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the phosphorylation could affect γ-cleavage of APP is an important question to be answered.

AD begins with an impairment of memory, which is caused by a disturbance of hippocampal synaptic function (32). In addition, cognitive decline in AD is correlated to the degree of synaptic loss (33), suggesting that the pathological alteration in the metabolism of synaptic protein is primarily involved in AD pathophysiology. It is known that abnormal increases in GSK3β level and activity have been associated with AD pathophysiology (6, 10). Taken together, our studies propose a causal link which may connect abnormal activation of GSK3β to the synaptic dysfunction in the following two ways; 1) Inhibition of cadherin-mediated PI3K/Akt signal transmission, thereby downregulating cell-survival signaling leading to neurodegeneration. Since synaptic plasticity should involve the process in which certain synapses survive and some others degenerate, dysregulation of this 'contact-mediated survival signal' should hinder synaptic plasticity as well. 2) Inhibition of N-cadherin ε-cleavage, thereby reducing the production of Ncad/CTF2. Since Ncad/CTF2 carries various signals transmitted from the cell-surface to the nucleus (14, 34), inhibition of Ncad/CTF2 production under abnormal activation of GSK3β should have negative impact on neuronal plasticity or its viability. Interestingly, FAD-linked mutations of PS1 have shown to inhibit ε-cleavage of N-cadherin (14, 34). Thus, from the view point of Ncad/CTF2 production, FAD-linked mutation and phosphorylation of PS1 act in a similar way. According to the previous report, conditional transgenic mice overexpressing GSK3β in the adult brain show decreased nuclear β-catenin, abnormally phosphorylated tau and clear evidence of neurodegeneration (35), indicating the possibility that altered N-cadherin metabolism or PS1/N-cadherin interaction could lead to neurodegeneration. Whether these mechanisms are actually involved in neurodegeneration *in vivo* especially in the case of 'sporadic' Alzheimer's disease should be elucidated in the future study.

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Fig. 1. Cell-surface expression of PS1 is enhanced by the cadherin-mediated cell-cell contact. (A-C) APPsw/Ncad-CHO cells were immunostained with anti-N-cadherin (red) and anti-PS1 antibodies (green). Both N-cadherin (A, arrow) and PS1 (B, arrowhead) immunoreactivities were concentrated at the sites of cell-cell contact, showing co-localization (C). (D-I)

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APPSw-CHO cells (D-F) and APPsw/Ncad-CHO cells (G-I) were immunostained with anti-β-catenin (red) and anti-PS1 (green) antibodies. In APPSw-CHO cells, both β-catenin (D, arrow) and PS1 (E, arrowhead) immunoreactivities were localized in the perinuclear region. Conversely, in APPsw/Ncad-CHO cells, both β-catenin (G, arrow) and PS1 (H, arrowhead) immunoreactivities were concentrated at the site of cell-cell contact, showing co-localization (I). (J) Native CHO cells were transiently transfected with human N-cadherin. 24 hours after transfection, then, cell-surface proteins were biotinylated. Cells lysates were precipitated by streptoavidin agarose and cell-surface proteins were analyzed by Western blot. The amount of cell-surface PS1 and nicastrin were increased in N-cadherin transfected cells (3rd lane), compared to those in control GFP transfected cells (2nd lane). The 1st lane is the negative control without biotinylation. (K) HEK293 cells, endogenously expressing human N-cadherin and PS1, were transfected with siRNA construct, targeting N-cadherin for 24 hours. In N-cadherin and β-catenin siRNA transfected cells, the amount of cell-surface nicastrin was reduced (3rd and 4th lane), compared to that of control cells (2nd lane), whereas the total amount of nicastrin remained unchanged. β-catenin knockdown had less impact on reduction of the cell-surface PS1 (3rd lane), compared to clear reduction of cell-surface PS1 shown by the N-cadherin knockdown (4th lane). N-cadherin knockdown reduced the amount of β-catenin (4th lane), whereas β-catenin knockdown did not change the amount of N-cadherin (3rd lane). The 1st lane is the negative control without biotinylation.

Fig. 2. GSK3β activity modulates the binding of PS1 to N-cadherin/β-catenin. (A) Schematic representation of PS1 mutants lacking the domain required for PS1/N-cadherin/β-catenin interaction (PS1Δ340-75 and Δ340-50). The phosphorylation sites of wtPS1 by GSK3β in indicated at the bottom (9). (B) Either wtPS1, deletion mutant (PS1Δ340-75 or Δ340-50) or pseudo-phosphorylation mutant (S353D or S357DPS1) was transfected into HEK293 cells. 24 hours after transfection, cells were lysed and lysates are immunoprecipitated with anti-PS1 NTF antibody, followed by the Western blot analysis. Control precipitation was done with normal rabbit IgG (1st lane). wtPS1 robustly associated with both N-cadherin and β-catenin (3rd lane). None of the deletion mutants significantly associated with N-cadherin or β-catenin (4th and 5th lanes). The association between pseudo-phosphorylation mutants (S353DPS1, 6th lane and S357DPS1, 7th lane) and N-cadherin/β-catenin was reduced, compared to wtPS1 (3rd lane), whereas the expression level of each PS1/N-cadherin/β-catenin complex component was similar in the total cell lysate (Lys). The bottom is the loading control, represented by the β-actin bands. (C) Either wtPS1 or one of pseudo-phosphorylation mutant (S353D or S357DPS1) was transfected into MEF PS-/- cells, followed by immunoprecipitation, using anti-PS1 NTF antibody and Western blot analysis. Control precipitation was done with normal rabbit IgG (1st lane). wtPS1 bound both N-cadherin and β-catenin (2nd lane). The association between pseudo-phosphorylation mutants (S353DPS1, 3rd lane and S357DPS1, 4th lane) and N-cadherin/β-catenin was reduced, compared to wtPS1. The expression level of each PS1/N-cadherin/β-catenin complex component was similar in the total cell lysate (Lys). The bottom is the loading control, represented by the β-actin bands. (D) Either control GFP (2nd lane) or constitutively active (S9A) GSK3β (3rd lane) was transfected into HEK293 cells, followed by immunoprecipitation, using anti-PS1 NTF antibody. Control precipitation was done with normal rabbit IgG (1st lane). Note that PS1-bound N-cadherin is reduced under the expression of S9AGSK3β (3rd lane), whereas the total level of N-cadherin did not change significantly (Lys). The bottom is the loading control, represented by the β-actin bands. (E) (Left) S9A-tet cells were treated by 1μg/ml tetracycline for 24 hours. Cells were lysed after treatment and analyzed by anti-Phospho-β-catenin (Ser 33/37/Thr 41) and anti-total-β-catenin antibodies. The magnitude of GSK3β expression was demonstrated by anti-GSK3β antibody. The induction of GSK3β expression was evident (GSK3β). Phosphorylation of β-catenin was

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enhanced (Phospho-β-catenin), whereas total β-catenin levels were reduced (Total β-catenin) 24 hours after treatment. The bottom is the loading control, represented by the β-actin bands. (Right) S9A-tet cells were treated by 1μg/ml tetracycline for 24 hours. Cells were lysed after treatment and immunoprecipitated by anti-phosphoserine antibody, followed by the Western blot, using anti-PS1 CTF. The PS1 CTF immunoprecipitated by anti-phosphoserine was increased after tetracycline treatment (Top, PS1 CTF), whereas the total levels of PS1 CTF were comparable between before and after treatment (Lys, PS1 CTF). The bottom is the loading control, represented by the β-actin bands. (F) S9A-tet cells were treated by 1μg/ml tetracycline for 24 hours. Cells were lysed after treatment and the lysates were immunoprecipitated by anti-PS1 NTF antibody. Control precipitation was done with normal rabbit IgG (1st lane). PS1/N-cadherin interaction was reduced after tet-on (3rd lane), compared to before tet-on (2nd lane). The expression levels of N-cadherin were demonstrated in the total cell lysates (Lys). The bottom is the loading control, represented by the β-actin bands.

Fig. 3. PS1/N-cadherin/β-catenin complex formation is required for the cell-surface expression of PS1/γ-secretase. (A) Either wtPS1 or one of deletion mutants (PS1Δ340-75 or Δ340-50) was transfected into MEF PS-/- cells. 24 hours after transfection, cells were fractionated into plasma membrane and ER fractions. Protein samples were analyzed by Western blot using anti-PS1 NTF antibody. The expression of deletion mutants in the plasma membrane fraction (3rd and 4th lanes, top) was reduced compared to that of wtPS1 (1st lane, top), whereas the expression in the ER fraction (bottom) was comparable. (B) Either wtPS1 or one of pseudo-phosphorylation mutants (S353D or S357DPS1) was transfected into MEF PS-/- cells. Control cells were transfected with GFP (1st lane). 24 hours after transfection, cell-surface proteins were biotinylated, precipitated by streptoavidin agarose and analyzed by Western blot. The cell-surface expression of PS1 as well as that of nicastrin (3rd and 4th lanes) was reduced in the cells transfected with pseudo-phosphorylation mutants, compared to that of wtPS1 (2nd lane), whereas the expression levels of these protein in the cell lysate were comparable (Total Nicastrin, Total PS1 NTF) . The asterisk designates the glycosylated 'mature' Nicastrin. The bottom is the loading control, represented by the β-actin bands.

Fig. 4. GSK3β activation downregulates PI3K/Akt cell survival signaling. (A) The ability of pseudo-phosphorylation mutants to facilitate PI3K/Akt signaling was tested by introducing wtPS1 or one of pseudo-phosphorylation mutants (S353D, S357D or S353/357D double mutant PS1) for 24 hours, followed by immunoblotting (bottom). Transfection of wtPS1 enhanced Akt phosphorylation (2nd lane). All the pseudo-phosphorylation mutants had reduced activity to enhance Akt phosphorylation (3rd -5th lanes), compared to wtPS1 (2nd lane). (B) Time course of the calcium switch assay (top). MEF PS-/- cells were treated with 4mM EGTA for 40 mins for calcium deprivation. After calcium deprivation, cells were cultured in calcium containing serum-free medium. The effect of the calcium switch assay on N-cadherin-based cell-cell contact is shown (bottom). Before EGTA treatment, N-cadherin in MEF PS-/- was seen at the sites of cell contacts as liner immunoreactivity (bottom, left, arrows). After EGTA treatment, N-cadherin concentration at the junction became weak and showed granular immunoreactivity (bottom, middle, arrowheads). 60 mins after calcium supplement, N-cadherin concentration at the cell-cell contact sites was restored and appeared as liner structures (bottom, right, arrows). (C) Either wtPS1 or PS1Δ340-50 was transfected into MEF PS-/- cells. 24 hours after transfection, cells were subjected to the calcium switch assay. Under wtPS1 transfection, Akt phosphorylation before calcium deprivation was prominent (wtPS1, Pre), which was diminished after EGTA treatment (wtPS1, 0) and gradually recovered after calcium supplement (wtPS1, 30, 90). Conversely, under PS1Δ340-50 transfection, Akt phosphorylation before calcium deprivation was not prominent and remained unchanged throughout the assay (PS1Δ340-50).

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(D) wtPS1 was transfected into MEF PS-/- cells in the presence or absence of S9A GSK3β. Control cells were transfected with GFP. 24 hours after transfection, cells were subjected to the calcium switch assay. In the absence of PS1 (GFP) or after co-transfection of wtPS1 and S9AGSK3β (wtPS1+S9AGSK3β), the phosphorylation state of Akt did not change significantly before and throughout the calcium switch assay. Conversely, wtPS1 transfection led to prominent Akt phosphorylation before the calcium switch assay (wtPS1, Pre), which is diminished after EGTA treatment (wtPS1, 0) and gradually recovered after calcium supplement (wtPS1, 30, 60).

Fig. 5. GSK3β-mediated phosphorylation of PS1 differentially regulates N-cadherin and APP cleavage. (A) PS1 constructs (wtPS1, pseudo-phosphorylation mutant (S353D or S357DPS1) or PS1Δ340-50) were transfected into MEF PS-/- cells. 24 hours after transfection. Cells were then collected and subjected to immunoblotting using anti-N-cadherin C-terminus antibody. The amount of Ncad/CTF1 was reduced after wtPS1 transfection (2nd lane), compared to control GFP transfection (1st lane). Neither pseudo-phosphorylation mutants (S353D, S357D) nor PS1Δ340-50 reduced Ncad/CTF1 (3rd-5th lanes). The bottom is the loading control, represented by the β-actin bands. (B) MEF PS-/- cells were transfected with PS1 constructs indicated. Then the ectodomain shedding of N-cadherin was stimulated by ionomycin treatment (10μM, 30mins). Cells were fractionated into membrane and cytoplasmic fractions. Protein samples were subjected to Western blot anlysis, using anti-N-cadherin C-terminus antibody. Ncad/CTF1 were reduced in the membrane fraction of wtPS1 transfected cells, compared to other transfectants (Top, 2nd lane), whereas Ncad/CTF2 production in the cytoplasmic fraction was observed only in wt PS1 transfected cells (bottom, 2nd lane). (C) Human APP and one of the PS1 constructs (wtPS1, pseudophosphorylation mutant or PS1Δ340-50) were co-transfected into MEF PS-/- cells. 24 hours after transfection, cells were collected and subjected to immunoblotting using anti-APP C-terminus antibody. All PS1 constructs equally reduced APP CTFα&β (2nd-5th lanes), compared to control GFP (1st lane). The bottom is the loading control, represented by the β-actin bands.

Fig. 6. Schematic presentation of cellular consequences caused by GSK3β-mediated PS1 phosphorylation. When PS1 is not phosphorylated, PS1 associates with N-cadherin/β-catenin at the cell-surface. This PS1/N-cadherin association recruits PI3K to the cell surface and PI3K/Akt signaling is activated, resulting in cell survival and GSK3β suppression (20) (left). After GSK3β activation, PS1 is phosphorylated at its loop domain, which leads to reduced PS1/N-cadherin/β-catenin complex formation, resulting in downregulation of PI3K/Akt signaling. PS1/γ-secretase is not tethered to the cell-surface after phosphorylation, which affects the cell survival signal negatively and further activates GSK3β. Inhibition of PS1/N-cadherin association leads to reduced N-cadherin cleavage as well.

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APPsw/Ncad-CHO cells

A B C

20μM

N-cadherin

β-catenin

Presenilin 1

Merge

APPsw-CHO cells

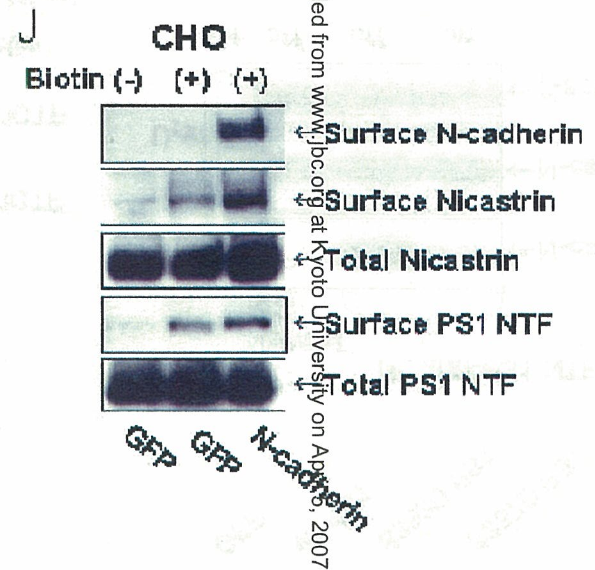
D E F

20μM

APPsw/Ncad-CHO cells

G H I

20μM



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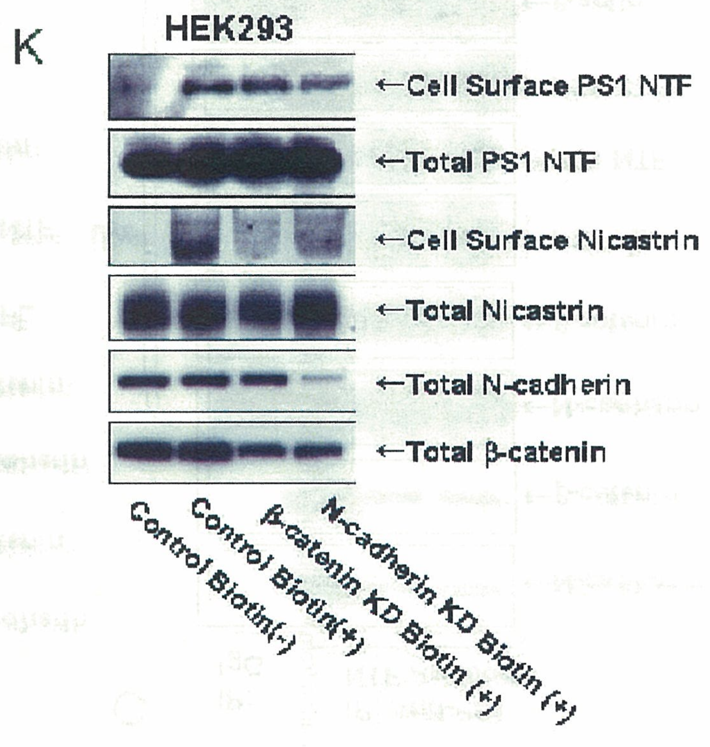

Figure 1.

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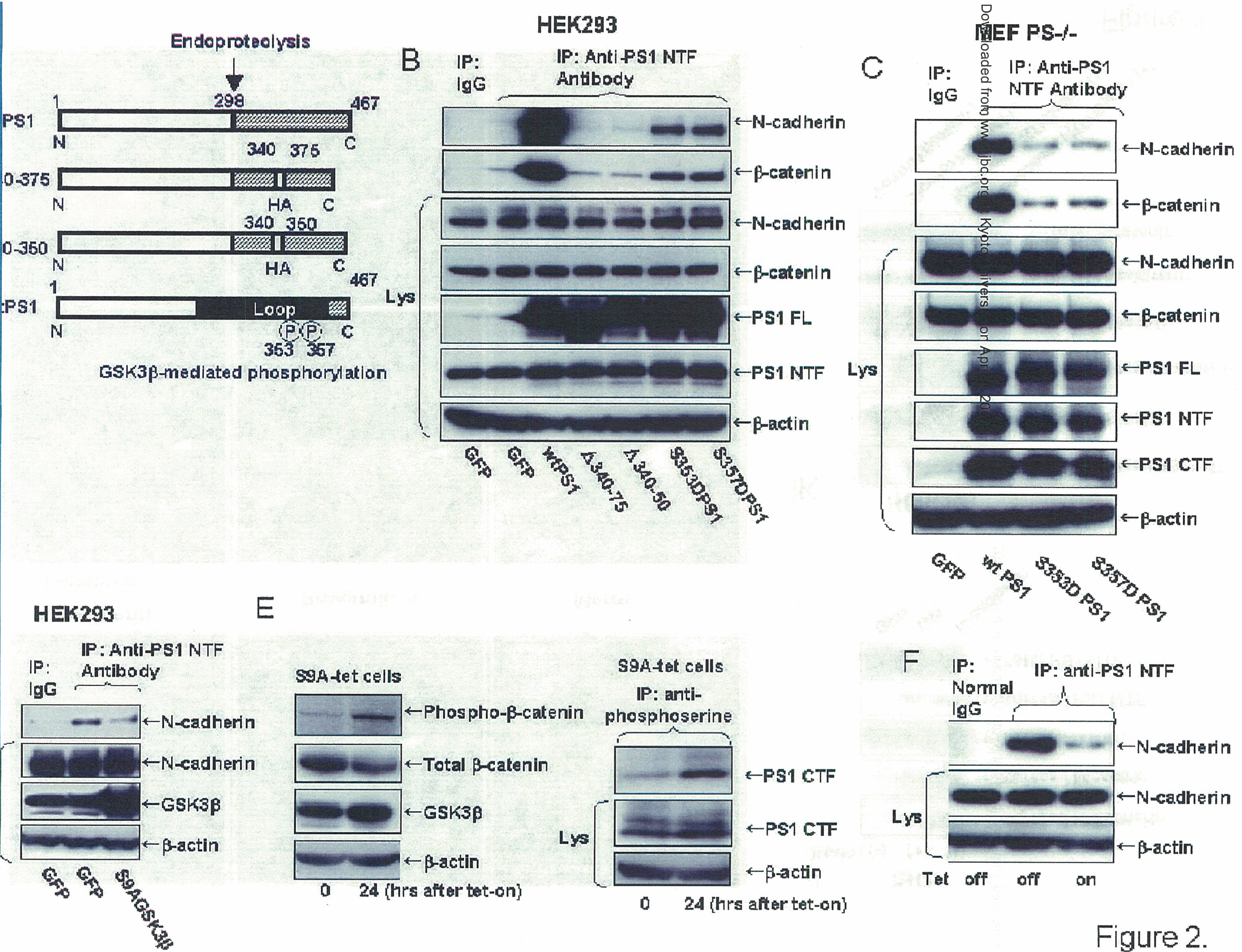

Figure 2.

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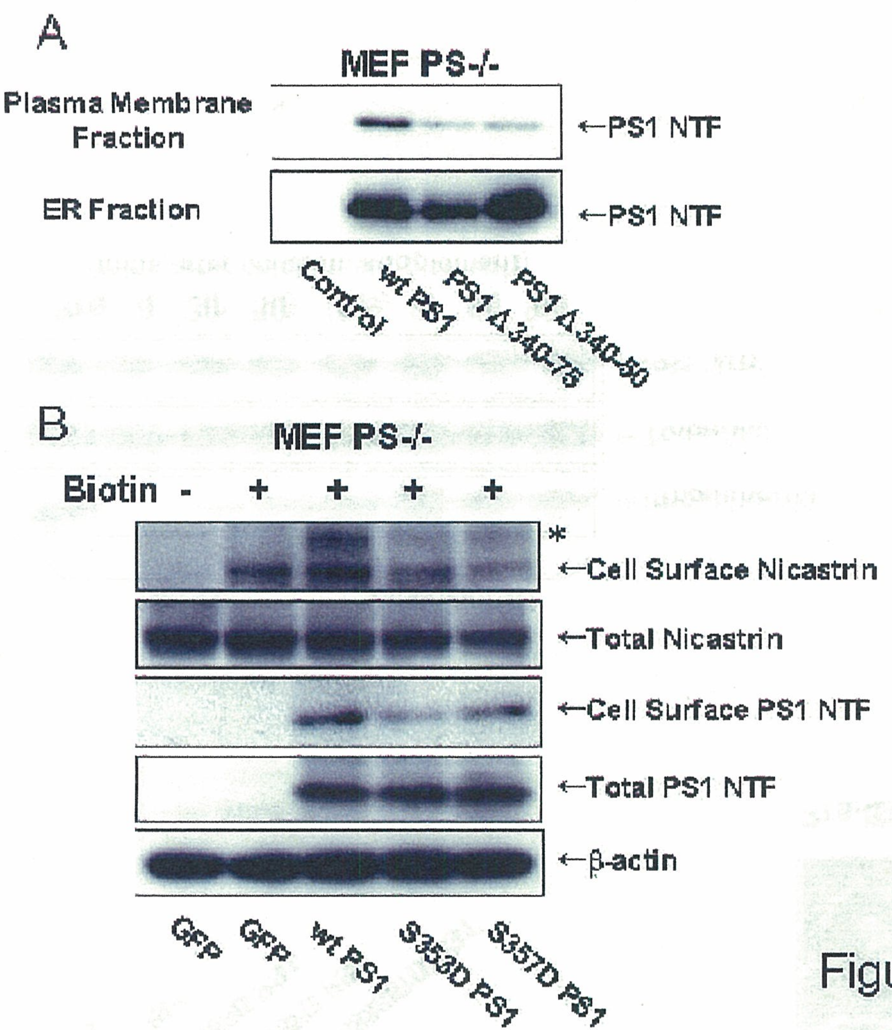

Figure 3.

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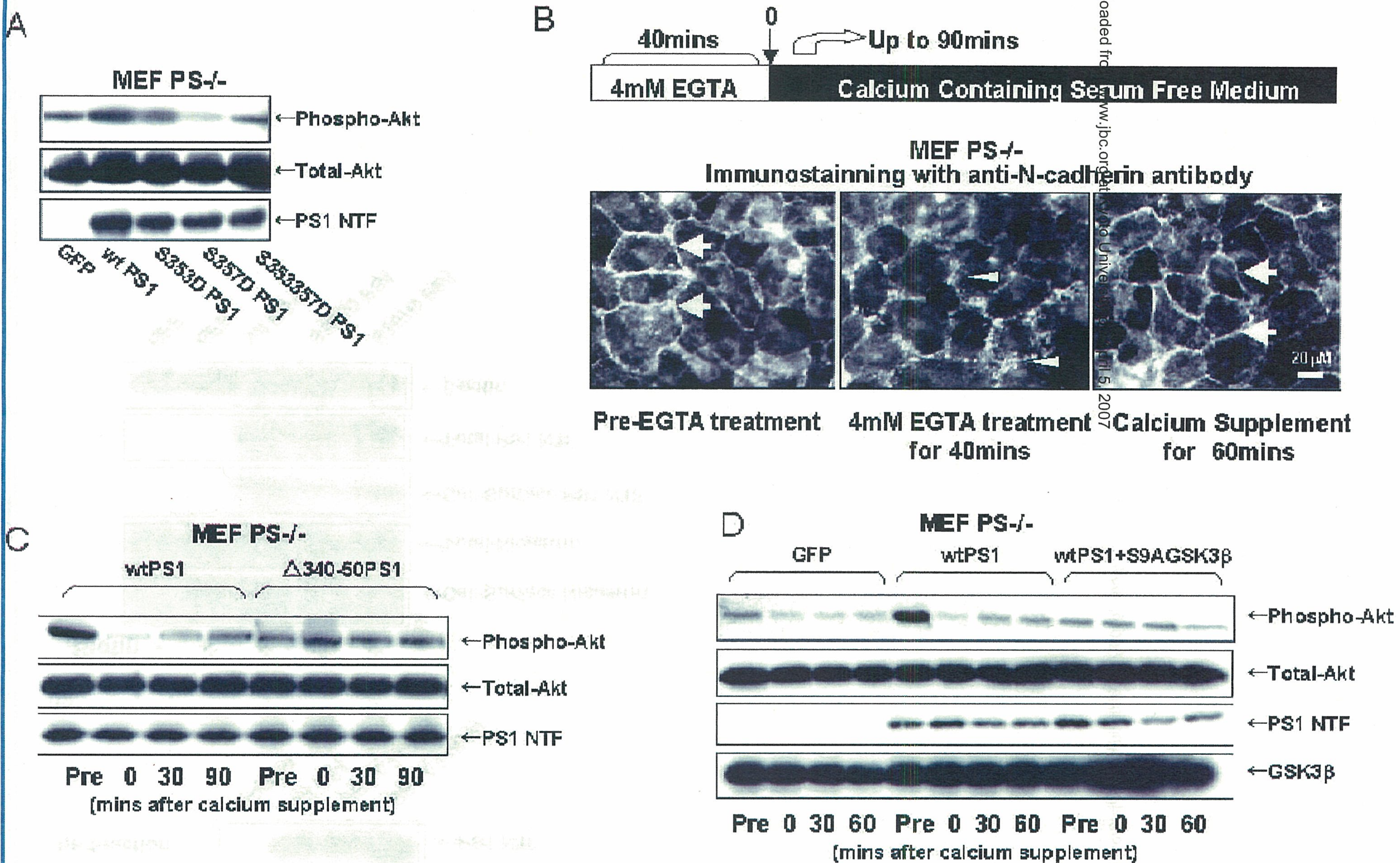

Figure 4.

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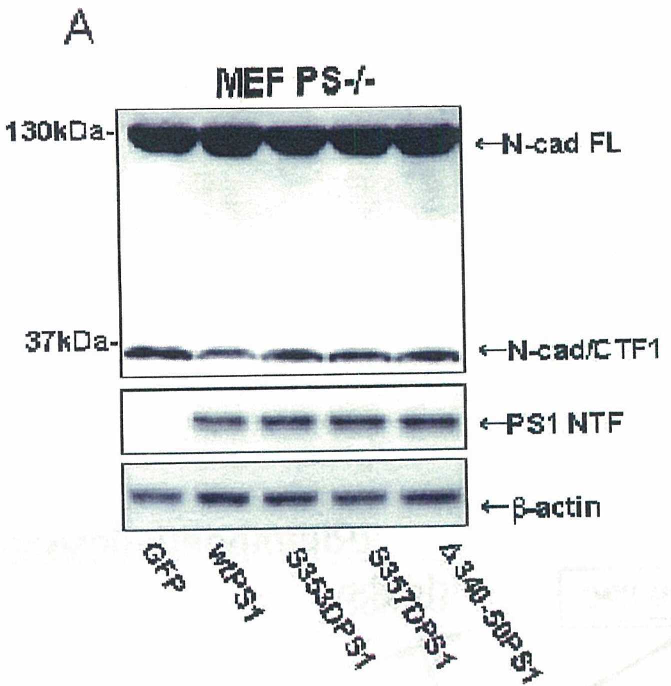

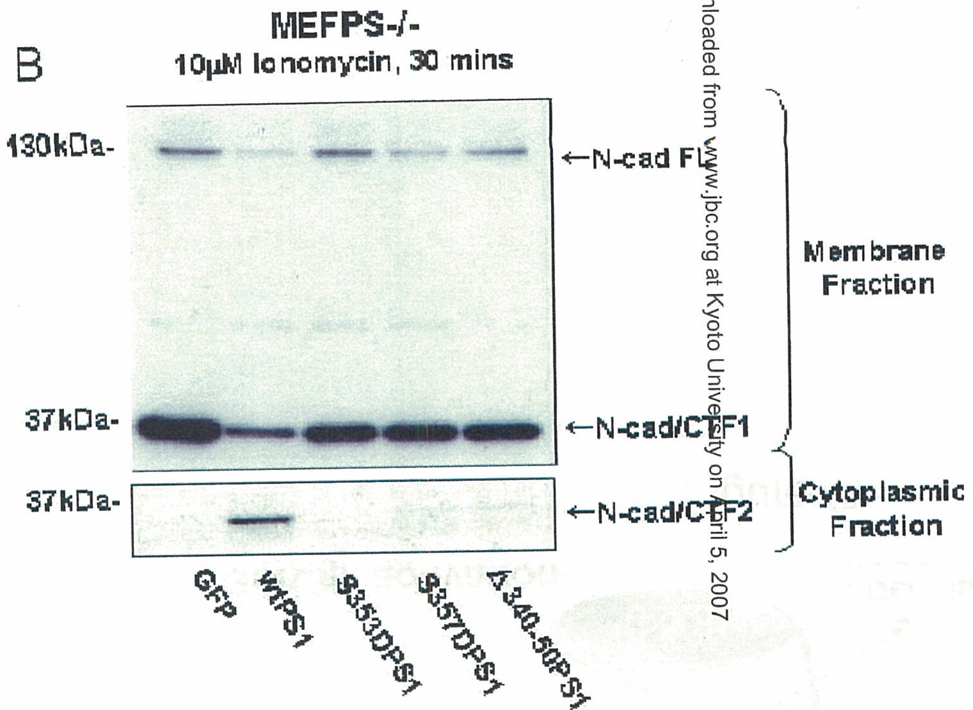

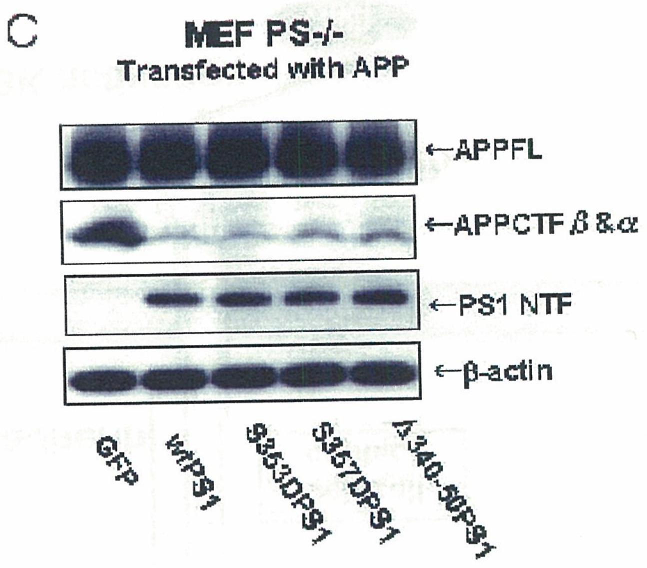

Figure 5.

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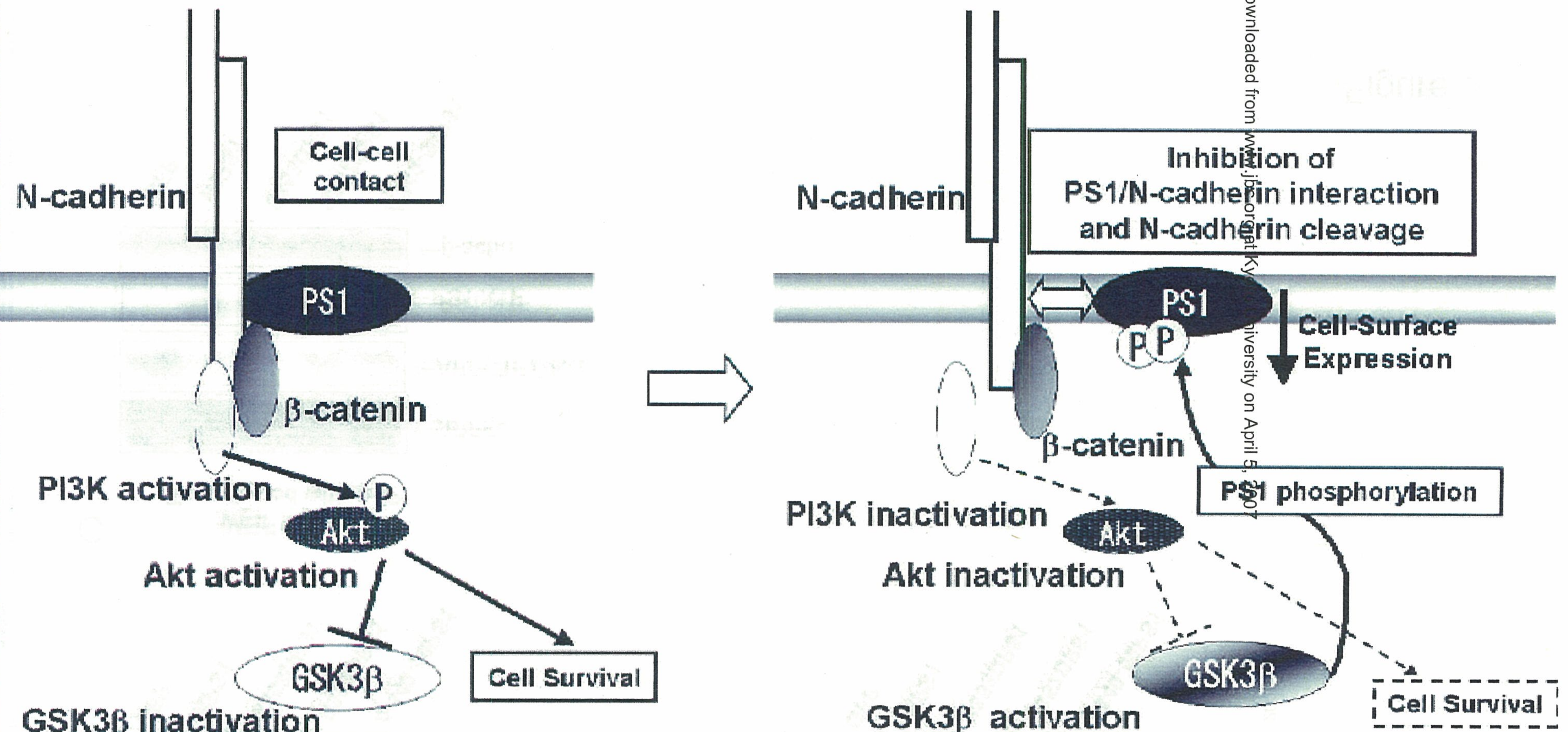

Figure 6.

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