Hypoxia-inducible Factor-1α Induces ErbB4 Signaling in the Differentiating Mammary Gland

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Running title: ErbB4 induction by HIF-1α

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Background: HIF-1α regulates mammary gland development by an unknown mechanism.
Results: HIF-1α promotes ErbB4 protein accumulation, activity, and ErbB4-dependent differentiation of mammary epithelial cells.
Conclusion: HIF-1α is a regulator of ErbB4 signaling *in vitro* and *in vivo*.
Significance: A mechanism involved in the regulation of mammary gland development by HIF-1α is described.

Conditional knock-out of *Hif1a* in the mouse mammary gland impairs lobuloalveolar differentiation during lactation. Here, we demonstrate that expression of ErbB4 was reduced in the lobulalveoli of mice with mammary gland-specific deletion of *Hif1a*. *Erbb4* was not, however, a direct target gene for transcriptional regulation by HIF-1α *in vitro*. HIF-1α overexpression or HIF accumulating prolyl hydroxylase inhibitors reduced ErbB4 endocytosis, promoted transcriptional co-regulatory activity of ErbB4, and stimulated ErbB4-induced differentiation of mammary carcinoma cells. Consistently, RNA interference-mediated down-regulation of HIF-1α resulted in reduced ErbB4 protein amount and reduced mammary carcinoma cell differentiation. These findings indicate that HIF-1α is a physiologically relevant regulator of ErbB4 and that ErbB4 is involved in HIF-regulated differentiation of the mammary gland.

Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor that binds specific hypoxia-response elements in the DNA with its basic helix-loop-helix domain. HIF-1α regulates transcription of several genes involved in angiogenesis, glycolysis, and pH regulation (1). HIF-1α protein degradation is suppressed in ischemic conditions and in malignancies, and HIF-1α is considered a promising drug target for cancer therapy (1).

HIF-1α is also necessary for normal development of several tissues, including the heart, vasculature, and the brain (2). In pregnant mice, *Hif1a* deficiency results in condensation of

the lobuloalveoli of the mammary gland and impaired production of milk during lactation (3).

ErbB4 is a growth factor receptor that mediates the signals of epidermal growth factor (EGF)-like ligands, such as the neuregulins (NRG), betacellulin, and heparin binding EGF-like growth factor (4). ErbB4 belongs to the ErbB/HER family of receptor tyrosine kinases (RTK), that also includes the EGF-receptor (EGFR, ErbB1, HER1), ErbB2 (HER2), and ErbB3 (HER3) (5). While EGFR and ErbB2 are clinically relevant cancer drug targets, both tumor-promoting and differentation-inducing roles have been proposed for ErbB4 (6). *Erbb4* is alternatively spliced generating functionally unique ErbB4 isoforms (7). These isoforms differ either at the extracellular juxtamembrane domain (isoforms JM-a and JM-b) or the intracellular cytoplasmic domain (isoforms CYT-1 and CYT-2), generating variability in proteolysis-dependent signaling or coupling to intracellular proteins, respectively (7).

In addition to critical roles in regulating cardiovascular (8), neural (8,9), and renal (10) development, ErbB4 has been demonstrated to be necessary for differentiation of the mouse mammary gland (9,11,12). Interestingly, mice deficient of either *Erbb4* or *Hif1a* in their mammary epithelia exhibit a similar failure in the formation and differentiation of the milk-producing lobuloalveoli (3,11,12).

The overlap of the phenotypes of *Hif1a*- and *Erbb4*-deficient mice implies that ErbB4 could be a novel HIF-1α-regulated gene with a function downstream of HIF-1α in the developing mammary gland. To test this hypothesis, we analyzed ErbB4 expression in mice deficient of HIF-1α in the mammary gland (*Hif1a*$^{Flox/Flox}$*MMTV-Cre*) and addressed the effect of *HIF1A* gain- and loss-of-function on ErbB4 expression and function in mammary epithelial cells *in vitro*. Here, we report that HIF-1α induces ErbB4 protein accumulation in the mammary epithelial cells both *in vivo* and *in vitro*, and that this process is necessary for the normal ErbB4-mediated differentiation of mammary gland epithelial cells.

EXPERIMENTAL PROCEDURES

Conditional knock-out mice and immunohistochemistry–Mice with mammary gland specific targeting of *Hif1a* (*Hif1a*$^{Flox/Flox}$*MMTV-Cre* line A) have been described earlier (3). Paraffin sections of the mammary glands from mice at pregnancy day 18 (P18) or lactating day 1 (L1) were immunostained with the rabbit polyclonal antibodies anti-ErbB4 (sc-283; Santa-Cruz Biotechnology) and anti-GLUT-1 (ab14683; Abcam). Immunohistochemical analysis was performed using HistomouseMax IHC staining kit (Invitrogen) following the manufacturer's protocol.

Cell culture–T-47D human breast cancer cells were maintained in RPMI supplemented with 10% FCS. HEK293 human embryonic kidney cells and MDA-MB-468 human breast cancer cells were maintained in DMEM supplemented with 10% FCS.

Ligands and inhibitors–To stimulate or block ErbB4 signaling, cells were treated with 50 ng/ml NRG-1β (R&D Systems) or 10 μM AG 1478 (Calbiochem), respectively. To stimulate HIF-1α with prolyl hydroxylase (PHD) inhibitors, cells were treated for 20 hours with 500 μM dimethyloxallylglycine (DMOG; Cayman Chemicals) or 100, 200 or 400 μM $CoCl_2$ (Sigma-Aldrich). All treatments were carried out in the absence of serum.

Expression plasmids and transfection–All pcDNA3.1*ERBB4* constructs have been described earlier (13-15). Plasmids encoding wild-type or P402A/P564A double-mutant HIF-1α with HA-tag were from Dr. William Kaelin (Addgene plasmids 18949 and 18955). STAT5a encoding plasmid pME18S-*STAT5a* (16) and pGL3-*β-casein-LUC* (17) (kindly provided by Dr. Edith Pfitzner, Institute for Biomedical Research, Frankfurt, Germany) have been described earlier. p*EGFP-C3* was obtained from Clontech. Transient transfectants of HEK293 cells were generated using Fugene 6 (Roche) and of T-47D cells using Lipofectamine 2000 (Invitrogen) following manufacturer's recommendations. MDA-MB-468 cells were transduced with empty (pBABE-puro) or ErbB4 (pBABE-puro*ErbB4JM-aCYT-1*) encoding retroviruses, as previously described (15).

RNA interference–HIF1A targeting siRNAs (#1, target sequence *5'-auggaauauauucugcguuua-3'*; #2, target sequence *5'-aggaagaacuaugaacauaaa-3'*), *RABEP1* targeting siRNAs (#1, target sequence *5'-uaccgugaggacaucauuaau-3'*; #2 target sequence *5'-cuggaggccucaaaggguuaaa-3'*) and negative control siRNA were purchased from Qiagen. The siRNAs (final concentration 33 nM) were transfected to cells at 80% confluency with Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

Western blotting–Western analyses were carried out, as previously described (18), using the following primary antibodies: anti-HIF-1α (clone 54 from BD Biosciences or ab2185 from Abcam), anti-actin (sc-1616; Santa Cruz Biotechnology), anti-ErbB4 (sc-283; Santa Cruz Biotechnology or E200; Abcam), anti-RABEP1 (sc-271069; Santa Cruz Biotechnology), anti-tubulin (sc-9104; Santa Cruz Biotechnology), anti-phospho-ErbB4 (Tyr1248; #4757; Cell Signaling Technology), anti-Erk1/2 (#9102; Cell Signaling Technology), anti-phospho-Erk1/2 (Thr202/Tyr204; #9101; Cell Signaling Technology), anti-Akt (sc-1618; Santa Cruz Biotechnology), and anti-phospho-Akt (Ser473; #9271; Cell Signaling Technology). The following HRP-conjugated secondary antibodies were used: goat anti-rabbit (sc-2004; Santa Cruz Biotechnology), goat anti-mouse (sc-2005; Santa Cruz Biotechnology), and rabbit-anti-goat (sc-2768; Santa Cruz Biotechnology). Streptavidin-HRP (Invitrogen) was used to detect biotinylated proteins.

Real-time RT-PCR–Total RNA was extracted from cell cultures using Trizol (Invitrogen) and subjected to real-time RT-PCR, as previously described (19). Primers and probes for *ERBB4* (isoform JM-a) (19), *β-actin* (19), and *GLUT1* (20) have been described earlier.

Immunofluorescence staining–T-47D cells as well as MDA-MB-468 transfectants expressing ErbB4 JM-a CYT-1 were plated on glass cover slips, and treated for 20 hours in DMEM with or without 500 μM DMOG followed by 30 min treatment with or without 50 ng/ml NRG-1. After fixing with ice-cold methanol, the cells were washed with PBS and placed in blocking solution (5% goat serum and 0.01% Tween-20 in PBS) for 1 hour at room temperature followed by overnight staining at 4°C with the anti-ErbB4 E200 diluted in blocking solution. After five washes with PBS, the secondary antibody goat anti-rabbit Alexa 488 (Invitrogen) and 0.5 μg/ml 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) diluted in blocking solution was applied for overnight incubation at 4°C. After five washes with PBS, the samples were mounted with Mowiol (Calbiochem). Samples were imaged with a LSM510 META (Carl Zeiss) confocal microscope using plan-apochromat NA1.4 63x objective. One micrometer thick optical sections were imaged through the middle plane of the cells. The localization of the anti-ErbB4 epitope was analyzed from unprocessed images using FIJI ImageJ software (21). First, the cell was outlined right outside of the plasma membrane using segmented line tool and fluorescence intensity was measured yielding total fluorescence intensity of the cell (I_{total}). Then, the segmented line was drawn right inside of the plasma membrane and the intensity was measured again yielding internal fluorescence intensity ($I_{internal}$). The amount of internalized signal was calculated using the following equation:

$$Internalized(\%) = 100\% \times \frac{I_{internal}}{I_{total}}$$

Antibody internalization assay–MDA-MB-468 cells expressing ErbB4 JM-a CYT-1 were plated on glass cover slips, and treated for 20 hours in DMEM with or without 500 μM DMOG. Next, 200 ng/ml of an antibody recognizing the extracellular domain of ErbB4 (clone H4.77.16; Thermo Scientific Pierce) was added and incubated with the cells for 2 hours at 37°C. The cells were washed with PBS and fixed in ice-cold methanol. Following a wash with PBS, the cells were incubated for 1 hour at room temperature in the presence of the secondary antibody goat anti-mouse Alexa 568 (Invitrogen) and 0.5 μg/ml DAPI. After four washes with PBS, the cells were mounted with Mowiol. Two micrometer thick optical sections were imaged through the middle plane of the cells. The amount of fluorescence signal reflecting the amount of internalized antibody was quantified as described above.

Cell surface biotinylation–MDA-MB-468 cells expressing ErbB4 JM-a CYT-1 were plated at 500,000 cells/60 mm dish. The following day, the medium was replaced with DMEM including 0 or 500 µM DMOG for 20 hours. The cells were washed three times with ice-cold PBS. The cell surfaces were biotinylated with 2 mM cell-impermeable biotinylation reagent Sulfo-NHS-LC-Biotin (Thermo Scientific Pierce) for 20 min at room temperature. The cells were washed twice with ice-cold PBS and once with ice-cold DMEM. Following biotinylation, the cells were treated with 50 ng/ml of NRG-1 for 0, 10 or 30 minutes. Subsequently, cell surface proteins were cleaved by incubation in trypsin solution (2.5 g/l trypsin (Lonza), 0.2 g/l K-EDTA (BDH Laboratory Supplies) and 1.0 g/l glucose (Amresco) in PBS) for 20 minutes on ice and 10 minutes at room temperature. Trypsinized cells were centrifuged and the cell pellet was resuspended in 200 µl of lysis buffer (1% SDS, 150 mM NaCl, 100 mM Tris-Cl, pH 7.4). The samples were incubated at 95°C for 5 min. Fifty microliters of each sample was diluted with 950 µl of TNN buffer (0.5% NP-40, 5 mM EDTA, 250 mM NaCl, 50 mM Tris-HCl, pH 7.4) and incubated together with streptavidin agarose beads at room temperature for 2 hours. After five 1 ml washes with TNN buffer, the beads were resuspended in sample buffer, incubated at 95°C for 5 minutes, cooled down, and subjected to Western analysis.

In vitro differentiation assay–MDA-MB-468 cells transduced with a retrovirus encoding ErbB4 (isoform JM-a CYT-1) or vector control cells (Vaparanta et al., in preparation) were cultured in Matrigel (BD Biosciences), as previously described (15). Briefly, the cells were trypsinized and resuspended in DMEM + 10% FCS at a density of 500,000 cells/ml. Thirty microliters of the cell suspension was combined with 180 µl of ice-cold Matrigel, and 50 µl of the mixture was added to 96-well plate wells. The gels were allowed to solidify for 30 min at 37°C, after which 150 µl of medium containing 50 ng/ml NRG-1β was added on top. After 7 days in culture, cell clusters were scored under a 20x objective with Olympus CK40 microscope (Olympus). For Western analyses, the cells were cultured at equivalent conditions on 24-well plates for 5 days. The cells were released from Matrigel using BD Cell Recovery Solution (BD Biosciences), as recommended by the manufacturer.

Luciferase reporter assays–To address the activity of the STAT5-sensitive *β-casein* promoter, HEK293 cells were plated on 24- or 96-well plates at a density of 1.5×10^4 cells/cm^2. The following day, the cells were transfected with 50 ng of empty pcDNA3.1 vector, pcDNA3.1*ERBB4JM-aCYT-2-HA* or pcDNA3.1*ERBB4JM-aCYT-2K751R-HA* together with 50 ng of pME18S-*STAT5a*, 100 ng of the reporter plasmid pGL3-*β-casein-LUC*, and 50 ng of the control plasmid p*EGFP-C3* (Clontech) (plasmid quantities given for 24-wells). The day after transfection, cells were subjected to treatment with 0 or 200 µM $CoCl_2$ and/or 0 or 10 µM AG 1478 for 24 hours. Luciferase activity was measured using Bright-Glo luciferase assay (Promega) following the manufacturer's protocol. The luminescence signal from the reporter plasmid was normalized by the fluorescence signal from the control plasmid.

Chromatin immunoprecipitation–T-47D cells were starved overnight in the absence of serum and then treated for 24 hours with 0 or 500 µM DMOG and for 45 min with 0 or 50 ng/ml of NRG-1. Chromatin immunoprecipitation was performed as previously described (22) using rabbit anti-ErbB4 (sc-283; Santa Cruz Biotechnology) to precipitate the intracellular domain of ErbB4, or rabbit IgG (Cell Signaling Technology) as a negative control. Immunoprecipitated chromatin was PCR amplified using PCR SuperMix (Life Technologies) and the primers 5'-*actgtcctccagtcattgtct*-3' and 5'-*tggtccatcagcttctgtgac*-3' previously shown to amplify a region of the human β-casein promoter that binds ErbB4 (23).

Statistical analyses–*In vitro* experiments were analyzed with two-sided Student's t-test or with one-way ANOVA associated with Dunnet's t post-hoc test calculated with IBM SPSS 20 software. Data about the effect of *HIF1A*-targeting siRNAs on ErbB4-mediated differentiation (shown in Fig. 6C) was analyzed using repeated measures ANOVA (http://faculty.vassar.edu/lowry/corr4.html) with Tukey HSD post-hoc test (http://faculty.vassar.edu/lowry/hsd.html) to

control for baseline variations between experiments.

RESULTS

HIF-1α promotes ErbB4 expression in the lactating mammary gland–Mice with mammary epithelium-specific knock-out of *Hif1a* (*Hif1a*$^{Flox/Flox}$*MMTV-Cre*) demonstrate defects in lactogenic differentiation and condensed lobuloalveoli (3). However, the molecular mechanisms by which HIF-1α promotes lactogenic differentiation have not been elucidated.

Loss of *Erbb4* in the lactating mammary gland leads to a similar phenotype as loss of *Hif1a* (3,11,12), implying that HIF-1α and ErbB4 reside in the same signaling pathway regulating mammary gland differentiation. To address whether HIF-1α regulates the level of ErbB4 protein *in vivo*, tissue sections from P18 mammary glands of the mice with conditionally targeted *Hif1a* alleles were analyzed by immunohistochemistry with an antibody against ErbB4. When compared to the wild-type control (Fig. 1A), immunoreactivity for ErbB4 was strongly reduced in the differentiating *Hif1a*$^{-/-}$ mammary epithelial cells (Fig. 1B). As a control, *Hif1a* knock-out was also shown to result in a significant suppression of immunoreactivity for the known (2) HIF-1α-regulated gene product glucose transporter-1 (GLUT-1) (Fig. 1C and D).

HIF-1α promotes accumulation of endogenous ErbB4 protein in vitro–To further test the effect of HIF-1α on ErbB4 expression, T-47D human breast cancer cells endogenously expressing ErbB4 (24) were transfected with an empty vector, or plasmids encoding wild-type HIF-1α or the HIF-1α mutant, HIF-1α P402A/P564A, resistant to prolyl hydroxylation-dependent proteolysis. Western analysis of the transfectants indicated that ectopic expression of both HIF-1α constructs increased ErbB4 protein level (Fig. 2A). Moreover, HIF-1α targeting siRNAs suppressed the ability of the known inducer of endogenous HIF stability, DMOG, to increase endogenous ErbB4 protein level in the T-47D cells (Fig. 2B).

To address the hypothesis that HIF-1α promoted ErbB4 protein accumulation as a direct transcriptional activator of the *ERBB4* gene, T-47D cells were treated with another chemical inducer of endogenous HIF-1α stability, $CoCl_2$, and the amount of *ERBB4* mRNA was measured by real-time RT-PCR. However, no up-regulation of *ERBB4* mRNA expression was observed, while $CoCl_2$ treatment significantly induced the expression of the known (2) HIF-1α-regulated gene *GLUT1* (Fig. 2C). Moreover, HIF over-expression did not stimulate *ERBB4* mRNA expression. *ERBB4* mRNA level was 0.3% of β-actin mRNA level 48 hour after transfection of a HIF plasmid as compared to 0.4% after transfection of a vector control. These data suggest that both endogenous and ectopically expressed HIF-1α can promote accumulation of the ErbB4 protein at the post-transcriptional level.

HIF-1α promotes accumulation of both the full-length ErbB4 as well as its cleaved intracellular domain fragment–The 180 kD full-length ErbB4 in T-47D cells is processed to an 80 kD intracellular domain (ICD) fragment with potential signaling activity (25). However, although considerable amounts of the 80 kD fragment is produced, it is also rapidly degraded in the T-47D background (26). To address the effect of HIF-regulation on the different ErbB4 species, HEK293 cells were transfected to express ErbB4 (cleavable isoform JM-a CYT-2). As expected for regulation taking place at the post-transcriptional level, treatment with $CoCl_2$ promoted accumulation of ectopically expressed ErbB4 (Fig. 3A, lane 4 *vs.* 2). Both the full-length 180 kD as well as the carboxy-terminal 80 kD ICD fragment of ErbB4 were accumulated in response to $CoCl_2$, and co-transfected HIF-1α siRNA suppressed the accumulation of both ErbB4 species (Fig. 3A). Consistently, also DMOG and over-expressed HIF accumulated ErbB4 in HEK293 cells (data not shown). The effect of $CoCl_2$ was not dependent on ErbB4 cleavage or on the type of the ICD of ErbB4 as it promoted accumulation of both cleavable (JM-a) and non-cleavable (JM-b) ErbB4 isoforms with two different types of ICDs (CYT-1 or CYT-2) (Fig. 3B).

HIF-1α induction reduces ErbB4 endocytosis–The steady-state levels of receptor tyrosine kinases at

the cell surface are regulated by the rate of receptor endocytosis and degradation (27). To assess the putative effect of HIF activation on ErbB4 internalization, T-47D cells endogenously expressing ErbB4 were treated with DMOG, and the localization of an ICD epitope of ErbB4 visualized by immunofluorescence microscopy (Fig. 4A). Indeed, more immunoreactivity for ErbB4 associated with the plasma membrane, as opposed to cytosol, in cells treated with DMOG. DMOG treatment also significantly suppressed ErbB4 internalization in cells in which ligand-induced receptor endocytosis was stimulated by NRG-1 (Fig. 4B). The effect of DMOG on ErbB4 localization was inhibited by siRNAs targeting HIF-1α, indicating that the DMOG effect was indeed dependent on HIF-1α (Fig. 4B). Similar findings were made when MDA-MB-468 breast cancer cell transfectants expressing ErbB4 JM-a CYT-1 were analyzed (data not shown).

Consistent observations of DMOG-suppressed ErbB4 internalization were also made when the MDA-MB-468 transfectants were cultured for two hours in the presence of an antibody recognizing the extracellular domain of ErbB4 (clone H4.77.16) followed by immunodetection of the subcellular localization of the antibody (Fig. 4C and D). Finally, DMOG reduced the amount of biotinylated ErbB4 translocating from the cell surface to a trypsin-insensitive intracellular compartment in response to stimulation with the NRG-1 ligand (Fig. 4E). These findings indicate that HIF induction promotes ErbB4 accumulation by reducing ErbB4 endocytosis.

We have previously demonstrated that trafficking of ErbB4 from the cell membrane to degradation occurs *via* Rab5-positive early endocytic vesicles (28). Interestingly, it was recently reported that Rab GTPase binding effector protein 1 (RABEP1, rabaptin-5), a critical regulator of Rab5 (29), is involved in HIF-promoted accumulation of EGFR (30). To address whether RABEP1 was also involved in the mechanism by which the HIF-activator DMOG suppressed ErbB4 internalization, the effect of *RABEP1* down-regulation by RNA interference was assessed. As predicted, the knock-down of RABEP1 inhibited ligand-induced endocytosis of ErbB4 biotinylated at the cell surface (Fig. 4F). RABEP1 was also necessary for the DMOG-induced accumulation of ErbB4 (Fig. 4G), suggesting that ErbB4 accumulation was, indeed, endocytosis-dependent.

HIF-1α promotes ErbB4 signaling–To evaluate the activity of the ErbB4 protein accumulated by HIF-1α, ligand-induced phosphorylation of endogenous ErbB4 in T-47D cells was analyzed by Western blotting using a phospho-specific anti-ErbB4 recognizing phosphorylated Tyr1248 at the carboxy-terminus of ErbB4. Overexpression of the degradation-resistant HIF-1α P402A/P564A mutant increased the duration of ErbB4 phosphorylation from 10 minutes to 30 minutes after NRG-1 stimulation (Fig. 5A). However, this prolongation in tyrosine phosphorylation was not associated with significantly altered kinetics of Erk or Akt phosphorylation at the same time points (Fig. 5A). This finding is consistent with previous observations indicating that in cells expressing both ErbB4 and ErbB2 (such as T-47D cells (24)) the Erk and Akt pathways are predominantly activated by the ErbB2 kinase in response to NRG-1 (15).

To more specifically analyze the effect of HIF-1α on a signaling output dependent on the ErbB4 kinase, a reporter luciferase assay measuring the transcriptional activity of the *β-casein* promoter was set up in HEK293 cells. This assay serves as a read-out of transcriptional co-activator activity of ErbB4 (31-33) as ErbB4 enhances activation of *β-casein* promoter via STAT5 (23). Overexpression of ErbB4 (JM-a CYT-2) significantly increased the activity of the *β-casein* promoter and the effect was further enhanced by the addition of $CoCl_2$ (Fig. 5B). Inhibition of ErbB kinase activity with the chemical inhibitor AG 1478 partially suppressed the increase achieved by ErbB4 overexpression, and introduction of a kinase-inactivating mutation (kinase-dead ErbB4 K751R) totally abolished the capability of $CoCl_2$ to increase ErbB4-stimulated promoter activity (Fig. 5B).

To assess the potential of HIF-1α to induce binding of the released ErbB4 ICD to endogenous β-casein promoter, chromatin immunoprecipitation experiments were carried out in the T-47D mammary cell background. Induction of endogenous HIF-1α by DMOG increased the NRG-1 ligand-induced binding of ErbB4 to the β-casein promoter (Fig. 5C). Taken

together, these findings indicate that HIF-activity can promote ErbB4 signaling.

HIF-1α promotes ErbB4-dependent mammary epithelial differentiation–The synergistic effect of HIF-inducers and ErbB4 on *β-casein* promoter indicated that regulation of ErbB4 by HIF-1α may also be relevant for milk protein synthesis and lactogenic differentiation of the mammary epithelial cells. To address the biological role, an *in vitro* model of mammary epithelial cell differentiation was set up. For these experiments we chose the MDA-MB-468 mammary carcinoma cells as these had previously been used to study ErbB4-dependent mammary epithelial differentiation (15,34). The MDA-MB-468 cells, lacking endogenous ErbB4 expression (15,35,36), were retrovirally transfected with the empty vector or a construct encoding ErbB4 JM-a CYT-1, an isoform known to promote mammary epithelial differentiation (31,32).

When the infected cell lines were cultured for seven days in Matrigel, they formed both clusters of randomly associated cells (non-acinar colonies), as well as acinus-like colonies demonstrating signs of epithelial organization into ball-like structures (Fig. 6A). As expected based on earlier reports (31,32), overexpression of ErbB4 JM-a CYT-1 significantly enhanced the percentage of acinar colonies of all the colonies (Fig. 6B), indicating enhanced differentiation. In contrast, simply treating the cells with 100 μM of the HIF-1α-inducing DMOG was not suffient to promote differentiation of the vector control cells lacking ErbB4 (Fig. 6B, white columns). When DMOG was administered to the cells expressing ErbB4, however, this compound promoted differentiation over the level achieved by ErbB4 overexpression alone (Fig. 6B, grey columns). Interestingly, experimentation with HIF-1α-targeting siRNAs demonstrated that the effect of ErbB4 expression on the formation of acinar colonies was also dependent of the presence of HIF-1α (Fig. 6C). These observations indicate that HIF-1α enhances ErbB4-mediated differentiation of mammary epithelial cells *in vitro*.

DISCUSSION

Here we provide evidence indicating that accumulation of ErbB4 is part of the mechanism by which HIF-1α promotes lactogenic differentiation of the mammary epithelium during pregnancy. Deletion of HIF-1α was shown to reduce ErbB4 protein level in the lactating mouse mammary gland *in vivo* and HIF-1α expression increased ErbB4 protein in human mammary epithelial cells *in vitro*. Experiments with an *in vitro* model of mammary epithelial differentiation indicated that HIF-1α activity was both sufficient and necessary for the ability of ErbB4 to induce differentiation. HIF-1α induction also promoted the ability of ErbB4 to bind and activate the promoter of the gene for β-casein, a major component of milk in the lactating mammary duct (37). This interaction between the HIF-1α and ErbB4 signaling pathways is expected to be biologically significant as mice carrying deletions of either *Erbb4* or *Hif1a* exhibit similar phenotypes in their lactating mammary glands (3,11,12).

HIF-1α is a transcription factor that is known to stimulate the expression of several genes by direct activation of specific response elements in the target gene promoter (1,38). However, the mechanism by which HIF-1α promoted the accumulation of ErbB4 protein did not seem to involve direct transcriptional activation. Treatment with the HIF-inducing $CoCl_2$ did not increase the amount of *ERBB4* transcript while a similar treatment promoted the accumulation of mRNA encoded by *GLUT1*, a well-known HIF target gene (2), by 80-fold. In addition, ErbB4 protein was accumulated when encoded by an expression vector lacking the regulatory sequences of the endogenous *ERBB4* locus.

In contrast, our data indicates that HIF-1α promotes ErbB4 accumulation as a result of reduced endocytosis. The PHD inhibitor DMOG reduced endocytosis of ErbB4 in MDA-MB-468 breast cancer cells and functional endocytosis was needed for the PHD inhibitor-promoted accumulation of ErbB4. Consistently with these findings, it has been reported that the loss of VHL, a protein recognizing PHD-modified HIF, reduces endocytosis of EGFR and fibroblast growth factor receptor-1 (FGFR1) in renal cell carcinoma cells (30,39). While the exact mechanism by which HIF-1α promotes ErbB4 accumulation remains to be elucidated, the accumulation of both the full-

length ErbB4 as well as its 80 kD carboxy-terminal fragment was dependent on RABEP1, a regulator of Rab5-mediated endocytosis (29).

Interestingly, HIF-1α has recently been shown to physically interact with the ICD of ErbB4 (20), raising a possibility that the direct molecular contact might have a role in regulating the stability of ErbB4. However, the interaction between HIF-1α and ErbB4 ICD is expected to be restricted to the nucleus and require proteolytic release of a the soluble ICD (20). Thus, our findings demonstrating that HIF-1α also promoted accumulation of the non-cleavable JM-b isoforms of ErbB4 are inconsistent with a model in which ErbB4 stability was regulated by a direct HIF-1α interaction. On the other hand, the observation that ErbB4 expression alone was not sufficient to promote MDA-MB-468 cell differentiation when HIF-1α expression was down-regulated by RNA interference, suggested that a direct or indirect interaction of the two proteins may also have a role in mediating the differentiation signaling downstream of ErbB4. As previous work has demonstrated that the interaction of HIF-1α with the ErbB4 ICD leads to enhanced stability of HIF-1α (20) , it is possible that HIF-1α and ErbB4 can reciprocally enhance each other's stability and signaling generating a positive feed-back loop (40). Given the similar mammary gland phenotypes of the *Hif1a*- and *Erbb4*-deficient mice, it is tempting to speculate that such a feed-back loop of HIF-1α and ErbB4 could be involved in the differentiation of the lactating mammary gland.

HIF-2α has also recently been reported to regulate ErbB4 signaling in breast cancer cells (41). The mechanism involves increased expression of the ErbB4 ligand amphiregulin and subsequent ErbB4 phosphorylation (41). Interestingly, HIF-2α, but not HIF-1α, protein expression has been associated with favorable survival and luminal epithelial differentiation in clinical breast cancer samples (41). However, while amphiregulin regulates mammary gland development in mice (42,43), no defects of mammary gland development have been reported for *Hif2a*/*Epas1* null mice. These findings imply that HIF-2α and HIF-1α may regulate ErbB4 by variable mechanisms in different biological contexts.

The induction of ErbB4 by HIF-1α in the HEK293 cells implied that ErbB4 may be induced by HIF-1α also outside of the context of the mammary gland. Indeed, in addition to mammary development, *Hif1a* deletion has been reported to produce similar phenotypes to *Erbb4* deletion during trabeculation of the heart (8,44,45) as well as during migration of the cells of the neural crest (45,46). It remains to be elucidated what kind of role, if any, the cross-talk between HIF-1α and ErbB4 plays in these processes. In addition to development, HIF-1α and ErbB4 may regulate each other in neoplastic tissues. Consistent with a widely distributed signaling role on the same pathway, our previous *in silico* analysis demonstrated that the expression of ErbB4 significantly associates with HIF-1α activity in a number of normal and malignant human tissues (20).

In conclusion, our findings indicate that HIF-1α may promote differentiation of the lactating mammary gland *via* a mechanism involving suppressed endocytosis of the ErbB4 receptor tyrosine kinase.

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FIGURE LEGENDS

FIGURE 1. **Expression of ErbB4 is dependent on *Hif1a* in the developing mammary gland.** Immunohistochemical analysis of ErbB4 (sc-283) (A, B) and GLUT-1 (ab14683) (C, D) expression in paraffin sections of P18 *Hif1a*$^{Flox/Flox}$*MMTV-Cre*$^{-/-}$ (wild-type) (A, C) and *Hif1a*$^{Flox/Flox}$*MMTV-Cre*$^{+/-}$ (*Hif1a*$^{-/-}$) (B, D) mice. Sections from three mice per genotype were analyzed.

FIGURE 2. **Regulation of endogenous ErbB4 expression by HIF-1α.** A) Western analysis of ErbB4 (E200) and HIF-1α (ab2185) expression in T-47D cells transfected with an empty vector or with plasmids encoding wild-type HIF-1α (wt) or a degradation-resistant mutant HIF-1α (P402A/P564A). Actin (sc-1616) expression was analyzed from the same lysates to control loading. B) Western analysis of ErbB4 (E200) and HIF-1α (clone 57) expression in T-47D cells transfected with a negative control siRNA (–) or HIF-1α-targeting siRNAs (#1 and #2) and cultured for 20 hours in the presence of 0 or 500 µM DMOG. The filter was reblotted with anti-actin (sc-1616) to control loading. C) Real-time RT-PCR analysis of *ERBB4* and *GLUT1* mRNA expression in T-47D cells treated for 20 hours with 0, 100 or 200 µM $CoCl_2$.

FIGURE 3. **Effect of HIF-1α on ErbB4 isoform expression and stability.** A) Western analysis of ErbB4 (sc-283) and HIF-1α (clone 57) expression in HEK293 cells transfected or not with a plasmid encoding ErbB4 JM-a CYT-2 together with a negative control siRNA (control) or HIF-1α-targeting siRNA (#2) and treated for 20 hours with 0 or 400 µM $CoCl_2$. The filter was reblotted with anti-actin (sc-1616) to control loading. n.s. = a non-specific band. B) Western analysis of HEK293 transfectants overexpressing the indicated full-length ErbB4 isoforms treated for 20 hours with 0, 100, or 200 µM $CoCl_2$. Western analyses of ErbB4 (sc-283) and actin (sc-1616) are shown.

FIGURE 4. **HIF-1α reduces endocytosis of ErbB4.** A-B) Confocal immunofluorescence analysis of ErbB4 internalization (A) and quantification of the data (B). T-47D cells were transfected with a negative control siRNA (–) or HIF-1α-targeting siRNAs (#1 and #2) and cultured for 20 hours in the presence of 0 or 500 µM DMOG followed by 30 min treatment with or without 50 ng/ml NRG-1. ErbB4 was detected with an antibody recognizing the ICD of ErbB4 (E200). HIF-1α-targeting siRNAs #1 and #2 reduced HIF-1α expression by 43% and 71% respectively, as assessed by quantifying immunofluorescence signal for anti-HIF-1α (clone 54). Scale bar in A: 5 µm. Columns in B represent the mean +/– standard error (n = 17 to 33 for different treatments). C-D) Confocal analysis of anti-ErbB4 antibody internalization (C) and quantification of the data (D). MDA-MB-468 cells expressing ErbB4 JM-a CYT-1 were cultured for 20 hours in the presence of 0 or 500 µM DMOG followed by 2 hour incubation with an antibody recognizing the extracellular domain of ErbB4 (clone H4.77.16). The subcellular localization of the ErbB4 antibody was determined by immunofluorescence staining. Arrow in C points to internalized anti-ErbB4 in vesicles. Scale bar in C: 5 µm. Columns in D represent the mean +/– standard error (n = 16). E) Western analysis of cell surface biotinylated proteins. MDA-MB-468 cells expressing JM-a CYT-1 were cultured for 20 hours in the presence of 0 or 500 µM DMOG. The cells were biotinylated using a cell-impermeable reagent followed by treatment with 50 ng/ml NRG-1 for 0, 10 or 30 minutes. The cells were trypsinized, and internalized biotin-labeled ErbB4 was detected by pull-down with streptavidin-agarose beads followed by Western blotting with anti-ErbB4 (E200). The filter was reblotted with streptavidin-HRP to control loading. RABEP1 (sc-271069) and actin (sc-1616) were analyzed by Western blotting from the cell lysates. F) Western analysis of cell surface biotinylated proteins. MDA-MB-468 cells expressing ErbB4 JM-a CYT-1 were transfected with a negative control siRNA (–) or RABEP1-targeting siRNAs (#1 and #2) and cultured for 20 hours in the presence of 0 or 500 µM DMOG. After biotinylation, the cells were treated for 30 min with NRG-1, trypsizined, and analyzed for ErbB4 endocytosis as in E. RABEP1 (sc-271069) and tubulin (sc-9104) were analyzed by Western blotting from parallel lysates. G) Western analysis of ErbB4 (E200) and RABEP1 (sc-271069) expression. MDA-MB-468 cells expressing ErbB4 JM-a CYT-1 were transfected with a negative control siRNA (–) or RABEP1-targeting siRNAs (#1 and #2) and cultured for

20 hours in the presence of 0 or 500 μM DMOG. The filter was reblotted with anti-actin (sc-1616) to control loading.

FIGURE 5. **HIF-1α promotes ErbB4-mediated signaling.** A) Western analyses of T-47D cells transfected with an empty vector (– HIF) or a plasmid encoding the degradation-resistant mutant HIF-1α P402A/P564A (+ HIF). The cells were starved overnight in 0% FCS and treated with 50 ng/ml NRG-1 for the indicated periods of time. Anti-ErbB4, E200; anti-pErbB4, Tyr1248, #4757; anti-pErk, Thr202/Tyr204, #9101; anti-Erk, #9102; anti-pAkt, Ser473, #9271; anti-Akt, sc-1618; anti-actin, sc-1616. B) A luciferase reporter assay of HEK293 cells transfected with plasmids encoding the luciferase reporter gene under a *β-casein* promoter, STAT5A, and EGFP, together with an empty control vector, or a plasmid encoding ErbB4 JM-a CYT-2 or the kinase-dead mutant ErbB4 JM-a CYT-2 K751R. Transfectants were treated with or without 200 μM $CoCl_2$ and/or 10 μM of the ErbB4 kinase inhibitor AG 1478 for 24 hours. Columns represent mean luciferase activity normalized with EGFP fluorescence +/– standard error from three independent experiments (n = 9; except for ErbB4 JM-a CYT-2 K751R, n = 6). Protein expression was controlled by Western analyses with anti-ErbB4 (E200) and anti-pErbB4 (Tyr1248, #4757). C) Chromatin immunoprecipitation. T-47D cells were starved overnight in 0% FCS and then treated for 24 hours with 0 or 500 μM DMOG followed by 45 min treatment with 0 or 50 ng/ml of NRG-1. Samples were immunoprecipitated with anti-ErbB4 (sc-283) or with the negative control IgG. Immunoprecipitated chromatin was analyzed by PCR amplifying the ErbB4 binding site of the human β-casein promoter.

FIGURE 6. **HIF-1α promotes ErbB4-mediated differentiation in vitro.** A) Representative images of colonies formed by control MDA-MB-468 cells cultured for seven days in Matrigel. A photograph of a differentiated acinus (left) and a non-differentiated cluster of cells (right) is shown. B) Quantitation of the percentage of acinar structures of all colonies (left). MDA-MB-468 cells infected with an empty vector or with a retrovirus encoding ErbB4 JM-a CYT-1 were cultured in Matrigel for seven days in the presence of 0 or 100 μM DMOG. Columns represent the mean +/– standard error of nine replicates from four independent experiments. Expression of ErbB4 (E200), HIF-1α (clone 57), and actin (sc-1616) was controlled by Western blotting (right). C) Quantitation of acinar colonies formed by the MDA-MB-468 transfectants as in B (left). The cells were infected with an empty vector or a retrovirus encoding ErbB4 JM-a CYT-1 together with a negative control siRNA (–) or HIF-1α-targeting siRNAs (#1 and #2). Columns represent the mean +/– standard error of eight replicates from two independent experiments. Expression of ErbB4 (E200), HIF-1α (clone 57), and actin (sc-1616) was controlled by Western blotting (right). All lanes are from a single gel.

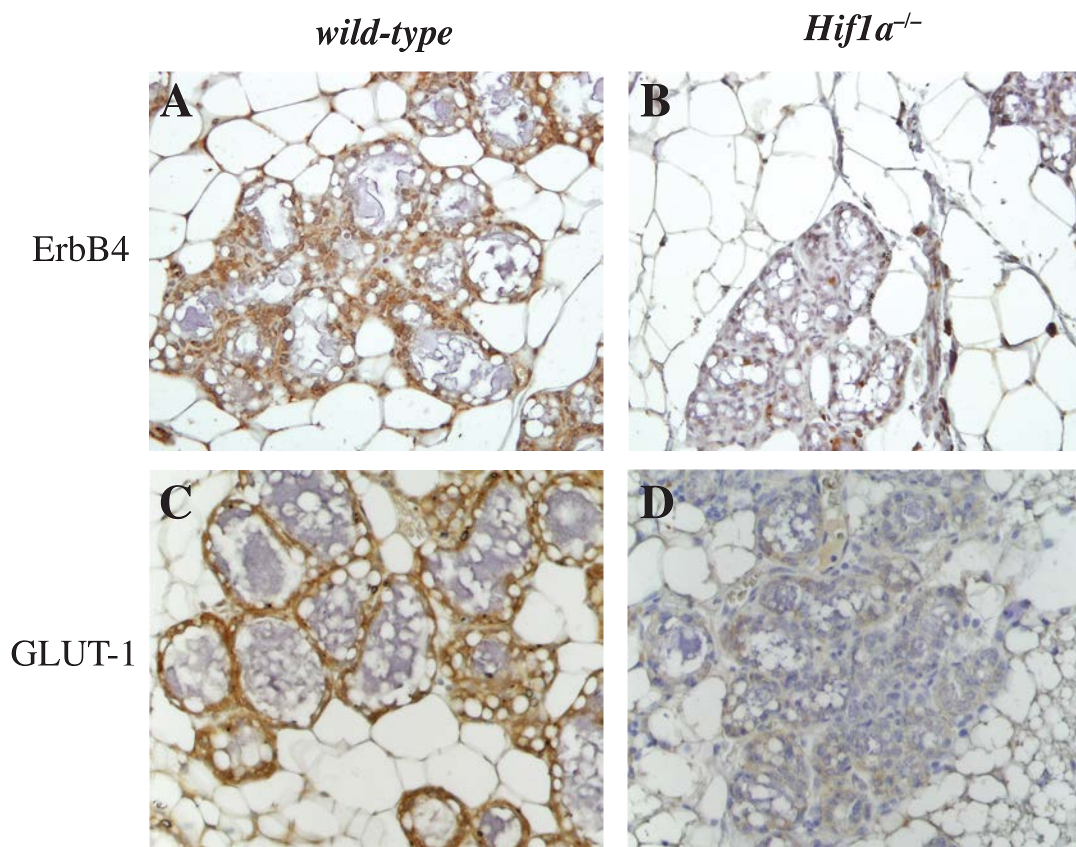
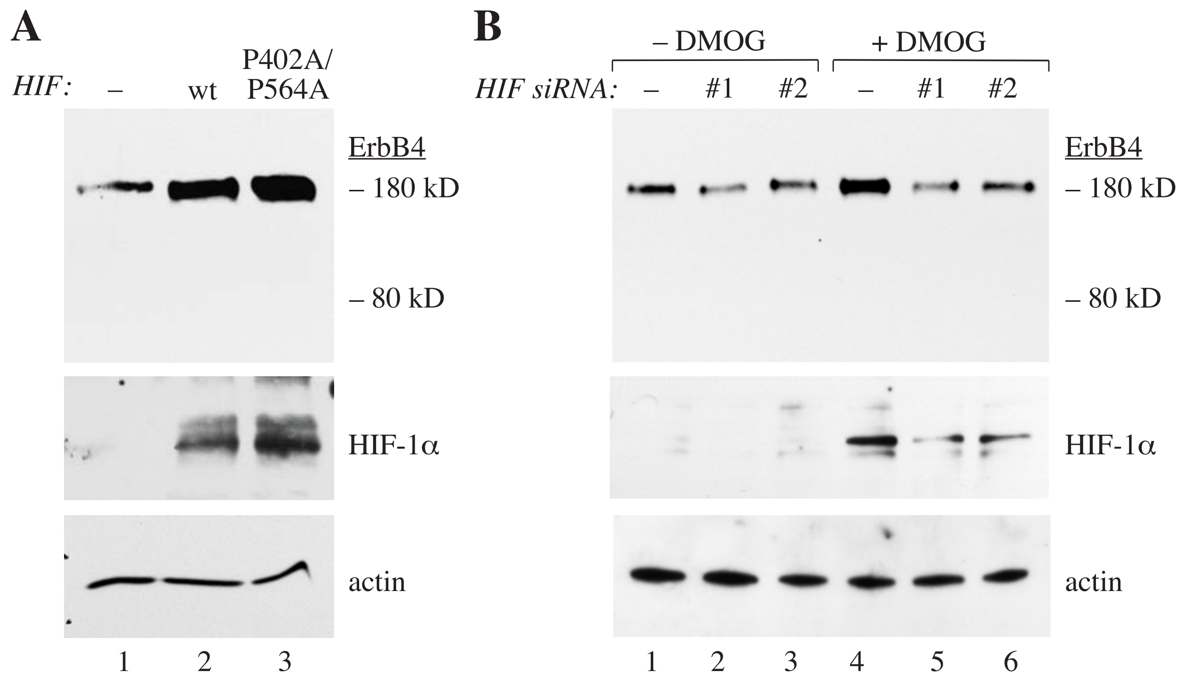


Fig. 1



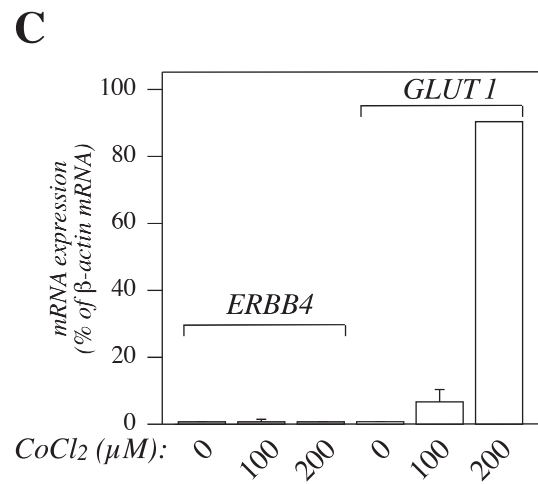


Fig. 2

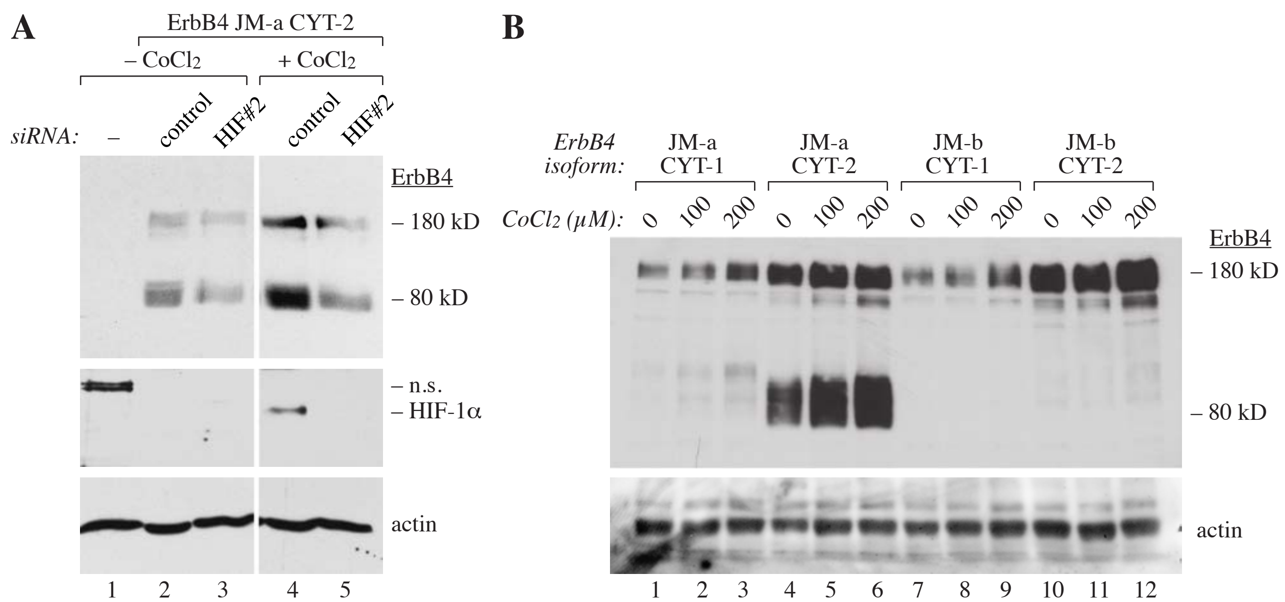


Fig. 3

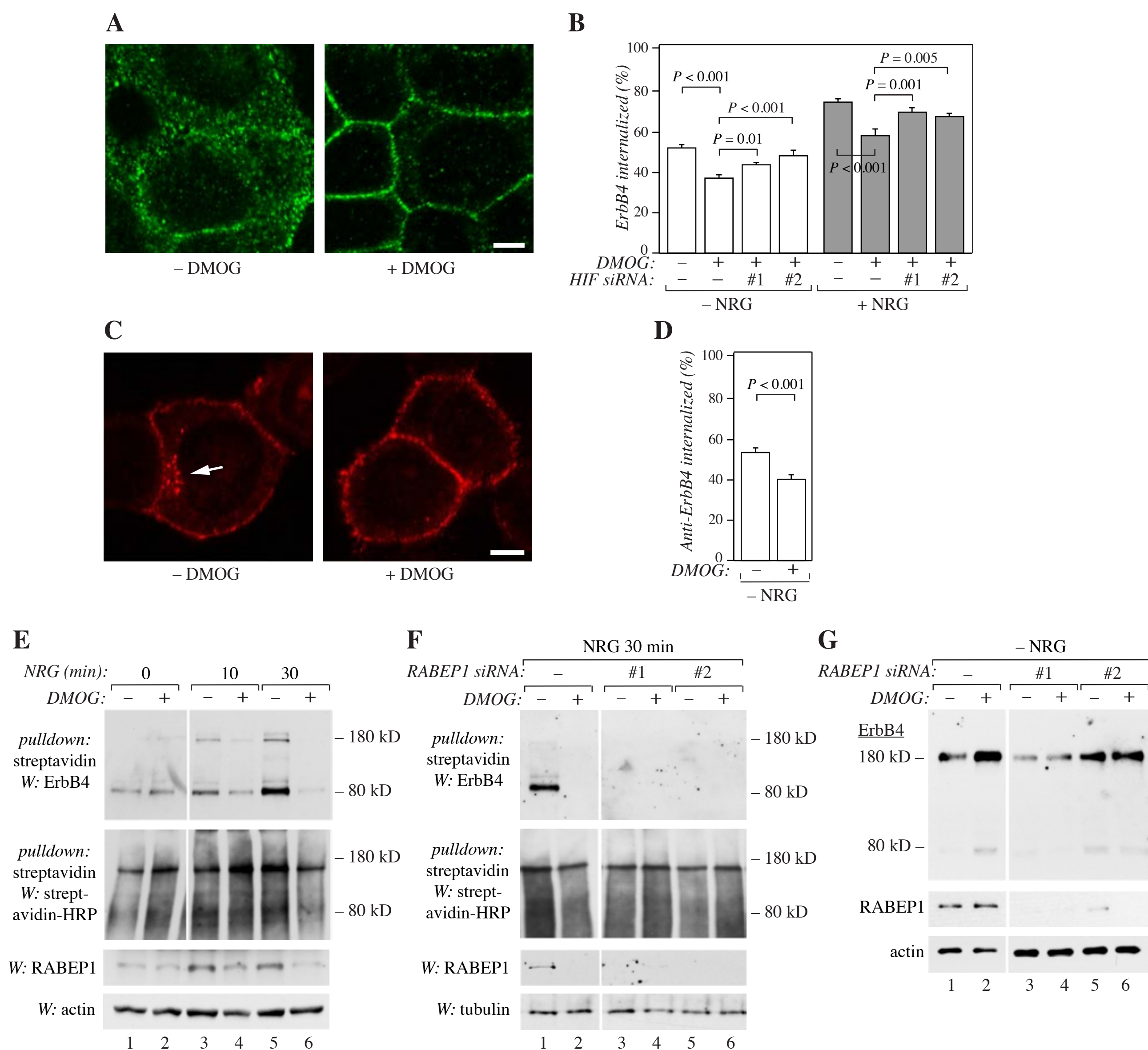
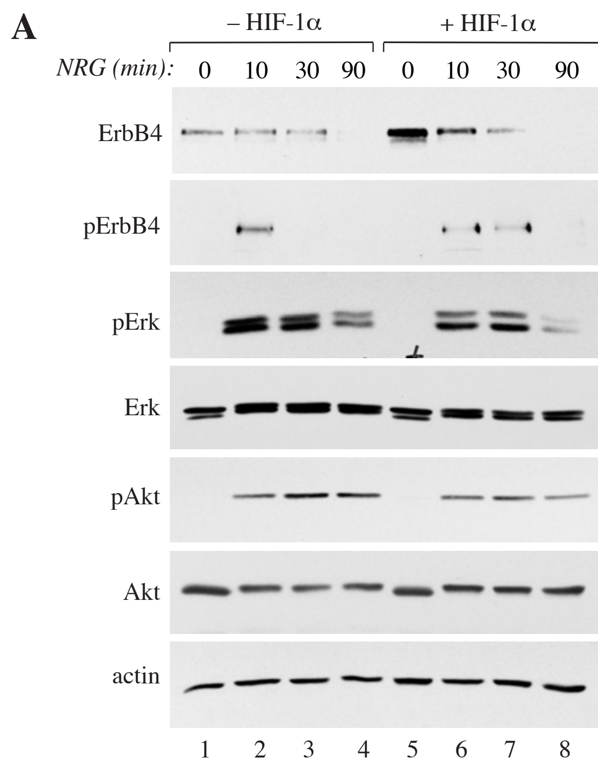
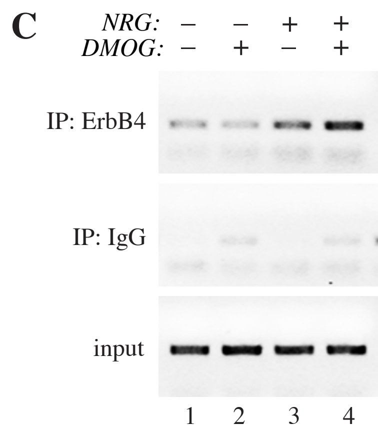


Fig. 4





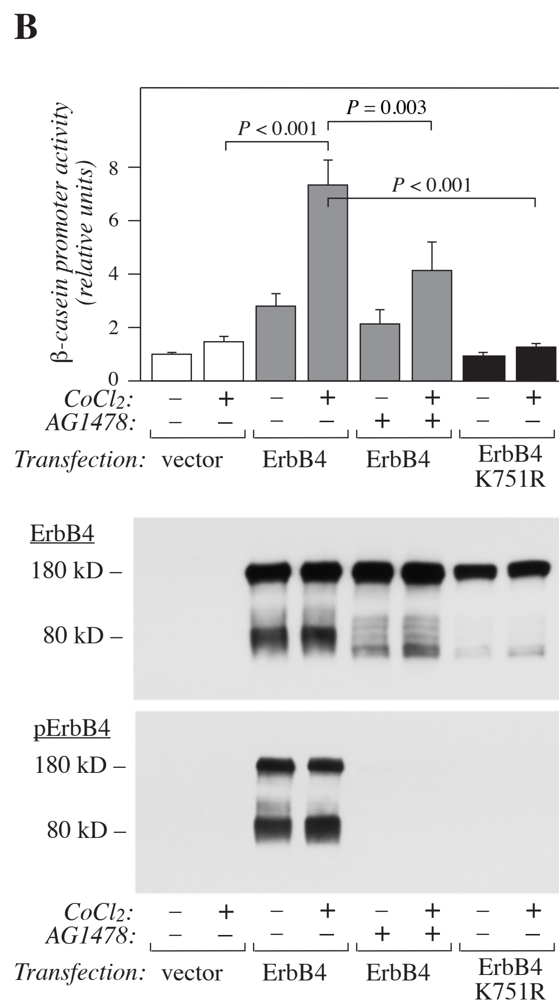


Fig. 5

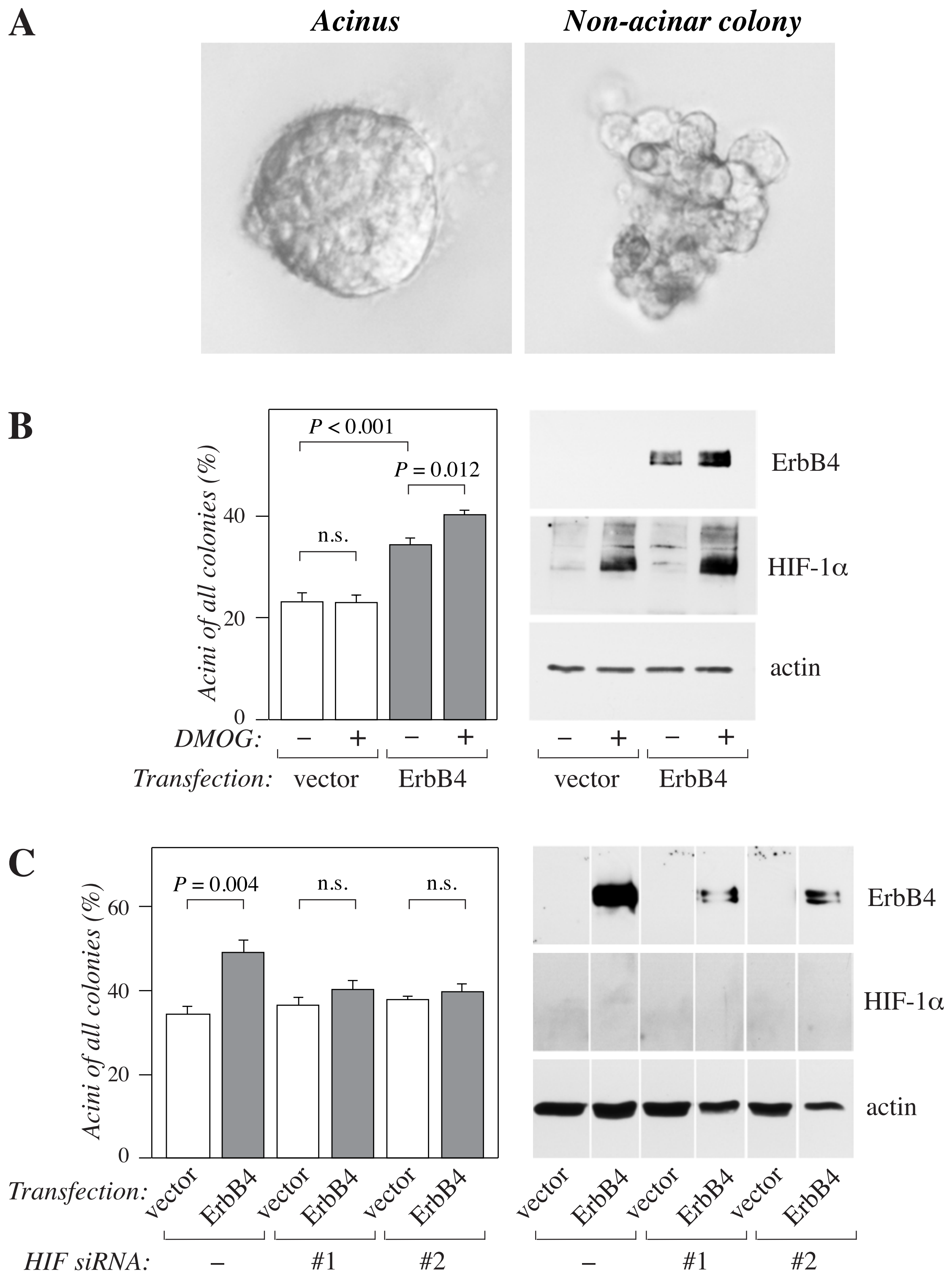


Fig. 6