamHI and NotI, and inserted into the BamHI-NotI site of the pPB-TRE3G-MCS-CEH-rtTA3-IB vector [19].

Transfection and establishment of stable cell lines

For the expression of Dox-inducible *AnxA2 res*, *S11A*, or *S25A* in endogenous *AnxA2*-knocked-down cells, pPB-TRE3G-MCS-CEH-rtTA3-IB/*AnxA2 res*, *S11A*, or *S25A*, respectively, was co-transfected with HyPBase into Dox-inducible *AnxA2* shRNA-expressing cells using FuGENE HD (Promega), as previously described [20]. Subsequently, the transfected cells were selected using 10 μg/mL blasticidin S (FUJIFILM Wako Pure Chemical Corporation).

Cell proliferation assay

Cell proliferation was assessed using the E-Click EdU Cell Proliferation Imaging Assay Kit (Elabscience), according to the manufacturer’s protocols. Briefly, the cells were seeded on chamber slides (Matsunami) and cultured in DMEM supplemented with 10% FBS in the presence or absence of Dox for 24 h. The cells were further cultured in serum-free DMEM in the presence or absence of Dox for 24 h. After adding 100 nM ET-1 and 10 μM 5-ethynyl-2’-deoxyuridine (EdU),

the cells were cultured for 24 h. Then, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.3% Triton X-100 in PBS. EdU was labeled with Elab Fluor 488. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Finally, confocal images were acquired using a laser-scanning microscope (Nikon-A1; Nikon).

Phosphorylation assay

The cells were cultured in serum-free DMEM for 24 h and stimulated with or without 100 nM ET-1 for 10 min. The, the cells were lysed with the ice-cold RIPA buffer (150 mM NaCl, 20 mM Tris-HCl [pH 7.4], 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM Na_3VO_4, 20 mM NaF, 10 mM sodium pyrophosphate, and Protease Inhibitor Cocktail for Use with Mammalian Cell and Tissue Extracts [Nacalai Tesque]). Cell lysates were sonicated and centrifuged at 21,900 × *g* for 10 min at 4 °C. After preclearing, the supernatants were incubated with anti-AnxA2 antibody and protein G PLUS-Agarose (Santa Cruz Biotechnology) for 5 h at 4 °C. The beads were washed four times with RIPA buffer, and the proteins were eluted from the beads using SDS sample buffer. Phosphorylation was detected via immunoprecipitation using the anti-phosphoserine or anti-phosphotyrosine antibodies together with the horseradish peroxidase-conjugated anti-mouse IgG antibody (Abcam).

Immunofluorescence microscopy

The cells were seeded on chamber slides (Matsunami) and cultured in DMEM supplemented with 10% FBS in the presence of Dox for 24 h. The cells were further cultured in serum-free DMEM in the presence of Dox for 24 h. After stimulating with 100 nM M ET-1 for 10 min, the cells were fixed with 4% paraformaldehyde in PBS, treated with 50 mM ammonium chloride in PBS, permeabilized with 0.5% saponin in PBS for 10 min, and blocked with 1% BSA in PBS for 30 min. Each antibody was diluted with the Can Get Signal immunostain (TOYOBO) and incubated overnight at 4°C. Nuclei were stained with DAPI (Nacalai Tesque). Confocal images were acquired using a laser-scanning microscope (Nikon-A1; Nikon). Colocalization of AnxA2 and ETRs was quantified using Coloc2 in the ImageJ Fiji software (National Institutes of Health).

Statistical analyses

All statistical analyses were conducted using the GraphPad Prism version 8 software (GraphPad Software, Inc.). Differences among multiple groups were analyzed using one-way analysis of variance, followed by the Tukey–Kramer test. Differences between two groups were analyzed using the Student's *t*-test.

Results and Discussion

Effects of *AnxA2* knockdown on ET-1-stimulated melanoma cells

ET-1 promotes the proliferation of melanoma cells [16]. Additionally, its receptors, ET_AR and ET_BR, bind to AnxA2 [17]. To examine whether AnxA2 is necessary for ET-1-stimulated proliferation of SK-MEL28 melanoma cells, we used a Dox-inducible knockdown system to minimize the effects of *AnxA2* knockdown during cell culture. Dox-induced *AnxA2* shRNA inhibited AnxA2 expression, with little changes in ET_AR and ET_BR expression levels in SK-MEL28 cells (Fig. 1A). Cell proliferation was examined by measuring EdU incorporation. Notably, cell proliferation was inhibited in *AnxA2*-knocked-down cells compared to that in *Irr* shRNA-expressing cells upon ET-1 stimulation, although these differences were negligible in the absence of Dox. (Fig. 1B, C). SK-MEL28 cell proliferation involves the PI3K/AKT pathway [21]. Therefore, we examined the effect of *AnxA2* knockdown on AKT activity after ET-1 stimulation. AKT activity was elevated in *Irr* shRNA-expressing cells upon ET-1 stimulation. In contrast, AKT activity was inhibited *AnxA2*-knocked-down cells, consistent with the cell proliferation results (Fig. 1D, E). These results suggest that AnxA2 is involved in ET-1-stimulated cell proliferation and the AKT activation in SK-MEL28 cells.

Phosphorylation of AnxA2 upon ET-1 stimulation

AnxA2 phosphorylation is involved in various processes, including cell cycle, regulation of actin rearrangement, and cell adhesion [1, 6, 8, 9]. Therefore, we examined the phosphorylation status of AnxA2 in SK-MEL28 cells upon ET-1 stimulation. Notably, ET-1 stimulation increased the serine phosphorylation of AnxA2; however, its effect on the tyrosine phosphorylation of AnxA2 was negligible (Fig. 2A, B).

Effect of AnxA2 Ser phosphorylation on AKT activity in ET-1-stimulated melanoma cells

Next, we examined the effect of serine phosphorylation of AnxA2 on cell proliferation in Dox-induced endogenous *AnxA2*-knocked-down cells using the rescue AnxA2 mutant (AnxA2 res), which retained the expression of AnxA2, or serine phosphorylation-deficient mutants (AnxA2 S11A or AnxA2 S25A), in which serine 11 and serine 25 of the rescue AnxA2 mutant, respectively, were changed to alanine. Endogenous AnxA2 expression was inhibited in these cells, but they expressed Myc-tagged AnxA2 res, AnxA2 S11A, and AnxA2 S25A. Notably, ET_AR and ET_BR expression levels were largely unchanged in SK-MEL28 cells (Fig. 3A). Proliferation of AnxA2 S25A-expressing SK-MEL28 cells was inhibited compared to that of AnxA2 res- and AnxA2 S11A-expressing cells (Fig. 3B, C). Furthermore, consistent with the cell proliferation results, AKT activity in AnxA2 S25A-expressing SK-MEL28 cells was inhibited compared to that in AnxA2 res- and AnxA2 S11A-expressing cells (Fig. 3D, E). These results suggest that

AnxA2 Ser25 phosphorylation is required for ET-1 stimulated cell proliferation and AKT activation in SK-MEL28 cells.

Subcellular localization of AnxA2 phosphorylation-deficient mutants in ET-1-stimulated melanoma cells

Phosphorylation of AnxA2 Ser25 is involved in its subcellular localization [22]. To understand the role of Ser25 phosphorylation in ET-1-stimulated melanoma cells, we examined the subcellular localization of AnxA2 and its phosphorylation-deficient mutants. AnxA2 res and AnxA2 S11A colocalized with ET_AR and ET_BR on the plasma membrane upon ET-1 stimulation (Fig. 4A, B). Although AnxA2 S25A was localized to the plasma membrane, the rate of colocalization of AnxA2 S25A and ETRs was lower than that of AnxA2 res and AnxA2 S11A upon ET-1 stimulation. (Fig. 4A, B). These results suggest that the phosphorylation of Ser25 in AnxA2 is required for colocalization with ETRs.

Since the dual phosphorylation of AnxA2 at Tyr23 and Ser25 has not yet been reported, these two phosphorylation sites are considered to be mutually exclusive in AnxA2 [1, 7]. Consistent with previous reports, we observed increased serine phosphorylation, but not tyrosine phosphorylation, of AnxA2 in ET-1-stimulated SK-MEL28 cells (Fig. 2A, B).

ET-1 stimulation activates PKC by increasing the intracellular Ca^{2+} level [12]. Furthermore, PKC has been reported to phosphorylate AnxA2 [1]. Serine phosphorylation of AnxA2 is involved in cell cycle, nuclear entry, basal barrier resistance, and cell migration in endothelial cells [8, 22]. Among all serine residues, Ser25 is the major phosphorylation site of AnxA2 in vivo and in vitro, and phosphorylation of AnxA2 Ser25 releases the N-terminal domain of AnxA2 from the C-terminal core domain, resulting in a conformational change in AnxA2 [4]. On the other hand, direct evidence of Ser11 phosphorylation in vivo is lacking [1]. Here, our results revealed the importance of Ser25 phosphorylation, but not Ser11 phosphorylation, for ET-1-stimulated cell proliferation, AKT activation, and colocalization with ET_AR and ET_BR (Fig. 3B-E and Fig. 4).

Whereas our study revealed a novel role for AnxA2 Ser25 phosphorylation at the plasma membrane, the same phosphorylation event has also been implicated in nuclear-cytoplasmic shuttling. Previous studies have reported that AnxA2 localizes in the nucleus in a cell cycle-dependent manner or in response to signaling events and is phosphorylated at residues yet to be identified [8, 23]. Liu *et al.* showed that nuclear localization of AnxA2 induced by the nuclear export inhibitor leptomycin B was significantly prevented by S11E/S25E double mutations, while neither S11A nor S25A mutations affected the leptomycin B-induced nuclear accumulation of AnxA2 [8]. These results suggest that phosphorylation at Ser11 and Ser25 promotes AnxA2 to function outside the nucleus. On the other hand, Luo *et al*. reported that AnxA2 is serine-phosphorylated in

the nucleus under signaling events induced by latent membrane protein 1 of Epstein-Barr virus, although the phosphorylation sites were not specified. They also showed that the S25E mutation excludes AnxA2 from the nucleus [23], which is actually in line with the above-mentioned findings by Liu *et al*. Serine to glutamic acid (SE) mutation is generally thought to mimic the phosphorylated state of serine [4, 8]. However, there are also known examples where the behavior differs between SE mutation and serine phosphorylation [24], and thus SE mutants may function as non-phosphorylated mutants, as Luo *et al.* assumed [23]. Regardless, these experimental observations do not contradict our findings and conclusion that Ser25 phosphorylation is involved in its protein-protein interactions on the plasma membrane: our results do not rule out the possibility that phosphorylation at Ser25 also regulates nuclear import or export of AnxA2. It should be noted that the relationship between phosphorylation of AnxA2 and nuclear localization may differ depending on cell type and stimuli to which the cells are exposed. The nuclear-cytoplasmic shuttling of AnxA2 under ET-1 stimulation, and roles of its serine phosphorylation, should be addressed elsewhere with careful consideration.

Previous studies have shown that ET-1 stimulation is involved in SK-MEL28 melanoma cell proliferation via the PI3K/AKT pathway [15, 16, 21], consistent with our findings. We previously revealed that AnxA2 binds to ET_AR and ET_BR [17]. Taken together, our results suggest

that phosphorylation of AnxA2 Ser25 is required for colocalization with ETRs and facilitates ET-1-stimulated proliferation via AKT activation in melanoma cells.

Abbreviations

AnxA2, annexin A2; Dox, Doxycycline; EdU, 5-ethynyl 2′-deoxyuridine; ERK, extracellular signal-regulated kinase; ET, endothelin; ETR, endothelin receptor; ET_AR, endothelin type A receptor; ET_BR, endothelin type B receptor; Irr, irrelevant; PI3K , Phosphoinositide 3-kinase; PKC, protein kinase C

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Figure Legend

Fig. 1. Effects of *AnxA2* knockdown on ET-1-stimulated cell proliferation and AKT activation. (A) Expression levels of AnxA2 and ETRs in *AnxA2*-knocked-down cells. (B) Representative immunofluorescence microscopic images of cell proliferation analysis. Blue, DAPI; green, Elab Fluor 488 EdU. Bar 50 μm. (C) Percentage of EdU-positive cells after ET-1 stimulation. Over 120 cells were analyzed in each experiment. $n = 4$. (D) Representative western blots for pAKT. Cells were cultured in the serum-free DMEM with or without Dox for 24 h. Then, the cells were stimulated with or without 100 nM ET-1 for 10 min. (E) Relative pAKT intensity. pAKT intensity in ET-1 stimulated Irr cells without Dox was set as 100%. $n = 3$. (C and D) ** $p < 0.01$; NS, not significant.

Fig. 2. Phosphorylation of AnxA2 upon ET-1 stimulation. (A) Representative western blots for AnxA2 phosphorylation. pS, phosphoserine; pY, phosphotyrosine. (B) Relative intensity of AnxA2 phosphorylation. Intensity of AnxA2 phosphorylation without ET-1 stimulation was set as 100%. ** $p < 0.01$; NS, not significant.

Fig. 3. Effects of AnxA2 Ser phosphorylation on ET-1-stimulated cell proliferation and AKT activation. (A) Expression levels of AnxA2 and ETRs in the phosphorylation-deficient mutants of

AnxA2. (B) Representative immunofluorescence microscopic image of cell proliferation analysis. Blue, DAPI; green, Elab Fluor 488 EdU. Bar 50 μm. (C) Percentage of EdU-positive cells after ET-1 stimulation. Over 200 cells were analyzed in each experiment. $n = 4$. (D) Representative western blots for pAKT. Cells were cultured in serum-free DMEM with or without Dox for 24 h. Then, the cells were stimulated with or without 100 nM ET-1 for 10 min. (E) pAKT intensity in ET-1 stimulated Myc-AnxA2 res cells without Dox was set as 100%. $n = 3$. (C and D) * $p < 0.05$; NS, not significant.

Fig. 4. Effect of the serine phosphorylation of AnxA2 on its colocalization with ET_AR and ET_BR. (A) Representative immunofluorescence microscopic images. Blue, DAPI; green, Myc; red, ETRs. Bar 50 μm. White arrowheads, colocalized site; black arrowheads, non-colocalized site. (B) Pearson's correlation coefficient for colocalization with Myc-AnxA2 and ETRs on the plasma membrane. Over 12 cells were analyzed in each experiment. ** $p < 0.01$; NS, not significant.

Fig. 1

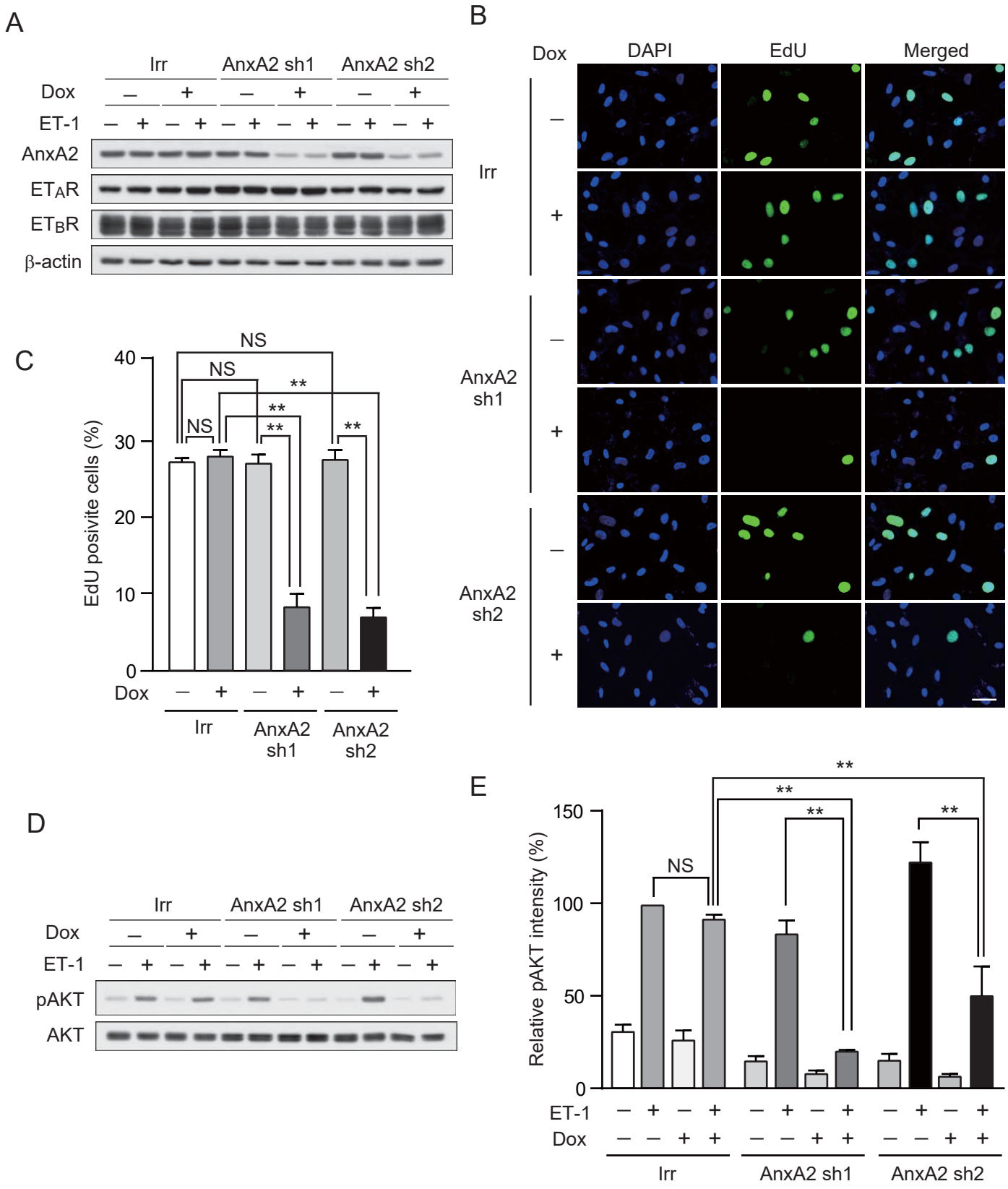
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Fig. 2

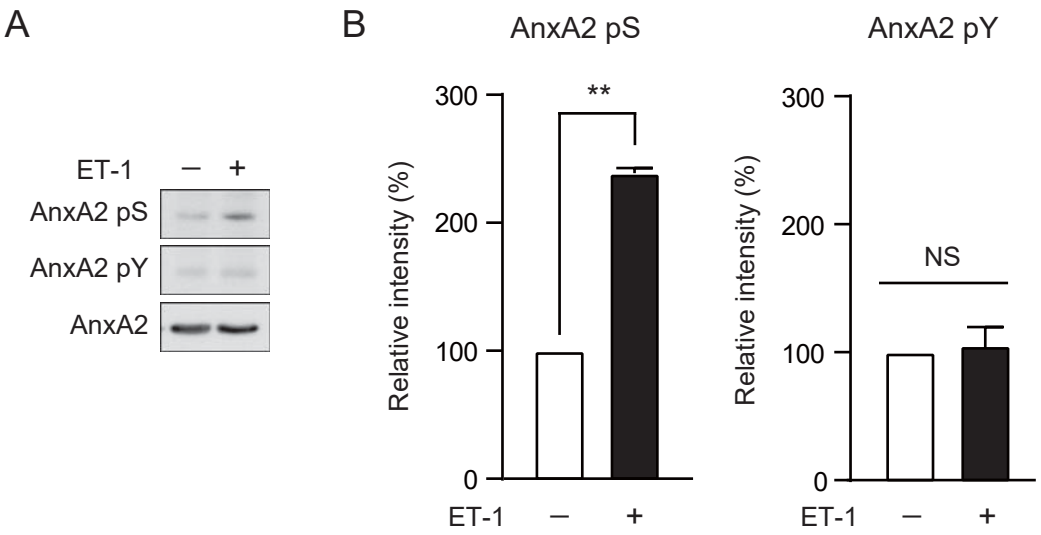
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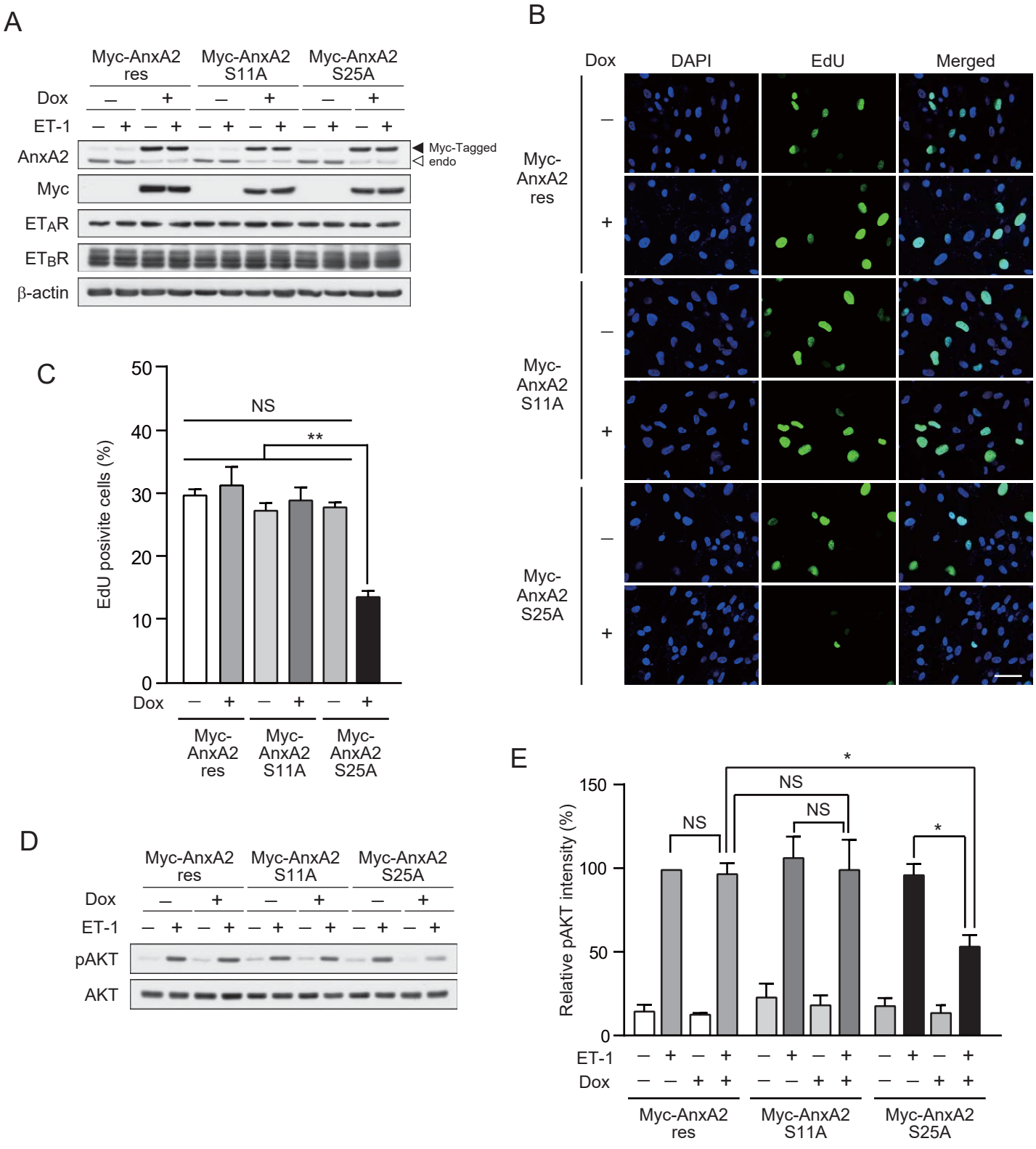
Fig. 3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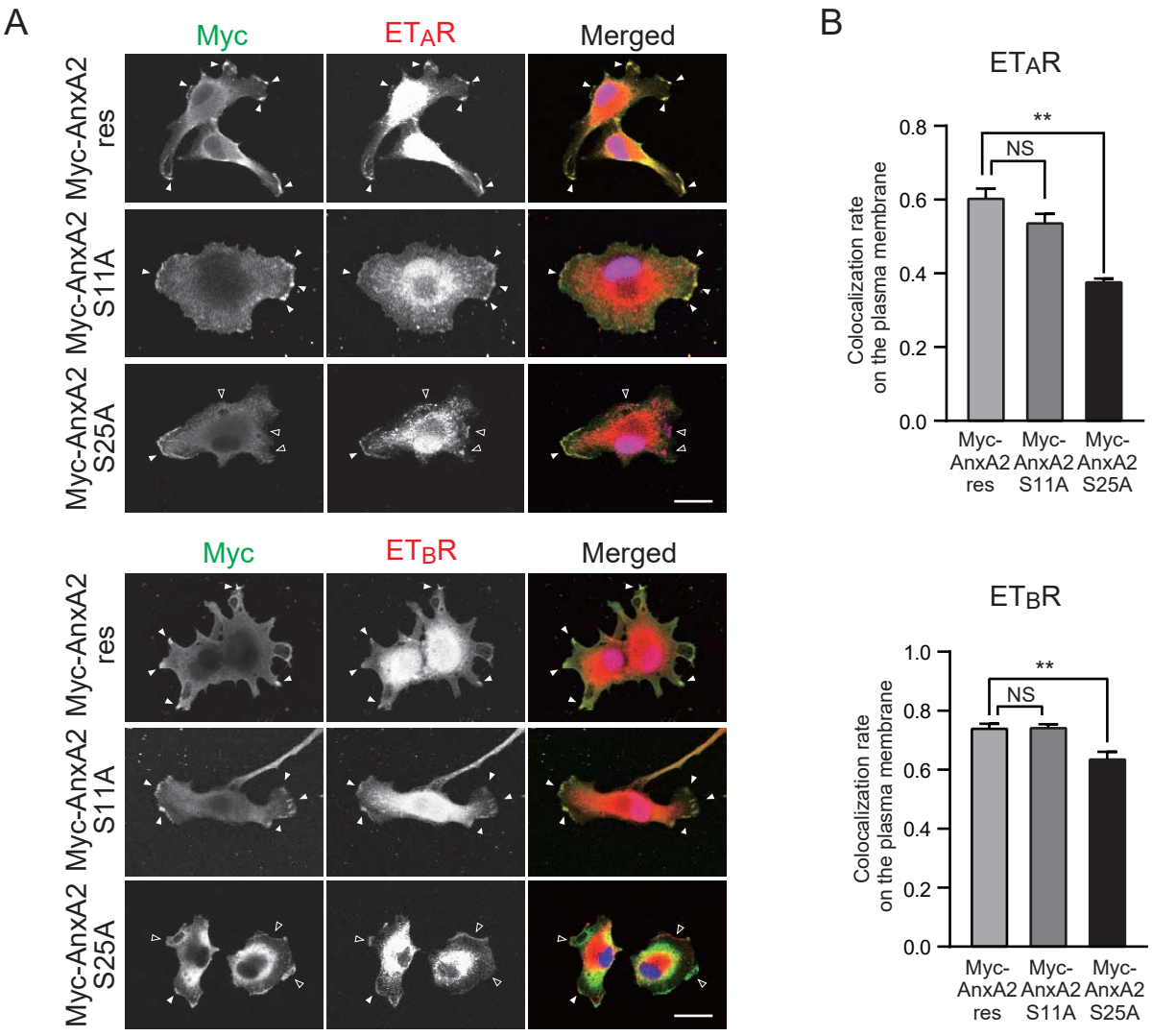
AnxA2
Myc
ETAR
ETBR
β-actin
Myc-Tagged
endo
B
Dox
DAPI
EdU
Merged
Myc-AnxA2 res
Myc-AnxA2 S11A
Myc-AnxA2 S25A
C
EdU posivite cells (%)
NS
**
D
pAKT
AKT
E
Relative pAKT intensity (%)
NS
*

Fig. 4


A
Myc
ETAR
Merged
Myc-AnxA2 res
Myc-AnxA2 S11A
Myc-AnxA2 S25A
Myc
ETBR
Merged
Myc-AnxA2 res
Myc-AnxA2 S11A
Myc-AnxA2 S25A
B
ETAR
Colocalization rate on the plasma membrane
0.0
0.2
0.4
0.6
0.8
**
NS
Myc-AnxA2 res
Myc-AnxA2 S11A
Myc-AnxA2 S25A
ETBR
Colocalization rate on the plasma membrane
0.0
0.2
0.4
0.6
0.8
1.0
**
NS
Myc-AnxA2 res
Myc-AnxA2 S11A
Myc-AnxA2 S25A