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**Supplemental Information**

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## Supplemental Information

# Non-transcriptional role of Hif-1α in activation of γ-secretase and Notch signaling in breast cancer

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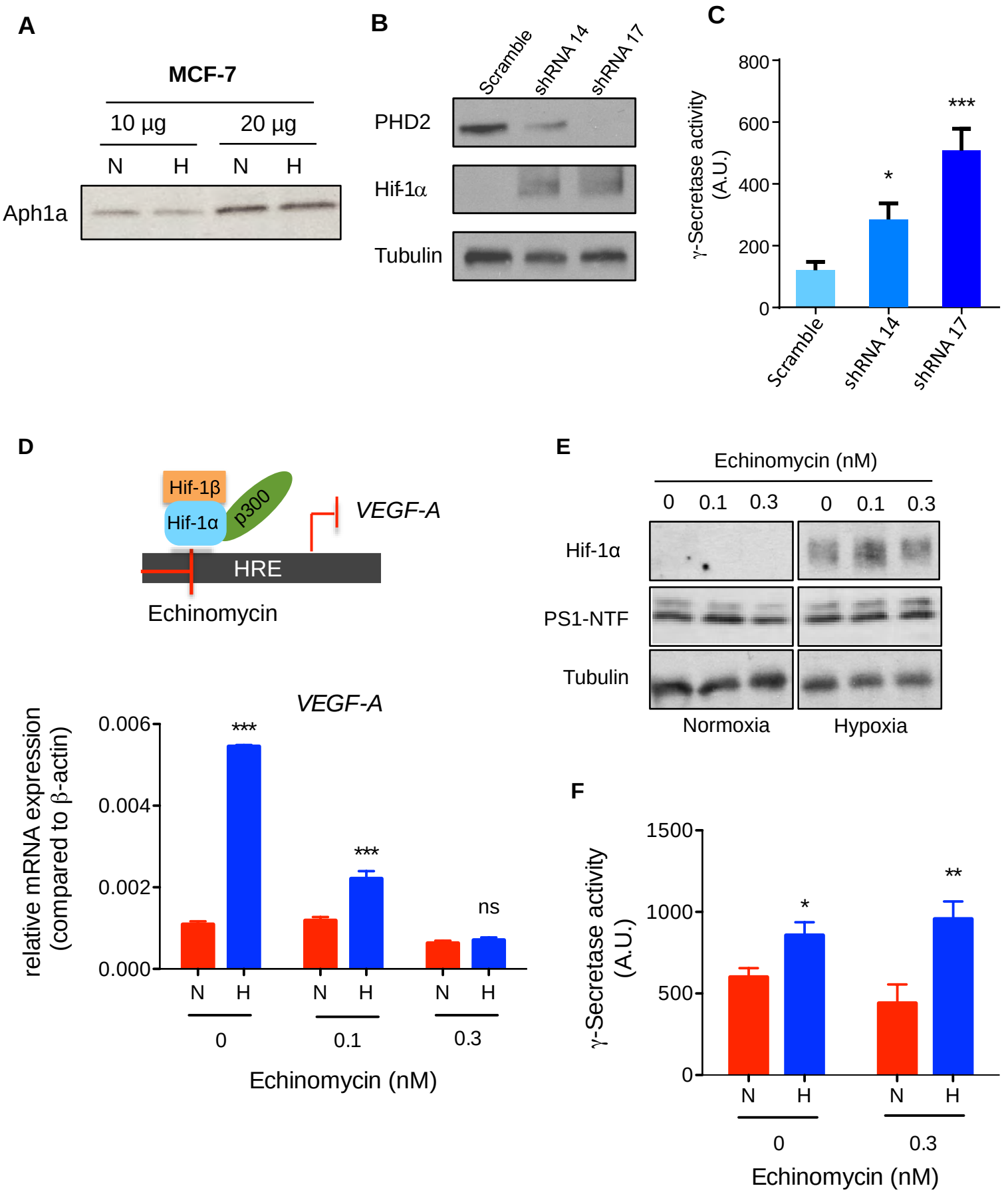
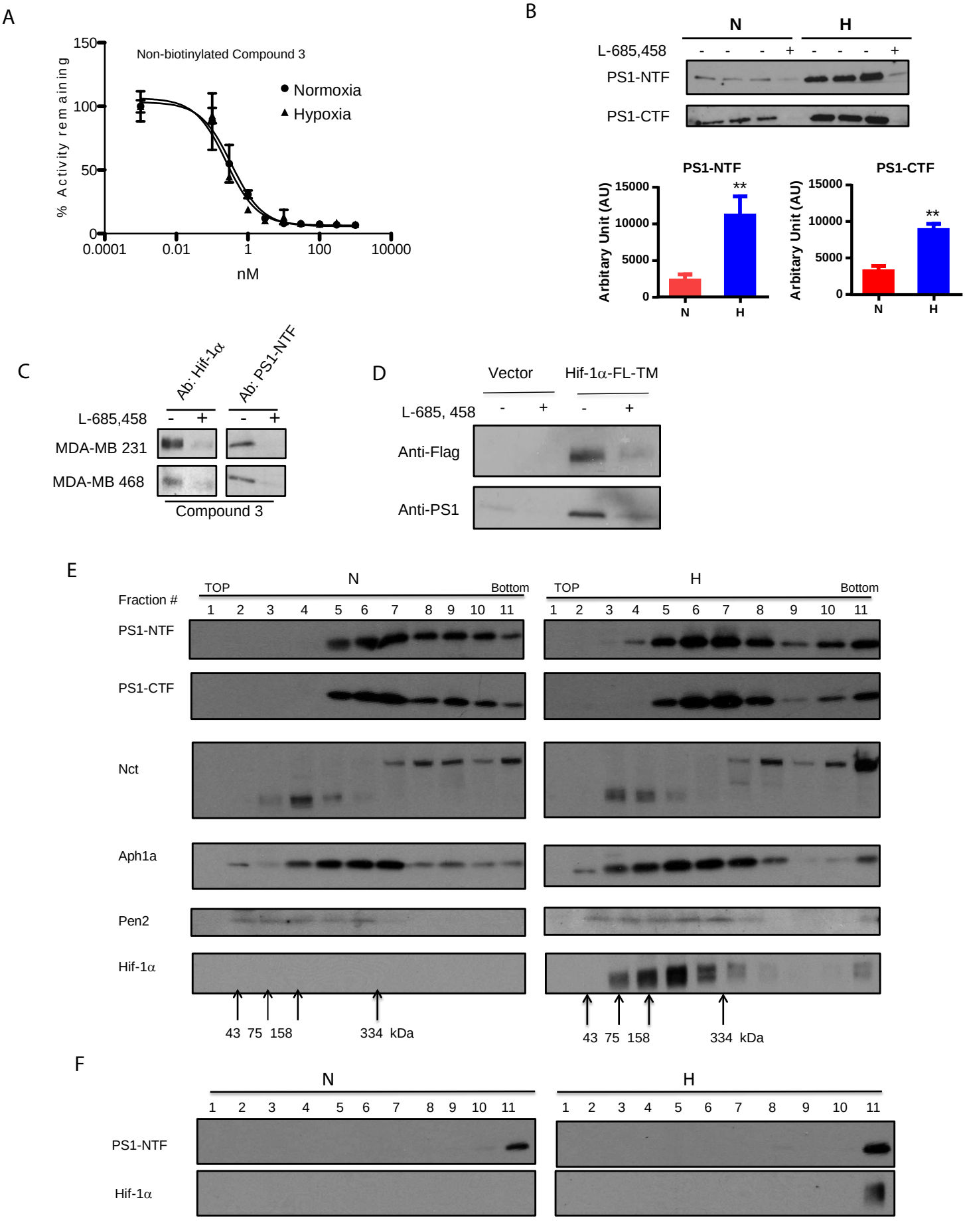
Supplementary materials include

1. Supplementary Figures S1 to S5 and Legends
2. Supplementary Procedures

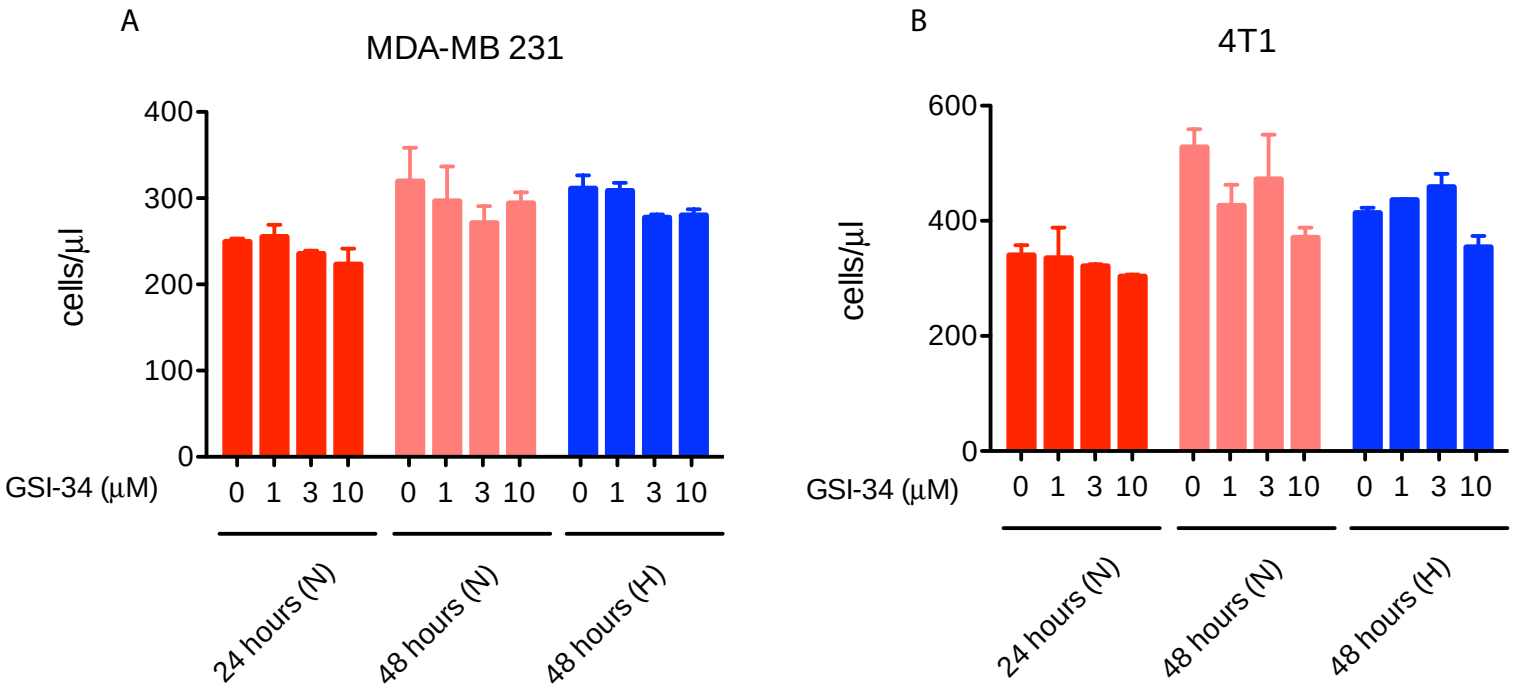
Supplemental Fig. S1



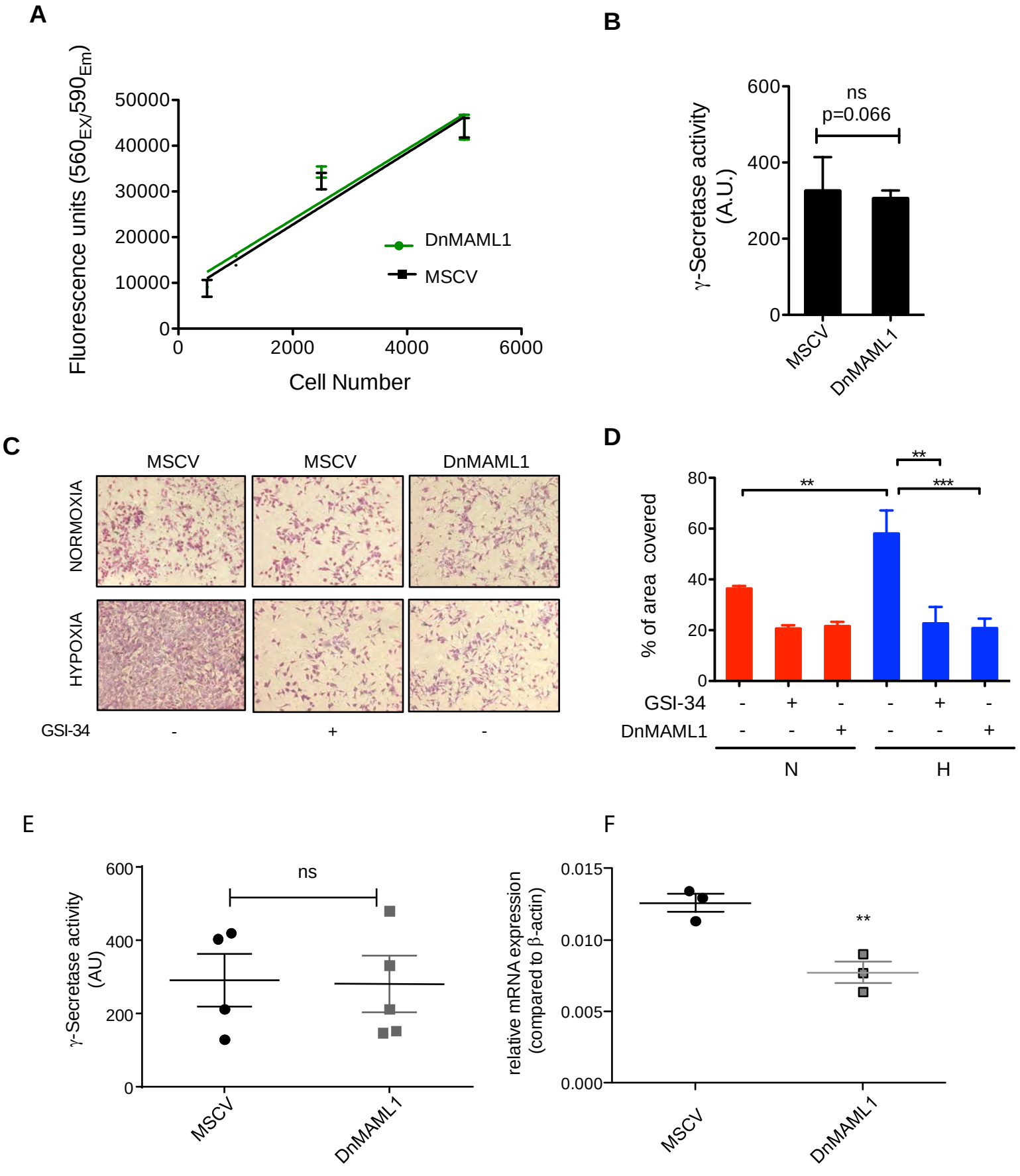
Supplemental Fig S2



Supplemental Fig. S3



Supplemental Fig. S4



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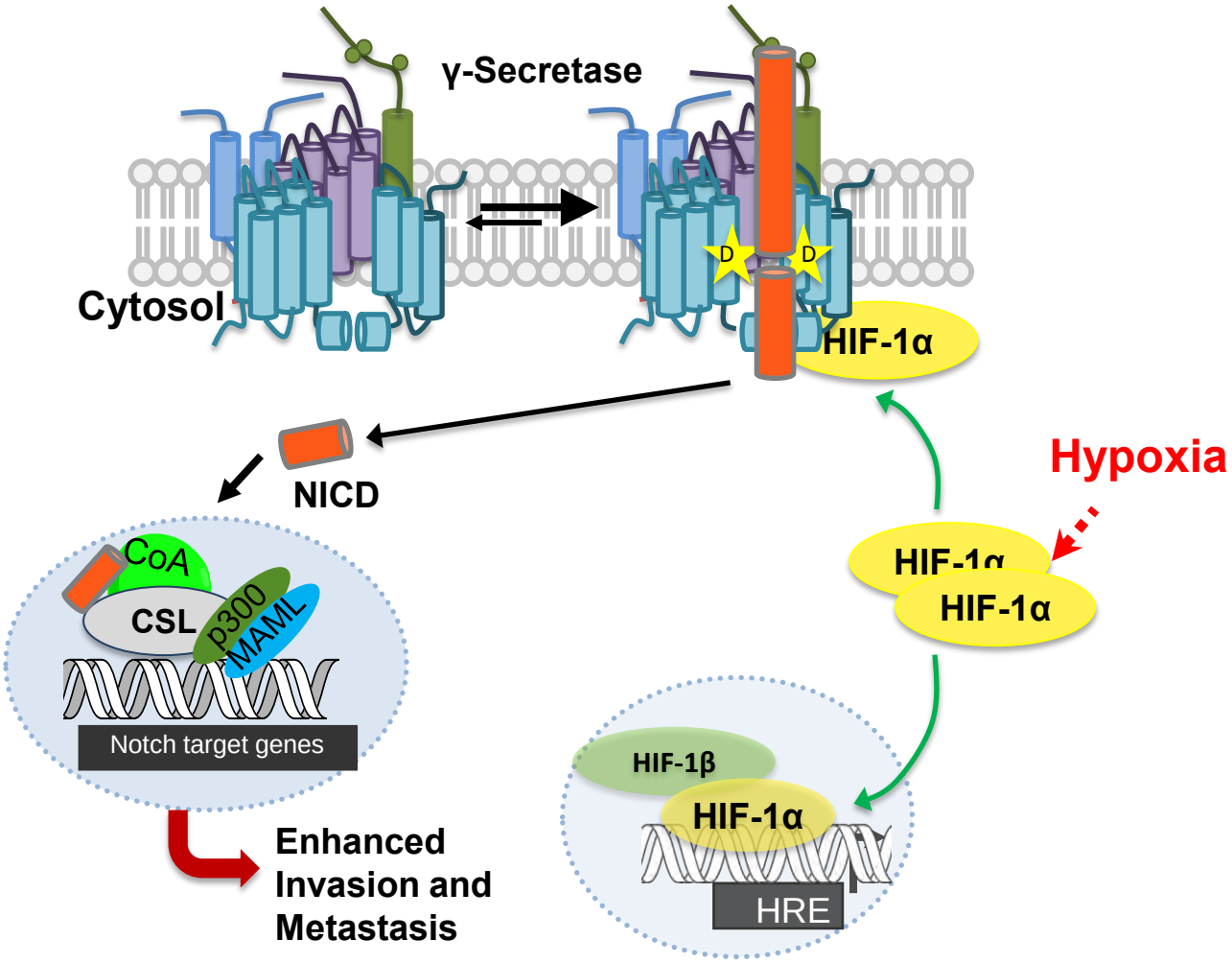

## Supplementary Figure Legends

**Figure S1. Related to Figure 2 and Figure 3. Effect of Hif1α on γ-secretase**

**(A)** Hypoxia does not increase the level of γ-secretase component Aph1a. Titration of total protein lysate from normoxic and hypoxic MCF-7 cells show that hypoxia does not stimulate the expression of Aph1a. Protein is loaded at 10 μg and 20 μg to validate dynamic range of detection. **(B)** Knockdown of PHD2 with shRNA14 and shRNA 17 and accumulation of Hif-1α were confirmed by western blot analyses. **(C)** Knockdown of PHD2 increased the level of γ-secretase activity shown in the exo-cell activity assay. Data are represented as arbitrary units (A.U.); n=3, and expressed as mean (±s.d.). Un-paired t-test, *P<0.05, *** P<0.001 (**D**). Schematic representation of Hif-1α transcriptional activity repression by echinomycin intercalating the complex binding to HRE resulting in downregulation of target genes, such as *VEGF-A* expression (Top). Quantitative RT-PCR analysis for Hif-1α target gene *VEGF-A* in echinomycin treated 4T1 cells grown in normoxia or hypoxia (Bottom). Data are expressed as relative mRNA level compared to β-actin, n=3, and plotted as mean ± (s.d.). Un-paired t-test, ***P<0.001. **(E)** Effect of echinomycin on the protein expression of Hif-1α and PS1-NTF represented by western analysis in 4T1 cells in normoxia and hypoxia. **(F)** Effect of echinomycin on γ-secretase activity using exo-cell assay in 4T1 cells treated under normoxia or hypoxia as measured by the cleavage of N1-Sb1 substrate. Data are expressed as arbitrary units (A.U.); mean ± (s.d.) and n=3. Unpaired t-test. *P<0.05, **P<0.01.

**Figure S2. Related to Figure 4. Hif-1α is associated with the γ-secretase complex**

**(A)** Exo-cell γ-secretase assay shows that non-biotinylated Compound 3 has the same IC50 values of 0.3 nM in normoxia and hypoxia. (**B**) Quantitative analysis of the amounts of the captured PS1-NTF and PS1-CTF by Compound 3 in both normoxia and hypoxia. The affinity capture was performed in the absence and the presence of L685,458. The captured catalytic subunit was analyzed by antibodies against PS1-NTF and PS1-CTF (top )

and quantitated by ImageJ for statistical analysis (bottom) Unpaired t-test. **P<0.01. **(C)** Compound 3 captured Hif-1α and PS1-NTF in the hypoxic lysates of MDA-MB 231 and MDA-MB 468 cells, which is blockable by L-685, 458. **(D)** Compound 3 captured both PS1-NTF and Hif-1α when Flag-tagged Hif-1α-FL was overexpressed under normoxia. **(E)** Analyses of Hif-1α and γ-secretase subunits after glycerol gradient fractionation. Each fraction (100μL) was precipitated by cold acetone, dissolved in the sample buffer and finally subjected to Western blot analysis against Hif-1α and γ-secretase. **(F)** Co-capture of PS1 and Hif-1α. Each fraction (200μL) was captured with compound 3, eluted by the sample buffer, and finally subjected to Western blot analysis against Hif-1α and PS-1 NTF.

**Figure S3. Related to Figure 5. GSI-34 treatment does not affect breast cancer cell proliferation**.

The effect of GSI-34 treatment on the proliferation of MDA-MB 231 cells (left), and 4T1 cells (right) after 24 hours and 48 hours. For the first 24 hours, cells were treated and cultured in normoxia and then transferred either to normoxia or hypoxia for another 24 hours with GSI-34 treatment. GSI-34 did not have significant effect on the number of live MDA-MB 231 or 4T1 cells.

**Figure S4. Related to Figure 7. Effect of downregulation of Notch signaling by DnMAML on 4T1 cell proliferation and migration**

**(A)** Comparison of proliferative rates between vector control 4T1 and DnMAML transduced 4T1 cells using Alamar Blue proliferation assay. Data are expressed as fluorescence units (560 $_{EX}$/590 $_{Em}$), n=3 and mean (±s.d). **(B)** Effect of DnMAML expression in 4T1 cells on the activity of γ-secretases validated using exo-cell assay and AlphaLISA detection for N1-Sb1 cleavage. Data are expressed as arbitrary units (A.U.) and experiment was performed in triplicates, n=3. Unpaired t-test indicates that the difference in γ-secretase activity between vector and DnMAML bearing 4T1 cells is not statistically significant (P=0.066). **(C)** Downregulation of Notch signaling by DnMAML1 decreased invasiveness in vitro. Matrigel invasion assay shows that hypoxia increased the invasive potential of 4T1 cells. Invasion was abrogated with GSI-34 treatment under hypoxia but was less significant in

normoxia. DnMAML1 4T1 cells exhibited similar invasiveness as cells treated with GSI-34. **(D)** The quantitation of invasion by Image J analysis under normoxia and hypoxia (Average ± SD n=3). Cell invasion expressed as % of area covered using Image J analysis; n=3, mean (±s.d.), un-paired t-test and one-way ANOVA used: **P<0.01, and ***P<0.001. **(E)** γ-Secretase activity assay using excised tumor membrane indicated that DnMAML1 and control tumors exhibited similar level of γ-secretase activity. **(F)** qRT-PCR analysis of RNA isolated from primary tumor samples showDnMAML1 tumors expressed decreased Notch effector Hes1 compared to control tumor samples (Average ± SEM, n=3).

**Figure S5. R**elated to discussion. Model of hypoxia mediated γ-secretase activation on Notch signaling.

Under hypoxic conditions, Hif-1α accumulates in the celland can travel to the nucleus and elicit transcription of Hif response genes. However, this study shows that Hif-1α can act outside of its canonical role as a temporal subunit of γ-secretase. The direct interaction of Hif-1α and γ-secretase drives the formation of active γ- secretase from the inactive complexes. The elevation of of active complexes enhances the cleavage of Notch, thus upregulating the production of NICD. NICD participates in Notch signaling transcriptional complex by recruiting coactivators (CoAs) and the DNA binding transcription factor CSL to promote enhanced invasion and metastasis in breast cancer cells.

## Supplemental Experimental Procedures

**Cell Culture and chemicals** Human breast cancer cell lines MCF-7, MDA-231, MDA-MB 468 and mouse mammary cancer line 4T1 were maintained in DME Hg F-12 NEAA supplemented with 10% FBS and 1% penicillin and streptomycin. Cells were cultured in normoxia (21% $O_2$, 5% $CO_2$) or hypoxia (1% $O_2$, 5% $CO_2$) for 24 hours. Echinomycin was purchased from Sigma Aldrich.

**Exo-cell γ-secretase activity assay** Breast cancer cell lines were seeded in 96-well culture plates and incubated either under normoxic (21% $O_2$, 5% $CO_2$) or hypoxic (1% $O_2$, 5% $CO_2$) setting for 24 hours. Media was then removed and cells were washed with PBS. Cleavage assay mixture included a final concentration of PIPES Buffer (50 mM PIPES, pH 7.0, 150 mM KCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$), 0.25% CHAPSO detergent, protease inhibitor cocktail, N1-Sb1 substrate (0.4 μM), and 0.1% DMSO or L-685,458 at 1 μM final concentration. Cleavage assay was performed at 37°C for 2.5 hours. Product of N1-Sb1 cleavage was recognized by AlphaLISA detection comprised of anti-activated Notch antibody SM320, Protein A-conjugated acceptor beads, and Streptavidin-conjugated donor beads (Perkin Elmer) (Chau et al., 2012). Activity readout was expressed as arbitrary units. Specific activity was plotted as arbitrary units minus the background signal and normalized to protein concentration. Cell AlphaLISA assay was performed with RIPA buffer solubilized cell lysate of N1-ΔE-myc transfected cells. NICD1 generation was detected with SM320 antibody, biotinylated anti-myc antibody, and conjugated-beads as described above.

**Activity-based photoaffinity labeling and capture** Activity-based photoaffinity labeling was performed with breast cancer cell lines in 12-well tissue culture dish with 10 nM of JC-8 in Phosphate buffer saline (PBS) pH 7.4, 0.25% CHAPSO in the presence and absence 1μM of L-685,458. Covalent cross-linking of benzophenone moiety to γ-secretase was performed at 350 nm for 30 min (Li et al., 2000b; Shelton et al., 2009). Reaction was then solubilized with RIPA buffer for 1 hour at room temperature and streptavidin ultra-link beads (Pierce) were added to capture labeled PS1 overnight at 4°C. Beads were pelleted, washed and eluted by boiling in 2X Laemmli sample buffer. Ensuing samples were resolved on SDS-PAGE followed by western blotting with PS1-NTF antibody. Capture of the active γ-secretase complex was first performed under non-denaturing conditions using compound 3 (Placanica et al., 2009). Breast cancer cells were solubilized with 1% CHAPSO in PBS containing protease inhibitors for 1 hour at 4°C (Li et al., 2000a). Solubilized γ-secretase was diluted with PBS to a final CHAPSO concentration of 0.25% (v/v) and captured by Compound 3 (20 nM) in the absence and presence of excess L-685,458. Then, the Compound 3-γ-secretase complex was isolated with Streptavidin-beads and eluted in 50 mM Tris-HCl pH 6.8, 10% β-mercaptoethanol, 0.5% SDS buffer, and 10% glycerol for 10 minutes at room temperature, and loaded onto SDS-PAGE for western blot analysis.

**Reconstitution studies** Full-length Hif-1α was cloned into the pIAD16 plasmid (McCafferty et al., 1997) that contains a Maltose Binding Protein (MBP). The pIAD16-MBP-Hif-1α or pIAD16-MBP constructs were transformed into BL21 (DE3) E. coli strain and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.1mM) for 5 hours at 20°C. Recombinant proteins were isolated by

amylose resin. Breast cancer cells (MCF-7 and 4T1) were co-solubilized with MBP or MBP-Hif-1α in PIPES buffer containing 1% CHAPSO for 1 hour at 4°C. Solubilized fractions were obtained by ultracentrifugation at 100,000 x g for 30 minutes at 4°C. Supernatant was then diluted to 0.25% CHAPSO and N1-Sb1 substrate was added to assay γ-secretase activity as previously described. For activity-based photolysis, JC-8 was added to the supernatant with L-685,458 or DMSO and labeling procedure was similar to previously described protocol.

**Wound-healing migration and Matrigel transwell invasion assay** Cells were seeded to monolayer confluence and incubated overnight in normoxia (Liang et al., 2007). The following day, a wound was made across the well with a 200 μl pipette tip. Cellular debris was washed away and media was replenished with or without GSI-34. Cells were then incubated for 24 hours under 21% or 1% oxygen. The following day, cells were washed briefly, and then stained with 2% crystal violet solution. Images were taken with a camera attached to a bright-field microscope. Wound space was quantitated using the ImageJ software for stained cells and defined as % of area covered given a constant area. BD Transwell inserts with 10 μg of Matrigel extracellular matrix (BD) (Li, 2001) was used for cell invasion assays. Cells in the upper chamber were seeded in media without serum and allowed to invade through the matrix for 24 hours in normoxia or hypoxia. Bottom of tissue culture wells were supplied with media containing serum. The following day, non-invaded cells in the upper chamber were removed by cotton swabs and washed with PBS. The underside of the insert was fixed by 4% paraformaldehyde in PBS and stained with 2% crystal violet solution, washed, and dried. Imaging and quantitation were the same as described above. Flash frozen primary tumor samples were thawed and homogenized in MES buffer with protease inhibitors using a Dounce homogenizer. Subsequently, debris was pelleted briefly and cleared membrane lysate was quantitated using the Biorad DC assay.

**Mouse mammary metastasis model and bioluminescence imaging**. 4T1 cells were prepared in PBS prior to injection. $1x10^6$ 4T1 luciferase expressing cells were injected into the mammary fatpad of 4-8 week old Balb/C female mice using sterile surgical methods. GSI-34 treated animals commenced on day 5 post 4T1 injection. Treatment continued 3-times a week for a two weeks period, and an additional treatment on day 19. Primary tumor size was measured on designated days with a caliper and translated to volume measurements by using the ellipsoid formula (LxW2)/2. To monitor metastatic progression, animals were injected by IP with D-luciferin (AnaSpec) at 1mg/mouse dissolved in saline solution. Imaging of each treatment group was consistently timed for 7 minutes post injection to ensure equal luciferin distribution. *In vivo* bioluminescent imaging was performed with the IVIS-200 optical imager (Xenogen) and acquired and analyzed by the Living Image 2.0 software (Caliper Life Sciences). Animals were sacrificed by $CO_2$ inhalation and lungs were harvested subsequently within a 20-minute period post initial luciferin injection to prevent signal degradation. Gross lung specimen was then imaged with the IVIS imager. Subsequently, lungs and primary were fixed in 4% paraformaldehyde (v/v) in PBS or flash frozen for additional analysis by histology or *in vitro* assays.

**Immunohistochemistry** Following fixation, samples were washed 3 times with PBS and incubated overnight at 4°C with 70% ethanol (v/v). Tumor and lung specimen were processed by the Molecular Cytology Facility and analyzed by the Comparative Pathology Facility at MSKCC. Tumor and lung samples were paraffin embedded and sections mounted to glass slides for immunohistological analysis. H&E staining was used to distinguish 4T1 metastasis in lung samples. Tumor samples were also stained for hypoxia marker Glut-1.

**Luciferase gene-reporter assay** MDA-MB 231 cells were co-transfected with wt8xCBF-TA-Fireflyluciferase (gift from Charles Eberhart, Johns Hopkins University); N1-ΔE-myc (gift from Dr. Raphael Kopan, University of Washington, St. Louis); and pGaussialuciferase vector, (gift from Dr Ronald Blasberg, MSKCC) as normalizing control using Fugene 6 transfection reagent (Promega). Transfected cells were then treated with GSI-34 and incubated in normoxia or hypoxia for 24 hours. Firefly- luciferase activity was measured using the luciferase gene assay kit (Roche) according to manufacturer's instructions and normalized to signal from secreted gaussia luciferase in the cell culture media.

**Western Blot and antibodies** Cells were lysed in RIPA buffer (50mM Tris, pH8.0, 150nM NaCl, 0.1% v/v Nonidet P-40, and 0.5% wt/v deoxycholic acid) with protease inhibitor cocktail. Protein lysate was separated by SDS-PAGE and transferred onto PVDF membrane (Millipore). Protein concentration was determined by the DC assay kit (Biorad). Antibodies for γ-secretase components are as follows: PS1-NTF and Nct antibodies were both produced from our laboratory, PS1-CTF (Millipore, MAB5232), Aph1a (Invitrogen, 38-3600), Pen2 (Abcam, 18189). Human Hif-1α antibody was purchased from Becton Dickinson (BD, 619958), and Mouse Hif-1α antibody (R&D, MAB1536). SM320 (activated Notch antibody) was generated in our laboratory (Chau et al., 2012). Mouse secondary antibodies were from Amersham and Ant-rabbit secondary was from GE. Standard electrochemiluminescence detection method was used.

**Immunoprecipitation** Immunoprecipitation assays were performed in lysates of MCF-7 cells co-transfected with HA-Hif-1α-ΔODD, and Flag-NICD1. Cells were lysed in lysis buffer containing 0.5% NP40, 50 mM Tris pH 7.4, 150 mM NaCl, and 1mM EDTA with protease inhibitor cocktail. Cell lysates were precleared with mouse IgG, (Vector Labs, I-2000), followed by immobilized Protein-G slurry (Pierce). Precleared lysates were then used for immunoprecipitation with anti-HA antibody (Covance, MMS-101R), anti-Flag antibody (Sigma, F9291), or mouse IgG control for 2 hours at 4°C, followed by Protein-G isolation overnight at 4°C. Beads were then washed 3X with NP40 lysis buffer, and eluted by boiling in Laemmli sample buffer. Immunoprecipitated proteins were then analyzed using SDS-PAGE and western blotting with HA and Flag antibodies.

**RNA isolation and real-time RT-PCR** Total RNA was isolated with the Qiagen RNeasy mini kit according to manufacturer's protocols. 1 μg of RNA was reverse transcribed to cDNA using the Superscript III 1$^{st}$ strand synthesis kit (Invitrogen). qRT-PCR analysis was performed with designated cDNA samples, Taqman Gene Expression Assay (Applied Biosystems). RNA samples were normalized to housekeeping gene β-actin. All real-time quantitative PCR was performed in triplicates on the Fast 7500 Real-time PCR system (Applied Biosystems). Prevalidated Taqman primers are as follows: Hes1 (Mm01342805_m1), VEGF-A (Mm01281449_m1), β-actin (Mm01205647_g1) from Applied Biosystems. Relative quantitation comparison between samples was made using the $\Delta\Delta C_T$ method.

**Cell count and Proliferation assay** Cells were treated with or without GSI-34 in triplicates for 24 and 48 hours. Cells were then trypsinized and stained with Trypan Blue (Thermo Scientific) reagent and counted for live cells using a hemocytometer. To access the proliferative rates of DnMAML1-4T1 and control cells, cells were seeded in increasing numbers overnight and incubated with alamarBlue reagent (AbD Serotec) diluted in media for 2 hours until color development is visible. Colorimetric readings for absorbance were detected using the SpectraMax M2 (Molecular Devices) at 600 nm.

**Viral transduction and cell transfections** Retroviral production was performed by the cotransfection

of packaging components containing VSV-G plasmid and pCL-Ampho packaging vector (gift from Dr. Pengbo Zhou, Weill Cornell Graduate School) with MigR1-GFP or MigR1-DnMAML1-GFP constructs (gift from Dr. Warren Pear, University of Pennsylvania) (Liu et al., 2010) into 293T cells. 4T1 cells were transduced with retrovirus for 48 hours and sorted for GFP expression by the Flow Cytometry Core Facility (MSKCC). Lentiviral production for knockdown studies and infection was performed by cotransfection of Hif-1α shRNA, or Hif-1β constructs obtained from the High-throughput Screening core facility (MSKCC) or non-targeting scramble shRNA control (Sigma, SHC002) with lentiviral packaging mix (Sigma) according to manufacturer's instructions. Hif-1α targeting shRNA oligos generated based on reference sequence NM_001530 are as follows:

#10: CCGGTGCTCTTTGTGGTTGGATCTACTCGAGTAGATCCAACCACAAAGAGCATTTTT

#90: CCGGCCGCTGGAGACACAATCATATCTCGAGATATGATTGTGTCTCCAGCGGTTTTT

#91: CCGGCCAGTTATGATTGTGAAGTTACTCGAGTAACTTCACAATCATAACTGGTTTTT

Hif-1β targeting shRNA oligos generated based on reference sequence NM_001669 are as follows:

#598: CCGGGCCTACACTCTCCAACACAATCTCGAGATTGTGTTGGAGAGTGTAGGCTTTTT

#599: CCGGCCTTTGTCTTTCTGTGTACTTCTCGAGAAGTACACAGAAAGACAAAGGTTTTT

Infection was performed over a 48-hour period and stable cells were selected for puromycin resistance for 10 days. Transient transfection of N1-ΔE-myc into breast cancer cells was performed using Fugene 6 reagent (Promega). Upon transfection, cells were then transferred immediately to normoxia or hypoxia for 24 hours. HA-Hif-1α-ΔODD-pcDNA3 (Addgene) or empty pcDNA3 control plasmid were transiently transfected using similar procedures described above.

The normoxia-stable Hif-1α-Triple Mutant-Flag-pcDNA3.1 (three point mutations at P420A, P557A, N813A), Hif-1α-N-terminal-Flag-pcDNA3.1 (amino acid 1-364), and Hif-1α-C-terminal-Flag-pcDNA3.1 (deletion of amino acid 10-370) constructs were obtained from Dr. Celeste Simon at University of Pennsylvania and have been previously described (Hu et al., 2007). Hif-1α-mutant bHLH-Flag-pcDNA3.1 was also provided by Dr. Celeste Simon and contains three point mutations at P420A, P557A, N813A to ensure stability in normoxia and contains four point mutations in DNA-binding residues (R24A, R26A, R27A and K29A) so it is unable to activate transcription, as has been previously described (Hu et al., 2006). MCF-7 cells were transfected with the normoxia-stable Hif-1α constructs using Fugene-6 (Promega). After 48 hours of transfection, cells were washed with PBS and the exo-cell γ-secretase activity assay was preformed (as previously described in Methods).

**Co-immunoprecipitation of Hif-1α via PS1 antibody** Human breast cancer cell line MCF-7 were seeded in 6-well culture plates and incubated under hypoxic conditions (1% O2, 5% CO2) overnight. Media was removed and cells quickly were washed with cold PBS Cells were then solubilized with 1% CHAPSO containing protease inhibitors for 1 hour at 4 C. Cell lysate was cleared by ultracentrifugation at 100,000 g for 1 hour at 4 C. Ensuing supernatant was diluted down to 0.25% CHAPSO using IP buffer (50mM PIPES, pH7, 5mM MgCl2, 150mM KCl) and was incubated with either 2ug of purified anti-PS1-NTF antibody or control rabbit IgG antibodies overnight in 4 C under constant rotation. Protein-AG magnetic beads were added to the samples and allowed to incubate in room temperature for 2 hours. Magnetic beads were washed three times with wash buffer (TBS, 0.05% Tween, 0.5M NaCl) and once with ddH20. Samples are eluted with 2x Laemmli Buffer (50mM Tris-HCl pH 6.8, 0.5% SDS

buffer, 10% Glycerol) and incubated for 10 minutes at room temperature and then loaded onto SDS-PAGE for western blot analyses.

**Glycerol gradient fractionation of γ-secretase and Hif-1α:** Breast cancer cell line MCF-7 were cultured under normoxic and hypoxic conditions and solubilized γ-secretase lysates were prepared as described above. Resulting supernatant (1 ml at 0.5 % CHAPSO) was applied to the top of an 11ml 13-39% (w/v) linear glycerol gradient in velocity buffer (25mM HEPES, 150mM NaCl pH 7.2, 0.5% CHAPSO) (Gu et al., 2004). Gradients were fractionated by centrifugation at 34,700 rpm at 4 C for 18 hours using the Beckman SW41 rotor. Fractions were carefully collected in 1ml volume from the top of the centrifugation tubes. Each fraction was analyzed for the components of γ-secretase complex and Hif-1α and capture for the active γ-secretase complex.

## Supplemental References


Chau, D.M., Crump, C.J., Villa, J.C., Scheinberg, D.A., and Li, Y.M. (2012). Familial Alzheimer Disease Presenilin-1 Mutations Alter the Active Site Conformation of gamma-secretase. The Journal of biological chemistry *287*, 17288-17296.

Gu, Y., Sanjo, N., Chen, F., Hasegawa, H., Petit, A., Ruan, X., Li, W., Shier, C., Kawarai, T., Schmitt-Ulms, G., *et al.* (2004). The presenilin proteins are components of multiple membrane-bound complexes that have different biological activities. The Journal of biological chemistry *279*, 31329-31336.

Hu, C.J., Iyer, S., Sataur, A., Covello, K.L., Chodosh, L.A., and Simon, M.C. (2006). Differential regulation of the transcriptional activities of hypoxia-inducible factor 1 alpha (HIF-1alpha) and HIF-2alpha in stem cells. Mol Cell Biol *26*, 3514-3526.

Hu, C.J., Sataur, A., Wang, L., Chen, H., and Simon, M.C. (2007). The N-terminal transactivation domain confers target gene specificity of hypoxia-inducible factors HIF-1alpha and HIF-2alpha. Mol Biol Cell *18*, 4528-4542.

Li, Y.M. (2001). Gamma-secretase: a catalyst of Alzheimer disease and signal transduction. Mol Interv *1*, 198-207.

Li, Y.M., Lai, M.T., Xu, M., Huang, Q., DiMuzio-Mower, J., Sardana, M.K., Shi, X.P., Yin, K.C., Shafer, J.A., and Gardell, S.J. (2000a). Presenilin 1 is linked with gamma-secretase activity in the detergent solubilized state. Proc Natl Acad Sci U S A *97*, 6138-6143.

Li, Y.M., Xu, M., Lai, M.T., Huang, Q., Castro, J.L., DiMuzio-Mower, J., Harrison, T., Lellis, C., Nadin, A., Neduvelil, J.G., *et al.* (2000b). Photoactivated gamma-secretase inhibitors directed to the active site covalently label presenilin 1. Nature *405*, 689-694.

Liang, C.C., Park, A.Y., and Guan, J.L. (2007). In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc *2*, 329-333.

Liu, H., Chi, A.W., Arnett, K.L., Chiang, M.Y., Xu, L., Shestova, O., Wang, H., Li, Y.M., Bhandoola, A., Aster, J.C., *et al.* (2010). Notch dimerization is required for leukemogenesis and T-cell development. Genes Dev *24*, 2395-2407.

McCafferty, D.G., Lessard, I.A., and Walsh, C.T. (1997). Mutational analysis of potential zinc-binding residues in the active site of the enterococcal D-Ala-D-Ala dipeptidase VanX. Biochemistry *36*, 10498-10505.

Placanica, L., Tarassishin, L., Yang, G., Peethumnongsin, E., Kim, S.H., Zheng, H., Sisodia, S.S., and Li, Y.M. (2009). Pen2 and Presenilin-1 Modulate the Dynamic Equilibrium of Presenilin-1 and Presenilin-2 {gamma}-Secretase Complexes. The Journal of biological chemistry *284*, 2967-2977.

Shelton, C.C., Zhu, L., Chau, D., Yang, L., Wang, R., Djaballah, H., Zheng, H., and Li, Y.M. (2009). Modulation of gamma-secretase specificity using small molecule allosteric inhibitors. Proc Natl Acad Sci U S A *106*, 20228-20233.