THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 52, pp. 35731–35742, December 26, 2014


# NGX6a Is Degraded through a Proteasome-dependent Pathway without Ubiquitination Mediated by Ezrin, a Cytoskeleton-Membrane Linker*

Received for publication, May 27, 2014, and in revised form, November 4, 2014 Published, JBC Papers in Press, November 5, 2014, DOI 10.1074/jbc.M114.584771

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**Background:** NGX6a, a novel isoform of the *NGX6* gene, was found down-regulated in nasopharyngeal carcinoma cells.
**Results:** NGX6a experienced proteasome-dependent degradation that is mediated by ezrin but without ubiquitination.
**Conclusion:** High expression of ezrin mediates the degradation of tumor suppressor NGX6a and contributes to the high metastasis potential of nasopharyngeal carcinoma cells.
**Significance:** These findings provide a novel molecular mechanism of NGX6a interacting with ezrin.

**Our previous study demonstrated that the *NGX6b* gene acts as a suppressor in the invasion and migration of nasopharyngeal carcinoma (NPC). Recently, we identified the novel isoform NGX6a, which is longer than NGX6b. In this study, we first found that NGX6a was degraded in NPC cells and that this degradation was mediated by ezrin, a linker between membrane proteins and the cytoskeleton. Specific siRNAs against ezrin increase the protein level of NGX6a in these cells. During degradation, NGX6a is not ubiquitinated but is degraded through a proteasome-dependent pathway. The distribution pattern of ezrin was negatively associated with NGX6a in an immunochemistry analysis of a nasopharyngeal carcinoma tissue microarray and fetus multiple organ tissues and Western blot analysis in nasopharyngeal and NPC cell lines, suggesting that ezrin and NGX6a are associated and are involved in the progression and invasion of NPC. By mapping the interacting binding sites, the seven-transmembrane domain of NGX6a was found to be the critical region for the degradation of NGX6a, and the amino terminus of ezrin is required for the induction of NGX6a degradation. The knockdown of ezrin or transfection of the NGX6a mutant CO, which has an EGF-like domain and a transmembrane 1 domain, resulted in no degradation, significantly reducing the ability of invasion and migration of NPC cells. This study provides a novel molecular mechanism for the low expression of NGX6a in NPC cells and an important molecular event in the process of invasion and metastasis of nasopharyngeal carcinoma cells.**

*NGX6* (nasopharyngeal cancer-related gene 6) is a candidate tumor metastasis suppressor gene that is cloned from the high frequency loss-of-heterozygosis region of chromosome 9p21-22 in nasopharyngeal carcinoma (1). Our previous studies demonstrated that the *NGX6* gene encodes a product of two isoforms, NGX6a and -b, from three different transcripts (2). NGX6b encodes 338 amino acids, which contain the extracellular domain of an EGF-like domain and two transmembrane domains, whereas NGX6a contains the extracellular domain of an EGF-like domain and seven transmembrane domains (3–5). NGX6b mRNA expression is reduced or absent in nasopharyngeal carcinoma and colon cancer and is associated with tumor metastasis (6–9). NGX6b expression in NPC 5-8F cells reduces the invasion capacity, increasing the rate of cell adhesion and restoring intercellular gap junction communication (10, 11); the tumor formation and lung metastases of NPC 5-8F cells that were transplanted in SCID mice *in vivo* were significantly inhibited by NGX6b expression. NGX6b can bind to the cell membrane via an intracellular region with ezrin and inhibit the cell proliferation, cell invasion, and metastasis of nasopharyngeal carcinoma through the EGF receptor signaling pathway (12, 13). NGX6b can also inhibit the invasion of colon cancer cells by inhibiting the Wnt/β-catenin signaling pathway (4, 5, 14). The isoform NGX6a was recently found to be expressed in various organs, mainly in epithelial cells and neuronal cells in the brain, nasopharynx, and lung, whereas NGX6b is expressed in the brain, heart, kidney, nasopharynx, and lung, and the expression levels of NGX6a are much higher than are those of NGX6b (3). However, the function of NGX6a is not well defined.

* This work was supported by National Natural Science Foundation of China Grants 30971147, 81101767, 81372366,81372907, and 81171934; New Century Excellent Talents in University of Ministry of Education of China Grant NCET-12-0544; the Overseas, Hong Kong, and Macao Scholars Collaborative Researching Fund of the National Natural Science Foundation of China Grant 81428018; Central South University Faculty Research Grant 2013JSJJ046; 111 Project Grant 111-2-12; 863 Plan Grant 2012AA02A206; the Hunan Province key laboratory program of non-resolving inflammation and cancer; and Specialized Research Fund for the Doctoral Program of Higher Education Grant 20110162120018.
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Ezrin is an important member of the ezrin/radixin/moesin (ERM)[3] family of eukaryotic membrane proteins-cytoskeleton bridge molecules (15, 16). Ezrin is involved in cell morphology, cell adhesion, movement, cytoskeleton remodeling, and signaling processes (10, 11, 17). The ezrin protein contains three main parts: a spherical highly conserved amino terminus (85% identical) that binds with the membrane protein; an extending $\alpha$ helix domain in the middle; and a positively charged carboxyl terminus, which binds to actin. When ezrin is present as a soluble monomer protein, the amino terminus binds with the carboxyl end but does not bind to actin protein when ezrin is in the inactivated state; when ezrin is activated, the binding sites are exposed, and it plays an important role as a bridge between membrane protein and cytoskeleton actin. Many studies have demonstrated that ezrin expression is abnormally regulated in tumors with or without metastasis and have indicated that ezrin plays a key role in tumor metastasis (18–21).

We aimed to examine what roles NGX6a plays in the invasion and metastasis of nasopharyngeal carcinoma cells and to determine the molecular link between NGX6a and ezrin. We found that NGX6a is degraded through the proteasome pathway mediated by ezrin in NPC cells but is not ubiquitinated. Seven transmembrane domains of NGX6a and the N-ERMAD domain of ezrin are required for the degradation of NGX6a. The knockdown of ezrin expression or the increase in NGX6a expression inhibits the invasion and metastasis of nasopharyngeal carcinoma cells.

## MATERIALS AND METHODS

*Antibodies, siRNAs, and Plasmids*—The monoclonal mouse antibody anti-FLAG(M2) and anti-ubiquitin (Sigma), mouse anti-c-Myc (Clontech, Mountain View, CA), anti-His (Novagen, Darmstadt, Germany), anti-GAPDH, mouse anti-$\beta$-actin, Protein G Plus-agarose (Santa Cruz Biotechnology, Inc.), rabbit anti-ezrin (Upstate), anti-ISG15, anti-NEDD8, anti-SUMO1 (Cell Signaling Technology, Danvers, MA), goat anti-mouse IgG(H+L) antibody (HRP), goat anti-rabbit IgG(H+L) antibody (HRP) (KPL), sheep anti-mouse IgG Cy3 conjugate antibody (Boster, Wuhan, China), and sheep anti-rabbit IgG FITC conjugate antibody (SABC, Beijing, China) were purchased from the companies indicated, and polymonoclonal anti-NGX6a antibody was prepared as described (3). pCMV-Myc-NGX6a and pIRES-neo3-NGX6a constructs were made by cloning the human NGX6a ORF coding region (accession number NM_001042589.2) into pCMV-Myc and pIRES-neo3 (Clontech). pcDNA3.1(+)-ezrin was built by cloning the human ezrin coding region (accession number NM_001111077.1) into pcDNA3.1(+) (Invitrogen). M1, M2, CO, and S mutants were amplified from full-length construct pCMV-Myc-NGX6a with specific primers from both termini of the deletion domain and were cloned into pCMV-Myc and pIRES-neo3, respectively. N-ERMAD, MC-ERMAD, MN-ERMAD, and C-ERMAD mutants were amplified from full-length construct pcDNA3.1(+)-ezrin with specific primers from both termini of the deletion domain by PCR. Transwells (Corning, Inc.), Matrigel basement membrane matrix, Bovine Fibrontin (BD Biosciences), Lipofectamine 2000 (Invitrogen), and MG132 (Calbiochem) were purchased.

*Cell Culture and siRNA Transfection*—The poorly differentiated NPC-derived cell lines HNE1, 6-10B, and 5-8F were established in our laboratory and at the Sun Yat-sen University Cancer Center (22, 23). The cells were grown in RPMI 1640 medium that was supplemented with 10% fetal calf serum and penicillin/streptomycin (Invitrogen) in a humidified incubator at 37 °C with 5% $CO_2$ and 95% air. The HNE1, 6-10B and 5-8F cell lines transfected with pIRES-FLAG-NGX6a and stably transfected pIRES-neo3 were established as described previously and cultured under the same conditions, with supplements of 200 $\mu$g/ml G418. The nasopharyngeal epithelial cell line NP, NP69, which is immortalized with an SV40 T-antigen, was a kind gift from Professor Sai Wah Tsao (Department of Anatomy, University of Hong Kong, China) and was maintained in keratinocyte serum-free medium (Invitrogen) with the addition of growth factor supplements (Invitrogen) (25). Human HEK293FT (human embryonal kidney cells that were transformed with the SV40 large T antigen) (Invitrogen) were cultured in DMEM containing 10% fetal calf serum and 1% penicillin/streptomycin at 37 °C in 5% $CO_2$ using standard protocols. Small interfering RNAs Silencer Select siRNA-ezrin (siEzrin) or negative control siRNAs (siCon) (Invitrogen) as negative controls were transfected into the 5-8F cell line with Lipofectamine 2000 (Invitrogen).

*Protein Extraction, Co-immunoprecipitation, and Western Blot Analysis*—To examine the interactions between NGX6a and ezrin, 5-8F cells were stably transfected with pIRES-FLAG-NGX6a and treated with MG132 at different concentrations (0, 10, 20, and 40 $\mu$M) for 24 h. The cells were washed with cold PBS solution, collected by manual scraping using a rubber policeman, and pelleted by centrifugation. The cell pellet was resuspended in gentle lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, 1× protease inhibitor mixture (Merck Millipore), 1 mM DTT, and 10 mg/ml RNase A) and incubated on ice for 15 min. The insoluble materials were removed by centrifugation at 13,400 × *g* in a microcentrifuge at 4 °C for 15 min. A total of 350 $\mu$l of the lysate was incubated with 20 $\mu$l of Protein A-Sepharose beads that were prebound with 10 $\mu$l of anti-Myc antibody or anti-ezrin antibody, preimmunoprecipitated with IgG, or 10 $\mu$g of anti-FLAG M2 antibody at 4 °C overnight. The next day, the beads were washed, and the bound fractions were eluted by 3× SDS-sample buffer and heating at 95 °C for 5 min. The proteins in the complex were resolved by SDS-PAGE, followed by Western blot analysis.

*Double-fluorescent Staining*—HEK 293FT cells ($6 \times 10^5$) were seeded on coverslips in a 6-well plate the day before transfection. On the second day, HEK 293 cells were transfected with pCMV-Myc-NGX6a or pcDNA3.1(+)-ezrin using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After incubation for 48 h at 37 °C (5% $CO_2$ in a humidified atmosphere), the cells were fixed in methanol and acetone (v/v, 1:1) for 30 min, permeabilized using 0.25% Triton X-100, and blocked using normal goat serum (Molecular Probes, Inc., Eugene, OR). The primary

[3] The abbreviations used are: ERM, ezrin/radixin/moesin; aa, amino acid(s); SUMO, small ubiquitin-like modifier.

antibodies, mouse anti-ezrin (Upstate) (1:1000) and rabbit anti-NGX6a (1:800), were added and incubated at 4 °C, followed by washing with PBS. The secondary antibodies, FITC-conjugated sheep anti-rabbit IgG (1:200) and Cy3-conjugated sheep anti-mouse IgG (1:200) (Boster) were then added and incubated at 37 °C for 1 h. The coverslips were washed four times with PBS and incubated with DAPI solution for 5 min at room temperature and washed. Control experiments were carried out without the primary antibody. The staining was observed using an Olympus laser confocal microscope. This experiment was also described previously (3).

*Immunohistochemistry and Immunostaining Evaluation*—The tissues were deparaffinized in xylene and rehydrated through graded alcohols (100, 90, 70, and 50% alcohol; 5 min for each). The endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. For antigen retrieval, sections were incubated in sodium citrate buffer (0.01 M, pH 6.0) for 20 min in a household microwave oven. After cooling to room temperature, the slides were washed in PBS (150 mM sodium chloride, 150 mM sodium phosphate, pH 7.2) and immersed in normal goat blocking serum (Maixin, Fuzhou, China) for 30 min. The anti-NGX6 serum (1:1500) was applied at 4 °C overnight. Polymerized HRP and anti-rabbit IgG (Maixin) were added according to the manufacturer's instructions. A color reaction was developed using diaminobenzidine chromogen solution (Maixin), and all of the slides were counterstained with hematoxylin. Negative control slides were included in the experiment. The immunohistochemical staining of these sections was scored microscopically (Olympus, Tokyo, Japan) at ×400 magnification in all of the available tumor cells or epithelial cells meeting the typical morphological criteria by two pathologists using the qualitative scale that is described in the literature (35).

*Scratch Wound Healing Assay and Matrigel Invasion Assay*—For the scratch wound healing assay, the same amount of cells from the different groups was grown to ~80% confluence in a 6-well dish. A "wound" in a cell monolayer was made by scratching the surface using a pipette tip, and the images were captured at the beginning and at 40 h during cell migration to close the wound. The images were compared to quantify the migration distance of the cells.

In each group, $1 \times 10^5$ cells were added to the upper compartments of 12-mm diameter cell culture inserts with 8-$\mu$m pores that were coated with BD Matrigel. Each insert was positioned into the well of the dish, with the bottom of the insert merged in medium containing 10% FBS as an attractant. After incubation for 24 h, the inserts were carefully removed. The cells and the gel within the upper compartment of the insert were gently removed with a cotton swab. The cells on the lower side of the insert membrane were fixed and stained with 1% crystal violet in 2% ethanol for 20 min. The cells were washed and counted randomly in five different views on the lower side of the filter under a microscope.

## RESULTS

*NGX6a Is Degraded in NPC Cell Lines and Tissues via the Ubiquitination-independent Proteasome Pathway*—Our previous studies have demonstrated that NGX6a is down-regulated in NPC biopsies and is associated with metastasis (3). Here, we assume that the recovery expression of NGX6a in the NPC cell lines may decrease the nasopharyngeal malignant phenotype and the invasion and metastasis ability. First, we analyzed the expression of NGX6a in nasopharyngeal carcinoma cell lines. Western blot analysis showed the high expression of NGX6a in nasopharyngeal epithelium, moderate expression in the normal nasopharyngeal epithelial cell line NP69, and a very low expression level in the three NPC cell lines 5-8F, 6-10B, and HNE1 (Fig. 1*A*). This result indicates that NGX6a has a similar distribution pattern in NPC as NGX6b.

We further established stable 5-8F cell lines that were transfected with the pIRES-neo3-NGX6a expression vector because its expression was significantly down-regulated in 5-8F cells; the 5-8F cell line is a nasopharyngeal carcinoma cell line with high tumorigenicity and a high metastasis capacity. The mRNA levels of NGX6a in cells that were stably transfected with pIRES-neo3-NGX6a were 20 times higher than those in 5-8F cells with pIRES-neo3, whereas at the protein level, no predicted protein was detectable with a specific anti-NGX6a antibody using a Western blot analysis. We tried to detect NGX6a in 6-10B, HNE1, and other nasopharyngeal carcinoma cell lines that were stably transfected with NGX6a/pIRES-neo3 and obtained the same results (Fig. 1*B*).

We presumed that NGX6a protein degradation occurs within these cells because the degradation of many proteins depends on the ubiquitin-proteasome pathway. When treated with the proteasome inhibitor MG-132, the cells that are stably transfected with pIRES-neo3-NGX6a had a detectable level of the NGX6a protein. Furthermore, the expression levels of NGX6a increased with increasing MG-132 concentration. Meanwhile, fluorescent signals were detected in the cells that were treated with more than 10 $\mu$M MG-132 for 24 h, and the signal increased with increasing MG-132 concentration (Fig. 1*C*). This result further confirms that transfected NGX6a protein degrades in nasopharyngeal carcinoma cells through the proteasome pathway.

When most proteins are degraded, they are ubiquitinated through the proteasome pathway. Ubiquitin is a small peptide that labels the target protein and mediates its entry into the proteasome, where the protein is degraded. We were interested in determining whether NGX6a is ubiquitinated as are other proteins. However, the repeatable results showed that the NGX6a protein that immunoprecipitated with the anti-Myc monoclonal antibody-conjugated agarose beads is not detectable by anti-ubiquitin antibody in pCMV-Myc-NGX6a transiently transfected 5-8F cells (Fig. 1*D*). These results suggest that NGX6a protein underwent degradation in the proteasome in nasopharyngeal carcinoma cells, but this degradation process may not depend on ubiquitination. To verify whether other ubiquitin-like proteins interact with NGX6a and mediate NGX6a degradation, total protein was extracted from pCMV-Myc-NGX6a/5-8F cells that were treated with MG-132 (20 $\mu$M) for 12 h and subjected to immunoprecipitation experiments with specific anti-Myc-agarose beads and then Western blot analysis with specific anti-NEDD8, anti-SUMO1, and anti-ISG15. There were no positive results (data not shown).

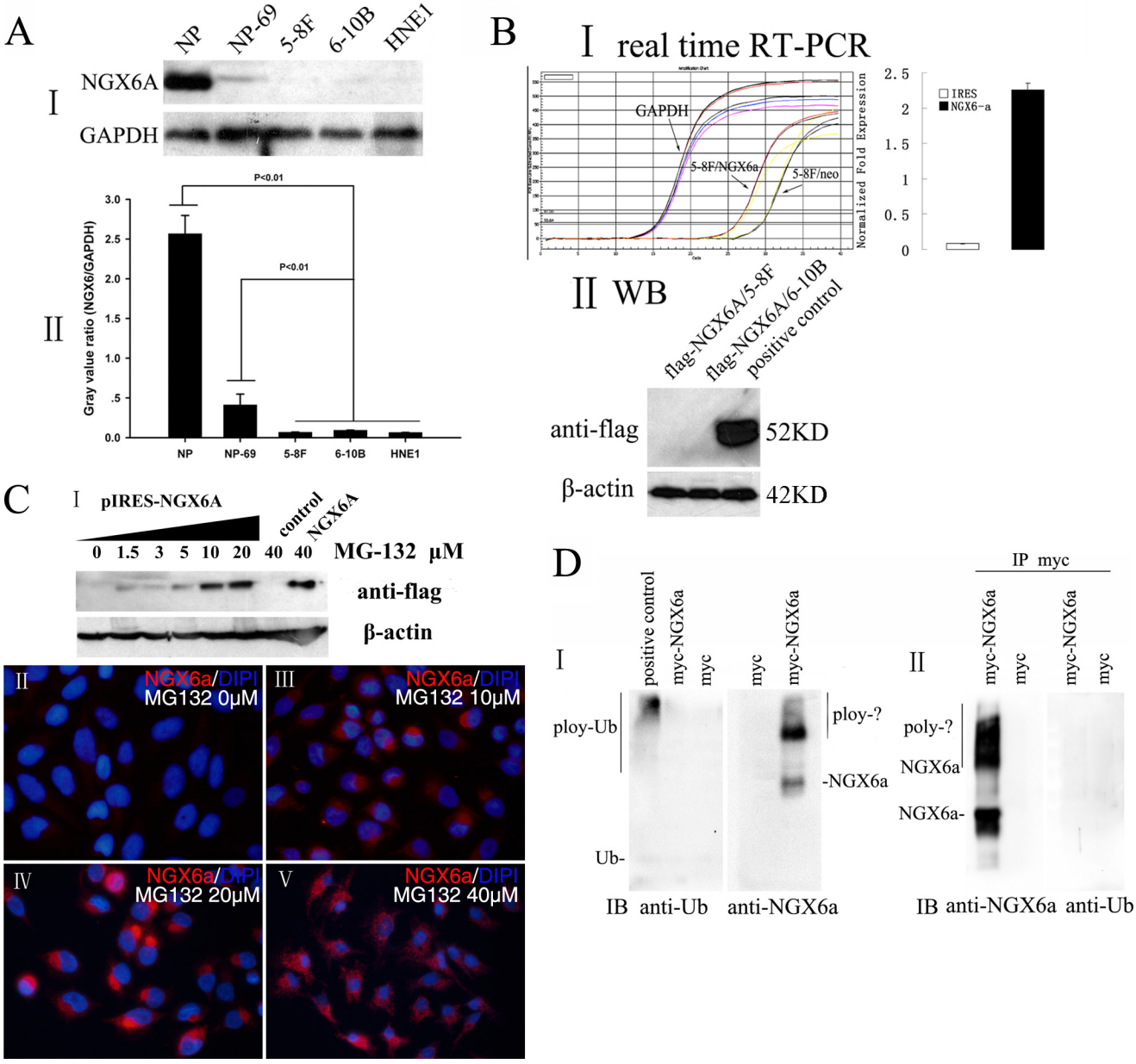


FIGURE 1. **NGX6 is down-regulated due to degradation via the proteasome pathway without ubiquitination in nasopharyngeal carcinoma.** *A*, *I*, total proteins were extracted from NP, NP-69, 5-8F, 6-10B, and HNE1 cells. The proteins were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by Western blot. The antibodies that were used in the Western blot analysis are indicated on the *left*. GAPDH was used as a loading control. *II*, the statistically significant differences are indicated by $p < 0.01$. *B*, *I*, total RNAs were isolated from human nasopharyngeal carcinoma 5-8F cells that were transfected with pIRES-neo3-NGX6a. The mRNA levels were determined using reverse transcription and quantitative real-time PCR. The relative mRNA levels of NGX6a are shown after normalization to GAPDH. The *numbers* represent the mean ± S.D. ($n = 3$). *II*, total proteins were extracted from 5-8F and 6-10B cells following transfection with pIRES-neo3-NGX6a cells and pIRES-neo3-FLAG-NOR1 (positive control), respectively. The proteins were analyzed by Western blot (*WB*). *C*, *I*, total proteins were extracted from 5-8F cells that were transfected with pIRES-neo3-NGX6a or pIRES-neo3 vector and treated with different concentrations (0, 1.5, 3, 5, 10, 20, and 40 μM) of the proteasome inhibitor MG132 for 24 h, after which the total proteins were analyzed by Western blot; *II–V*, immunofluorescence test of the pIRES-neo3-NGX6a/5-8F cells that were treated with different concentrations (0, 10, 20, and 40 μM) of the proteasome inhibitor MG132 for 24 h. *D*, the ubiquitination of NGX6a is not detectable. *I*, total proteins were extracted from 5-8F cells transiently transfected with pCMV-NGX6a or pCMV-Myc, respectively. The proteins were analyzed by Western blot (*IB*) with anti-ubiquitin and anti-NGX6a. *I*, *left*, positive control ubiquitinated protein (the cells that were treated with 1.5 μM MG132), lysate of 5-8F cells that were transfected with pCMV-Myc-NGX6a and pCMV-Myc; *right*, lysate of 5-8F cells that were transfected with empty pCMV-Myc or pCMV-Myc-NGX6a. *II*, the cell lysates of 5-8F cells that were transfected with pCMV-Myc-NGX6a or pCMV-Myc were immunoprecipitated (*IP*) with anti-Myc monoclonal antibody and then detected with anti-ubiquitin or anti-NGX6a antibodies.

*NGX6a Interacts with Ezrin*—Our previous studies reported that NGX6b interacts with ezrin, which is an important cytoskeleton bridge molecule. Ezrin plays an important regulatory role in cell attachment, mobility, and metastasis. The full-length NGX6a protein contains 476 amino acids, and the NGX6b protein contains 338 amino acids with a highly homologous 272-amino acid region at the 5′-amino terminus. NGX6a and NGX6b contain similar structures (an EGF-like domain and a different number of transmembrane domains). Therefore, the predicted similar domains of NGX6a indicate that this domain potentially interacts with ezrin. This hypothesis was confirmed by the co-localization of these proteins in 5-8F cells

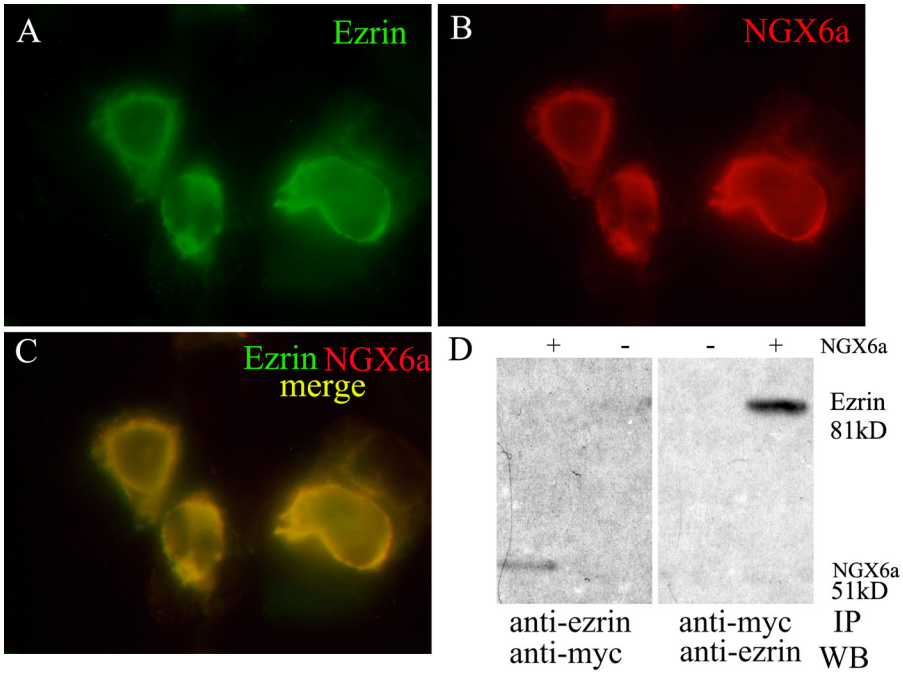


FIGURE 2. **NGX6a interacts with ezrin in nasopharyngeal carcinoma cell lines that were transiently transfected with pCMV-Myc-NGX6a that were treated with MG132.** *A* and *B*, immunofluorescence test using anti-ezrin (*green*) and anti-NGX6a (*red*) antibodies. *C*, a *merged image* of *A* and *B*. *D*, NGX6a is included in the complex of anti-ezrin antibody, and ezrin is included in the complex of anti-Myc (tagged to NGX6a), the antibody protein. The total proteins from 5-8F cells that were transfected with the pCMV-Myc-NGX6 or pCMV-Myc vector were subjected to an immunoprecipitation (*IP*) assay 48 h later using an anti-ezrin monoclonal antibody and then subjected to the Western blot (*WB*) analysis with an anti-Myc (tagged to NGX6a protein) monoclonal antibody or *vice versa*.

that were transfected with pCMV-Myc-NGX6a and treated with MG132 by an immunofluorescence test (Fig. 2, *A*–*C*). The total protein that was extracted from the above cells was immunoprecipitated with an anti-ezrin antibody or an anti-Myc antibody and subjected to Western blot analysis with an anti-Myc antibody or an anti-ezrin antibody. These data demonstrate that NGX6a interacts with ezrin (Fig. 2*D*).

*NGX6a Expression Is Negatively Associated with Ezrin in Different Tissues and Cell Lines*—A low ezrin expression level was found in nasopharyngeal epithelium and in the normal nasopharyngeal epithelial cell line NP69, in which NGX6a is relatively abundant, whereas a high expression of ezrin was detected in a variety of nasopharyngeal carcinoma cell lines in which NGX6a is rare. The evidence indicates that the higher ezrin level is, the less abundant NGX6a will be, and *vice versa* (Fig. 3*A*).

We also analyzed expression of ezrin and NGX6a in NPC biopsies. The expression pattern of NGX6a in nasopharyngeal biopsy tissues was significantly negatively associated with that of ezrin with statistical significance (Fig. 3*B*).

The expression pattern of ezrin and NGX6a in normal tissue is also very interesting. The Western blot analysis of ezrin and NGX6a in various normal tissues and organs produced a similar pattern as that in nasopharyngeal cells. Ezrin is highly expressed in the kidney, small intestine, colon, rectum, testis, and thymus, where NGX6a is expressed at relatively low levels. Ezrin expression is low or absent in the nasopharynx, pancreas, adipose, bladder, uterus, and liver, whereas the expression of NGX6a in these tissues is relatively high (Fig. 3*C*). However, in some organs, both ezrin and NGX6a are expressed at relatively high levels, such as in the nervous system. However, the distribution of NGX6a and ezrin are different. An immunohistochemistry analysis showed that NGX6a was expressed in neuronal cells where ezrin expression was not detectable, that ezrin expression occurred mainly in astrocytes and nerve fibers in cerebella molecular layer slices where there was no staining with anti-NGX6a, and that ezrin was undetectable in deep nerve fibers and in the Purkinje cell layer where NGX6a was enriched (Fig. 3*D*). This evidence demonstrates that the pattern of NGX6a expression distribution is negatively associated with that of ezrin.

*Ezrin Mediates the Degradation of NGX6a in NPC Cells*—We observed that transfected exogenous NGX6a degrades in NPC cells, that NGX6a interacts with ezrin, and that the expression of NGX6a is negatively associated with ezrin in cell lines and tissues. We hypothesized that ezrin may mediate NGX6a degradation. The different amounts of pcDNA3.1(+)-ezrin (0, 0.2, 0.5, 1, and 2 $\mu$g) together with same amount of pCMV-Myc-NGX6a (2 $\mu$g) were co-transfected into HEK 293FT cells. We confirmed that the NGX6a level decreased with the increasing of amount of plasmid pcDNA3.1(+)-ezrin. A Western blot analysis showed that with the increase in ezrin expression, the NGX6a level decreased in turn (Fig. 4*A*). Because ezrin was highly expressed in NPC cell lines, we used RNAi technology to knock down the expression of ezrin and to determine whether NGX6a degradation occurs in these cells. When ezrin expression was high, NGX6a is undetectable by FLAG antibody, even when the cells were transfected with the pIRES-neo3-NGX6a vector. Once the expression of ezrin was knocked down, NGX6a was detectable (Fig. 4*B*). Similarly, with ezrin knockdown in the nasopharyngeal carcinoma cell 5-8F, the expression of endogenous NGX6a increased (Fig. 4*C*).

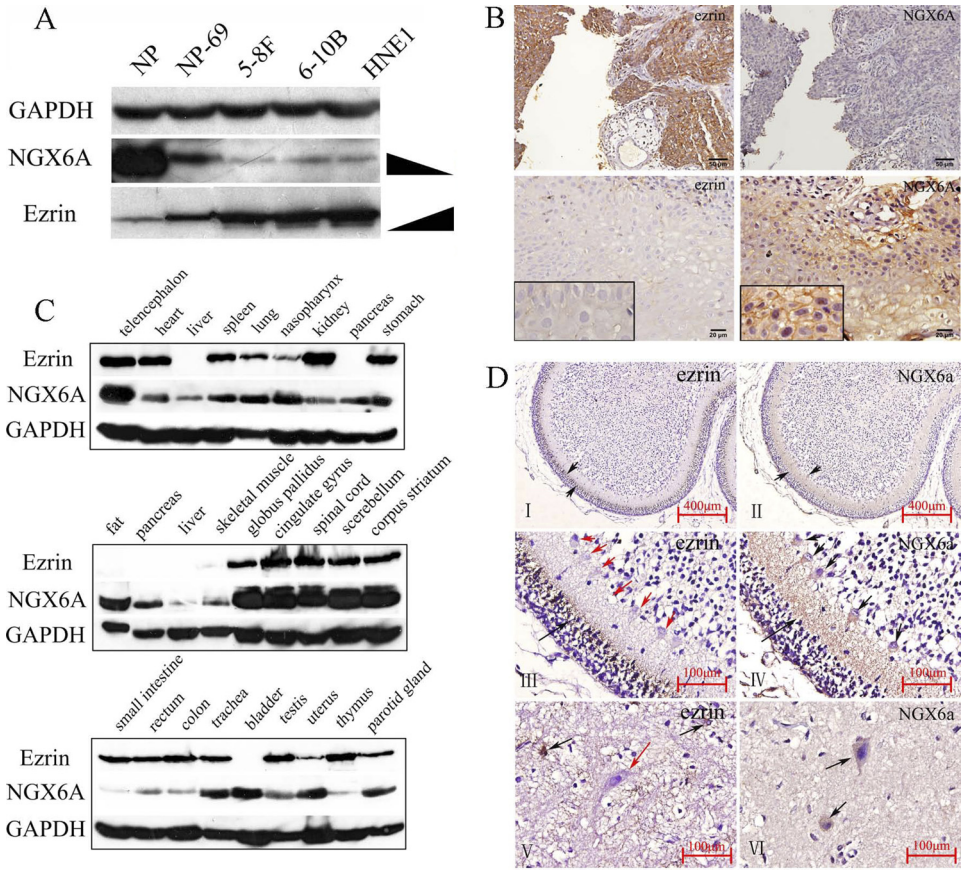


FIGURE 3. **The expression and distribution of NGX6a were negatively associated with ezrin in cell lines and tissues.** *A*, total proteins were extracted from NP, NP-69, 5-8F, 6-10B, and HNE1 cells. The proteins were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by Western blot. The antibodies that were used in the Western blot analysis are indicated on the *left*. GAPDH was used as a loading control. *B*, immunohistochemistry analysis of NGX6a and ezrin in human NPC tissues. *Left* and *right*, representative positive staining with anti-ezrin and NGX6a, respectively. *C*, Western blot analysis of NGX6a and ezrin in various normal tissues. GAPDH was used as a loading control. *D*, immunohistochemistry analysis of human nervous system. *I* and *III*, in the cerebellum, staining in the astrocytes and nerve fibers of the cerebella indicated a shallow molecular layer with an antibody that was specific for ezrin (*black arrow*) and no staining of deep nerve fibers of Purkinje cells (*red arrow*). *II* and *V*, in cerebellum, staining was shown in deep nerve fibers and Purkinje cells using an antibody that was specific for NGX6a (*black arrow*). *V*, in the telencephalon, staining was shown in some glial cells with an anti-ezrin antibody (*black arrow*), and no staining was observed in the neurons (*red arrow*). *VI*, in the telencephalon, staining was shown in neuronal cells using anti-NGX6a antibody (*black arrow*).

*The Seven-transmembrane Domain of NGX6a Is the Key Domain for Its Degradation*—In order to clarify the key domains that are required for NGX6a intracellular degradation, we constructed a series of deletion mutants of NGX6a. M1 is a deletion mutant of the amino-terminal deletion of amino acids 1–227 (deletion of the EGF-like domain); M2 is a deletion of amino acids 228–472 (deletion of the seven-transmembrane region); S is a deletion of amino acids 1–273, which are homologous to NGX6b; and CO is a deletion of the specific sequence of NGX6a amino acids 274–472 (Fig. 4*D*). The individual mutants were transfected into 5-8F cells. The Western blot analysis verified their expression. M2 had a very strong expression, whereas the expression of CO, containing 228–273 aa more than M2, was much weaker than that of M2. The M1 mutant has 228–273 aa more than the S mutant, and S expression was slightly higher than that of M1 but was weaker than that of CO and M2. Among the four mutants, M1 expression was the weakest and was similar to that of full-length NGX6a. Furthermore, the degree of possible multimerization of M1 and S with the deletion of the amino terminus was greatly reduced (Fig. 4*E*), which indirectly indicates that the amino terminus of NGX6a is a critical domain for multimerization.

When the pCMV-NGX6a mutants and pcDNA3.1(+)-ezrin were co-transfected into HEK 293FT cells, 2 $\mu$g was used for each mutant, and 0, 0.2, 0.5, 1, or 2 $\mu$g was used for pcDNA3.1(+)-ezrin, with the empty vector added for a total amount of 4 $\mu$g. The cells were harvested 48 h after transfection, and the lysate of the cell extract was subjected to Western blot analysis. The results showed that the expression of only the M1 mutant decreased with increasing ezrin expression, whereas that of the other mutants did not respond to the increase in ezrin expression (Fig. 4*F*). Although mutant S has 228–273 aa fewer than M1, it did not degrade in response to the increase in ezrin. CO has a 228–273-aa region but did not degrade in response to ezrin. This evidence indicates that the 228–273-aa region together with other transmembrane domains is a critical domain for NGX6a degradation. Taken together, M1 contains the recognition domain, the seven-transmembrane domain for NGX6a degradation by the proteasome.

*The N-ERMAD Domain of Ezrin Is Required for the Induction of NGX6a Degradation*—In order to clarify the critical domain of ezrin that is required for NGX6a degradation, we generated a series of constructs containing the domains of ezrin (Fig. 5*A*). MC-ERMAD (aa 1–468) contains a carboxyl-terminal deletion of

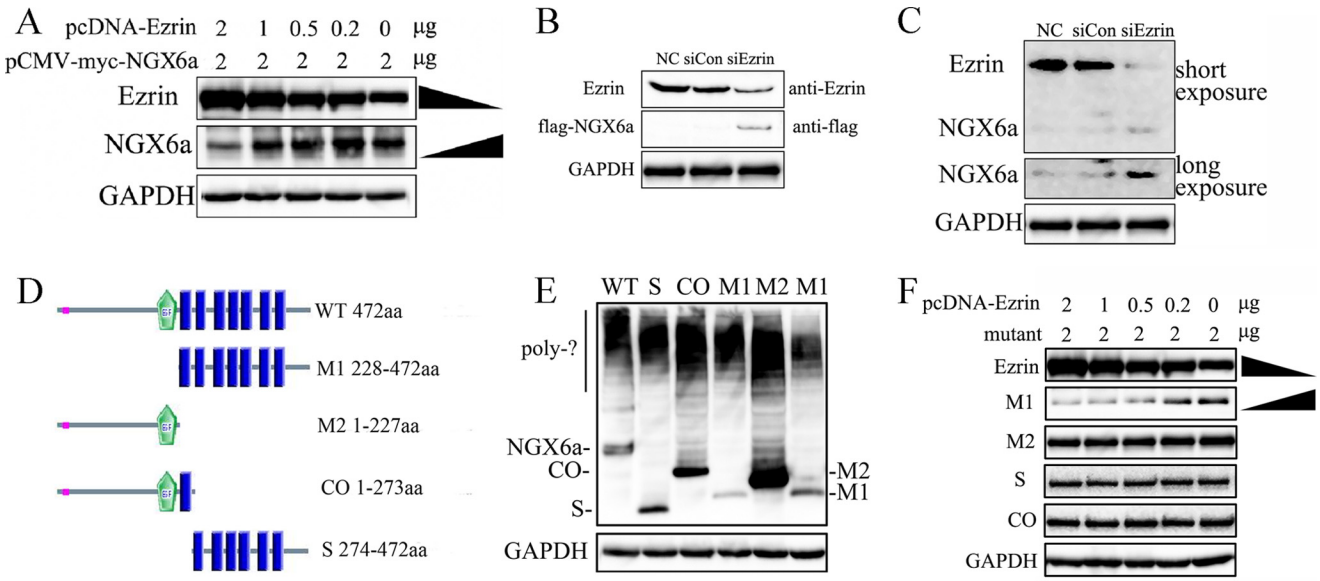


FIGURE 4. **Ezrin mediates the intracellular degradation of NGX6a.** *A*, the pcDNA3.1(+)-ezrin and pCMV-Myc-NGX6a were co-transfected into HEK 293FT cells. The cells were harvested, and the cell lysates were subjected to Western blot analysis with anti-ezrin, NGX6a. GAPDH was used as a loading control. *B*, the cell lysate of pIRES-neo3-NGX6a/5-8F was transiently transfected with siEzrin or siCon and subjected to Western blot analysis for NGX6a and ezrin. *C*, endogenous NGX6a expression of 5-8F after the transfection of siEzrin. *D*, schematic representation of NGX6a mutants with the deletion of different domains, respectively. *E*, total proteins were extracted from 5-8F cells that were transfected with deletion mutant constructs of pIRES-neo-NGX6a. The proteins were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with an anti-FLAG antibody. GAPDH was used as a loading control. The same amount of each plasmid and the Lipofectamine 2000 was used. *F*, the seven-transmembrane region is a key domain for *NGX6a* degradation. The increased ezrin expression induced the degradation of *NGX6a* mutant with the seven-transmembrane region, whereas when even one (*S*) or six (*CO*) transmembranes or the entire transmembrane region (*M2*) were deleted, their levels did not vary with the increased ezrin concentration.

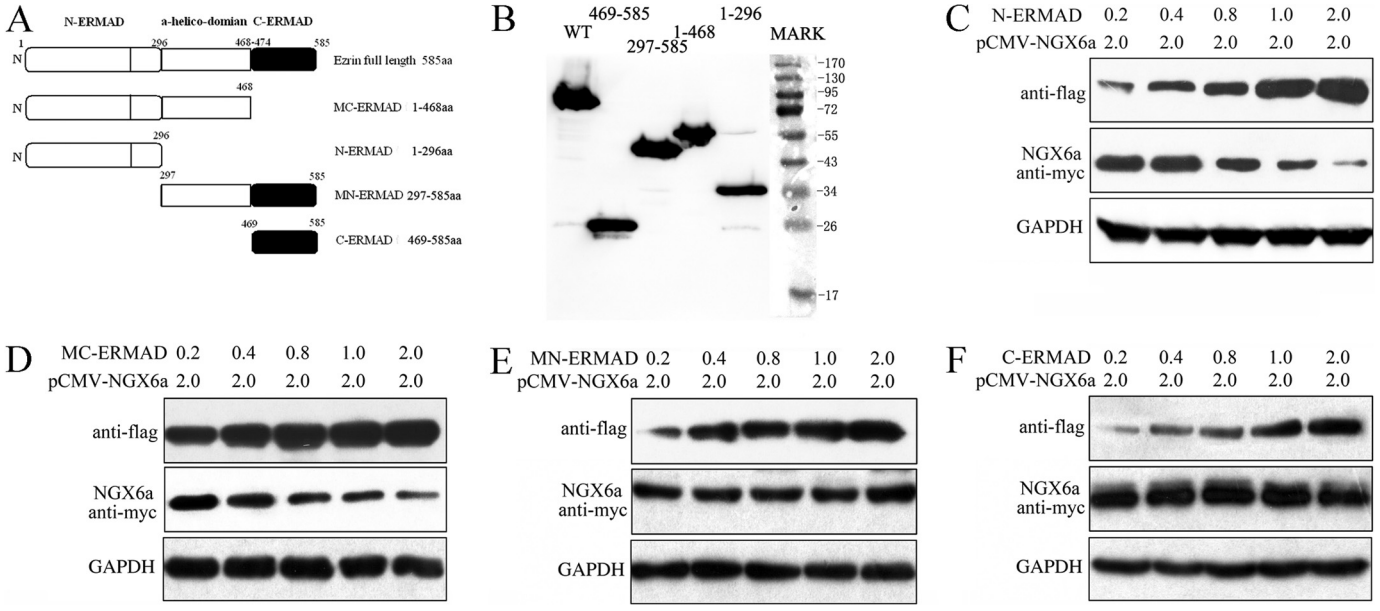


FIGURE 5. **The N-ERMAD domain of ezrin is critical for the degradation NGX6a.** *A*, schematic representation of ezrin mutants with the deletion of different domains that were inserted into pcDNA3.1 that was tagged with FLAG. *B*, total proteins were extracted from 293T cells that were transfected with wild type pcDNA3.1(+)-ezrin or pcDNA3.1(+)-FLAG-C or MN-ERMAD, MC-ERMAD, or N-ERMAD. The proteins were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by Western blot using an anti-FLAG antibody. *C–G*, the wild-type ezrin or mutant constructs with increased concentrations and pCMV-Myc-NGX6a were co-transfected into HEK 293FT cells. The cells were harvested, and the cell lysates were subjected to Western blot analysis with anti-FLAG and anti-Myc antibodies. GAPDH was used as a loading control. MC- and N-ERMAD ezrin mutants mediated NGX6a degradation in the cells; however, the increased expression of MN- and C-ERMAD did not change the NGX6a levels.

amino acids 469–585; N-ERMAD (aa 1–296) contains a deletion of amino acids 297–585; MN-ERMAD (aa 297–585) contains a deletion of amino acids 1–296; and C-ERMAD (aa 469–585) contains a deletion of amino acids 1–468. The full-length ezrin was amplified by PCR with the FLAG sequence at the amino-terminal end using the fetal brain cDNA library as a template. The fragment was inserted into the pcDNA3.1(+) plasmid, and then each mutant fragment was amplified from the full-length ezrin construct and inserted into the pcDNA3.1(+) plasmid. The individual mutants were transfected into 293T cells and subjected to Western blot analysis. The results confirmed that the mutants were successfully constructed (Fig. 5*B*).

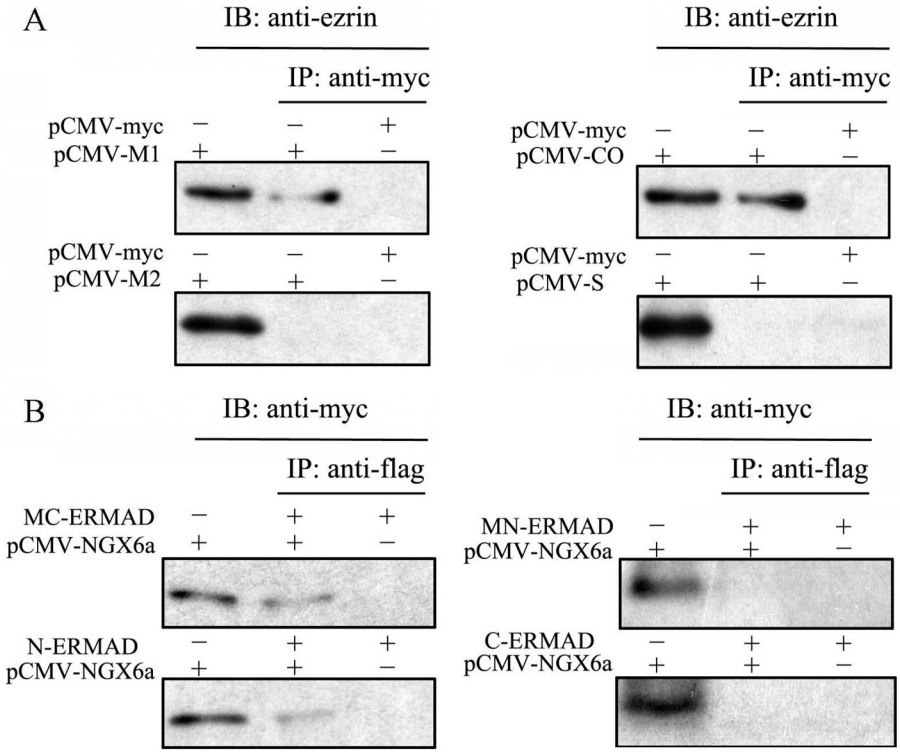


FIGURE 6. **Identification of the binding sites of NGX6a interacting with ezrin.** *A*, total proteins were extracted from 293FT cells that were transfected with pCMV-M1, pCMV-M2, pCMV-CO, or pCMV-S and treated with the proteasome inhibitor MG132 (5 $\mu$M) for 12 h, after which the total proteins were immunoprecipitated (*IP*) with anti-Myc-agarose beads and analyzed by Western blot (*IB*) with anti-ezrin polyclonal antibody. *B*, total proteins were extracted from 293FT cells that were co-transfected with pcDNA3.1(+)-FLAG-C or MN-, MC-, or N-ERMAD and pCMV-NGX6a (tagged with Myc tag) and then treated with the proteasome inhibitor MG132 (5 $\mu$M) for 12 h, after which the total proteins were immunoprecipitated with anti-FLAG-agarose beads and analyzed by Western blot with anti-Myc antibody.

The deletion mutants, full-length ezrin constructs (0.2, 0.4, 0.8, 1, and 2 $\mu$g), and pCMV-Myc-NGX6a (2.0 $\mu$g) were co-transfected into HEK 293T cells. Empty vector was supplemented to a total amount of 4 $\mu$g. At 48 h after transfection, the cells were harvested in cell lysis buffer. The Western blot analysis showed that NGX6a expression decreased only in response to the increase in the ezrin deletion mutants MC-ERMAD (aa 1–468) and N-ERMAD (aa 1–296), whereas there was no effect on the NGX6a protein level when the expression of the mutants MN-ERMAD (aa 297–585) and C-ERMAD (aa 469–585) increased (Fig. 5, *C–F*). These results demonstrate that the N-ERMAD (aa 1–296) domain of ezrin is required for NGX6a degradation.

*Identification of Binding Site of NGX6a Interacting with Ezrin*—In order to determine whether the binding sites of NGX6a and ezrin interact, an immunoprecipitation assay and Western blot analysis were performed. Total proteins were extracted from 293FT cells that were transfected with pCMV-M1, pCMV-M2, pCMV-CO, or pCMV-S and, 12 h after the transfection, treated with the proteasome inhibitor MG132 (5 $\mu$M) for 12 h, after which the total proteins were immunoprecipitated with anti-Myc-agarose beads and analyzed by Western blot with anti-ezrin polyclonal antibody. Mutants M1 and CO still interact with ezrin, whereas M2 and S in which transmembrane region 1 was deleted lost the interaction, indicating that transmembrane region 1 may be the binding site (Fig. 6A). In the same way, total proteins were extracted from 293FT cells that were co-transfected with pcDNA3.1(+)-FLAG-C or MN-, MC-, or N-ERMAD and pCMV-NGX6a (tagged with Myc tag) and, 12 h after the transfection, treated with the proteasome inhibitor MG132 (5 $\mu$M) for 12 h, after which the total proteins were immunoprecipitated with anti-FLAG-agarose beads and analyzed by Western blot with anti-Myc antibody. Mutants MC and N still interacted with NGX6a, whereas MN and C, which deleted the amino terminus, lost the interaction with ezrin, suggesting that the amino terminus of ezrin is the critical site with NGX6a (Fig. 6*B*).

*Ezrin Promotes the Invasion and Metastasis of Nasopharyngeal Carcinoma Cells by Inducing NGX6a Degradation*—Because nasopharyngeal carcinoma cells express high levels of ezrin, transfected exogenous NGX6a is undetectable due to the rapid degradation that is mediated by ezrin in these cell lines. After treatment with MG-132 (1.5 $\mu$M) for 24 h, the number of cells in the pCMV-Myc-NGX6a/5-8F group that migrated through the scratch line after 40 h was significantly lower compared with those of the pCMV-Myc/5-8F group (Fig. 7, *A* and *B*), demonstrating that NGX6a inhibited the invasion and migration of nasopharyngeal carcinoma cells.

To examine whether the knockdown of ezrin can inhibit the migration of 5-8F cells, small interfering RNA siEzrin or siCon was transfected into stably transfected pIRES-neo3/5-8F or pIRES-neo3-NGX6a/5-8F cell lines. After 48 h of cell culture, the two groups were subjected to a Matrigel invasion assay. The number of cells in the pIRES-neo3-NGX6a/5-8F group that migrated through the Transwells was significantly lower compared with that of the pIRES-neo3-NGX6a/

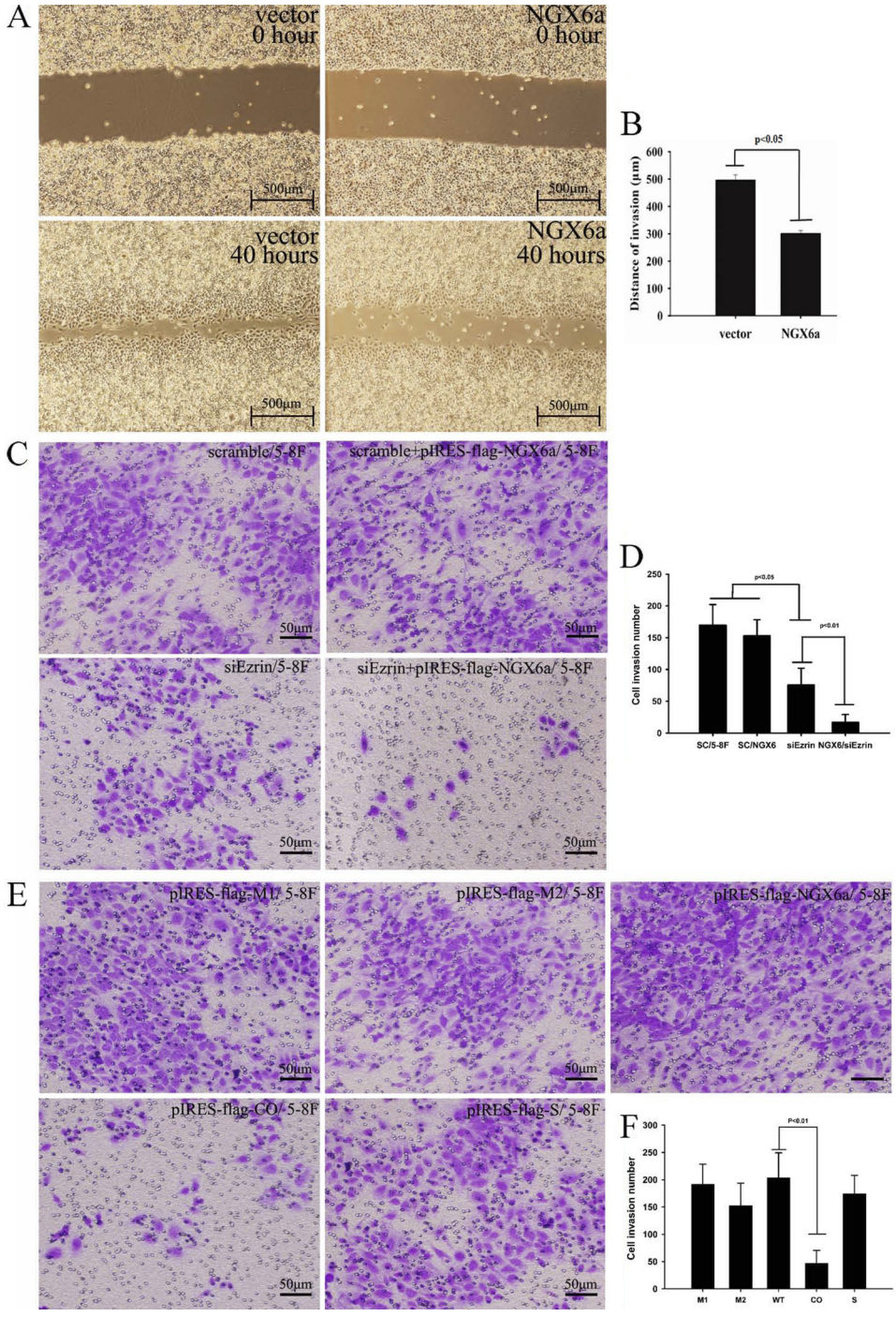


FIGURE 7. **Ezrin promotes the invasion of nasopharyngeal carcinoma cells by mediating the degradation of NGX6a through a scratch wound healing assay and Matrigel invasion assay.** *A*, scratch wound healing assay. 5-8F cells that were transfected with empty pIRES-neo3 or pIRES-neo3-NGX6a plasmids were seeded in a 6-well dish, in which the monolayer was gently and slowly scratched with a new 10-$\mu$l pipette tip across the center of the well 24 h later. The detached cells were gently washed twice with medium and removed. Following an incubation period (40 h), the cells were fixed with 3.7% paraformaldehyde for 30 min. The stained monolayer was photographed under a microscope. The gap distance was quantitatively evaluated using ImageJ software (*B*). The statistically significant differences are indicated by $p < 0.05$. *C* and *D*, cell invasion assay. *C*, $1 \times 10^5$ cells were added to the upper compartment of cell culture inserts with a 12-mm diameter and 8-$\mu$m pores. The insert was positioned into the well, with the bottom of the insert merged in medium containing 10%FBS as an attractant. After the incubation period, the insert was carefully removed. The cells and the gel within the upper compartment of the insert were gently removed with a cotton swab 24 h later. The cells on the lower side of the insert membrane were fixed and stained with 1% crystal violet in 2% ethanol for 20 min. The cells were washed and counted randomly in five different views on the lower side of the filter under the microscope. *D*, statistically significant differences are indicated by $p < 0.05$ or $p < 0.01$. *E*, cell invasion assay of 5-8F cells that were transfected with pIRES-neo3-NGX6a, M1, M2, CO, and S. *F*, statistically significant differences are indicated by $p < 0.05$. *Error bars*, S.D.

5-8F group transfected with siCon (Fig. 7, *C* and *D*). These data indicate that the knockdown of ezrin expression inhibits the invasion and migration of nasopharyngeal carcinoma cells. The combination of NGX6a level promotion and the knockdown of ezrin synergistically inhibits the invasion and migration of cells.

We further investigated whether the degradation of NGX6a facilitates invasion and migration in NPC cells. The deletion mutants of NGX6a that were tagged with FLAG were transfected into 5-8F cells. Compared with the full-length of NGX6a, the CO group had a significantly lower number of cells that migrated through the Transwell, which was fewer than in the other three groups (transfected with pIRES-neo3-S, pIRES-neo3-M1, and pIRES-neo3-M2), which did not differ (Fig. 7, *E* and *F*). Because the deletion mutant M1 was degraded in the presence of ezrin in 5-8F cells, it did not inhibit the migration of the 5-8F cells; therefore, the cells were comparable with the full-length NGX6a. Although M2 and S did not degrade in the presence of ezrin, they were incomplete in the absence of the EGF-like domain or transmembrane domain 1, which may be responsible for receiving external signals and for the transduction of signals into intracellular cascades. CO has an EGF-like domain and transmembrane domain 1 but with the absence of transmembrane domains 2–6, which are critical domains for the degradation of NGX6a. NGX6a does not degrade and possesses the ability to interact with ezrin and inhibit the signaling of the invasion and migration of NPC cells. These data indicate that NGX6a degradation as mediated by ezrin contributes to the invasion and migration of NPC cells, as well as the EGF-like domain and transmembrane region 1, which are indispensable for NGX6a function. This result further demonstrates that transmembrane domains 1–7 are critical domains for the degradation of NGX6a as mediated by ezrin.

## DISCUSSION

The *NGX6* gene was cloned from chromosome 9 using a positional candidate cloning approach from a susceptible gene region of nasopharyngeal carcinoma. At first, only the first variant (NGX6b) was identified. This variant inhibits the invasion and migration in nasopharyngeal carcinoma and colorectal cancer (1, 5, 9, 22–25). NGX6a is primarily expressed in nasopharyngeal epithelium cells compared with NGX6b but expressed at a very low level in nasopharyngeal carcinoma cells. More interesting, the NGX6a protein is not detectable in 5-8F cells with the stable transfection of pIRES-neo3-NGX6a, which contains the full-length NGX6a ORF tagged with FLAG, although the mRNA level is 20-fold higher than that of the control vector. However, the NGX6a protein is detectable in 5-8F cells when transfected transiently with the pCMV-NGX6a vector. These results are contradictory, and we had difficulty explaining this paradox. This study demonstrated that the NGX6a protein experiences degradation mediated by ezrin through the proteasome pathway in nasopharyngeal carcinoma cells, but its degradation is independent of ubiquitin modification. A feasible explanation is that pCMV-Myc-NGX6a is transiently transfected into cells in which protein expression is induced by a strong enhancer. These factors resulted in the speed of protein synthesis being much higher than that of the degradation that is mediated by ezrin, so that NGX6a recombinant proteins are transiently high and detectable by Western blot analysis. By contrast, in 5-8F cells that are transfected with pIRES-neo3-NGX6a stably, the protein expression is lower and is degraded through long term screening tests mediated by a high level of ezrin of 5-8F cells; therefore, it is feasible that NGX6a is much lower or undetectable.

When the lysates of nasopharyngeal carcinoma cells that were transiently transfected with pCMV-Myc-NGX6a were subjected to Western blot analysis, an interesting phenomenon occurred; a 51 kDa band was predominant, and a smear band greater than 51 kDa also existed, which was similar to the polyubiquitination of most ubiquitinated protein. This type of protein band is characteristic of protein degradation. However, we found that NGX6a is not ubiquitinated. NGX6a may be labeled by some unidentified multimeric molecule, which is progressively larger but is not ubiquitin. Current evidence suggests that some target proteins also can be bound by other ubiquitin-like proteins. The ubiquitin-like protein family contains small ubiquitin-like modifier (SUMO) proteins (26), NEDD8 (neural precursor cell-expressed developmentally down-regulated protein 8) (27), and ISG15 (interferon-stimulated 15-kDa protein) (28). Ubiquitin is the predominant regulator for the degradation of a wide range of target proteins, whereas SUMO, NEDD8, and ISG15 modify a limited set of substrates to regulate various other biological processes. We attempted to determine whether NGX6a was labeled by these ubiquitin-like proteins, but the results were negative (data not shown). Our hypothesis is as follows; some other cellular protein, similar to but not the ubiquitin molecule, is covalently bound for multimerization and to label target proteins and to transition them into proteasome-dependent degradation. It is likely that one or more ubiquitin-proteasome pathway-parallel signaling pathways may exist in cells and that the protein NGX6a degrades via a novel but undefined pathway.

Ezrin belongs to the intracellular ERM family, which includes ezrin, radixin, moesin, Merlin, and other members. Our previous results demonstrated that ezrin interacts with NGX6b and that the NGX6b Cyto domain is a key domain for its interaction with ezrin (5, 25). In this study, we used an immunofluorescence test and a coimmunoprecipitation assay to confirm the interaction of NGX6a and ezrin. At the same time, this study confirmed that ezrin mediates the degradation of NGX6a and that the N-ERMAD domain of ezrin and the seven-transmembrane region of NGX6a are the key domains for the degradation of NGX6a. The binding of N-ERMAD of ezrin with NGX6a mediates NGX6a degradation. NGX6a is a membrane protein with seven transmembrane domains, whereas NGX6b not only is located on the membrane but also is one of a number of membrane structure-containing organelles of the cytoplasm and nucleus.

Many studies have documented that ezrin binds membrane proteins (CD43, CD44, ICAM-1, ICAM-2, and ICAM-3) via the N-ERMAD domain and links cytoskeletal proteins by the carboxyl terminus (29–33). The molecular events that occur to membrane proteins after interacting with ezrin are still not very clear. According to the pattern of NGX6a mediated by ezrin, a hypothesis can be made; ezrin may be a novel ubiquitin-ligase like ligase, which binds multiple membrane proteins via the N-ERMAD domain and labels these membrane proteins with an unknown protein that is similar to ubiquitin. The labeled membrane proteins are then degraded by the proteasome path-

way. This hypothesis provides a novel pathway of membrane protein degradation.

Abnormal ezrin expression in tumor tissue with metastasis suggests that this may be involved in the invasion and metastasis of tumor cells (12–13, 34). In this study, ezrin expression knockdown in NPC cells increased endogenous NGX6a, and the invasion and metastasis of nasopharyngeal carcinoma cells were inhibited. When NGX6a expression was recovered, the invasion and metastasis of NPC cells were further inhibited. These results indicate that ezrin mediates NGX6a degradation, which may be an important mechanism of the invasion and metastasis of NPC cells. However, there are several questions we still cannot answer (*e.g.* what is the molecular mechanism of ezrin-mediated NGX6a degradation, what cofactors are involved in the degradation events, what roles does ezrin play in the process of degradation, and what type of modifications were made to label the NGX6a protein prior to degradation?). The degradation of NGX6a is characterized by a proteasome-dependent pathway; however, we cannot exclude the possibility that part of NGX6a degradation may be lysosome-dependent. These questions remain unclear and warrant further study.

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