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Jung-Mao Hsu

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CROSSTALK BETWEEN R1175 METHYLATION AND Y1173 PHOSPHORYLATION NEGATIVELY MODULATES EGFR-MEDIATED ERK ACTIVATION

By

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CROSSTALK BETWEEN R1175 METHYLATION AND Y1173 PHOSPHORYLATION NEGATIVELY MODULATES EGFR-MEDIATED ERK ACTIVATION

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DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

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in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

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Houston, Texas

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Abstract

Post-translational protein modifications are critical regulators of protein functions as they expand the signaling potentials of the modified proteins, leading to diverse physiological consequences. Currently, increasing evidence suggests that protein methylation is as important as other post-translational modifications in the regulation of various biological processes. This drives us to ask whether methylation is involved in the EGFR (epidermal growth factor receptor) signaling, a biological process extensively regulated by multiple post-translational modifications including phosphorylation, glycosylation and ubiquitination. We found that EGFR R1175 is methylated by a protein arginine methyltransferase named PRMT5. During EGFR activation, PRMT5-mediated R1175 methylation

specifically enhances EGF-induced EGFR autophosphorylation at Y1173 residue. This novel modification crosstalk increases SHP1 recruitment to EGFR and suppresses EGFR-mediated ERK activation, resulting in inhibition of cell proliferation, migration, and invasion of EGFR-expressing cells. Based on these findings, we provide the first link between arginine methylation and tyrosine phosphorylation and identify R1175 methylation as an inhibitory modification specifically against EGFR-mediated ERK activation.

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CHAPTER 1 INTRODUCTION

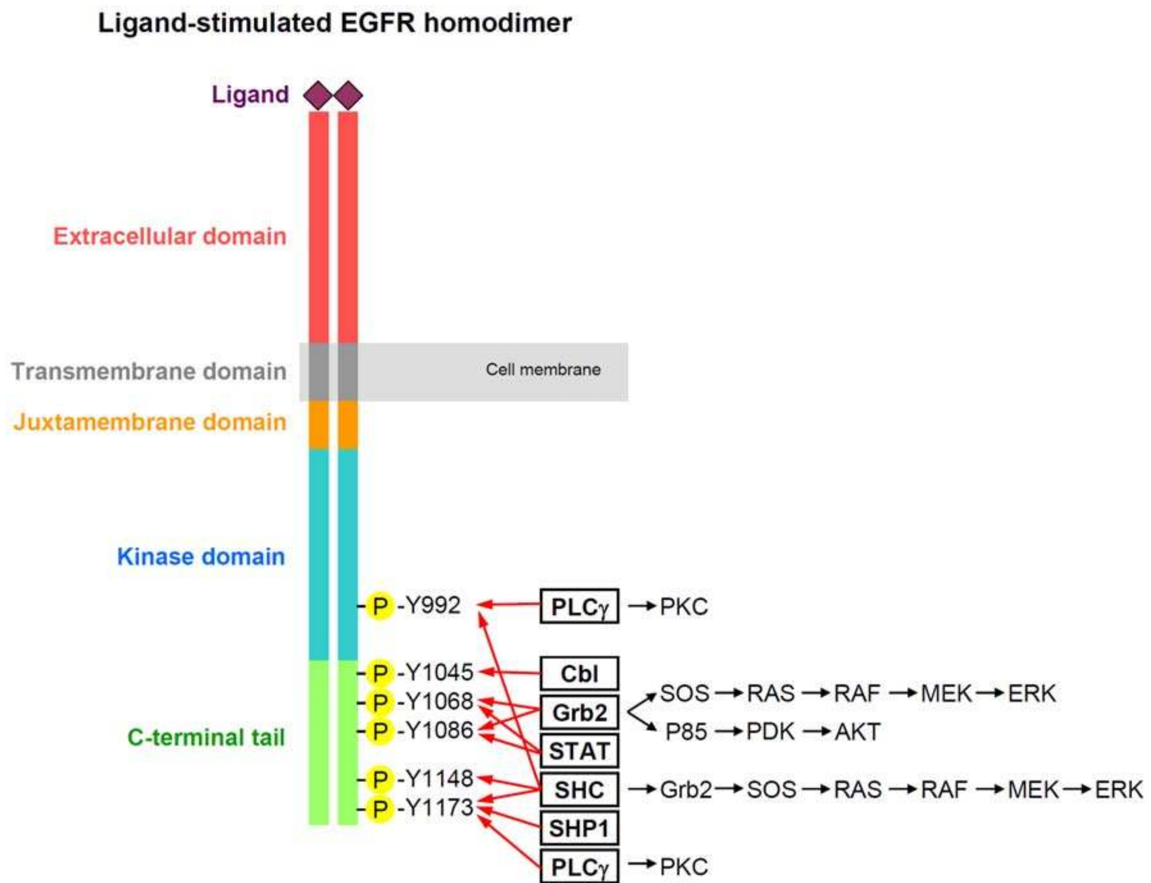
1.1 EGFR signaling and its biological effects

The epidermal growth factor receptor (EGFR) is a transmembrane cell-surface receptor of the ErbB (erythroblastic leukemia viral oncogene homolog) receptor tyrosine kinase family consisting of ErbB1 (EGFR), ErbB2 (Her2), ErbB3 and ErbB4. It is composed of an extracellular domain that provides ligand-binding sites, a single transmembrane domain and a cytosolic region that contains a juxtamembrane domain, a tyrosine kinase domain and a C-terminal tail segment[1, 2] (Figure 1). As a cell surface receptor, EGFR is able to convert extracellular cues into intracellular effectors, leading to specified cellular responses. A general accepted theory of EGFR activation is that binding of ligands, including EGF (epidermal growth factor), TGF-α (transforming growth factor-α), HB-EGF (heparin-binding EGF-like growth factor), AREG (amphiregulin), BTC (betacellulin), EPG (epigen) and EPR (epiregulin) to the EGFR extracellular domains causes structural change of the domains, exposure of dimerization arms and induces dimerization of two receptor monomers[2-6]. EGFR can form either homo-dimers or hetero-dimers with other family members[2, 7, 8]. Receptor

dimerization leads to activation of intrinsic tyrosine kinase domains[9] and subsequent autophosphorylations on multiple tyrosine (Y) residues of the C-terminal tail segments, including Y992, Y1045, Y1068, Y1086, Y1148 and Y1173[10] (Figure 1). These tyrosine phosphorylations create docking motifs for different cytosolic signaling molecules containing SH2 (Src homology 2) and PTB (phosphotyrosine binding) domains[11]. Through recruiting these molecules, EGFR initiates several downstream signaling cascades including the RAS -RAF-MEK-ERK pathway (Ras, rat sarcoma viral oncogene homologue; RAF, v-raf murine sarcoma viral oncogene homologue; MEK, MAPK/ERK activator kinase; ERK, extracellular signal-regulated kinase), the PI3K-AKT pathway (PI3K, phosphoinositide 3-kinase), the PLCγ-PKC pathway (PLCγ, phospholipase C-γ; PKC, protein kinase C) and the STATs (signal transducer and activator of transcription) pathway (Figure 1). These signalings finally culminate in cell proliferation, migration, adhesion, invasion, cell cycle progression and differentiation[4, 12-14].

EGFR has been shown to play important roles in development. In genetically engineered mouse models, mice lacking EGFR die within the first month of birth and multiple developmental defects can be observed in mammary duct, skin,

Figure 1. The EGFR signaling. Schematic representation of the functional domains of EGFR, the tyrosine (Y) phosphorylations induced by ligand stimulation and the downstream signaling cascades activated by recruiting cytosolic signaling molecules to the phospho-tyrosine residues. Red arrows indicate bindings between the phospho-tyrosines and the cytosolic molecules.



central nervous system, lung, pancreas and gastrointestinal tract[15-20]. EGFR has also been linked to stem cell renewal and proliferation[21-26]. Besides, aberrant EGFR activation caused by EGFR gene amplification, mutation and/or ligand overexpression is involved in the pathogenesis and progression of various cancer types, especially breast cancer, lung cancer and colon cancer[12, 27-36].

1.2 Regulation of EGFR signaling by post-translational modifications

Post-translation modifications (PTMs) play central roles in the activation and regulation of EGFR signaling. As mentioned above, ligand-stimulated EGFR tyrosine autophosphorylations are essential to transmit extracellular stimuli into intracellular responses. Each of the phospho-tyrosines and its flanking amino acid residues form a peptide motif to selectively bind the SH2 or PTB domains of one or more cytosolic signaling molecules. Similarly, with very few exceptions, each of the cytosolic signaling molecules binds EGFR through more than one phospho-tyrosine with different affinities[37-40] (Figure 1). Given that different ligand stimulations or dimerization partners can induce different tyrosine phosphorylation patterns on EGFR[41-43]. This redundancy in binding sites between phospho-tyrosines and cytosolic signaling molecules allows the

activation of various downstream signaling cascades to be combinatorially regulated by different stimulation conditions or cellular contents[38, 39]. In addition to the positive roles in initiating EGFR downstream signalings, autophosphorylated tyrosine residues also mediate inhibitory mechanisms against EGFR activity. One well-characterized example is Y1045, which serves as a docking site for Cbl (Casitas B-cell lymphoma), the primary E3 ubiquitin ligase of EGFR. Recruitment of Cbl to EGFR through phosphorylated Y1045 promotes receptor ubiquitination and degradation, resulting in downregulation of EGFR activity[44]. Receptor with defective Y1045 phosphorylation escapes from Cbl-induced ubiquitination and degradation[45-47].

Many other kinases also involve in the EGFR signaling through directly phosphorylating EGFR. Some of the best known include: growth hormone-activated JAK2 (Janus kinase 2) can phosphorylate EGFR at Y1068 and specifically trigger EGFR-mediated ERK activation [48]. Src phosphorylates EGFR at Y845 and Y1101, resulting in enhanced receptor signaling[49]. Serine/threonine phosphorylation by PKC, ERK and CaMKII (calmodulin-dependent protein kinase II) modulates receptor tyrosine kinase activity and internalization[50-52].

Besides phosphorylation, EGFR is also subjected to non-phosphorylation post-translational modifications. One of the well-known is N-glycosylation of the EGFR extracellular domain, which is essential for the maturation and membrane transport of nascent receptor, and for the ligand binding activity of mature surface receptor[53-57]. Moreover, the E3 ubiquitin ligase Cbl-mediated ubiquitination and neddylation have been demonstrated as a primary attenuation mechanism of EGFR signaling. Both modifications coordinately serve as sorting signals to promote lysosomal degradation of activated EGFR and control the duration of EGFR activation[58-63]. Recently identified acetylation of EGFR further expands the content of EGFR PTM network as acetylation was demonstrated to enhance endocytosis or tyrosine phosphorylation of the receptor[64, 65].

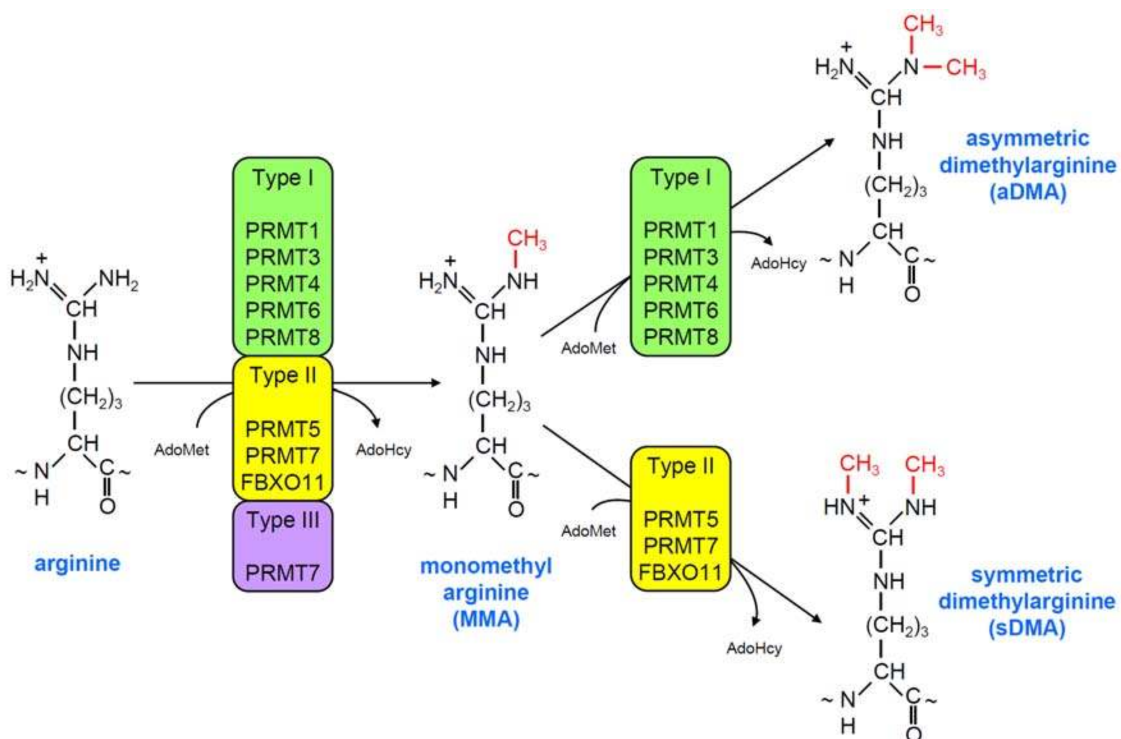
1.3 Protein arginine methylation in regulating cellular processes

Protein arginine methylation is a post-translational modification that adds one or two methyl groups to the guanidino nitrogen atoms of arginine. Methyl groups can be added to the internal (δ) or the terminal (ω) nitrogen atoms. So far, δ-N^G-methylarginine residues have been observed only in *Saccharomyces cerevisiae.* In eukaryotes, three types of methylarginine have been identified,

including ω-N^G-monomethylarginines (MMA), ω-N^G, N^G-asymmetric dimethylarginines (aDMA), and ω-N^G, N'^G-symmetric dimethylarginines (sDMA) [66-68] (Figure 2).

Arginine is a positively charged amino acid important in mediating hydrogen bonding and amino-aromatic interactions. Although methylation does not change the overall charge of an arginine residue, addition of methyl groups removes amino hydrogens that be involved in hydrogen bonds and increases steric hindrance. Therefore, arginine methylation may inhibit intra- or inter-molecular interactions. On the other hand, arginine methylation can also act as a positive regulator of protein-protein interactions. The Tudor domain has been recognized as a methyl-binding protein structure motif specifically recognizing di-methyl arginines. Several Tudor domain-containing proteins have been shown to interact with their binding partners in a methylarginine-dependent manner[69-71]. Besides protein-protein interactions, arginine methylation may modulate protein function through regulating the subcellular localization of targeting proteins[72-75]. Through these regulatory mechanisms, arginine methylation has been shown to be involved in transcriptional regulation, RNA processing, signaling transduction and DNA damage repair[67, 68, 76, 77].

Figure 2. Arginine methylation and the protein arginine methyltransferase (PRMT) family. Monomethylarginine (MMA) can be generated by all types of PRMTs. Type I and type II PRMTs further catalyze the formation of asymmetric dimethylarginine (aDMA) and symmetric dimethylarginine (sDMA), respectively. S-adenosyl methionine (AdoMet) is the methyl group donor and converted into S-adenosyl homocysteine (AdoHcy) after reactions.



1.4 Protein arginine methyltransferases (PRMTs), demethylase and deiminase

Protein arginine methylation is mediated by enzymes of the protein arginine *N*-methyltransferase (PRMT) family. All family members share a core arginine methyltransferases region which catalyze the transfer of a methyl group from the methyl donor, S-adenosyl methionine (AdoMet) to arginine[67, 68, 76-78]. PRMT members are further classified into four major groups according to the type of methylarginine they generate. Type I, II and III PRMTs methylate the terminal (ω) guanidino nitrogen atoms. Type I and type II PRMTs both catalyze the formation of MMA, and type I PRMTs further catalyze the production of aDMA, whereas type II PRMTs catalyze the formation of sDMA. Type III PRMTs catalyze only MMA (Figure 2). Besides, type IV PRMTs methylate the internal (δ) guanidino nitrogen atom of arginine[67, 68, 76, 77].

Currently, ten mammalian PRMTs have been identified within which PRMT1, 3, 4, 6 and 8 belong to type I PRMTs, whereas PRMT5, 7 and FBXO11 exhibit type II enzymatic activity. In addition, PRMT7 also shows type III activity on certain substrates. On the contrary, no activity has been demonstrated for PRMT2 and PRMT9, and no type IV enzyme has been identified in mammals to date [67, 68,

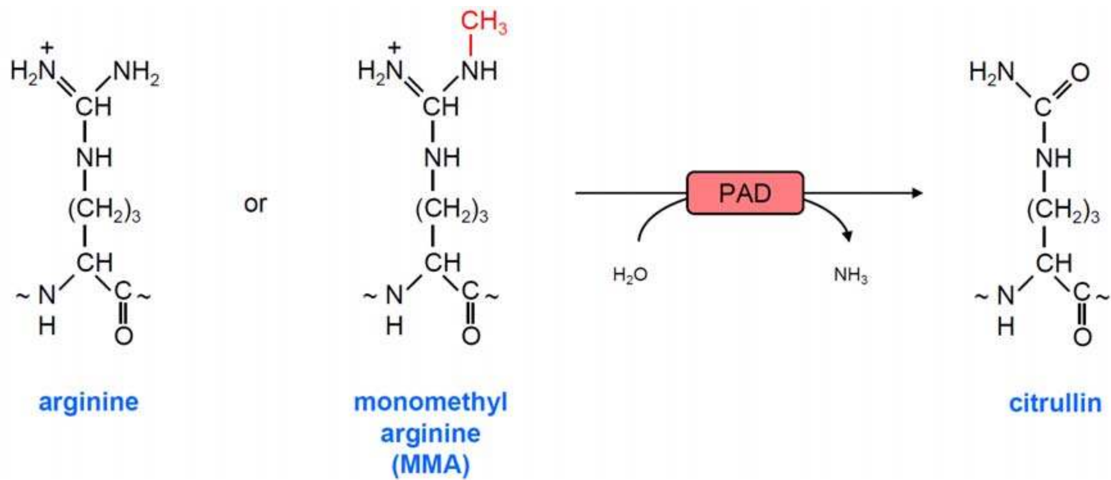
77] (Figure 2). Proteins harboring GAR (glycine- and arginine-rich) or PGM (proline-, glycine-, methionine-, arginine-rich) motifs are often candidate targets for PRMTs[69, 79].

In contrast to the action of PRMTs, methylation of an arginine can be removed by demethylase or deiminase. JMJD6 (Jumonji domain–containing 6 protein) is the only demethylase identified to data, which demethylates histone H3 at arginine 2 and histone H4 at arginine 3, and reverses methylated arginine back to arginine[80]. Besides, the peptidyl arginine deiminase can block methylation on an arginine residue by converting it to citrulline. PAD4 (peptidylarginine deiminase 4) deiminase has been shown to catalyze the deimination of both arginine and monomethylarginine, but not dimethylarginine, to citrulline and prevent dimethylation formation on histone H3 arginine 17 and histone H4 arginine 3 by PRMT4 and PRMT1, respectively[81] (Figure 3).

1.5 PRMT5

PRMT5 was originally cloned as a JAK2-binding protein with type II arginine methyltransferase activity[82, 83]. Subsequent investigations indicate that PRMT5 functions in various protein complexes localized to both the cytoplasm and the

Figure 3. Inhibition of arginine methylation by the peptidyl arginine deiminase (PAD). PAD blocks arginine methylation by converting arginine or monomethyl arginine (MMA) to citrulline.



nucleus. Nuclear PRMT5 is linked to several roles in transcription regulation. It forms complexes with the hSWI/SNF (human switch/sucrose non-fermenting) chromatin-remodeling proteins BRG1 (Brahma-related gene 1) and BRM (Brahma) to methylate histone H3 at arginine 8, resulting in the repression of tumor suppressor genes ST7 (suppression of tumorigenicity 7) and NM23 (nonmetastatic 23) and promotion of a tumorigenic state in NIH3T3 cells[84]. It also methylates histone H4 at arginine 3 to recruit DNA methyltransferase DNMT3A (DNA cytosine methyltransferase 3A) for gene silencing[85]. Besides, nuclear PRMT5-mediated arginine methylation of p53 disposes p53 to trigger cell-cycle arrest rather apoptosis[86]. In the cytoplasm, PRMT5 is found in the methylosome, where it methylates several spliceosomal Sm proteins to promote the assembly and stability of the splicesome and regulate snRNP (small nuclear ribonucleoprotein) biogenesis[87-89]. During the derivation of embryonic stem cells, PRMT5 is upregulated in the cytosol and methylates histone H2A at arginine 3 to maintain stem cell pluripotency[90]. In yeast model, cytoplasmic PRMT5 also has been shown to inhibit cell cycle progression through binding with cell cycle inhibitor cdc25p[91] and suppress the MAPK pathway by inhibiting STE20p kinase[92].

Currently, little is known about the regulations of the expression, stability or activity of PRMT5. Recent studies indicate that PRMT5 activation requires PRMT5 homo-dimerization and association with a cofactor, Mep50 (methylosome protein 50)[68]. Moreover, the subcellular location of Mep50 varies under different physiological and pathological conditions[93-95]. These findings suggest that PRMT5 activity may be regulated by the expression or subcellular distribution of Mep50. In addition, tyrosine phosphorylation of PRMT5 by a JAK2 constitutively active mutant JAK2V617F disrupts PRMT5-Mep50 association and impairs PRMT5 methyltransferase activity[96]. Besides, the substrate specificity of PRMT5 can be regulated by its binding partners. RioK1 (Rio kinase 1) and pICln mutually exclusively bind with PRMT5. They complete for binding to PRMT5 and coexist with PRMT5-Mep50 in two distinct protein complexes, in which they serve as adapters to recruit different PRMT5 substrates. The RioK1-containing PRMT5-Mep50 complex methylates the RNA-binding protein nucleolin and the pICln-containing complex methylates the spliceosomal Sm proteins[97].

1.6 Hypothesis

Although protein arginine methylation and arginine methyltransferase have

been known since 1968[98, 99], the cellular processes affected by arginine methylation only have begun to be elucidates in the last decade, and a growing body of evidence suggests that methylation may parallel other PTMs in its role in the regulation of various biological processes[67, 68, 76, 77, 100, 101]. Here, we hypothesize that protein methylation may involve in the EGFR signaling, a biological process extensively regulated by multiple PTMs. If this is the case, identification and elucidation of the role of methylation in EGFR signaling will comprise a new level of EGFR regulation.

CHAPTER 2 MATERIAL AND METHOD

2.1 Constructs, antibodies, reagents, and peptides

All GFP-PRMT plasmids were kindly provided by Dr. Mark T. Bedford. PRMT3, PRMT5, and PRMT8 cDNAs were further subcloned into a modified pCMV5 vector containing an N-terminal HA tag. Full-length EGFR cDNA was cloned into a pCDNA3 vector. PRMT5 and EGFR intracellular domain (ICD, amino acid 645-1186) were further subcloned into a modified pCMV5 vector containing an N-terminal GST tag for the purification of recombinant protein. EGFR R1175K and PRMT5 R368A mutagenesises were generated using the QuickChange Site-Directed Mutagenesis Kit according to the manufacturer's protocol (Stratagene). Epidermal growth factor (EGF) and U0126 were purchased from Sigma and Cell Signaling, respectively, and prepared according to the manufacturer's instructions. The following peptides were chemically synthesized from QCB for antibody production in mice, dot blots, peptide competition assay, and *in vitro* methylation assay. Unmodified peptide: NH_2-CAEYLRVAPQSSE-COOH; Methylated peptides: NH_2-CAEYL(monomethyl-R)VAPQSSE-COOH, NH_2-CAEYL(symmetric

dimethyl-R)VAPQSSE-COOH and NH_2-CAEYL(asymmetric dimethyl-R)VAPQSSE-COOH. Histone H4 peptide with monomethyl R3 was purchased from Abcam. Anti-EGFR (Ab-12, 1:5000) antibody from Thermo Scientific and anti-EGFR (06-847, 1:5000) antibody from Millipore were used to detect full-length EGFR and EGFR peptides, respectively. For detection of EGFR tyrosine phosphorylations, antibody to phosphotyrosine (4G10, 1:5000) from Millipore was used to detect total tyrosine phosphorylations, and site-specific antibodies against phospho-Y845, Y992, Y1045, Y1068, Y1086, Y1148, and Y1173 from Cell Signaling and Abcam were used (1:2000) to detect individual phosphotyrosine. Antibodies to ERK (1:5000), SHP1 (1:2000) and SHC (1:5000) from Millipore, antibodies to STAT3 (1:2000) and SOS (1:2000) from Santa Cruz, and antibodies to AKT (1:2000), PLC-γ (1:2000), phospho-ERK (1:5000), phospho-AKT (1:2000), phospho-STAT3 (1:2000), phospho-PLC-γ (1:2000) and Grb2 (1:2000) from Cell Signaling were used to detect the EGFR downstream pathways. Anti-PRMT5 (1:5000) and anti-tubulin (1:5000) antibodies were from Sigma. Anti-GFP (1:5000) antibody was from Thermo Scientific. For immunofluorescence staining, antibodies were diluted 1:200. For immunoprecipitation, 5μg of anti-EGFR (Ab-13, Thermo Scientific), anti-Grb2,

anti-SHC or anti-SOS antibodies were used per 1mg of total protein in 1 ml of cell lysates.

2.2 *In vivo* methylation assay

For *in vivo* methylation of EGFR, a procedure, modified from the method described by Qing Liu et al.[102], was used. A431 cells were incubated 1 hr in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% FBS, 100 μg/ml cycloheximide (Sigma), and 40 μg/ml chloramphenicol (Sigma). Then, cells were washed twice with methionine-depleted DMEM (GIBCO) and incubated in the same medium containing 10 μCi/ml L-[*methyl*-^{3}H]methionine (Amersham Biosciences), 10% dialyzed FBS (GIBCO), 100 μg/ml cycloheximide, and 40 μg/ml chloramphenicol. After labeling for 5 hr, endogenous EGFR was immunopurified and analyzed by SDS-PAGE. ^{3}H-methyl incorporation was visualized by fluorography. For monitoring the effect of protein synthesis inhibitors, A431 cells were also labeled with 10 μCi/ml L-[^{35}S]methionine (MP Biomedicals) using a procedure almost exactly the same as the one described above, with or without protein synthesis inhibitors. After labeling, whole-cell lysates were prepared, analyzed by SDS-PAGE, and detected by autoradiography.

2.3 *In vitro* methylation assay

HA-PRMT3, HA-PRMT5, and HA-PRMT8 were expressed in HEK293 cells and immunopurified using HA-agarose (Sigma). The enzymes immobilized on the beads were then incubated with unmodified peptide (50 μg) in the presence of 2.2 μCi *S*-adenosyl-L-[*methyl*-^{3}H]methionine (85 Ci/mmol from a 0.55 mCi/ml stock solution) (MP Biomedicals) for 1 hr at 30℃ in a final volume of 50 μl of phosphate-buffered saline. One microgram of peptide was spotted onto PVDF membranes and detected using anti-EGFR or anti-EGFR me-R1175 antibodies. Five micrograms of peptide were spotted onto P81 papers, washed, and counted by liquid scintillation.

In vitro methylation assay was also performed as following. GST-PRMT5 and GST-EGFR (ICD) were expressed in HEK293 cell and purified using glutathione resin (GE Healthcare). GST-PRMT5 and GST-EGFR (ICD) proteins were incubated in the presence of 2.2 μCi *S*-adenosyl-L-[*methyl*-^{3}H]methionine (85 Ci/mmol from a 0.55 mCi/ml stock solution) for 1 hr at 30℃ in a final volume of 50 μl of phosphate-buffered saline. Subsequently, samples were analyzed by SDS-PAGE and EGFR methylation was detected using fluorography and

anti-EGFR me-R1175 antibody.

2.4 *In vitro* kinase assay

A procedure, modified from the method described by Jaeho Lee et al.[103], was used. HA-EGFR was expressed in HEK293 cells and immunopurified using HA-agarose. The EGFR proteins immobilized on the beads were then incubated with unmodified or monomethylated peptides (50 μg) in a total volume of 50 μl of reaction buffer containing 5 mM HEPES (pH 7.4), 50 μM Na_3VO_4, 5 mM $MnCl_2$, 2 mM $MnCl_2$, 40 μg/ml BSA, 250 mM ammonium sulfate, 25 μM ATP, and 62.5 μCi/ml [γ-^{32}P]ATP (MP Biomedicals). Reactions were performed at 30℃ and stopped using 8.5% phosphoric acid. One microgram of peptide was spotted onto PVDF membranes and detected using anti-EGFR or anti-EGFR p-Y1173 antibodies. Five micrograms of peptide were spotted onto P81 papers, washed, and counted by liquid scintillation.

2.5 siRNA transfection and siRNA-resistant mutant of PRMT5

Cells were transfected individually with three PRMT5 siRNA oligonucleotides (#1: 5’-UGGCACAACUUCCGGACUUUU-3’, #2:

5’-CAACAGAGAUCCUAUGAUU-3’ or #3: 5’-CGAAAUAGCUGACACACUA-3’) or two EGFR siRNA oligonucleotides (#1: 5’-CAAAGUGUGUAACGGAAUA-3’ or #2: 5’-CCAUAAAUGCUACGAAUAU-3’) with DharmaFECT 1 (Dharmacon), and used for experiments 96 hr after transfection. A non-targeting siRNA (5’-UGGUUUACAUGUCGACUAA-3’) was used as control. To rescue the phenotype of PRMT5 siRNA, an siRNA-resistant mutant of PRMT5 (RR-PRMT5) was created by substituting five nucleotides in the PRMT5 siRNA #1 targeting region (C570T, C573T, C576T, C577A and G579A).

2.6 Mass spectrometry

EGFR was isolated by immunoprecipitation with anti-EGFR antibody and analyzed by SDS-PAGE. The protein band corresponding to EGFR was excised and subjected to in-gel digestion with trypsin. After being isolated from gel, samples were analyzed by nanoelectrospray mass spectrometry which was performed using an Ultimate capillary LC system (LC Packings, Amsterdam, The Netherlands) coupled to a QSTAR^{XL} quadrupole-time of flight (Q-TOF) mass spectrometer (Applied Biosystem/MDS Sciex, Foster City, CA).

2.7 Confocal microscopy analysis

Cultured cells were washed three times with PBS, fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 15 min, and incubated with 5% bovine serum albumin for 1 h. Cells were then incubated with the primary antibodies overnight at 4°C. Cells were washed with PBS and then further incubated with the appropriate secondary antibody diluted at 1:500 and tagged with fluorescein isothiocyanate (FITC), Texas red, or Alexa 647 [104] for 45 min at room temperature. Nuclei were stained with DAPI before mounting. Confocal fluorescence images were captured using a Zeiss LSM710 laser microscope. In all cases, optical sections through the middle planes of the nuclei as determined using nuclear counterstaining were obtained.

2.8 Cell proliferation assay

Cells (5×10^3 cells per well) were seeded in 96-well plates, and relative cell amounts were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric method on a daily basis. MTT (Sigma) at 1 mg/ml was added to each well. After 2-hr incubation, the medium was removed, and the MTT was solubilized in 100 μl of DMSO. The absorbance was measured

at 570 nm, and the relative proliferation index for each day was determined using the absorbance at day 0 as the standard.

2.9 Migration and invasion assay

Cell migration and invasion were analyzed using Biocoat Control inserts and Biocoat Matrigel invasion chambers (BD Biosciences), respectively. Cells (2×10^5) in DMEM medium with 0.1% FBS were added to the upper chamber and allowed to penetrate a porous (8 μm), uncoated membrane or a Matrigel-coated membrane to the bottom chamber containing DMEM medium with 10% FBS. Cells on the top surface of the membrane were removed 72 hr after incubation, and the remaining cells on the bottom surface were fixed with 4% paraformaldehyde, stained with 0.5 % crystal violet, and counted from four random fields of each membrane using a bright-field microscope. The average cell number per field for each membrane was used to calculate the mean and s.d. for triplicate membranes. Migration value is shown as "number of migrated cells per field". Invasion value is reported as the "invasion index = number of invaded cells per field / number of migrated cells per field".

2.10 Mouse model

In vivo cell growth was analyzed in an orthotopic breast cancer mouse model[105]. Briefly, cells (5×10^6 cells) were injected into the mammary fat pads of nude mice, and the tumor volumes were measured weekly.

2.11 Statistics

All quantitative results are presented as the mean and s.d. of independent experiments. Statistical differences between two groups of data were analyzed by Student's *t*-test.

CHAPTER 3 EGFR R1175 IS MONOMETHYLATED

3.1 EGFR is methylated *in vivo*

To determine whether EGFR is a target of methylation, we performed an *in vivo* methylation assay in which cells were metabolically labeled with L-[*methyl*-^{3}H]methionine in the presence of protein synthesis inhibitors (Figure 4A, lanes 1-6), and endogenous EGFR proteins were immunopurified, followed by analysis with fluorography. We observed a radioactive signal corresponding to the size of EGFR in the immuno-products of anti-EGFR antibody (Figure 4A, lane 2) but not in those of the control antibody (Figure 4A, lane 1). Simultaneously, we also labeled cells with L-[^{35}S]methionine to monitor the activity of protein synthesis inhibitors (Figure 4A, lanes 7-10). No L-[^{35}S]methionine incorporation was detected in the presence of the inhibitors (Figure 4A, compare lane 10 to lane 9), indicating that the radiolabeling in lane 2 was resulted from post-translational modification, rather than from translational incorporation. Taken together, these results suggest that endogenous EGFR is a target of protein methylation.

3.2 EGFR R1175 is monomethylated

Next, we used mass spectrometry analysis to identify methylation site(s) of endogenous EGFR immunopurified from cells and result shows that EGFR R1175 is monomethylated (Figure 4B). This finding further supports EGFR methylation and indicates methylation of R1175.

3.3 Generation and characterization of the EGFR monomethylated-R1175 antibody

To assist detection of EGFR R1175 monomethylation, we generated a polyclonal antibody against a synthetic methylated EGFR peptide antigen. This antibody specifically recognized a monomethylated EGFR peptide (amino acid 1171-1182), in which R1175 residue is monomethylated, but not unmodified and dimethylated peptides (Figure 5A). In addition, this antibody only recognized ectopic full-length EGFR wild type (wt) and not methylation-site mutant (R1175K) in cells (Figure 5B). In peptide competition assays, only the monomethylated EGFR peptide neutralized the activity of antibody (Figure 5C). Therefore, this antibody is capable of specifically recognizing R1175-methylated EGFR. In addition to exogenous EGFR proteins, this antibody is suitable for endogenous EGFR detection (Figures 5D and 5E). Methylated EGFR is mainly located at the

cell membrane region.

Figure 4. EGFR R1175 is monomethylated

A. *In vivo* methylation of EGFR. A431 cells were metabolically labeled with L-[*methyl*-^{3}H]methionine (left panel) or L-[^{35}S]methionine (right panel) in the presence or absence of protein synthesis inhibitors, as indicated. Immunoprecipitates of EGFR or control antibodies from L-[*methyl*-^{3}H]methionine-labeled cells were analyzed by fluorography (lanes 1 and 2), coomassie blue staining (lanes 3 and 4), or western blotting with EGFR antibody (lanes 5 and 6). Whole-cell lysates of L-[^{35}S]methionine-labeled cells were analyzed by coomassie blue staining (lanes 7 and 8) or autoradiography (lanes 9 and 10).

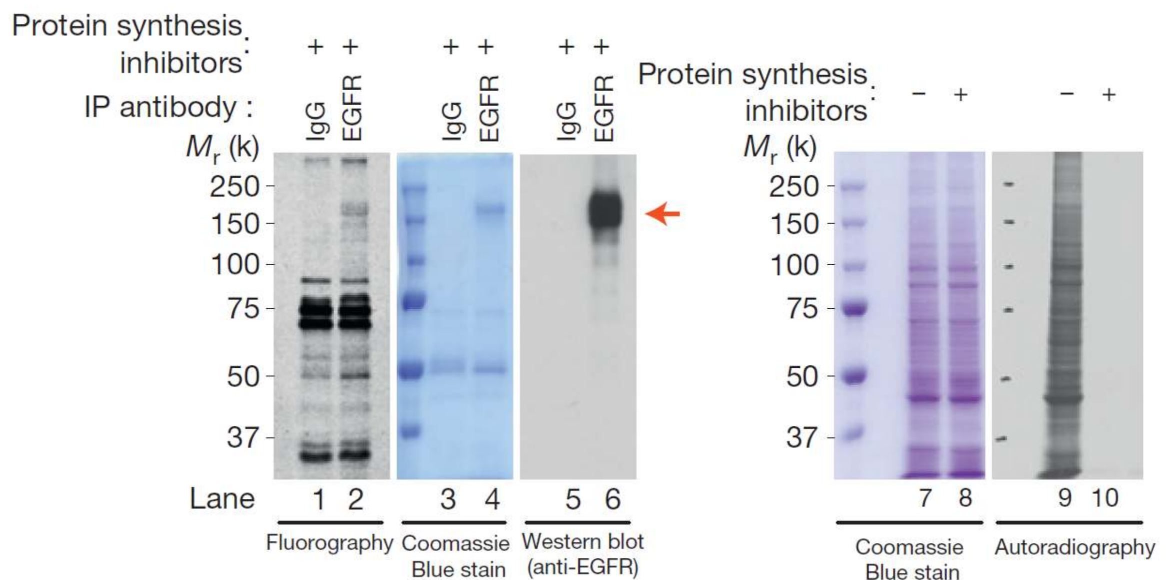


Figure 4. EGFR R1175 is monomethylated

B. Mass spectrometry analysis of endogenous EGFR immunopurified from A431 cells.

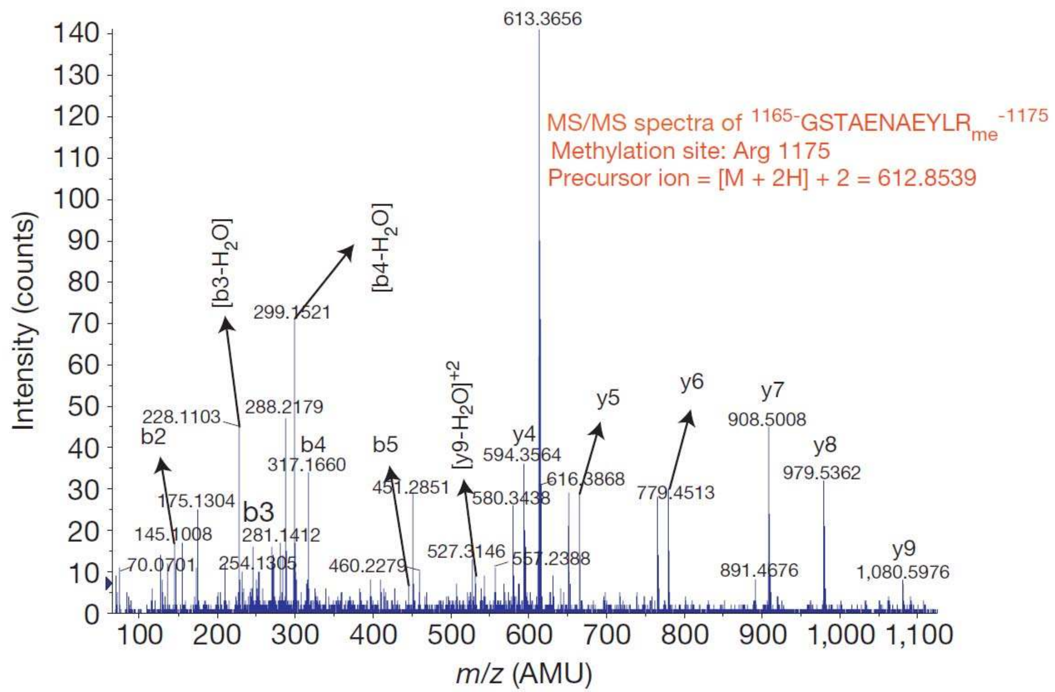


Figure 5. Generation and characterization of the antibody specifically recognizing EGFR R1175 methylation

A. Amino acid sequence of peptides corresponding to the EGFR 1171-1182 region in which R1175 is unmodified, monomethylated or dimethylated. Different amounts of peptides were spotted on PVDF membranes and detected by anti-EGFR (Millipore #06-847) or anti-EGFR me-R1175 antibodies.

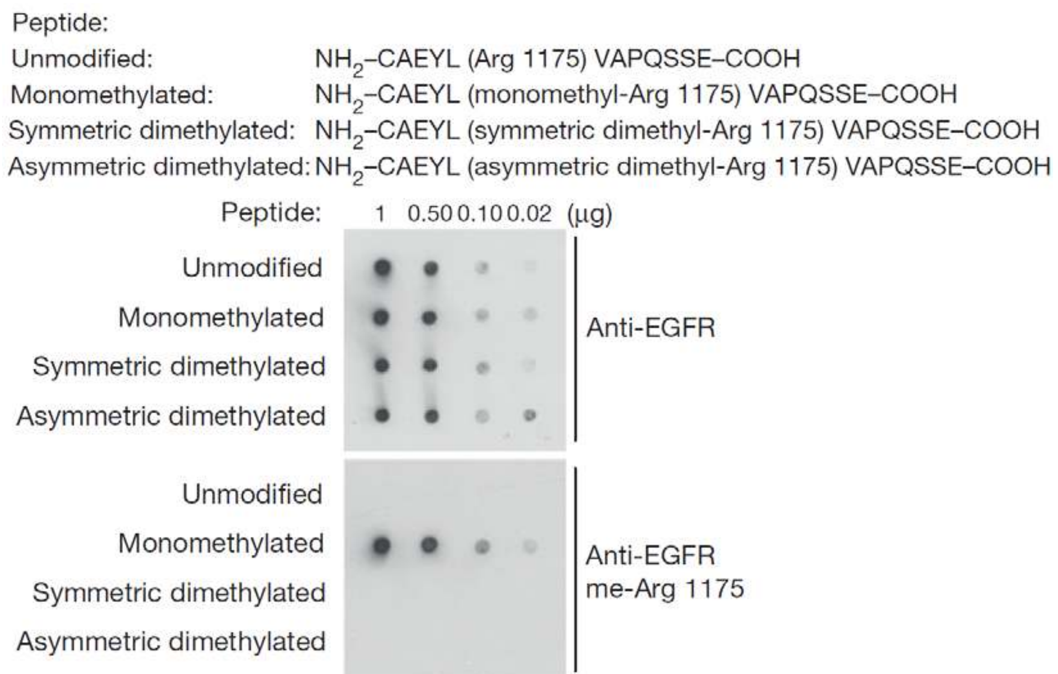


Figure 5. Generation and characterization of the antibody specifically against EGFR R1175 methylation

B. Western blot analysis of exogenous EGFR in HEK293 cells transfected with control vector, EGFR (wt) or EGFR (R1175K).

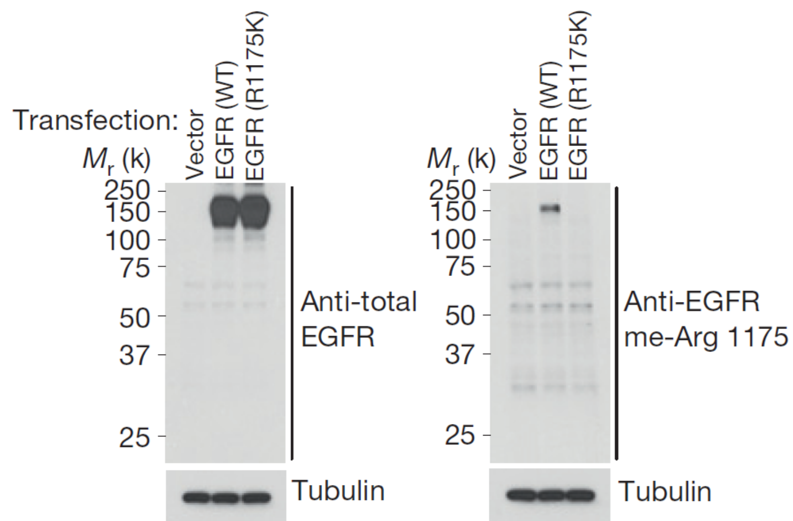
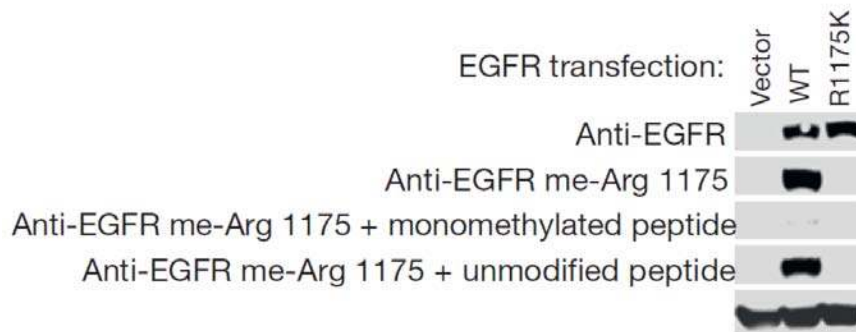


Figure 5. Generation and characterization of the antibody specifically against EGFR R1175 methylation

C. Western blot analysis of exogenous EGFR in HEK293 cells transfected with empty vector, EGFR (wt) or EGFR (R1175K). Anti-EGFR me-R1175 antibody was pre-incubated with peptides, as indicated prior to use.



D. Western blot analysis of endogenous EGFR in MDA-MB-468 cells transfected with control or EGFR siRNAs.

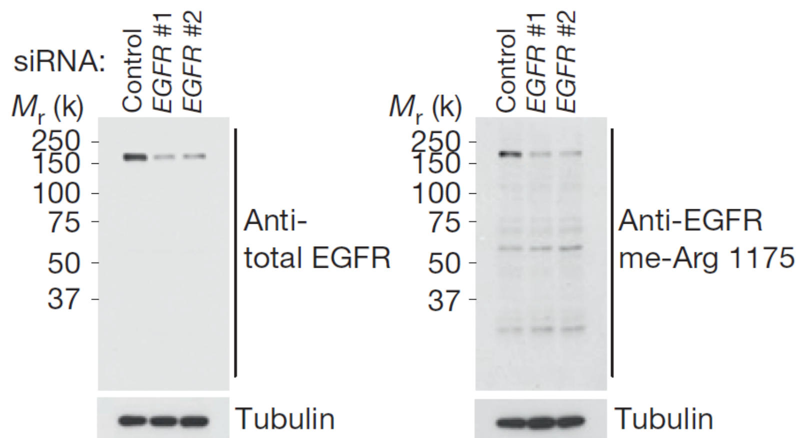
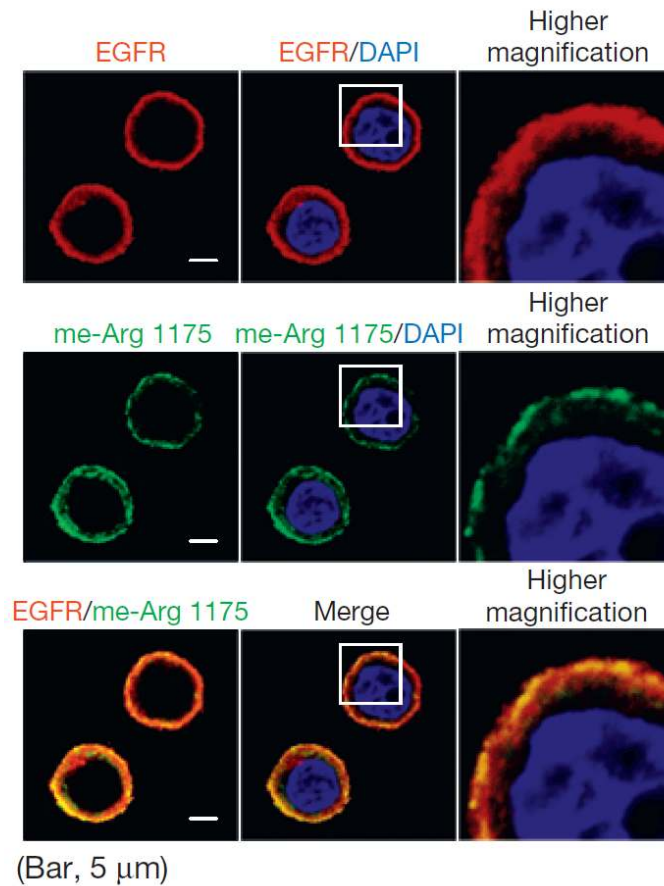


Figure 5. Generation and characterization of the antibody specifically against EGFR R1175 methylation

E. Confocal microscopy analysis of MDA-MB-468 cells stained with total endogenous EGFR (red), me-R1175 (green) and DAPI (blue).



CHAPTER 4 PRMT5 METHYLATES EGFR R1175 *IN VITRO* AND *IN VIVO*

4.1 PRMT5 interacts with EGFR and methylates R1175

Members of the protein arginine methyltransferase (PRMT) family are the only enzymes responsible for protein arginine methylation[67]. To identify the upstream enzyme responsible for EGFR R1175 methylation, we screened the interaction between EGFR and several PRMT family members by using co-immunoprecipitation assays and found that EGFR bound with PRMT5 and PRMT8 (Figure 6A). Next, *in vitro* methylation assays were used to determine whether PRMT5 and/or PRMT8 could methylate EGFR. The unmodified EGFR peptide was incubated with affinity-purified PRMT3, PRMT5 or PRMT8 in the presence of S-adenosyl-L-[*methyl*-^{3}H]methionine as a methyl donor and methylation was detected using R1175 methylation-specific antibody and scintillation counting. We observed that PRMT5 and PRMT8, but not PRMT3, methylated R1175 (Figure 6B). In human tissues, PRMT5 is ubiquitously expressed while PRMT8 is restricted in the brain [103, 106, 107]. In human breast cancer cell, EGFR is mainly associated with the cell membrane region, where some PRMT5 is also found (Figure 6C). Suppression of endogenous PRMT5

expression by short interfering RNAs (siRNAs) decreased R1175 methylation and reintroduction of an siRNA-resistant PRMT5 mutant (RR-PRMT5) rescued the effect of siRNA (Figure 6D). Collectively, these data indicate that PRMT5 is an enzyme responsible for EGFR R1175 methylation. Using *in vitro* methylation assays with recombinant EGFR proteins as substrates, we further confirmed that PRMT5 methylates EGFR only at R1175 residue (Figure 6E).

4.2 EGF stimulation and EGFR kinase activity are not required for R1175 methylation

Similarly to the interactions between other PRMTs with their substrates[77, 78, 106], PRMT5 binds with EGFR mainly through its catalytic core domain (Figure 7). Moreover, the PRMT5-EGFR binding (Figure 8, top panel) and the R1175 methylation status (Figure 8, bottom panel) are independent of EGF stimulation, indicating that EGF stimulation and EGFR kinase activity are not required for R1175 methylation.

Figure 6. PRMT5 interacts with EGFR and methylates R1175

A. Western blot analysis of exogenous EGFR and PRMTs in the input and anti-EGFR immunoprecipitates from HEK293 cells transfected with EGFR and GFP-PRMTs, as indicated.

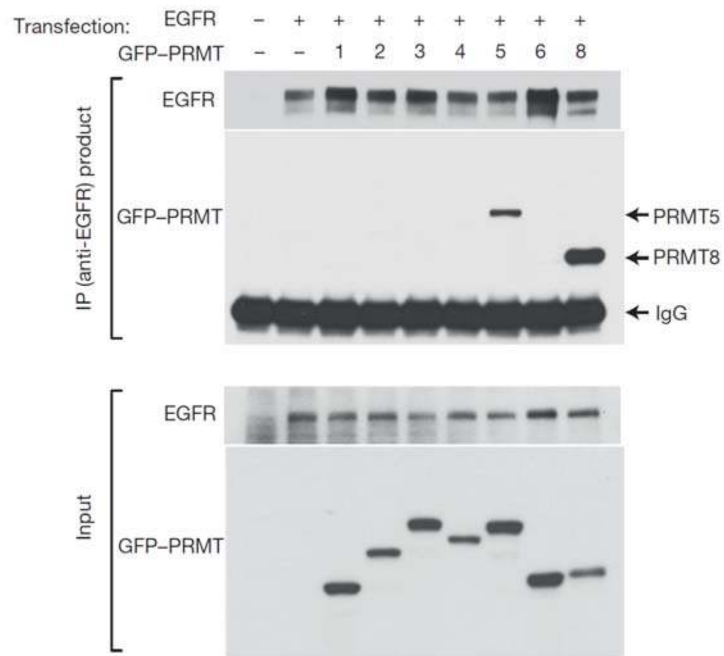


Figure 6. PRMT5 interacts with EGFR and methylates R1175

B. *In vitro* methylation assay of unmodified EGFR peptide by immunopurified HA-PRMT3, 5, or 8. Methylation of peptides was detected by western blotting (top panel) and scintillation counting (bottom panel). Error bars represent s.d. (n = 3).

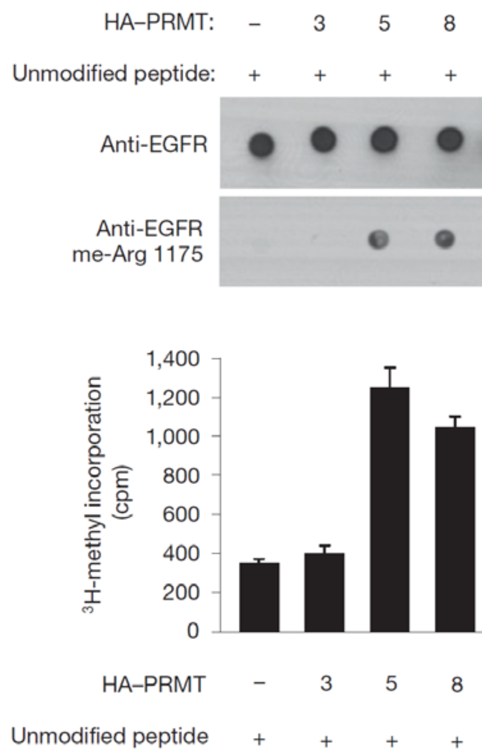


Figure 6. PRMT5 interacts with EGFR and methylates R1175

C. Confocal microscopy analysis of MDA-MB-468 cells stained with endogenous EGFR (red), PRMT5 (green) and DAPI (blue).

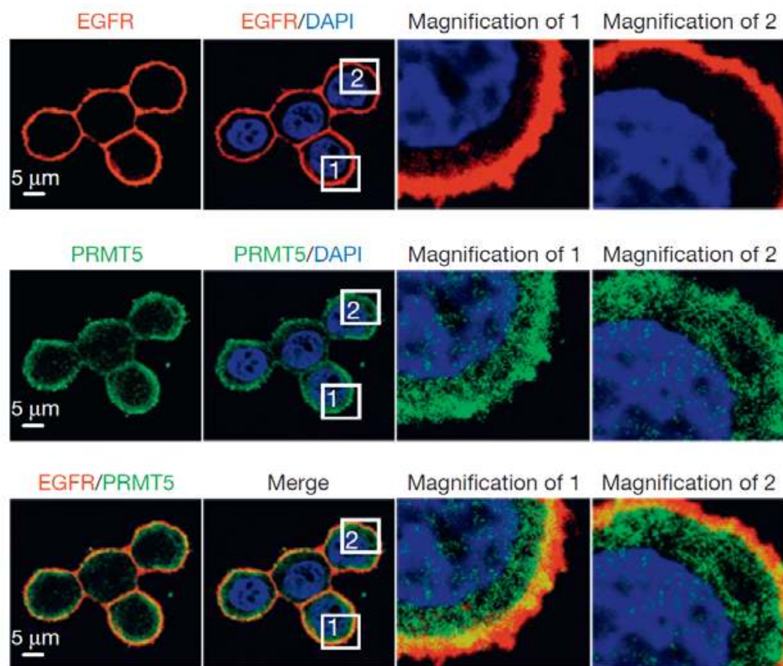


Figure 6. PRMT5 interacts with EGFR and methylates R1175

D. Western blot analysis of endogenous EGFR and total PRMT5 of the MDA-MB-468 cells in which endogenous PRMT5 was knocked down by three PRMT5 siRNAs (lane 1-4) and then rescued with an siRNA-resistant PRMT5 mutant (RR-PRMT5) (lane 5).

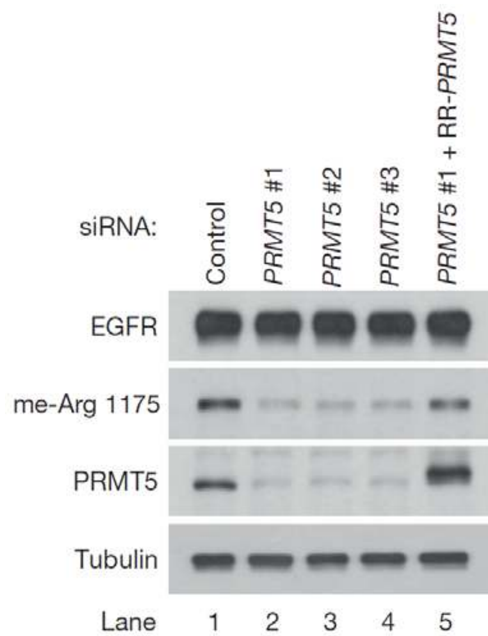


Figure 6. PRMT5 interacts with EGFR and methylates R1175

E. *In vitro* methylation assay of EGFR intracellular domain (ICD) wild type (wt) or R1175K mutant by PRMT5 wild type (wt) or inactive mutant (R368A). Methylation of EGFR (ICD) was detected by fluorography and western blotting using anti-EGFR me-R1175 antibody.

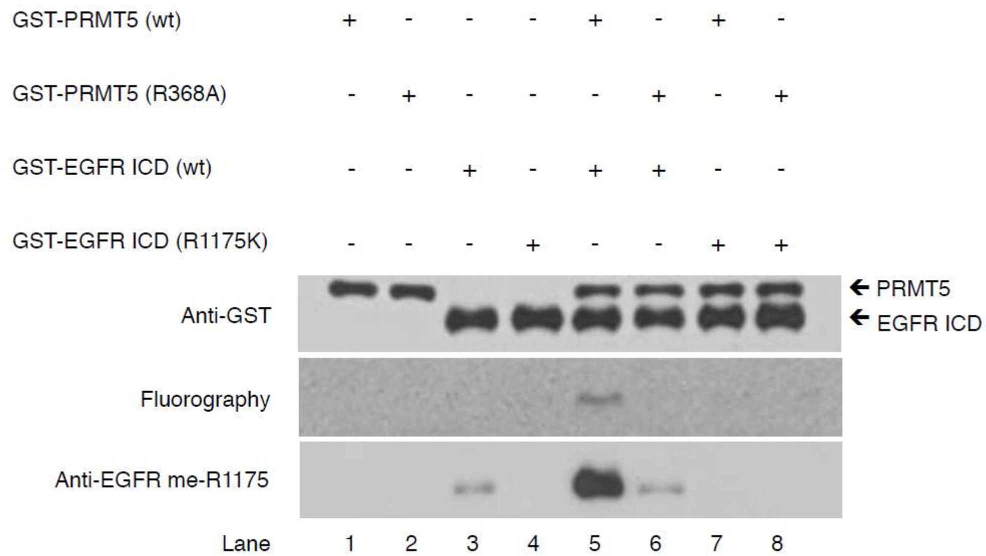


Figure 7. PRMT5 associates with EGFR through the catalytic core domain

Left panel: Schematic representation of the PRMT5 domain structure containing catalytic core, pre-core and post-core domains. PRMT5 truncation mutants without pre-core domain, post-core domain or both domains are assigned as ∆N, ∆C or ∆NC, respectively. Arabic numbers indicate amino acid residues. Right panel: Western blot analysis of exogenous EGFR and PRMT5 in the input and anti-EGFR immunoprecipitates from HEK293 cells transfected with EGFR and various PRMT5 truncation mutants.

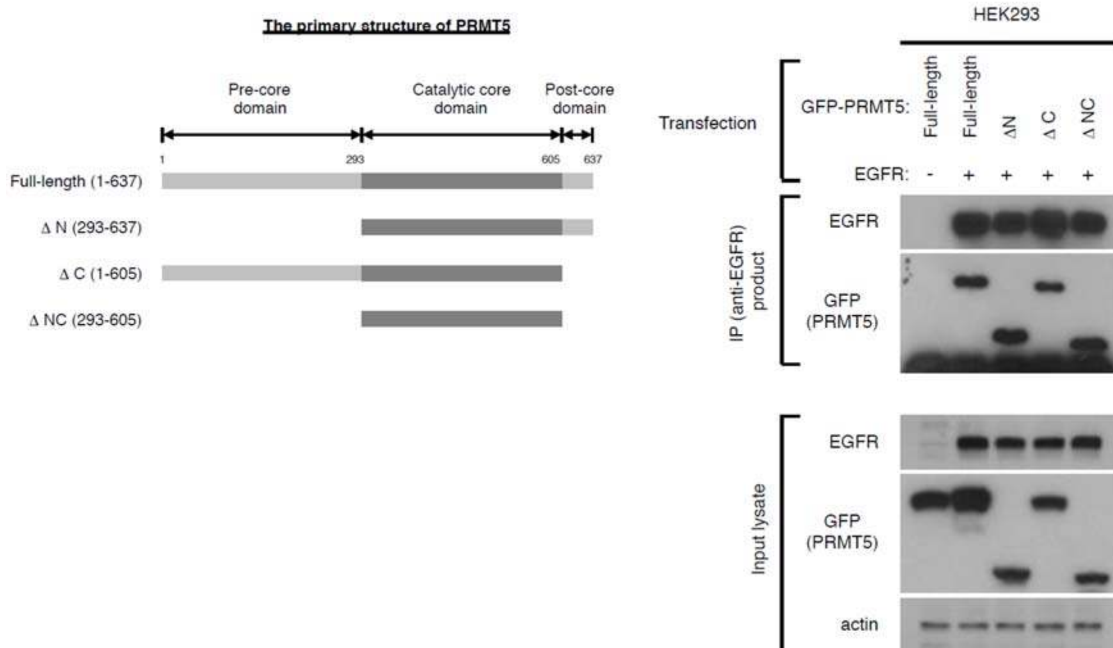
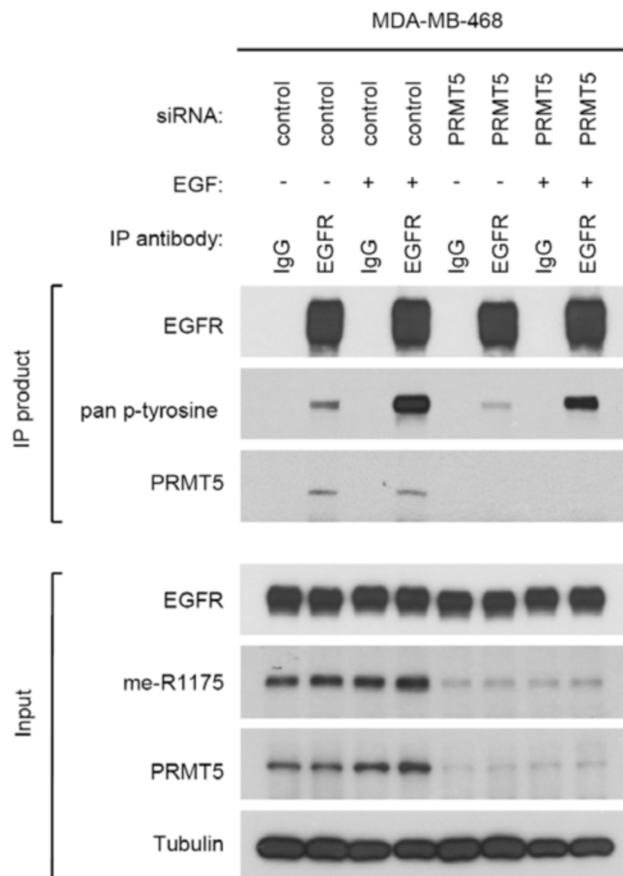


Figure 8. EGF stimulation and EGFR kinase activity are not required for R1175 methylation

Western blot analysis of endogenous EGFR and PRMT5 in the input (bottom panel) and immunoprecipitates of indicated antibodies (top panel) from EGF-stimulated and siRNA-transfected MDA-MB-468 cells.



CHAPTER 5 R1175 METHYLATION NEGATIVELY MODULATES EGFR FUNCTIONALITY

5.1 Suppression of R1175 methylation promotes EGFR-mediated cell proliferation, migration, and invasion

As mentioned in the introduction, multiple lines of the EGFR downstream signalings ultimately culminate in cell proliferation, migration, invasion, and tumorigenicity[14, 39, 40]. To investigate if EGFR R1175 methylation participates in the EGFR functionality, we created three stable transfectants of human MCF7 breast cancer cells with EGFR (wt), EGFR (R1175K), or empty vector (designated as MCF7-EGFR [wt], MCF7-EGFR [R1175K], and MCF7-vector, respectively) for serial functional studies (Figure 9A). First, to evaluate their *in vitro* cell growth abilities, cells were cultured in DMEM supplemented with 10% FBS and relative cell amounts were determined by the MTT colorimetric method on a daily basis. The results showed MCF7-EGFR (R1175K) proliferated faster than MCF7-EGFR (wt), as compared with MCF7-vector control (Figure 9A). Moreover, we used an orthotopic tumor cell growth model to assay the *in vivo* cell growth of these cells. Cells were injected into the mammary fat pads of nude mice and the tumor

volumes were measured weekly. Consistently, we found that MCF7-EGFR (R1175K) cells were more efficient than MCF7-EGFR (wt) and MCF7-vector cells at inducing mammary tumor formation (Figure 9B). On the other hand, the cell motility or invasiveness of these cells was analyzed using a Transwell chamber system with a porous, uncoated membrane or a Matrigel-coated membrane respectively. The cells placed in the upper chamber were induced to migrate across 8.0 μm membrane pores to the lower chamber in response to the chemoattractant. In cell motility, we observed EGFR (R1175K)-expressing cells migrated more efficiently than the control cells (220.2 ± 46.6% of MCF7-vector control), even though MCF7-EGFR (wt) had only slightly positive effects on cell migration (132.8 ± 24.8% of MCF7-vector control) under experimental condition (Figure 9C, left panel). In invasion assay, no significant effect was observed in MCF7-EGFR (wt) cells. In contrast, MCF7-EGFR (R1175K) cells exhibited significantly increased invasion ability (254.4 ± 80.1% of MCF7-vector control) (Figure 9C, right panel). Taken together, these results suggest an inhibitory role of R1175 methylation in the EGFR functionality.

Figure 9. Suppression of R1175 methylation promotes EGFR-mediated cell proliferation, migration, and invasion

A. Western blot analysis of MCF7 stable transfectants expressing EGFR (wt), EGFR (R1175K), or empty vector. *In vitro* cell proliferation rates were assayed using the MTT colorimetric method. Error bars represent s.d. (n = 5).

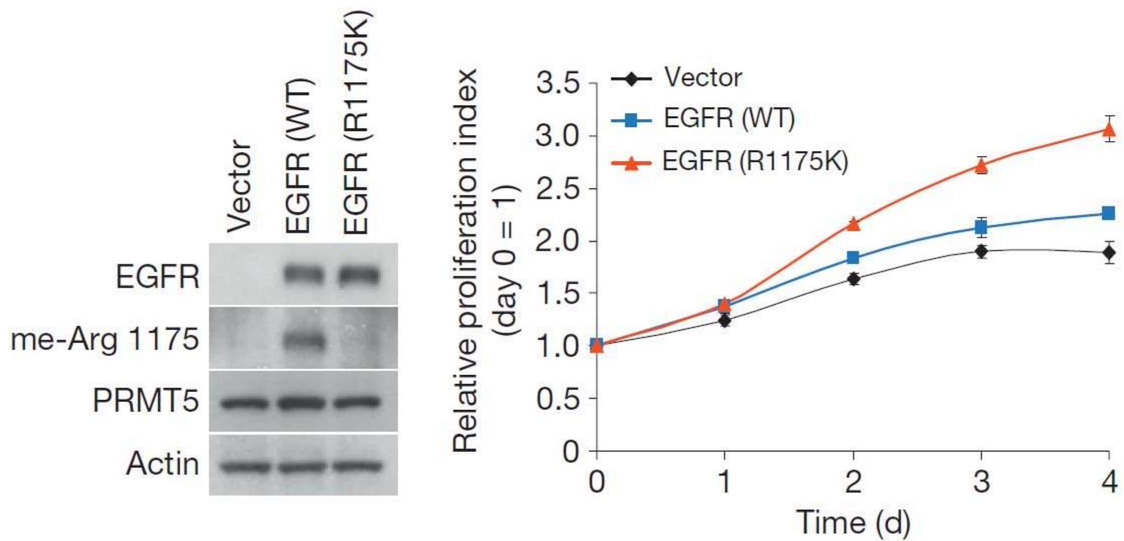


Figure 9. Suppression of R1175 methylation promotes EGFR-mediated cell proliferation, migration, and invasion

B. *In vivo* cell proliferation was measured using an orthotopic breast cancer mouse model. Error bars represent s.d. (n = 10).

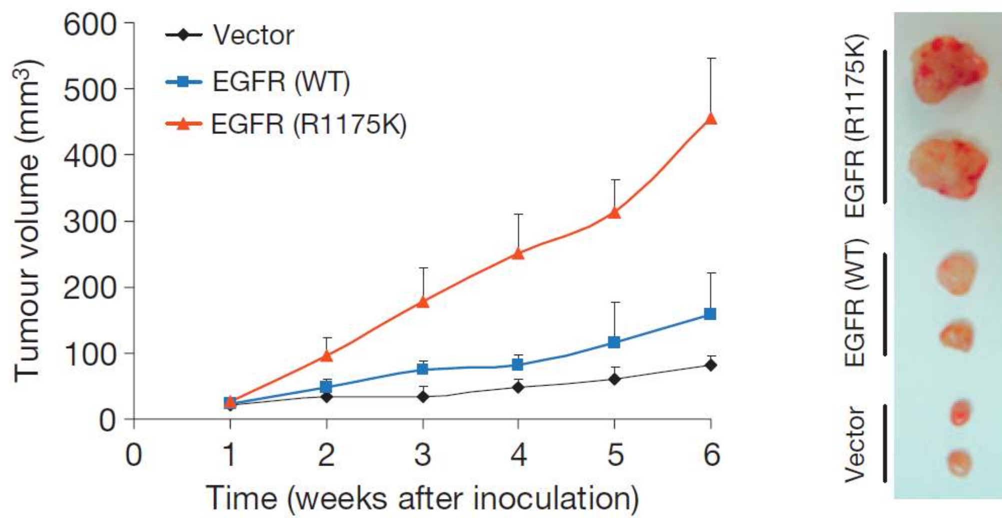
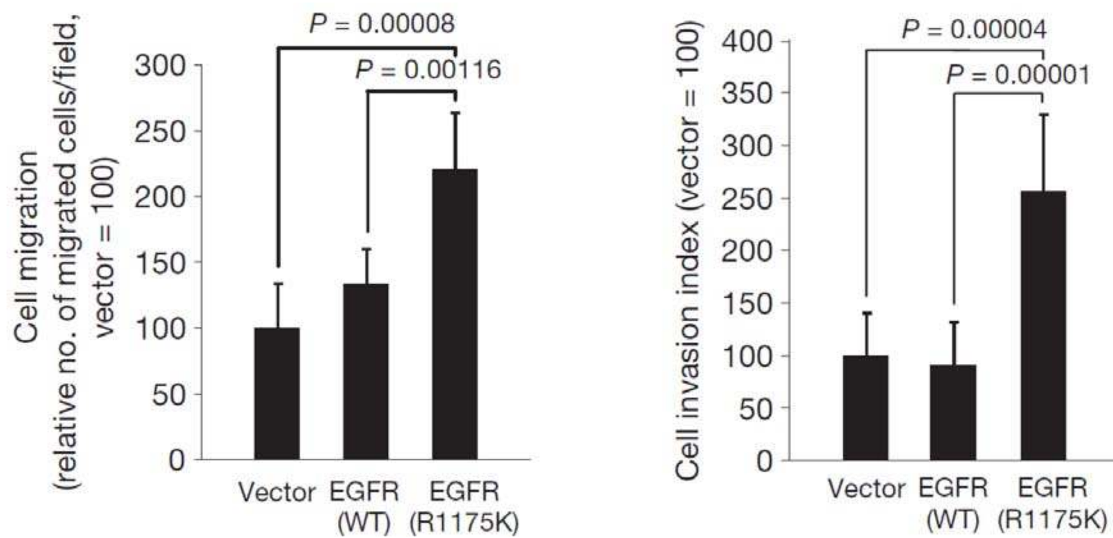


Figure 9. Suppression of R1175 methylation promotes EGFR-mediated cell proliferation, migration, and invasion

C. Migration assay (left) and invasion assay (right) of these stable transfectants. Statistical analysis was performed with Student's *t*-test. Error bars represent s.d. (n = 3).



CHAPTER 6 R1175 METHYLATION CROSSTALKS WITH Y1173 PHOSPHORYLATION

6.1 R1175 methylation upregulates ligand-stimulated EGFR autophosphorylation at Y1173

Regulatory crosstalk usually occurs between two closely spaced post-translational modifications. To further explore how R1175 methylation is involved in EGFR functionality, we noticed that R1175 residue is close to several tyrosine residues that are autophosphorylated during EGFR activation. This gives us a clue that R1175 methylation might regulate EGFR through crosstalk with these tyrosine phosphorylations. Thus, we activated EGFR (wt) and EGFR (R1175K) with EGF and then compared their tyrosine phosphorylation status using several site-specific antibodies against phospho-Y845, Y992, Y1045, Y1068, Y1086, Y1148, and Y1173. Interestingly, compared with EGFR (wt), EGFR (R1175K) got fully phosphorylated at all tyrosine residues tested except Y1173 (Figure 10A). To rule out the possibility that the change of Y1173 phosphorylation was caused by protein conformational misfolding of R1175K mutagenesis, EGFR R1175 methylation status was also manipulated by using multiple PRMT5 siRNAs

as a comparison. Consistently, PRMT5 knockdown specifically inhibited EGF-induced phosphorylation at Y1173, and not other tyrosine residues (Figure 10B). These results indicate that R1175 methylation positively modulates Y1173 phosphorylation.

6.2 R1175 methylation upregulates Y1173 phosphorylation by EGFR *in vitro*

EGFR Y1173 phosphorylation is mediated by EGFR itself. Thus, we further check whether R1175 methylation affects Y1173 phosphorylation by *in vitro* kinase assays in which EGFR peptides with or without R1175 monomethylation were used as substrates for EGFR. Results show EGFR phosphorylated the monomethylated peptide more efficiently than the unmodified peptide (Figure 11), further supporting previous finding that R1175 methylation upregulates Y1173 phosphorylation.

Figure 10. R1175 methylation upregulates ligand-stimulated EGFR autophosphorylation at Y1173

A. Left panel: Western blot analysis of exogenous EGFR in EGF-stimulated MCF7-EGFR (wt) and MCF7-EGFR (R1175K) stable transfectants. Right panel: Densitometry of phospho-EGFR Y1173 (p-Y1173) blot. Error bars represent s.d. (n = 3).

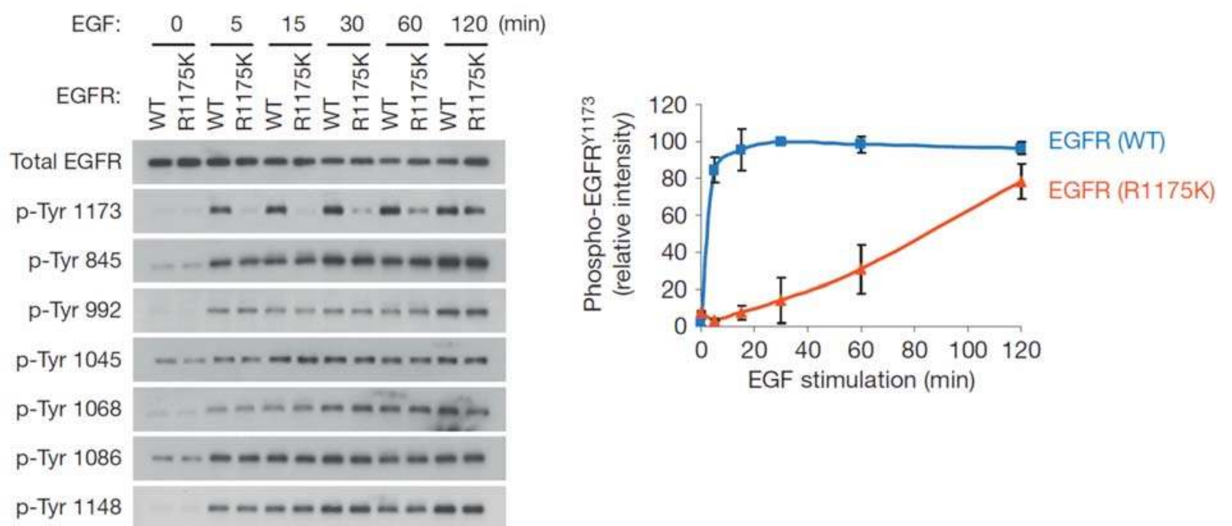


Figure 10. R1175 methylation upregulates ligand-stimulated EGFR autophosphorylation at Y1173

B. Left panel: Western blot analysis of endogenous EGFR in EGF-stimulated MDA-MB-468 cells transfected with control or PRMT5 siRNA #1. Right panel: Densitometry of phospho-EGFR Y1173 (p-Y1173) blot. Error bars represent s.d. (n = 3).

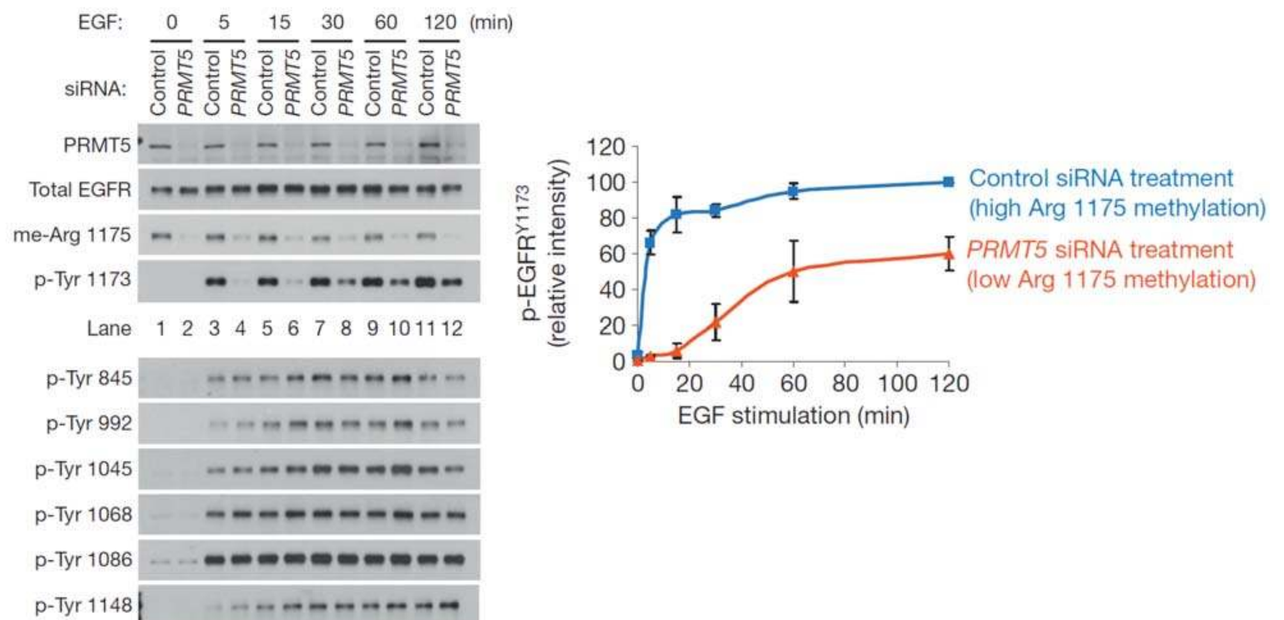
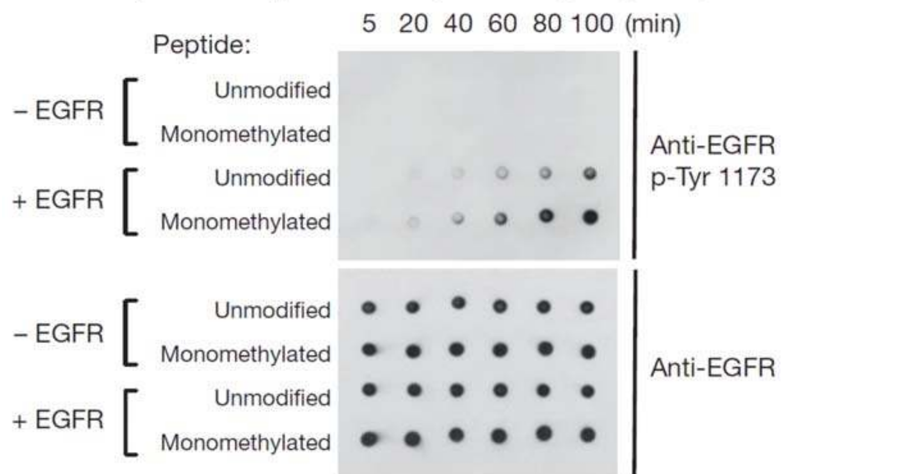
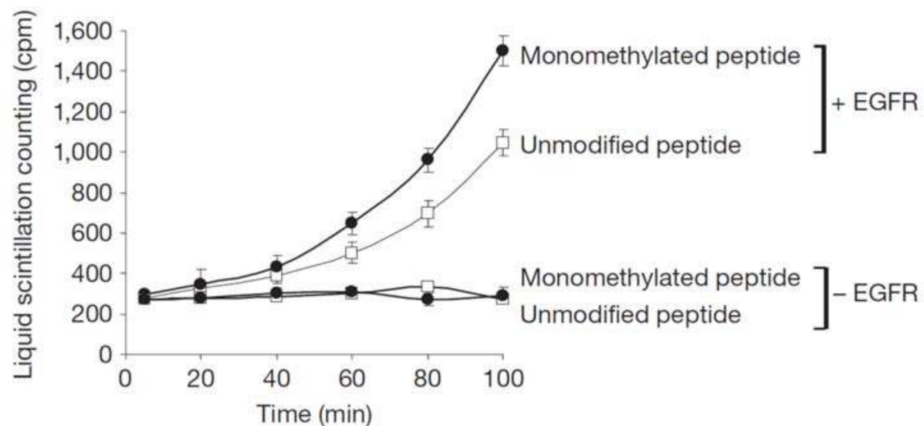


Figure 11. R1175 methylation upregulates Y1173 phosphorylation by EGFR *in vitro*

In vitro kinase assay of unmodified and monomethylated EGFR peptides by immunopurified EGFR proteins. Phosphorylation of peptides was detected by western blotting using anti-EGFR p-Y1173 antibody (top panel) and scintillation counting (bottom panel). Error bars represent s.d. (n = 3).





CHAPTER 7 CROSSTALK BETWEEN R1175 METHYLATION AND Y1173 PHOSPHORYLATION RESULTS IN DOWNREGULATION OF ERK ACTIVATION

7.1 Suppression of R1175 methylation inhibits SHP1 recruitment by EGFR

During EGFR activation, phospho-Y1173 is one of the binding sites for the cytosolic signaling molecules SHC and Grb2 to elicit downstream ERK activation[108-110]. Phospho-Y1173 also serves as the major docking site for SHP1, an SH2 domain-containing protein tyrosine phosphatase. In contrast to the effect of SHC and Grb2 binding, recruitment of SHP1 to EGFR leads to attenuation of EGFR-dependent ERK activation[111]. Since R1175 methylation positively regulates Y1173 phosphorylation, we were motivated to investigate whether it modulates the binding between EGFR and these cytosolic molecules. Using a co-immunoprecipitation assay, we found that downregulation of R1175 methylation by R1175K mutagenesis or by PRMT5 siRNA treatment inhibited only EGFR-SHP1 binding, and not EGFR-Grb2 and EGFR-SHC associations (Figures 12A and 12B), suggesting that R1175 methylation improves SHP1 binding to EGFR and may inhibit EGFR-mediated ERK activation.

7.2 Suppression of R1175 methylation prolongs EGFR-mediated ERK activation

We also studied the effect of EGFR R1175 methylation on the four major EGFR downstream pathways by monitoring the activation of key signaling molecules, including ERK1 (p-ERK1 T202/Y204) and ERK2 (p-ERK2 T185/Y187) in the RAS-RAF-MEK-ERK module, AKT (p-AKT S473) in the PI3K-AKT module, PLC-γ1 (p-PLC-γ1 T783) in the PLC-γ-PKC module, and STAT3 (p-STAT3 T705) in the STATs module. In line with previous results, we observed that inhibition of R1175 methylation affected only EGFR-mediated activation of ERK, and not AKT, PLC-γ or STAT3 (Figures 13A and 13B). In EGFR (wt) cells, upon EGF stimulation, ERK activation was transiently upregulated and then rapidly deactivated. In contrast, ERK activation lasted longer when EGFR R1175 methylation was downregulated by R1175K mutagenesis or by PRMT5 siRNA transfection (Figures 13A and 13B, compare even lanes and odd lanes). These results show that R1175 methylation specifically inhibits EGFR-mediated ERK activation. In supporting of this notion, ERK inhibitor treatment diminished the enhanced cell growth, migration, and invasion abilities of MCF7-EGFR (R1175K) cells (Figures

14A and 14B).

Figure 12. Suppression of R1175 methylation inhibits SHP1 recruitment by EGFR

A. Left panel: Western blot analysis of EGFR, SHP1, Grb2 and SHC in the input and anti-EGFR immunoprecipitates from EGF-stimulated MCF7-EGFR (wt) and MCF7-EGFR (R1175K) stable transfectants. Right panel: Densitometry of EGFR-bound SHP1 blot. Error bars represent s.d. (n = 3).

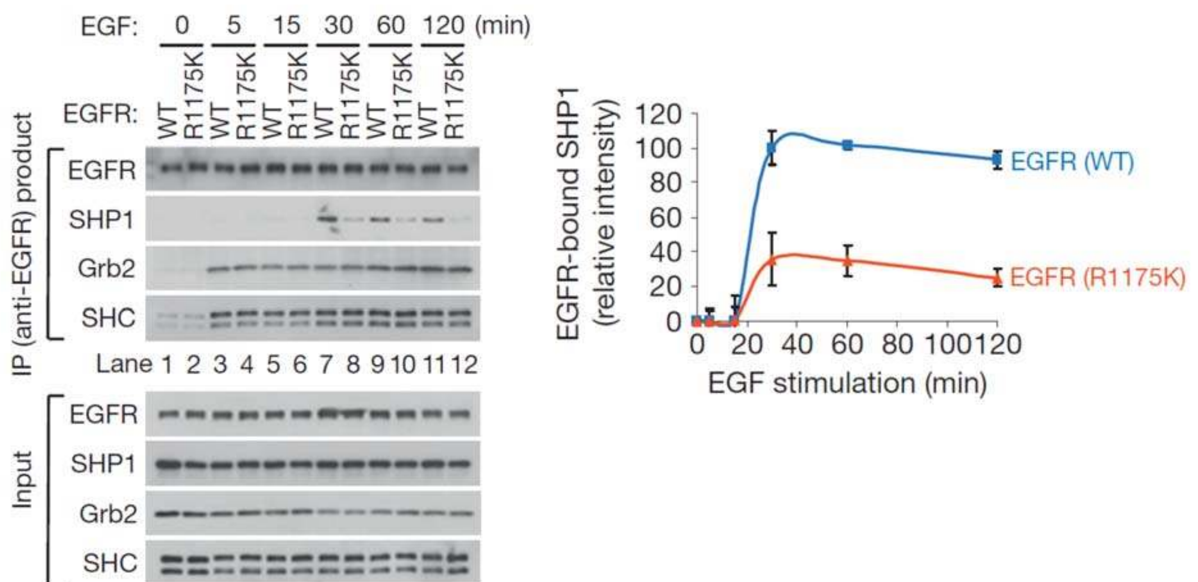


Figure 12. Suppression of R1175 methylation inhibits SHP1 recruitment by EGFR

B. Left panel: Western blot analysis of endogenous EGFR, PRMT5, SHP1, Grb2 and SHC in the input and anti-EGFR immunoprecipitates from EGF-stimulated MDA-MB-468 cells transfected with control or PRMT5 siRNA #1. Right panel: Densitometry of EGFR-bound SHP1 blot. Error bars represent s.d. (n = 3).

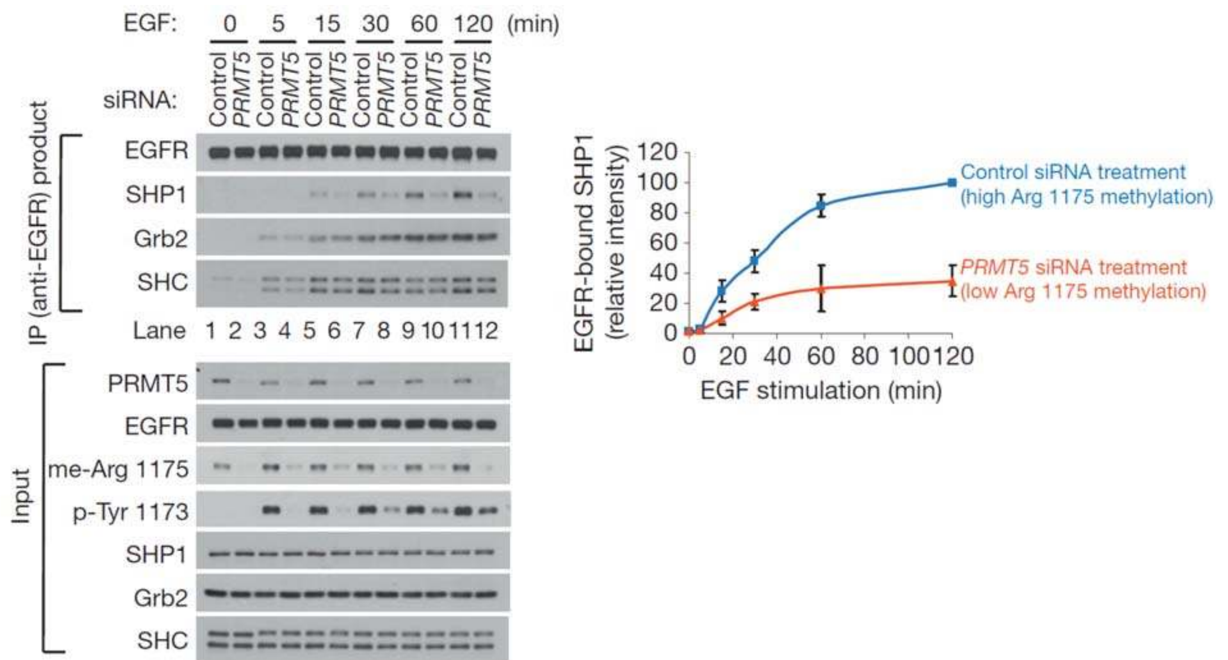


Figure 13. Suppression of R1175 methylation prolongs EGFR-mediated ERK activation

A. Left panel: Western blot analysis of endogenous ERK, PLC-γ, STAT3 and AKT in EGF-stimulated MCF7-EGFR (wt) and MCF7-EGFR (R1175K) stable transfectants. Right panel: Densitometry of phospho-ERK (p-ERK) blot. Error bars represent s.d. (n = 3).

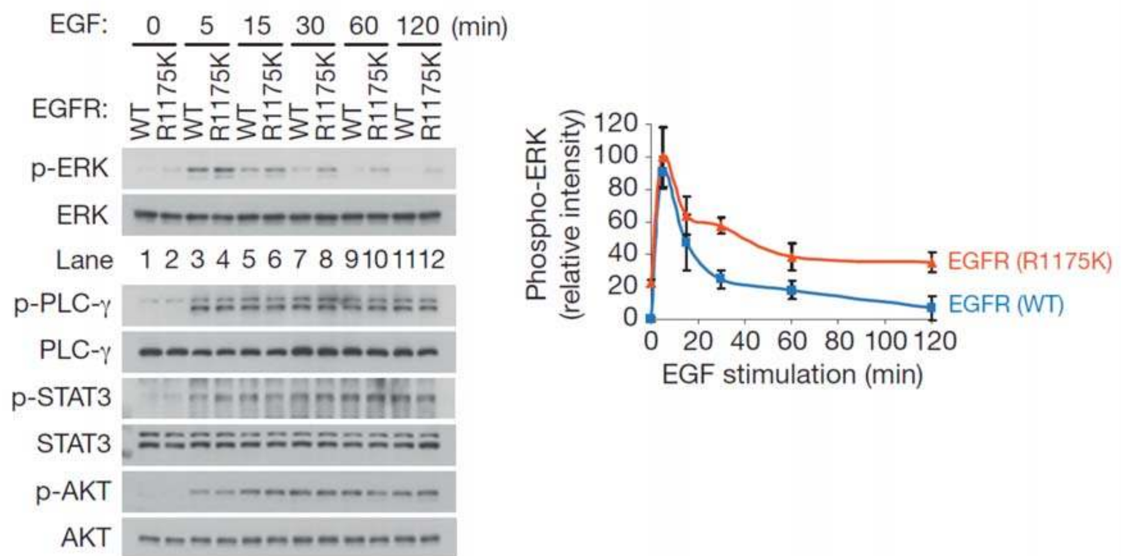
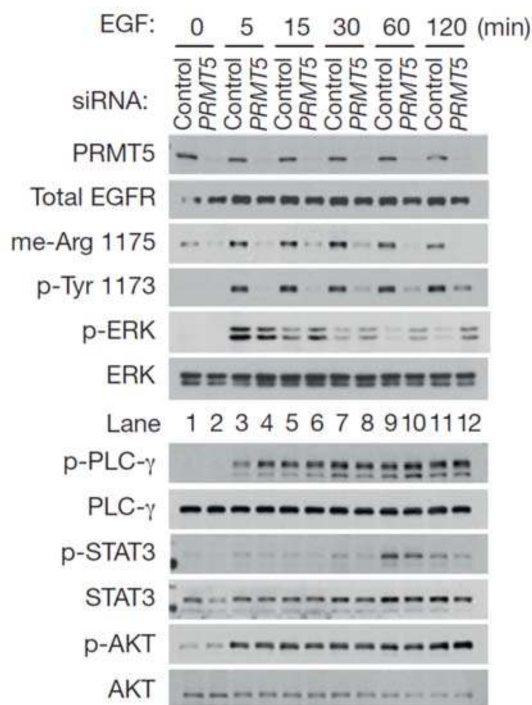


Figure 13. Suppression of R1175 methylation prolongs EGFR-mediated ERK activation

B. Left panel: Western blot analysis of endogenous EGFR, PRMT5, ERK, PLC-γ, STAT3 and AKT in EGF-stimulated MDA-MB-468 cells transfected with control or PRMT5 siRNA #1. Right panel: Densitometry of phospho-ERK (p-ERK) blot. Error bars represent s.d. (n = 3).



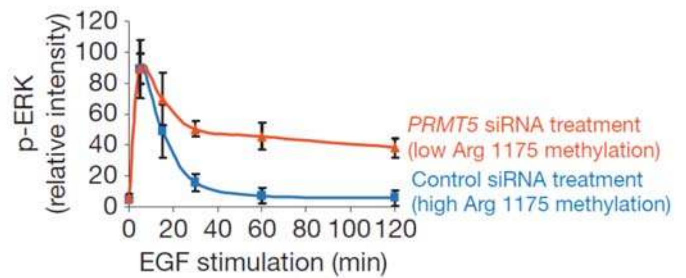


Figure 14. ERK inhibitor treatment impairs the enhanced cell growth, migration, and invasion abilities of MCF7-EGFR (R1175K) cells

A. *In vitro* cell proliferation of MCF7-EGFR (wt), MCF7-EGFR (R1175K), and MCF7-vector cells were performed in the presence or absence of the ERK inhibitor U0126. Error bars represent s.d. (n = 3).

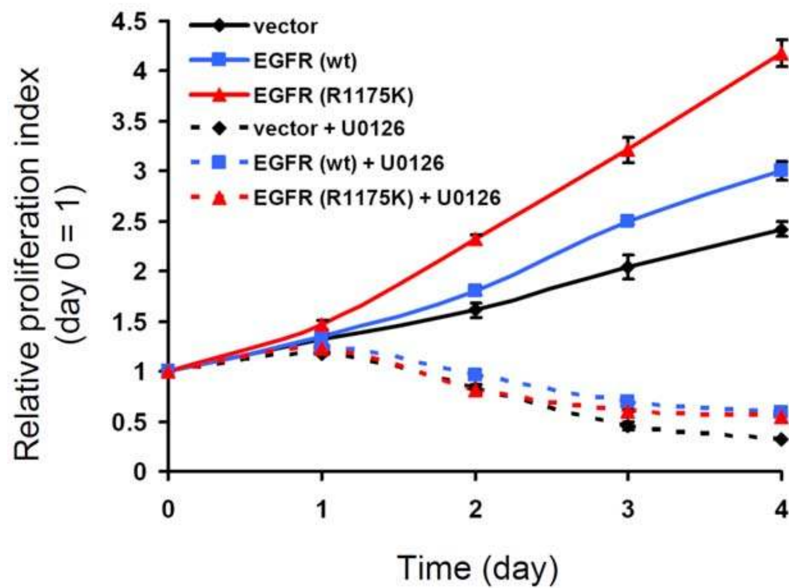
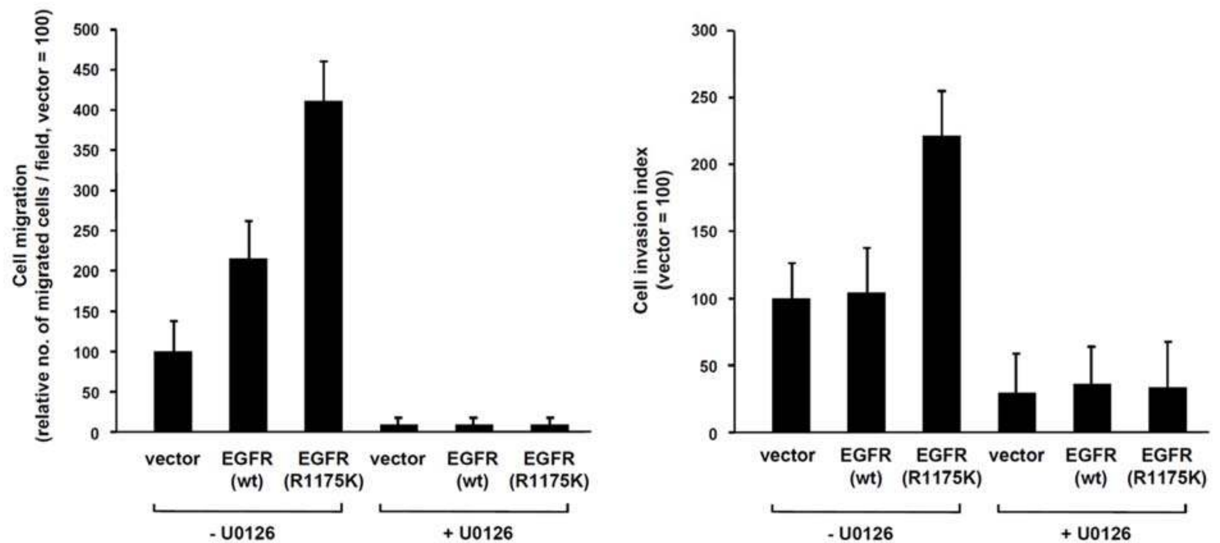


Figure 14. ERK inhibitor treatment impairs the enhanced cell growth, migration, and invasion abilities of MCF7-EGFR (R1175K) cells

B. Migration (left) and invasion (right) assays of MCF7-EGFR (wt), MCF7-EGFR (R1175K), and MCF7-vector cells were performed in the presence or absence of the ERK inhibitor U0126. Error bars represent s.d. (n = 3).



CHAPTER 8 SUMMARY OF CHAPTER 3 TO 7 AND DISCUSSION

We observe that PRMT5-mediated EGFR R1175 methylation (Figures 4, 5 and 6) upregulates EGF-induced EGFR autophosphorylation at Y1173 (Figures 10 and 11). This modification crosstalk positively modulates EGFR-SHP1 binding (Figure 12). In line with the published literatures[111], the increased EGFR-SHP1 binding results in suppression of EGFR-mediated ERK activation (Figure 13). Accordingly, the methylation defective mutant, EGFR (R1175K), increases its activity to promote cell proliferation, migration, invasion and tumorigenicity (Figure 9). Thus, we propose a new link between arginine methylation and tyrosine phosphorylation regulate EGFR functionality. This regulatory mechanism is significant for the following reasons. First, it indicates that EGFR methylation could differentially regulate the activation of EGFR downstream pathways. We might expect that any signaling event leading to a change in R1175 methylation status would specifically modulate the EGF-EGFR-ERK signaling axis. Second, it suggests EGFR might contain a protein modification code, which is reminiscent of the histone code composed of abundant cross-regulated histone modifications[112, 113]. In addition to R1175 methylation, seven potential lysine

and arginine methylation sites were also identified in the kinase domain and C-terminal tail of EGFR (Figure 15A). In the protein primary sequence, some of them are close to, or even overlap with, other known modifications (Figure 15B). Further study of individual methylation and their interrelationships with other modifications would expand our knowledge of the EGFR signaling network. In addition to EGFR, the current study may also open an avenue to understand the regulation of other receptor tyrosine kinases by arginine methylation.

Our results suggest that EGFR Y1173 phosphorylation has a suppressive effect on ERK signaling. Previous literature gave phospho-Y1173 two opposite roles in EGFR-mediated ERK activation and indicated that phospho-Y1173 may coact with phospho-Y992 ,Y1068, Y1086 and Y1148 to activate ERK signaling through recruiting SHC and Grb2[108-110, 114], or may work alone to inhibit ERK activation through SHP1 binding[111]. However, to the best of our knowledge, the interrelationships between these two groups of proteins and phospho-Y1173 have not been well-characterized. Here, we first demonstrate that EGFR recruits these two groups of signaling molecules in a time-dependent manner (Figure 12). Upon EGF stimulation, SHC and Grb2 bind to EGFR immediately while SHP1 is recruited to EGFR only at the later stage (~30 min later after stimulation).

Downregulation of phospho-Y1173 only suppresses the recruitment of SHP1 but not SHC and Grb2 (Figure 12). These results indicate that the major function of phospho-Y1173 is recruiting SHP1 to deactivate ERK at the later stage of EGFR activation instead of recruiting SHC and Grb2 for ERK activation, which, as mentioned above[108-110, 114], is coregulated by multiple phosphotyrosine residues. The minor influence of Y1173 phosphorylation on SHC and Grb2 recruitment could be due to the redundancy in both specificity and function of the different EGFR phosphorylation sites[38, 39]. To further address the role of phospho-Y1173 in ERK activation, we generated a MCF7-EGFR (Y1173F) stable transfectant and found that it exhibited higher proliferation and tumor formation abilities than the MCF7-EGFR (wt) cells (Figures 16A, 16B and 16C), supporting the suggestion that Y1173 phosphorylation plays a suppressive role in EGFR functionality.

The association of SHP1 with EGFR results in suppression of EGFR-mediated ERK activation. However, present knowledge has different interpretations of the action of SHP1 on the EGFR signaling such that SHP1 binding to the EGFR can cause an overall decrease in tyrosine phosphorylation status of the receptor and attenuation of the receptor signaling both in transient

coexpression systems and in stably SHP1-transfected cells[111, 115, 116]. However, other studies have shown that repression of endogenous SHP1 expression by SHP1 siRNA does not affect full EGFR tyrosine phosphorylation[117]. This contradiction raises question of how endogenous SHP1 is involved in the EGFR signaling regulation. To clarify this issue, we knocked down endogenous SHP1 expression by SHP1 siRNA and examined its effect on EGF-stimulated EGFR phosphorylation and downstream signaling activation (Figure 17A). The results show that SHP1 knockdown extends ERK activation and, in line with previous studies, does not affect the EGFR tyrosine phosphorylation status, suggesting that endogenous SHP1 may dephosphorylate other molecules rather than EGFR to attenuate ERK activation.

EGFR activates ERK through EGFR-SHC-Grb2-SOS-Ras-RAF-MEK-ERK pathway. Upon EGF stimulation, SHC-Grb2-SOS complex is recruited to EGFR. It has been reported that these three molecules are subject to tyrosine phosphorylation[118-120]. Next, we tested whether they are potential targets of SHP1. We found EGF stimulation induces tyrosine phosphorylation of SHC and SOS and knockdown of endogenous SHP1 can extend the phosphorylation status of SOS (Figure 17B), suggesting SOS may be a potential target of endogenous

SHP1 to reduce the activity of ERK. Given that phospho-Y1173 is the major binding site of SHP1, we further tested whether Y1173 is involved in the regulation of SOS tyrosine phosphorylation and found SOS phosphorylation status lasted longer in the EGFR (Y1173F) cells than in the EGFR (wt) cells (Figure 17C). Taken together, these data imply that phospho-Y1173 recruits endogenous SHP1 to attenuate ERK activation through reducing the phosphorylation of SOS, rather than EGFR. Moreover, a similar pattern also could be observed in the EGFR (R1175K) cells (Figure 17C), further supporting that R1175 methylation downregulates ERK activation through enhancing Y1173 phosphorylation.

In this study, we found that EGFR R1175 methylation status is consistent during EGF stimulation, raising question of how EGFR R1175 methylation can be regulated. During our manuscript preparation, other studies indicate that the methyltransferase activity of PRMT5 is controlled by Mep50. Mep50 is first identified as an interacting protein of PRMT5 from a yeast-two hybrid screening and subsequent studies show that Mep50 binding to PRMT5 is required for the methyltransferase activity of PRMT5[68, 121]. Moreover, Mep50 has different subcellular distribution patterns at various pathological stages of breast cancer[122]. In malignant breast epithelia, Mep50 prefers nuclear localization,

whereas in their benign counterparts, Mep50 is located at cytoplasm. Cytoplasmic Mep50 has been linked to cell growth inhibition[122], but the mechanism is unclear. As our results indicate PRMT5 methylates EGFR and suppresses EGFR-mediated cell growth, it is reasonable to predict that cytoplasmic Mep50 may inhibit cell growth through upregulating PRMT5-mediated EGFR methylation. To verify this hypothesis, we first tested whether cytoplasmic Mep50 is involved in EGFR methylation. Following the method used in the original study[122], we observed that NES (nuclear exporting signal)-fused Mep50, rather than NLS (nuclear localization signal)-fused Mep50, increased EGFR R1175 methylation in human breast cancer cell (Figure 18A), suggesting cytoplasmic Mep50 is involved in EGFR R1175 methylation. Next, we evaluated the effect of EGFR methylation in cytoplasmic Mep50-mediated cell growth suppression and found that MCF7-EGFR (wt) cells were more susceptible than MCF7-EGFR (R1175K) cells to NES-Mep50-induced cell growth arrest (Figure 18B), implying cytoplasmic Mep50 may suppress cell growth through upregulating EGFR R1175 methylation. Taken together, these preliminary data imply that the subcellular distribution of Mep50 is a regulatory factor for EGFR R1175 methylation and future work will be directed towards elucidation of the role of EGFR methylation at different

pathological stages of breast cancer.

Figure 15. Summary of EGFR post-translational modifications

A. Mass spectrometry identification of EGFR methylation sites. In addition to R1175 monomethylation, another seven potential EGFR methylation sites were identified in our mass spectrometry analysis, including one dimethylated lysine (K704), three monomethylated lysines (K713, K946 and K1037), two monomethylated arginines (R752 and R962) and one dimethylated arginine (R1076).

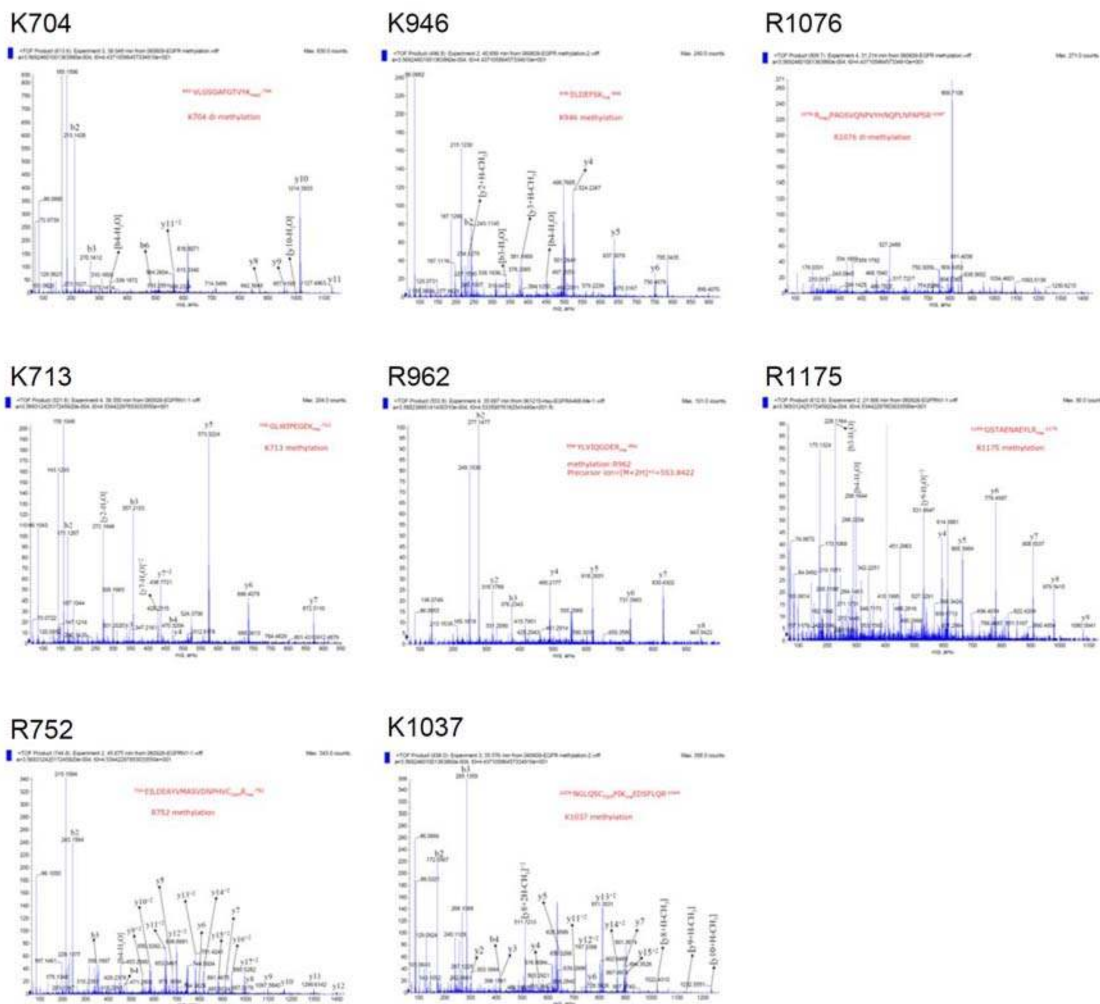


Figure 15. Summary of EGFR post-translational modifications

B. Schematic representation of the functional domains of EGFR intracellular domain (amino acid 645-1186), including a juxtamembrane domain (JM, amino acid 645-683), a tyrosine kinase domain (KD, amino acid 683-959) and a C-terminal tail region (CT, amino acid 959-1186). The relative positions of known EGFR post-translational modifications[10, 64], including phosphorylation (P), ubiquitination (U), acetylation (A) and also the methylation (M) sites we identified are indicated. Alphabets indicate the amino acid residues subjected to modifications (T, threonine; S, serine; Y, tyrosine; K, lysine; R, arginine). Arabic numbers indicate amino acid positions.

Figure 16. MCF7-EGFR (Y1173F) cells exhibit lower cell growth and tumor formation abilities than MCF7-EGFR (wt) cells

A. Western blot analysis of MCF7 stable transfectants expressing EGFR (wt), EGFR (Y1173F), EGFR (R1175K) or empty vector.

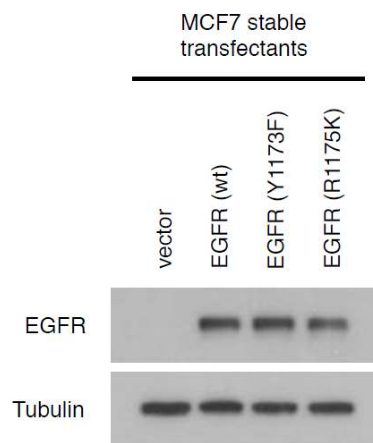
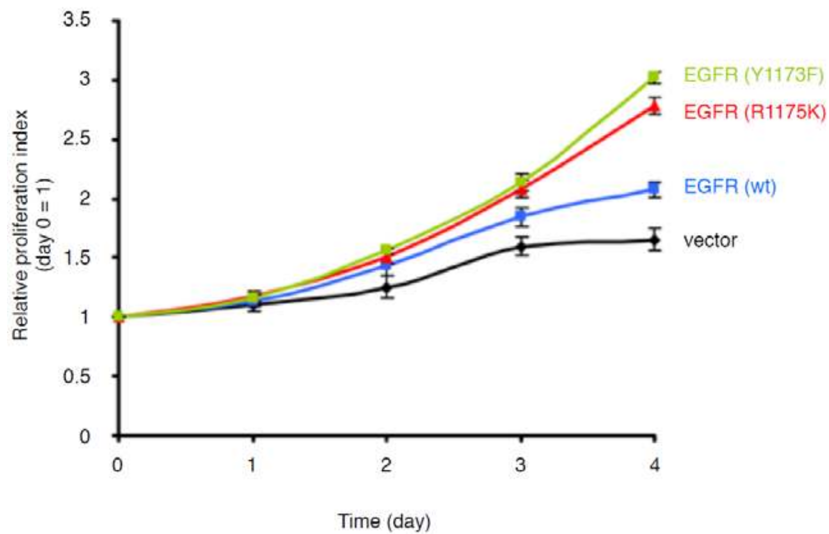


Figure 16. MCF7-EGFR (Y1173F) cells exhibit lower cell growth and tumor formation abilities than MCF7-EGFR (wt) cells

B. *In vitro* cell proliferation assay of the stable transfectants using the MTT colorimetric method. Error bars represent s.d. (n = 5).



C. *In vivo* cell proliferation of the stable transfectants in an orthotopic breast cancer mouse model. Error bars represent s.d. (n = 10).

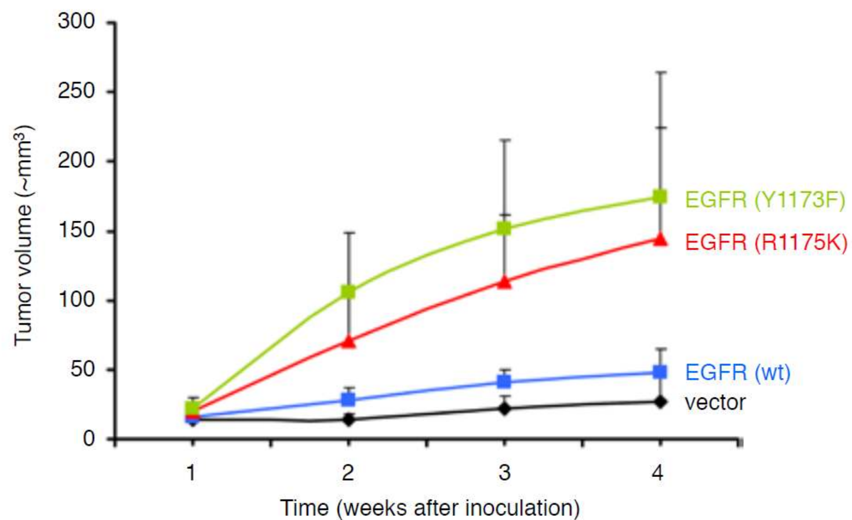


Figure 17. Knockdown of endogenous SHP1 enhances EGF-stimulated SOS phosphorylation and ERK activation

A. Western blot analysis of endogenous EGFR, ERK and AKT in EGF-stimulated MDA-MB-468 cells transfected with control or SHP1 siRNA.

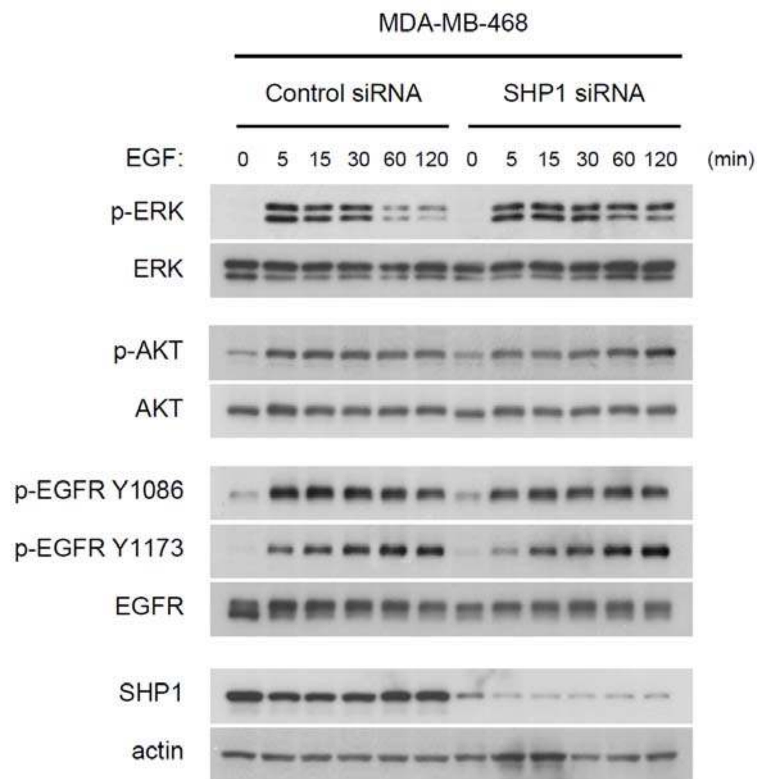


Figure 17. Knockdown of endogenous SHP1 enhances EGF-stimulated SOS phosphorylation and ERK activation

B. Western blot analysis of the tyrosine phosphorylation status of SOS, SHC and Grb2 immunoprecipitated from EGF-stimulated MDA-MB-468 cells transfected with control or SHP1 siRNA.

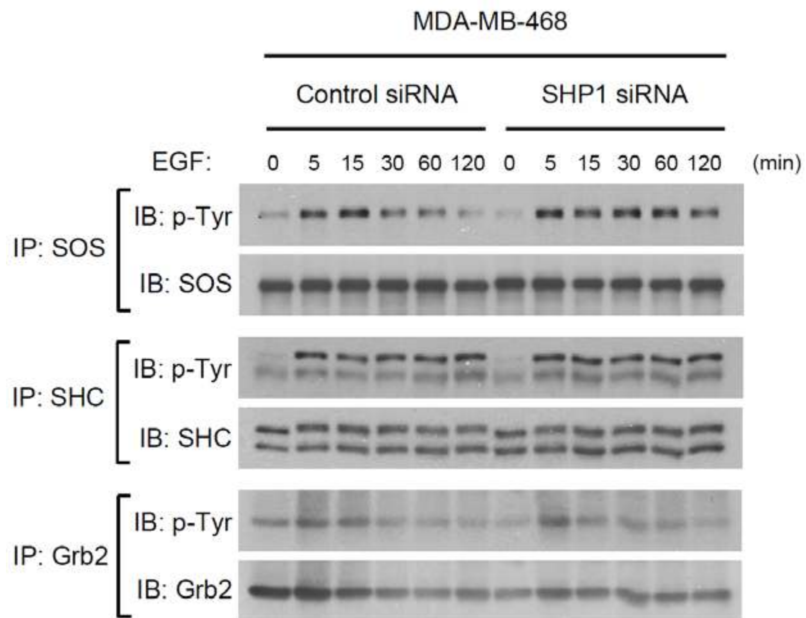


Figure 17. Knockdown of endogenous SHP1 enhances EGF-stimulated SOS phosphorylation and ERK activation

C. Western blot analysis of the tyrosine phosphorylation status of SOS, SHC and Grb2 immunoprecipitated from EGF-stimulated MCF7-EGFR (wt), MCF7-EGFR (Y1173F) and MCF7-EGFR (R1175K) stable transfectants.

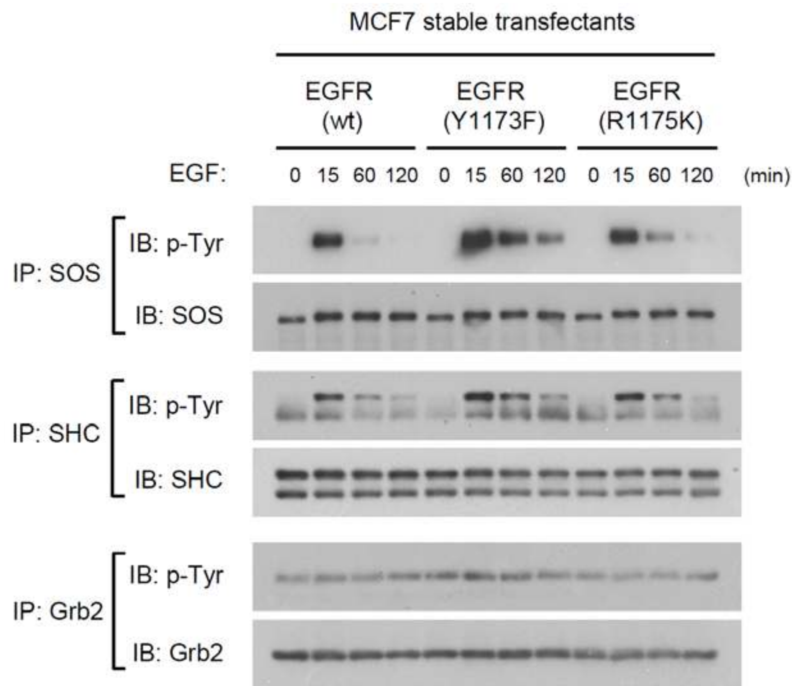
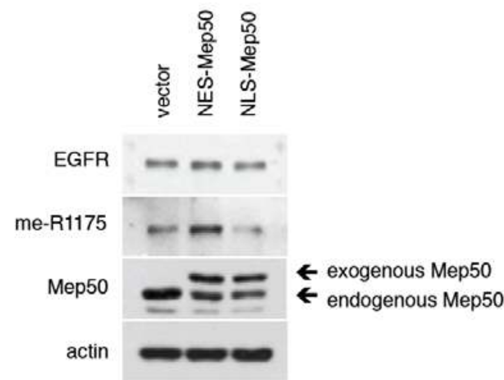
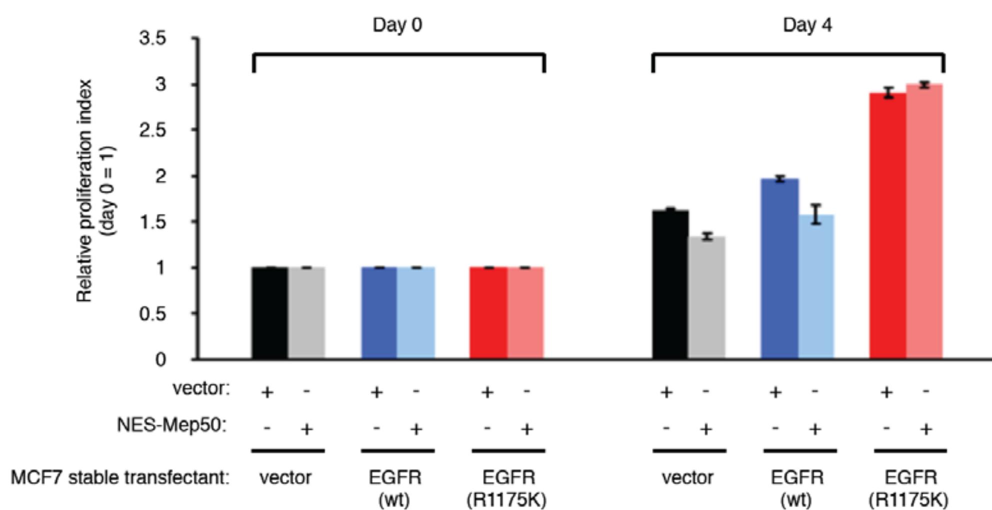


Figure 18. Expression of cytosolic Mep50 enhances R1175 methylation and inhibits cell growth

A. Western blot analysis of EGFR and Mep50 in the MCF7-EGFR (wt) cells ectopically expressed with NES (nuclear exporting signal)-fused Mep50, NLS (nuclear localization signal)-fused Mep50, or empty vector.



B. *In vitro* cell proliferation assay of the MCF7-EGFR stable transfectants expressed with NES-Mep50 or vector. Error bars represent s.d. (n = 5).



CHAPTER 9 FUTURE DIRECTIONS

9.1 Further elucidation of the functions of individual methylations or the interrelationships between methylations and other modifications

In addition to R1175 methylation, several methylation sites are also identified in current study, some of which are close to, or even overlap with, other known modifications that play critical roles in the regulation of EGFR functionalities (Figure 15). This suggests that protein methylation may have more in-depth participation in the EGFR signaling and this field is worthwhile to be pursued further to make the EGFR signalosome more comprehensive.

9.2 Identification of the regulatory mechanism and physiological relevance of EGFR R1175 methylation

Our current study shows that EGFR R1175 methylation is involved in the downstream ERK activation. However, it is still unclear clear what kind of extracellular stimulus can lead to change of the EGFR methylation status and which type of biological process is regulated by EGFR methylation. Our data imply that EGFR R1175 methylation could be controlled by the cytoplasmic distribution

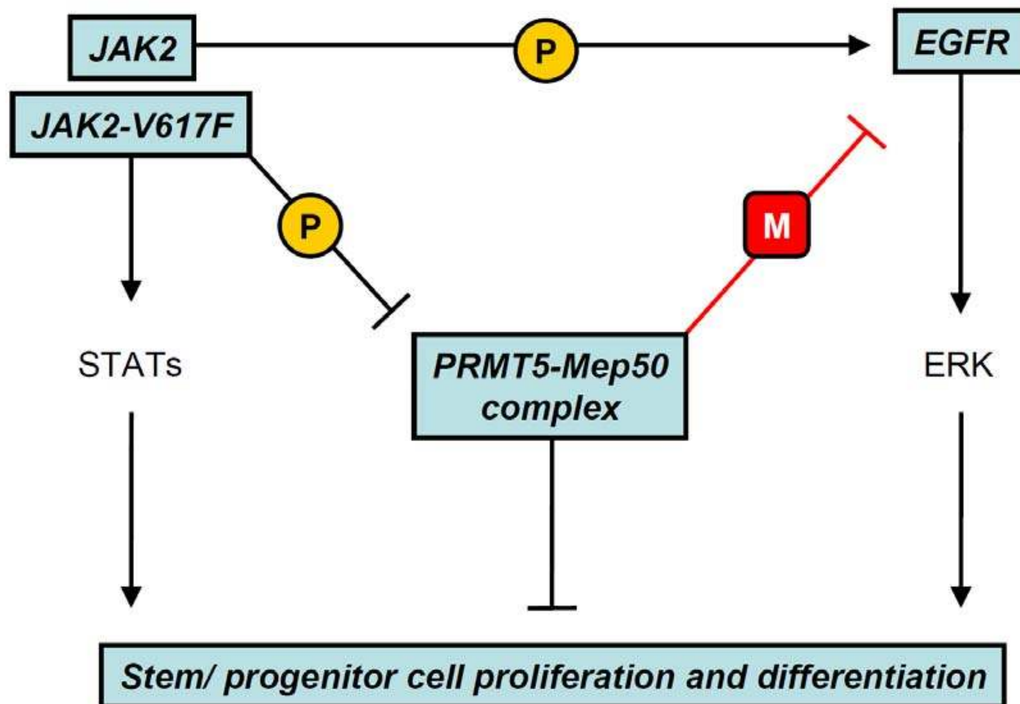
of Mep50 (Figure 18) and may involve in the regulation of breast cancer cell growth in different pathological stages. The role of EGFR methylation in breast cancer progression will be further studied.

In addition, EGFR methylation might also involve in the proliferation and differentiation of stem cells. In Drosophila models, EGFR-RAS-ERK and JAK-STAT pathways are identified as two important participants in mediating intestinal stem cell proliferation and differentiation and maintaining midgut epithelial homeostasis in response to damage or stress[123, 124]. Later studies indicate that these two signalings work cooperatively. JAK-STAT pathway-induced stem cell proliferation is dependent on EGFR signaling[125], but the exact mechanism is unclear. Recently, two separate studies reveal that PRMT5-Mep50 complex suppresses embryonic stem cell differentiation[90] and JAK2V617F, a constitutively active mutant of JAK2, promotes hematopoietic stem cell proliferation and differentiation through phosphorylating PRMT5, disrupting PRMT5-Mep50 association and inactivating PRMT5[96]. Moreover, our finding show that PRMT5-mediated EGFR methylation is a negative modulator of EGFR-RAS-ERK signaling. Taken all these results together, we predict that EGFR methylation might be regulated by JAK2 phosphorylation-mediated PRMT5

inactivation and involve in the regulation of stem cell proliferation and differentiation (Figure 19).

Figure 19. Potential connections between JAK2 signaling, EGFR signaling and PRMT5-Mep50 complex in regulating stem cell proliferation and differentiation.

The PRMT5-Mep50 complex methylates EGFR and inhibits EGFR-mediated ERK activation. JAK2 kinase may activate the EGFR-ERK pathway through directly phosphorylating EGFR, or disrupting the association between PRMT5 and Mep50.



BIBLIOGRAPHY

1. Yarden, Y. (2001). The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. Eur J Cancer *37 Suppl 4*, S3-8.
2. Olayioye, M.A., Neve, R.M., Lane, H.A., and Hynes, N.E. (2000). The ErbB signaling network: receptor heterodimerization in development and cancer. Embo J *19*, 3159-3167.
3. Ferguson, K.M., Berger, M.B., Mendrola, J.M., Cho, H.S., Leahy, D.J., and Lemmon, M.A. (2003). EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. Mol Cell *11*, 507-517.
4. Yarden, Y., and Sliwkowski, M.X. (2001). Untangling the ErbB signalling network. Nat Rev Mol Cell Biol *2*, 127-137.
5. Garrett, T.P., McKern, N.M., Lou, M., Elleman, T.C., Adams, T.E., Lovrecz, G.O., Zhu, H.J., Walker, F., Frenkel, M.J., Hoyne, P.A., Jorissen, R.N., Nice, E.C., Burgess, A.W., and Ward, C.W. (2002). Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha. Cell *110*, 763-773.

6. Ogiso, H., Ishitani, R., Nureki, O., Fukai, S., Yamanaka, M., Kim, J.H., Saito, K., Sakamoto, A., Inoue, M., Shirouzu, M., and Yokoyama, S. (2002). Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. Cell *110*, 775-787.

7. Gullick, W.J. (2001). The Type 1 growth factor receptors and their ligands considered as a complex system. Endocr Relat Cancer *8*, 75-82.

8. Schlessinger, J. (2000). Cell signaling by receptor tyrosine kinases. Cell *103*, 211-225.

9. Zhang, X., Gureasko, J., Shen, K., Cole, P.A., and Kuriyan, J. (2006). An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. Cell *125*, 1137-1149.

10. Linggi, B., and Carpenter, G. (2006). ErbB receptors: new insights on mechanisms and biology. Trends Cell Biol *16*, 649-656.

11. Yaffe, M.B. (2002). Phosphotyrosine-binding domains in signal transduction. Nat Rev Mol Cell Biol *3*, 177-186.

12. Zandi, R., Larsen, A.B., Andersen, P., Stockhausen, M.T., and Poulsen, H.S. (2007). Mechanisms for oncogenic activation of the epidermal growth factor receptor. Cell Signal *19*, 2013-2023.

13. Jorissen, R.N., Walker, F., Pouliot, N., Garrett, T.P., Ward, C.W., and Burgess, A.W. (2003). Epidermal growth factor receptor: mechanisms of activation and signalling. Exp Cell Res *284*, 31-53.

14. Bogdan, S., and Klambt, C. (2001). Epidermal growth factor receptor signaling. Curr Biol *11*, R292-295.

15. Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R.C., and et al. (1995). Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science *269*, 230-234.

16. Xie, W., Paterson, A.J., Chin, E., Nabell, L.M., and Kudlow, J.E. (1997). Targeted expression of a dominant negative epidermal growth factor receptor in the mammary gland of transgenic mice inhibits pubertal mammary duct development. Mol Endocrinol *11*, 1766-1781.

17. Wiesen, J.F., Young, P., Werb, Z., and Cunha, G.R. (1999). Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development. Development *126*, 335-344.

18. Miettinen, P.J., Berger, J.E., Meneses, J., Phung, Y., Pedersen, R.A., Werb, Z., and Derynck, R. (1995). Epithelial immaturity and multiorgan failure in

mice lacking epidermal growth factor receptor. Nature *376*, 337-341.

19. Sibilia, M., and Wagner, E.F. (1995). Strain-dependent epithelial defects in mice lacking the EGF receptor. Science *269*, 234-238.

20. Sibilia, M., Steinbach, J.P., Stingl, L., Aguzzi, A., and Wagner, E.F. (1998). A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor. Embo J *17*, 719-731.

21. Yun, S.P., Lee, M.Y., Ryu, J.M., and Han, H.J. (2009). Interaction between PGE2 and EGF receptor through MAPKs in mouse embryonic stem cell proliferation. Cell Mol Life Sci *66*, 1603-1616.

22. Biteau, B., and Jasper, H. (2011). EGF signaling regulates the proliferation of intestinal stem cells in Drosophila. Development *138*, 1045-1055.

23. Hu, Q., Zhang, L., Wen, J., Wang, S., Li, M., Feng, R., Yang, X., and Li, L. (2010). The EGF receptor-sox2-EGF receptor feedback loop positively regulates the self-renewal of neural precursor cells. Stem Cells *28*, 279-286.

24. Shih, C.C., Weng, Y., Mamelak, A., LeBon, T., Hu, M.C., and Forman, S.J. (2001). Identification of a candidate human neurohematopoietic stem-cell population. Blood *98*, 2412-2422.

25. Casper, D., Mytilineou, C., and Blum, M. (1991). EGF enhances the survival of dopamine neurons in rat embryonic mesencephalon primary cell culture. J Neurosci Res *30*, 372-381.

26. O'Keeffe, G.C., Tyers, P., Aarsland, D., Dalley, J.W., Barker, R.A., and Caldwell, M.A. (2009). Dopamine-induced proliferation of adult neural precursor cells in the mammalian subventricular zone is mediated through EGF. Proc Natl Acad Sci U S A *106*, 8754-8759.

27. Normanno, N., De Luca, A., Bianco, C., Strizzi, L., Mancino, M., Maiello, M.R., Carotenuto, A., De Feo, G., Caponigro, F., and Salomon, D.S. (2006). Epidermal growth factor receptor (EGFR) signaling in cancer. Gene *366*, 2-16.

28. Normanno, N., Bianco, C., Strizzi, L., Mancino, M., Maiello, M.R., De Luca, A., Caponigro, F., and Salomon, D.S. (2005). The ErbB receptors and their ligands in cancer: an overview. Curr Drug Targets *6*, 243-257.

29. Uberall, I., Kolar, Z., Trojanec, R., Berkovcova, J., and Hajduch, M. (2008). The status and role of ErbB receptors in human cancer. Exp Mol Pathol *84*, 79-89.

30. Holbro, T., Civenni, G., and Hynes, N.E. (2003). The ErbB receptors and

their role in cancer progression. Exp Cell Res *284*, 99-110.

31. Abd El-Rehim, D.M., Pinder, S.E., Paish, C.E., Bell, J.A., Rampaul, R.S., Blamey, R.W., Robertson, J.F., Nicholson, R.I., and Ellis, I.O. (2004). Expression and co-expression of the members of the epidermal growth factor receptor (EGFR) family in invasive breast carcinoma. Br J Cancer *91*, 1532-1542.

32. Normanno, N., Bianco, C., De Luca, A., and Salomon, D.S. (2001). The role of EGF-related peptides in tumor growth. Front Biosci *6*, D685-707.

33. Salomon, D.S., Brandt, R., Ciardiello, F., and Normanno, N. (1995). Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol *19*, 183-232.

34. Saeki, T., Salomon, D.S., Johnson, G.R., Gullick, W.J., Mandai, K., Yamagami, K., Moriwaki, S., Tanada, M., Takashima, S., and Tahara, E. (1995). Association of epidermal growth factor-related peptides and type I receptor tyrosine kinase receptors with prognosis of human colorectal carcinomas. Jpn J Clin Oncol *25*, 240-249.

35. Normanno, N., De Luca, A., Salomon, D.S., and Ciardiello, F. (1998). Epidermal growth factor-related peptides as targets for experimental

therapy of human colon carcinoma. Cancer Detect Prev *22*, 62-67.

36. Sharma, S.V., Bell, D.W., Settleman, J., and Haber, D.A. (2007). Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer *7*, 169-181.

37. Seet, B.T., Dikic, I., Zhou, M.M., and Pawson, T. (2006). Reading protein modifications with interaction domains. Nat Rev Mol Cell Biol *7*, 473-483.

38. Schulze, W.X., Deng, L., and Mann, M. (2005). Phosphotyrosine interactome of the ErbB-receptor kinase family. Mol Syst Biol *1*, 2005 0008.

39. Citri, A., and Yarden, Y. (2006). EGF-ERBB signalling: towards the systems level. Nat Rev Mol Cell Biol *7*, 505-516.

40. Hynes, N.E., and Lane, H.A. (2005). ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer *5*, 341-354.

41. Guo, L., Kozlosky, C.J., Ericsson, L.H., Daniel, T.O., Cerretti, D.P., and Johnson, R.S. (2003). Studies of ligand-induced site-specific phosphorylation of epidermal growth factor receptor. J Am Soc Mass Spectrom *14*, 1022-1031.

42. Hynes, N.E., Horsch, K., Olayioye, M.A., and Badache, A. (2001). The ErbB receptor tyrosine family as signal integrators. Endocr Relat Cancer *8*,

151-159.

43. Olayioye, M.A., Graus-Porta, D., Beerli, R.R., Rohrer, J., Gay, B., and Hynes, N.E. (1998). ErbB-1 and ErbB-2 acquire distinct signaling properties dependent upon their dimerization partner. Mol Cell Biol *18*, 5042-5051.

44. Grovdal, L.M., Stang, E., Sorkin, A., and Madshus, I.H. (2004). Direct interaction of Cbl with pTyr 1045 of the EGF receptor (EGFR) is required to sort the EGFR to lysosomes for degradation. Exp Cell Res *300*, 388-395.

45. Willmarth, N.E., Baillo, A., Dziubinski, M.L., Wilson, K., Riese, D.J., 2nd, and Ethier, S.P. (2009). Altered EGFR localization and degradation in human breast cancer cells with an amphiregulin/EGFR autocrine loop. Cell Signal *21*, 212-219.

46. Han, W., Zhang, T., Yu, H., Foulke, J.G., and Tang, C.K. (2006). Hypophosphorylation of residue Y1045 leads to defective downregulation of EGFRvIII. Cancer Biol Ther *5*, 1361-1368.

47. Grandal, M.V., Zandi, R., Pedersen, M.W., Willumsen, B.M., van Deurs, B., and Poulsen, H.S. (2007). EGFRvIII escapes down-regulation due to impaired internalization and sorting to lysosomes. Carcinogenesis *28*,

1408-1417.

48. Yamauchi, T., Ueki, K., Tobe, K., Tamemoto, H., Sekine, N., Wada, M., Honjo, M., Takahashi, M., Takahashi, T., Hirai, H., Tushima, T., Akanuma, Y., Fujita, T., Komuro, I., Yazaki, Y., and Kadowaki, T. (1997). Tyrosine phosphorylation of the EGF receptor by the kinase Jak2 is induced by growth hormone. Nature *390*, 91-96.

49. Biscardi, J.S., Maa, M.C., Tice, D.A., Cox, M.E., Leu, T.H., and Parsons, S.J. (1999). c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function. J Biol Chem *274*, 8335-8343.

50. Li, X., Huang, Y., Jiang, J., and Frank, S.J. (2008). ERK-dependent threonine phosphorylation of EGF receptor modulates receptor downregulation and signaling. Cell Signal *20*, 2145-2155.

51. Aifa, S., Frikha, F., Miled, N., Johansen, K., Lundstrom, I., and Svensson, S.P. (2006). Phosphorylation of Thr654 but not Thr669 within the juxtamembrane domain of the EGF receptor inhibits calmodulin binding. Biochem Biophys Res Commun *347*, 381-387.

52. Feinmesser, R.L., Wicks, S.J., Taverner, C.J., and Chantry, A. (1999).

Ca2+/calmodulin-dependent kinase II phosphorylates the epidermal growth factor receptor on multiple sites in the cytoplasmic tail and serine 744 within the kinase domain to regulate signal generation. J Biol Chem *274*, 16168-16173.

53. Konishi, A., and Berk, B.C. (2003). Epidermal growth factor receptor transactivation is regulated by glucose in vascular smooth muscle cells. J Biol Chem *278*, 35049-35056.

54. Soderquist, A.M., and Carpenter, G. (1984). Glycosylation of the epidermal growth factor receptor in A-431 cells. The contribution of carbohydrate to receptor function. J Biol Chem *259*, 12586-12594.

55. Takahashi, M., Yokoe, S., Asahi, M., Lee, S.H., Li, W., Osumi, D., Miyoshi, E., and Taniguchi, N. (2008). N-glycan of ErbB family plays a crucial role in dimer formation and tumor promotion. Biochim Biophys Acta *1780*, 520-524.

56. Whitson, K.B., Whitson, S.R., Red-Brewer, M.L., McCoy, A.J., Vitali, A.A., Walker, F., Johns, T.G., Beth, A.H., and Staros, J.V. (2005). Functional effects of glycosylation at Asn-579 of the epidermal growth factor receptor. Biochemistry *44*, 14920-14931.

57. Tsuda, T., Ikeda, Y., and Taniguchi, N. (2000). The Asn-420-linked sugar chain in human epidermal growth factor receptor suppresses ligand-independent spontaneous oligomerization. Possible role of a specific sugar chain in controllable receptor activation. J Biol Chem *275*, 21988-21994.

58. Marmor, M.D., and Yarden, Y. (2004). Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases. Oncogene *23*, 2057-2070.

59. Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W.Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998). c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev *12*, 3663-3674.

60. Levkowitz, G., Waterman, H., Ettenberg, S.A., Katz, M., Tsygankov, A.Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999). Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. Mol Cell *4*, 1029-1040.

61. Sorkin, A., and Von Zastrow, M. (2002). Signal transduction and

endocytosis: close encounters of many kinds. Nat Rev Mol Cell Biol *3*, 600-614.

62. Huang, F., Kirkpatrick, D., Jiang, X., Gygi, S., and Sorkin, A. (2006). Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. Mol Cell *21*, 737-748.

63. Oved, S., Mosesson, Y., Zwang, Y., Santonico, E., Shtiegman, K., Marmor, M.D., Kochupurakkal, B.S., Katz, M., Lavi, S., Cesareni, G., and Yarden, Y. (2006). Conjugation to Nedd8 instigates ubiquitylation and down-regulation of activated receptor tyrosine kinases. J Biol Chem *281*, 21640-21651.

64. Goh, L.K., Huang, F., Kim, W., Gygi, S., and Sorkin, A. (2010). Multiple mechanisms collectively regulate clathrin-mediated endocytosis of the epidermal growth factor receptor. J Cell Biol *189*, 871-883.

65. Song, H., Li, C.W., Labaff, A.M., Lim, S.O., Li, L.Y., Kan, S.F., Chen, Y., Zhang, K., Lang, J., Xie, X., Wang, Y., Huo, L.F., Hsu, S.C., Chen, X., Zhao, Y., and Hung, M.C. (2010). Acetylation of EGF receptor contributes to tumor cell resistance to histone deacetylase inhibitors. Biochem Biophys Res Commun *404*, 68-73.

66. Gary, J.D., and Clarke, S. (1998). RNA and protein interactions modulated

by protein arginine methylation. Prog Nucleic Acid Res Mol Biol *61*, 65-131.

67. Bedford, M.T. (2007). Arginine methylation at a glance. J Cell Sci *120*, 4243-4246.

68. Krause, C.D., Yang, Z.H., Kim, Y.S., Lee, J.H., Cook, J.R., and Pestka, S. (2007). Protein arginine methyltransferases: evolution and assessment of their pharmacological and therapeutic potential. Pharmacol Ther *113*, 50-87.

69. Cheng, D., Cote, J., Shaaban, S., and Bedford, M.T. (2007). The arginine methyltransferase CARM1 regulates the coupling of transcription and mRNA processing. Mol Cell *25*, 71-83.

70. Cote, J., and Richard, S. (2005). Tudor domains bind symmetrical dimethylated arginines. J Biol Chem *280*, 28476-28483.

71. Charier, G., Couprie, J., Alpha-Bazin, B., Meyer, V., Quemeneur, E., Guerois, R., Callebaut, I., Gilquin, B., and Zinn-Justin, S. (2004). The Tudor tandem of 53BP1: a new structural motif involved in DNA and RG-rich peptide binding. Structure *12*, 1551-1562.

72. Sinha, R., Allemand, E., Zhang, Z., Karni, R., Myers, M.P., and Krainer, A.R. (2010). Arginine methylation controls the subcellular localization and

functions of the oncoprotein splicing factor SF2/ASF. Mol Cell Biol *30*, 2762-2774.

73. Smith, W.A., Schurter, B.T., Wong-Staal, F., and David, M. (2004). Arginine methylation of RNA helicase a determines its subcellular localization. J Biol Chem *279*, 22795-22798.

74. Anne, J. (2010). Arginine methylation of SmB is required for Drosophila germ cell development. Development *137*, 2819-2828.

75. Mostaqul Huq, M.D., Gupta, P., Tsai, N.P., White, R., Parker, M.G., and Wei, L.N. (2006). Suppression of receptor interacting protein 140 repressive activity by protein arginine methylation. Embo J *25*, 5094-5104.

76. Bedford, M.T., and Richard, S. (2005). Arginine methylation an emerging regulator of protein function. Mol Cell *18*, 263-272.

77. Pahlich, S., Zakaryan, R.P., and Gehring, H. (2006). Protein arginine methylation: Cellular functions and methods of analysis. Biochim Biophys Acta *1764*, 1890-1903.

78. McBride, A.E., and Silver, P.A. (2001). State of the arg: protein methylation at arginine comes of age. Cell *106*, 5-8.

79. Najbauer, J., Johnson, B.A., Young, A.L., and Aswad, D.W. (1993).

Peptides with sequences similar to glycine, arginine-rich motifs in proteins interacting with RNA are efficiently recognized by methyltransferase(s) modifying arginine in numerous proteins. J Biol Chem *268*, 10501-10509.

80. Chang, B., Chen, Y., Zhao, Y., and Bruick, R.K. (2007). JMJD6 is a histone arginine demethylase. Science *318*, 444-447.

81. Wang, Y., Wysocka, J., Sayegh, J., Lee, Y.H., Perlin, J.R., Leonelli, L., Sonbuchner, L.S., McDonald, C.H., Cook, R.G., Dou, Y., Roeder, R.G., Clarke, S., Stallcup, M.R., Allis, C.D., and Coonrod, S.A. (2004). Human PAD4 regulates histone arginine methylation levels via demethylimination. Science *306*, 279-283.

82. Branscombe, T.L., Frankel, A., Lee, J.H., Cook, J.R., Yang, Z., Pestka, S., and Clarke, S. (2001). PRMT5 (Janus kinase-binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins. J Biol Chem *276*, 32971-32976.

83. Pollack, B.P., Kotenko, S.V., He, W., Izotova, L.S., Barnoski, B.L., and Pestka, S. (1999). The human homologue of the yeast proteins Skb1 and Hsl7p interacts with Jak kinases and contains protein methyltransferase activity. J Biol Chem *274*, 31531-31542.

84. Pal, S., Vishwanath, S.N., Erdjument-Bromage, H., Tempst, P., and Sif, S. (2004). Human SWI/SNF-associated PRMT5 methylates histone H3 arginine 8 and negatively regulates expression of ST7 and NM23 tumor suppressor genes. Mol Cell Biol *24*, 9630-9645.

85. Zhao, Q., Rank, G., Tan, Y.T., Li, H., Moritz, R.L., Simpson, R.J., Cerruti, L., Curtis, D.J., Patel, D.J., Allis, C.D., Cunningham, J.M., and Jane, S.M. (2009). PRMT5-mediated methylation of histone H4R3 recruits DNMT3A, coupling histone and DNA methylation in gene silencing. Nat Struct Mol Biol *16*, 304-311.

86. Jansson, M., Durant, S.T., Cho, E.C., Sheahan, S., Edelmann, M., Kessler, B., and La Thangue, N.B. (2008). Arginine methylation regulates the p53 response. Nat Cell Biol *10*, 1431-1439.

87. Friesen, W.J., Paushkin, S., Wyce, A., Massenet, S., Pesiridis, G.S., Van Duyne, G., Rappsilber, J., Mann, M., and Dreyfuss, G. (2001). The methylosome, a 20S complex containing JBP1 and pICln, produces dimethylarginine-modified Sm proteins. Mol Cell Biol *21*, 8289-8300.

88. Meister, G., Eggert, C., Buhler, D., Brahms, H., Kambach, C., and Fischer, U. (2001). Methylation of Sm proteins by a complex containing PRMT5 and

the putative U snRNP assembly factor pICln. Curr Biol *11*, 1990-1994.

89. Friesen, W.J., Wyce, A., Paushkin, S., Abel, L., Rappsilber, J., Mann, M., and Dreyfuss, G. (2002). A novel WD repeat protein component of the methylosome binds Sm proteins. J Biol Chem *277*, 8243-8247.

90. Tee, W.W., Pardo, M., Theunissen, T.W., Yu, L., Choudhary, J.S., Hajkova, P., and Surani, M.A. (2010). Prmt5 is essential for early mouse development and acts in the cytoplasm to maintain ES cell pluripotency. Genes Dev *24*, 2772-2777.

91. Gilbreth, M., Yang, P., Wang, D., Frost, J., Polverino, A., Cobb, M.H., and Marcus, S. (1996). The highly conserved skb1 gene encodes a protein that interacts with Shk1, a fission yeast Ste20/PAK homolog. Proc Natl Acad Sci U S A *93*, 13802-13807.

92. Fujita, A., Tonouchi, A., Hiroko, T., Inose, F., Nagashima, T., Satoh, R., and Tanaka, S. (1999). Hsl7p, a negative regulator of Ste20p protein kinase in the Saccharomyces cerevisiae filamentous growth-signaling pathway. Proc Natl Acad Sci U S A *96*, 8522-8527.

93. Peng, Y., Li, Y., Gellert, L.L., Zou, X., Wang, J., Singh, B., Xu, R., Chiriboga, L., Daniels, G., Pan, R., Zhang, D.Y., Garabedian, M.J., Schneider, R.J.,

Wang, Z., and Lee, P. (2010). Androgen receptor coactivator p44/Mep50 in breast cancer growth and invasion. J Cell Mol Med *14*, 2780-2789.

94. Peng, Y., Chen, F., Melamed, J., Chiriboga, L., Wei, J., Kong, X., McLeod, M., Li, Y., Li, C.X., Feng, A., Garabedian, M.J., Wang, Z., Roeder, R.G., and Lee, P. (2008). Distinct nuclear and cytoplasmic functions of androgen receptor cofactor p44 and association with androgen-independent prostate cancer. Proc Natl Acad Sci U S A *105*, 5236-5241.

95. Liang, J.J., Wang, Z., Chiriboga, L., Greco, M.A., Shapiro, E., Huang, H., Yang, X.J., Huang, J., Peng, Y., Melamed, J., Garabedian, M.J., and Lee, P. (2007). The expression and function of androgen receptor coactivator p44 and protein arginine methyltransferase 5 in the developing testis and testicular tumors. J Urol *177*, 1918-1922.

96. Liu, F., Zhao, X., Perna, F., Wang, L., Koppikar, P., Abdel-Wahab, O., Harr, M.W., Levine, R.L., Xu, H., Tefferi, A., Deblasio, A., Hatlen, M., Menendez, S., and Nimer, S.D. (2011). JAK2V617F-mediated phosphorylation of PRMT5 downregulates its methyltransferase activity and promotes myeloproliferation. Cancer Cell *19*, 283-294.

97. Guderian, G., Peter, C., Wiesner, J., Sickmann, A., Schulze-Osthoff, K.,

Fischer, U., and Grimmler, M. (2010). RioK1, a new interactor of protein arginine methyltransferase 5 (PRMT5), competes with pICln for binding and modulates PRMT5 complex composition and substrate specificity. J Biol Chem *286*, 1976-1986.

98. Paik, W.K., and Kim, S. (1967). Enzymatic methylation of protein fractions from calf thymus nuclei. Biochem Biophys Res Commun *29*, 14-20.

99. Paik, W.K., and Kim, S. (1968). Protein methylase I. Purification and properties of the enzyme. J Biol Chem *243*, 2108-2114.

100. Paik, W.K., Paik, D.C., and Kim, S. (2007). Historical review: the field of protein methylation. Trends Biochem Sci *32*, 146-152.

101. Boisvert, F.M., Chenard, C.A., and Richard, S. (2005). Protein interfaces in signaling regulated by arginine methylation. Sci STKE *2005*, re2.

102. Liu, Q., and Dreyfuss, G. (1995). In vivo and in vitro arginine methylation of RNA-binding proteins. Mol Cell Biol *15*, 2800-2808.

103. Lee, J., Sayegh, J., Daniel, J., Clarke, S., and Bedford, M.T. (2005). PRMT8, a new membrane-bound tissue-specific member of the protein arginine methyltransferase family. J Biol Chem *280*, 32890-32896.

104. Countaway, J.L., McQuilkin, P., Girones, N., and Davis, R.J. (1990).

Multisite phosphorylation of the epidermal growth factor receptor. Use of site-directed mutagenesis to examine the role of serine/threonine phosphorylation. J Biol Chem *265*, 3407-3416.

105. Chang, J.Y., Xia, W., Shao, R., Sorgi, F., Hortobagyi, G.N., Huang, L., and Hung, M.C. (1997). The tumor suppression activity of E1A in HER-2/neu-overexpressing breast cancer. Oncogene *14*, 561-568.

106. Bachand, F. (2007). Protein arginine methyltransferases: from unicellular eukaryotes to humans. Eukaryot Cell *6*, 889-898.

107. Braun, M.C., Kelly, C.N., Prada, A.E., Mishra, J., Chand, D., Devarajan, P., and Zahedi, K. (2004). Human PRMT5 expression is enhanced during in vitro tubule formation and after in vivo ischemic injury in renal epithelial cells. Am J Nephrol *24*, 250-257.

108. Okabayashi, Y., Kido, Y., Okutani, T., Sugimoto, Y., Sakaguchi, K., and Kasuga, M. (1994). Tyrosines 1148 and 1173 of activated human epidermal growth factor receptors are binding sites of Shc in intact cells. J Biol Chem *269*, 18674-18678.

109. Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P.G., and et al. (1992).

Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. Nature *360*, 689-692.

110. Batzer, A.G., Rotin, D., Urena, J.M., Skolnik, E.Y., and Schlessinger, J. (1994). Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. Mol Cell Biol *14*, 5192-5201.

111. Keilhack, H., Tenev, T., Nyakatura, E., Godovac-Zimmermann, J., Nielsen, L., Seedorf, K., and Bohmer, F.D. (1998). Phosphotyrosine 1173 mediates binding of the protein-tyrosine phosphatase SHP-1 to the epidermal growth factor receptor and attenuation of receptor signaling. J Biol Chem *273*, 24839-24846.

112. Latham, J.A., and Dent, S.Y. (2007). Cross-regulation of histone modifications. Nat Struct Mol Biol *14*, 1017-1024.

113. Sims, R.J., 3rd, and Reinberg, D. (2008). Is there a code embedded in proteins that is based on post-translational modifications? Nat Rev Mol Cell Biol *9*, 815-820.

114. Okutani, T., Okabayashi, Y., Kido, Y., Sugimoto, Y., Sakaguchi, K., Matuoka, K., Takenawa, T., and Kasuga, M. (1994). Grb2/Ash binds directly to

tyrosines 1068 and 1086 and indirectly to tyrosine 1148 of activated human epidermal growth factor receptors in intact cells. J Biol Chem *269*, 31310-31314.

115. You, M., and Zhao, Z. (1997). Positive effects of SH2 domain-containing tyrosine phosphatase SHP-1 on epidermal growth factor- and interferon-gamma-stimulated activation of STAT transcription factors in HeLa cells. J Biol Chem *272*, 23376-23381.

116. Tomic, S., Greiser, U., Lammers, R., Kharitonenkov, A., Imyanitov, E., Ullrich, A., and Bohmer, F.D. (1995). Association of SH2 domain protein tyrosine phosphatases with the epidermal growth factor receptor in human tumor cells. Phosphatidic acid activates receptor dephosphorylation by PTP1C. J Biol Chem *270*, 21277-21284.

117. Montano, X. (2009). Repression of SHP-1 expression by p53 leads to trkA tyrosine phosphorylation and suppression of breast cancer cell proliferation. Oncogene *28*, 3787-3800.

118. Soler, C., Alvarez, C.V., Beguinot, L., and Carpenter, G. (1994). Potent SHC tyrosine phosphorylation by epidermal growth factor at low receptor density or in the absence of receptor autophosphorylation sites. Oncogene

9, 2207-2215.

119. Li, S., Couvillon, A.D., Brasher, B.B., and Van Etten, R.A. (2001). Tyrosine phosphorylation of Grb2 by Bcr/Abl and epidermal growth factor receptor: a novel regulatory mechanism for tyrosine kinase signaling. Embo J *20*, 6793-6804.

120. Sini, P., Cannas, A., Koleske, A.J., Di Fiore, P.P., and Scita, G. (2004). Abl-dependent tyrosine phosphorylation of Sos-1 mediates growth-factor-induced Rac activation. Nat Cell Biol *6*, 268-274.

121. Anne, J., and Mechler, B.M. (2005). Valois, a component of the nuage and pole plasm, is involved in assembly of these structures, and binds to Tudor and the methyltransferase Capsuleen. Development *132*, 2167-2177.

122. Peng, Y., Li, Y., Gellert, L.L., Zou, X., Wang, J., Singh, B., Xu, R., Chiriboga, L., Daniels, G., Pan, R., Zhang, D.Y., Garabedian, M., Schneider, R., Wang, Z., and Lee, P. (2009). Androgen receptor coactivator p44/Mep50 in breast cancer growth and invasion. J Cell Mol Med.

123. Jiang, H., Patel, P.H., Kohlmaier, A., Grenley, M.O., McEwen, D.G., and Edgar, B.A. (2009). Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut. Cell *137*, 1343-1355.

124. Jiang, H., and Edgar, B.A. (2009). EGFR signaling regulates the proliferation of Drosophila adult midgut progenitors. Development *136*, 483-493.

125. Jiang, H., Grenley, M.O., Bravo, M.J., Blumhagen, R.Z., and Edgar, B.A. (2011). EGFR/Ras/MAPK signaling mediates adult midgut epithelial homeostasis and regeneration in Drosophila. Cell Stem Cell *8*, 84-95.

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