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THE MUCIN Muc4 POTENTIATES NEUREGULIN SIGNALING BY INCREASING THE CELL SURFACE POPULATIONS OF ErbB2 AND ErbB3

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Mucins provide a protective barrier for epithelial surfaces, and their overexpression in tumors has been implicated in malignancy. We have previously demonstrated that Muc4, a transmembrane mucin that promotes tumor growth and metastasis, physically interacts with the ErbB2 receptor tyrosine kinase and augments receptor tyrosine phosphorylation in response to the NRG1β growth factor. In the present study we demonstrate that Muc4 expression in A375 human melanoma cells, as well as MCF7 and T47D human breast cancer cells, enhances NRG1β signaling through the PI-3 kinase pathway. In examining the mechanism underlying Muc4-potetiated ErbB2 signaling, we found that Muc4 expression markedly augments NRG1β binding to A375 cells without altering the total quantity of receptors expressed by the cells. Cell surface protein biotinylation experiments and immunofluorescence studies suggest that Muc4 induces the re-localization of the ErbB2 and ErbB3 receptors from intracellular compartments to the plasma membrane. Moreover, Muc4 interferes with the accumulation of surface receptors within internal compartments following NRG1β treatment by suppressing the efficiency of receptor internalization. These observations suggest that transmembrane mucins can modulate receptor tyrosine kinase signaling by influencing receptor localization and trafficking, and contribute to our understanding of the mechanisms by which mucins contribute to tumor growth and progression.

The ErbB family of receptor tyrosine kinases (RTKs) includes ErbB1/epidermal growth factor receptor (EGFR), ErbB2/Her2/Neu, ErbB3 and ErbB4. The EGF-like growth factor family of ligands binds the extracellular domain of the ErbB receptors leading to the formation of both homo- and heterodimers. Receptor dimer formation is followed by autophosphorylation of receptor intracellular domains, recruitment of intracellular signaling proteins, and the initiation of a number of signaling cascades that regulate cellular growth, motility and survival. ErbB receptors play critical roles both in development and tissue maintenance (1), and their overexpression and aberrant activation has been implicated in the genesis and progression of a variety of human tumors (2).

While in general receptor heterodimerization is thought to diversify ErbB signaling (1), heterodimerization with ErbB2 is required for ErbB3 signaling in response to the neuregulin-1 (NRG1) growth factor. No soluble growth factor ligand has been identified that binds to ErbB2, but it is the preferred dimerization partner of the other ErbB family members. Thus, it is thought that its function is to heterodimerize with the other ErbB family members to enhance signaling.

ErbB3 is a binding receptor for NRG1, but lacks intrinsic kinase activity (3) and is not capable of signaling on its own. The ErbB2-ErbB3 heterodimer efficiently activates the ras/Erk pathway, and the C-terminal domain of ErbB3 contains six binding sites for the p85 subunit of PI-3 kinase that very efficiently couple the activated receptor heterodimer to this pathway (4).

Muc4 is a member of the mucin gene family, and is a heterodimeric complex of two non-covalently linked subunits arising from a single gene (5). The large (~600 kDa) extracellular mucin subunit ASGP-1 is heavily O-glycosylated, and this portion of the molecule is thought to provide physical protection to epithelial surfaces. The membrane-associated subunit ASGP2 (120 kDa) includes two EGF-like domains in its extracellular portion, a single transmembrane domain and a short cytoplasmic tail. Muc4 is expressed in epithelia of the airway, ocular surface, lacrimal gland, female reproductive tract, oral cavity, and mammary gland, where its expression increases dramatically during pregnancy and lactation (6).

It has been previously demonstrated that Muc4, also known as sialomucin complex, exists in a complex with ErbB2 in a number of tissues and cell lines, including rat mammary gland (6), lacrimal gland (7), ocular surface epithelia (8), and female reproductive tract (9), as well as in the highly malignant rat ascites 13762 mammary adenocarcinoma cell line. Our previous expression studies demonstrate that the interaction of Muc4 and ErbB2 is mediated by their extracellular regions, and the amino terminal EGF-like domain of ASGP2 is required for their association (10). Moreover, the co-expression of the two proteins in the same cell is necessary for their interaction, suggesting that soluble or shed forms of Muc4 may not interact with ErbB2 *in vivo*.

One functional consequence of Muc4 interaction with ErbB2 is the potentiation of signaling in response to NRG1. Expression of Muc4 in A375 human melanoma cells results in the augmented tyrosine phosphorylation of ErbB2 and ErbB3 in response to growth factor (10). In addition, Muc4 expression in these cells enhances their growth rate as tumors (11) and their metastasis (12) in nude mice, suggesting that Muc4-augmented signaling through ErbB receptors may contribute to tumor progression. In this regard, it is interesting that Muc4 is frequently co-expressed with ErbB2 in highly aggressive human breast cancers (13), suggesting that overexpression of the mucin in tumors could augment ErbB2/ErbB3 signaling and tumor progression.

In the present study we uncover the mechanism underlying the ability of Muc4 to enhance ErbB2/ErbB3 response to ligand. We demonstrate that Muc4 prevents the intracellular accumulation of ErbB2 and ErbB3, thereby increasing the amount of receptor localized to the plasma membrane. This correlates with enhanced coupling of ErbB2/ErbB3 to the PI-3 kinase pathway and augmented cellular proliferation.

Experimental Procedures

Materials. Reagents were purchased as follows: Protein G Plus/Protein A Agarose from Oncogene Research Products; recombinant human EGF from Upstate Biotechnology; recombinant horseradish peroxidase-linked anti-phosphotyrosine antibody RC20 from BD Transduction Laboratories; anti-phospho-ErbB2 (Tyr1248) and (Tyr877) antibodies, anti-phospho-Akt Ser473 antibody, anti-phospho-p70 S6 Kinase Thr389 antibody, anti-phospho-p90rsk antibody, anti-phospho-FKHR antibody and anti-phospho-Erk1/2 antibody from Cell Signaling Technology; mouse monoclonal anti-actin AC-15 antibody, anti-glutathione-S-transferase-peroxidase conjugate antibody and reduced glutathione-Sepharose from Sigma; monoclonal anti-c-erbB3 antibody Ab4, rabbit

anti c-erbB2 antibody Ab21, monoclonal anti-EGFR antibody Ab1 (528) from NeoMarkers; anti-ErbB3 antibody C17 and anti-ICAM-1 antibody C19 from Santa Cruz Biotechnology; goat-anti-rabbit secondary antibody from Chemicon; goat-anti-mouse antibody from Zymed; AlexaFluor fluorescent secondary antibodies from Molecular Probes; inhibitors LY294002 and U0126, Biotin-X-NHS, and horseradish peroxidase conjugated streptavidin from Calbiochem; Oragami B competent cells, BugBuster and benzonase from Novagen; PreScission protease from Amersham Biosciences; FuGENE 6 transfection reagent and monoclonal anti-HA antibody from Roche. Monoclonal anti-ASGP-2 blotting antibody 4F12 has been previously described (14), monoclonal anti-ASGP-2 immunoprecipitating antibody 1G8 has previously been described (15), and anti-p85 polyclonal antibody was a generous gift of Dr. Lewis Cantley.

Recombinant GST-NRG1β fusion protein production. Recombinant beta isoform of neuregulin-1 was constructed by expressing the EGF-like domain of the beta isoform of NRG1 (sequence TSHLIKCAEKEKTFCVNGGECFMVKDLS NPSRYLCKCPNEFTGDRCQNYVMASFY KHLGIEFMEAEELYQK) as a C-terminal GST fusion protein. Briefly, the EGF-like domain region was amplified from murine cDNA clones and inserted into the EcoRI/BamH1 sites of pGEX-6P-2 (Amersham). Recombinant plasmids were introduced into Origami B competent cells, which support disulfide bond formation required for biologically active EGF-like domains. Expression was induced with 0.04mM IPTG for 2 hours at 25°C prior to harvest. Frozen bacterial pellets were lysed using BugBuster supplemented with 200 mM AEBSF and Benzonase (17 units/ml). GST fusion proteins were bound to reduced Glutathione Sepharose beads and washed 5 times with ice-cold PBS, 0.2% Tween-20, 0.2% Triton X-100, 10 mM EDTA followed by 5 washes with PBS, 1.0% Triton X-114 at 4ºC to remove bacterial endotoxins, and 5 rinses with PBS to remove excess Triton X-114, prior to 3 rounds of dialysis in 1X phosphate-buffered saline at 4ºC. Factor concentrations were estimated by comparative Coomassie staining with bovine serum albumin.

Cell Culture and Muc4 Induction. Construction of A375-Rep8, A375-Rep5 and MCF7-Rep5 cell lines expressing Muc4 under tetracycline regulation has been previously described (16). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 5% penicillin/streptomycin, 0.8 mg/ml G418, 0.3 mg/ml hygromycin. Cells were grown to 60% confluence in the presence of 2 μg/ml tetracycline, and then serum starved in 0.1% fetal calf serum for 48 hours in the presence or absence of 2 μg/ml tetracycline to induce Muc4 expression.

Analysis of Receptor Phosphorylation and Downstream Signaling. Induced, serum starved cells in 60 mm dishes were treated with 50 nM NRG1β for various times at 37°C. For pathway inhibition experiments cells were treated with inhibitors for 30 minutes prior to growth factor addition. Cells were rinsed twice in ice-cold PBS saline and lysed in 500 μl RIPA (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.1% SDS, 1% Trition-X-100, 1 mM Na_3VO_4, 1 mM NaF, 10 mM β-glycerophosphate, 5 mM tetrasodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride and 4 μg/ml each aprotinin, leupeptin and pepstatin). Lysates were cleared by microcentrifugation at 12,000xg for 10 min and added to SDS sample buffer. Lysates were resolved by 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Blots were cut horizontally into strips of the appropriate size ranges for detecting specific signaling proteins, and immunoblotted as previously described (17).

Blotted proteins were visualized by chemiluminesence using an Alpha Innotech Digital Imaging Station. NRG1β titration analysis was carried out using GraphPad Prism software. Each figure depicts a representative of at least three experiments.

Transient Transfections. Human embryonic kidney HEK-293T cells, or human T47D breast cancer cells, were grown in 100-mm dishes and transfected using FuGENE 6 reagent as recommended by the manufacturer. For co-immunoprecipitation experiments, HEK-293T cells were transfected with ErbB receptors without or with Muc4 construct Rep8 (16). For signaling experiments, T47D cells were transfected with HA-tagged Akt and HA-tagged Erk without and with Muc4 Rep8. Cells were serum starved overnight prior to growth factor treatment, and cell lysates were collected as described above.

Immunoprecipitation Experiments. Cells were lysed in co-immunoprecipitation buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1mM $MgCl_2$, 1% Nonidet P-40, 10% glycerol, 1 mM Na_3VO_4, 1 mM NaF, 10 mM β-glycerophosphate, 5 mM tetrasodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride and 4 μg/ml each aprotinin, leupeptin and pepstatin), and immunoprecipitation was carried out as previously described (17).

Proliferation Analysis. 150,000 A375-Rep8 cells were seeded into 60 mm dishes and simultaneously serum starved and treated with or without tetracycline for 12 hours. Cells were then treated for 24 hours with or without 50 nM NRG1β in the absence or presence of 20 μM LY294002 or 10 μM U0126. Cell number was determined using a Coulter counter after harvesting in 1X Trypsin-EDTA.

Immunofluoresence. Serum-starved and induced cells on 25 mm round cover slips were pre-incubated with 1/500 dilutions of primary antibodies (mouse monoclonal anti-ErbB3 Ab4, rabbit anti-ErbB2 Ab21, mouse monoclonal anti-EGFR Ab1, mouse monoclonal anti-ASGP2 4F12) in PBS for 1 hour at 4°C. Cells were then treated with growth factors at 37°C for 20 minutes, rinsed with ice-cold PBS and fixed with 4% paraformaldehyde. Cells on cover slips were then blocked, incubated with fluorescent secondary antibodies and mounted as previously described (18). Receptors were visualized using an Olympus BX61 fluorescence microscope by capturing 12 z-plane images at 0.5 μm intervals encompassing the depth of the cell. Flat field corrected image stacks were deconvolved by the constrained iterative method to mathematically remove out-of-focus light from the image stack using SlideBook 4.1 software (Intelligent Imaging Innovations), resulting in confocal quality images of receptor localization in three dimensions. PS-Speck green and orange fluorescent beads (Molecular Probes) were used to generate the point spread functions used in deconvolutions.

Preparation of Cell Membranes and Dephosphorylation Experiments. Induced cells were harvested in PBS, 1 mM EDTA and incubated in hypotonic buffer (20 mM HEPES, pH 7.4) containing protease inhibitors for 30 min at 4°C. Cells were homogenized with 80 strokes in a Dounce homogenizer, and nuclei were pelleted at 3000x*g* for 5 min. Supernatants were then centrifuged at 20,000x*g* for 45 min, and the membranous pellets were resuspended in 20 mM HEPES pH 7.4, 100 mM NaCl, 1 mM DTT with protease inhibitors. Preparations were kept frozen at -80°C until use. For dephosphorylation assays, membranes were incubated with 5 mM $MnCl_2$ and 100 μM ATP for 10 min at room temperature to allow receptor phosphorylation. The phosphorylation reaction was terminated with the addition of EDTA to a final concentration of 10 mM, to elicit dephosphorylation. To promote receptor dimerization, membranes were incubated for various times in the absence or presence of NRG1β. In some cases calf intestinal phosphatase (CIP) was added.

Samples were collected in 2X SDS sample buffer at various time points after termination of the phosphorylation reaction and analyzed by immunoblotting.

Preparation of Non-GST NRG1β and NRG1β Binding Assay. The GST moiety was cleaved from GSH-Sepharose-bound GST-NRG1β using PreScission Protease (Amersham) per the manufacturer's instruction. Induced A375-Rep8 cells were treated with saturating concentrations of GST-NRG1β in combination with either increasing concentrations of non-GST NRG1β or GST alone, for 60 min on ice. Cells were extensively washed using ice-cold 1X PBS to remove unbound growth factor, and lysates were immunoblotted for GST. Bands were quantified by densitometry.

Receptor Internalization Assay. GST-NRG1β was bound to induced, serum starved A375-Rep8 cells as described above. After extensive washing with ice-cold PBS to remove unbound growth factor, cells were incubated at 37°C for various times to initiate ligand internalization. Cells were then washed with ice-cold PBS and then treated on ice for 20 minutes with 0.2M acetic acid, 0.5M NaCl, to dissociate remaining surface receptor-bound GST-NRG1β. Lysates were collected as described above and immunoblotted for GST. The density of each band was obtained using AlphaInnotech FluorChem software and used in the determination of internalization percentage.

Receptor Biotinylation. Induced and uninduced cells were washed with ice-cold 1X PBS and incubated with 0.5 mg/ml of Biotin-X-NHS dissolved in a borate buffer (10 mM boric acid, 150 mM NaCl, pH 8.0) for 1 hr at 4°C. Cells were extensively washed with ice-cold 1X PBS containing 15 mM glycine to terminate biotin coupling. Receptors were then immunoprecipitated and blotted with horseradish peroxidase-conjugated streptavidin, or precipitated with streptavidin agarose and blotted with receptor antibodies.

Results

Muc4 specifically potentiates NRG1β-stimulated ErbB2 tyrosine phosphorylation and PI-3 kinase signaling. We have previously shown that Muc4 expression potentiates NRG1β-stimulated tyrosine phosphorylation of ErbB2 and ErbB3, and that these effects cannot be accounted for by an increase in overall receptor expression levels in cells (10). To examine the mechanisms underlying Muc4 potentiation of ErbB receptor signaling, we analyzed the properties of A375 human melanoma cells stably expressing rat Muc4 under tetracycline control (A375-Rep8 cells; 16). As shown in Figure 1, tetracycline withdrawal from A375-Rep8 cells potently induced Muc4 expression as determined by immunoblotting whole cell lysates with an antibody to the ASGP2 subunit. Muc4 expression enhanced the tyrosine phosphorylation of ErbB receptors after stimulation with saturating concentrations (50 nM) of NRG1β, as observed by blotting for phosphotyrosine, but did not augment basal receptor phosphorylation. Blotting with specific anti-phospho-ErbB2 antibodies revealed that Muc4 expression induced the hyperphosphorylation of ErbB2 at Y1248 and Y877, sites that have been implicated in ErbB2 signaling (19, 20). Importantly, tyrosine phosphorylation of ErbB2 at Y1248 is an independent predictor of poor prognosis in human breast cancer (21).

ErbB2 Y1248 serves as a docking site for the adaptor protein Grb2, which links the activated receptor to signaling by the Erk serine/threonine kinases. Interestingly, while Y1248 was hyperphosphorylated in the presence of Muc4, Erk signaling was unaffected as shown by the phosphorylation of Erk1/2 and its downstream target p90RSK (Figure 2A). However, Muc4 significantly potentiated the phosphorylation of the serine/threonine kinase Akt and its downstream transcription factor target

Forkhead (FKHR). Phosphorylation of the mTor target p70S6 kinase was also enhanced. These observed hyperphosphorylation events could not be explained by increased levels of the signaling proteins (data not shown), and point to the possibility that Muc4 selectively potentiates ErbB receptor signaling through the PI-3 kinase pathway. In contrast, Muc4 failed to potentiate Akt phosphorylation in response to EGF (Figure 2B) and insulin (Figure 2C), indicating that it specifically modulates ErbB2/ErbB3 receptor signaling.

To determine whether these results may be extended beyond a single cell line, we examined the impact of MUC4 expression on NRG1 signaling in the human breast cancer cell lines MCF7 and T47D. As illustrated in Figure 3A, Muc4 similarly potentiated NRG1β-induced stimulation of ErbB2/ErbB3 and Akt phosphorylation but not Erk phosphorylation in MCF7 cells stably transfected with tetracycline-regulated rat Muc4 (MCF7-Rep5; 16). Likewise, transient co-transfection of T47D cells with Muc4 and either HA-tagged Akt or HA-tagged Erk1 revealed that MUC4 selectively potentiated NRG1β-stimulated Akt but not Erk phosphorylation in these cells as well (Figure 3B). Together these observations suggest that potentiation of ErbB2/ErbB3 signaling to Akt is a general feature of Muc4 expression.

Since previous reports suggest Akt phosphorylation may be augmented in a PI-3 kinase-independent manner (e.g. ref 22), we first examined whether the Muc4-mediated Akt hyperphosphorylation was dependent on PI-3 kinase activity using the specific inhibitor LY294002. As illustrated in Figure 4A, addition of LY294002 abolished all NRG1β-stimulated Akt phosphorylation, indicating that Muc4 mediates Akt hyperphosphorylation through the PI-3 kinase pathway. As PI-3 kinase activation by the ErbB2/ErbB3 heterodimeric complex is thought to be mediated largely by ErbB3 with its six p85 subunit binding sites (23), we examined whether Muc4 could enhance PI-3 kinase association with ErbB3. Figure 4B demonstrates that Muc4 expression resulted in enhanced recruitment of p85 to NRG1β-stimulated ErbB3, with no impact on the levels of ErbB3 protein.

As illustrated in Figure 4C, PI-3 kinase pathway but not Erk pathway signaling is necessary for the proliferation of A375 cells. In this experiment 150,000 serum-starved cells were grown in the presence and absence of tetracycline or NRG1β, and cell number was determined after 24 hours. Expression of Muc4 and treatment with 50 nM NRG1β each stimulated the growth of A375 cells, and the two treatments together further augmented cellular growth. Proliferation was potently inhibited under all conditions by Ly294002, but was unaffected by the MEK inhibitor U0126, even though each inhibitor very effectively suppressed its respective pathway (not shown). These observations indicate that the NRG1β-stimulated proliferation of these cells is strictly PI-3 kinase-dependent, and that the selective potentiation of PI-3 kinase signaling by Muc4 drives enhanced cellular growth responses to growth factor stimulation.

To better understand the mechanism by which Muc4 potentiates NRG1-stimulated PI-3 kinase signaling, we further investigated its interaction with ErbB2 and ErbB3 in the presence and absence of NRG1β. Co-immunoprecipitation of ErbB receptors with Muc4 could not be detected in A375 cells, possibly because receptor levels are relatively low. However, we observed that Muc4 could be co-immunoprecipitated with either ErbB2 or ErbB3, or both, in transiently transfected 293T cells, and the interactions could be detected independently of NRG1β treatment (Figure 5).

Taken together, the observations illustrated in Figures 1-5 point to a model whereby Muc4 interacts specifically with ErbB2 and ErbB3 receptors to mediate their hyperphosphorylation. Potentiated receptor

activity does not significantly impact Erk activation, either because events following receptor phosphorylation restrain Erk signaling, or because the level of receptor phosphorylation in the absence of Muc4 expression is sufficient to fully activate this pathway. However, Muc4-induced receptor hyperphosphorylation does lead to increased PI-3 kinase recruitment to ErbB3 and hyperactivation of Akt, which in turn contributes to Muc4-induced cellular proliferation. We consider below several possible mechanisms by which Muc4 could potentiate ErbB receptor tyrosine phosphorylation.

Muc4 does not alter the sensitivity of NRG1β signaling or protect receptors from dephosphorylation. One possible explanation for Muc4-potentiated receptor phosphorylation is that mucin expression enhances the sensitivity of cell surface receptors for ligand, yielding a greater number of receptors phosphorylated at moderate ligand concentrations. This seems unlikely because our experiments were carried out under conditions of saturating growth factor ligand concentrations where all surface receptors should have been fully activated. However, to address this possibility we examined the dose-response of receptor and Akt phosphorylation to NRG1β.

A375-Rep8 cells were treated with and without tetracycline to induce Muc4 expression, and were then treated with increasing concentrations of NRG1β. As shown in Figure 6A, Muc4 expression resulted in enhanced receptor phosphorylation and Akt activation at all concentrations of ligand. Receptor and Akt phosphorylation was quantified and values were plotted to obtain the dose of NRG1β required for half-maximal receptor and Akt phosphorylation. As shown in Figure 6B, Muc4 expression had no effect on the NRG1β dose required for half-maximal receptor and Akt phosphorylation but enhanced the overall response of receptors to ligand, even under receptor saturating conditions. These observations suggest either that each receptor is responding more robustly to a given concentration of NRG1β in the presence of Muc4, or that Muc4 makes more receptors available for stimulation.

Another explanation that could account for the augmented response of receptors to NRG1β is that Muc4 limits receptor access to phosphatases. It has been reported, for example, that ligand stimulation of PDGF β-receptor protects it from tyrosine dephosphorylation, possibly due to steric hindrance imposed by receptor dimerization (24).

We employed a similar method to determine whether Muc4 expression alone or in conjunction with NRG1β-induced receptor dimerization influences phosphatase access to ErbB receptors. In the experiment depicted in Figure 7, isolated plasma membranes were prepared from A375-Rep8 cells treated with or without tetracycline. Receptors were first phosphorylated *in vitro*, and the phosphorylation reaction was stopped with the addition of a chelating agent. NRG1β was then added to one set of reactions to generate receptor dimers. The rate of receptor dephosphorylation in membranes by endogenous membrane-associated phosphatases or exogenously added calf intestinal phosphatase (CIP) was then examined over a twenty minute time course. While Muc4 expression markedly enhanced the tyrosine phosphorylation of receptors in membranes, it did not protect the receptors from dephosphorylation either in the absence (Figure 7A) or presence (Figure 7B) of added NRG1β, as indicated by phosphotyrosine blotting. These observations suggest that neither NRG1β-induced receptor dimerization nor Muc4 expression protect receptors from phosphatases.

Muc4 increases NRG1β binding by enhancing cell surface associated ErbB2 and ErbB3. A third possible explanation for

potentiated ErbB2/ErbB3 signaling is that Muc4 expression makes more receptors available at the cell surface. In the experiment illustrated in Figure 8A, recombinant GST-tagged NRG1β was allowed to bind to surface receptors under conditions where internalization was prevented. Unbound growth factor was then removed by extensive washing, and NRG1β binding was assessed by blotting whole cell lysates for GST. In order to establish specificity in growth factor binding, GST-NRG1β was competed off by titration of increasing amounts of recombinant non-GST-tagged NRG1β. We observed that Muc4 expression resulted in a substantial increase in the specific binding of GST-NRG1β, suggesting that Muc4 increases the number of cell surface receptors available for growth factor binding. Quantification of these results (Figure 8B) revealed that Muc4 increased NRG1β binding to cell surface receptors by greater than 5 fold.

To determine whether Muc4 augments the number of receptors at the cell surface, biotinylation experiments were carried out. Cell surface ErbB2 and ErbB3 receptors were biotinylated under conditions preventing internalization, and receptor immunoprecipitates were blotted with streptavidin (Figure 9A). Muc4 expression increased the amounts of cell surface associated ErbB2 and ErbB3, as determined by an increase in biotinylated receptors, without altering the total cellular levels of these receptors observed in lysates. Likewise, immobilized streptavidin precipitated more ErbB2 and ErbB3, but not EGF receptor, after expression of Muc4 (Figure 9B). Our observations that Muc4 increases surface ErbB2 and ErbB3 without changing receptor levels indicate that Muc4 induces a relocalization of receptors from intracellular stores to the cell surface. Immunofluorescence microscopy has confirmed that Muc4 expression in A375-Rep8 cells results in the loss of ErbB2 and ErbB3 from punctate intracellular structures (not shown), providing further support for Muc4-mediated ErbB relocalization. Thus, enhanced NRG1β stimulation of receptor phosphorylation, as well as enhanced signaling through PI-3 kinase, may result from the increased presence of ErbB2 and ErbB3 receptors at the cell surface.

Muc4 inhibits the intracellular accumulation of activated ErbB2 and ErbB3. To determine the mechanism by which Muc4 mediates ErbB receptor re-localization, we examined the internalization of cell surface receptors. In the experiment depicted in Figure 10A, surface EGF receptor, ErbB2 or ErbB3 in A375-Rep8 cells were labeled with their respective antibodies at 4^{o}C, conditions under which internalization is prevented. Cells were then simultaneously shifted to 37^{o}C and treated with growth factor for 20 minutes to elicit receptor endocytosis. Cells were then fixed and permeabilized, and the localization of receptors originally at the surface was determined by three-dimensional deconvolution immunofluorescence microscopy. Before the addition of growth factor, each of the ErbB receptors were present in a punctate pattern at the cell surface. Addition of EGF for 20 minutes induced the accumulation of a fraction of the surface EGF receptors into punctate perinuclear structures. Likewise, 20 minute treatment with NRG1 induced the accumulation of the bulk of ErbB2 and ErbB3 receptors into similar structures. Muc4, expressed in a more uniform pattern at the cell surface, had no effect on EGF-induced EGF receptor internalization but completely blocked NRG-stimulated ErbB2 and ErbB3 intracellular accumulation. These observations suggest that Muc4 mediates the cell surface retention of receptors by inhibiting their internalization and accumulation in internal compartments.

To quantify the effect of Muc4 on ErbB internalization, we examined the rate of

NRG1 accumulation inside cells. In these experiments GST-NRG1β was bound to the cell surface receptors at 4°C. After extensive washing, receptor/GST-NRG1β complex internalization was initiated by shifting the cells to 37°C for various times. Cells were then incubated with a mildly acidic solution to remove any non-internalized growth factor from the cell surface, and internalized NRG1β was determined by blotting cell lysates for GST. Quantification of acid-resistant, cell-associated GST revealed that over 40% of the growth factor originally bound to the cell surface was internalized over 15 minutes in the absence of Muc4, while only ~7% of the cell surface factor was internalized when Muc4 was expressed.

Taken together, the immunofluorescence and ligand uptake studies indicate that Muc4 expression potently suppresses the NRG1-induced accumulation of receptors within cells. These observations suggest that Muc4-induced translocation of receptors from intracellular compartments to the cell surface arises from a retention of receptors at the cell surface and a prevention of their accumulation in intracellular compartments.

Discussion

A growing number of studies indicate that a disparate array of plasma membrane proteins can physically interact with growth factor receptors to augment their signaling capacity. For example, CD44, a transmembrane protein that acts as a receptor for hyaluronan and functions in cellular adhesion and motility (25), has been demonstrated to physically interact with ErbB2 and ErbB3 in Schwann cells and human tumor cells to potentiate signaling in response to NRG1 (26, 27). Recently FLRT3, a transmembrane protein possessing extracellular leucine-rich repeat and fibronectin domains, has been demonstrated to physically interact with FGF receptors in *Xenopus laevis*. In this instance, FLRT3 association appears to promote FGF receptor signaling, particularly through the Erk pathway (28). While these observations underscore a role for transmembrane modulator proteins in augmenting receptor tyrosine kinase activity and influencing signaling pathway utilization, the mechanisms underlying receptor potentiation are not understood.

In this study we demonstrate that Muc4 enhances ErbB2/ErbB3 receptor tyrosine phosphorylation by making more receptors available at the plasma membrane for interaction with the NRG1 ligand, without affecting the total quantity of receptors expressed by the cell. We have observed that in contrast with the EGF receptor, significant amounts of ErbB2 and ErbB3 accumulate within internal compartments in many cell types. Our observations suggest that Muc4 expression induces the relocalization of these receptors to the cell surface, markedly augmenting the number of NRG1 binding sites and the efficiency of NRG1 signaling.

It should be noted that previous studies have suggested that Muc4 might act as a growth factor ligand for ErbB2, stimulating the phosphorylation of Y1248 in the absence of added growth factor (29). In contrast, we find no evidence for Muc4-stimulated receptor tyrosine phosphorylation. While the reasons underlying the discrepancy are unclear, it is possible that Muc4 expression is sufficient to unmask receptor responses to low levels of autocrine factors in some cell lines.

It should also be noted that previous studies indicated that Muc4 interacts specifically with ErbB2 and not other members of the ErbB family when co-expressed in insect cells (10). Here we demonstrate that Muc4 can also interact with ErbB3 when expressed in mammalian cells, suggesting that cell type-dependent factors may influence Muc4/receptor interactions.

The precise mechanism by which Muc4 induces ErbB2 and ErbB3 translocation is not clear. Our observations suggest that Muc4 expression impairs receptor internalization and accumulation in intracellular compartments. Since Muc4 is found almost exclusively at the cell surface, one possibility would be that the physical association of receptors with Muc4 causes them to adopt the localization and trafficking patterns of the mucin. Thus, Muc4 could interfere with the accumulation of receptors in internal compartments by preventing receptor internalization. Alternatively, Muc4 expression could affect ErbB2 and ErbB3 trafficking independent of its physical interaction with receptors by directly affecting trafficking machinery. Such an effect must be specific for ErbB2 and ErbB3 trafficking, however, because Muc4 does not affect EGF receptor intracellular accumulation. Future studies will be aimed at elucidating mechanisms of Muc4-mediated receptor trafficking.

Muc4 enhances the recruitment of PI-3 kinase to ErbB3 and augments ErbB2/ErbB3 signaling through the PI-3 kinase pathway. Since activation of the PI-3 kinase pathway occurs at the plasma membrane, the ability of Muc4 to trap receptors at the plasma membrane likely underlies its preferential effects on PI-3 kinase signaling. The PI-3 kinase pathway is more effectively activated by surface receptors because receptors in intracellular compartments have poor access to the lipid substrates required for PI-3 kinase signaling (30). In fact, the PI-3 kinase substrate PtdIns(4,5)P_2 is absent in endosomes, and endosomal EGF receptors are unable to signal through the PI-3 kinase pathway (31). Indeed, receptor trafficking plays a critical role in determining which signaling proteins are encountered and the pattern of pathways activated (32, 33). Accordingly, any disruption in trafficking would be expected to perturb the overall pattern of signaling.

The PI-3 kinase pathway is an important regulator of cellular proliferation and survival, and it has recently been demonstrated that PI-3 kinase is essential for ErbB2/ErbB3 mediated breast tumor cell proliferation (34). Consistent with this, our observations indicate that PI-3 kinase activity is essential for Muc4- and NRG1-stimulated proliferation of A375 cells. Several PI-3 kinase pathway components are dysregulated in cancers, and aberrant pathway activation contributes to tumor progression (4). For example, aberrant PI-3 kinase signaling has been implicated in malignant melanoma development (35, 36) and in therapeutic resistance in breast tumors (37). Therefore, our findings that Muc4 amplifies PI-3 kinase signaling by the ErbB2/ErbB3 heterodimer could have important clinical implications.

Muc4 overexpression is frequently observed in numerous cancers, including pancreatic adenocarcinomas (38) where its expression is associated with metastatic phenotype (39), lung adenocarcinomas (40), intrahepatic cholangiocarcinoma-mass forming type where its co-expression with ErbB2 correlated with short survival time (41), and non-small-cell lung cancer, where its expression is also associated with ErbB2 overexpression and early postoperative recurrence (42). Our observations suggest that Muc4 overexpression could contribute to tumor progression by augmenting PI-3 kinase activity through ErbB2/3 hyperactivation.

While Muc4 is commonly found co-overexpressed with ErbB2 in various tumors, our findings also underscore a potential role for Muc4 in augmenting the progression of tumors that express low ErbB2 levels. As demonstrated in the A375, MCF7 and T47D cells, Muc4 potentiates ErbB signaling in tumor cells that express modest levels of ErbB receptors. In addition, Muc4 expression alters receptor trafficking, effectively increasing levels of cell surface receptors available for signaling. In this regard, aberrant Muc4 could

mimic ErbB2 overexpression and augment the growth properties of the 70-80% of breast cancers that do not overexpress this receptor.

Previous studies indicate that other transmembrane mucins can also modulate receptor signaling. Muc1 is a member of the transmembrane mucin family whose aberrant expression has been linked to highly aggressive carcinomas. Unlike Muc4, Muc1 lacks EGF-like domains and possesses a much larger cytoplasmic domain containing several potential sites for tyrosine phosphorylation. Phosphorylation of Muc1 by activated EGF receptor enhances recruitment of c-src and β-catenin to the mucin (43) and potentiates EGF-stimulated ERK1/2 signaling (44). Recently the novel transmembrane mucin Muc20 was found to interact with the Met receptor tyrosine kinase, preventing Grb2 recruitment to the activated receptor and negatively modulating hepatocyte growth factor induced Erk1/2 signaling without affecting the PI-3 kinase pathway (45). These observations suggest that the modulation of receptor tyrosine kinase activity and signaling pathway usage by transmembrane mucins may represent a general theme.

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Legends

Fig. 1. Potentiation of ErbB activity by Muc4 expression. A375-Rep8 cells were treated with and without tetracycline to induce Muc4 expression, and then treated with NRG1β for the indicated times. Cell lysates were immunoblotted in the 170-225 kDa size range with antibodies to phosphotyrosine (pY) or ErbB2 tyrosine phosphorylation sites Y877 and Y1248. Lysates were also blotted with antibodies to Muc4 subunit ASGP2 and actin.

Fig. 2. Selective potentiation of the Akt signaling pathway by Muc4. (A) A375-Rep8 cells were treated with and without tetracycline and NRG1β, and lysates were immunoblotted with phospho-specific antibodies to the indicated signaling proteins. Lysates from EGF **(B)** or insulin **(C)** treated A375-Rep8 cells were blotted with the indicated antibodies.

Fig. 3. Muc4 selectively potentiates the Akt signaling pathway in breast cancer cell lines. (A) MCF7-Rep5 cells were treated with and without tetracycline or NRG1β, and blotted with the indicated antibodies. **(B)** T47D cells were co-transfected as indicated with Muc4 (Rep8), HA-Akt and HA-Erk1 and treated with NRG1β for the indicated times. Lysates and anti-HA immunoprecipitates were blotted with the indicated antibodies.

Fig. 4. Muc4-potentiated Akt signaling and NRG1β stimulated proliferation is dependent on PI-3 kinase. (A) The potentiation assay was carried out in the absence and presence of 20 μM LY294002, as indicated. **(B)** Cells were treated without and with tetracycline and NRG1β, as indicated, lysates (lower panel) were immunoprecipitated with anti-ErbB3, and precipitates (upper panels) or lysates were blotted with the indicated antibodies. **(C)** $1.5x10^5$ cells were treated without or with tetracycline, 50 nM NRG1β, 20 μM Ly294002 or 10 μM U0126, as indicated, for 24 hours and the cell number determined. Shown are averages and standard errors of triplicate samples.

Fig. 5. Muc4 interacts with ErbB2 and ErbB3 independent of receptor activation. HEK-293T cells were transfected as indicated, treated without or with NRG1β, and lysates were immunoprecipitated using anti-ASGP2 monoclonal antibody 1G8.

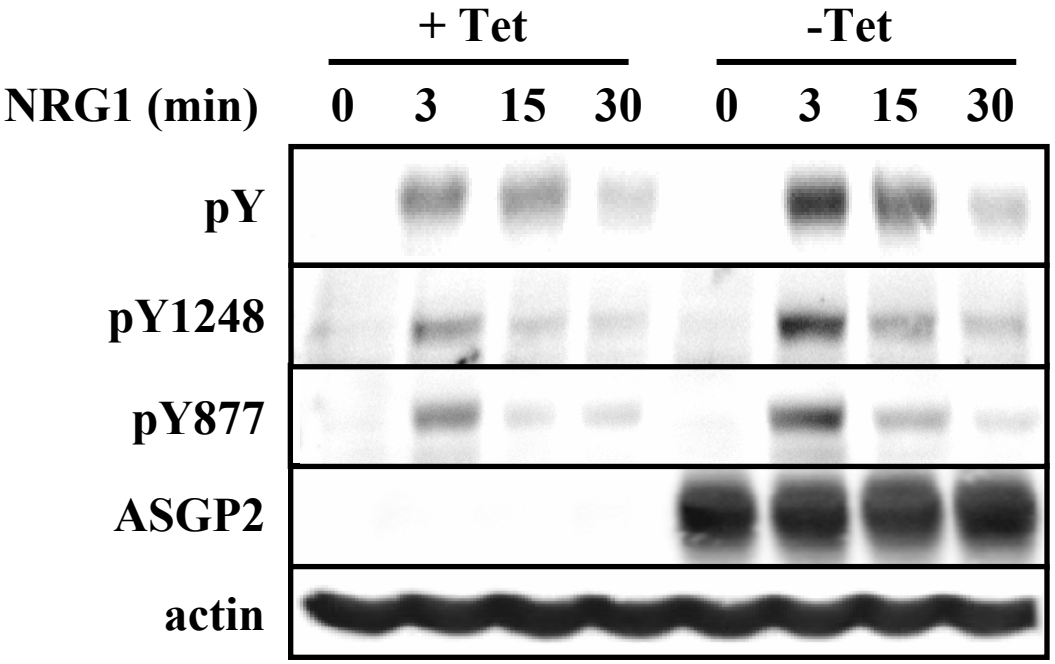
Fig. 6. Muc4 does not sensitize receptors to lower concentrations of NRG1β. (A) Cells treated with and without tetracycline were incubated with the indicated concentrations of NRG1β, and lysates were blotted with the indicated antibodies. **(B)** Quantification of the ErbB (left panel) and Akt (right panel) phosphorylation data from **(A)**. The concentration of NRG1β required for half-maximal response (K_{app}) was determined by fitting the data to the equation for a single class of binding sites.

Fig. 7. MUC4 does not affect receptor sensitivity to phosphatases. Membranes isolated from A375-Rep8 cells were phosphorylated with the addition of $MnCl_2$ and ATP, and then treated with EDTA (upper panels) or EDTA and calf intestine phosphatase (middle panels) for the indicated times to promote dephosphorylation. Dephosphorylation was carried out in the absence **(A)** and presence **(B)** of added NRG1β.

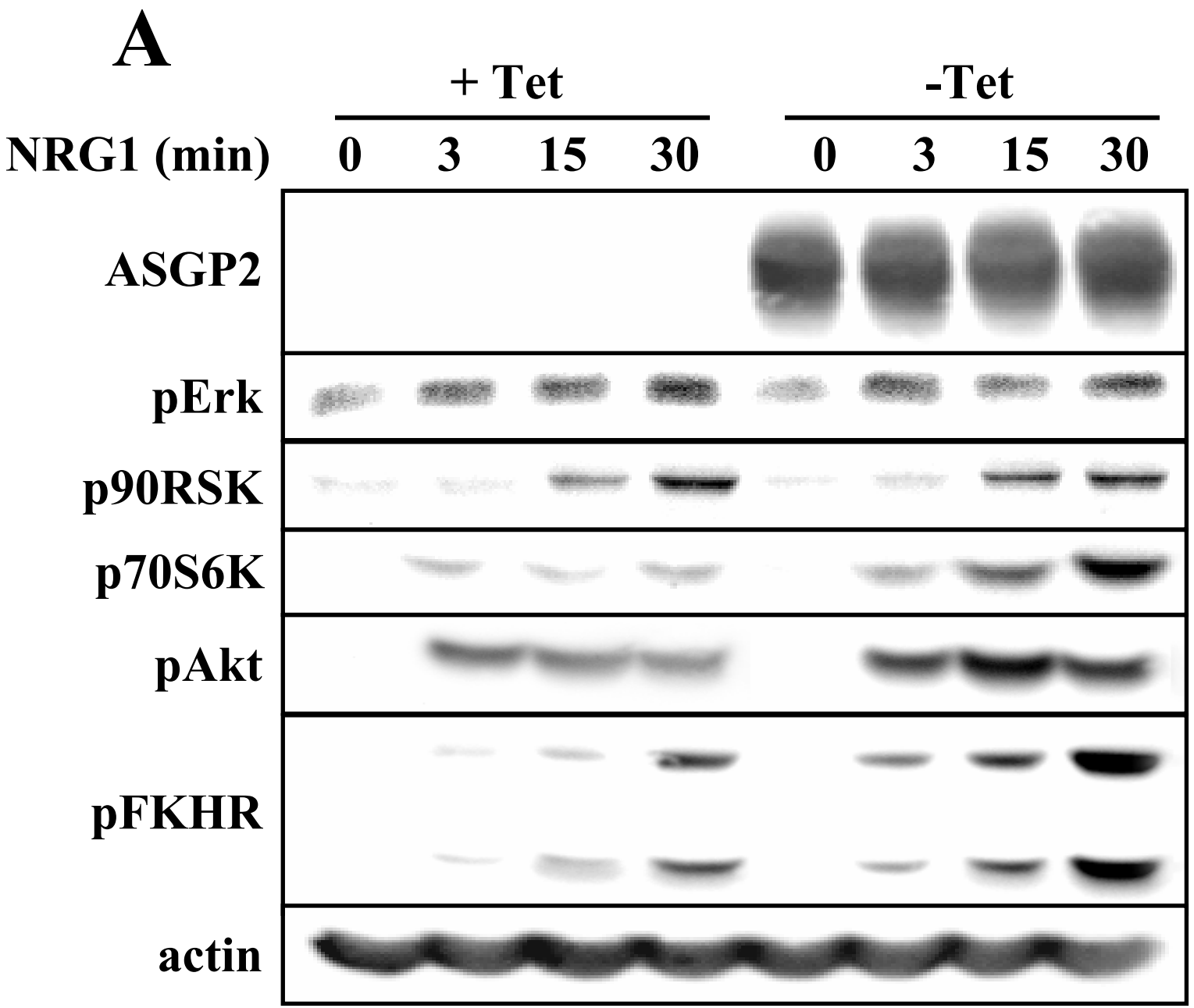
Fig. 8. Muc4 expression augments NRG1β binding sites. A) Cells were treated without and with tetracycline to induce Muc4 expression. Cells were then incubated at 4°C with nothing, or with GST-tagged NRG1β in the presence of increasing levels of non-tagged NRG1β or in the presence of GST. Lysates from washed cells were blotted with the indicated antibodies. **B)** The anti-GST bands in panel A were quantified and plotted.

Fig. 9. Muc4 increases surface ErbB2 and ErbB3 levels without affecting total cellular receptor levels. Cells were treated with and without tetracycline, and cell surface proteins were biotinylated for 1 hour at 4°C. **(A)** ErbB2 and ErbB3 receptors from lysates (lower panels) were immunoprecipitated, and blotted with streptavidin or anti-receptor antibodies. **(B)** Biotinylated proteins were precipitated with streptavidin agarose and blotted with anti-receptor antibodies.

Fig. 10. Muc4 expression interferes with ligand-stimulated accumulation of ErbB2 and ErbB3 in internal compartments. (A) A375-Rep8 cells treated with and without tetracycline were incubated at 4°C with antibodies to the indicated receptors to specifically label cell surface receptors. Cells were then simultaneously treated with growth factors and switched to 37°C to promote internalization of receptor/antibody complexes. After 20 minutes cells were fixed, and the localization of receptors originally at the cell surface was determined by three dimensional deconvolution immunofluorescence microscopy. **(B)** Localization of Muc4 was similarly determined. **(C)** GST-tagged NRG1β was pre-bound to surface receptors of A375-Rep8 cells at 4°C. Cells were switched to 37°C for indicated times, and then acid washed to dissociate surface bound growth factor. Lysates from washed cells were blotted for GST, the GST-NRG1β band was quantified by densitometry, and the fraction of the initial surface-bound growth factor at time 0 that was internalized plotted as a function of time.



Funes et al., Fig. 1



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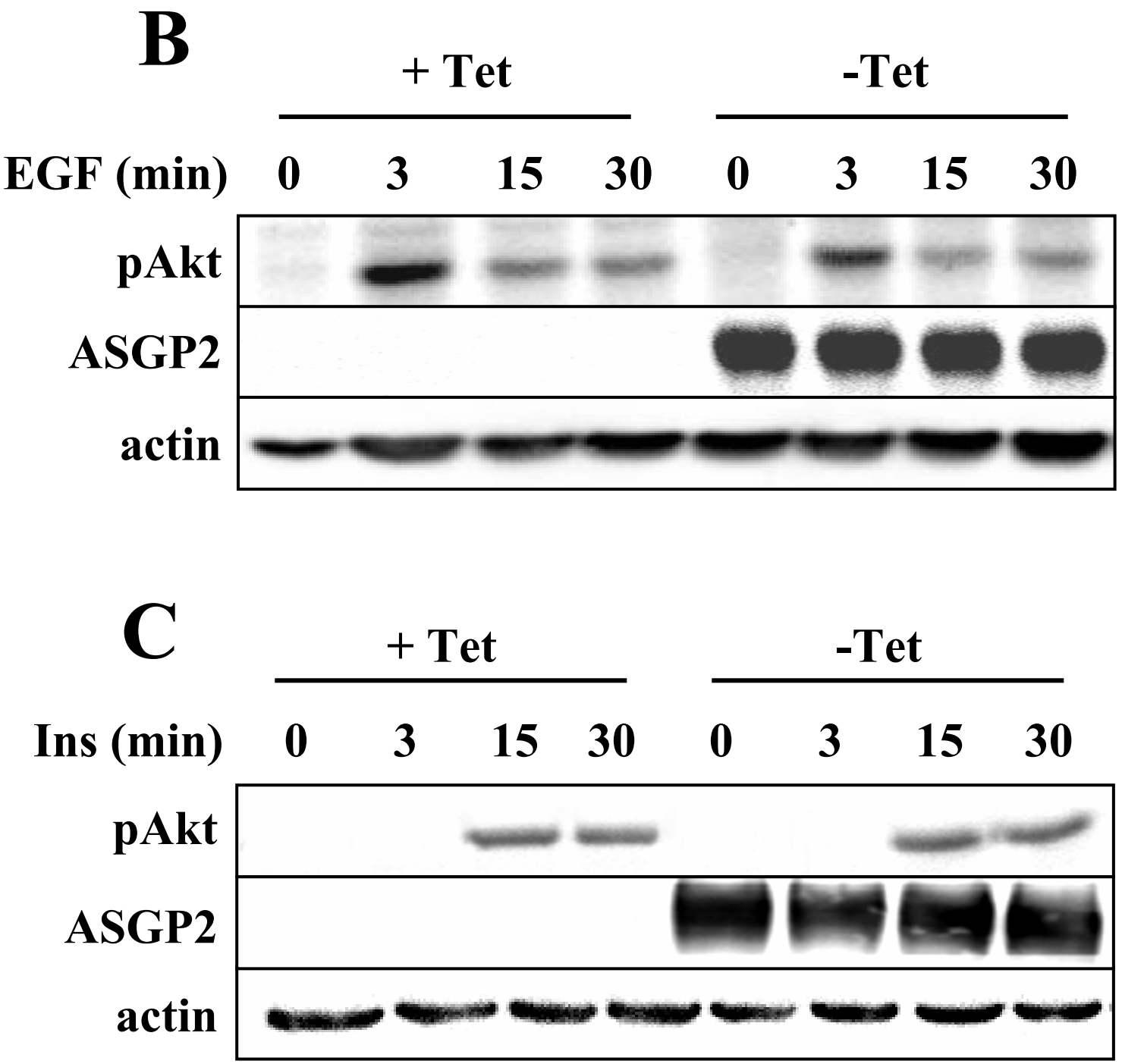
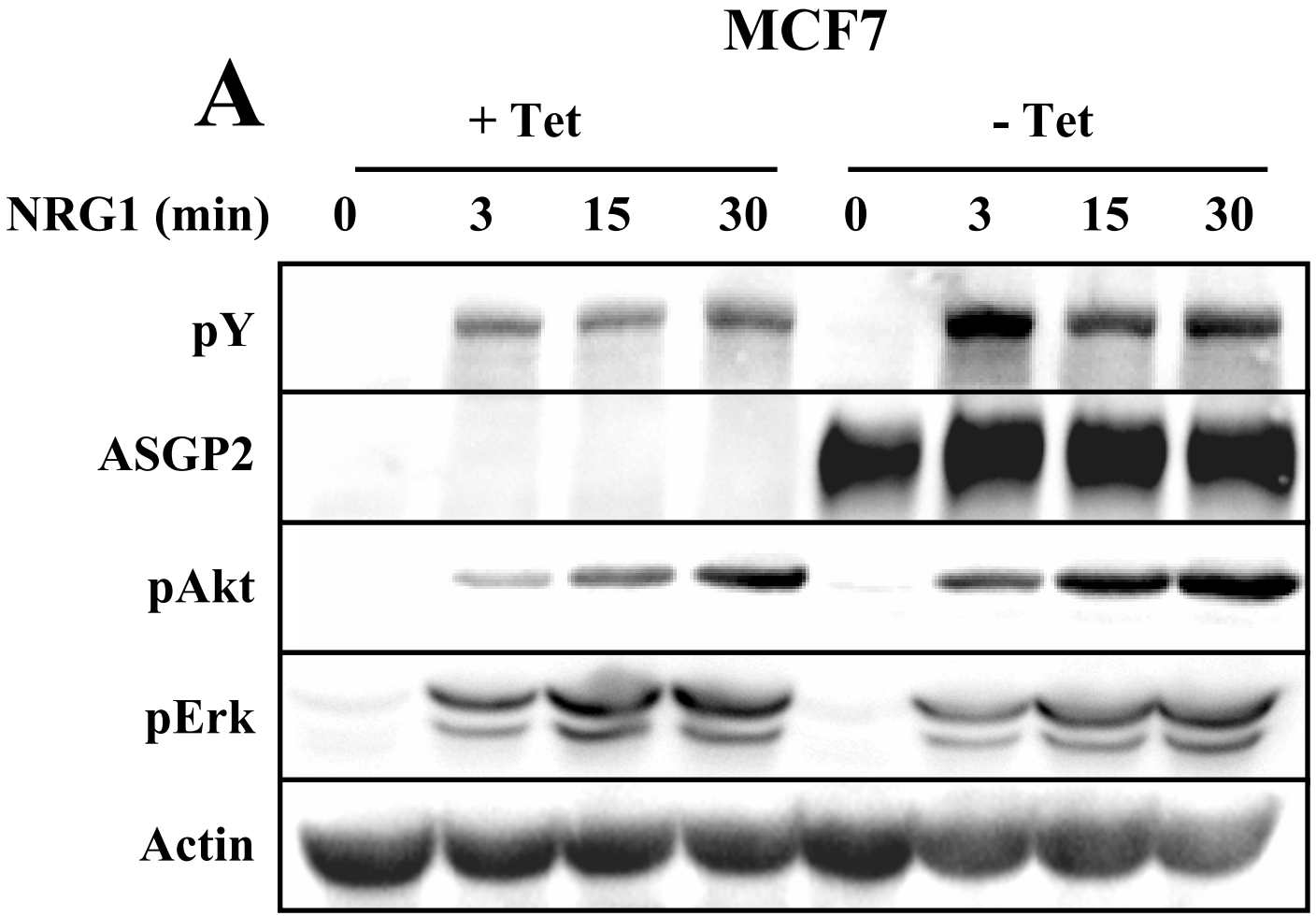


Funes et al.,
Figs. 2B&C



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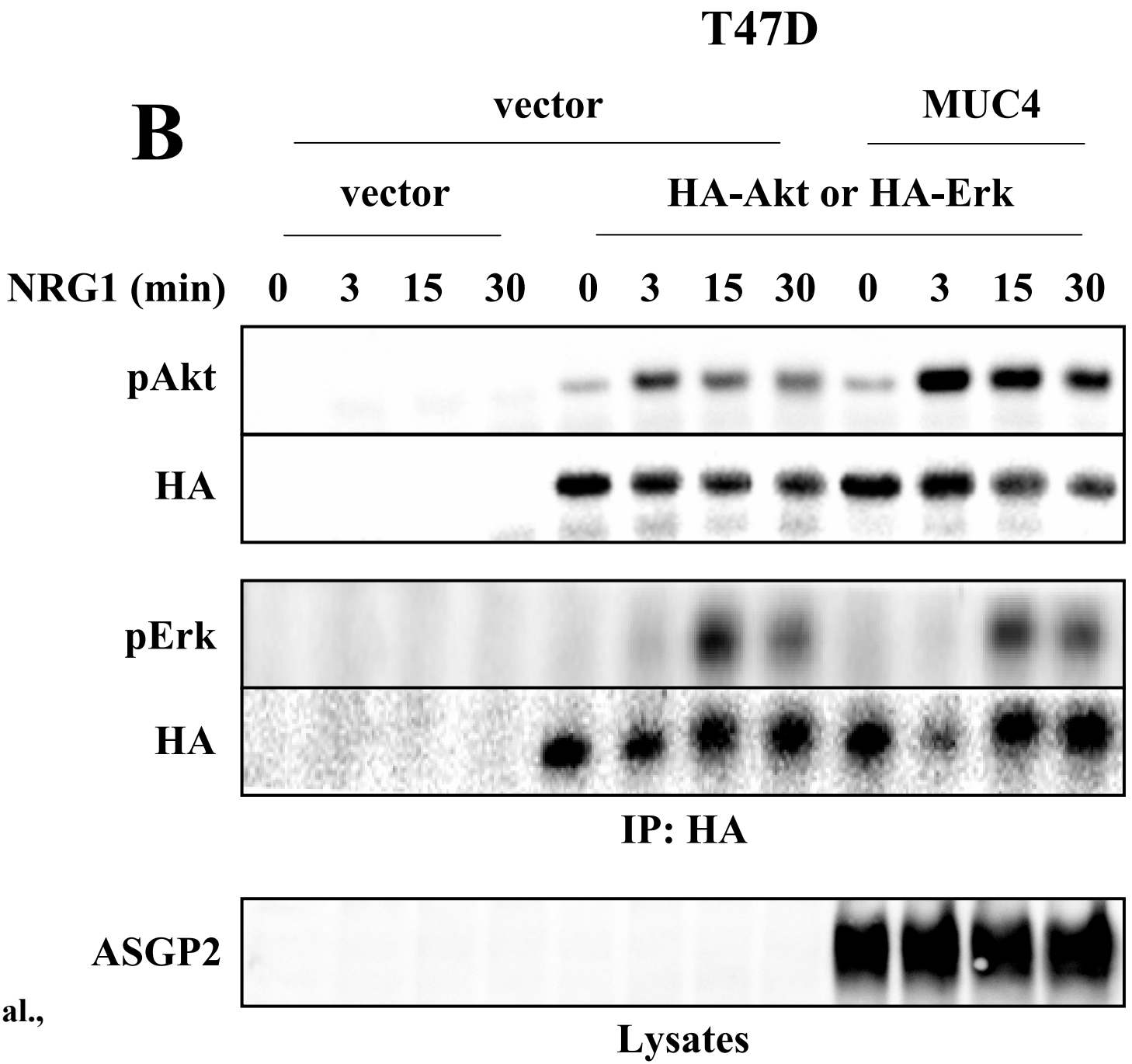

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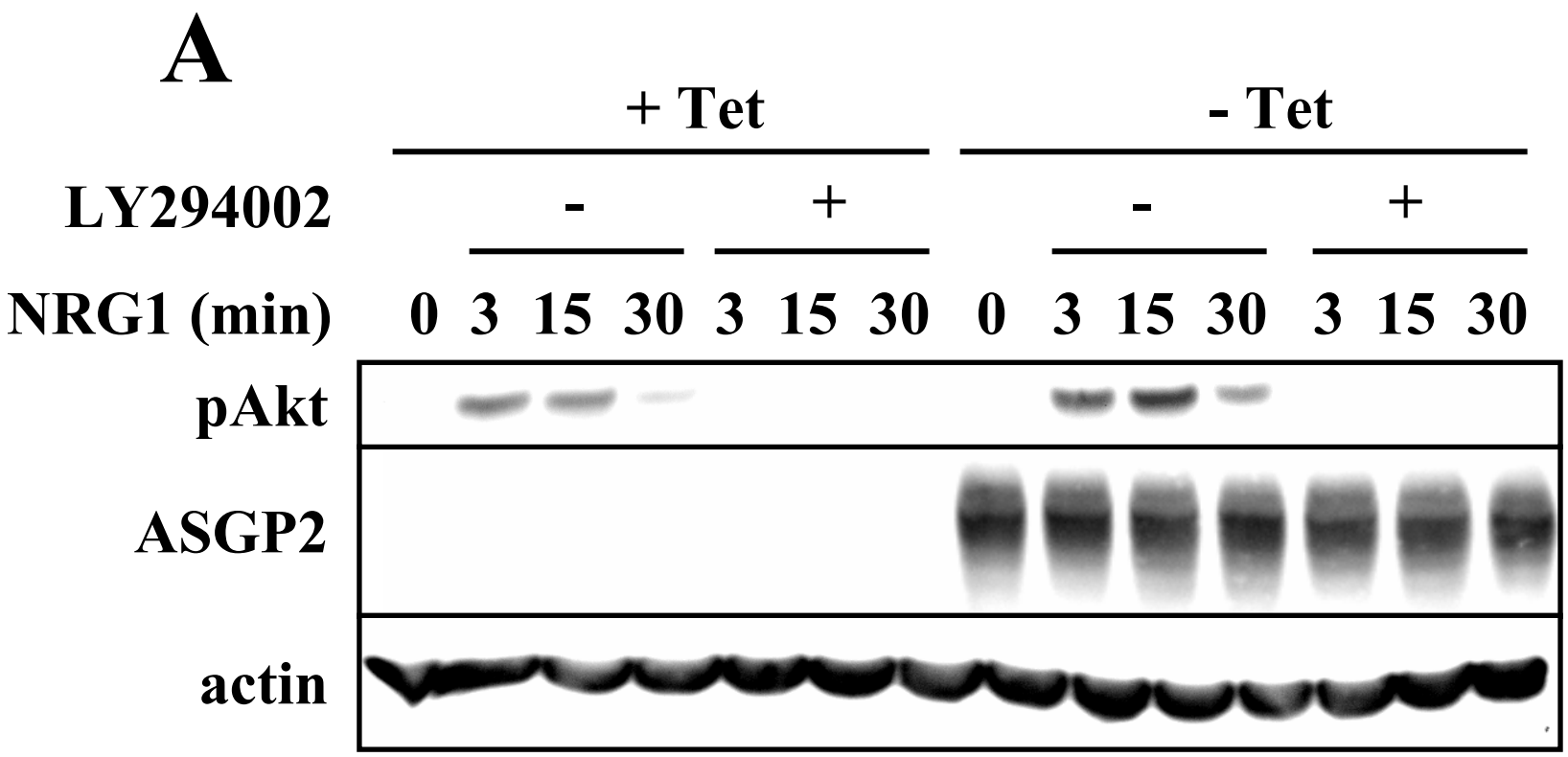
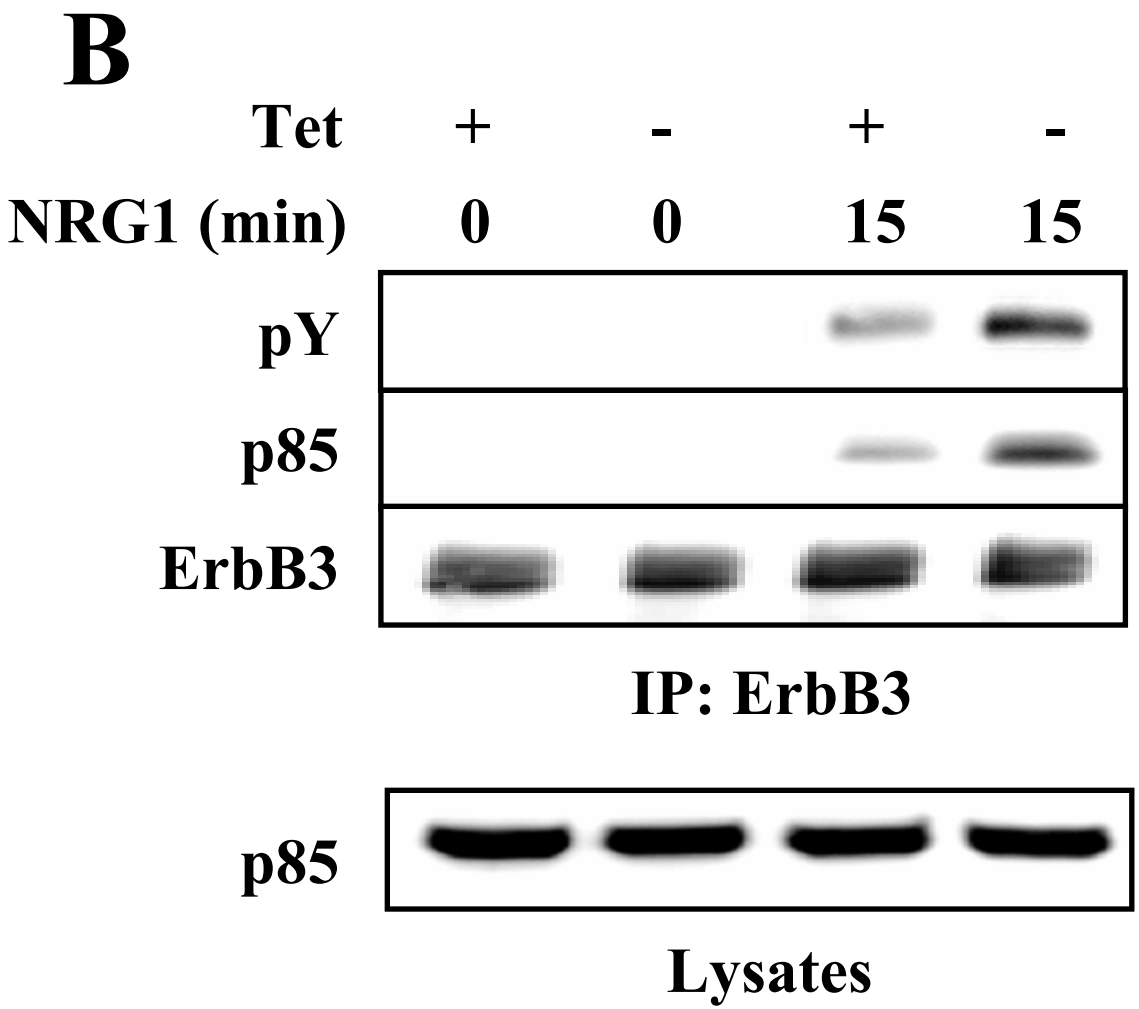
Fig. 3B



Funes et al., Fig. 4A



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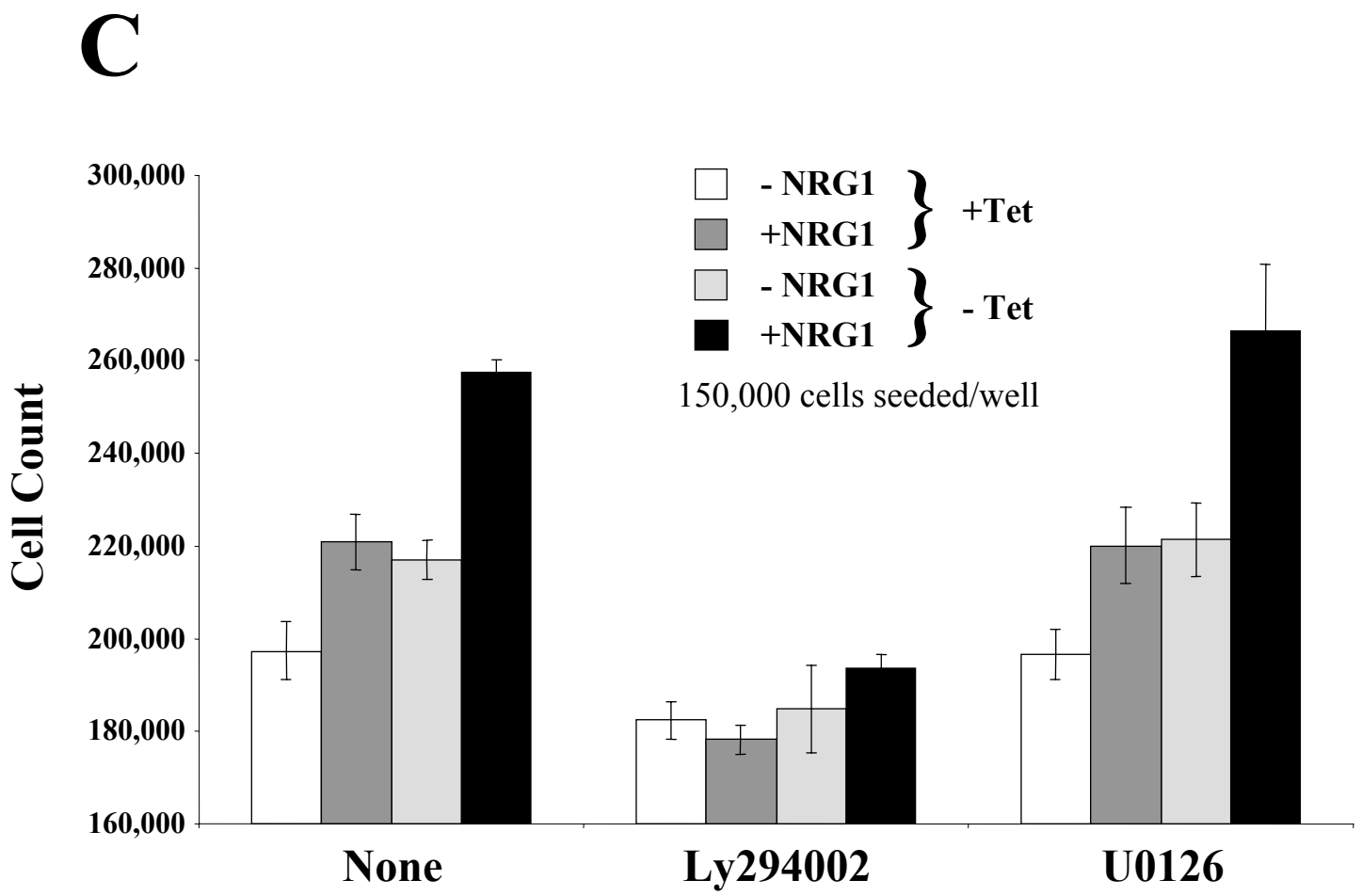

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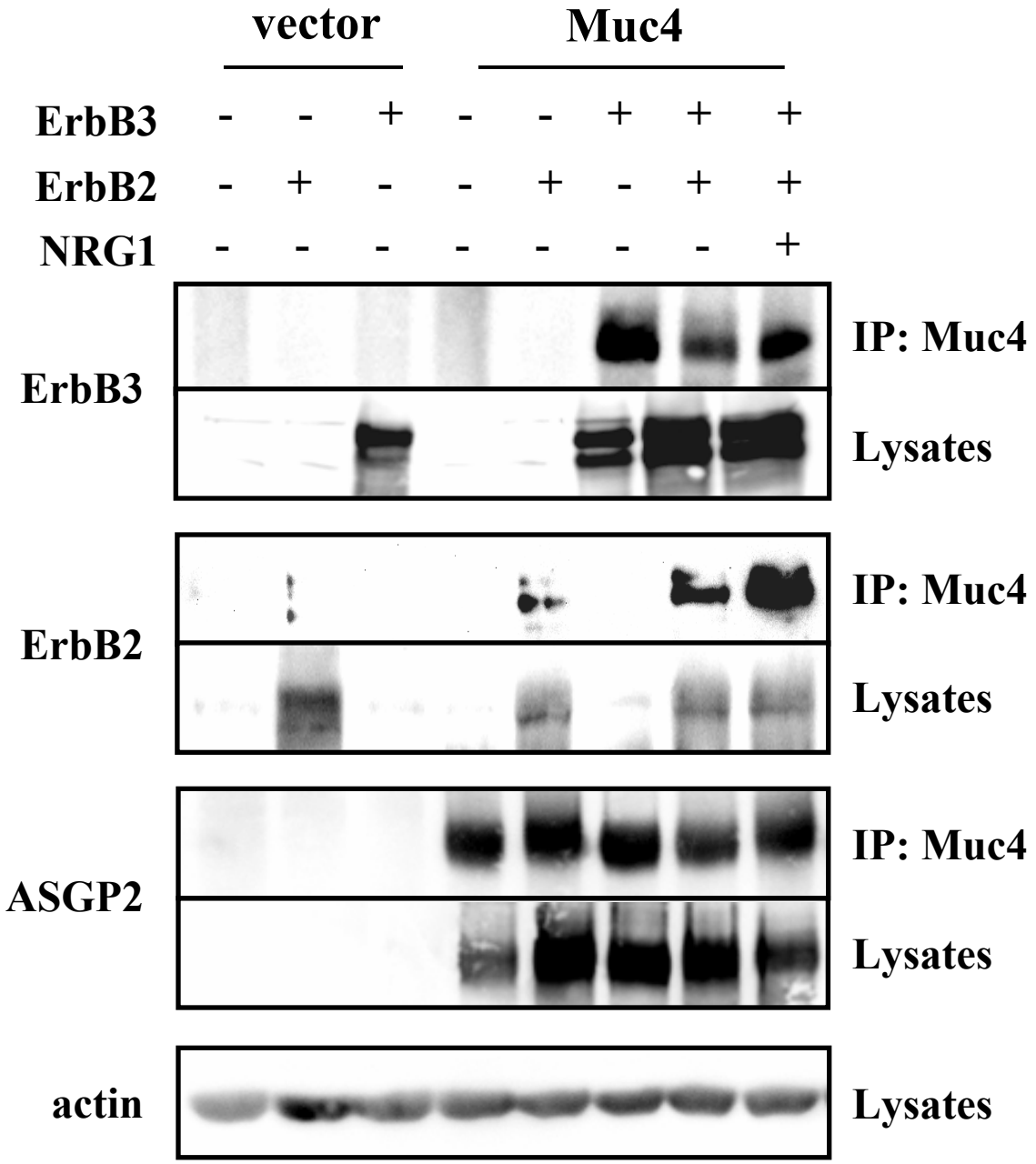

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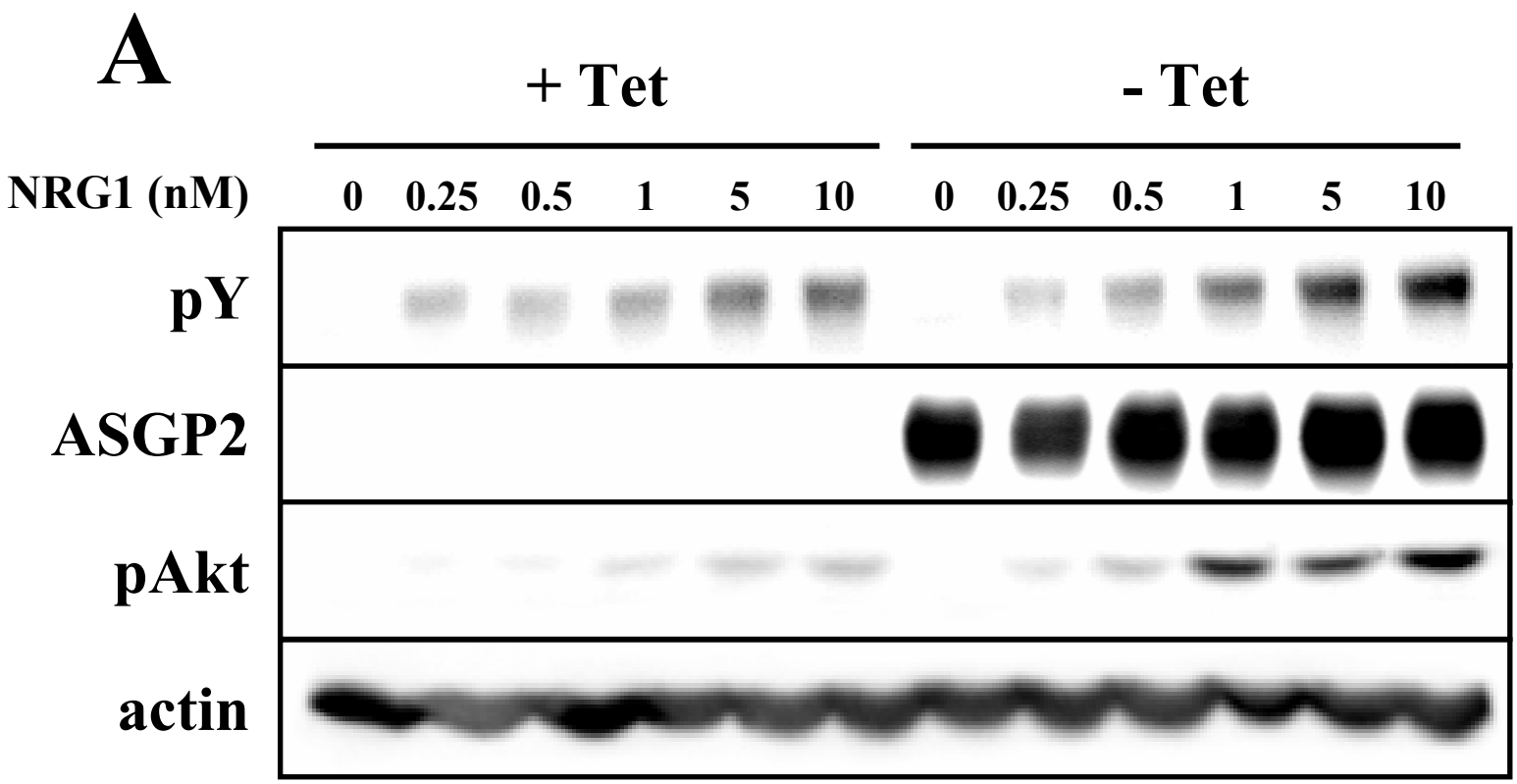
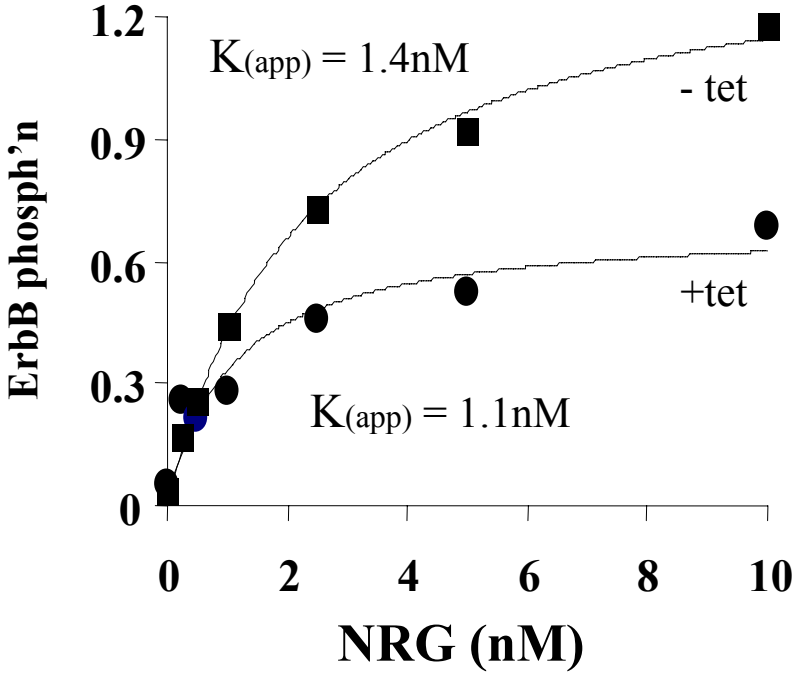
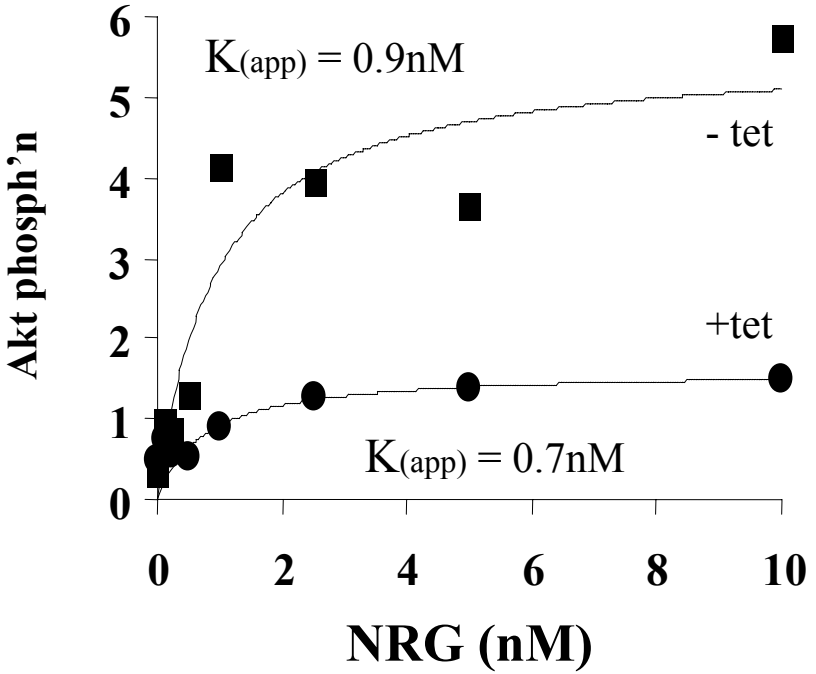


Funes et al., Fig. 6A

B





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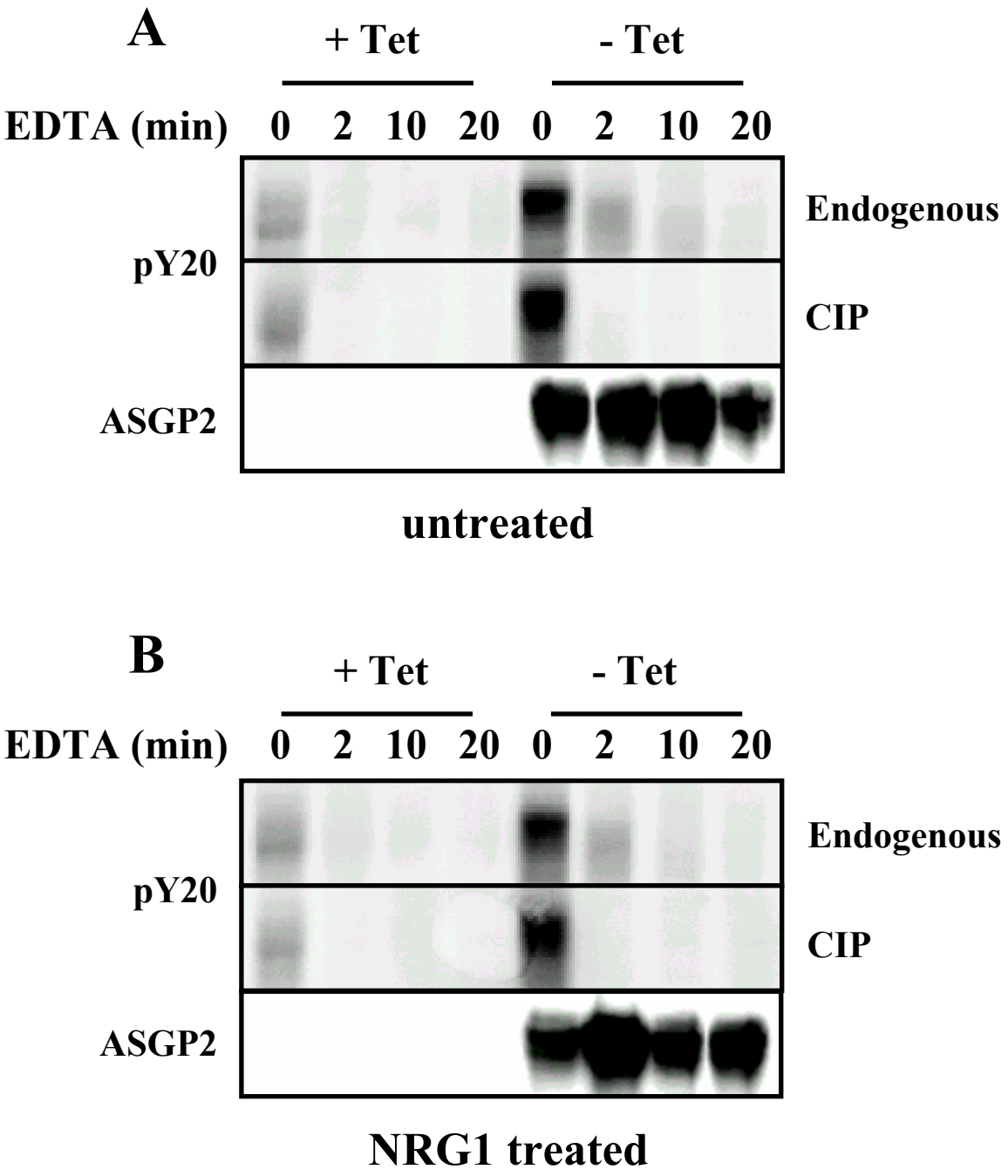

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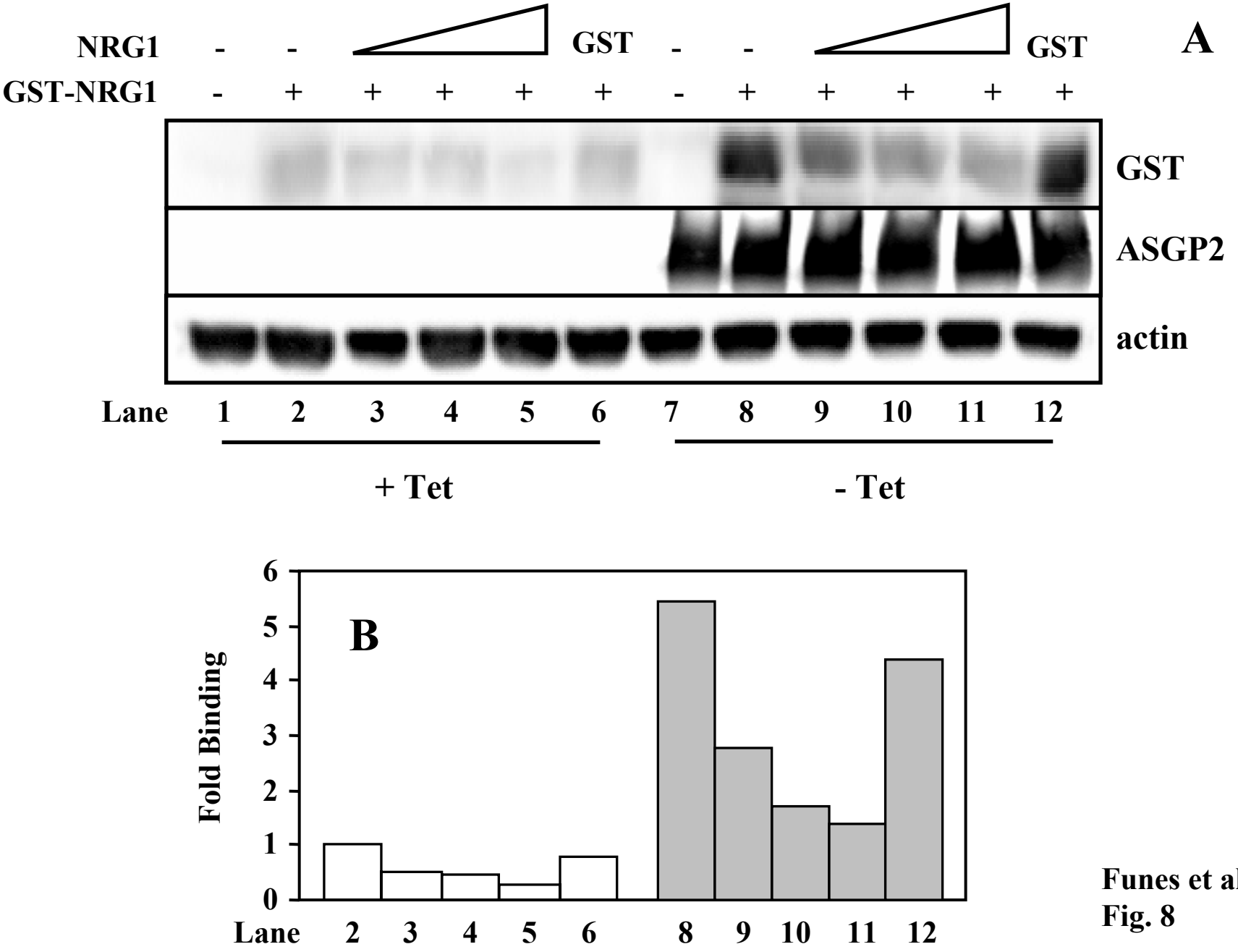

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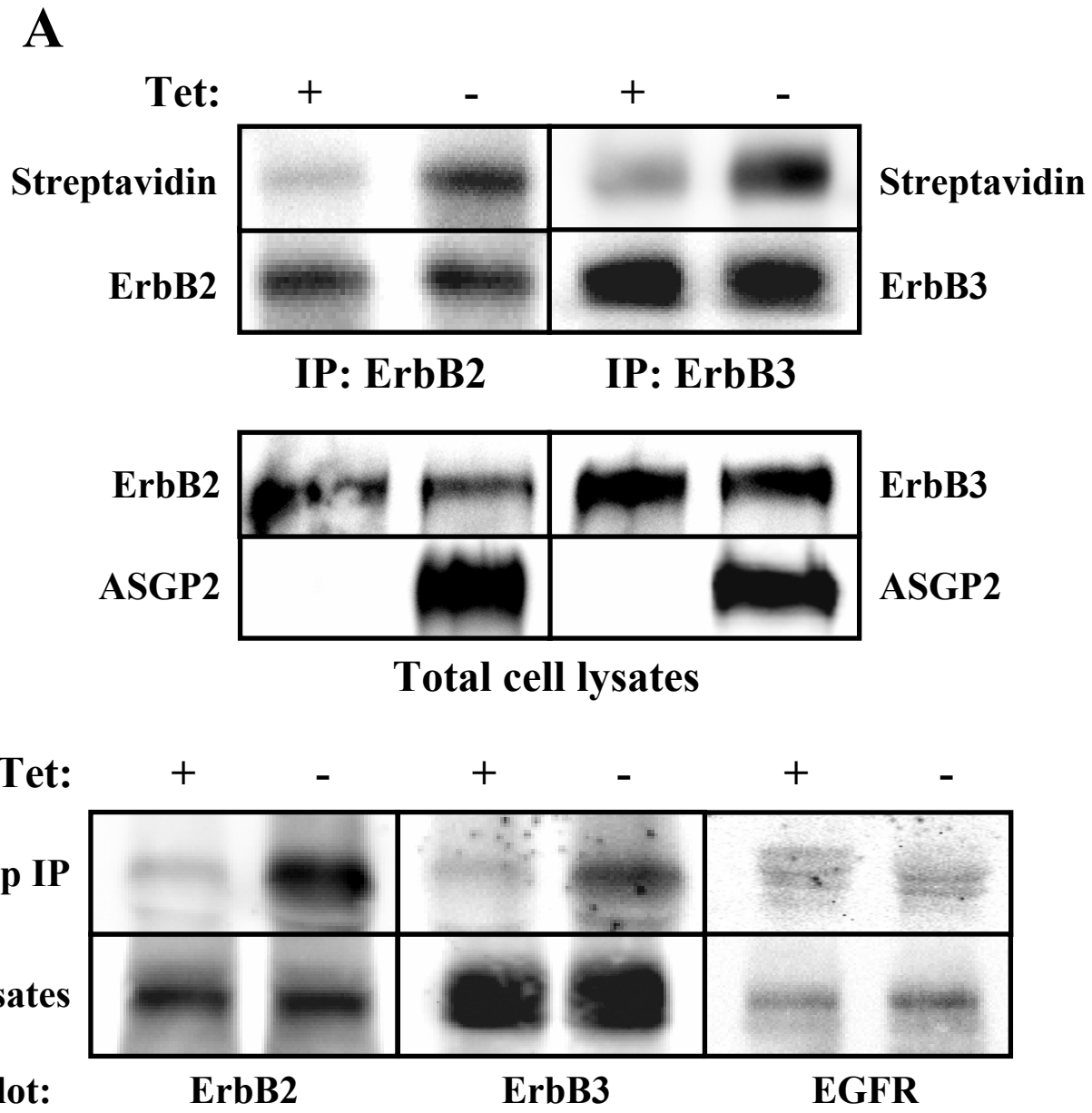
Fig. 8



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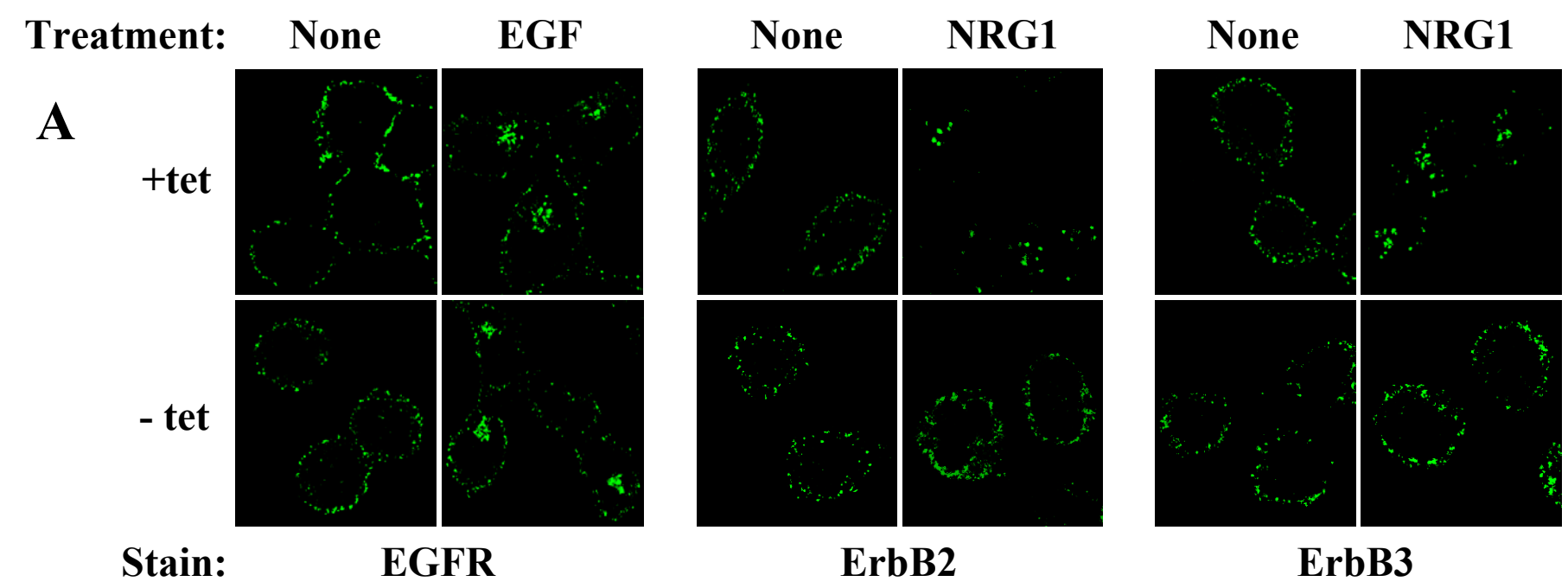

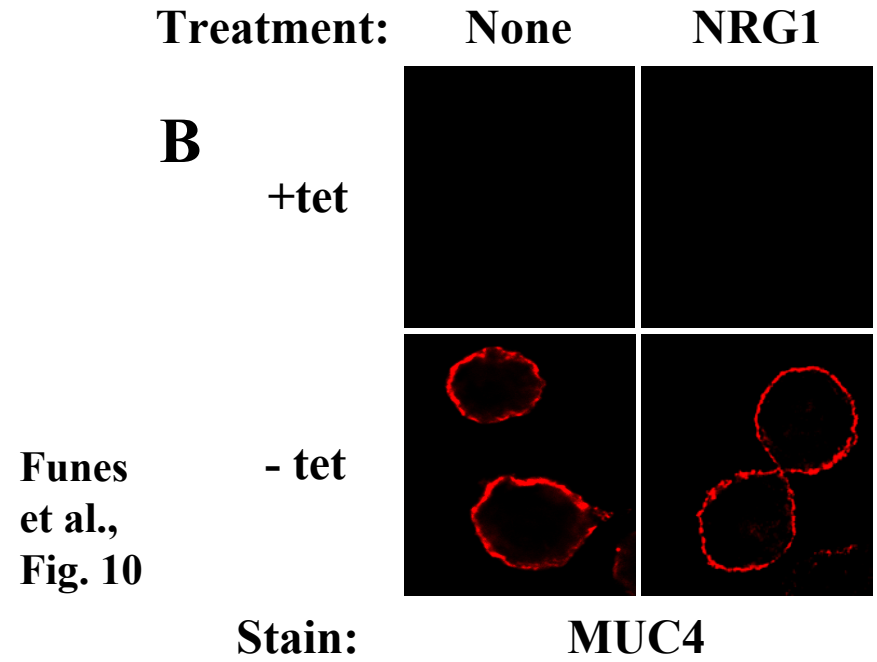

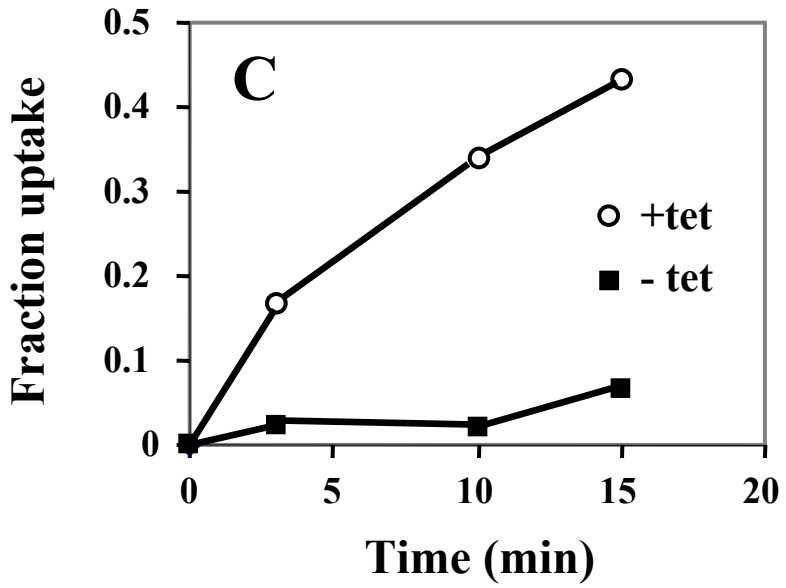

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