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Title Page

Neuroglobin, a Novel Intracellular Hexa-coordinated Globin, Functions as a Tumor Suppressor in Hepatocellular Carcinoma via Raf/MAPK/Erk

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Running Title Page

Running title: Neuroglobin functions as a tumor suppressor

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ABBREVIATIONS: ANT, adjacent non-tumor tissue; EdU, 5-ethynyl-2-deoxyuridine; DMEM, Dulbecco's modified Eagle's medium; Erk, extracellular signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide; Ngb, neuroglobin; PCR, polymerase chain reaction; ROS, reactive oxygen species; sh-RNA, short hairpin RNA.

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ABSTRACT

Hypoxia and oxidative stress are critical factors in carcinogenesis and exists throughout cancer development, however, the underlying mechanisms are far from clear. Here, for the first time, we reported that neuroglobin (Ngb), an intracellular hexa-coordinated globin serving as an oxygen/reactive oxygen species (ROS) sensor, functions as a tumor suppressor in hepatocelluar carcinoma (HCC). Ngb protein and mRNA expression were significantly down-regulated in tumor tissues as compared to its adjacent non-tumor tissues of human HCC samples and normal liver tissues. Knock-down of Ngb by RNA interference promoted human HCC cell line (HepG2) growth, proliferation, G0/G1-S transition *in vitro* and tumor growth *in vivo*. On the contrary, overexpression of Ngb suppressed HepG2 cell growth, proliferation, G0/G1-S transition, colony formation *in vitro* and tumorigenicity *in vivo*. These results established a tumor suppressor function of Ngb in HCC. The underlying mechanisms were further investigated. Overexpression of Ngb suppressed Raf/MEK/Erk while knock-down of Ngb enhanced Raf/MEK/Erk activation in HepG2 cells *in vitro* and *in vivo*. GST pull-down showed that Ngb interacted with c-Raf-1 in HepG2 cells. Overexpression of Ngb suppressed serum- and H_2O_2-stimulated Erk activation in HepG2 cells. Pharmacological inhibition of Erk activation abolished the proliferative effect of Ngb-knock down in HepG2 cells. Mutation of Ngb at its oxygen-binding site (H64L) abolished the inhibitory effects of Ngb on Erk activation and HepG2 cell proliferation. Therefore, we propose that Ngb controls HCC development by linking oxygen/ROS signals to oncogenic Raf/MAPK/Erk signaling. Our data suggest that neuroglobin could be a new target for cancer therapy.

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Introduction

Liver cancer is the sixth most common malignant tumor and the third leading cause of cancer-related death in the world. Hepatocellular carcinoma (HCC) is the most common primary liver malignant tumor in adults. Hepatocarcinogenesis caused by virus infection, liver injury or inflammation usually involves hepatocytic death and abnormal reconstruction (Severi et al., 2010; Pollicino et al., 2011), in which hypoxia or oxidative stress plays an important role. Hypoxia and oxidative stress might be independent initial factors in hepatocarcinogenesis by activating pro-oncogenic genes or causing genetic instability (El-Serag et al., 2007; Aravalli et al., 2008; Zender et al., 2010) which finally induces cellular transformation and promotes tumor cell growth. For example, diethylnitrosamine, a well known liver carcinogen, induces hepatocyte injury and compensatory proliferation by accumulating reactive oxygen species (ROS) (Aravalli et al., 2008; Sakurai et al, 2008; Zender et al., 2010). The major signaling pathways involving in HCC genesis or development includes Raf/mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/AKT and Wnt/β-catenin (Aravalli et al., 2008; El-Serag et al., 2007; Whittaker et al., 2010). Nevertheless, the exact mechanisms underlying hypoxia- or oxidative stress-induced HCC remain far from understood.

Neuroglobin (Ngb), a newly discovered intracellular respiratory globin with a hexa-coordinated heme (Burmester et al., 2000; Trent et al., 2001), plays an important role in protecting brain cells from ischemic (Sun et al., 2001; Chen et al., 2005b) or oxidative stress (Ye et al., 2009; Antao et al., 2010). Since Ngb shows a higher binding affinity to oxygen (O_2) and nitric oxide (NO), it is hypothesized the Ngb may function as the oxygen carrier, NO/ROS scavenger or oxygen/ROS sensor (Trent et al., 2001; Brunori et al., 2005; Burmester et al., 2009; Herold et al., 2004). Structural studies reveal that the

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distal histidine (His^{64}) residue of Ngb is critical for its heme-structure stability and ligand-binding activities (Nienhaus et al., 2004; Vallone et al., 2004). Mutation of His^{64} locks Ngb in a penta-coordinated state, increasing its catalytic nitrite binding and reductase activity. In addition, Ngb binds to G-protein subunit (Wakasugi et al., 2003), 14-3-3ζ (Jayaraman et al., 2011) or cytochrome C (Raychaudhuri et al., 2010), which is regulated by hypoxic or oxidative conditions. These evidences suggest that Ngb may regulate signal transduction by sensing changes of intracellular O_2 or ROS signals. On the other hand, the expression (Zhu et al., 2002) and phosphorylation (Raychaudhuri et al., 2010) of Ngb under ischemic or hypoxic conditions are also regulated by intracellular signaling pathways such as Erk activation. These data suggest a complicated relationship between Ngb and Raf/MAPK/Erk cascades under physiological or pathological conditions.

There are emerging evidences showing that the expression levels of hexa-coordinated globins are associated with tumor genesis or development. Emara et al (2010) reports that neuroglobin (Ngb) and cytoglobin (Cygb) are detected in most types of tumors by microarray analysis. Oleksiewicz et al (2011) reports that Ngb is significantly up-regulated in lung cancer. However, Gorr et al (2011) reports that neither Ngb nor Cygb is systematically up-regulated in tumors. In the non-small cell lung cancer, the down-regulation of Ngb is found to be associated with Ngb promoter hypermethylation (Oleksiewicz et al., 2011). Hypermethylation of Cygb promoter has also been found in several tumors (Shivapurkar et al., 2008). These evidences indicate that Ngb expression in tumors may depend on specific cellular conditions such as hypoxia or hypermethylation. Until now, the functional role of Ngb in tumor cells has not been directly studied while the function of other globin members in tumor cells has already been reported. Since Ngb is a native oxygen or ROS sensor, clarifying the functional role of Ngb in tumor cells is important for understanding the role of hypoxia and ROS in tumor

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genesis and development.

Here, we reported that Ngb was down-regulated in HCC. Overexpression of Ngb suppressed HCC cell growth *in vitro* and tumor growth *in vivo* while knock-down of Ngb showed the opposite effects in HCC cells. Further, we found that Ngb controls HCC cell proliferation via regulating Raf/MEK/Erk activities negatively.

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Materials and methods

Tissue samples. Sixty male human HCC samples and lung cancer samples were used for immunohistochemical analysis. These samples were purchased from the Department of Pathology, Tongji Hospital, Huazhong University of Science and Technology (HUST). All samples were surgically resected during 2008 to 2009 in Tongji Hospital, HUST and were diagnosed clinically and pathologically by Tongji Hospital, HUST. Among the 60 HCC samples, 48 samples had their corresponding adjacent non-tumor tissues (ANT). All human (male) normal liver samples were obtained from the Department of Forensic Medicine, HUST.

Seventeen fresh male HCC tissues with their corresponding adjacent non-tumor tissues were used for quantitative mRNA analysis. All samples were purchased from the Department of Hepatobiliary Surgery, Union Hospital, HUST and were diagnostic confirmed clinically and pathologically by Union Hospital, HUST.

Immunohistochemical analysis and assessment. The expression of Ngb in HCC was examined by immunohistochemistry (IHC) using a rabbit polyclonal antibody against Ngb (Santa Cruz Biotechnology, USA). Four-micrometer sections were cut from paraffin-embedded HCC tissue blocks, deparaffinized, rehydrated, and put into endogenous peroxidase-blocking solution. The sections were then boiled for 10 minutes (min) in 10 mmol/L citrate-buffer (pH 6.0) in a water bath. Thereafter, slides were incubated with Ngb antibody at a dilution of 1:100 in 4°C overnight. The expression of Ngb was detected by using corresponding rabbit EnVision System-HRP (DAB) kit (DAKO, Demark) according to the manufacturer’s instruction. Slides were then washed in water, counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany), and coverslipped. Negative controls without primary antibody were performed in parallel. Positive staining was envisionized as brown particles in

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the cytoplasm. Total immunostaining scores of each slide were calculated by multiplying the positive cell percentage scores and the intensity scores as previously described (Huynh, 2004). The positive cell percentage scores (0-4) were defined by the percentage of positively stained cells in tissues (0, none; 1, <10%; 2, 10-50%; 3, 50-80%; 4, >80%). The intensity scores (0-3) represented the relative staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong). The total score ranged from 0 to 12. Ngb positive staining was defined as a total score greater than 4 (including 4).

Reverse transcriptional polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qPCR). Total RNA was extracted by using TRIzol reagent according to the manufacturer's instructions (Invitrogen, USA). Conventional and quantitative RT-PCR was performed as previously described (Chen et al., 2005b; Ye et al., 2009). qPCR was performed by using the Platinum SYBR Green qPCR superMix-UDG Detection Kit (Invitrogen Life Technology, USA) and the Applied Biosystems StepOnePlus™ Real-Time PCR Systems (Applied Biosystems Inc., USA). The Ngb primers were 5'-GAAGCACCGGGCAGTG-3' and 5'-AGACACTTCTCCAGCATGTAGAG-3'. GAPDH was used as an internal control and its primers were 5'-AACGGATTTGGTCGTATTG-3' and 5'-GGAAGATGGTGATGGGATT-3'. Triple Ct values were analyzed by using the comparative Ct ($\triangle\triangle$Ct) method following the manufacturer's instructions (Applied Biosystems Inc. USA). Relative Ngb expression level was expressed as the ratio of Ngb/GAPDH.

Cell Lines, plasmids and transfection. Human HCC cell line (HepG2 cells) was cultured in DMEM plus 10% FBS (Sigma, USA) at 37^{o}C with 95% humidified atmosphere containing 5% CO_2 in an incubator (Heal Force Inc., USA). The Ngb overexpression plasmids (p-Ngb-EGFP-N1) were constructed previously (Chen et al., 2005b). Ngb mutants (Y44D, H64A, H64L, E53Q, E118Q, K119N) were constructed by using the Fast Mutagenesis System (Bejing TransGen Biotech Co.,

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China) using p-Ngb-EGFP-N1 as the template. The Ngb short hairpin RNA-expressing plasmids (p-Ngb-shRNA-Genesil-1, sh-Ngb) for Ngb knock-down were constructed by inserting the a specific Ngb-shRNA DNA fragment into the p-Genesil-1 vector (Wuhan Genesil Biotechnology, China) as previously reported (Ye et al., 2009). The target sequence of Ngb-shRNA was 5'-GGTGATGCTCGTGATTGATGC-3'. A non-specific DNA fragment was used as negative control (N-con). All plasmids were confirmed by sequencing. Transfection of HepG2 cells at 80-90% confluence were performed by using FuGENE HD reagent (Roche, Switzerland). Stably transfected HepG2 cell lines were established by neomycin (G418) (Merck KGaA, Germany).

Western blot analysis. Total soluble proteins were extracted from tissues or cultured cells and equal amount of total proteins were used for Western blot analysis as previously reported (Chen et al., 2005a). After blocking with 5% of non-fat milk in phosphate buffered saline (PBS), the blots were incubated with primary antibodies against Ngb, c-Raf-1, β-actin (Santa Cruz Biotechnology, USA), p-Raf-1 Ser338, p-MEK1/2 Ser217/221, p-Erk1/2 Thr202/Tyr204, Erk1/2, p-GSK-3β Ser9, GSK-3β, p-PTEN Ser380/Thr382/383, PTEN, p-β-catenin Ser33/37/Thr41 and β-catenin (Cell Signaling Tech, USA). The blots were visualized with corresponding fluorescent secondary antibodies and the bands were quantified by using the Odyssey Infrared Imaging System (LI-COR Bioscience, USA).

Cell viability assay. Cell viability was measured by MTT assay as previously reported (Ye et al., 2009). Stably transfected HepG2 cells were seeded in 96-well plates (2000 cells/well) and MTT (1 mg/ml of final concentration, Amresco Inc., USA) was added to each well at a fixed time every day. The culture plates were incubated for 4 hours (hrs) at 37^{o}C and 150 μl of dimethyl sulfoxide (DMSO) were applied to dissolve the blue formazan crystals. The optical density of solution was measured by absorbance spectrometry at 490 nm using a microtiter plate reader (Synergy 2, BioTek, USA).

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Colony formation assay in soft agar. Soft agar plates were prepared in 35-mm culture dishes with 1 ml of a bottom layer of 0.6% (V/V) agar (Sigma, USA) in DMEM containing 10% FBS. Stably transfected HepG2 cells were trypsinized into single cell suspension and 2000 cells were resuspended with 1 ml of top agarose layer containing 0.7 % (V/V) agarose in DMEM with 10% FBS. The plates were then cultured under normal conditions for 3 weeks continuously (Huynh, 2004). The colonies larger than 50 μm were counted from the whole plates under the microscope after the cells in the plates were stained with 0.5 ml of 0.005% crystal violet.

EdU-incorporation assay and cell cycle re-entry assay. For cell proliferation assay, stably or transiently transfected HepG2 cells were seeded in 96-well plates (1×10^4 cells/well) for 10 hrs and were incubated with 50 μM of 5-ethynyl-2-deoxyuridine (EdU, RiboBio Co. LTD, Guangzhou, China) for another 3 hrs according to the manufacturer's instructions. The nuclei were stained with Hoechst 33342 (RiboBio Co. LTD, Guangzhou, China). Micrographs were taken randomly from at least six different fields in each 96-well with a conventional inverted fluorescent microscope. The numbers EdU positive cells as well as total cells (determined by Heochst staining) in all fields were counted and the percentage of EdU positive cells (representing proliferating cells) was calculated.

For cell cycle re-entry assay, stably transfected HepG2 cells were seeded in 6-well plates (2×10^4 cells/well) and starved with DMEM without serum for 48 hrs. The cultures were then replaced with fresh DMEM plus 10% FBS to allow cells re-entering into cell cycle. HepG2 cells were harvested at various time points (8, 12 or 16 hrs), fixed in ice-cold 70% ethanol at 4^oC overnight, washed with PBS and re-suspended with the binding buffer containing 0.2% (V/V) Triton X-100 and 1% (W/V) bovine serum albumin (BSA) in PBS at a final density of 1×10^5 cells/ml. Subsequently, RNase at a final concentration of 1 μg/μl was added and cells were stained with propidium iodide (PI, 0.1mg/ml,

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Sigma, USA) for 15 min. All cells were subjected to fluorescent-activated cell sorting (FACS) analysis in triplicate on a FACSCalibur system (Becton Dickinson, USA).

Tumor xenograft in nude mice. All animal experiments were performed under the approval of the HUST Animal Welfare Committee. The protocol and procedures were approved by the Ethics Committee of HUST and performed with humane care. Female BALB/c nude mice (four to five-week-old and 10-12 g of body weight) were purchased from Beijing HFK Bioscience Company, LTD (Beijing, China) and housed in specific pathogen free (SPF) barrier system of animal centre, Union hospital, HUST. Stably transfected parental HepG2 cells (2×10^6 cells in 200 μl of PBS) were injected subcutaneously into both flanks of mice (Wang et al., 2010). Tumor volume was measured with calipers at 2 or 3 days interval and the tumor volume was calculated by using the following formula: tumor volume=$0.5\times\text{length}\times\text{width}^2$ (Deng et al., 2010). Mice were sacrificed one month later and tumors were removed completely, weighed and photographed.

Glutathione S-transferase (**GST) pull-down.** Equal amount of p-GST-c-Raf-1 was co-transfected with p-Ngb-EGFP-N1 wide type (WT) or its mutants in HepG2 cells and whole cell lysate (WCL) was extracted with GST-lysis buffer (150 mM NaCl, 10 mM HEPES (pH 7.45), 1% Nonident P-40, 5 mM Na Pyrophosphate, 5 mM NaF, 2 mM Na Orthovanadate, 10 mg/L Aprotinin, 10 mg/L Leupeptin and 1 mM PMSF) two days after transfection. After centrifugation, four hundreds of total soluble proteins were incubated with 40 μl of GST agarose-beads (Thermo Fisher Scientific Inc, USA) for 4 hr at cold room with gentle rotation. After extensive washing with GST-lysis buffer, the immunoprecipitates were subejected with Western blotting analysis with anti-GST or anti-GFP antibody (Santa Cruz Biotechnology, USA).

Statistical analysis. For animal experiments, 8 animals/group were randomly selected. Cell

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experiments were repeated for at least 3 times independently. Categorical data were evaluated with Chi-square and Fisher's exact test. The weight and volume of xenografted tumor were evaluated by Mann-Whitney test. Other values were expressed as mean±SEM and evaluated with a 2-way ANOVA followed by the Student-Newman-Keuls test. *p* value less than 0.05 was considered statistically significant.

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Results

Ngb is down-regulated in HCC. The expression of Ngb in human HCC tissues was examined by IHC. Fig. 1A and 1B showed that the Ngb antibody we used recognized Ngb protein specifically in Western blot analysis and fluorescent cytoimmunostaining assay. Using lung cancer as positive control (Emara et al., 2010; Oleksiewicz et al., 2011), the up-regulation of Ngb in human lung cancer was verified by IHC (Fig. 1C). Fig. 1D showed the representative micrographs of IHC from normal human liver and three different HCC samples with adjacent non-tumor tissues. Clearly, the intensity of Ngb staining in tumor tissues (T) was much lower than that of their adjacent non-tumor tissues (ANT). The percentage of Ngb-positive tumor tissues (22/60 cases, 36.7%) was significantly decreased as compared to that of Ngb-positive adjacent non-tumor tissues (36/48 cases, 75.0%) and normal liver tissues (20/20, 100%) (Fig. 1E). Further, the expression levels of Ngb mRNA in HCC (17 cases) were measured by conventional (inserted graphs, Fig. 1F) and quantitative RT-PCR (Fig. 1F). Statistic analysis demonstrated that the relative level of Ngb mRNA in tumor tissue (T) was significantly decreased than that of corresponding adjacent non-tumor tissues (Fig. 1F). These data established a negative relationship between Ngb expression level and HCC.

Knock-down of Ngb promotes HCC cell growth in vitro and tumor growth in vivo. The apparent low expression of Ngb in HCC prompted us to investigate the functional role of Ngb knock-down in HCC cell growth. Endogenous Ngb in HepG2 cells was knocked down by RNA interfering technique. Results of qPCR (left upper panel, Fig. 2A), conventional RT-PCR (left lower panel, Fig. 2A) and Western blot (right panels) confirmed that the expression level of Ngb was significantly decreased in human HepG2 cells stably transfected with the p-Ngb-shRNA-Genesil-1 plasmids (sh-Ngb) as compared to that of negative control cells (N-con, transfected with the

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non-specific plasmids). MTT assay demonstrated that knock-down of Ngb (sh-Ngb) significantly enhanced HepG2 cell viability (representing total cell number) in the culture as compared to that of corresponding negative controls (N-con) (Fig. 2B), suggesting that the loss of Ngb promoted HCC cell growth. We further determined the effect of Ngb knock-down on tumor growth *in vivo*. Subcutaneous injection of parental HepG2/sh-Ngb cells (stably expressing sh-Ngb) developed much larger tumor xenograft in the nude mouse (left side, Fig. 2C) than the tumor xenograft derived from same amounts of parental HepG2/N-con cells (right side, Fig. 2C) 30 days after cell injection. Statistical analysis demonstrated that the tumor volume derived from HepG2/sh-Ngb cells was significantly increased as compared to that derived from HepG2/N-con cells in the consecutive measurement for 30 days (Fig. 2D). The weight of tumor derived from HepG2/sh-Ngb cells was also significantly heavier than that derived from HepG2/N-con control cells (Fig. 2E). The reduction of Ngb in tumor xenograft derived from HepG2/sh-Ngb cells was confirmed by RT-PCR (inserted graphs, Fig. 2E). These data demonstrated that Ngb down-regulation promoted HepG2 cell growth *in vitro* and *in vivo*.

Overexpression of Ngb suppresses HCC cell growth in vitro and tumor growth in vivo. After establishing a causative relationship between the loss of Ngb and HCC cell growth, we then asked whether increasing Ngb artificially might suppress HCC cell growth. HepG2 cells were stably transfected with p-Ngb-EGFP-N1 or p-EGFP-N1 plasmids and the overexpression of Ngb in HepG2/Ngb cells (stably overexpressing Ngb) was verified by Western blot analysis (Fig. 3A) and RT-PCR (Fig. 3B). MTT assay demonstrated that overexpressing Ngb significantly reduced HepG2 cell viability (representing total cell number) as compared to its corresponding vector control cells (GFP) along the 5 days of cell growth in the culture (Fig. 3C). We further investigated the effect of

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Ngb overexpression on anchorage-independent growth of HCC cells, a characteristic phenotype of malignant transformed cells *in vitro*. Colony formation assay in soft agar showed that overexpressing Ngb evidently reduced HepG2 cell colonies as compared to its vector control (Fig. 3D). Statistic analysis demonstrated that the colony number derived from parental HepG2/Ngb cells (190.5±40.3 colonies/dish) were significantly less than that derived from same amount of parental HepG2/vector cells (582.0±113.8 colonies/dish) (Fig. 3E). The effect of Ngb overexpression on HepG2 tumor growth *in vivo* was further studied by subcutaneous injection of same amounts of HepG2/Ngb or HepG2/vector cells into either side of the same nude mouse. Four weeks later, the tumor derived from parental HepG2/Ngb cells (Ngb, left side, Fig. 3F) was evidently smaller than that derived from parental HepG2/GFP cells (GFP, right side, Fig. 3F). The growth kinetic curve of HepG2/Ngb tumors was compared to that of HepG2/GFP tumors, which showed a significant reduced growth speed in HepG2/Ngb tumors (Fig. 3G). Finally, the weight of tumors derived from HepG2/Ngb cells was significantly decreased as compared to that derived from HepG2/GFP cells (Fig. 3H). The overexpression of Ngb in tumors derived from HepG2/Ngb cells was confirmed by Western blot analysis (inserted blots, Fig. 3H). These data demonstrated an inhibitory effect of Ngb on HCC cell growth *in vitro* and *in vivo*.

Ngb suppresses proliferation and G1/G0-S transition in HCC cells. Since Ngb controlled HCC cell growth, we further analyzed the functional role of Ngb in HepG2 cell division and cell cycle. EdU incorporation assay showed that overexpressing Ngb reduced EdU-positive stained cells (Fig. 4A and 4B). The average percentage of EdU-positive HepG2/Ngb cells was significantly reduced as compared to that of EdU-positive HepG2/GFP cells (Fig. 4B). On the contrary, knock-down of Ngb (sh-Ngb) significantly increased the percentage of EdU-positive HepG2 cells as compared to its

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control (N-con, Fig. 4C and 4D). These data suggested that the inhibitory effect of Ngb on HCC cell growth was a direct inhibitory effect of Ngb on cell proliferation.

We then studied the effect of Ngb overexpression or knock-down on HepG2 cell cycle. Synchronized HepG2 cells were induced to re-enter the cell cycle by serum supplementation. FACS analysis showed that Ngb overexpression reduced the percentage of HepG2 cells in the S phase (38.4% *vs* 49.2% of control), increased that of HepG2 cells in the G1/G phase (51% *vs* 41.1% of control) but did not alter the percentage of HepG2 cells in the G2/M phase after 12 hrs of serum stimulation (Fig. 4E). Statistic analysis demonstrated that the percentage of HepG2 cells in the S phase was significantly reduced in HepG2/Ngb cells as compared to its corresponding vector controls after 12 and 16 hrs of serum stimulation (Fig. 4F). On the contrary, knock-down of Ngb increased the percentage of HepG2/sh-Ngb cells in the S phase (32.9% *vs* 22.5% of control, 12 hrs), decreased the percentage of HepG2/sh-Ngb cells in the G1/G0 phase (55.7% *vs* 66.5%, 12 hrs) but did not alter the percentage of HepG2/sh-Ngb cells in the G2/M phase (Fig. 4G). Statistic analysis demonstrated that the percentage of HepG2/sh-Ngb cells in the S phase was significantly increased after 12 and 16 hrs of serum stimulation as compared to that of corresponding N-con (Fig. 4H). These data demonstrated that Ngb suppressed HepG2 cells entering into the S phase from the G1/G0 phase.

Ngb controls HCC cell proliferation via regulating the Raf/MEK/Erk signaling. To understand the molecular mechanisms by which Ngb controlled HCC cell growth or proliferation, we investigated the effect of Ngb on several key signal pathways, which are closely related to the cellular proliferation of HCC cells (Huynh, 2004; Newell et al., 2009). Western blot analysis (Fig. 5A) showed that Ngb had a prominent effect on Raf/MEK/Erk signaling cascades but had no effect on GSK-3β or β-catenin signaling in HepG2 cells under normal culture conditions. Overexpression of Ngb evidently

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reduced the expression levels of p-Raf-1 Ser338, p-MEK and p-Erk Thr202/Tyr204 in HepG2 cells but did not altered total Erk and Raf-1 levels as compared to its GFP controls (Fig. 5A). On the contrary, knock-down of Ngb (sh-Ngb) clearly increased the expression levels of p-Erk Thr202/Tyr204, p-MEK and p-Raf-1 Ser338/Raf-1 but did not alter the expression levels of total Erk and Raf-1 in HepG2 cells as compared to its N-con (Fig. 5A). In HepG2 cell-derived tumor xenografts, activation of Erk was either enhanced by Ngb knock-down (Fig. 5B) or suppressed by Ngb overexpression (Fig. 5C) as compared to their corresponding controls. To explore the possible mechansim by which Ngb may regulate Raf/MEK/Erk cascade, the interaction of Ngb and c-Raf-1 was examined. GST pull-down assay showed that Ngb bound with c-Raf-1 and mutation of Ngb at its oxygen-bnding site (H64L) altered their interactions (Fig. 5D). Further, we investigated the effects of Ngb on Erk signaling upon serum stimulation (Fig. 6A) and H_2O_2 treament (Fig. 6B), which mimic physilogical and pathological microenvironments. Western blot analysis showed that overexpression of Ngb suppressed Erk activation under both stimulants (Fig. 6A and 6B). These *in vitro* and *in vivo* data together demonstrated that Ngb negatively regulated the oncogenic Raf-1/MEK/Erk signaling in HepG2 cells. Finally, we demonstrated that Erk activation was responsible for Ngb-mediated HCC cell proliferative. Pharmacological inhibition of Raf-1/MEK/Erk signaling by using the MEK specific inhibitor U0126 decreased the p-Erk levels prominently in both HepG2/N-con and HepG2/sh-Ngb cells (Fig. 6C). Simultaneously, the proliferative effect of Ngb knock-down in HepG2 cells was abolished as determined by the EdU incorporation assay (Fig. 6D). Therefore, oncogenic Raf-1/MEK/Erk pathway was a down-stream target of Ngb signaling in HCC cells.

Ngb regulates HCC cell proliferation by linking oxygen/ROS signals to Erk signaling. Present evidence suggests that Ngb plays a major role in sensing oxygen or ROS signals (Trent et al., 2001;

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Herold et al., 2004; Burmester et al., 2009). Our data also showed that Ngb suppressed H_2O_2-induced Erk activation in HepG2 cells. To link Ngb's ability in sensing oxygen/ROS signal directly to Erk signaling and HCC cell proliferation, the effect of Ngb mutants, including its oxygen/NO binding site (i.e., H64L) (Nienhaus et al., 2004; Vallone et al., 2004) and G-protein binding site (i.e., E53Q and E118Q) (Wakasugi et al., 2003), on Erk activation and HCC cell proliferation were investigated. Western blot analysis showed that Ngb^{H64L} reversed the inhibitory effect of wide type (WT) Ngb on Erk activation while mutation of Ngb^{E53Q} and Ngb^{E118Q} had no effect (Fig. 7A and 7B). Consistently, mutation of Ngb at its oxygen/ROS binding site but not G-protein binging site abolished the inhibitory effect of Ngb^{WT} on HepG2 cell proliferation (Fig. 7C). This evidence supported that Ngb controlled HCC proliferation by linking oxygen/ROS signals to Erk signaling cascades.

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Discussion

In the present study, we reported that the loss of Ngb was associated to HCC development. We demonstrated a causative role of Ngb in HCC cell growth and proliferation *in vitro* and *in vivo* by the gain of function or knock-down of Ngb. Moreover, we identified a novel Ngb-regulated signaling pathway, i.e., Raf/MEK/Erk, which controls HCC cell proliferation. Further, our data suggest that Ngb is a molecular linker between oxygen/ROS signals and Raf/MEK/Erk cascade.

Recently, Ngb is found to be up-regulated in many tumors (Emara et al, 2010), however, this up-regulation is challenged by others (Gorr et al, 2011). Our finding that Ngb is expressed in normal liver tissues is consistent to Gorr's result (2011). However, we demonstrated that Ngb was significantly down-regulated in HCC tissues. The inconsistence of our data with that of Gorr's results (2011) may be caused by the difference of human tissue samples and the amount of tissue samples used.

Hypoxia and oxidative stress are initial or stimulatory factors in the process of cancer genesis or development (Severi et al., 2010). However, the mechanisms underlying these processes are far from understood. Presently, HIF1α is considered to be the major oxygen sensor which can certainly sense hypoxic signal and functions as a transcriptional factor in various tumor cells (Dewhirst et al., 2008). Ngb is the first hexa-coordinated oxygen-binding protein originally identified in mammalian brains and is considered to be a respiratory protein (Burmester et al., 2000). Although oxygen binds to Ngb with higher affinity as compared to hemoglobin (Hb), it is difficult for oxygen to be disassociated from Ngb (Trent et al., 2001). Therefore, it is unlikely for Ngb to function mainly as an oxygen supplier as Hb or myoglobin does. Since Ngb has a native oxygen-binding property, it can serve as an intracellular oxygen sensor. Indeed, the expression of Ngb in brain cells is sensitive to severe ischemic

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or hypoxic conditions (Sun et al., 2001; Chen et al., 2005b). In tumor cells, the expression of Ngb could be either increased (Emara et al., 2010) or decreased (Oleksiewicz et al., 2011), suggesting a complicated regulation of Ngb in tumor cells. The increase of Ngb might represent the mechanism of hypoxic response of Ngb (Sun et al., 2001) while the decrease of Ngb might be caused by the hypermethylation of its promoter (Oleksiewicz et al., 2011). Hypermethylation of cytoglobin (Cygb) promoter, another hexa-coordinated member of Hb families, is also found in several malignant tumors (Shivapurkar et al., 2008). The decrease of Ngb in HCC might be caused by the hypermethylation mechanism of its promoter during prolonged hypoxia. Further investigating the status Ngb promoter hypermethylation will elucidate the mechanism by which Ngb was down-regulated in HCC.

The altered expression of Ngb in tumor cells suggests a functional role of Ngb in cancer genesis or development, however, direct evidence demonstrating its function is missing. Here, we demonstrated that the decrease of Ngb promoted HCC cell growth (Fig. 2) and proliferation (Fig. 4D and 4H) while the increase of Ngb inhibited HCC cell growth (Fig. 3) and proliferation (Fig. 4B and 4F) *in vitro* and *in vivo*. These data clearly showed a negative causative relationship between the expression level of Ngb and HCC. Previous studies have shown a protective role of Ngb upon ischemic death in primary cultures of neurons (Sun et al., 2001) and astrocytes (Chen et al., 2005b). However, administrating Ngb directly to human neuronal SH-SY5Y cell line does not confer protection from oxygen and glucose deprivation (OGD) (Peroni et al., 2007). These evidences indicate that the biological functions of Ngb in distinct cellular contexts or tissues could be different. In malignant tumor cells, the energy metabolism pattern and key signaling transduction pathways are altered fundamentally as a result of chronic hypoxia or oxidative stress. It is likely that the pathological functions of Ngb in malignant tumor cells are different form that in brain cells. Whether Ngb plays a specific inhibitory

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role in HCC cell growth or proliferation or has a similar function in other tumor cells deserves further investigations.

Ngb binds not only to oxygen but also to signaling proteins, such as the alpha subunit of heterotrimeric G protein (Gαi) (Wakasugi et al., 2003), 14-3-3 protein (Jayaraman et al., 2011) or cytochrome C (Raychaudhuri et al., 2010). The interaction of Ngb with Gαi/14-3-3/cytochrome C is controlled by hypoxic conditions (i.e., cellular oxygen signals) although the exact working model and function remain unclear. These evidences suggest that Ngb may function as a signaling molecule by coupling oxygen/ROS signals to intracellular signaling cascades. We found that Ngb interacted with c-Raf-1 and regulated the oncogenic Raf/MEK/Erk pathway in HCC in vitro and in vivo (Fig. 5). Blocking Erk signaling abolished the effect of Ngb knock-down on HCC cell proliferation (Fig. 6). Therefore, Raf/MEK/Erk was a down-stream signaling pathway of Ngb in HCC cells. We have previously observed an early and robust activation of the Raf/MEK/Erk pathway in the primary cultures of cerebral cortical astrocytes but not in the primary cultures of cerebral cortical neurons upon OGD (Chen et al., 2005a). This suggests that the Raf/MEK/Erk signaling is not only sensitive to hypoxic signal but also specific to the cell type. Hypoxia is a common pathological factor in tumor cells and the Raf/MEK/Erk pathway is a well-known oncogenic proliferative signaling pathway in various tumors including HCC (Huynh, 2004; Newell et al., 2009). The distal histidine of Ngb (i.e., His^{64}) is the binding site of oxygen. In vitro studies reveal that Ngb mutation at this site alters its oxygen-binding affinity (Vallone et al., 2004) although its physiological importance is unknown. We found that H64L mutation affected the binding of Ngb with c-Raf-1 (Fig. 5D), abolished the inhibitory effects of Ngb^{WT} on Erk activation and HCC proliferation (Fig. 7), providing a direct link between oxygen sensing and Raf/MEK/Erk signaling. Based on these finding, we proposed a novel hypoxic

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signaling pathway in HCC cells, i.e., O_2/Ngb/Raf/MEK/Erk, which links oxygen signal directly to the oncogenic signaling pathway. In addition, Ngb may regulate Raf/MEK/Erk activity via other proteins such as 14-3-3 proteins (Zhao et al., 2011), suggesting a complicated regulatory machinery of Ngb on Raf/ME/Erk cascades. It is known that p21-activated kinase (PAK) can phosphorylate Raf-1 at Ser338 and regulates Raf-1 activity positively (King et al., 1998). It is likely that the interaction of Ngb with Raf-1 may modulate Raf-1 phosphorylation and activity by PAK. Further dissecting this pathway may provide insights in the understanding of the role of hypoxia in carcinogenesis and cancer development. It may also provide potential therapeutic targets for HCC.

Ngb is not only an oxygen-binding protein but also a NO-binding protein (Brunori et al., 2005). Altering the hexa-coordinated feature of Ngb to a penta-coordinated property greatly enhances its reductive activity for nitrite (Tiso et al., 2011). We have previously reported that endogenous Ngb is required for protecting neuronal cells from H_2O_2-induced damage (Ye et al., 2009). In the present study, we also demonstrated that Ngb suppressed H_2O_2-induced Erk activation in HepG2 cells. These evidences support an important role of Ngb in oxidative stress, probably as an oxidative stress sensor or ROS scavenger (Herold et al., 2004; Brunori et al., 2005; Burmester et al., 2009; Antao et al., 2010). Oxidative stress is a critical mechanism in HCC genesis or development. It is likely that Ngb may exert its biological functions through mediating oxidative stress in the process of HCC development. Recent study has shown that Ngb overexpression could alter the spectrum of gene expression in neurons upon ischemic conditions (Yu et al., 2009). We have also reported previously that Ngb alters the expression level of 14-3-3gamma protein in neuronal cells (Ye et al., 2009). 14-3-3 protein are signaling hubs among intracellular signaling networks and are heavily involved in cell survival/death and growth/proliferation in tumor cells (Zhao et al., 2011). It is interesting to investigate whether Ngb

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may regulate gene expression in HCC cells as an oxidative stress sensor or scavenger in order to explore the role of Ngb in hepatocarcinogenesis. Further analyzing the relationships between Ngb expression and HCC clinical data (i.e., staging, grading, the survival time) together with other biomarkers such as α-fetoprotein and HIF-1 is valuable to evaluate whether Ngb could be used as a prognosis predicting biomarker or not. Since Ngb plays an important role in hypoxia/ROS-mediated signaling which is essential for HCC development, manipulating Ngb expression or targeting to Ngb-mediated signaling might provide novel therapeutic approaches for advanced HCC.

In summary, we found that the loss of Ngb was associated HCC development via suppressing Raf/MEK/Erk. Our data first suggested that Ngb, an intracellular respiratory protein, might function as a tumor suppressor in HCC by coupling oxygen/ROS signals to the oncogenic Raf/MEK/Erk signaling.

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Authorship Contributions

Participated in research design: Zhang and Chen.

Conducted experiments: Zhang, Lan, QR Liu and JM Liu.

Performed data analysis: Zhang and Chen.

Wrote or contributed to the writing of the manuscript: Zhang and Chen

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This article has not been copyedited and formatted. The final version may differ from this version.

References

Antao ST, Duong TT, Aran R, Witting PK (2010) Neuroglobin overexpression in cultured human neuronal cells protects against hydrogen peroxide insult via activating phosphoinositide-3 kinase and opening the mitochondrial K_{ATP} channel. *Antioxid Redox Signal* **13**: 769-781.

Aravalli RN, Steer CJ, Cressman EN (2008) Molecular mechanisms of hepatocellular carcinoma. *Hepatology* **48**: 2047-2063.

Brunori M, Giuffre A, Nienhaus K, Nienhaus GU, Scandurra FM, Vallone B (2005) Neuroglobin, nitric oxide, and oxygen: functional pathways and conformational changes. *Proc Natl Acad Sci USA* **102**: 8483-8488.

Burmester T, Hankeln T (2009) What is the function of neuroglobin? *J Exp Biol* **212**: 1423-1428.

Burmester T, Weich B, Reinhardt S, Hankeln T (2000) A vertebrate globin expressed in the brain. *Nature* **407**: 520–523.

Chen XQ, Fung YW, Yu AC (2005a) Association of 14-3-3gamma and phosphorylated bad attenuates injury in ischemic astrocytes. *J Cereb Blood Flow Metab* **25**: 338-347.

Chen XQ, Qin LY, Zhang CG, Yang LT, Gao Z, Liu S, Lau LT, Fung YW, Greenberg DA, Yu AC (2005b) Presence of neuroglobin in cultured astrocytes. *Glia* **50**: 182-186.

Deng Q, Wang Q, Zong WY, Zheng DL, Wen YX, Wang KS, Teng XM, Zhang X, Huang J, Han ZG (2010) E2F8 contributes to human hepatocellular carcinoma via regulating cell proliferation. *Cancer Res* **70**: 782-791.

Dewhirst MW, Cao Y, Moeller B (2008) Cycling hypoxia and free radicals regulate angiogenesis and radiotherapy response. *Nat Rev Cancer* **8**: 425-437.

El-Serag HB, Rudolph KL (2007) Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* **132**: 2557–2576.

Emara M, Turner AR, Allalunis-Turner J (2010) Hypoxic regulation of cytoglobin and neuroglobin expression in human normal and tumor tissues. *Cancer Cell Int* **10**: 33.

Gorr TA, Wichmann D, Pilarsky C, Theurillat JP, Fabrizius A, Laufs T, Bauer T, Koslowski M, Horn S, Burmester T, Hankeln T, Kristiansen G (2011) Old proteins - new locations: myoglobin, haemoglobin, neuroglobin and cytoglobin in solid tumours and cancer cells. *Acta Physiol (Oxf)* **202**: 563-581.

Herold S, Fago A, Weber RE, Dewilde S, Moens L (2004) Reactivity studies of the Fe(III) and Fe(II)NO forms of human neuroglobin reveal a potential role against oxidative stress. *J Biol Chem* **279**: 22841–22847.

Huynh H (2004) Overexpression of tumour suppressor retinoblastoma 2 protein (pRb2/p130) in hepatocellular carcinoma. *Carcinogenesis* **25**: 1485-1494.

Jayaraman T, Tejero J, Chen BB, Blood AB, Frizzell S, Shapiro C, Tiso M, Hood BL, Wang X, Zhao X, Conrads TP, Mallampalli RK, Gladwin MT (2011) 14-3-3 binding and phosphorylation of neuroglobin during hypoxia modulate six-to-five heme pocket coordination and rate of nitrite reduction to nitric oxide. *J Biol Chem* **286**: 42679-42689.

King AJ, Sun H, Diaz B, Barnard D, Miao W, Bagrodia S, Marshall MS (1998) The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. *Nature* **396**:180-183.

Newell P, Toffanin S, Villanueva A, Chiang DY, Minguez B, Cabellos L, Savic R, Hoshida Y, Lim KH, Melgar-Lesmes P, Yea S, Peix J, Deniz K, Fiel MI, Thung S, Alsinet C, Tovar V, Mazzaferro V, Bruix J, Roayaie S, Schwartz M, Friedman SL, Llovet JM (2009) Ras pathway activation in hepatocellular carcinoma and anti-tumoral effect of combined sorafenib and rapamycin in vivo. *J Hepatol* **51**: 725-733.

Nienhaus K, Kriegl JM, Nienhaus GU (2004) Structural dynamics in the active site of murine neuroglobin and its effects on ligand binding. *J Biol Chem* **279**: 22944–22952.

Oleksiewicz U, Daskoulidou N, Liloglou T, Tasopoulou K, Bryan J, Gosney JR, Field JK, Xinarianos G (2011) Neuroglobin and myoglobin in non-small cell lung cancer: expression, regulation and prognosis. *Lung Cancer* **74**: 411-418.

Peroni D, Negro A, Bähr M, Dietz GP (2007) Intracellular delivery of Neuroglobin using HIV-1 TAT protein transduction domain fails to protect against oxygen and glucose deprivation. *Neurosci Lett* **421**: 110-114.

Pollicino T, Saitta C, Raimondo G (2011) Hepatocellular carcinoma: the point of view of the hepatitis B virus. *Carcinogenesis* **32**: 1122-1132.

Raychaudhuri S, Skommer J, Henty K, Birch N, Brittain T (2010) Neuroglobin protects nerve cells from apoptosis by inhibiting the intrinsic pathway of cell death. *Apoptosis* **15**: 401-411.

Sakurai T, He G, Matsuzawa A, Yu GY, Maeda S, Hardiman G, Karin M (2008) Hepatocyte necrosis

induced by oxidative stress and IL-1 alpha release mediate carcinogen-induced compensatory proliferation and liver tumorigenesis. *Cancer Cell* **14**: 156–165.

Severi T, van Malenstein H, Verslype C, van Pelt JF (2010) Tumor initiation and progression in hepatocellular carcinoma: risk factors, classification, and therapeutic targets. *Acta Pharmacol Sin* **31**: 1409-1420.

Shivapurkar N, Stastny V, Okumura N, Girard L, Xie Y, Prinsen C, Thunnissen FB, Wistuba II, Czerniak B, Frenkel E, Roth JA, Liloglou T, Xinarianos G, Field JK, Minna JD, Gazdar AF (2008) Cytoglobin, the newest member of the globin family, functions as a tumor suppressor gene. *Cancer Res* **68**: 7448-7456.

Sun Y, Jin K, Mao XO, Zhu Y, Greenberg DA (2001) Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury. *Proc Natl Acad Sci USA* **98**: 15306-15311.

Tiso M, Tejero J, Basu S, Azarov I, Wang X, Simplaceanu V, Frizzell S, Jayaraman T, Geary L, Shapiro C, Ho C, Shiva S, Kim-Shapiro DB, Gladwin MT (2011) Human neuroglobin functions as a redox-regulated nitrite reductase. *J Biol Chem* **20**: 18277–18289.

Trent JT 3rd, Watts RA, Hargrove MS (2001) Human neuroglobin, a hexacoordinate hemoglobin that reversibly binds oxygen. *J Biol Chem* **276**: 30106-30110.

Vallone B, Nienhaus K, Brunori M, Nienhaus GU (2004) The structure of murine neuroglobin: Novel pathways for ligand migration and binding. *Proteins* **56**: 85–92.

Wakasugi K, Nakano T, Morishima I (2003) Oxidized human neuroglobin acts as a heterotrimeric Gα protein guanine nucleotide dissociation inhibitor. *J Biol Chem* **278**: 36505-36512.

Wang YC, Chen YL, Yuan RH, Pan HW, Yang WC, Hsu HC, Jeng YM (2010) Lin-28B expression promotes transformation and invasion in human hepatocellular carcinoma. *Carcinogenesis* **9**: 1516–1522.

Whittaker S, Marais R, Zhu AX (2010) The role of signaling pathways in the development and treatment of hepatocellular carcinoma. *Oncogene* **29**: 4989–5005.

Ye SQ, Zhou XY, Lai XJ, Zheng L, Chen XQ (2009) Silencing neuroglobin enhances neuronal vulnerability to oxidative injury by down-regulating 14-3-3gamma. *Acta Pharmacol Sin* **30**: 913-918.

Yu Z, Liu J, Guo S, Xing C, Fan X, Ning M, Yuan JC, Lo EH, Wang X (2009) Neuroglobin-overexpression alters hypoxic response gene expression in primary neuron culture following oxygen glucose deprivation. *Neuroscience* **162**: 396-403.

Molecular Pharmacology Fast Forward. Published on March 11, 2013 as DOI: 10.1124/mol.112.083634
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Zender L, Villanueva A, Tovar V, Sia D, Chiang DY, Llovet JM (2010) Cancer gene discovery in hepatocellular carcinoma. *J Hepatol* **52**: 921-929.

Zhao J, Meyerkord CL, Du Y, Khuri FR, Fu H (2011) 14-3-3 proteins as potential therapeutic targets. *Semin Cell Dev Biol* **22**: 705-712.

Zhu Y, Sun Y, Jin K, Greenberg DA (2002) Hemin induces neuroglobin expression in neural cells. *Blood* **100**: 2494–2498.

Molecular Pharmacology Fast Forward. Published on March 11, 2013 as DOI: 10.1124/mol.112.083634
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Footnotes

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Figure Legends

Fig. 1. Ngb is down-regulated in human HCC. (A) Representative results of Western blot showing the specificity of Ngb antibody. 293T cells were transiently transfected with either pcDNA-Ngb or control vector for 2 days. Equal amount of total proteins extracted from transfected cells were subjected for Western blot analysis with anti-Ngb antibody. β–actin was used as a loading control. (B) Representative results of fluorescent cytoimmunostaining showed that the Ngb antibody recognized Ngb specifically. HepG2 cells were transiently transfected with either p-Ngb-EGFP or p-EGFP plasmids for 2 days. Cells in the cultures were fixed for fluorescent cytoimmunostaining with anti-Ngb antibody (red). Transfected cells were viewed by GFP fluorescence. (C) Results of immunohistochemical stainging showed that Ngb was elevated in human lung cancer tissue but not in normal human lung tissue. (D) Representative results of immunohistochemical staining of Ngb in human HCC samples. The upper panels showed Ngb staining in normal human liver and HCC samples at lower magnifications (20X). The lower panels showed Ngb staining of another two HCC samples at higher magnifications (40X). T, tumor; ANT, adjacent non-tumor tissue. (E) Statistic analysis of IHC results of Ngb staining in human HCC samples. The percentage of Ngb-positive stained tumor (T) was compared to that of Ngb-positive adjacent non-tumor tissues (ANT) and normal liver tissues (Nor). ***$p<0.001$, Chi-square test. (F) Conventional (inserted graphs) and quantitative PCR results of Ngb in human HCC samples. The expression levels of Ngb mRNA in HCC tumor tissues (T) and their adjacent non-tumor tissues (ANT) were measured quantitatively and normalized to that of GAPDH. The relative Ngb expression level (Ngb/GPDH) in HCC tumor tissues (T) was significantly lower than that in adjacent non-tumor tissues (ANT). *$p<0.05$, paired student *t*-test, n=17.

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Fig. 2. Knock-down of Ngb promotes HCC cell growth *in intro* and *in vivo*. (A) Effect of Ngb knockdown in HepG2 cells. HepG2 cells were stably transfected with p-Ngb-shRNA-Genesil-1 plasmids (sh-Ngb) or non-specific control plasmids (N-con). The relative expression level of Ngb (Ngb/GAPDH or Ngb/β-actin) was examined by either qPCR (left upper panel, *p<0.05, n=3), conventional RT-PCR (left lower panel, representative results) or Western blot analysis (right panels). GAPDH or β-actin was used as an internal control. (B) Results of MTT assay. Cell viability (representing total cell number) of HepG2/sh-Ngb cells (sh-Ngb) or HepG2/N-con cells (N-con) was determined by MTT assay at the various time points (1, 2, 3, 4, and 5 days) after initial seeding (day 0) with same amount of cells in the cultures. *p<0.05 and **p<0.01 *vs* corresponding vector controls, n=5. (C) Representative tumor-bearing nude mouse and the xenografted tumor derived from either HepG2/sh-Ngb cells (left side) or HepG2/N-con cells (right side). Equal amount of HepG2/sh-Ngb or HepG2/N-con cells were injected subcutaneously into left or right flank of nude mice simultaneously. The tumor size was measured every 2 or 3 days and the animal was sacrificed at day 30. The tumors were removed completely from tissues and weighted. (D) Statistic analysis demonstrated that the tumor volume derived from HepG2/sh-Ngb cells (sh-Ngb) was significantly increased as compared to that derived from HepG2/N-con cells (N-con) at various time points. *p<0.05, **p<0.01, Mann-Whitney test, n=8. (E) Statistic analysis demonstrated that the weight of tumors derived from HepG2/sh-Ngb cells was significantly increased as compared to that derived from HepG2/N-con cells. **p<0.01, Mann-Whitney test, n=8. Inserted RT-PCR results showed the expression of Ngb in tumor derived from HepG2/sh-Ngb cells and its N-con.

Fig. 3. Overexpression of Ngb suppressed HCC cell growth *in vitro* and *in vivo*. Representative Western blot (A) and RT-PCR (B) results showed the expression level of Ngb in HepG2 cells stably transfected

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with p-Ngb-GFP-N1 (Ngb) or p-EGFP-N1 (GFP). All experiments were repeated for least three times. The relative level of Ngb (Ngb/β-actin) in Ngb-stably transsfected HepG2 cells (Ngb) was around 3 folds of that of GFP control cells. ** *P*<0.01 *vs* GFP control. (C) Results of MTT assay. Cell viability (representing total cell number) of HepG2/Ngb (Ngb) or HepG2/GFP (GFP) cells was determined by MTT assay at various time points (1, 2, 3, 4, and 5 days) after initial seeding (day 0) with same amount of cells in the cultures. **P*<0.05 and ***P*<0.01 *vs* corresponding GFP controls, n=5. (D) Representative results of colony formation in soft agar derived from same amounts of parental HepG2/Ngb or HepG2/GFP cells. The experiments were repeated for three times. (E) Statistic analysis demonstrated that the average number of HepG2/Ngb cell colonies (Ngb) was significantly reduced as compared to that of HepG2/GFP cell colonies (GFP). **p*<0.05, n=3. (F) Representative tumor-bearing nude mouse and the xenografted tumor derived from either HepG2/Ngb cells (left side) or HepG2/GFP cells (right side). (G) Statistic analysis demonstrated that the tumor volume derived from parental HepG2/Ngb cells (Ngb) was significantly decreased as compared to that of parental HepG2/GFP cells (GFP). **p*<0.05, ***p*<0.01 *vs* corresponding GFP controls, Mann-Whitney test, n=8. (H) Statistic analysis demonstrated that the tumor weight derived from HepG2/Ngb cells was significantly decreased as compared to that of HepG2/GFP controls. ***p*<0.01, Mann-Whitney test, n=8. Inserted Western blot showed that Ngb was overexpressed in tumors derived from HepG2/Ngb cells.

Fig. 4. Ngb prevents HCC cell proliferation and G0/G1-S transition. (A-B) Representative micrographs (A) and statistic analysis (B) of EdU incorporation assay demonstrated that overexpression of Ngb reduced cell proliferation in HepG2/Ngb cells. ***P*<0.01, n=4. (C-D) Representative micrographs (C) and statistic analysis (D) of EdU incorporation assay demonstrated that overexpression of sh-Ngb plasmids enhanced

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HepG2/shNgb cell proliferation. $^{**}p<0.01$, n=4. (E-F) Representative FACS results (E, 12 hrs of serum stimulation) and statistic analysis (F) demonstrated that Ngb overexpression (Ngb) increased the percentage of HepG2 cells in the G0/G1 phase but decreased the percentage of HepG2 cells in the S phase as compared to its GFP controls. $^{*}p<0.05$, n=3. (G-H) Representative FACS results (G, 12 hrs of serum stimulation) and statistic analysis (H) demonstrated that knocking-down Ngb (sh-Ngb) decreased the percentage of HepG2 cells in the G0/G1 phase but increased the percentage of HepG2 cells in the S phase as compared to its N-con. $^{**}p<0.01$, n=3.

Fig. 5. Ngb regulates Raf-1/MEK/Erk signaling and interacts with c-Raf-1. (A) Representative Western blot results showed that overexpression of Ngb (Ngb) decreased the expression levels of p-Erk 1/2, p-MEK and p-Raf-1 Ser338 while knockdown of Ngb (sh-Ngb) increased their expression level as compared to corresponding controls. The expression levels of p-GSK-3β/GSk-3β, p-β-catenin/β-catenin were not altered evidently. (B) Representative Western blot results showed that knocking-down of Ngb (sh-Ngb) enhanced Erk activation in tumor xenografts derived from HepG2/sh-Ngb cells as compared to its contorl tumor xenografts (N-con) from different nude mice (No. 1 and 2). (C) Representative Western blot results showed that overexpression of Ngb (Ngb) suppressed Erk activation in tumor xenografts derived from HepG2/Ngb cells as compared to its contorl tumor xenografts (GFP) from different nude mice (No. 1 and 2). (D) Representative results of GST pull-down showed that Ngb bound to c-Raf-1. WCL, whole cell lysate. IB, immunobloting.

Fig. 6. Ngb suppresses serum- and H_2O_2-stimulated Erk activation in HepG2 cells and controls HepG2 cell proliferation via Erk signaling. (A) Representative Western blot results showed that p-Erk was decreased in

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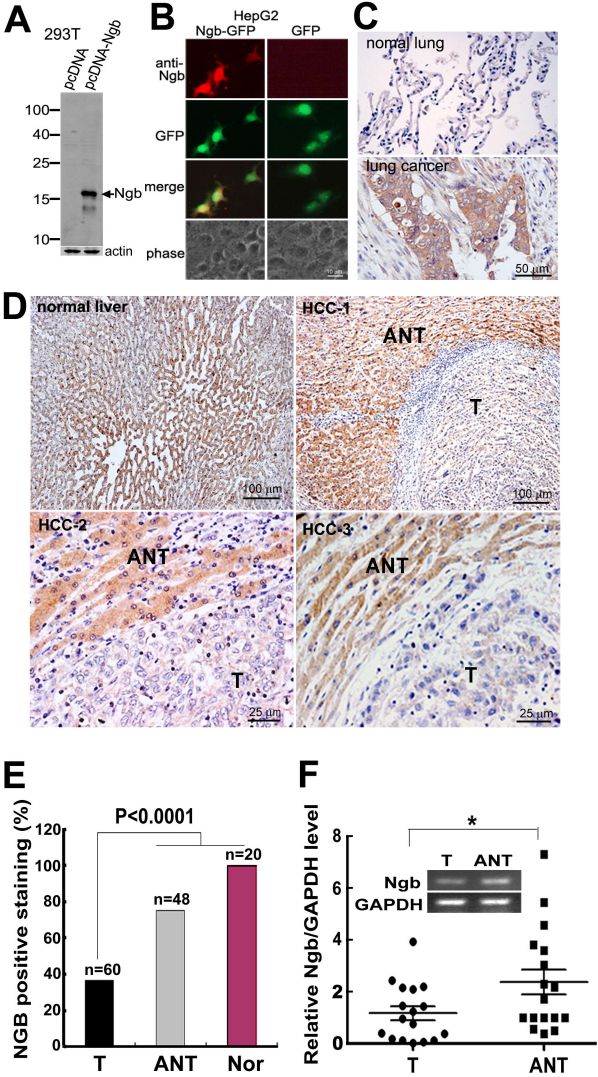
HepG2/Ngb cells upon serum supplementation as compared to its corresponding HepG2/GFP control cells at various time points. The experiment was repeated for three times. (B) Representative Western blot results showed that p-Erk was decreased in HepG2/Ngb cells upon 2 hr of H_2O_2 treatment at various concentrations as compared to its corresponding HepG2/GFP control cells. The experiment was repeated for three times. (C) Pharmacologically inhibition of Erk signaling by U0126 (1 μM) blocked the activation of Erk in HepG2 cells with or without Ngb knock-down. The experiments were repeated for three times. (D) Statistic analysis of EdU incorporation assay demonstrated that U0126 (1 μM) blocked the proliferative effect of HepG2/sh-Ngb cells. *p<0.05, n=3.

Fig. 7. Effects of Ngb mutants on Erk activation and HepG2 cell proliferation. (A) Representative Western blot results showed the effect of stably transfected Ngb mutations on Erk activation in HepG2 cells. The experiments were repeated for three times. (B) Statistic analysis of p-Erk/Erk ratio from Western blot results. *, p<0.05, n=3. (C) Effects of stably transfected Ngb mutants on HepG2 cell proliferation. *, p<0.05, n=3.

Figure 1


A
293T
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anti-Ngb
GFP
merge
phase
C
normal lung
lung cancer
50 μm
D
normal liver
100 μm
HCC-1
ANT
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100 μm
HCC-2
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T
25 μm
HCC-3
ANT
T
25 μm
E
P<0.0001
n=60
n=48
n=20
NGB positive staining (%)
120
100
80
60
40
20
0
T
ANT
Nor
F
*
T
ANT
Ngb
GAPDH
Relative Ngb/GAPDH level
8
6
4
2
0
T
ANT

Figure 2

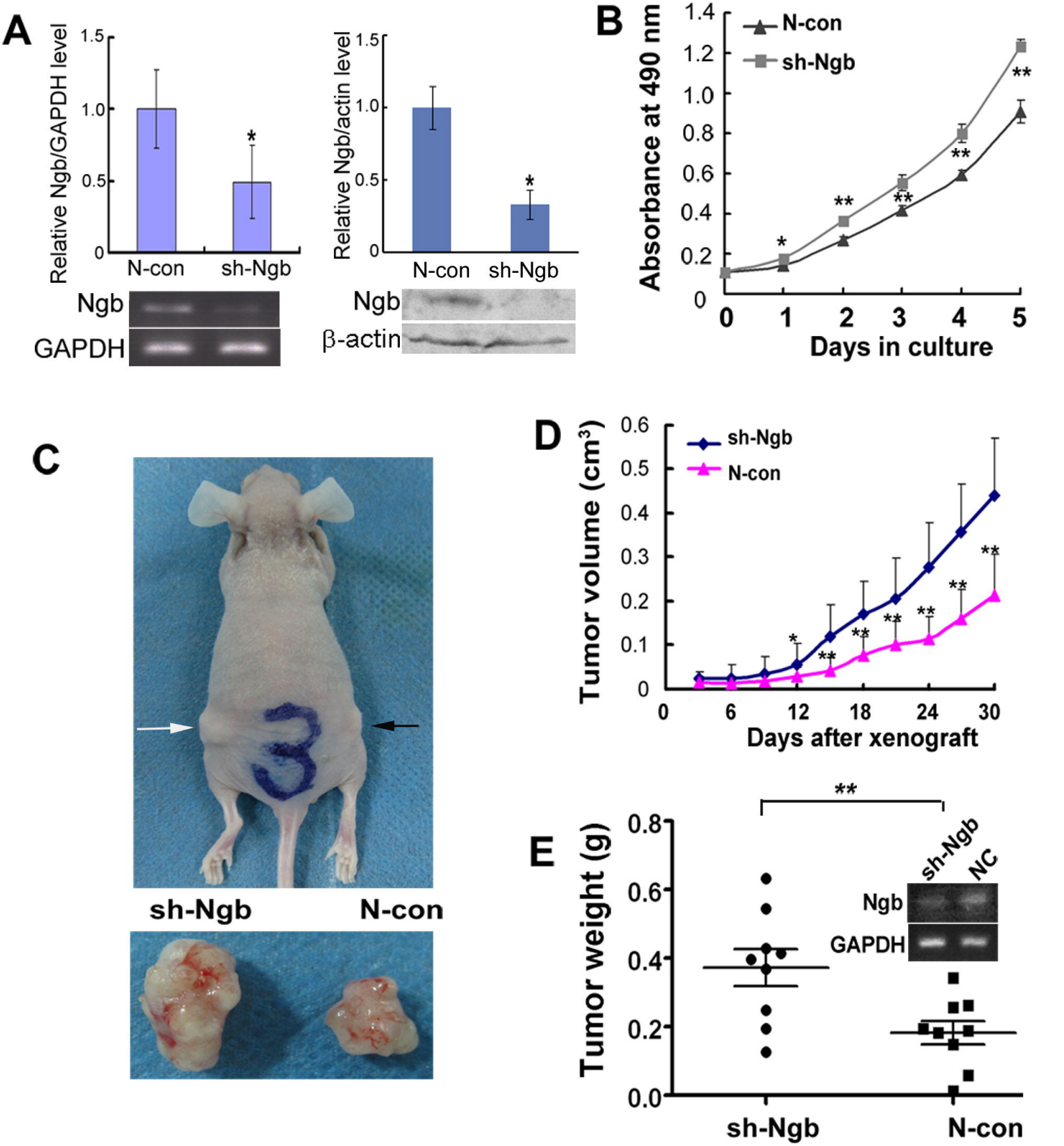

A
Relative Ngb/GAPDH level
1.5
1.0
0.5
0
*
N-con
sh-Ngb
Ngb
GAPDH
Relative Ngb/actin level
1.5
1.0
0.5
0
*
N-con
sh-Ngb
Ngb
β-actin
B
Absorbance at 490 nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
N-con
sh-Ngb
*
**
**
**
**
0
1
2
3
4
5
Days in culture
C
3
sh-Ngb
N-con
D
Tumor volume (cm³)
0.6
0.5
0.4
0.3
0.2
0.1
0
sh-Ngb
N-con
*
**
**
**
**
**
0
6
12
18
24
30
Days after xenograft
E
Tumor weight (g)
0.8
0.6
0.4
0.2
0.0
**
sh-Ngb
NC
Ngb
GAPDH
sh-Ngb
N-con

Figure 3

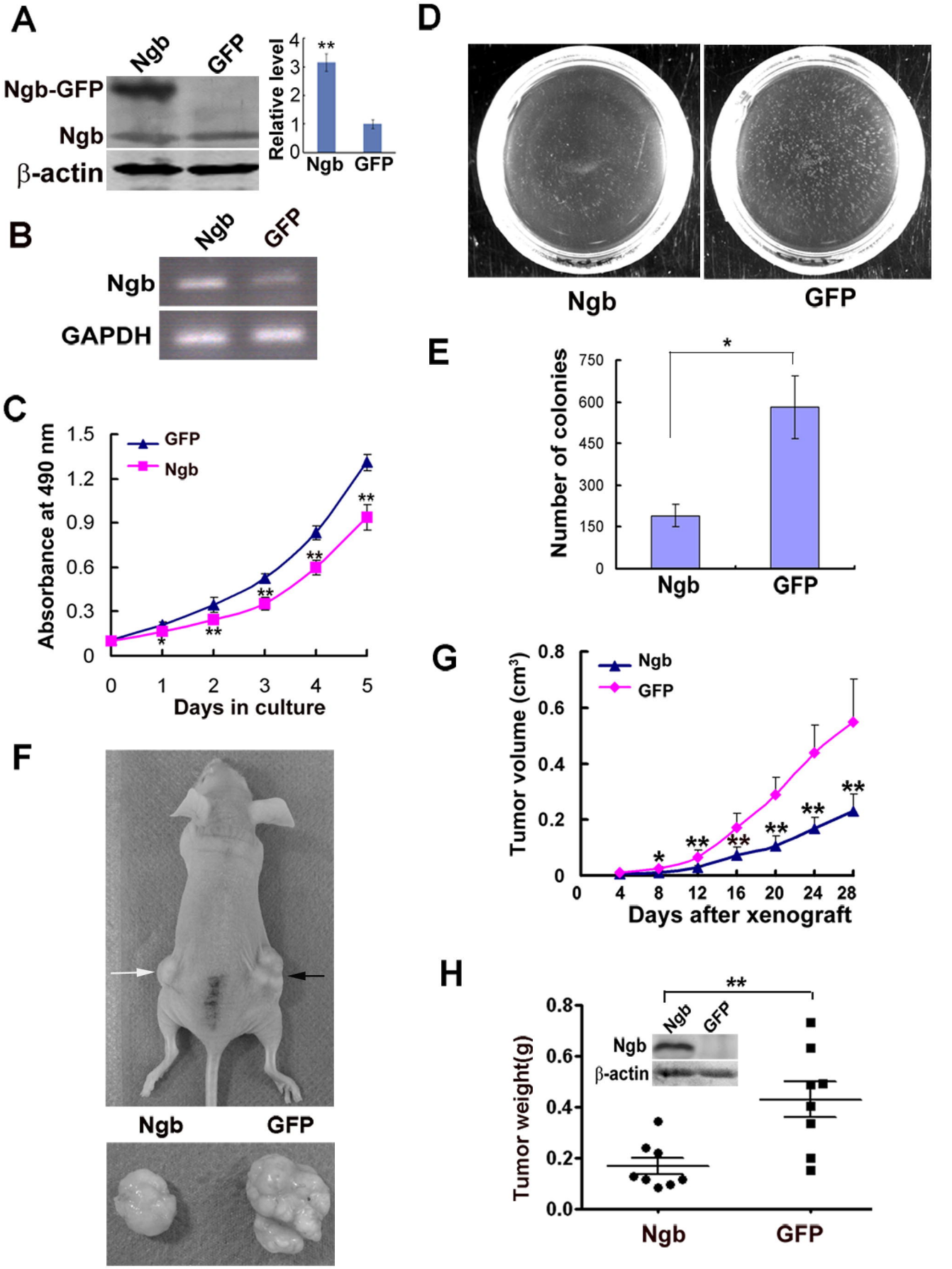

A
Ngb
GFP
Ngb-GFP
Ngb
β-actin
Relative level
**
Ngb
GFP
B
Ngb
GFP
Ngb
GAPDH
C
GFP
Ngb
Absorbance at 490 nm
Days in culture
D
Ngb
GFP
E
Number of colonies
*
Ngb
GFP
F
Ngb
GFP
G
Ngb
GFP
Tumor volume (cm³)
Days after xenograft
H
**
Ngb
GFP
Ngb
β-actin
Tumor weight(g)
Ngb
GFP

Figure 4

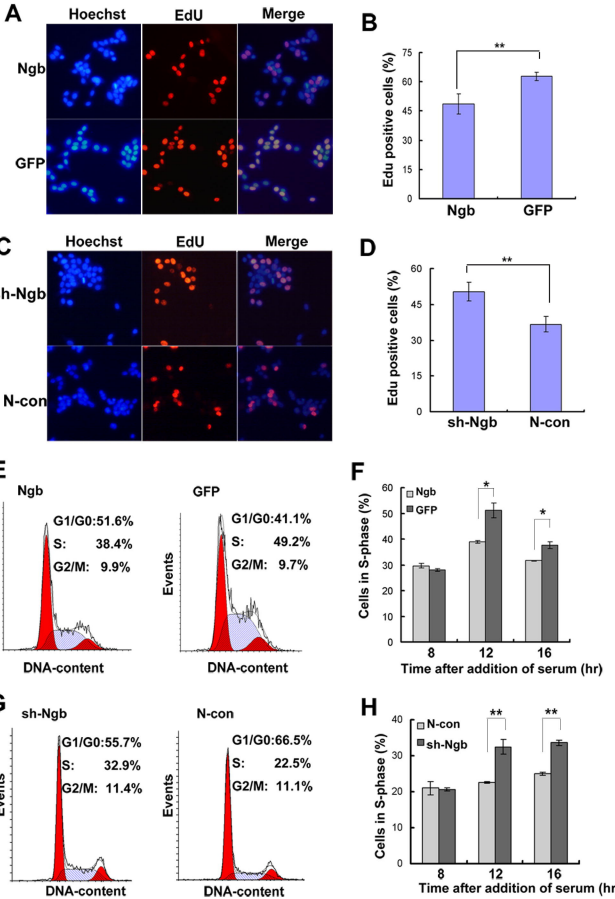

A
Hoechst
EdU
Merge
Ngb
GFP
B
Edu positive cells (%)
75
60
45
30
15
0
**
Ngb
GFP
C
Hoechst
EdU
Merge
sh-Ngb
N-con
D
Edu positive cells (%)
60
45
30
15
0
**
sh-Ngb
N-con
E
Ngb
G1/G0:51.6%
S: 38.4%
G2/M: 9.9%
Events
DNA-content
GFP
G1/G0:41.1%
S: 49.2%
G2/M: 9.7%
Events
DNA-content
F
Ngb
GFP
Cells in S-phase (%)
60
50
40
30
20
10
0
*
*
8
12
16
Time after addition of serum (hr)
G
sh-Ngb
G1/G0:55.7%
S: 32.9%
G2/M: 11.4%
Events
DNA-content
N-con
G1/G0:66.5%
S: 22.5%
G2/M: 11.1%
Events
DNA-content
H
N-con
sh-Ngb
Cells in S-phase (%)
40
30
20
10
0
**
**
8
12
16
Time after addition of serum (hr)

Figure 5

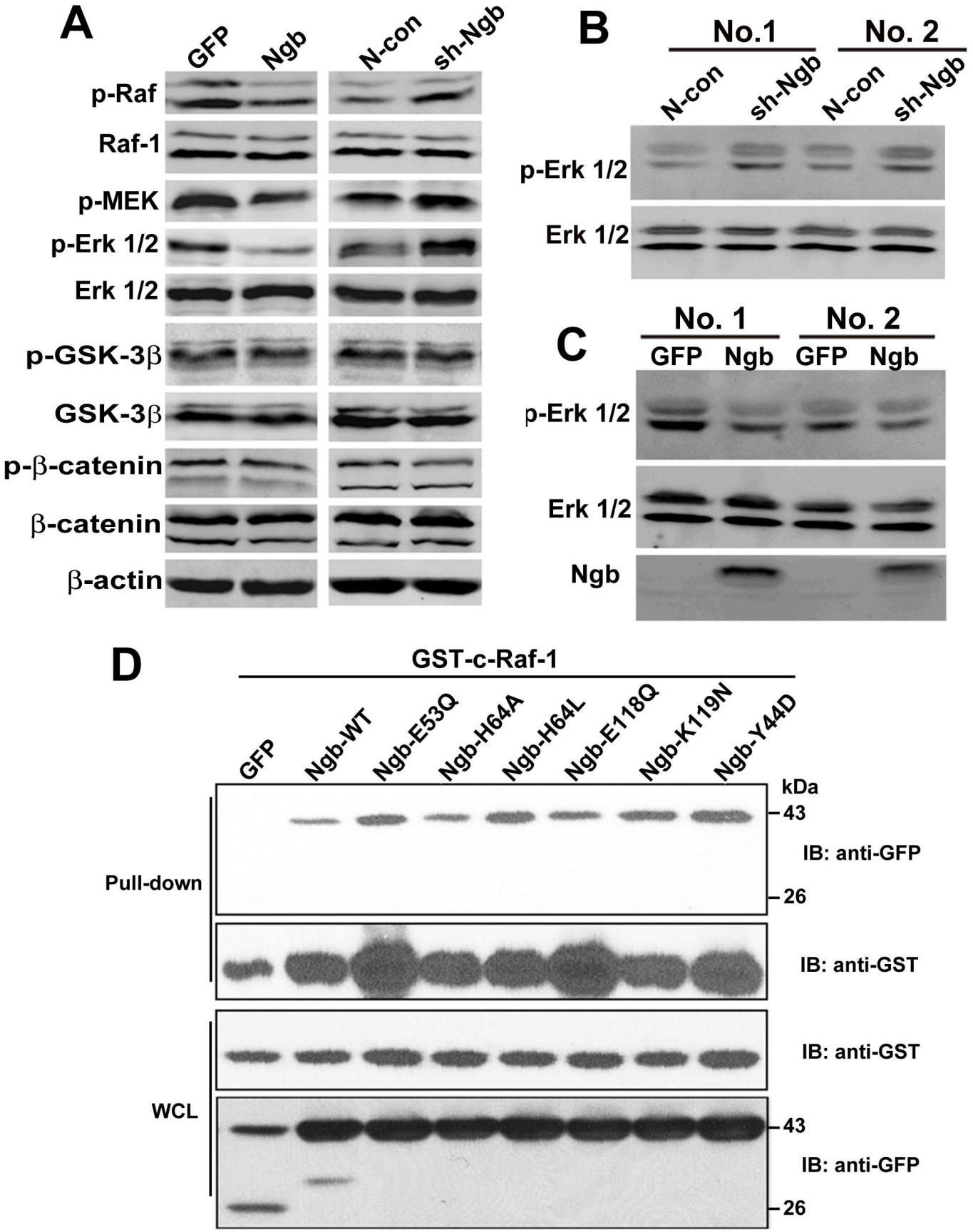

A
GFP
Ngb
N-con
sh-Ngb
p-Raf
Raf-1
p-MEK
p-Erk 1/2
Erk 1/2
p-GSK-3β
GSK-3β
p-β-catenin
β-catenin
β-actin
B
No.1
No. 2
N-con
sh-Ngb
N-con
sh-Ngb
p-Erk 1/2
Erk 1/2
C
No. 1
No. 2
GFP
Ngb
GFP
Ngb
p-Erk 1/2
Erk 1/2
Ngb
D
GST-c-Raf-1
GFP
Ngb-WT
Ngb-E53Q
Ngb-H64A
Ngb-H64L
Ngb-E118Q
Ngb-K119N
Ngb-Y44D
kDa
43
26
Pull-down
IB: anti-GFP
IB: anti-GST
IB: anti-GST
WCL
43
IB: anti-GFP
26

Figure 6

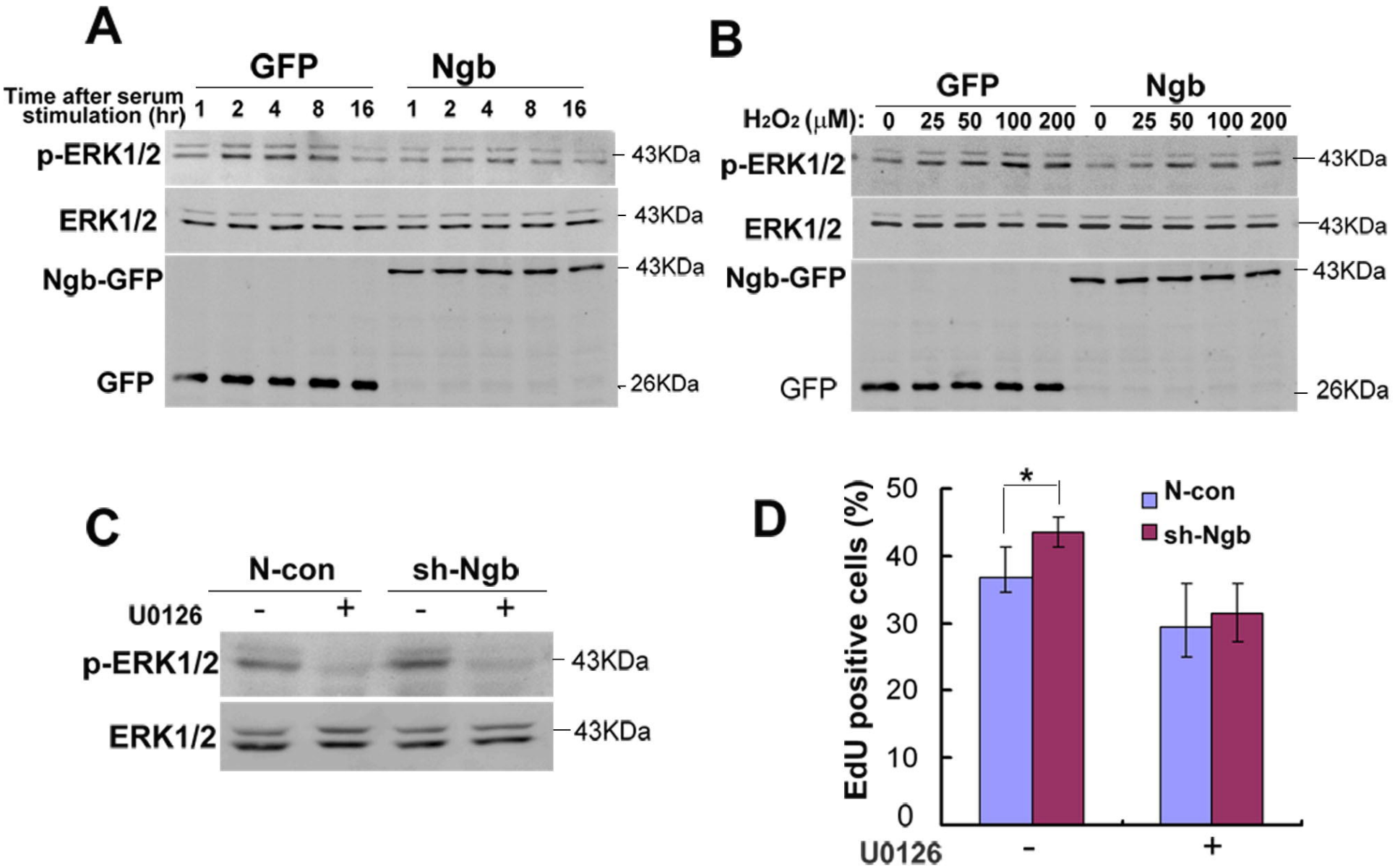
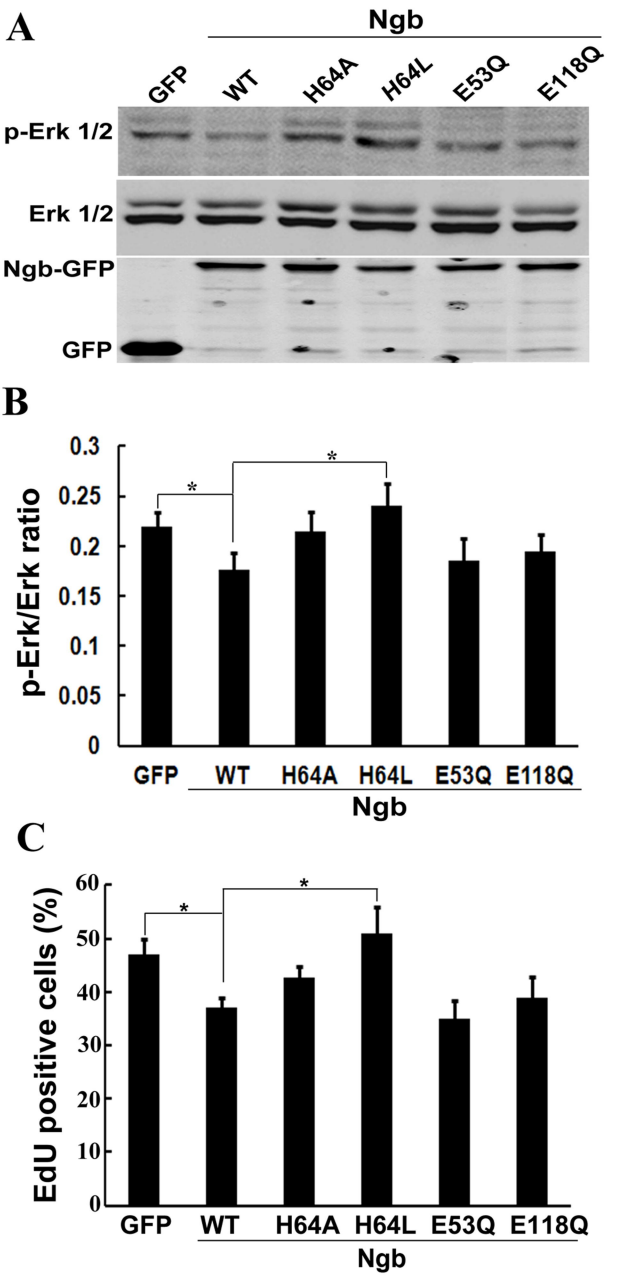

A
GFP
Ngb
Time after serum stimulation (hr)
1 2 4 8 16 1 2 4 8 16
p-ERK1/2
ERK1/2
Ngb-GFP
GFP
43KDa
43KDa
43KDa
26KDa
B
GFP
Ngb
H2O2 (μM): 0 25 50 100 200 0 25 50 100 200
p-ERK1/2
ERK1/2
Ngb-GFP
GFP
43KDa
43KDa
43KDa
26KDa
C
N-con
sh-Ngb
U0126 - + - +
p-ERK1/2
ERK1/2
43KDa
43KDa
D
EdU positive cells (%)
50
40
30
20
10
0
*
N-con
sh-Ngb
U0126 - +

Figure 7


A
Ngb
GFP
WT
H64A
H64L
E53Q
E118Q
p-Erk 1/2
Erk 1/2
Ngb-GFP
GFP
B
p-Erk/Erk ratio
0.3
0.25
0.2
0.15
0.1
0.05
0
*
*
GFP
WT
H64A
H64L
E53Q
E118Q
Ngb
C
EdU positive cells (%)
60
50
40
30
20
10
0
*
*
GFP
WT
H64A
H64L
E53Q
E118Q
Ngb