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Insight into the roles of interleukin-33 and LPIN1 in epithelial cell transformation and mammary tumorigenesis

조선대학교 대학원

약 학 과

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List of Abbreviations

AP-1	Activator protein-1
BME	Basal medium Eagle
BrdU	5-bromo-2'-deoxyuridine
CHX	Cycloheximide
COT	Cancer Osaka Thyroid
CQ	Chloroquine
DG	Diacylglycerol
DMSO	Dimethylsulfoxide
ERK	Extracellular signal-regulated kinase
IGF-1	Insulin-like rrowth fator 1
IgG	Immunoglobulin G
IL-1	Interleukin-1
IL-33	Interleukin 33
IRS1	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
MAP	Mitogen-activated protein
MAP3K8	Mitogen-activated protein kinase kinase kinase 8
MEM	Eagle's minimal essential medium

MG132	Carbobenzoxy-Leu-Leu-leucinal
PA	phosphatidic acid
PAP1	phosphatidate phosphatase
PBS	Phosphate-buffered saline
PC	phosphatidylcholine
PE	Phosphatidylethanolamine
PROP	Propranolol
PVDF	Polyvinylidene difluoride
RIPA buffer	Radioimmunoprecipitation assay buffer
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SOFT AGAR	Anchorage-independent cell transformation
STAT3	signal transducer and activator of transcription 3
TG	Triacylglycerol
TKI	TPL2 kinase inhititor
TNFα	Tumor necrosis factor α
Ub	Ubiquitin

국문 초록

Interleukin-33 과 LPIN1 이 상피세포 형질전환과 유방암 발생에 미치는 영향

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지도교수: 최홍석

약학과

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종양 미세환경은 종양의 발생과 성장, 침습과 전이에 미치는 영향에 주요한 인자로 인식되고 있다. 종양 미세환경의 변화는 암을 매개한 염증과 상관관계가 있으며, 비만도 마찬가지로 염증 환경에서 종양형성에 기여한다.이러한 염증과 비만은 여러가지 기전을 통하여 유방종양을 일으킨다. 그러나 유방암의 생성 및 발달과정에서 염증 사이토카인 Interleukin-33 (IL-33)과 지질 생합성 조절 효소인 LPIN1의 역할은 알려지지 않았다. 본 연구에서는 유방에서의 상피세포의 증식과 암 형성을 조절하는 데 있어 IL-33과 LPIN1이 중요한 역할을 수행하고 있다고 제안한다.

IL-1α/β, IL-18과 같은 interleukin-1 (IL-1) family의 cytokine들은 생체방어

와 질환에 있어서 선천적 면역반응에 대한 다면활성을 가지고 있다. 그러므로 다양한 interleukin의 생물학적 기능에 대한 인식은 인간의 염증성 질환을 치료하는데 새로운 치료학적 방법을 제시해왔다. IL-33은 IL-1 interleukin family의 구성원으로 분류되었으며, ST2 수용체에 결합하는 특징을 가지고 있다. 그러나 유방암의 종양성장 및 전이에서 IL-33/ST2 axis의 역할은 알려지지 않았다. 본 연구에서는 유방에서의 암 형성과 내피세포의 증식을 조절하는 데 있어 IL-33이 중요한 역할을 수행하고 있다고 제안하였다. 정상 상피세포와 유방암 세포에서 ST2-COT 상호작용을 통해 IL-33의 농도 및 시간 의존적으로 COT의 인산화를 증가시켰다. IL-33/ST2/COT cascade는 MEK-ERK, JNK-cJun 과 STAT3의 활성화를 유도함으로써, AP-1 및 STAT3 전사활성을 증가시켰다. 그리고 ST2 및 COT의 siRNA가 세포내로 도입되었을 때, IL-33에 의해 유도되는 AP-1과 STAT3 활성은 대조군에 비하여 유의성 있게 감소하였다. COT의 활성을 억제한 결과 상피세포에서의 형질전환이 감소되었다. 유방암 세포에서 IL-33, ST2 및 COT의 knockdown은 유방암 세포에서의 종양형성을 억제시켰다. 이러한 관찰들과 일관성을 보이며, ST2와 COT의 발현 수준은 인간 유방암 조직에서 유의적으로 증가하였음을 규명하였다.

나아가 본 연구에서는 유방에서 암 형성과 내피세포의 증식을 조절하는 데 있어 LPIN1이 IRS1과 단백-단백 상호작용을 하는 분자 생물학적 기전을 규명하였다. 또한, 유방암 세포주인 SK-BR3와 MDA-MB231 세포에

서 LPIN1을 과다발현 시켰을 때 IGF-1에 의해 내인성 IRS1의 발현을 대조군에 비하여 증가시켰다. 반면에 LPIN1의 kockdown은 IGF-1이 유도하는 IRS1의 발현을 억제시켰다. LPIN1의 kockdown에 의해 IRS1의 mRNA 발현에 영향을 미치지 않았으나, LPIN1의 과다발현은 cycloheximide 처리에 의해 감소되는 IRS1 발현을 억제하였다. 이러한 결과는 LPIN1이 IGF-1 의존적으로 IRS1 stability에 영향을 끼치는 것으로 사료된다. 그러므로 In vitro에서 LPIN1이 IRS1의 ubiquitination에 미치는 영향을 살펴본 결과 LPIN1의 과다발현은 ubiquitination의 억제를 통해 IRS1의 proteasome 의존적 단백질 분해를 억제시키는 것으로 보인다. 또한, chloroquine에 의해 lysosome의 활성 억제는 LPIN1의 과다발현에 의한 증가된 IRS1 발현을 대조군에 비하여 더욱 상승시켰다. 이러한 결과들은 LPIN1이 lysosome과 proteasome 의존적 경로에 의하여 IRS1 단백질의 분해를 억제하는 것으로 사료된다. 나아가, LPIN1은 IRS1에 의해 유도한 MAPK 경로에 영향을 미치는지 알아본 결과 LPIN1 과다발현 세포에서 IGF-1에 의한 RAF1, MEK1/2, ERK1/2, p90RSK의 인산화를 대조군에 비하여 더욱 증가시킨 반면에, LPIN1 kockdown은 IGF-1에 의해 증가된 RAF1, MEK1/2, ERK1/2, p90RSK의 인산화를 억제하였다. LIPIN1 억제제인 propranolol과 IRS1 억제제인 NT157은 농도 의존적으로 IGF-1에 의한 RAF1 뿐만아니라 downstream kinases의 인산화를 감소시켰다. 또한, SK-BR3와 MDA-MB231 세포에서 propranolol과 NT157는 IGF-1에 의해 촉진된 *c-fos*와 AP-1 promoter 활성을 억제시켰다. 나아가 xenograft

model에서 IGF-1에 의하여 형성된 종양이 propranolol과 NT157를 처리하였을 때 억제되었다. 본 연구에서는 LPIN1이 IRS1 stability의 upregulation을 통하여 유방암 생성에 중요한 조절현상으로 규명하였다. 위의 결과를 종합적으로 볼 때, Interleukin-33과 LPIN1은 종양 미세환경에서 발암 및 암화 진행에 밀접한 관계가 있는 것으로 사료한다.

I. Introduction

1. Tumor microenvironment and cancer progression

A normal epithelial tissue can undergo hyperplasia and acquire tumorigenic properties that promote the development of a benign, non-invasive solid tumor known as carcinoma in situ. Normal epithelial tissues and non-invasive carcinoma in situ tumors are separated from a supportive stromal compartment by basement membrane. Carcinoma in situ can progress to a malignant, invasive carcinoma, the most common form of human cancer. Inflammation is a major component of the tumor microenvironment and a driving force in cancer initiation, promotion, and progression (Ridnour et al., 2013; Smith and Kang, 2013). An inflammatory component is present in the microenvironment of most neoplastic tissues. The contributing factors of inflammation include specific genetic background and long-term exposure to various environmental stresses. All these risk factors finally reveal on the accumulation of molecular changes in cells, which contributes to the initiation of carcinogenesis. Therefore, the tumor microenvironment is recognized as an important participant of tumor progression and response to treatment (Smith and Kang, 2013).

2. Cytokines involved in tumor microenvironment

The main cytokines present in the inflammatory tumor microenvironment include inflammatory cytokines such as tumor necrosis factor α (TNF-α), interferon-gamma (IFN-γ), interleukin-6 (IL-6), IL-1, IL-8, and transforming growth factor-beta (TGF-β), growth factors such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and other factors such as matrix metalloproteinases. The main non-tumor cells present in the inflammatory tumor microenvironment include inflammatory cells such as lymphocytes, macrophages, and myeloid-derived suppressor cells, vascular endothelial cells and tumor-associated stromal cells such as tumor-associated fibroblasts (TAFs). The roles of two other inflammatory cytokines, IL-6 and IL-1, were also addressed in breast carcinoma. Breast cancer cell lines produce IL-6, of which ER-positive cells secrete lower levels than ER-negative cells. In addition, IL-6 induces proliferation and a more aggressive phenotype in ER-positive cells (Sasser and others 2007). Depending on the tumor microenvironment, cytokines can modulate an anti-tumoral response, but during chronic inflammation, they can also induce cell transformation and malignancy (Zamarron and Chen, 2011). Therefore, the understanding of inflammatory cytokines could be fundamental for the proposal of new therapeutic approaches to particularly cancer and other cancer-related disorders.

3. Significance of interleukins in breast cancer

The interleukin (IL)-1 family of cytokines (IL-lα, IL-1β), the IL-1 receptor antagonist (IL-1Rα) and receptors (IL-1RI and IL-1RII) have been found to be frequently expressed in breast cancer cell lines and tissues within the tumour microenvironment (Miller et al., 2000; Singer et al., 2003). This local expression of IL-1/IL-1R cytokine family can be controled via autocrine and paracrine mechanisms. The tumour cell expression of other cytokines, such as the expression of IL-8, subsequently contribute to tumour proliferation and local tumour invasion (Pantschenko et al., 2003; Singer et al., 2003). It has been reported that IL-1β signalling pathway may be different in ER positive MCF-7 versus ER negative MDA-MB231 breast carcinoma cells (Liu et al., 2002). IL-6 has been found in high concentrations in human breast cancer cell lines (Honma et al., 2002; Kurebayashi, 2000). In addition, IL-6 promotes tumour growth by upregulating anti-apoptotic and angiogenic proteins in tumour cells (Kovacs, 2001; Trikha et al., 2003). Concomitant gene expression for IL-12 and interferon γ was demonstrated by reverse transcriptase-polymerase chain reaction in all 10 cases of infiltrating ductal carcinoma (Vitolo et al., 2000). Elevated serum levels of IL-1β correlated with a high rate of recurrence in patients with breast cancer (Mettler et al., 2004). An association of prognostically favourable factors with higher serum values of soluble IL-2 receptors has been reported (Sabbioni et al., 2000).

4. Novel cytokine: IL-33

4.1 Biological function of IL-33

IL-33, a ligand for IL-1RL1 (also called ST2, T1, Der4 and fit-1), which is a member of the Toll-IL-1 receptor superfamily (Schmitz et al., 2005). IL-33 is primarily expressed in non-hematopoietic cells, including fibroblasts, epithelial cells, and endothelial cells, but is also present in cells of hematopoietic origin, particularly in macrophages and dendritic cells (DCs) (Kim et al., 2014). IL-33 is also known to be identical to DVS27, a gene transcript that is upregulated in vasospastic cerebral arteries after subarachnoid hemorrhage (Onda et al., 1999), and nuclear factor from high endothelial venules (NF-HEV), a transcript expressed in the nucleus of endothelial cells (Baekkevold et al., 2003). IL-33 is localized in the nucleus due to its association with heterochromatin via a helix-turn-helix motif within the N-terminal part where it acts as a transcriptional repressor, at least *in vitro* (Carriere et al., 2007; Roussel et al., 2008).

IL-33 is a member of the IL-1 superfamily of cytokines, a determination based in part on the molecules β-trefoil structure, a conserved structure type described in other IL-1 cytokines, including IL-1α, IL-1β, IL-1Ra and IL-18 (Lingel et al., 2009). In addition, IL-33 was initially believed to be cleaved by caspase-1 to release a “mature” 18 kD form corresponding to the C-terminal cytokine domain. However, it became clear that the IL-33 protein only contains the cleavage sites of caspase-3 and caspase-

7 but not the cleavage sites of caspase-1 (Cayrol and Girard, 2009; Luthi et al., 2009).

4.2 IL-33 receptor, ST2

IL-33 binds to the IL-33 receptor, ST2, which is also called IL-1RL1 and T1, and belongs to the Toll-like receptor (TLR)/IL-1R superfamily. IL-33-bound ST2 then forms a complex with the IL-1R accessory protein (IL-1RAcP). ST2 is a member of the IL-1 receptor family, which has the Toll/IL-1R domain in the cytoplasmic region which was first identified in oncogene- or serum-stimulated fibroblasts (Tominaga, 1989). ST2 has two major forms: sST2 and ST2L (Lin et al., 2013). sST2 is a soluble ST2, which has no transmembrane sequence, so it can be excreted outside the cells. But ST2L is the transmembrane ST2 for having transmembrane sequence (Oboki et al., 2010). The transmembrane form IL-1RL1 is considered to be a functional component of IL-33R, whereas soluble form IL-1RL1 is regarded as a decoy receptor for IL-33, like soluble IL-1R for IL-1α and IL-1β (Ohno et al., 2012). ST2 is expressed on various cells including hematopoietic cells, such as mast cells, basophils, eosinophils, Th2 cells, macrophages, dendritic cells, NK cells, NKT cells, and type 2 innate lymphoid cells (ILC2), which contribute to the induction and development. Regarding intracellular events after the activation of ST2 by IL-33, the heterodimeric ST2/IL-1RAcP receptor complex activates an adaptor protein, MyD88 (myeloid differentiation primary response gene 88), and IRAK-1/4 (interleukin-1 receptor-

associated kinase 1/4) and MAP kinases. IL-33/ST2 signaling affects tumor growth and metastasis and mechanisms of antitumor immunity. $ST2^{-/-}$ mice with mammary tumors have attenuated tumor growth and metastasis, with increased circulating levels of proinflammatory cytokines and activated NK and $CD8^{+}$ T cells (Jovanovic et al., 2011).

4.3 IL-33 involved in tumor microenvironment

IL-33 is a dual-function protein that acts as both a cytokine and a nuclear factor. It is constitutively expressed by the epithelial and endothelial cells of many organs (Mirchandani et al., 2012), and it is expressed by some innate immune cells such as dendritic cells and macrophages (Ohno et al., 2009; Talabot-Ayer et al., 2012). IL-33 can increase the expression of IL-5 and IL-13 in Th2 cells (Kondo et al., 2008; Kurowska-Stolarska et al., 2008). Howevere, IL-33 is not necessary for the differentiation of Th2 cells. Native CD4 and Th1 cells do not express IL-33R, culture of resting Th2 cells in medium containing IL-33 combined with IL-2, IL-7 and cause upregulation of IL-33R expression (Guo et al., 2009). IL-33 is a ligand that binds to a high affinity receptor family member ST2 (Stampalija et al., 2014). IL-33 can also induce basophils to produce Th2-type cytokines and chemokines at the same time and then increases the expression of cell adhesion molecules and CD11b on basophils both in human and mice (Schneider et al., 2009). It was reported that Th2

lymphocytes, mast cells, macrophages, dendritic cells, $CD8^+$ T cells, and B cells and some granulocytes such as basophils and eosinophils are possible to express membrane-type ST2 molecules (Baba et al., 2012; Suzukawa et al., 2008). IL-33 could induce the production of many cytokines such as IL-5, IL-13, TNF-α, IFN-γ, and IL-2 when binding with ST2 receptors on the surface (Milovanovic et al., 2012). Accumulating evidence suggests a crucial role of IL-33/ST2 in inducing and modifying host immune responses against a variety of pathogens including parasites, bacteria, viruses, and fungi as well as sterile insults of endogenous and exogenous source (Sattler et al., 2013).

4.4 IL-33/ST2 signaling pathway

IL-33/ST2 signaling pathway was involved in T cell mediated immune response and was a potential medium for a variety of inflammatory diseases. IL-33 may function as a modulator of NF-κB and canonical Toll-like receptor/IL-1 receptor signaling (Kakkar and Lee, 2008). IL-33 can transfer extracellular information by binding with the ST2 receptor complex when serving as a conventional cytokine (Sattler et al., 2013). Furthermore, IL-33 can act as an intracellular nuclear factor which inhibits the transcription. The specific transcriptional targets or the biological effects of nuclear IL-33 are unclear at present (Haraldsen et al., 2009). It has been recognized that IL-33 signaling pathways are different from the typical Th2 cytokines

mediated pathway. IL-33 binds with the receptor complex containing ST2 and IL-1RAcp and acts through the Toll/IL-1 receptor domain of IL-1 receptor accessory protein (IL-1RAcp), which is shared by other IL-1 family members such as IL-1R and IL-18R, to transduce the IL-33/ST2 signal (Chackerian et al., 2007; Palmer et al., 2008). This causes the recruitment of MyD88, IRAK, and IRAK4 by the intracellular TIR domain of IL-1RAcP (Hong et al., 2011). Then the complex activates the transcription factor NF-κB and AP-1 through mitogen activated protein kinases (MAPK) or TNF receptor associated factor 6 (TRAF6), finally causing inflammatory responses (Schmitz et al., 2005). However, the underlying molecular mechanisms and the specific signaling pathway of the IL-33/ST2 axis during tumor development remain to be elucidated.

4.5 The role of IL-33 in tumourigenesis

IL-33/ST2 signaling pathway is a potent inducer of production of pro-inflammatory cytokines and chemokines by mast cells, particularly IL-1, IL-6, TNF, and CCL2, which have been implicated in both development and progression of cancer in some preclinical models (Ali et al., 2007; Moulin et al., 2007). Immune cells, which often infiltrate tumors and preneoplastic lesions, produce a variety of cytokines and chemokines that propagate a localized inflammatory response and enhance the growth and survival of premalignant cells by activating transcription factors such as NF-κB

(Lin, Karin, 2007; Pikarsky et al., 2004). In this study, we demonstrate that COT acts as a critical signal transducer in the IL-33/ST2 signaling axis. IL-33 markedly induced the phosphorylation of COT through increased interaction between ST2 and COT. Furthermore, IL-33-induced COT phosphorylation increased the activity of ERK1/2, JNK1/2 and STAT3, resulting in increased transcriptional activities of AP-1 and stat3. Ablation of COT effectively inhibited its role in epithelial cell transformation and tumorigenesis in the breast, demonstrating the critical oncogenic function of these IL-33-activated transcription factors.

5. LPIN1

5.1 The LPIN proteins family and function

The *LPIN1* gene family encodes the proteins LPIN-1, LPIN-2, and LPIN-3. The *LPIN1* gene was first isolated from the fatty liver dystrophy (fld) mutant mouse strain (Langner et al., 1989; Langner et al., 1991), where LPIN1 deficiency was identified as the cause of lipodystrophy, insulin resistance and neonatal fatty liver in these animals (Péterfy et al., 2001). Similarly, the human LPIN1 has two isoforms (LPIN1α, LPIN1β) derived from alternative mRNA splicing (Han et al., 2010). LPIN1α appears to be predominantly nuclear, whereas LPIN1β resides mostly in the cytoplasm (Peterfy et al., 2005).

LPIN1 family proteins are present in species ranging from mammals to yeast, and all have highly conserved regions known as the N-LIP and C-LIP domains. The current understanding is that all of the mammalian LPIN1 proteins are phosphatidate phosphatase (PAP) enzymes, which convert phosphatidate to diacylglycerol. Two LPIN1 protein isoforms are generated by alternative mRNA splicing, giving rise to proteins with predicted sizes of ~98 and 102 kDa (Péterfy et al., 2005). The role of nuclear LPIN1 may be related to its function as a transcriptional coactivator (Finck et al., 2006). In addition, component of a transcriptional complex with peroxisome proliferator-activated receptor α (PPARα) and PPARγ coactivator 1α (PGC-1α) to regulate fatty acid metabolism in liver (Finck et al., 2006). Amino acid motifs

required for LPIN1 PAP activity (DIDGT) and transcriptional coactivator activity (LXXIL) reside in the C-LIP domain (Wang et al., 2011; Finck et al., 2006). The role of LPIN1 in adipose tissue has been studied extensively and found to be required both for expression of key adipogenic genes during adipocyte differentiation, and for triacylglycerol (TAG) accumulation (Phan et al., 2004). In addition, LPIN1 in skeletal muscle has also been demonstrated through studies of muscle-specific LPIN1 transgenic mice (Phan and Reue, 2005). There are several key differences in LPIN proteins biology between yeast and mammals, and many of the key phosphorylation sites that regulate LPIN1 activity in yeast are not found in the mammalian LPIN1 (Grimsey et al., 2008). All LPIN1 proteins contain a C-LIP domain. However, the PAP1 activity of LPIN1β is higher than that of LPIN1α (Han and Carman, 2010; Donkor et al., 2007).

5.2 LPIN1 proteins as PAP enzymes required for lipid synthesis

Triacylglycerol plays a key role in the maintenance of energy homeostasis, as the major energy storage molecule, and TAG accumulation in adipose tissue allows animals to survive under conditions of food deprivation. TAG is primarily synthesized via the sequential acylation of glycerol phosphate in the Kennedy pathway (Kennedy, 1958). Phosphatidic acid, which is a common precursor of glyceride synthesis is dephosphorylated by phosphatidate phosphatase enzymes catalysis to form

diacylglycerol (DAG), which is a key substrate for the synthesis of TAG, phosphatidylethanolamine (PE) and phosphatidylcholine (PC). The LPIN proteins usually reside in the cytosol and translocate to the endoplasmic reticulum (ER) membrane in response to elevated fatty acid levels, where they bind to PA and catalyze its conversion to DAG (Cascales et al., 1984, Figure 1).

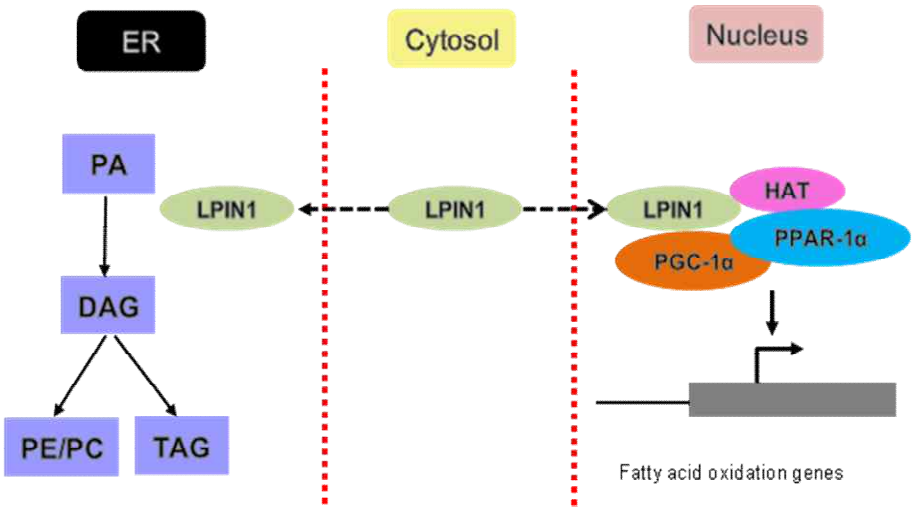

Figure 1. LPIN1 proteins as PAP enzymes required for lipid synthesis

(Chen et al., 2009)

5.3 Regulation of LPIN1 proteins activity and expression

The LPIN proteins activity is regulated at several levels, including mRNA transcription, protein phosphorylation, and subcellular localization. All three LPIN genes are expressed in liver. It has been known for decades that PAP activity in liver is induced by glucocorticoid treatment, which increases its capacity to store TAG for subsequent assembly into lipoproteins or use in beta-oxidation (Reue and Brindley, 2008). It was reported that dexamethasone increased LPIN1 mRNA, but not LPIN2 or LPIN3 and this resulted in increased LPIN1 protein synthesis and PAP activity (Manmontri et al., 2008). Dexamethasone induces LPIN1 expression and PAP activity during adipocyte differentiation, which is mediated by glucocorticoid receptor binding to a DNA sequence upstream of LPIN1 (Zhang et al., 2008). LPIN1 gene transcription during adipocyte differentiation is also regulated by binding of CAAT/enhancer binding protein α in the LPIN1 upstream region (Koh et al., 2008).

LPIN1 expression, rather than LPIN2 or LPIN3, is activated by synthetic glucocorticoids during adipocyte differentiation. LPIN1 expression is also positively correlated with insulin sensitivity, because LPIN1 expression in the adipose tissue is induced by insulin-sensitizing compounds such as thiazolidinediones and harmine (Festuccia et al., 2009; Yao-Borengasser et al., 2006). The increase in LPIN1 expression in fasting probably provides the capacity for the increased synthesis of TAG (Harris et al., 2007). The glucocorticoid-induced LPIN1 accumulation occurring

in obesity is associated with increased TAG accumulation in adipose tissue. Several studies have demonstrated that intracellular sterols modulate the expression of human LPIN1 via the sterol regulatory response element binding protein 1 (SREBP-1) and nuclear factor Y (NF-Y), and the expression of LPIN1 induced by sterol depletion controls TG accumulation through its PAP1 activity in the cytosol (Ishimoto et al., 2009).

5.4 The role of LPIN1 in insulin signaling and tumourigenesis

LPIN1 has been demonstrated to play an important role in the regulation of triglyceride and phospholipid biosynthesis by catalyzing the dephosphorylation of phosphatidate into diacylgrlycerol (Han et al., 2006). Loss of LPIN1 prevents normal adipose tissue development, leading to lipodystrophy and insulin resistance, whereas its overexpression leads to obesity (Phan and Reue, 2005; Peterfy et al., 2001). Interestingly, the high expression of LPIN1 was frequently observed in various human cancer cell lines (Brohee et al., 2015). In addition, LIPIN1 is involved in various pathways in lipid metabolism in diverse cell types such as liver, adipose tissues, muscle, and neuronal cells (Phan and Reue, 2005; Reue and Zhang, 2008). Moreover, various factors involved in LPIN1 pathway, noted that LPIN1 promotes hepatic insulin receptor signaling dysfunction via enhanced DAG production, triglyceride formation, and VLDL secretion (Chanda et al. 2012). However, numerous enzymes

are involved in lipid biosynthesis and the specific role of many of them during cancer progression is still unknown.

6. The role of mitogen-activated protein (MAP) kinase in breast cancer tumourigenesis

Mitogen-activated protein kinase (MAP kinase) is a key signal transducing protein which transmits signals involved in both cell proliferation and apoptosis. Consequently, it serves as an indicator of the intensity of trafficking of cell signaling pathways. Recent studies have found that breast cancers frequently contain an increased proportion of cells with the activated form of MAP kinase. For these reasons, MAP kinase is a key molecule regulating breast cancer growth and apoptosis. Mitogen-activated protein are the most thoroughly studied signal transduction systems. The activities of ERK1 and ERK2 had been routinely measured with two substrates, myelin basic protein (MBP) and microtubule-associated protein-2 (MAP2). As a result, they had been called MBP and MAP2 kinases (Ray and Sturgill, 1988; Ahn and Krebs, 1990). ERK signalling is activated by numerous extracellular signals. The pathway whereby growth factors and mitogens activate ERK signalling is of particular relevance to cancer. Most cancer-associated lesions that lead to constitutive activation of ERK signalling occur at these early steps of the pathway, namely, overexpression of receptor tyrosine kinases, activating mutations in receptor tyrosine kinases, sustained autocrine or paracrine production of activating ligands, Ras mutations and B-Raf mutations. In addition to being activated by phosphorylation, MAPKs are regulated by protein phosphatases that inactivate MAPKs and regulate

the strength and duration of MAPK activity (Keyse et al., 1998). Recently, increased activated MAPK was found in another cell line model of apparently estrogen-independent proliferation (Jeng et al., 2000). MAPK has been implicated in the ligand-independent activation of ERα because it can directly phosphorylate ERα on serine 118, leading to ligand-independent ER activation (Kato et al., 1995). Treatment of cells with EGF or IGF-I that activates the Ras/Raf/MAPK pathway also activates the ERs in a ligand independent fashion (Kato et al., 1995).

7. Role of STAT3 in tumorigenesis

The STAT family members, STAT3 is most often correlated to tumorigenesis, and is considered as an oncogene (Bromberg et al., 1999) as it is the point of convergence of many signaling pathways triggered by cytokines, growth factors and oncogenes. Accumulating evidence strongly implicates the critical role of aberrant STAT3 activation in malignant transformation and tumorigenesis. In contrast to the transient nature of STAT3 activation in normal cells, persistent activation of STAT3 has been reported in a variety of human tumor cell lines and primary human tumors, including leukemias, lymphomas, multiple myeloma, glioma, melanoma, head and neck, breast, ovarian, endometrial, colon, pancreatic, lung , brain, renal, and prostate cancers.

Activation of STAT3 signal transduction cascade by distinct stimuli such as growth factors, cytokines and other extrinsic agents. In addition, constitutive activation of STAT3 is involved in many cellular processes including proliferation, survival, inflammation, invasion, metastasis and angiogenesis. STAT proteins are a family of cytoplasmic transcription factors that are activated by tyrosine kinases and mediate cellular response to inflammatory and proliferative signals (Zhong et al., 1994, Bromberg and Darnell, 2000). Evidence indicates that constitutive STAT3 signalling is associated with upregulation of cyclin D1 and cMyc expression, contributing to accelerated cell-cycle progression. Consistent with its role in cellular proliferation, various studies have demonstrated that STAT3 signaling provides

survival signals and suppresses the apoptosis in cancerous cells. These effects are mediated through the expression of Bcl2, BclxL, Mcl1, surviving (Bhattacharya et al., 2005). In addition to pro-apoptotic function of STAT3, some studies suggested that loss of STAT3 promotes cellular proliferation and transformation (De La Iglesia et al., 2008). In cutaneous squamous cell carcinoma, overexpression of phosphorylated STAT3 correlated with increased invasion, and metastasis (Suiqing et al., 2005).

8. Intracellular protein degradation systems; Ubiquitin-proteasome system (UPS) and autophagy

8. 1 The ubiquitin-proteasome system (UPS)

Ubiquitin is a 76-residue protein that is highly evolutionarily conserved in all eukaryotes (Nandi et al., 2006; Hershko and Ciechanover, 1998). Intracellular proteolysis plays a crucial role in the cell cycle, signal transduction, gene expression, development, maintenance of proper protein folding and other cellular processes (Kirschner, 1999). The 'housekeeping' 26S proteasomes are ATP-driven, multisubunit proteolytic machines that preferentially degrade proteins tagged with polyubiquitin chains (Hershko and Ciechanover, 1998; Elsasser and Finley, 2005). Conjugation is accomplished in three sequential reactions, which are catalyzed by a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin-protein ligase (E3). After the formation of the ubiquitin-substrate isopeptide bond, a second ubiquitin can be conjugated to a specific lysine residue, typically Lys48, of the first ubiquitin. Repetition of this reaction leads to assembly of a polyubiquitin chain on the proteolytic substrate that targets the protein for degradation by the 26S proteasome. The 20S core is an abundant particle that has been highly conserved from yeast to humans (Löwe et al., 1995). Four α- and β-rings surround a barrel-shaped cavity in the 20S core (Groll et al., 1997). The two inner –β rings form a central chamber that harbors the proteolytic centers containing chymotryptic, tryptic

and caspase-like activities. Therefore, ubiquitinated cytosolic proteins undergo degradation via the proteasome or the lysosome, which comprise the two major intracellular proteolytic systems in mammalian cells routes (Kirkin et al., 2009).

8.2 Autophagy system

"Autophagy", literally "self-eating", describes a catabolic process in which cell constituents such as organelles and proteins are delivered to the lysosomal compartment for degradation. Autophagy is an evolutionarily conserved process whose primary task in lower organisms is the maintenance of metabolic homeostasis in the face of changing nutrient availability (Abeliovich and Klionsky, 2001). Recent advances have demonstrated that autophagy also serves a surprisingly diverse array of additional functions, including organelle clearance, antigen presentation, elimination of microbes, as well as regulation of development and cell death (Mizushima, 2005). Autophagy has been considered a less selective degradative pathway than the UPS and is frequently illustrated as the engulfment of large portions of cytoplasm and delivery of the contents to the lysosome in bulk.

Microautophagy consists of direct engulfment of small volumes of cytosol by lysosomes (Ahlberg et al., 1982), whereas chaperone-mediated autophagy (CMA) involves selective, receptor-mediated translocation of proteins into the lysosomal lumen (Dice et al., 1990). Even within the category of macroautophagy, there appears

to be capability for selectivity, as autophagic processes have been observed that appear to be specific for mitochondria (mitophagy), portions of the nucleus (nucleophagy), endoplasmic reticulum (reticulophagy), microorganisms (xenophagy), ribosomes (ribophagy) or protein aggregates (aggrephagy). While it remains unclear how substrates are specifically marked for degradation by macroautophagy, the identification of a family of autophagy-related (Atg) genes in yeast and their homologues in higher organisms has permitted minute dissection of the general process by which autophagy engulfs and degrades its targets. The process of autophagy is controlled by parallel activation cascades that involve ubiquitin-like (UBL) protein modification, strikingly similar to the activation cascade that regulates the UPS. Autophagosomes and autolysosomes can be distinguished morphologically, as autophagosomes contain contents with densities similar to cytosol, while autolysosomes appear as electron-dense material with a hollow rim beneath the limiting membrane. For the autophagosomes formation, Pro-LC3 is cleaved co-translationally to create a form of LC3 denoted “LC3-I”. LC3-I becomes conjugated to phosphatidylethanolamine (PE) to form “LC3-II” and thereby covalently associates with the phagophore. Therefore, microtubule-associated protein 1 light chain 3 (LC3) facilitate autophagic/lysosomal degradation via its interaction with ubiquitinated proteins (Pankiv et al., 2007; Kirkin et al., 2009, Figure 2).

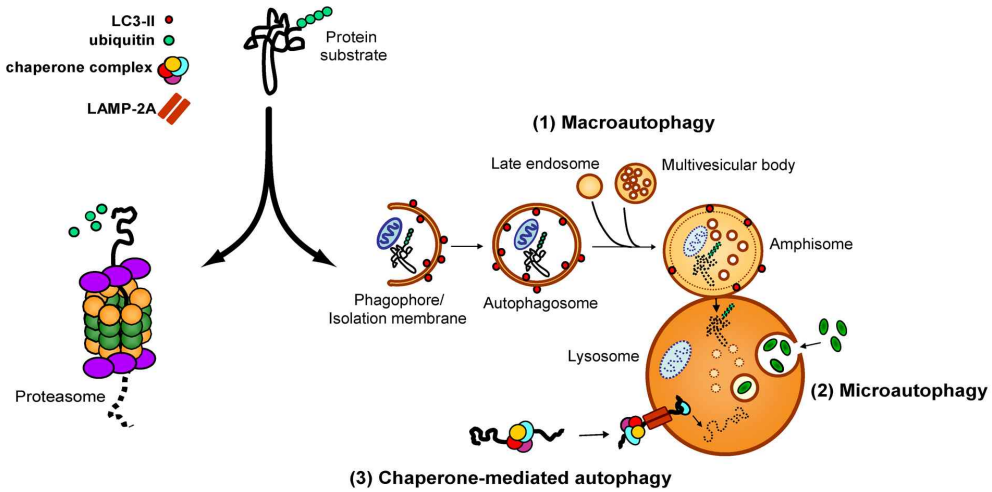

Figure 2. Intracellular protein degradation systems; Ubiquitin-proteasome system(UPS) and autophagy (Nedelsky et al., 2008)

8.3 Diseases related with dysregulation of proteosomal and lysosomal proteolysis

The pathologic states associated with the UPS can be classified into two mechanism-based groups: those that result from a mutation in a UPS enzyme or a target substrate, leading to stabilization of certain proteins, and those that result from accelerated degradation of the target protein. This section presents several studied examples of aberrations in the UPS that are directly linked to human diseases (Betarbet et al., 2005). The accumulation of insoluble protein deposits in the affected cells is a common feature of many age-related neurodegenerative disorders, such as tauopathies (e.g. Alzheimer's and Pick's disease), synucleinopathies (e.g. Lewy body disease) and polyglutamine diseases (Huntington's disease, and several spinocerebellar ataxias). In addition, in non-neuronal disorders inclusions are present as well (e.g. alcoholic liver disease, inclusion body myositis and α-antitrypsin deficiency) (Spillantini et al., 1997). Although the deposits vary in protein composition, shape and localization, each of these structures (e.g. aggregates, aggresomes or inclusions) is mainly composed of insoluble misfolded proteins (e.g. huntingtin, α-synuclein or hyperphosphorylated tau), different molecular chaperones (e.g. heat shock proteins) and various components of the ubiquitin-proteasome.

Neurodegenerative disease-causing proteins are frequently degraded by autophagy was demonstrated by a series of in vitro studies which showed that pharmacological induction or inhibition of macroautophagy alters the rate of turnover of a number of diseaserelated proteins including polyglutamine-expanded proteins, polyalanine-expanded proteins, as well as wild type and mutant forms of α-synuclein (Ravikumar

et al., 2002; Webb et al., 2003). In addition, CMA has also been found to contribute to the degradation of α-synuclein (Cuervo et al., 2004). Collectively, these studies suggested that autophagy contributes to the degradation of multiple disease proteins and the efficiency of this pathway could relate to the onset or progression of disease. The relative contribution of autophagy and the UPS to degrading disease-related substrates, and the relationship of this to the onset and progression of various diseases, remains to be elucidated.

9. Activator protein-1 (AP-1) in tumorigenesis

9.1 AP-1 family proteins, structures, and functions

The Activator protein-1 (AP-1) family of transcription factors is composed of homodimers and heterodimers of Jun (v-Jun, c-Jun, JunB, and JunD), Fos (v-Fos, c-Fos, FosB, Fral, and Fra2), ATF (ATF2, ATF3/LRF1, B-ATF, JDP1, and JDP2), and MAF (c-Maf, MafB, MafA, MafG/F/K, and Nrl) protein families (Hai et al., 1998; Angel and Karin, 1992), which are characterized by highly conserved dimeric basic leucine zipper (bZIP) DNA-binding domains. AP-1 is one of the first mammalian sequence-specific transcription factors recognized (Angel et al., 1987; Bohmann et al., 1987). Moreover, AP-1 was first known as a 12-O-tetradecanoylphorbyl-13-acetate (TPA) inducible transcription factor, since the TPA response element (TRE) was identified as a binding site for AP-1 in many cellular and viral genes (Angel et al., 1987). AP-1 belongs to the dimeric basic region-leucine zipper (bZIP) protein group composed of Jun, Fos, and activating transcription factor (ATF) protein family members and AP-1 transcription factor is a dimer and the complexity of AP-1 begins with the transcription factor itself. Moreover, AP-1 is composed of many different combinations of hetero- or homodimers and the composition of AP-1 determines the genes that it regulates. AP-1 is regulated on multiple levels. The expression of AP-1 proteins is regulated by controlling the transcription of their genes. AP-1 function is also dependent on dimer composition in the DNA binding complex. In addition, AP-1

proteins are regulated by posttranslational modifications. The most common posttranslational modification known to regulate protein activity is phosphorylation and AP-1 proteins are phosphorylated by MAPKs.

9.2 Jun family proteins, structures, and functions

c-Jun is the best characterized AP-1 component. c-Jun is a nuclear protein which is expressed in many cell types at low levels, but its expression is upregulated by growth factors, cytokines, and UV irradiation. c-Jun is fairly conserved among different species. Human *c-jun* is a 3.1 kb proto-oncogene and lacks introns (Hattori et al., 1988). The c-Jun protein is composed of 334 amino acids. It has three main domains that are particularly well conserved among the different Jun and Fos family members; the leucine zipper (bZIP) domain, the basic region and the transactivation domain. Among them, the bZIP domain, containing two parallel α-helixes that form a coiled coil in the C-terminus is responsible for the dimerization of AP-1 proteins (Landschulz et al., 1988). The N-terminus of c-Jun contains also a δ-domain, which is the docking site for JNK and mediates ubiquitin-dependent degradation of c-Jun (Treier et al., 1994).

c-Jun is an immediate-early gene. It is activated rapidly and transiently without the need for new protein synthesis. Cytokines, growth factors, environmental stress, bacterial and viral infections, and oncogenes activate c-Jun and induce its expression

in various cellular contexts. Expression in both proliferating and differentiating cells suggests that c-jun is associated with both cell proliferation and differentiation. As for all bZIP transcription factors, dimerization of c-Jun is required prior to binding to DNA. c-Jun forms homodimers, or heterodimers with other Jun, Fos or ATF proteins (Smeal et al., 1989). The c-Jun enhancer contains two AP-1 binding sites, one of which has been reported to mediate positive autoregulation of the c-jun gene by c-Jun (Angel et al., 1988). c-Jun induction is usually mediated through two TPA-response elements. These are preferentially recognized by c-Jun-ATF-2 heterodimers and MAPK-dependent phosphorylation of these sites stimulates transcriptional activation of c-Jun (Smeal et al., 1991; Derijard et al., 1994). c-Jun and JunB have been to shown to act antagonistically in controlling cell transformation, differentiation and expression of AP-1-dependent target genes. JunD is the most ubiquitously expressed of the AP-1 proteins (Hirai et al., 1989). c-Jun and JunD have been proposed in cell cycle regulation and cell proliferation.

9.3 Fos family proteins, structures, and functions

An important step in defining the AP-1 transcription factor came with the discovery that the viral oncoprotein c-Fos binds to the same DNA sequence as c-Jun (Rauscher et al., 1988). c-Fos is an immediate-early proto-oncogene with rapid and transient transcriptional activation following mitogenic stimuli (Greenberg and Ziff, 1984).

Like c-Jun, c-Fos is involved in numerous cellular processes such as proliferation, differentiation, transformation, and apoptosis. The 4 kb mammalian c-fos gene has four exons and transcribes a 2.2 kb mRNA. c-Fos protein is composed of 381 amino acids (Piechaczyk and Blanchard, 1994). Fos family members are able to form heterodimers with Jun proteins, with varying affinities (Hai and Curran, 1991). c-Fos also contains a more recently discovered ERK-docking site, the DEF domain, in the C-terminus (Murphy et al., 2002). c-Fos is associated with a variety of biological processes, from cell-cycle progression and cell differentiation to cell transformation and tumorigenesis (Shaulian and Karin, 2001). Tumorigenic properties of c-Fos have been demonstrated by overexpression, which causes osteosarcomas by transforming chondroblasts and osteoclasts (Grigoriadis et al., 1993).

Like Jun proteins, Fos proteins must dimerize upon activation. Fos proteins cannot form homodimers, but can heterodimerize with Jun proteins (Halazonetis et al., 1988). Fos-oncoproteins include several family members in addition to c-Fos, FosB, Fra-1, and Fra-2 (Zerial et al., 1989; Matsui et al., 1990). The expression of FosB has been localized to neuronal tissue and bone during embryonic development, although no known essential function during embryonic development has been identified (Gruda et al., 1996). Another Fos-family member, fos related antigen-1 (Fra-1), lacks the C-terminal transactivation domain and has therefore been proposed to be a negative regulator of AP-1 activity. Overexpression of fra-1 has a growth inhibitory effect and

induces apoptosis in glioma cells (Shirsat and Shaikh, 2003). In addition, Fra-2 has a peculiar expression pattern compared to those observed in other fos-related genes, suggesting that Fra-2 has a unique role in cellular differentiation during fetal development. Fra-2 expression has been identified in differentiating epithelia, developing cartilage and in the central nervous system during embryonic development (Carrasco and Bravo, 1995).

II. Materials & Methods

1. Cell lines and establishment of stable cell lines

JB6 Cl41 mouse epidermal cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 5% fetal bovine serum (FBS). MDA-MB231 human breast cancer cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 5% fetal bovine serum (FBS). Human embryonic kidney 293 (HEK293), MCF7 human breast cancer cells were grown in Dulbecco's modified Eagle's medium (DMEM) EMEM supplemented with 10% FBS, respectively. SKBR3 and 4T1 breast cancer cells were grown in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS, respectively. All cell lines were cultured and maintained at 37°C in humidified air containing 5% CO_2. For establishment of IL-33-stably-overexprssing cell lines, human IL-33 expression plasmid, pCMV/Myc-IL-33, was transfected into mouse 4T1 cells. At 48 h after transfection, cells were cultured in medium containing 600 μg/ml of neomycin. Each colony that grew after G418 selection was picked and expended. We designated the 4T1 cells transfected with the IL-33 expression vector as 4T1/Myc-IL33 cells. For establishment of stable cell lines, 4T1 metastatic mouse breast carcinoma cells were transfected with pcDNA4 and FLAG-LPIN1, respectively. The selection of these transfected cells was performed with 300 μg/ml zeocin for 1 wk and with 100 μg/ml zeocin until the formation of a sufficient colony. Stable cell clones or pools were checked for protein expression by immunoblotting analysis with respective antibodies

to confirm the expression of expected proteins.

2. Reagents and antibodies

Recombinant human interleukin-33 (IL-33) was obtained from R&D Systems (Minneapolis, MN, USA). The NT157 was obtained from Axon Medchem (Groningen, NL). The COT inhibitor [TKI (Tpl2 kinase inhibitor), 4-(3-chlor-4-fluorophenylamino)-6-(pyridin-3-yl-methylamino)-3-cyano-1, 7-naphthylridine], and WP1066, PD98059, and SP600125 were purchased from Calbiochem-Novabiochem (San Diego, CA, USA). The phospho-specific and total antibodies against c-RAF, MEK1/2, ERK1/2, P90RSK, JNK1/2, STAT3, COT, c-Jun (Ser 63), and IRS1 (Ser 307) were acquired from Cell Signaling Technology, Inc. (Beverly, MA, USA); antibody against ST2 was purchased from Abcam (Cambridge, MA, USA); LPIN1, IRS1, c-Jun, COT, and mouse IgG were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Propranolol, Insulin-like growth factor-1 (IGF-1), MG132, Cycloheximide, Chloroquine diphosphate salt and anti-c-Myc antibody, anti-FLAG and anti-ST2 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Anti-LC3 antibodies were purchased from Novus Biologicals (Littleton, CO, USA). The anti-IL-33 antibody was purchased from Millipore (Billerica, MA, USA).

3. Construction of mammalian expression and small interfering RNA (siRNAs)

The WT and mutants (S400A) of the pRK-Myc-COT plasmids were provided by Warner C. Greene (University of California, San Francisco, CA, USA). The human IL-33 expression plasmid, pCMV/Myc-IL-33 was purchased from OriGene

Technology, Inc. (Rockville, MD, USA). IL-33 (accession number: NM_001199640), ST2 (accession number: NM_010743), and COT (accession number: NM_005204), LPIN1 (accession number: NM_001130412), IRS1 (accession number: NM_005544). were silenced by transfecting the ON-TARGET plus small interfering RNA (siRNA) SMART pool-specific or nonspecific control pool double-stranded RNA oligonucleotides (Dharmacon, Chicago, IL, USA) using Lipofectamine® 2000 Reagent (Invitrogen).

4. RNA Isolation and Semiquantitative RT-PCR

Total RNA was isolated from cells using the RNeasy micro kit (Qiagen, Valencia, CA, USA). cDNAs were prepared from the above RNAs by using the ImProm-IIreverse transcription system (Promega). RT-PCR was performed with Step One (Applied Biosystems, Foster City, CA, USA) using a SYBR green premix according to the manufacturer's instructions (Applied Biosystems). The following primers were used for RT-PCR: human IRS1, sense, 5'-CAAGACCATCAGCTTCGTGA-3' and antisense: 5'-AGAGTCATCCACCTGCATCC-3'. The relative level of PCR products was determined on the basis of the threshold cycle value. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalized reference gene. All data were normalized to GAPDH as an internal control according to the manufacturer's instructions.

5. Immunofluorescence staining

Cells cultured on cover slips were allowed to recover for adequate times and then fixed with 4% paraformaldehyde and 98% methanol, followed by permeabilization with 0.3% Triton X-100. After permeabilization, coverslips were blocked with 5% BSA in PBS and then immunostained with primary antibodies and Alexa Fluor 488- (green, Molecular Probe) or Alexa Fluor 594- (red, Molecular Probe) conjugated secondary antibodies. After washing, the coverslips were mounted onto slides using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Fluorescence images were taken using a confocal microscope (Zeiss LSM 510 Meta; Carl Zeiss) and analyzed with Zeiss microscope image software ZEN (Carl Zeiss).

6. Cell proliferation assay (BrdU incorporation)

Cells (5×10^3 cells) were seeded in 96-well plates in 100 μl of cell suspension in each well. After 24 h, they were treated with or without IL-33 for 24 h, labeled with 10 μL/well BrdU labeling solution, and then incubated for 4 h at 37°C in a 5% CO_2 atmosphere. Cell proliferation was estimated by measuring the absorbance at 370 nm.

7. Immunoblot analysis

The cells were disrupted in radioimmunoprecipitation assay (RIPA) lysis buffer. The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF. For detecting chemiluminescence, a LAS

4000 imaging system (GE Healthcare Biosciences, Pittsburgh, PA, USA) was used.

8. Measurement of cytokines

The cells were plated in 96 well plates at 5 $\times 10^3$ cells/well overnight and treated with IL-33 for an additional 24 h. Culture media was harvested for measurement of IL-6, MCP-1, and TNF-α concentration. The ELISA was performed according to the manufacturer instructions (R&D Systems, Minneapolis, MN, USA). The absorbance of the reaction solution was measured at 450 nm on a Microplate Autoreader ELISA (Bio-Tek Instruments Inc., Winooski, VT, USA).

9. Anchorage-independent cellular transformation assay

Briefly, 8 × 10^3 cells were exposed to different doses of IL-33 in 1 mL of 0.3% basal medium Eagle's (BME) agar containing 10% FBS, 2 mM L-glutamine, and 25 μg/mL gentamicin. The cultures were maintained at 37°C in a 5% CO_2 incubator for 14 days, and scores of the cell colonies were determined using an Axiovert 200 M fluorescence microscope and the Axio Vision software (Carl Zeiss, Inc., Thornwood, NY, USA).

10. Reporter gene assays

To detect firefly luciferase activity, the reporter gene assay was performed using lysates from c-*fos*-, *c-jun*-, AP-1-, or *stat3*-luc-transfected JB6 Cl41 cells and c-*fos*-, AP-1-luc-transfected SKBR3 and MDA-MB231 cells. The reporter gene vector pRL-TK-luciferase plasmid (Promega) was co-transfected into each cell line and the *Renilla* luciferase activity generated by this vector was used to normalize the results

for transfection efficiency. Cell lysates were mixed with luciferase assay II reagent, and firefly luciferase light emission was measured using the GloMax®-Multi Detection System (Promega). Subsequently, *Renilla* luciferase substrate was added to normalize the firefly luciferase data. The *c-fos-luc* promoter (pFos-WT GL3) and *c-jun-luc* promoter (JC6GL3) constructs were kindly provided by Dr Ron Prywes (Columbia University, New York, NY). The AP-1 and *stat3* luciferase reporter plasmid (−73/+63 collagenase-luciferase) were kindly provided by Dr Dong Zigang (Hormel Institute, University of Minnesota, Austin, MN) and Dr K-Y Lee (Chonnam National University, South Korea).

11. Tumor samples

The breast tissues that were selected for immunohistochemical staining were collected from a breast cancer group of 60 patients (age range: 42–72 y). The normal breast group included mammary infiltrating duct carcinoma patients who had undergone mastectomy with adjuvant hormone therapy and had no subsequent local recurrence or metastasis within 5 y, and the breast cancer group included mammary infiltrating duct carcinoma patients who had undergone mastectomy with adjuvant hormone therapy and lapsed into a subsequent bone metastasis. Informed consent was obtained from all the patients, and research protocols were approved by the ethics committee of Chosun University Hospital (South Korea).

12. Immunohistochemical staining analysis

All tumors investigated in the study were tested using the ST2, IL-33, and COT

mouse monoclonal antibodies. Immunolocalization for each antibody was performed using a Polink-2 HRP Plus mouse diaminobenzidine detection system (Golden Bridge International, WA, USA), according to the supplier's protocol. Slides were incubated for 1 h with anti-IL-33 and anti-ST2 antibodies and overnight with anti-COT antibody in a moist chamber at 37°C. Instead of a primary antibody, normal goat serum was used in the negative control. Distinct cytoplasmic staining for COT, ST2, and IL-33 were considered to indicate positive immune reactivity.

13. Tumorigenicity assay in nude mice

Six-week-old female BALB/c mice (18–20 g) were obtained from Samtako Co. (South Korea), acclimatized for 1 week, and maintained in a clean room at the College of Pharmacy, Chosun University. The protocols for the animal studies were approved by the Animal Care Committee of Chosun University (Gwangju, South Korea). Mice were randomly divided into two or four groups of 20 animals each, and 4T1 cells, control-transfected 4T1 cells (4T1/mock), IL-33-transfected 4T1 cells (4T1/Myc-IL33) were trypsinized, washed with PBS, resuspended in PBS, and adjusted to a concentration of 1×10^6/100 μl in PBS. Then the cell suspensions were injected into the mammary gland of the mice in the presence or absence of IL-33 and 100 μM TKI and/or WP1066 then allowed to grow until the formation of tumors. 4T1 metastatic mouse breast carcinoma cells stably expressing mock, LPIN1 (mock-4T1, LPIN1-4T1) and mouse breast carcinoma cells (4T1) treated with 400 ng/ml IGF-1 or 400 ng/ml IGF-1 combined with 20 μM NT157 or 500 μM propranolol were injected into the mammary glands of the mice and allowed to grow until tumor formation (14

days). The tumor volume was calculated using the formula: $V = (ab^2)/2$, in which 'a' was the longest diameter and 'b' was the shortest diameter of the tumor.

14. Statistical analysis

Fisher's exact test with two-sided probability values ($P < 0.001$) was used to analyze correlations between IL-33, ST2, and COT in breast cancer patients. Data from the BrdU incorporation assay, the reporter gene assay and the soft agar assay were analyzed using unpaired *t*-tests, and *P*-values of <0.05 were considered significant. Statistical calculations were carried out with Prism 4.0 for Macintosh (GraphPad Software, Inc., La Jolla, CA, USA). Results are expressed as the mean ± standard error of triplicate measurements of three independent experiments.

III. Results

Part I : Interleukin-33/ST2 axis promotes epithelial cell transformation and breast tumorigenesis via upregulation of COT activity

1. IL-33 promotes anchorage-independent transformation and tumorigenesis in breast.

To understand the pathological relevance of IL-33 in breast tumorigenesis, immunohistochemistry was performed on human normal and breast cancer tissues using IL-33 and ST2 antibodies. Knowing that IL-33 is a member of the IL-1 family and an extracellular ligand for the orphan IL-1 receptor family member ST2 (Schmitz et al., 2005), immunohistochemistry was performed on human normal and breast cancer tissues using IL-33 and ST2 antibodies. Four of the 20 normal breast samples had a low amount of IL-33, whereas all of 20 breast cancer samples contained high amounts of IL-33 (Figure 3a). In contrast, eighteen of the 20 normal breast samples had a low amount of ST2, whereas thirteen of 20 breast cancer samples contained high amounts of ST2, indicating that ST2 expression was significantly increased in breast cancer (Figure 3a). To further investigate the expression of IL-33 and ST2 proteins in breast cancer, we used immunoblotting to examine cells from the normal breast cells, MCF10A, and breast cancer cell lines BT-474, MDA-MB231, MCF7,

and SK-BR3. IL-33 and ST2 protein expression were detected in all breast cancer cell lines (Figure 3b). Interestingly, ST2 expression in all breast cancer cell lines was higher than normal breast cells (Figure 3b). To examine the effects of IL-33 on cell proliferation and anchorage-independent cell transformation, the BrdU incorporation and soft agar assays were performed. IL-33 treatment significantly and dose-dependently induced proliferation of JB6 Cl41 cells, in which IL-33 and ST2 were normally expressed (Figure 3c). Furthermore, IL-33 treatment significantly increased not only colony numbers but also colony sizes in JB6 Cl41 cells in a dose-dependent manner (Figure 3d). To investigate the environmental effects of IL-33 on production of proinflammtory cytokines, IL-6, MCP-1, and TNF-α levels were analyzed in culture supernatant of IL-33-stimulated MCF7 and MDA-MB231 cells. IL-33 treatment led to a significant increase of IL-6 and MCP-1, but not TNF-α, in culture supernatant of MCF7 and MDA-MB231 cells (Figure 3e-3g). However, the culture supernatant of IL-33-stimulated cells did not influence proliferation of MCF7 and MDA-MB231 cells, while IL-33 treatment significantly induced the proliferation of MCF7 and MDA-MB231 cells (Figure 3h and 3i). The effects of IL-33 on tumor development *in vivo* were studied in a syngeneic mouse 4T1 breast tumor metastasis model. IL-33 treatment significantly induced proliferation of 4T1 cells in a dose-dependent manner (Figure 3j). Representative tumor images demonstrated that IL-33 significantly induced mammary gland tumor development (Figure 3k).

Figure3 (a)

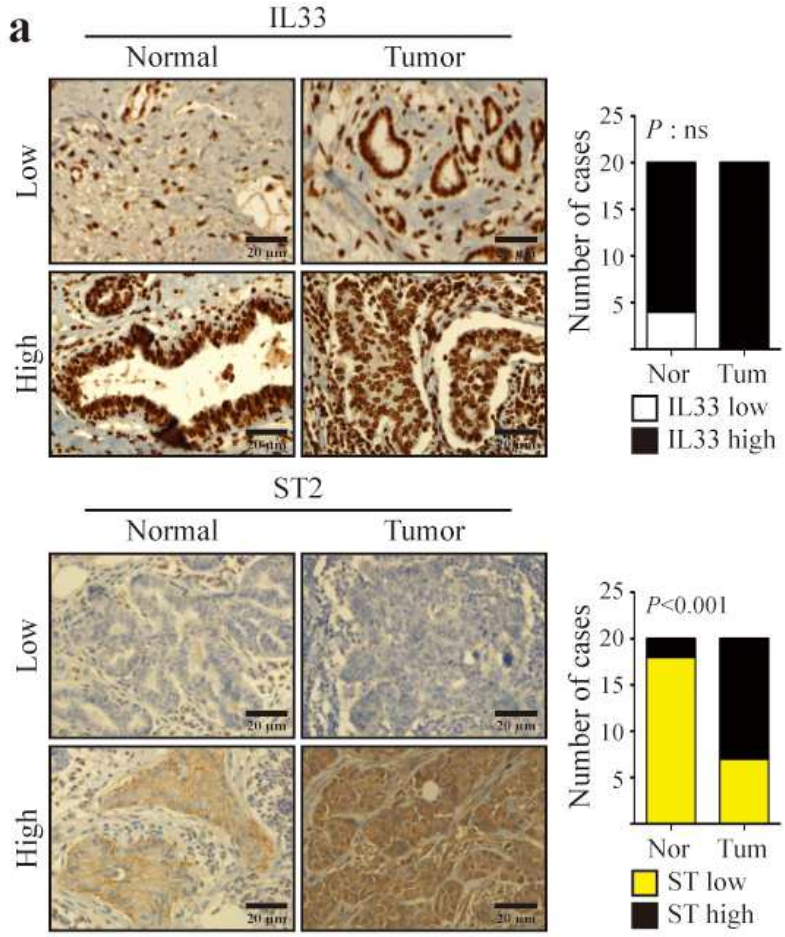

Figure3 (b-d)

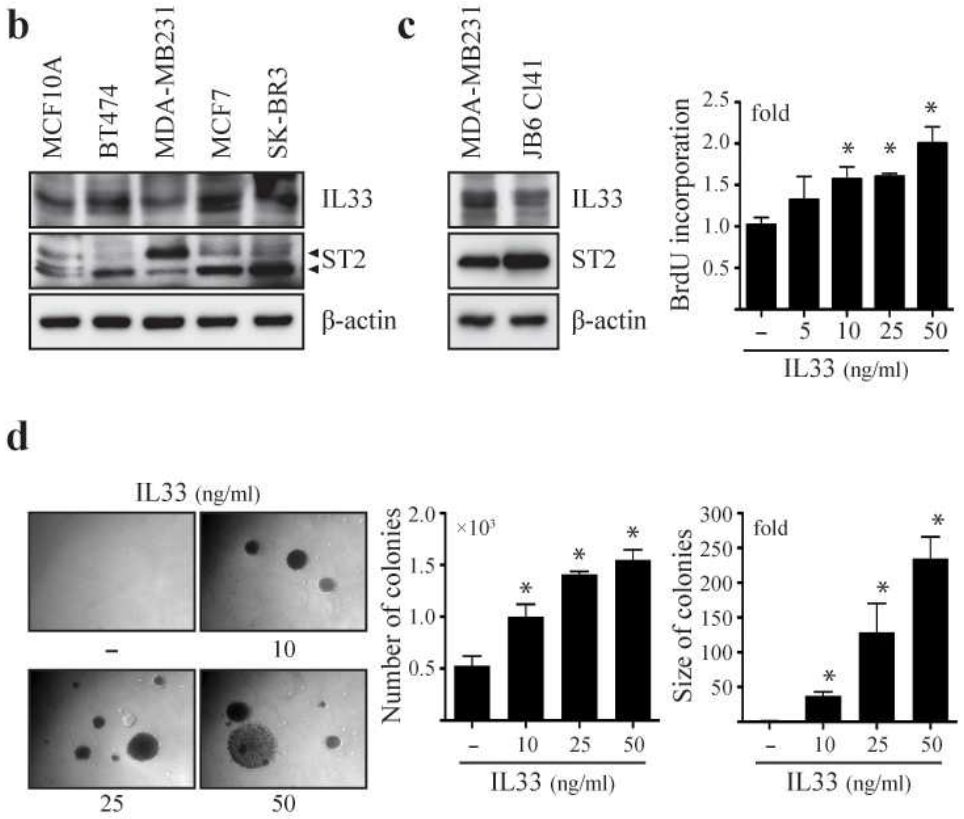

Figure3 (e-g)

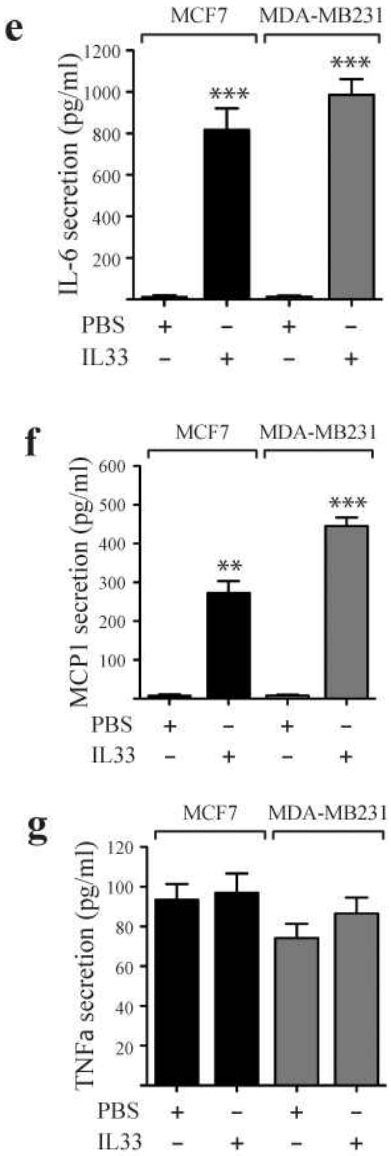

Figure3 (h-i)

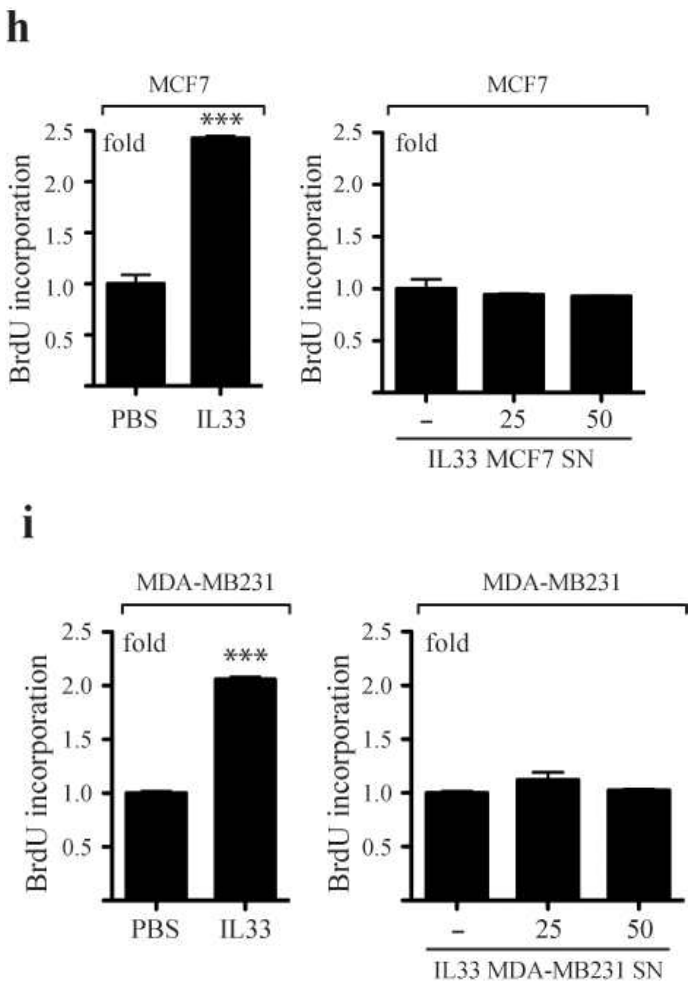

Figure3 (j-k)

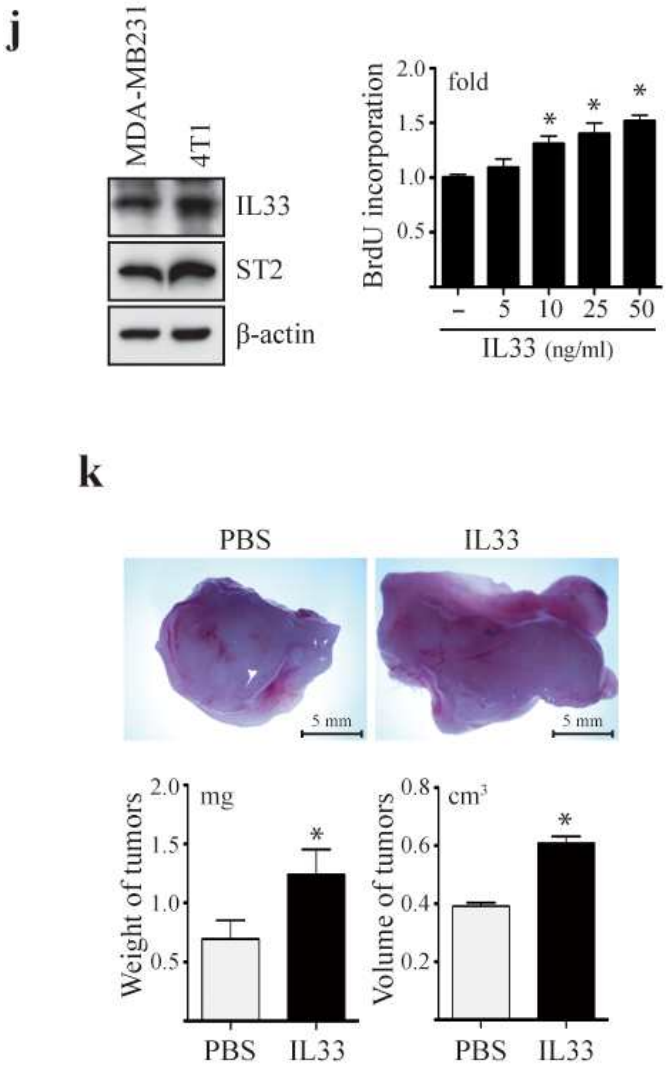

Figure 3. IL-33 promotes epithelial cell transformation and breast tumorigenesis.

(a) Representative samples showing the results of immunohistochemical analysis of human normal breast tissues and breast infiltrating duct carcinoma performed using the indicated antibodies. Their correlation was analyzed using Fisher's exact test.

(b) MCF10A, BT-474, MDA-MB231, MCF7, or SK-BR3 cells were harvested and lysed. Immunoblotting analysis was performed using specific antibodies against the corresponding proteins and figure is representative of at least three separate experiments that yielded similar results.

(c) Left, expression levels of IL-33 and ST2 in MDA-MB231 cells or JB6 Cl41 cells; right, JB6 Cl41 cells were seeded, treated with different concentrations of IL-33 for 24 h, and then cell proliferation was estimated using a BrdU assay.

(d) JB6 Cl41 cells were exposed to various concentrations of IL-33 in soft agar matrix, and incubated at 37°C in a 5% CO_2 atmosphere for 14 days.

(e − g) Production of IL-6, MCP-1, and TNF-α in MCF-7 and MDA-MB-231 cells in response to IL-33. The cells were treated with IL-33 for 24 h. IL-6, MCP-1, and TNF-α levels in the culture supernatant were measured by ELISA, as indicated in Materials and Methods. Data are shown as means ± SEM of triplicate samples from one experiment representative of three independent experiments performed. **, $P < 0.01$, ***, $P < 0.001$.

(h and i) Cell proliferation was estimated using a BrdU assay. Left panels, MCF7

cells (h) or MDA-MB231 cells (i) were seeded, maintained for 24 h, and then treated or not treated with 50 ng/ml IL-33 for 24 h; right panels, MCF7 cells (h) or MDA-MB231 cells (i) were seeded, maintained for 24 h Then the cells were stimulated with the supernatants from IL-33-treated MCF7 cells (IL33 MCF7 SN) and MDA-MB231 cells (IL33 MDA-MB231 SN) for 24 h, which were treated with/without 25 ng/ml or 50 ng/ml IL33 for 24 h, respectively. Data are represented as mean ± SEM from one experiment representative of three independent experiments performed.

(j) Left, expression levels of IL-33 and ST2 in MDA-MB231 cells or 4T1 cells; right, 4T1 cells were seeded, treated with different concentrations of IL-33 for 48 h, and then cell proliferation was estimated using a BrdU assay.

(k) 4T1 cells were injected into the mammary gland of BALB/c mice in the presence or absence of 250 ng/mL IL-33, and allowed to grow until tumors formed (14 days). Data in c, d, j and k are represented as mean ± SEM from one experiment representative of three independent experiments performed. *, $P < 0.05$.

2. ST2 mediates IL-33-induced COT phosphorylation via the interaction of ST2 with COT.

To investigate the relationship between ST2 and COT in human breast cancer, we performed immunohistochemistry using specific antibodies against human ST2 and COT in human breast cancer tissues. Five of the 7 breast cancer samples that showed low amounts of ST2 presented with lower expression COT, whereas all 13 breast cancer samples that contained a high amount of ST2 correspondingly had higher expression of COT (Figure 4a). To examine whether COT is a critical signal transducer in the IL-33 signaling pathway, we first tested whether ST2 interacts with COT in cells. Reciprocal immunoprecipitation/immunoblotting using anti-Myc and anti-ST2 antibodies showed that endogenously expressed ST2 interacted with Myc-COT *in vitro* (Figure 4b). Previously, we reported that COT could be phosphorylated on the Ser400 residue of its C-terminus by immune cytokines such as IL-17A and IL-22 (Kim et al., 2013; Kim et al., 2014). Thus, this study examined the possibility that mutation of the Ser400 residue at the COT carboxyl terminus affects COT's interaction with ST2. To determine the site responsible for COT's interaction with ST2, we mutated the Ser400 of COT to Ala and investigated the ability of this non-phosphorylated mutant to bind to ST2 in immunoprecipitation/immunoblotting analyses. The results showed that mutation of the Ser400 site in COT led to a significant loss of binding between COT and endogenous ST2 (Figure 4c). In addition,

endogenous ST2 interacted with COT in IL-33-stimulated cells, but not in un-stimulated cells (Figure 4d). To further determine whether IL-33 phosphorylates COT, JB6 Cl41 cells were treated with IL-33, and immunoblotting was performed using a specific antibody for phospho-COT (Ser400). The results showed that IL-33 markedly induced phosphorylation of COT in a dose- and time-dependent manner (Figure 4e). However, knockdown of ST2 suppressed IL-33-induced phosphorylation of COT (Figure 4f). Given the positive correlation between ST2 and COT abundance in human breast cancer tissues and the phosphorylation-dependent interaction between ST2 and COT, we next examined the effect of IL-33 on the phosphorylation of COT in MDA-MB231 and MCF7 cells. The results showed that IL-33 markedly induced the phosphorylation of COT in both cell types (Figure 4g).

Figure 4 (a)

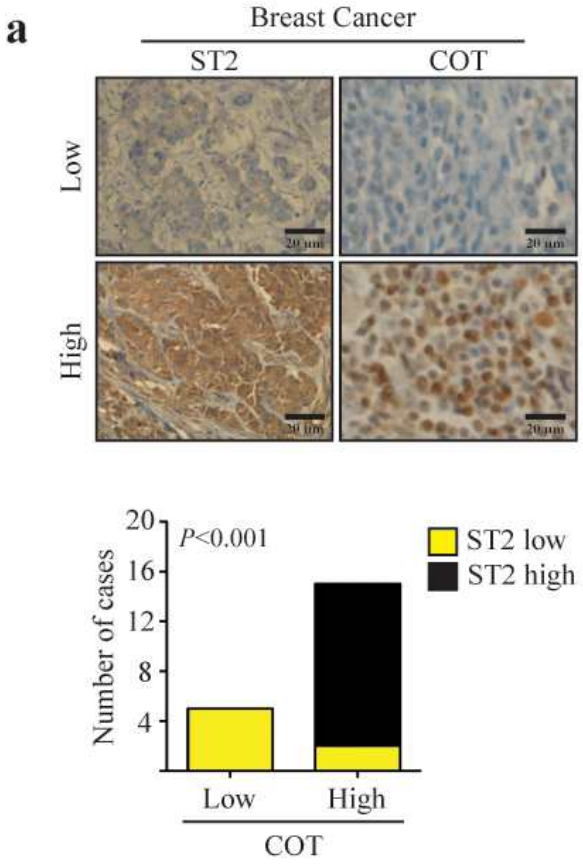

Figure 4 (b-d)

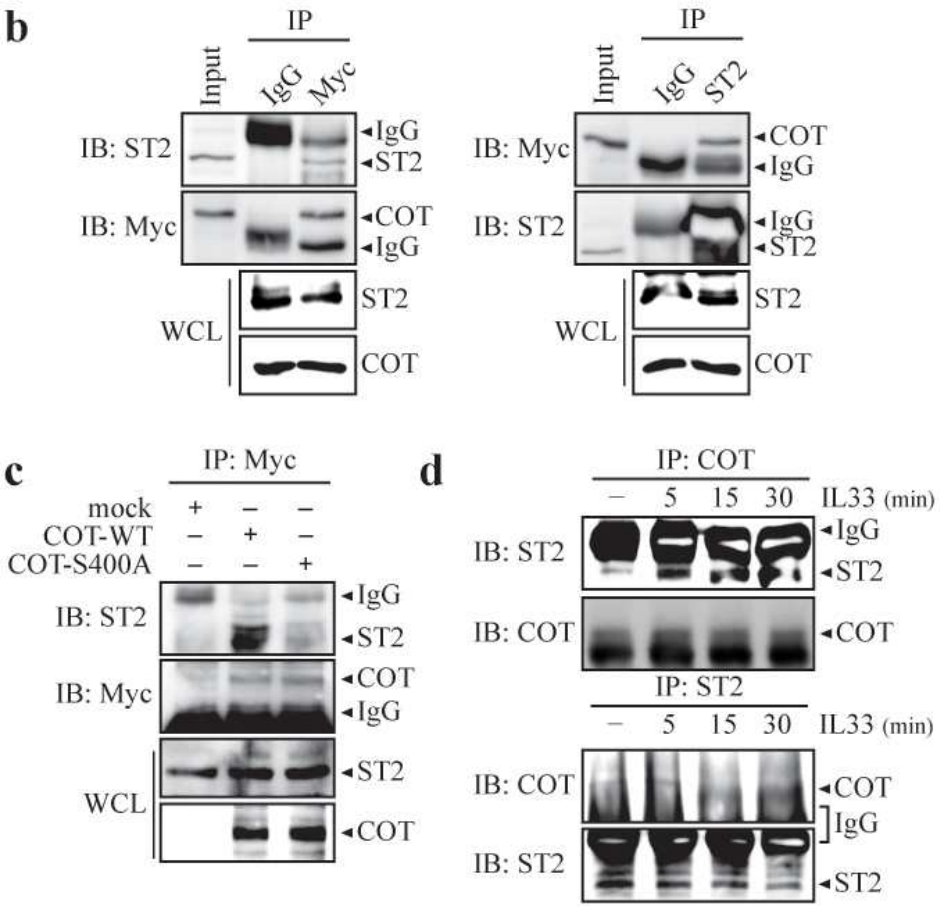

Figure 4 (e-g)

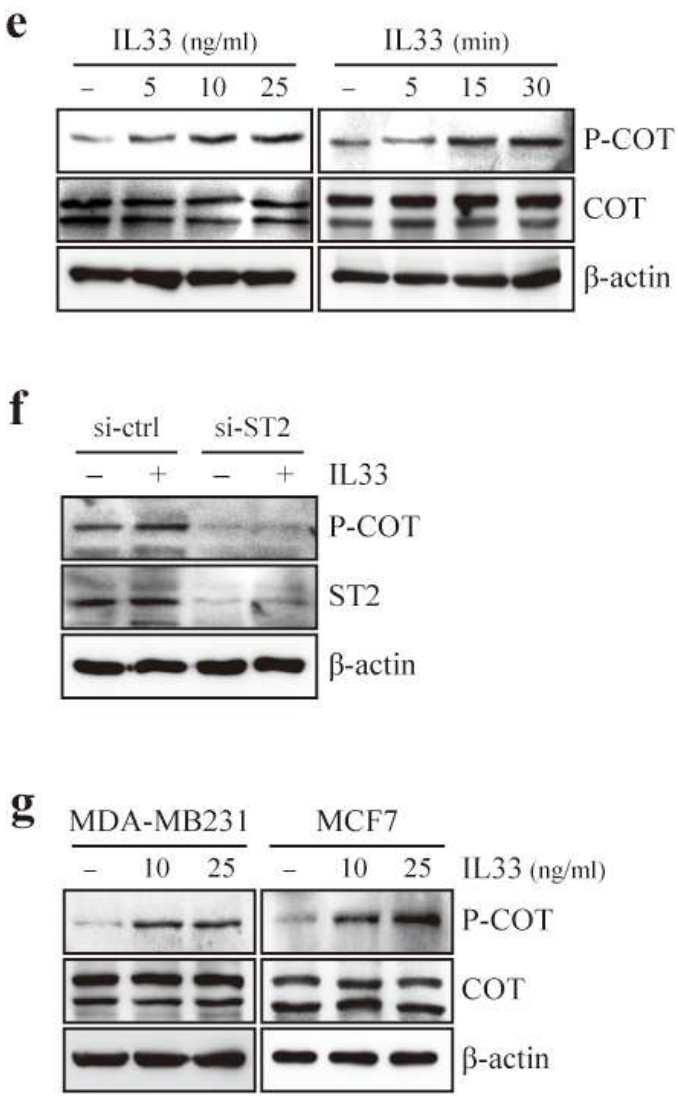

Figure 4. ST2 abundance is positively correlated with COT expression in human breast cancer tissues and ST2 mediates IL-33-induced COT phosphorylation.

(a) Representative samples showing the results of immunohistochemical analysis of infiltrating duct carcinoma of the breast, performed using the indicated antibodies on adjacent sections of samples. Correlations were analyzed using Fisher's exact test.

(b) HEK293 cells expressing endogenous ST2 with Myc-COT were subjected to immunoprecipitation with anti-myc or anti-ST2 antibodies, followed by immunoblotting with anti-ST2 or anti-Myc antibodies. Normal IgG antibody was used as a negative control for immunoprecipitation.

(c) JB6 Cl41 cells were transfected with COT-WT or COT-S400A, and then subjected to immunoprecipitation with anti-Myc, followed by immunoblotting with anti-ST2 antibodies.

(d) JB6 Cl41 cells were starved for 24 h and stimulated with 25 ng/mL IL-33 for the indicated time. Immunoprecipitation was performed to precipitate endogenous COT (upper) or ST2 (lower), and then immunoblotting analysis was performed using anti-ST2 (upper) or anti-COT (lower) antibodies, respectively.

(e) JB6 Cl41 cells were serum-starved for 24 h, treated with 25 ng/mL IL-33 with the indicated doses of IL-33 for 30 min (left) or for the indicated times (right), harvested, lysed, and immunoblotted.

(f) JB6 Cl41 cells were transfected with siRNA-ST2. After 48 h, cells were serum-

starved for 24 h, treated or not treated with 25 ng/mL IL-33 for 30 min, harvested, lysed, immunoblotted.

(g) MDA-MB231 or MCF7 cells were serum-starved for 24 h, treated with or without 25 ng/mL IL-33 for 30 min, harvested, lysed, immunoblotted. The figures are representative of at least three separate experiments that yielded similar results.

3. COT mediates the MEK1/2-ERK1/2 and JNK1/2-cJun signaling pathways induced by IL-33

Given the role of COT as an important signal transducer of IL-33/ST2 axis, we examined whether COT might regulate the IL-33-induced MEK1/2-ERK1/2 and JNK1/2-cJun signaling pathways. The results showed that IL-33 induced phosphorylation of MEK1/2 and ERK1/2 in a dose- and time-dependent manner (Figure 5a and 5b), as well as JNK1/2 and cJun (Figure 5c and 5d). Furthermore, treatment with TKI, a COT inhibitor, inhibited the IL-33-induced phosphorylation of MEK1/2, ERK1/2, JNK1/2, and cJun (Figure 5e). Similarly, PD98059, a specific MEK1/2 inhibitor, and SP600125, a specific JNK inhibitor, also inhibited the IL-33-induced phosphorylation of ERK1/2 and cJun, respectively (Figure 5f), thus suggesting that COT mediates the IL-33-induced MEK1/2-ERK1/2 and JNK1/2-cJun signaling pathways through COT activation in JB6 Cl41 cells. Consistent with these results, we observed that MEK1/2, ERK1/2, JNK1/2, and cJun were phosphorylated by IL-33 in MDA-MB231 and MCF7 cells (Figure 5g and 5h), suggesting that COT might play an important role in IL-33-induced epithelial cell proliferation and mammary tumorigenesis.

Figure 5 (a-d)

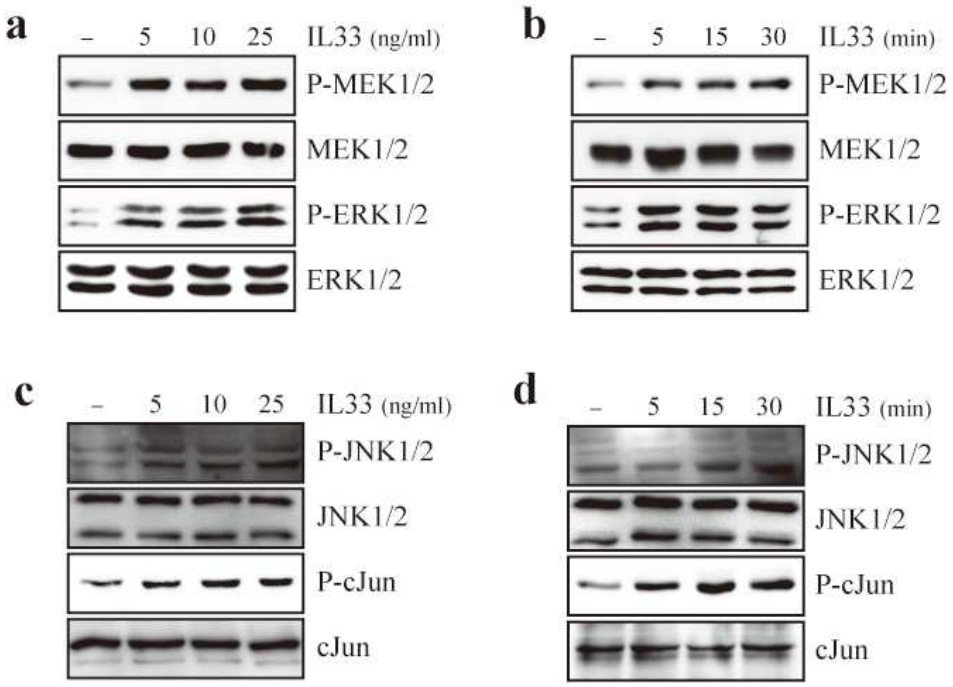

Figure 5(e-h)

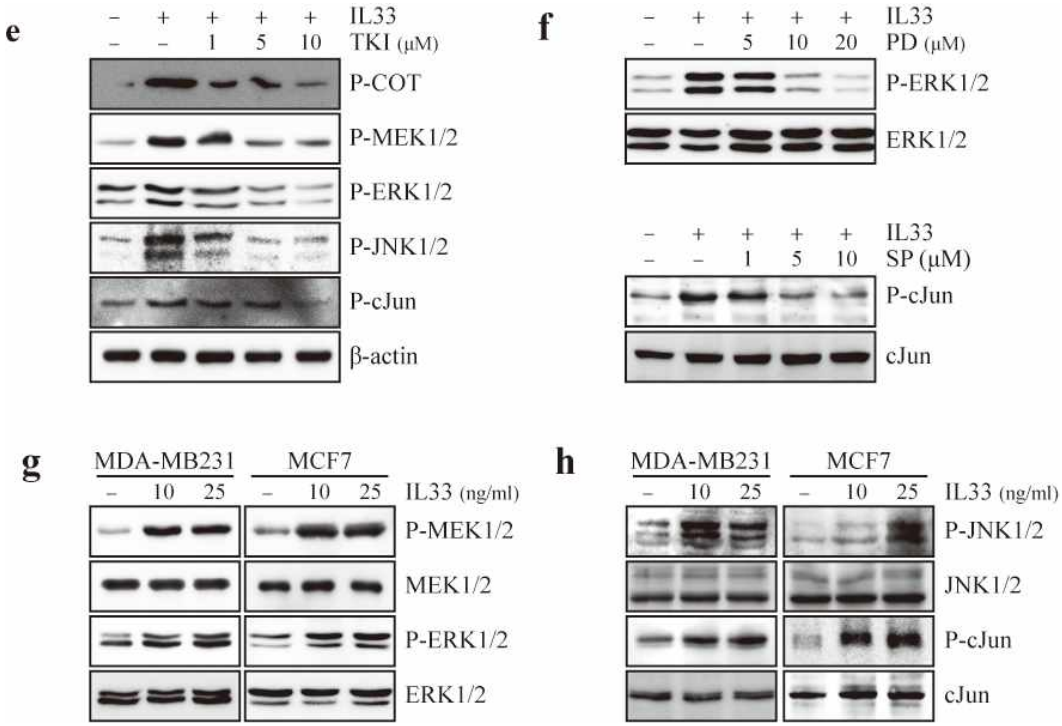

Figure 5. COT mediates MEK-ERK and JNK-cJun signaling induced by IL-33.

(a and b) JB6 Cl41 cells were serum-starved for 24 h, treated with the indicated doses of IL-33 for 30 min as in *a* or 25 ng/mL IL-33 for the indicated times as in *b*, harvested, lysed, and immunoblotted.

(c and d) JB6 Cl41 cells were serum-starved for 24 h, treated with the indicated doses of IL-33 for 30 min as in *c* or 25 ng/mL IL-33 for the indicated times as in *d*, harvested, lysed, and immunoblotted.

(e) JB6 Cl41 cells were serum-starved for 24 h, pretreated with the indicated concentrations of TKI for 2 h, exposed to 25 ng/mL IL-33 for 30 min, harvested, lysed, and immunoblotted.

(f) JB6 Cl41 cells were serum-starved for 24 h, treated with either PD98059 (upper) or SP600125 (lower). At 1 h after pretreatment, cells were exposed to 25 ng/mL IL-33 for 30 min, harvested, lysed, and immunoblotted.

(g and h) MDA-MB231 and MCF7 cells were serum-starved for 24 h, treated with or without 25 ng/mL IL-33 for 30 min, harvested, lysed, and immunoblotted. The figures are representative of at least three separate experiments that yielded similar results.

4. COT mediates STAT3 phosphorylation via its interaction with STAT3

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that is activated by the actions of many autocrine and paracrine growth factors, including IL-6 and epidermal growth factor (EGF), in human cancers (Grivennikov et al., 2009; Itoh et al., 2006), suggesting that IL-33 may play a regulatory role in STAT3 activation. Given the ST2 and COT specific interaction induced by IL-33, it is important to understand whether COT mediates IL-33-induced STAT3 phosphorylation. The interaction detected between Myc-COT and STAT3 in *in vitro* binding assays (Figure 6a and 6b) supports the hypothesis that STAT3 is a possible COT target. In addition, endogenous COT interacts with STAT3 in IL-33-stimulated cells, but not in unstimulated cells (Figure 6c). To determine whether IL-33-induced STAT3 activation is mediated by COT, we first examined the IL-33-induced phosphorylation of STAT3 in JB6 Cl41 cells. The results showed that IL-33 dose- and time-dependently increased the phosphorylation of STAT3 in JB6 Cl41 cells (Figure 6d and 6e). In addition, the phosphorylation of STAT3 induced by IL-33 was suppressed in siRNA-ST2-transfected cells compared with siRNA-control-transfected cells (Figure 6f). Furthermore, the knockdown of COT inhibited the IL-33-induced phosphorylation of STAT3 (Figure 6g). TKI also inhibited the IL-33-induced phosphorylation of STAT3 in a dose-dependent manner (Figure 6h). Consistent with these results, we observed that STAT3 phosphorylation was induced by IL-33 in

MDA-MB231 and MCF7 cells (Figure 6i), indicating that COT positively regulates the STAT3 signaling pathway induced by IL-33 in human breast cancer cells.

Figure 6 (a-g)

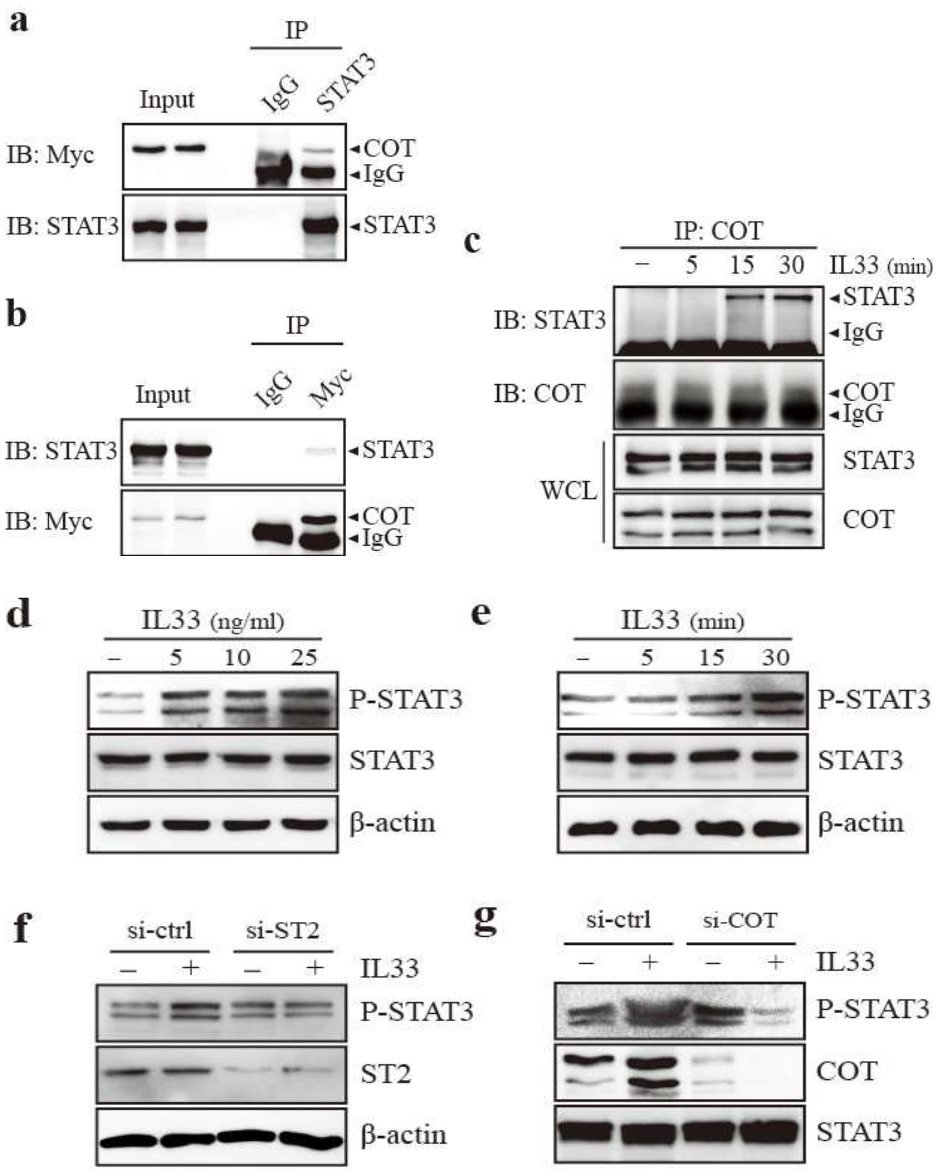

Figure 6 (h-i)

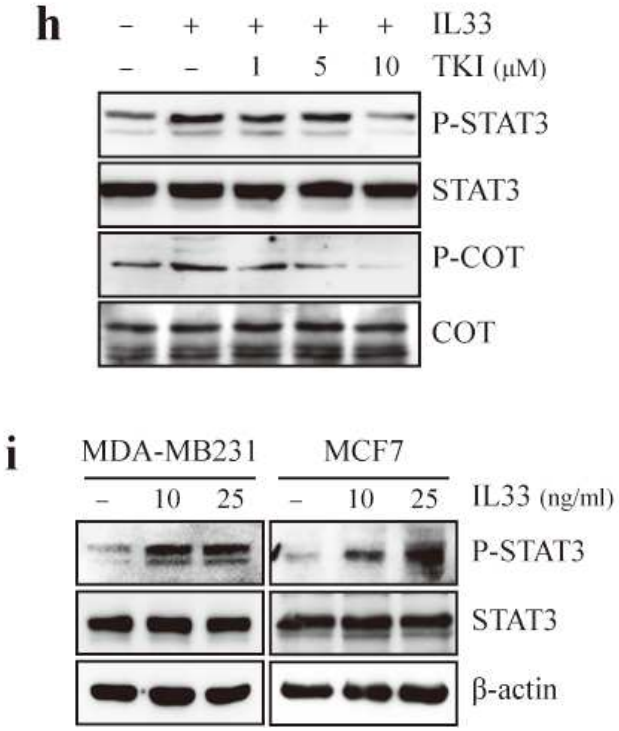

Figure 6. IL-33 induces STAT3 phosphorylation via the interaction between STAT3 and COT.

(a and b) HEK293 cells expressing endogenous STAT3 with Myc-COT were subjected to immunoprecipitation with anti-STAT3 (*a*) or anti-Myc (*b*) antibodies, followed by immunoblotting with anti-Myc (*a*) or anti-STAT3 (*b*) antibodies. Normal IgG antibody was used as a negative control for immunoprecipitation.

(c) JB6 Cl41 cells were starved for 24 h and stimulated with 25 ng/mL IL-33 for the indicated times. Immunoprecipitation was performed to precipitate endogenous COT, and then immunoblotting analysis was performed using anti-STAT3 antibody.

(d and e) JB6 Cl41 cells were serum-starved for 24 h, treated with the indicated doses of IL-33 for 30 min (*d*) or 25 ng/mL IL-33 for the indicated times (*e*), harvested lysed, and immunoblotted.

(f and g) JB6 Cl41 cells were cultured for 24 h, then transfected with siRNA-ST2 (*f*) or siRNA-COT (*g*), respectively. After 48 h, cells were serum-starved for 24 h, treated or not treated with 25 ng/mL IL-33 for 30 min, harvested, lysed, and immunoblotted.

(h) JB6 Cl41 cells were serum-starved for 24 h, pretreated with the indicated concentrations of TKI for 2 h, exposed to 25 ng/mL IL-33 for 30 min, harvested, lysed, and immunoblotted.

(i) MDA-MB231 and MCF7 cells were serum-starved for 24 h, treated with or without 25 ng/mL IL-33 for 30 min, harvested, lysed, and immunoblotted.

5. COT is required for IL-33-induced AP-1 and *stat3* activation

The AP-1 transcription factor is a dimeric complex of homodimers or heterodimers of Jun, Fos, activating transcription factor (ATF), and musculoaponeurotic fibrosarcoma protein family members that are activated by the MAPK signaling pathway (Eferl and Wagner, 2003; Karin, 1995). To determine the effects of IL-33 on AP-1 activity, we first examined the effects of IL-33 on the *c-fos* and *c-jun* promoter. The transcriptional activity of *c-fos* and *c-jun* was significantly increased by treatment with IL-33 in JB6 Cl41 cells in a dose-dependent manner (Figure 7a and 7b). In addition, treatment with IL-33 was found to significantly induce AP-1 transactivation in JB6 Cl41 cells (Figure 7c). Given the increased phosphorylation of STAT3 induced by IL-33, we next examined the effect of IL-33 on the transcriptional activity of STAT3 in JB6 Cl41 cells using a *stat3* reporter gene assay. The results showed that IL-33 treatment significantly enhanced *stat3* transcriptional activity (Figure 7d). To further confirm that COT regulates the AP-1 and *stat3* transcriptional activity induced by IL-33, we assessed IL-33-induced AP-1 and *stat3* activity in the presence or absence of TKI in JB6 Cl41 cells. As expected, TKI treatment inhibited the IL-33-induced AP-1 and *stat3* activity in JB6 Cl41 cells (Figure 7e and 7f). To further determine the effects of COT knockdown on *stat3* transcriptional activation, MDA-MB231 cells were transfected with siRNA-COT, and then exposed to IL-33. Consistent with a potential positive role for COT in the IL-33-induced MEK-ERK,

JNK-cJun, and STAT3 signaling pathways, depletion of endogenous COT in MDA-MB231 cells suppressed the IL-33-induced phosphorylation of ERK1/2, cJun, and STAT3 (Figure 7g). More importantly, depletion of COT in MDA-MB231 cells profoundly reduced the AP-1 and *stat3* activity induced by IL-33, compared to the untreated control group (Figure 7h and 7i), suggesting that IL-33-induced AP-1 and *stat3* transcriptional activity is mediated by COT signaling.

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Figure 7 (a-d)

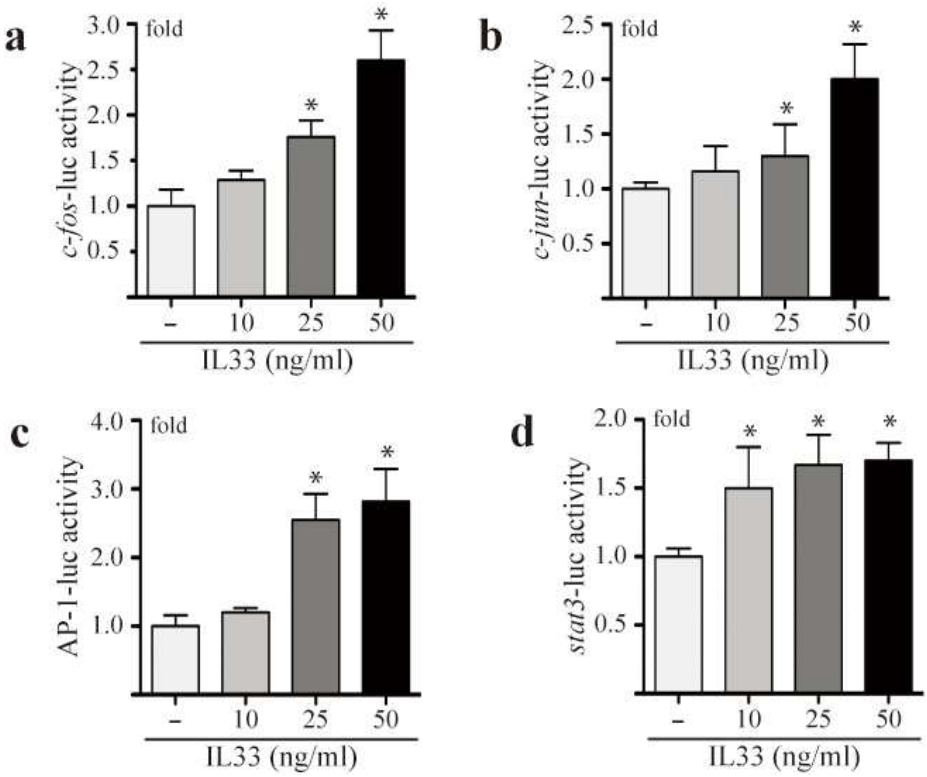


Figure 7 (e-i)

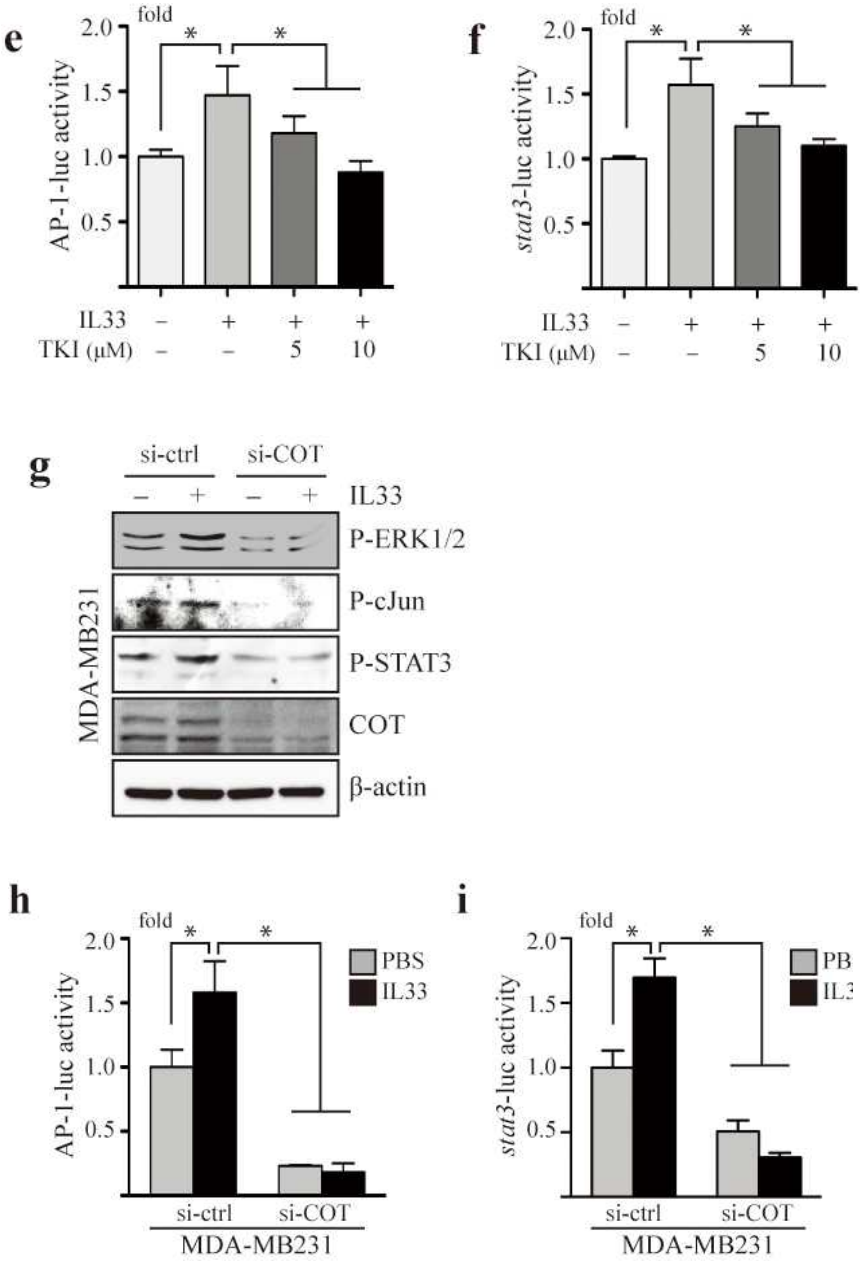


Figure 7. Effects of inhibition of COT on IL-33-induced AP-1 and *stat3* promoter activity.

(a–d) JB6 Cl41 cells were co-transfected with a luciferase reporter, *c-fos*-luc (*a*), *c-jun*-luc (*b*), AP-1-luc (*c*), or *stat3*-luc (*d*), and the pRL-TK vector. At 30 h after transfection, cells were serum-starved for 24 h, and then treated with the indicated doses of IL-33 for 24 h.

(e and f) JB6 Cl41 cells were co-transfected with a luciferase reporter, AP-1-luc (*e*) or *stat3*-luc (*f*), and the pRL-TK vector, respectively. After 30 h, cells were serum-starved for 24 h and then treated with 25 ng/mL IL-33 in the presence or absence of the indicated doses of TKI for 30 h.

(g) MDA-MB231 cells were transfected with siRNA-control or siRNA-COT. After 48 h, cells were serum-starved for 24 h, treated or not treated with 25 ng/mL IL-33 for 30 min, harvested, lysed, and immunoblotted. The figure is representative of at least three separate experiments that yielded similar results.

(h and i) MDA-MB231 cells were co-transfected with a luciferase reporter, AP-1-luc (*h*) or *stat3*-luc (*i*), and siRNA-COT. After 30 h, cells were serum-starved for 24 h and then treated with 25 ng/mL IL-33. Data in *a-f*, *h* and *i* are represented as mean ± SEM from one experiment representative of three independent experiments performed. *, $P < 0.05$.

6. An IL-33/ST2/COT axis promotes epithelial cell transformation and breast mammary tumorigenesis

To investigate whether COT and STAT3 are essential for IL-33-induced epithelial cell transformation, we first examined the effect of TKI and WP1066 in JB6 Cl41 cells on IL-33-induced transformation. The results showed that TKI and WP1066 treatment significantly inhibited the IL-33-induced transformation of JB6 Cl41 cells (Figure 8a). Given the ability of IL-33 to induce epithelial cell transformation via the interaction between ST2 and COT, we wondered whether inhibition of this signaling pathway contributes to suppression of breast tumorigenesis. Compared to the control group, knockdown of ST2, IL-33, and COT in MCF7 cells significantly attenuated the number of colonies in soft agar (Figure 8b). Next, we studied the correlation between COT activity and IL-33 on tumorigenicity of MCF7 cells using a soft agar assay. The results showed that COT knockdown greatly inhibited colony formation of MCF7 cells (Figure 8c, *top*). Consistent with these results, depletion of COT in MCF7 cells profoundly reduced IL-33-induced tumor growth, compared to the untreated control group (Figure 8c, *bottom*). The effects of COT on IL-33-induced tumor development *in vivo* were studied in a nude mice syngeneic model with 4T1 metastatic mouse breast carcinoma cells. Representative tumor images demonstrated that IL-33 significantly induced mammary gland tumor development, whereas treatment with TKI significantly inhibited mammary gland tumor development, suggesting that COT

might play an important role in IL-33-induced mammary tumorigenesis (Figure 8d). Knowing that COT activates STAT3 via its interaction with STAT3, we also examined the effects of WP1066 on IL-33-induced tumor development *in vivo*. The results showed that STAT3 inhibition greatly suppressed mammary gland tumor development (Figure 8d), indicating that the COT-STAT3 signaling pathway plays a pivotal role in breast tumorigenesis. To better determine the role of IL-33 on the mammary tumorigenesis, we established human IL-33-stably-transfected 4T1 cell lines (4T1/Myc-IL33) and evaluated the tumorigenicity of IL-33-overexpressing cells in nude mice model. The results showed that IL-33 overexpression significantly enhanced mammary gland tumor development compared with control in nude mice (Figure 8e, *top right panels*). In addition, compared to the control group, knockdown of human IL-33 in 4T1/Myc-IL33 cells significantly attenuated the tumor growth in nude mice (Figure 8e, *middle right panels*).

Figure 8 (a-b)

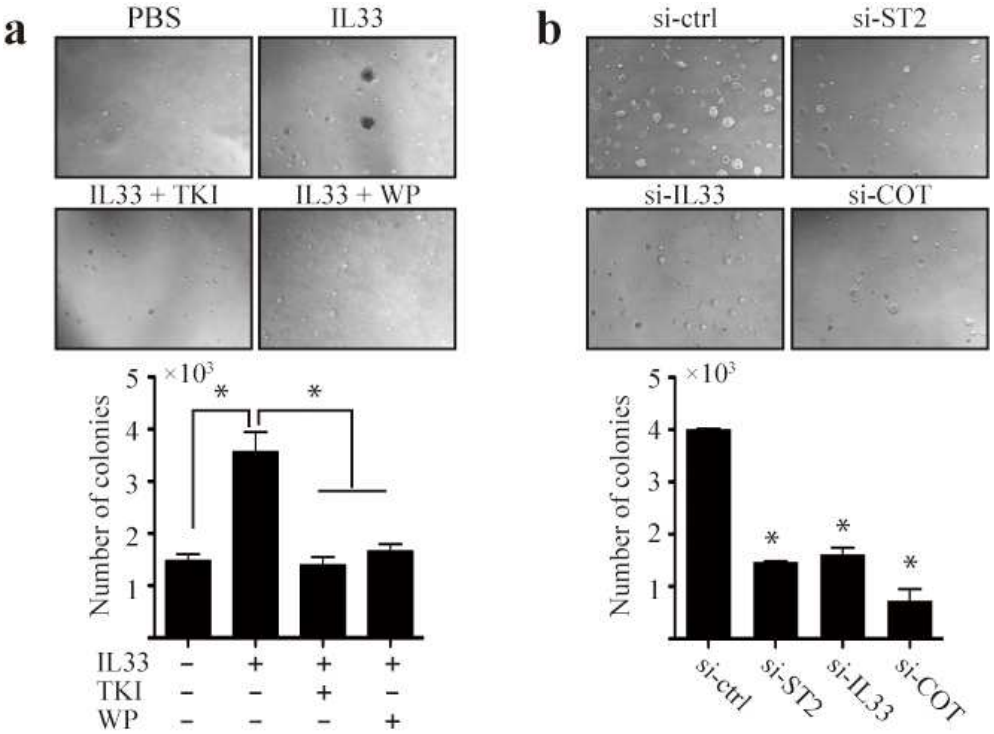


Figure 8 (c)

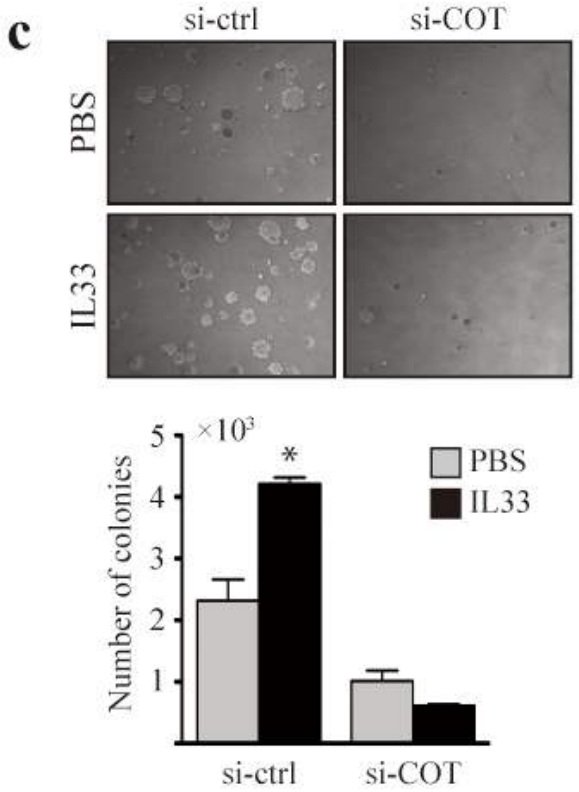


Figure 8 (d)

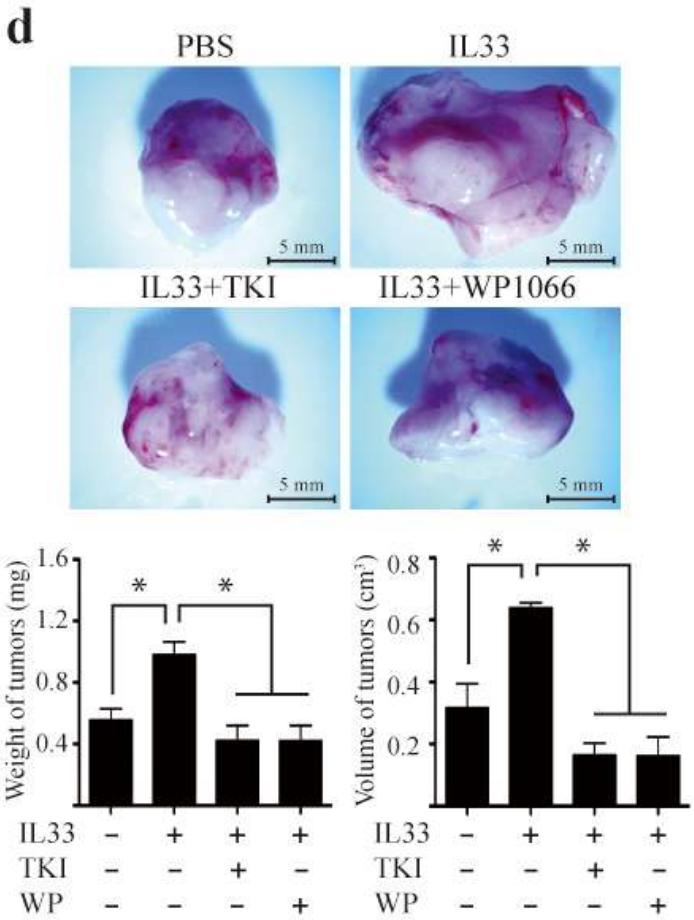


Figure 8 (e)

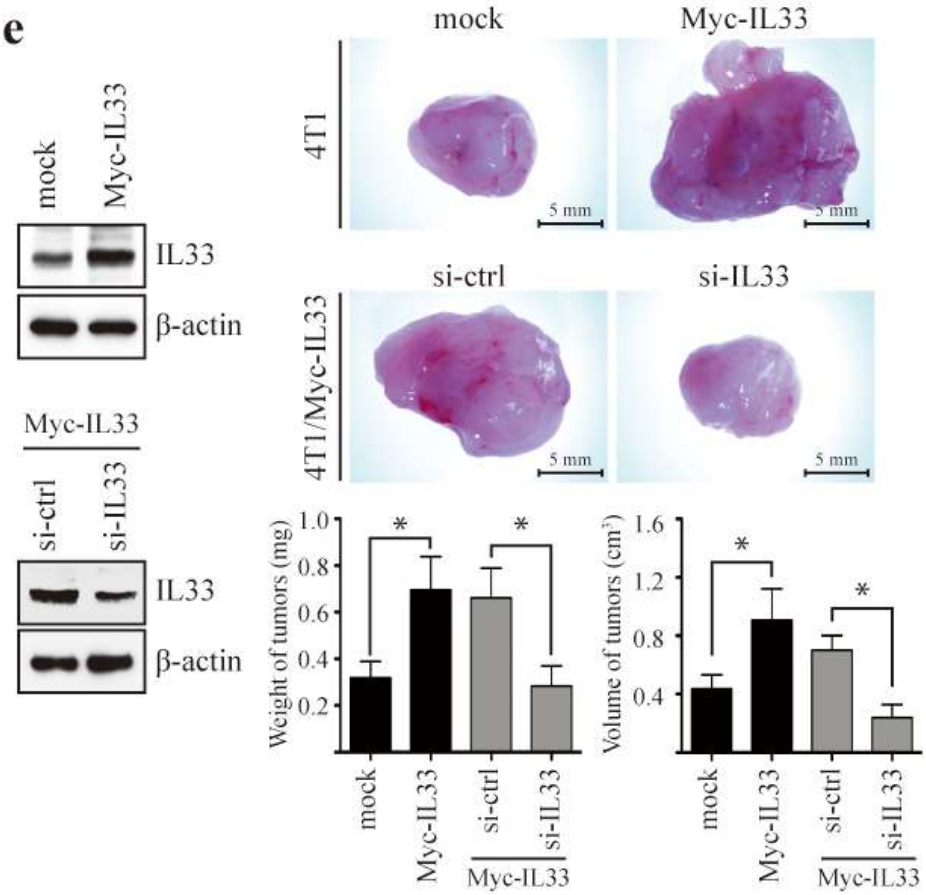


Figure 8. Effects of the ST2/COT axis on IL-33-induced epithelial cell transformation and breast mammary tumorigenesis

(a) JB6 Cl41 cells were treated with 50 ng/mL IL-33 with/without treatment of 10 μM TKI or 10 μM WP1066 as indicated in soft agar matrix, and incubated at 37°C in a 5% CO_2 atmosphere for 14 days.

(b) MCF7 cells were transfected with siRNA-ST2, siRNA-IL-33, or siRNA-COT. At 48 h after transfection, cells were treated with/without 50 ng/mL IL-33 in soft agar.

(c) MCF7 cells were transfected with either siRNA-control or siRNA-COT. At 48 h after transfection, cells were treated with/without 50 ng/mL IL-33 in soft.

(d) 4T1 cells were injected into the mammary gland of BALB/c mice in the presence or absence of 250 ng/mL IL-33 with 100 μM TKI or WP1066, respectively, and allowed to grow until tumors formed (14 days).

(e) Top left, expression levels of IL-33 in 4T1/mock and 4T1/Myc-IL-33; bottom left, expression levels of IL-33 in 4T1/Myc-IL-33 trasfected with si-RNA-control or siRNA-IL-33; right, 4T1/Myc-IL33 cells were transfected with siRNA-control or siRNA-IL-33. At 48 h after transfection, 4T1/mock, 4T1/Myc-IL33, or siRNA-transfected 4T1/Myc-IL33 cells were injected into the mammary gland of BALB/c mice and allowed to grow until tumors formed (14 days). Data in *a*−*e* are represented as mean ± SEM from one experiment representative of three independent experiments performed. *, $P < 0.05$.

Part Ⅱ : LPIN1 promotes breast tumorigenesis via upregulation of insulin receptor substrate 1 stability

7. LPIN1 promotes epithelial cell transformation and tumorigenesis in breast

To investigate the pathoetiology of human LPIN1 in breast tumorigenesis, we performed immunohistochemistry analysis on human normal and breast cancer tissues using LPIN1 antibodies. Notably, we observed that all of the 30 normal breast samples had a low amount of LPIN1, whereas sixteen of 36 breast cancer samples contained high amounts of LPIN1 (Figure 9a), supporting a possible pathological correlation between the increased expression of LPIN1 and breast tumorigenesis. To investigate the effects of LPIN1 on anchorage-independent cell transformation and tumor promotion, we next performed the soft agar assays. JB6 Cl41 cells expressing FLAG-LPIN1 showed a significantly increased colony numbers and colony sizes in soft agar (Figure 9b). Moreover, overexpression of LPIN1 also significantly enhanced anchorage-independent colony growth of SK-BR3 cells (Figure 9c). As previous studies have shown that the LPIN1 enzymatic activity requires magnesium and can be inhibited by propranolol, N-ethylmaleimide (NEM), and bromoenol lactone (BEL) (Grkovich et al., 2006; Han et al., 2010). To further examine the role of LPIN1 on cell proliferation and anchorage-independent cell transformation, the 5-bromo-2-

deoxyuridine incorporation and soft agar assays were performed in the presence of propranolol. We found that propranolol treatment significantly and dose-dependently inhibited proliferation of SK-BR3 cells in (Figure 9d). Furthermore, propranolol treatment significantly inhibited not only colony numbers but also colony sizes of SK-BR3 cells in soft agar (Figure 9e). The effects of LPIN1 on tumor development in vivo were studied in a syngeneic model of 4T1 cell implantation into the mammary gland of female BAKB/c mice. LPIN1-overexpressing 4T1 tumors showed significant increase in mammary gland tumor development compared to control tumors (Figure 9f), suggesting that increased levels of LPIN1 may lead to a comparable enhancement of tumor growth via increased cell proliferation.

Figure 9 (a)

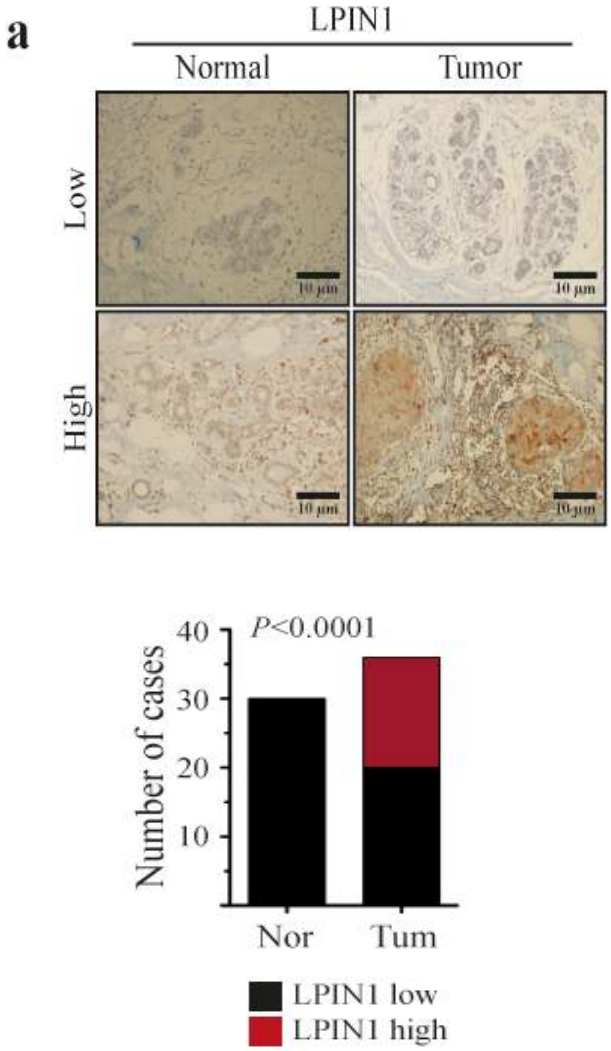


Figure 9 (b-c)

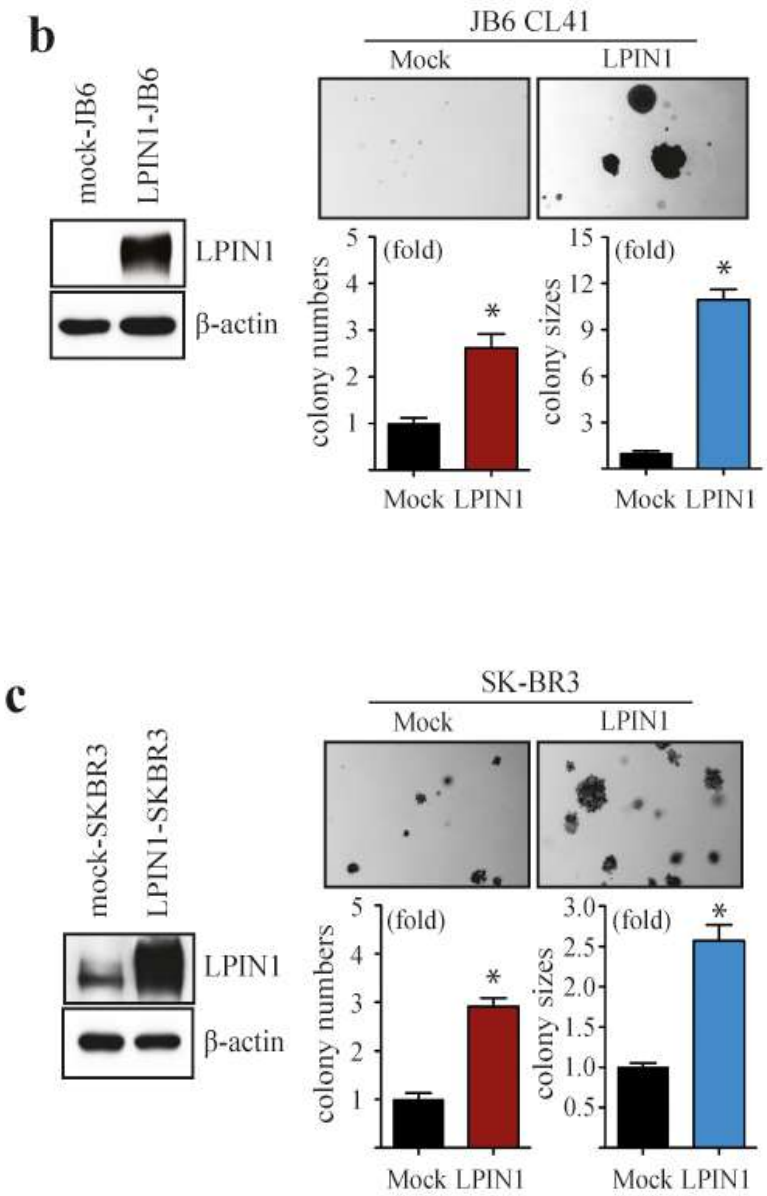


Figure 9 (d-f)

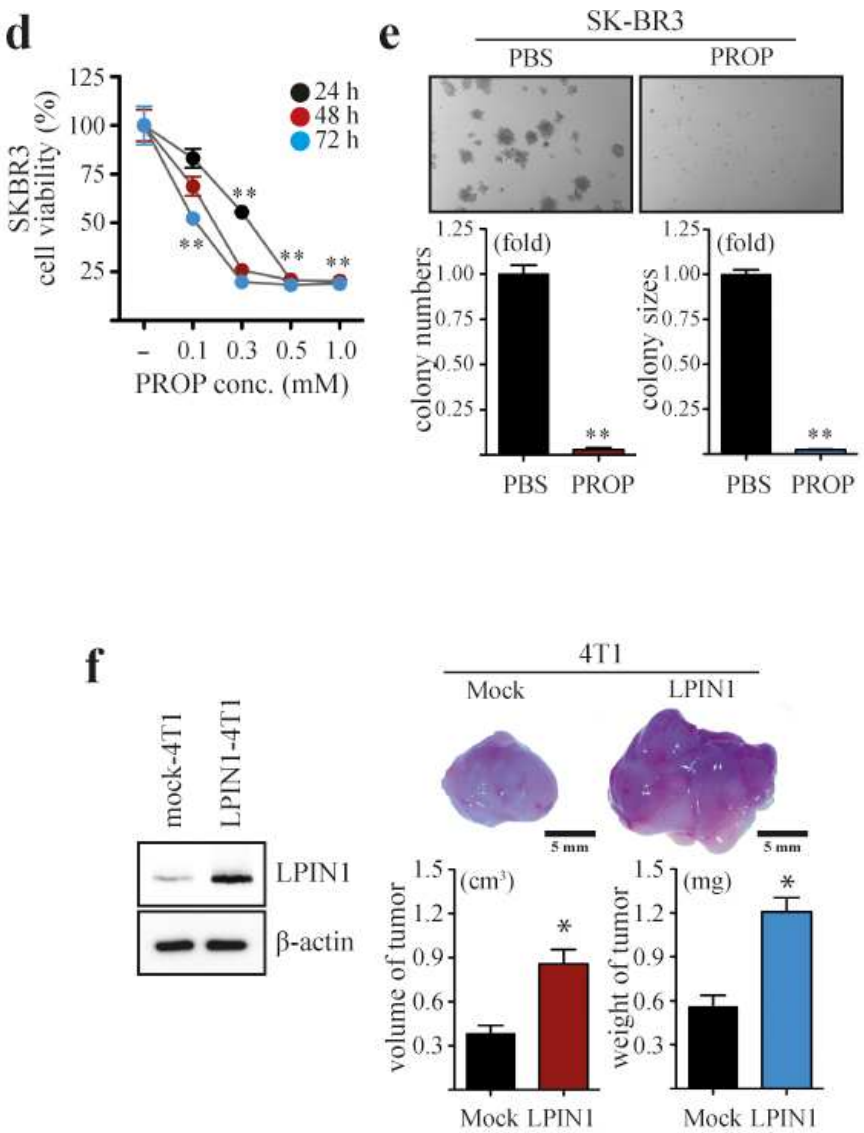


Figure 9. Overexpression of LPIN1 enhanced epithelial cell transformation and breast tumorigenesis.

(a) Human normal breast tissue and infiltrating ductal carcinoma of the breast were subjected to immunohistochemistry with anti-LPIN1. In each sample, LPIN1 expression was semiquantified in a double-blind manner a high or low according to the standards presented in (top) and statistical analysis was performed using Fisher's exact test (botton, $p < 0.001$).

(b) Overexpression of LPIN1 increased epithelial cell transformation in soft agar. Left, expression levels of LPIN1 in Mock-transfected (mock-JB6) and LPIN1-overexpressing (LPIN1-JB6) cells; right, Mock-JB6 or IRS1-JB6 cells were seeded in soft agar and incubated at 37°C in a 5% CO_2 atmosphere for 14 d. Results shown are mean ± standard deviation (S.D.), n = 3. **$p < 0.002$.

(c) Overexpression of LPIN1 increased anchorage-independent cell growth in soft agar. Left, expression levels of LPIN1 in Mock-transfected (mock-SKBR3) and LPIN1-overexpressing (LPIN1-SKBR3) cells; right, Mock-SKBR3 or LPIN1-SKBR3 cells were seeded in soft agar and incubated at 37°C in a 5% CO_2 atmosphere for 14 d. Results shown are mean ± S.D., n = 3. **$p < 0.001$.

(d) Inhibition of LPIN1 suppressed cell proliferation of SKBR3 cells. Cells were seeded and treated with various concentrations of propranolol, as indicated, and then cultured for 24 h, 48 h and 72 h at 37°C in a 5% CO_2 atmosphere. Cell viability was

measured by the MTT assay, as described in Methods. Results shown are mean ± S.D., n = 3. **p < 0.001.

(e) Propranolol suppressed anchorage-independent cell growth in soft agar. SKBR3 cells were treated with 0.1mM propranolol in soft agar and incubated at 37°C in a 5% CO_2 atmosphere for 14 d. Results shown are mean ± S.D., n = 3. **p < 0.001.

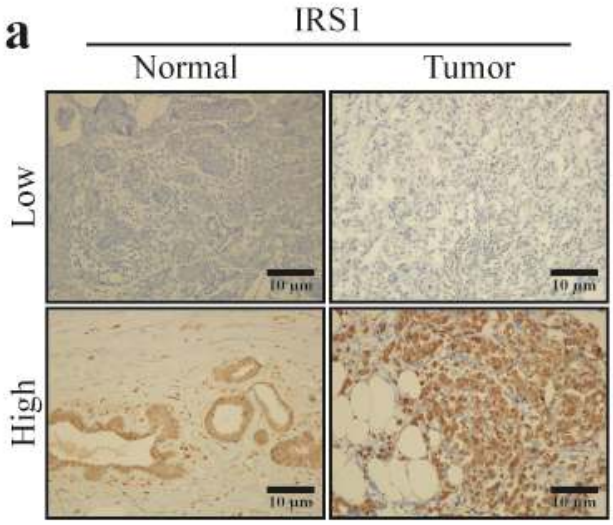
(f) Overexpression of LPIN1 enhances mammary gland tumorigenesis. Left, expression levels of LPIN1 in mock-4T1 and LPIN1-4T1; right, The mock-4T1 and LPIN1-4T1 cells were injected into the mammary gland of BALB/c mice and allowed to grow until the formation of tumors for 10 d. Results shown are representative pictures of tumor and mean ± S.D., n = 30. **p < 0.001.

8. LPIN1 colocalizes and interacts with IRS1 in breast cancer cells

In investigating whether others have previously observed the various factors involved in LPIN1 pathway, we noted that LPIN1 promotes hepatic insulin receptor signaling dysfunction via enhanced DAG production, triglyceride formation, and VLDL secretion (Chanda et al., 2012). This finding was interesting to us because, previously, the insulin receptor substrate 1 (IRS1) was shown to be a predominant signaling molecule activated by insulin-like growth factor 1 (IGF-1) in breast cancer cells (Jackson et al., 1998), suggesting that LPIN1 might be involved in regulating the IRS1 signaling pathway. However, it remains largely unknown what mechanisms may contribute to enhance IRS1 functions in LPIN1-dependent breast tumorigenesis. Notably, we observed an IRS1 expression was significantly increased in breast cancer tissues (Figure 10a). To investigate the relationship between LPIN1 and IRS1 in human breast cancer, we next performed immunohistochemistry using specific antibodies against human LPIN1 and IRS1 in human breast cancer tissues. Seven of the 14 breast cancer samples that showed low amounts of LPIN1 presented with lower expression IRS1, whereas fourteen of 16 breast cancer samples that contained a high amount of LPIN1 correspondingly had higher expression of IRS1 (Figure 10b). Since LPIN1 appeared to affect the processing of IRS1 pathway, we examined whether all these proteins reside within the same cellular compartment. Using confocal microcopy analysis, we found that in untreated SK-BR3 and MDA-MB231

cells, LPIN1 is present in cytoplasm as well as in the nucleus, while IRS1 were mostly cytoplasmatic (Figure 10c), indicating that a significant fraction of LPIN1 colocalized with IRS1 in the cytoplasm. Although recent studies suggest that LPIN1-deficient fatty liver dystrophy (fld) mice develop insulin resistance (Reue et al., 2000), but transgenic mice with adipose tissue–specific overexpression of LPIN1 exhibit enhanced insulin sensitivity (Phan and Reue, 2005), it is largely unknown whether LPIN1 may regulate it by IRS1. Thus, we screened its interactions with IRS1. In support of IRS1 being a possible LPIN1 target, we detected the interaction between LPIN1 and IRS1 (Figure 10d) using in vitro binding assay. We next examined whether the interaction of LPIN1 and IRS1 was detectable at the endogenous level and found that endogenous LPIN1 interacted with IRS1 in IGF-1-treated cells but not in untreated cells (Figure 10e).

Figure 10 (a)



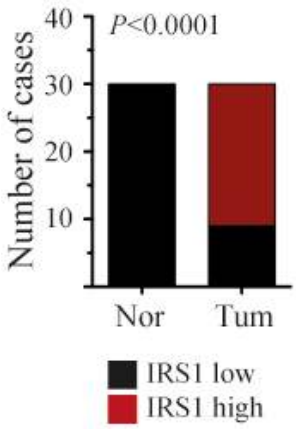


Figure 10 (b)

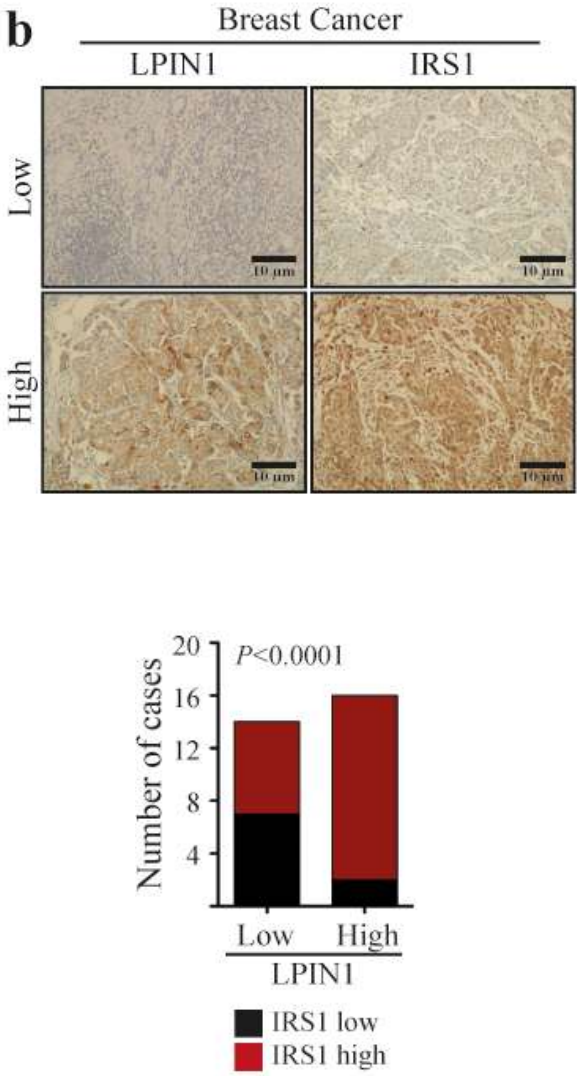


Figure 10 (c-d)

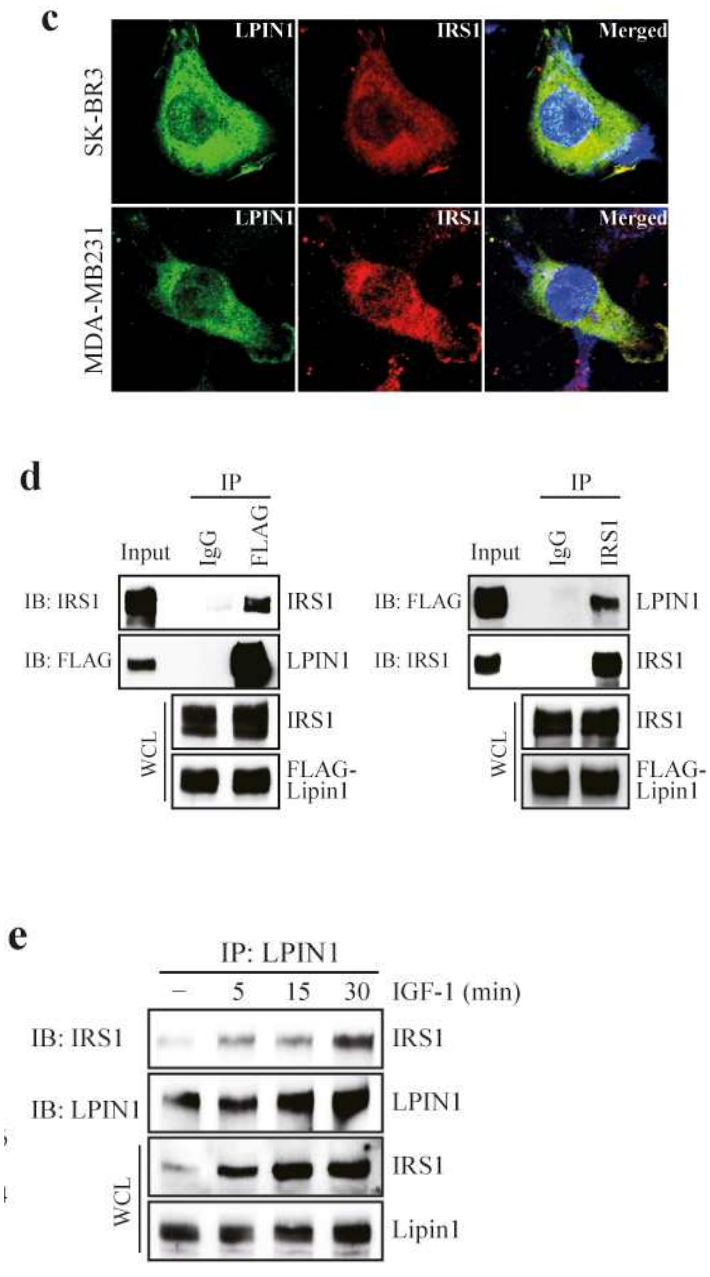


Figure 10. LPIN1 interacts with IRS1 and is positively correlated with IRS expression in human breast cancer tissues.

(a) Human normal breast tissue and infiltrating ductal carcinoma of the breast were subjected to immunohistochemistry with anti-IRS1. In each sample, IRS1 expression was semiquantified in a double-blind manner a high or low according to the standards presented in (top) and statistical analysis was performed using Fisher's exact test (botton, $p < 0.001$).

(b) Representative samples showing the results of immunohistochemical analysis of infiltrating duct carcinoma of the breast, performed using the anti-LPIN1 or anti-IRS1 on adjacent sections of samples. In each sample, the expression of IRS1 and LPIN1 were semiquantified in a double-blind manner a high or low according to the standards presented in (top) and statistical analysis was performed using Fisher's exact test (botton, $p < 0.001$).

(c) Immunofluorescence colocalization of LPIN1 and IRS1. SKBR3 (top) and MDA-MB231 (bottom) cells expressing FLAG-LPIN1 and pCMV-IRS1 were subjected to immunofluorosence analysis with anti-FLAG (green) and anti-IRS1(red) immunostaining against FLAG-LPIN1 and IRS1, and then with the appropriate secondary antibodies conjugated with FITC and Cy3, respectively. Fluorescence on the mounted slides was captured with a confocal microscope system.

(d) Coimmunoprecipitation of expressed LPIN1 and IRS1. HEK293 cells expressing

FLAG-LPIN1 or pCMV-IRS1 were subjected to immunoprecipitattion with anti-FLAG or anti-IRS1 antibodies, followed by immunblot with anti-IRS1 or anti-FLAG, respectively. Normal IgG antibody was used as a negative control for immunoprecipitation.

(e) Association between endogenous LPIN1 and IRS1. MDA-MB231 cells were starved for 24 h and stimulated with 100 ng/mL IGF-1 for the indicated time. Immunoprecipitation was performed to precipitate endogenous LPIN1, and then immunoblotting analysis was performed using anti-IRS1 antibody.

9. LPIN1 participates in the upregulation of IRS1 levels induced by IGF-1

Given the positive correlation between LPIN1 and IRS1 abundance in human clinical cancer samples and the IGF-1-induced interaction between LPIN1 and IRS1, we next examined whether LPIN1 could directly regulate IRS1 stability. We found that LPIN1 overexpression in SK-BR3 and MDA-MB231 cells increased IRS1 expression (Figure 11a). Reciprocally, depleting endogenous LPIN1 in both cells led to reduced IRS1 levels (Figure 11b). Next, various doses of FLAG-LPIN1 were transfected into SK-BR3 and MDA-MB231 cells and the cells were incubated for 48 h. The overexpression of LPIN1 increased IRS1 levels in a dose-dependent way (Figure 11c). Given that the increased interaction between LPIN1 and IRS1 by IGF-1 treatment, we next examined the effect of LPIN1 expression on the IGF-1-induced IRS1 levels. The results showed that the increased endogenous IRS1 levels by IGF-1 treatment were augmented by LPIN1 overexpression in SK-BR3 and MDA-MB231 cells, respectively (Figure 11d and 11e). In contrast, silencing of LPIN1 attenuated the IRS1 levels increased by IGF-1 in both cells (Figure 11f). In addition, real-time PCR showed that the IRS1 mRNA level was not affected by knockdown of LPIN1 in SK-BR3 and MDA-MB231 cells (Figure 11g), suggesting that LPIN1 might be associated with IRS1 protein stability. We next examined whether the elevated IRS1 levels were because of alterations in IRS1 half-life, using the cycloheximide chase. In contrast to vector control cells where IRS1 was unstable, IRS1 was much more stable in LPIN1-

overexpressing MDA-MB231 cells (Figure 11h). In confirmation of a regulatory role for LPIN1 in IRS1 stability, IRS1 exhibited decreased half-life in LPIN1-depleted SK-BR3 and MDA-MB231 cells compared to those normally expressing LPIN1 (Figure 11i). Thus, LPIN1 appears to be a regulator of IRS1 by presumably interacting with IRS1 to inhibit its degradation.

Figure 11 (a-c)

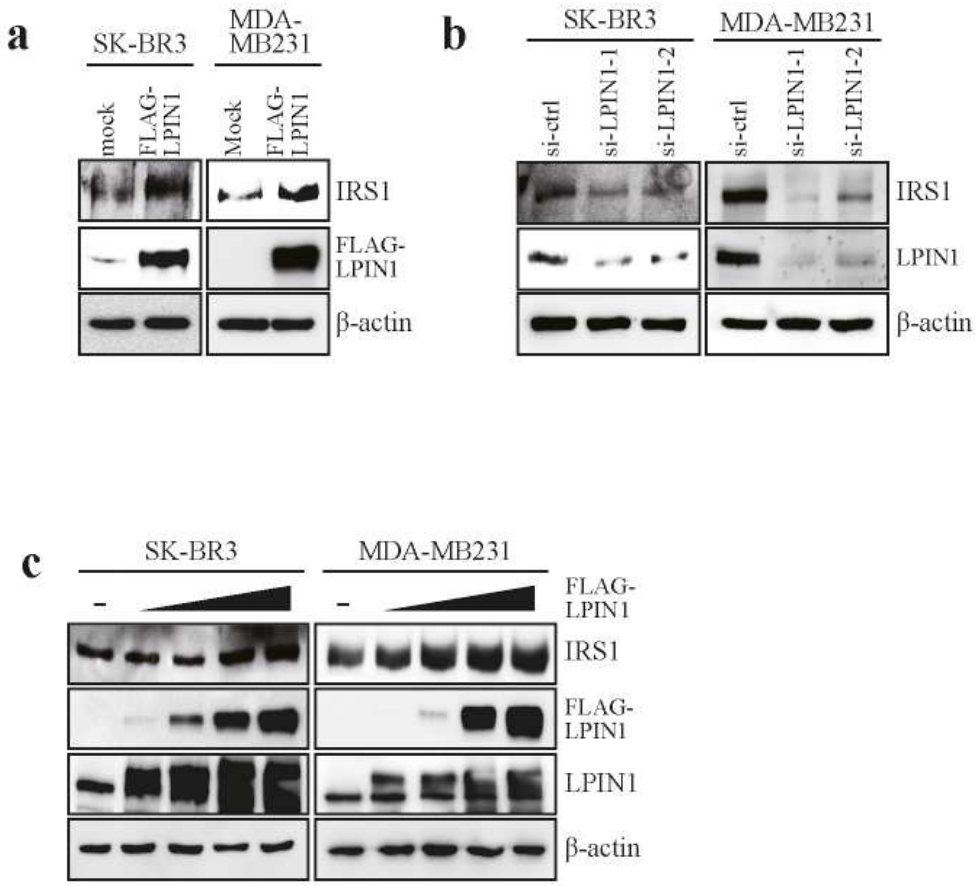


Figure 11 (d-f)

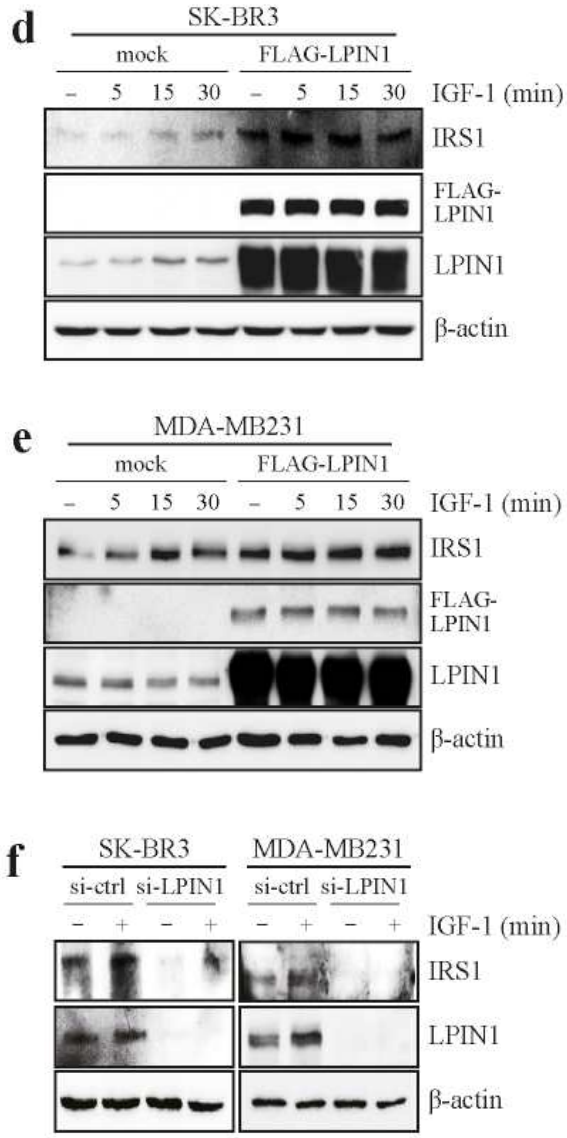


Figure 11 (g-i)

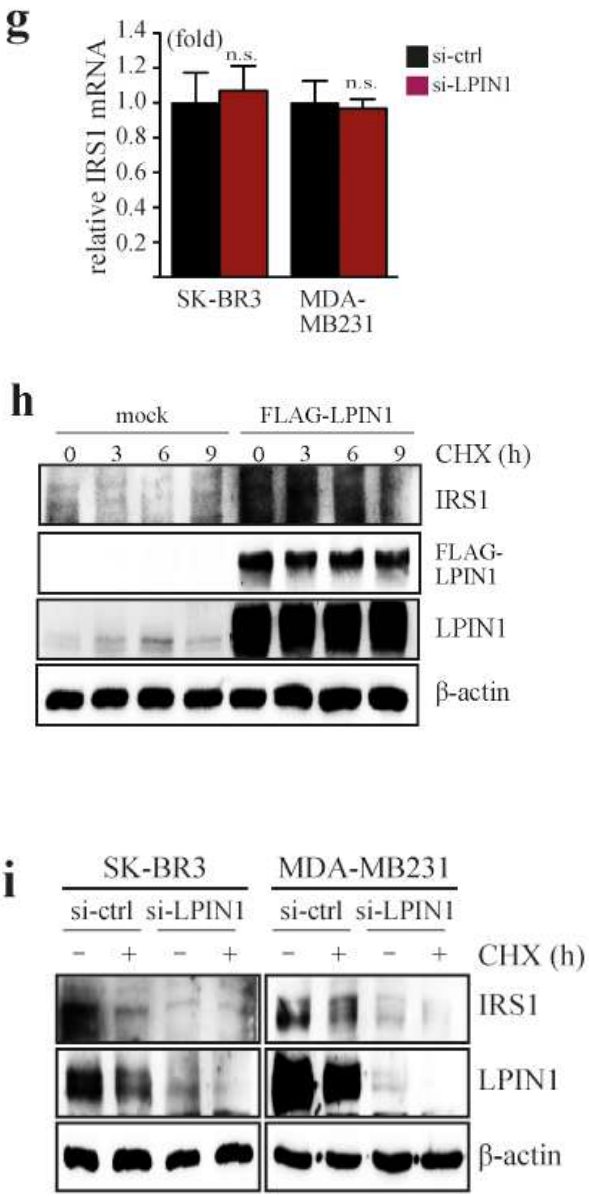


Figure 11. LPIN1 participates in the upregulation of IRS1 levels induced by IGF-1.

(a) Overexpression of LPIN1 leads to elevated IRS1 levels. SKBR3 or MDA-MB231 cells expressing mock or FLAG-LPIN1 were harvested, lysed and immunoblotted.

(b) Depletion of LPIN1 inhibits IRS1 abundance. siRNA-LPIN1-1 and -2 targeted against various LPIN1 sequences or siRNA-control were transfected into SKBR3 or MDA-MB231 Cells. At 48 h after transfection, the cells were harvested, lysed and immunoblotted.

(c) Ectopically expressed FLAG-LPIN1 dose-dependently increases IRS1. SKBR3 or MDA-MB231 cells were transfected with different amounts of FLAG-LPIN1, incubated for 48 h, harvested and immunoblotted.

(d and e) Overexpression of LPIN1 enhances IGF-1-induced IRS1 levels. SK-BR3 (d) and MDA-MB231 (e) cells were transfected with FLAG-LPIN1 or mock plasmid. At 24 h after transfection, the cells were serum starved, treated with or without 100 ng/mL IGF-1 for the indicated times, harvested, lysed and immunoblotted.

(f) Depletion of LPIN1 suppresses IGF-1-induced IRS1 levels. SKBR3 or MDA-MB231 cells were transfected with siRNA-LPIN1 and incubated for 24 h. Cells were exposed to 100 ng/ml IGF-1 for 30 min, harvested, and immunoblotted.

(g) Depletion of LPIN1 does not affect on the level of IRS1 mRNA. SKBR3 or MDA-MB231 cells were transfected with siRNA-LPIN1. Levels of IRS1 mRNA

were assessed by real time-PCR analysis. Corresponding signal intensities of each mRNA were determined and normalized to GAPDH. Results shown are mean ± S.D., n = 3. p = n.s.

(h) Overexpression of LPIN1 increases the half-life of IRS1. Mock plasmid and FLAG-LPIN1 were transfected into SKBR3 cells. After 48 h, the cells were treated with cycloheximide (CHX, 100 μg/ml) for different periods, harvested and immunoblotted.

(i) Depletion of LPIN1 downregulates IRS1 stability. siRNA-control and siRNA-LPIN1 were transfected into SKBR3 or MDA-MB231 cells. After 48 h, the cells were treated with CHX (100 μg/ml) for 9 hour, harvested and immunoblotted.

10. LPIN1 inhibits IRS1 degradation by the proteasomal and autophagic/lysosomal pathways

Given the tight regulation of IRS1 turnover by LPIN1, we examined the mechanisms underlying the LPIN1-mediated up-regulation of IRS1 stability. The IRS1 protein level was increased by the addition of MG132, a proteasome inhibitor, in a time-dependent manner (Figure 12a). Next, we asked whether the ubiquitin-mediated proteolytic pathway is responsible for IRS1 degradation by comparing protein stability in the absence or presence of MG132 with/without transfection of FLAG-LPIN1 in SK-BR3 and MDA-MB231 cells. MG132 strikingly enhanced the elevated IRS1 levels in LPIN1-overexpressing cells (Figure 12b). To examine whether LPIN1 regulates IRS1 ubiquitination in vitro, FLAG-LPIN1 was cotransfected with HA-ubiquitin in MDA-MB231 cells. The results showed that LPIN1 overexpression inhibited the ubiquitination of IRS1 (Figure 12c), suggesting that LPIN1 promotes IRS1 stability via inhibition of its ubiquitination.

To evaluate to what extent lysosome contribute to proteolysis of IRS1, we next used a specific lysosomal inhibitor, chloroquine, which accumulate in lysosome and raise intralysosomal pH (Seglen et al., 1983). Treatment of chloroquine in MDA-MB231cells time-dependently caused the increased IRS1 protein levels (Figure 12d). In addition, the lysosomal inhibition by chloroquine substantially enhanced the elevated IRS1 levels by LPIN1 in SK-BR3 and MDA-MB231 cells (Figure 12e).

Given that microtubule-associated protein 1 light chain 3 (LC3) facilitate autophagic/lysosomal degradation via its interaction with ubiquitinated proteins (Pankiv et al., 2007; Kirkin et al., 2009), we further examined the role of LC3 on the LPIN1-mediated up-regulation of IRS1 stability. The results showed that autophagy induction by LC-3 overexpression reduced IRS1 stability, whereas LPIN1 overexpression inhibited autophagic/lysosomal degradation of IRS1 by LC3 (Figure 12f). Consistent with this tight regulation of LPIN1 on the lysosomal degradation of IRS1, the starvation-mediated activation of LC3 time-dependently reduced IRS1 turnover, whereas LPIN1 overexpression inhibited starvation-induced IRS1 degradation (Figure 12g). Moreover, the combination of MG132 and chloroquine yielded a synergistic increase in IRS1 turnover MDA-MB231 cells (Figure 12h). Interestingly, the simultaneous inhibition of proteasome and lysosome function by treatment of MG132 and chloroquine enhanced the elevated IRS1 levels in LPIN1-overexpressing MDA-MB231 cells (Figure 12i). These results indicate that these two proteolytic processes for IRS1 are negatively regulated by LPIN1.

Figure 12 (a-c)

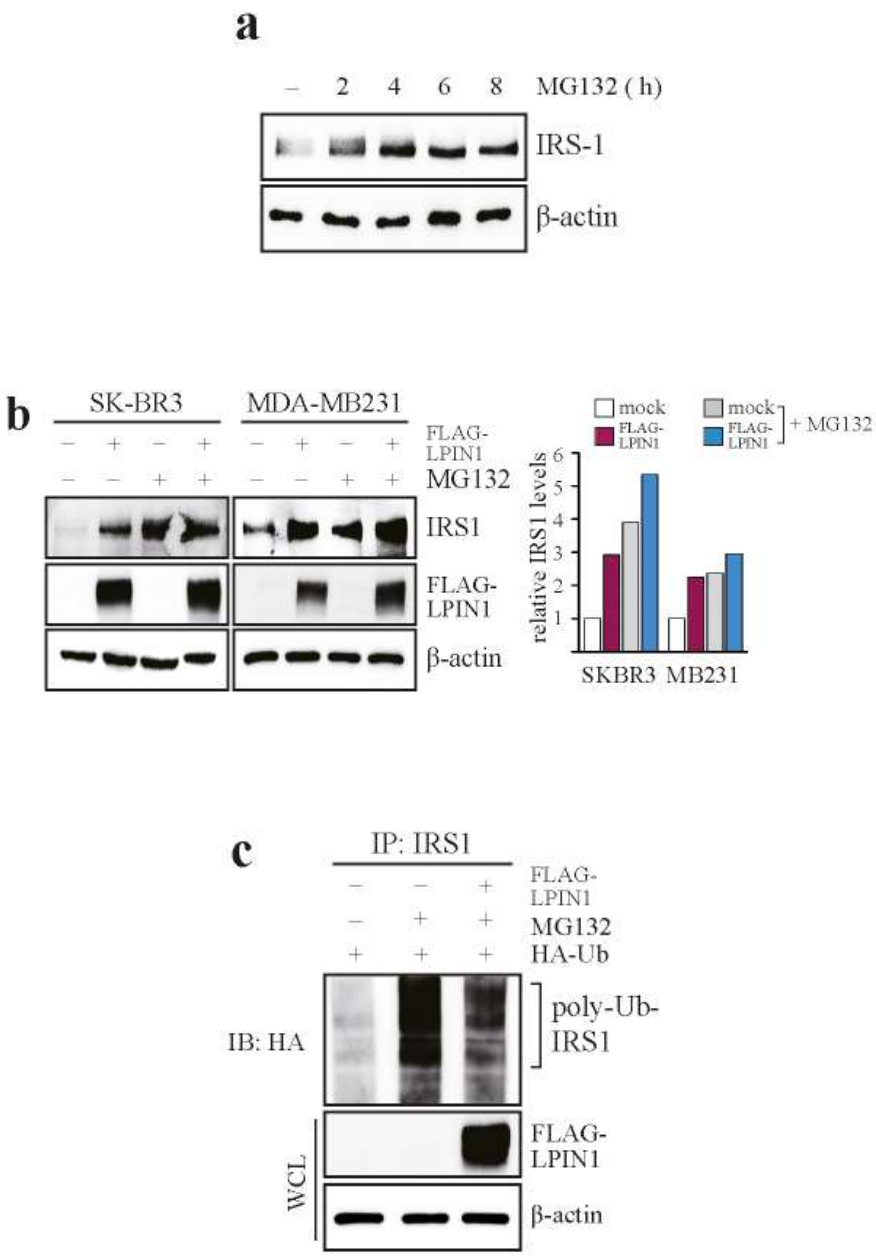
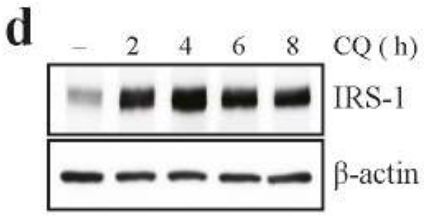
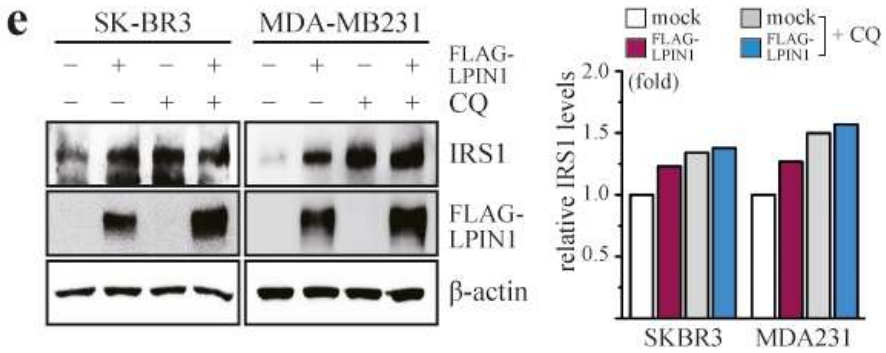


Figure 12 (d-f)





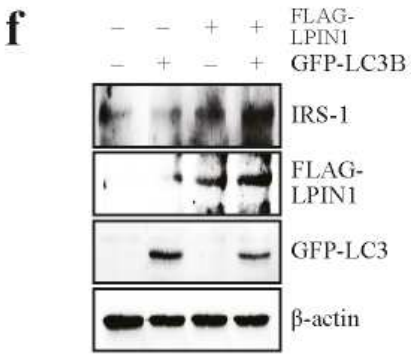


Figure 12 (g-i)

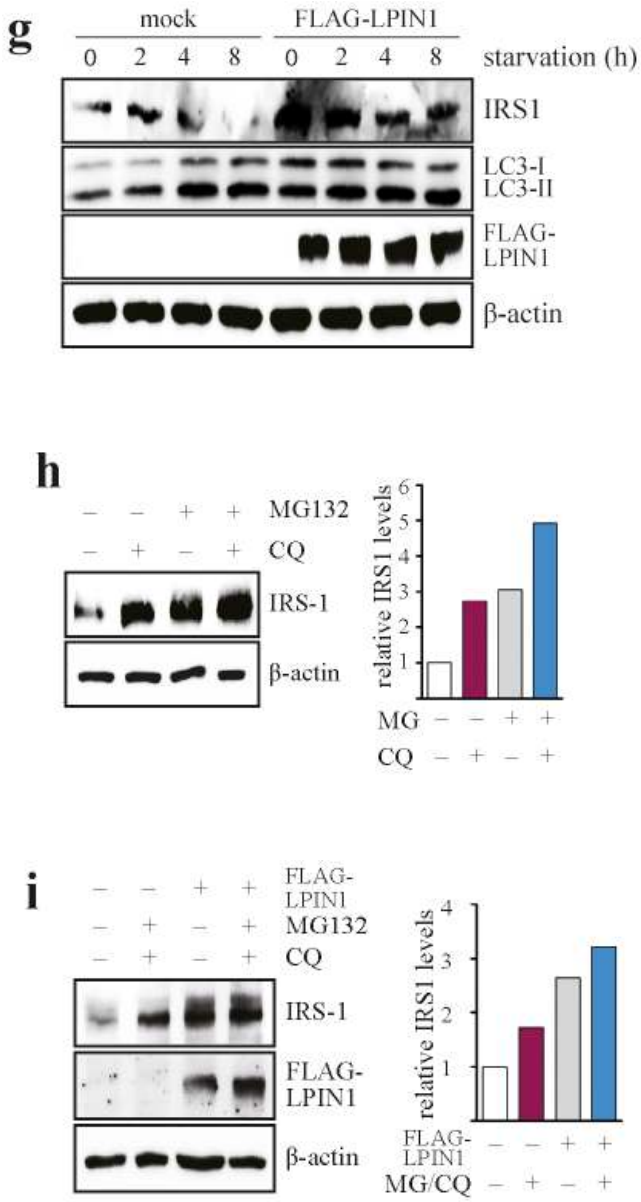


Figure 12. LPIN1 coordinately increases IRS1 stability by the inhibition of its proteasomal and autophagic/lysosomal degradation.

(a) Chase analysis of IRS1 expression after the treatment with MG132. MDA-MB231 cells were treated with 20 μM MG132 for indicated times, harvested and immunoblotted.

(b) Overexpression of LPIN1 inhibits the proteasomal degradation of IRS1. SKBR3 or MDA-MB231 cells expressing FLAG-LPIN1 were treated with 20 μM MG132 for 8 h, followed by immune blot analysis with the indicated antibodies (left) and a semiquantification with β-actin as a loading control and relative IRS1 levels (right).

(c) Overexpression of LPIN1 inhibits IRS1 ubiquitination. MDA-MB231 cells were cotransfected with vectors expressing FLAG-LPIN1 and HA-Ub, singly or in combination. At 48 h posttransfetion, cells were treated with MG132 (20 μM) for 8 hours, followed by immunoprecipitation with anti-FLAG antibody and immunoblot analysis with the indicated antibodies.

(d) Chase analysis of IRS1 expression after the treatment with Chloroquine (CQ). MDA-MB231 cells were treated with chloroquine (CQ, 100 μM) for indicated times, harvested and immunoblotted.

(e) Overexpression of LPIN1 inhibits the lysosomal degradation of IRS1. SKBR3 or MDA-MB231 cells expressing FLAG-LPIN1 were treated with CQ (100 μM) for 8 h, followed by immune blot analysis with the indicated antibodies (left) and a

semiquantification with β-actin as a loading control and relative IRS1 levels (right).

(f) Overexpression of LPIN1 inhibits the autopagic/lysosomal degradation of IRS1. MDA-MB231 cells were cotransfected with vectors expressing FLAG-LPIN1 and GFP-LC3, singly or in combination. At 48 h posttransfetion, the cells were harvested, lysed and immunoblotted.

(g) Impairment of starvation-induced lysosomal degradation of IRS1 by LPIN1 overexpression. MDA-MB231 cells expressing FLAG-LPIN1 were subjected to starvation for the indicated duration, followed by immunoblot analysis with the indicated antibodies.

(h) Synergistic enhancement of IRS1 stability by inhibiting both proteasomal and lysosomal pathway. MDA-MB231 cells were treated with MG132 (20 μM) for 2 h, and then exposed to CQ (100 μM) for 6h, followed by immune blot analysis with the indicated antibodies (left) and a semiquantification with β-actin as a loading control and relative IRS1 levels (right).

(i) Overexpression of LPIN1 increases IRS1 stability by simultaneous inhibition of its proteasomal and lysosomal degradation. MDA-MB231 cells expressing FLAG-LPIN1 were treated with MG132 (20 μM) for 2 h, and then exposed to CQ (100 μM) for 6h, followed by immune blot analysis with the indicated antibodies (left) and a semiquantification with β-actin as a loading control and relative IRS1 levels (right).

11. Activation of RAF1 is associated with increased IRS1 stability by LPIN1

Given that activation of IRS-1 by the IGF-1 results in the formation of an IRS1-Grb2-Sos complex for control of Ras signaling, which results in the activation of mitogen-activated protein kinase pathway (Skolnik et al., 1993), a key question is whether LPIN1 affects the MAPK pathway induced by IRS1. We observed that LPIN1 overexpression in SK-BR3 and MDA-MB231 cells upregulated RAF1/MEK/ERK/p90RSK signaling cascade (Figure 13a). Human LPIN1 silencing in both cells led to reduced phosphorylation of RFA1 as well as its downstream kinases (Figure 13b). Next, various doses of FLAG-LPIN1 were transfected into SK-BR3 and MDA-MB231 cells and the cells were incubated for 48 h. The overexpression of LPIN1 dose-dependently increased the phosphorylation of RAF1 and MEK protein kinases (Figure 13c). Because the Ras/RAF1/MAPK pathway primarily mediates the cell proliferative response to growth factors such as IGF-1 (Samani et al., 2007; Levine et al., 2006), we were interested in whether LPIN1 might contribute to the regulation of RAF1 and MAPK signaling cascade induced by IGF-1. To determine the regulatory role of LPIN1 on the phosphorylation of RAF1 and its downstream kinases, FLAG-LPIN1 overexpressing SK-BR3 and MDA-MB231 cells were exposed to IGF-1. The levels of RAF1, MEK1/2, ERK1/2, and p90RSK phosphorylation in LPIN1 overexpressing cells were enhanced compared with the control cells (Figure 13d and 13e). In contrast, we observed the

downregulated RAF1/MEK/ERK/p90RSK signaling cascade in both human LPIN1-silenced cell lines (Figure 13f). To further determine if the inhibition of LPIN1 or IRS1 downregulates RAF1 phosphorylation induced by IGF-1, the SK-BR3 and MDA-MB231 cells were pretreated with propranolol, a LPIN1 inhibitor (Han et al., 2010), or a tyrphostins NT157, an inhibitor of IRS1 (Reuveni et al., 2013), and either exposed or not exposed to IGF-1. Propranolol and NT157 dose-dependently decreased the IGF-1-induced phosphorylation of RAF1 as well as its downstream kinases, respectively (Figure 13g and 13h). Overall, these data indicate that LPIN1 enhances the IGF-1-induced phosphorylation of RAF1 and MEK1/2 through increased IRS1 stability by LPIN1. Because the RAF1-dependent signal transduction pathway promotes *c-fos* expression and subsequently induces AP-1 transactivation (Bruder et al., 1992, Karin et al., 1997), we next investigated whether LPIN1 might regulate *c-fos* and AP-1 promoter activity. As shown in figure 13i and 13j, *c-fos* and AP-1 transcriptional activity was significantly inhibited in LPIN1-silenced SK-BR3 and MDA-MB231 cells compared with control cells. Moreover, propranolol and NT157 significantly inhibited the IGF-1-promoted *c-fos* and AP-1 promoter activity in both cells, respectively (Figure 13k-13n). LPIN1-IRS1-RAF1 axis promotes the induction of the *c-fos* gene and subsequent AP-1 transactivation, which leads to a comparable enhancement of tumor growth via increased cell proliferation.

Figure 13 (a-c)

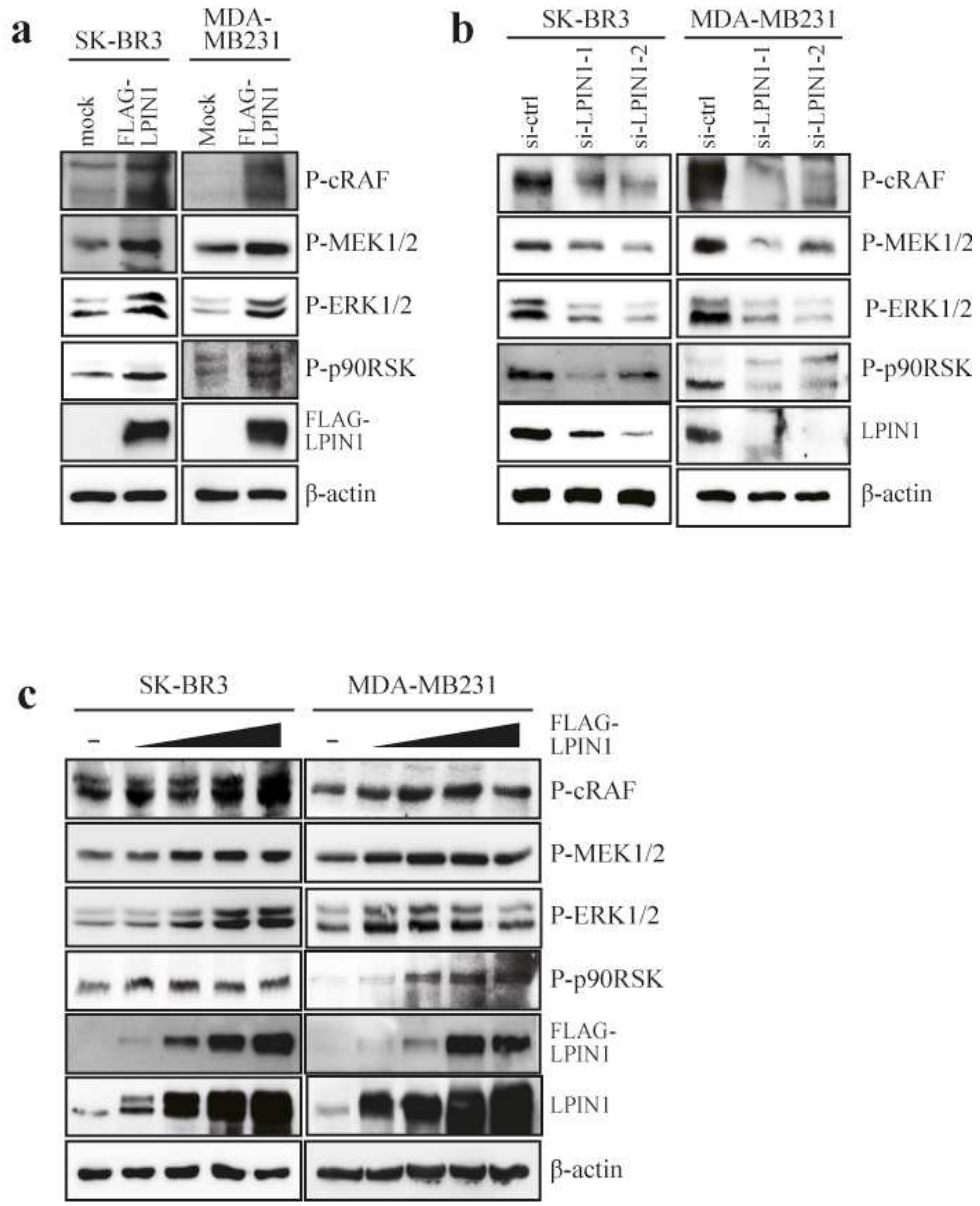


Figure 13 (d-f)

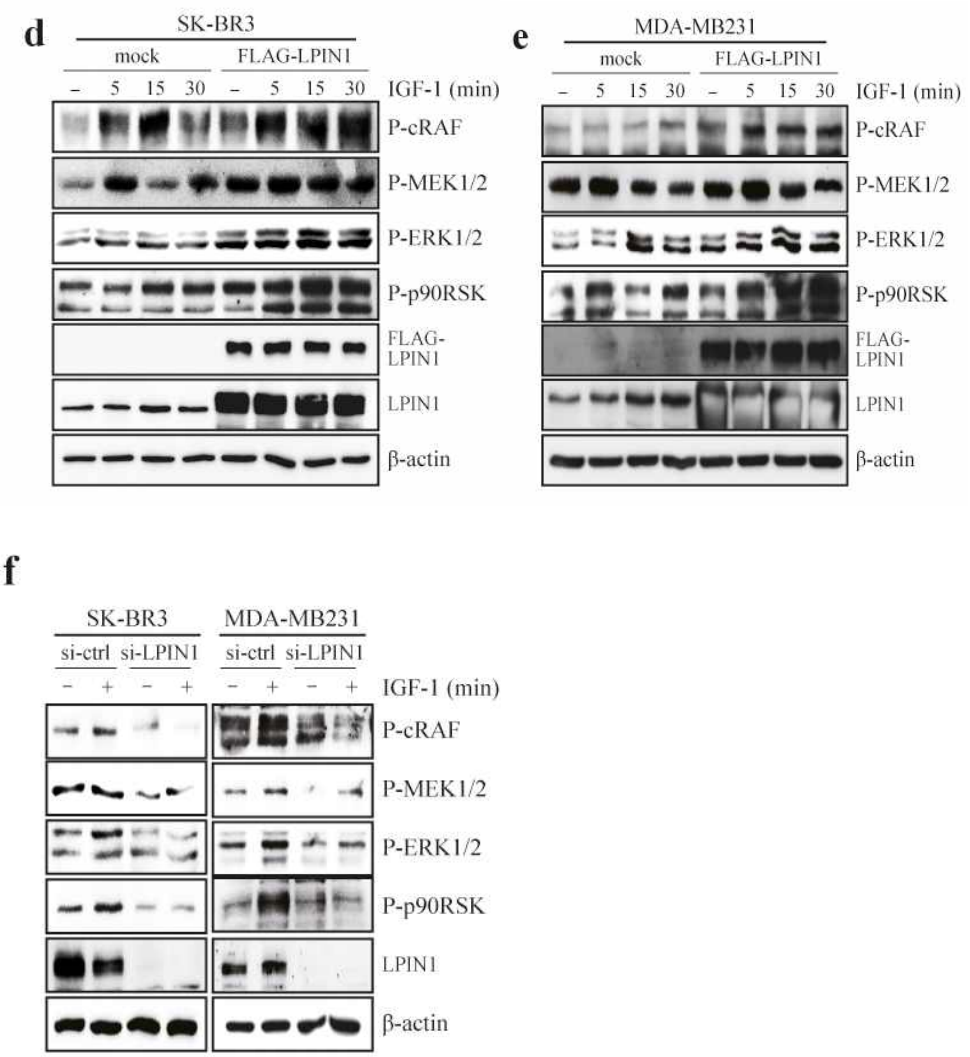


Figure 13 (g-h)

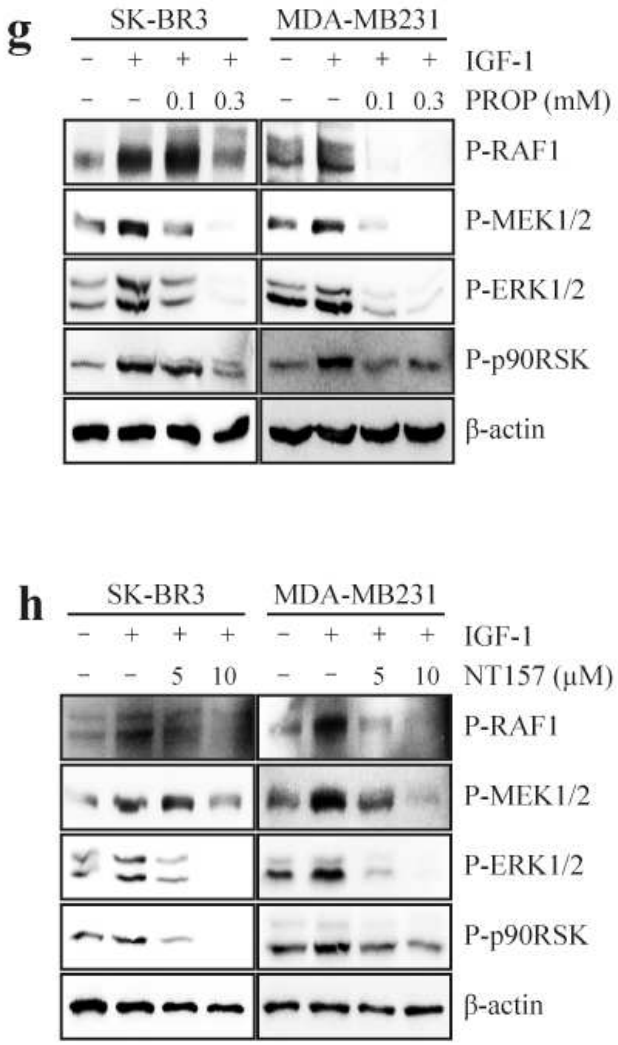


Figure 13 (i-n)

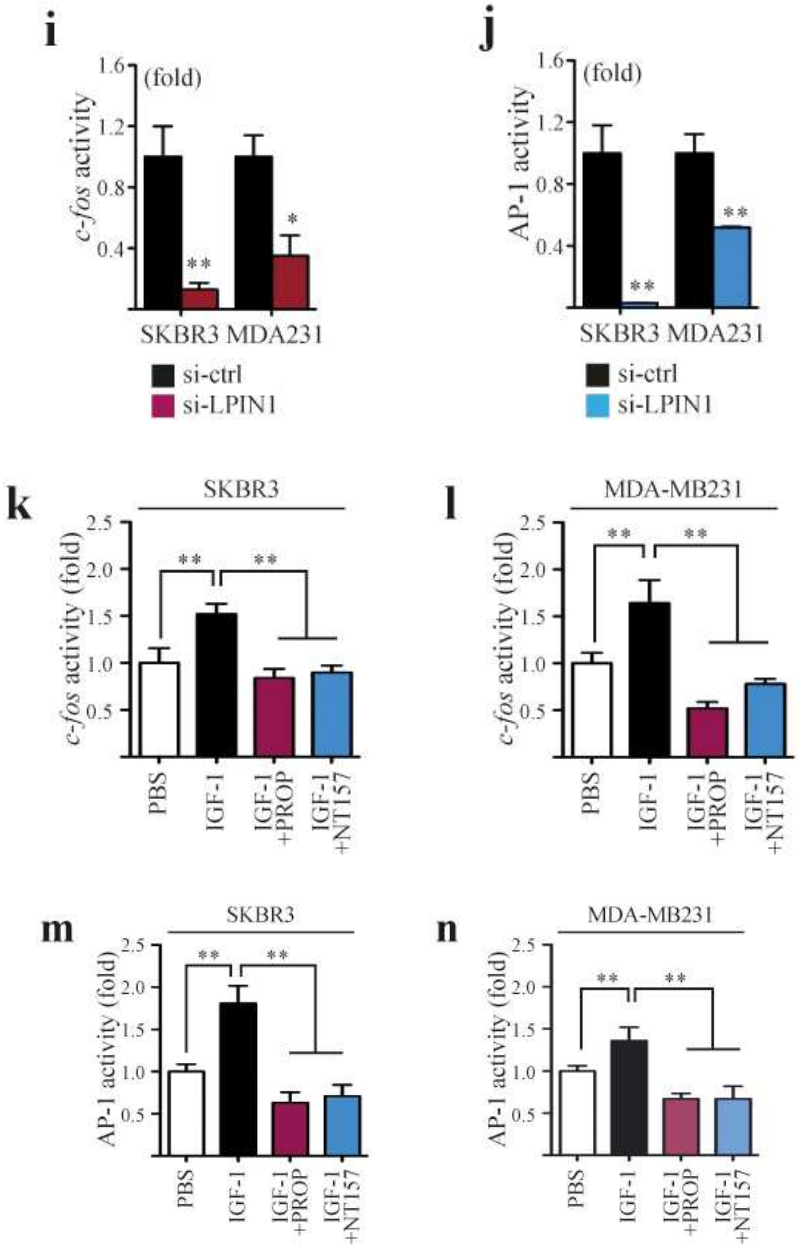


Figure 13. LPIN1 enhances IRS1-mediated activation of mitogenic signaling pathway.

(a) Overexpression of LPIN1 leads to enhancement of mitogen-activated protein (MAP) kinases activation. SKBR3 (left) or MDA-MB231 (right) cells expressing mock or FLAG-LPIN1 were harvested, lysed and immunoblotted.

(b) Depletion of LPIN1 suppresses phosphorylation of MAP kinases. siRNA-LPIN1-1 and -2 targeted against various LPIN1 sequences or siRNA-control were transfected into SKBR3 (left) or MDA-MB231 (right) cells. At 48 h after transfection, the cells were harvested, lysed and immunoblotted.

(c) Ectopically expressed FLAG-LPIN1 dose-dependently increases phosphorylation of MAP kinases. SKBR3 (left) or MDA-MB231 (right) cells were transfected with different amounts of FLAG-LPIN1 (0.5, 1, 3, and 5 μg), incubated for 48 h, harvested and immunoblotted.

(d and e) Overexpression of LPIN1 enhances IGF-1-induced phosphorylation of MAP kinases. SK-BR3 (d) and MDA-MB231 (e) cells were transfected with FLAG-LPIN1 or mock plasmid. At 24 h after transfection, the cells were serum starved, treated with or without 100 ng/mL IGF-1 for the indicated times, harvested, lysed and immunoblotted.

(f) Depletion of LPIN1 suppresses IGF-1-induced phosphorylation of MAP kinases. SKBR3 or MDA-MB231 cells were transfected with siRNA-LPIN1 and incubated for

24 h. Cells were exposed to 100 ng/ml IGF-1 for 30 min, harvested, and immunoblotted.

(g and h) Inhibition of LPIN1 and IRS1 suppresses IGF-1-induced MAP kinases activation. SKBR3 or MDA-MB231 cells were seeded and treated with various concentrations of propranolol (g) and NT157 (h), as indicated, for 24 h, and then exposed to 100 ng/mL IGF-1 for 30 min, followed by immune blot analysis with the indicated antibodies.

(i and j) Depletion of LPIN1 suppresses activities of *c-fos* and *AP-1* promoters. SKBR3 or MDA-MB231 cells were co-transfected with a mixture containting the *c-fos*- (i) or *AP-1*-luciferase promoter (j), pRL-TK gene with siRNA-LPIN1. At 48 h after transfection, the firefly luciferase activity was determined in cell lysates and normalized to renilla luciferase activity. Results shown are mean ± S.D., n = 4. *p < 0.05, **p < 0.001.

(k and l) Inhibition of LPIN1 and IRS1 suppresses *c-fos* promoter activity induced by IGF-1. SKBR3 (k) or MDA-MB231 cells (l) were co-transfected with a mixture containting the *c-fos*-luciferase promoter and pRL-TK gene. At 48 h after transfection, cells were treated with 0.1 mM propranolol and 0.1 μM NT157 for 24 h, respectively, and then exposed to 100 ng/mL IGF-1 for 12 h. Results shown are mean ± S.D., n = 4. **p < 0.001.

(m and n) Inhibition of LPIN1 and IRS1 suppresses AP-1 promoter activity induced

by IGF-1. SKBR3 (m) or MDA-MB231 cells (n) were co-transfected with a mixture containting the *AP-1*-luciferase promoter and pRL-TK gene. At 48 h after transfection, cells were treated with 0.1 mM propranolol and 0.1 μM NT157 for 24 h, respectively, and then exposed to 100 ng/mL IGF-1 for 12 h. Results shown are mean ± S.D., n = 4. **$p < 0.001$.

12. Epithelial cell transformation and breast tumor growth is efficiently inhibited by propranolol and NT157

Given the ability of LPIN1-IRS1 axis to induce epithelial cell transformation and breast tumor growth via the activation of RAF1, we examined whether inhibition of this signal transduction contributes to the suppression of breast tumourigenesis. To understand the effect of IRS1 overexpression on cell transformation and breast turmorigenesis, we established JB6 Cl41 and SK-BR3 cells stably overexpressing IRS1 (IRS1-JB6 and IRS1-SKBR3) and mock-transfected cells (mock-JB6 and mock-SKBR3), respectively (Figure 14a and 14b, left panels). Using these cell lines, we first examined differences in cell transformation of JB6 Cl41 cells and colony growth of SK-BR3 cells in a soft agar matrix. As a result, IRS1-JB6 cells showed significantly enhanced neoplastic cell transformation compared with mock-JB6 cells (Figure 14a, right panels). In addition, overexpression of IRS1 also significantly enhanced anchorage-independent colony growth of SK-BR3 cells (Figure 14b, right panels). To further confirm the role of IRS1 on breast tumorigenesis, the soft agar assays were performed in the presence of NT157. We observed that NT157 treatment significantly inhibited not only colony numbers but also colony sizes of SK-BR3 cells in soft agar (Figure 14d). To test the effects of inhibition of LPIN1 and IRS1 on the IGF-1-promoted breast tumor growth, SK-BR3 cells were treated with IGF-1 in a presence or absence of propranolol and NT157, respectively and incubated at 37°C in

a 5% CO_2 incubator for 14 days. The results showed that the treatment of propranolol and NT157 significantly inhibited the formation of IGF-1-promoted colonies compared with control cells (Figure 14e). The inhibition was evident not only in colony number but also in colony size. Subsequently, the effects of propranolol and NT157 on tumor development induced by IGF-1 in vivo were studied in the syngeneic mouse 4T1 metastatic breast cancer model. Representative tumor images demonstrated that a profound reduction in the weight and volume of tumors was mediated by treatment of propranolol and NT157, compared with only IGF-1-promoted tumor (Figure 14f).

Figure 14 (a-b)

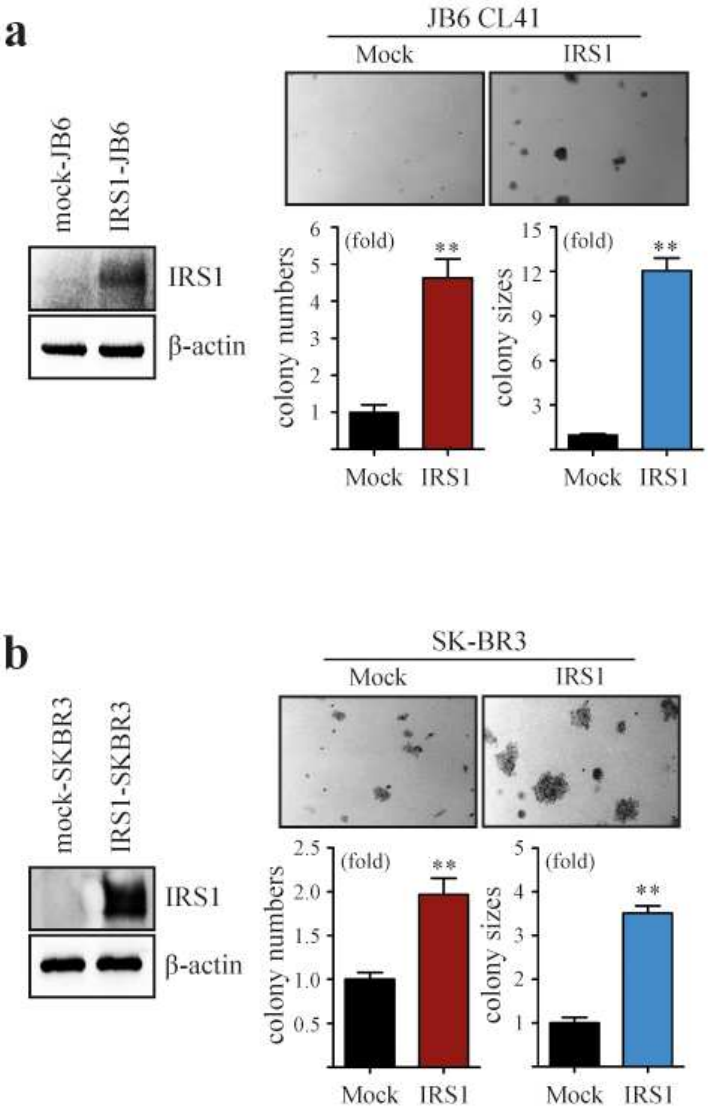


Figure 14 (c-d)

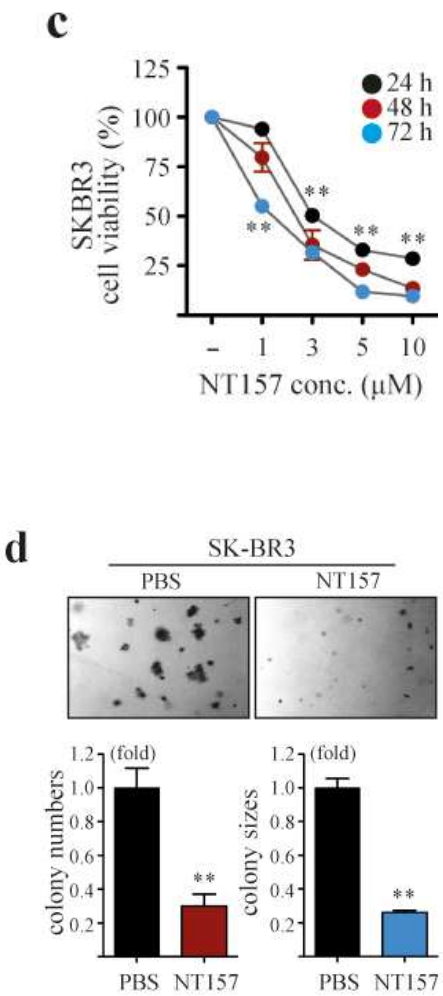


Figure 14 (e)

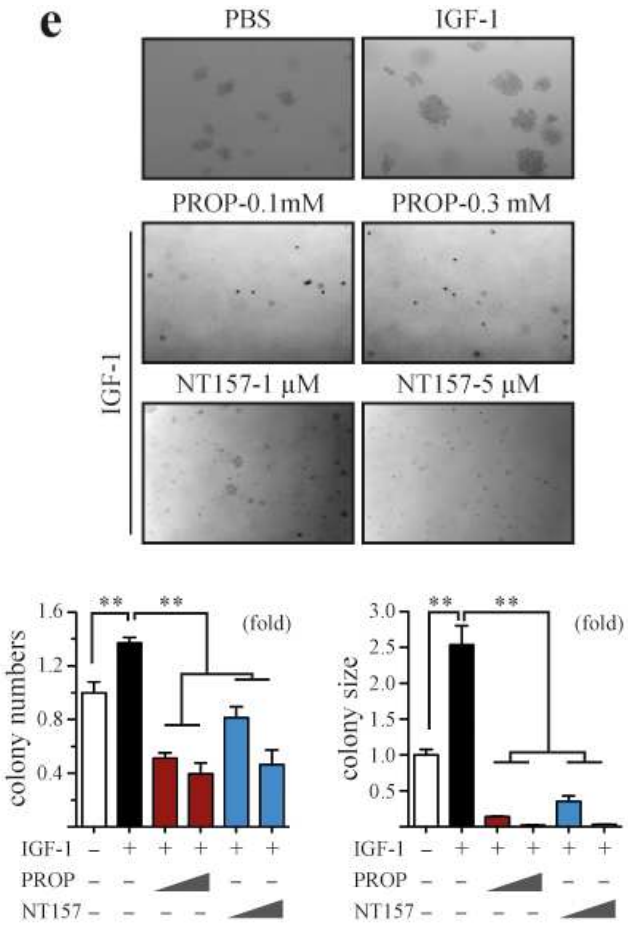


Figure 14 (f)

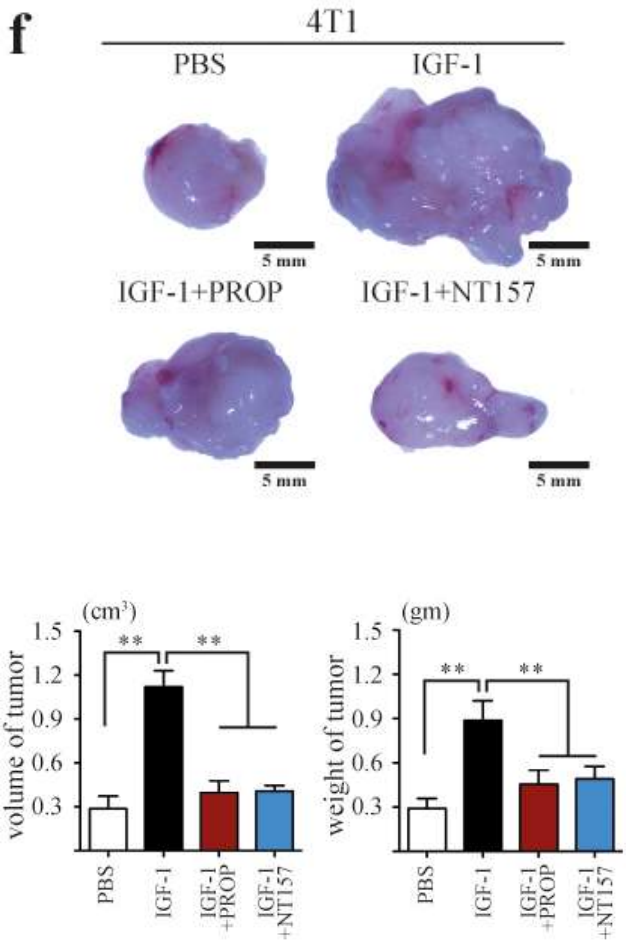


Figure 14. Treatment with propranolol and NT157 inhibits epithelial cell transformation and breast tumorigenesis in vitro and in vivo.

(a) Overexpression of IRS1 increases epithelial cell transformation in soft agar. Left, expression levels of IRS1 in Mock-transfected (mock-JB6) and IRS1-overexpressing (IRS1-JB6) cells; right, Mock-JB6 or IRS1-JB6 cells were seeded in soft agar and incubated at 37°C in a 5% CO_2 atmosphere for 14 d. Results shown are mean ± standard deviation (S.D.), n = 3. **p < 0.001.

(b) Overexpression of IRS1 increases anchorage-independent cell growth in soft agar. Left, expression levels of IRS1 in Mock-transfected (mock-SKBR3) and IRS1-overexpressing (IRS1-SKBR3) cells; right, Mock-SKBR3 or IRS1-SKBR3 cells were seeded in soft agar and incubated at 37°C in a 5% CO_2 atmosphere for 14 d. Results shown are mean ± S.D., n = 3. **p < 0.001.

(c) NT157 treatment suppresses cell proliferation of SKBR3 cells. Cells were seeded and treated with various concentrations of NT157, as indicated, and then cultured for 24 h, 48 h and 72 h at 37°C in a 5% CO_2 atmosphere. Cell viability was measured by the MTT assay, as described in Methods. Results shown are mean ± S.D., n = 3. **p < 0.001.

(d) NT157 suppresses anchorage-independent cell growth in soft agar. SKBR3 cells were treated with 5 μM NT157 in soft agar and incubated at 37°C in a 5% CO_2 atmosphere for 14 d. Results shown are mean ± S.D., n = 3. **p < 0.001.

(e) Treatment of propranolol and NT157 inhibits IGF-1-induced anchorage-independent growth of SKBR3 cells. SKBR3 cells were treated with 100 ng/mL IGF-1 with/without treatment of different doses of propranolol or NT157 as indicated in soft agar and incubated at 37°C in a 5% CO_2 atmosphere for 14 d. Results shown are mean ± S.D., n = 3. **p < 0.001.

(f) Treatment of propranolol and NT157 inhibits mammary gland tumorigenesis in mice. The 4T1 cells cells were injected with treatment of 400 ng/mL IGF-1 in the presence or absence of 0.5 mM propranolol or 20 μM NT157, as indicated, into the mammary gland of BALB/c mice and allowed to grow until the formation of tumors for 10 d. Results shown are representative pictures of tumor and mean ± S.D., n = 30. **p < 0.001.

IV. Discussion

Part I : Interleukin-33/ST2 axis promotes epithelial cell transformation and breast tumorigenesis via upregulation of COT activity

Although cell-autonomous events, such as proliferation and death evasion, control tumor development, the tumor microenvironment also makes a major contribution and influences the physiology of malignant cells (Radisky and Bissell, 2004). Nearly all tumors contain inflammatory and immune cells, such as dendritic cells, macrophages, and lymphocytes, which produce cytokines and other factors that promote tumor growth and survival (Coussens and Werb, 2003; Lin and Karin, 2007; Balkwill et al., 2005). The most obvious tumor-promoting role of immune cells is manifested in inflammation-associated cancers, where tumors arise and grow at sites of chronic inflammation. Here, we provide the first direct evidence that IL-33 leads to the activation of COT via its interaction with ST2, which results in the induction of neoplastic cellular transformation through its downstream AP-1 and STAT3 signaling pathways. Combined with our increasing understanding of the IL-33/ST2 signaling pathway, this work highlights how COT regulates IL-33-induced tumorigenesis.

IL-33 is a member of the IL-1 cytokine family, originally described as a nuclear

protein in cerebral arteries (Onda et al., 1999) and later as NF-HEV, a nuclear factor expressed in human high endothelial venules in secondary lymphoid organs (Baekkevold et al., 2003). Recently, IL-33 was identified as the ligand for the orphan receptor, ST2 (IL-1RL1) (Tominaga et al., 1991). ST2 molecules are members of the IL-1 receptor family and exist in two forms: a transmembrane full-length form (ST2L) and a soluble secreted form (sST2) due to differential splicing of ST2 mRNA (Bergers et al., 1994). ST2 associates with IL-1R accessory protein (IL-1RAcP) to form an IL-33 receptor (IL-33R1), and IL-33 signals via this heterodimer (Schmitz et al., 2005). The biding of IL-33 to ST2 results in the recruitment of MyD88, IRAK1, and IRAK4 to the receptor complex in the cytoplasmic region of ST2 (Palmer et al., 2008), which induces activation of various singling proteins, including NF-κB, IκBa, ERK1/2, p38, and JNK1/2, leading to the induction of the inflammatory mediators IL-1β, IL-3, IL-6, TNF, IL-5, and IL-13 (Moulin et al., 2007; Pushparaj et al., 2009; Allakhverdi et al., 2007). Furthermore, TNF receptor-associated factor 6 (TRAF6) has been shown to be a critical adaptor molecule in both the IL-1 and IL-33 signaling pathways (Cao et al., 1996; Funakoshi-Tago et al., 2008). IL-33 rapidly induces the formation of an ST2L complex containing IRAK; however, the absence of TRAF6 abolishes the recruitment of IRAK to ST2L (Funakoshi-Tago et al., 2008). Consequently, the IL-33-induced activation of p38, JNK, and NF-κB is completely inhibited in TRAF6-deficient MEFs (Funakoshi-Tago et al., 2008). Interestingly, ST2

deletion attenuates tumor growth and metastasis in 4T1 breast tumor model and IL-33 promotes breast cancer progression through increased intratumoral accumulation of immunosuppressive cells and by diminishing innate antitumor immunity (Jovanovic et al., 2011; Jovanovic et al., 2014). Here, a demonstration of the clinical relevance of IL-33/ST2 axis was provided by our results in breast cancer patients. This analysis revealed that the prognostic value of IL-33, ST2 and COT levels, is readily detected in human breast cancer tissues. Furthermore, ST2 levels were positively correlated with COT levels in breast cancer tissues, indicating that the IL-33/ST2-axis contributed to breast carcinogenesis via COT activation. Furthermore, ST2 directly interacts with wild-type COT, but not with its mutant S400A. Indeed, IL-33 increases the interaction between ST2 and COT, suggesting that COT is an important signal transducer for the IL-33/ST2 signaling pathway. As a result, IL-33 strongly induced phosphorylation of COT at Ser400, followed by the activation of the MEK-ERK and JNK-cJun signaling pathways. In addition, the knockdown of ST2 lowered IL-33-induced COT phosphorylation, indicating that COT activation depends on the IL-33/ST2 signaling axis. Moreover, TKI, PD98059, and SP600125, which are COT, MEK, and JNK inhibitors, respectively, strongly suppressed the IL-33-induced MEK-ERK and JNK-cJun signaling pathways. Overall, these data indicate that COT functions as an important mediator of the IL-33-ST2 signaling pathway, which contributes breast carcinogenesis.

The pathway from Ras through Raf and MEK (MAPK and ERK kinase) to ERK/MAPK (extracellular signal-regulated kinase/mitogen-activated protein kinase) regulates many fundamental cellular processes that govern that govern cell transformation, differentiation, proliferation, and survival (Kolch, 2005). Interestingly, lipopolysaccharide (LPS), a component of Gram-negative bacteria, and the proinflammatory cytokines tumor necrosis factor α (TNFα) and interleukin-1 (IL-1) do not require Raf, but instead require a distinct COT to promote tumor development and growth (Harmey et al., 2002; Gelin et al., 1991; Dumitru et al., 2000). In addition, the neoplastic transforming activity of COT results from the coordinated activat ion of chromatin remodeling by histone H3, which ultimately converges on the regulation of *c-fos* transcriptional activity, followed by AP-1 transcriptional acti vity (Choi et al., 2008). AP-1 is a major transcription factor in epithelial cell tra nsformation and tumor development induced by various tumor promoters, such as epidermal growth factor (EGF) and 12-*O*-tetradecanoylphorbol 13-acetate (TP A) (Khanal et al., 2011). Despite the vital role of COT in chronic inflammatory diseases and tumor development, the precise mechanisms that regulate the IL-33 signaling pathway in epithelial cell transformation and tumorigenesis in the breast have not yet been well explored. The data presented in this report show that the IL-33/ST2/COT pathway promotes *c-fos* and *c-jun* promoter activity, resulting in the induction of AP-1 activity in JB6 Cl41 cells. The silencing of COT, and also the i

nhibition of COT activity by TKI, dramatically suppressed IL-33-induced AP-1 activity in JB6 Cl41 and MDA-MB231 cells. These findings, coupled with our recently published work showing that COT conveys oncogenic signals to prom ote aggressiveness in human breast cancers, indicates that COT may play an es sential role in epithelial cell transformation and tumor development in the brea st. Interestingly, ST2 levels were positively correlated with COT expression in breast cancer tissues. These results define an IL-33/ST2 signaling axis that conveys an oncogenic signal to promote aggressiveness in human breast cancers through the induction of COT activity. Consistent with these observations, our results also showed that knockdown of IL-33, ST2, and COT decreased the tumorigenicity of MCF7 cells, suggesting that COT might be an important molecular target for IL-33-mediated development of breast cancer

STAT3 is constitutively activated in more than 50% of primary breast tumors and tumor-derived cell lines (Leslie et al., 2006; Gritsko et al., 2006; Dolled-Filhart et al., 2003). Clinical studies have demonstrated that elevated levels of tyrosine-phosphorylated STAT3 in patients with stage II breast cancer are correlated with an incomplete response to neo-adjuvant chemotherapy (Diaz et al., 2006). The evidence that activated STAT3 participates in a causal manner in breast tumorigenesis is based on studies from cell lines expressing high levels of phosphorylated STAT3. Inhibiting or removing STAT3 leads to increased apoptosis, chemosensitivity, and decreased

angiogenesis both in cell culture and in xenograft models (Leslie et al., 2006; Alvarez et al., 2005). Furthermore, the expression of a constitutively active form of STAT3 (STAT3C) in immortalized human breast epithelial cells mediates *de novo* tumorigenesis, demonstrating the sufficiency of this transcription factor in promoting cellular transformation (Dechow et al., 2004). STAT3 activation can occur through the actions of many autocrine and paracrine growth factors, including IL-6, epidermal growth factor, platelet-derived growth factor, heregulin, vascular endothelial growth factor, hepatocyte growth factors, and the Src non-receptor tyrosine kinase, leading to the prediction that it would be phosphorylated in breast cancer via these multiple, redundantly acting growth factors (Li and Shaw, 2002; Berclaz et al., 2001; Garcia et al., 2001). However, little is known about whether and how STAT3 activity is regulated by IL-33/ST2 signaling during tumor development. Here, we provide that endogenous COT interacts with STAT3 in an IL-33-dependent manner. Moreover, IL-33 strongly induced phosphorylation of STAT3 at Tyr 705, indicating that a homodimer of phosphorylated STAT3 induced by IL-33 then translocates to the nucleus and induces the transcription of several target genes promoting proliferation, cell growth, and the inhibition of apoptosis. In addition, the knockdown of ST2 as well as COT decreased IL-33-induced STAT3 phosphorylation. The treatment with TKI, a COT inhibitor, strongly suppressed IL-33-induced STAT3 phosphorylation, resulting from the inhibition of COT activity, indicating that COT functions as an

important mediator of the IL-33/ST2 signaling pathway to induce STAT3 transcriptional activity. This study documents a previously unknown role of IL-33 in human breast cancer by displaying its promotional role in proliferation, cell survival, transformation, and tumor development. Moreover, these results collectively reveal COT as an attractive therapeutic target in the tumor microenvironment, inhibition of which could potentially attenuate the aggressiveness of breast cancer. Hence, our study not only reveals a novel mechanism underlying IL-33 activity but also provides a novel treatment strategy against metastatic breast cancer.

Part Ⅱ : LPIN1 promotes breast tumorigenesis via upregulation of insulin receptor substrate 1 stability

Lipid biosynthesis is essential for the maintenance of cellular homeostasis. The lipids produced by cells are used an energy source/reserve, as building blocks for membrane biosynthesis, as precursor molecules for the synthesis of various cellular products, and as signaling molecules (Laplante and Sabatini, 2009). Accumulating evidence suggests that defects in lipid synthesis or processing contribute to the development of cancer (Mashima et al., 2009), emerging as the important aspect of the lipogenic enzymes in cancer cell metabolism. However, numerous enzymes involved in lipid biosynthesis and the specific role of many of them during cancer progression is still

unknown. LPIN1 has been demonstrated to play an important role in the regulation of triglyceride and phospholipid biosynthesis by catalyzing the dephosphorylation of phosphatidate into diacylgrlycerol (DAG) (Han et al., 2006). Loss of LPIN1 prevents normal adipose tissue development, leading to lipodystrophy and insulin resistance, whereas its overexpression leads to obesity (Phan and Reue, 2005; Peterfy et al., 2001). Interestingly, the high expression of LPIN1 was frequently observed in various human cancer cell lines (Brohee et al., 2015). These studies led us to define an unexpected regulatory role for LPIN1 in modulating IRS1 stability through control of its lysosomal and protasomal degradation. The regulated control IRS1 by LPIN1 is interesting in light of previous work showing that IRS1 were overexpressed in some primary breast tumors (Rocha et al., 1997; Lee et al., 1999; Myers et al., 1996) and higher levels of IRS1 is associated with decreased disease-free survival and a higher recurrence in a subset of patients with breast tumor (Rocha et al., 1997). Our data also illustrates that the inhibition of LPIN1/IRS1 axis suppresses RAF1-mediated MAPK signaling pathway as well as *c-fos* and AP-1 transcriptional activity in the context of physiologic and pathophysiologic condition that led to induction of epithelial transformation and breast tumorigenesis.

The IGF system regulates cell proliferation, differentiation, and apoptosis in normal tissues including the mammary gland (Khandwala et al., 2000; Sachdev and Yee, 2001). The IGF system comprises a complex network of ligands, IGF-1 and IGF-

2, and their cognate receptor, IGF-1R and IGF-2R (Khandwala et al., 2000). There is abundant evidence that IGF-1 is potent mitogen for breast cancer cells, acts synergistically with estrogen to stimulate cell growth, and protect breast cancer cells from apoptosis (Rubin and Baserga, 1995). It has been reported that activation of phosphatidylinositol 3-kinase (PI3K) and the Akt/protein kinase B (PKB) pathway mediate the protection from apoptosis by IGF-1 (Kulik et al., 1997). In addition, both Akt and MAPK pathways are induced by IGF1 to promote cell survival (Parrizas et al., 1997). One of the major adapter proteins activated by IGF-1 to transduce its signal is IRS1 (Sun et al., 1999). IRS1 binds to IGF1 through its phosphotyrosine-binding (PTB) domain and serves to connect to its downstream pathways to transduce IGF signal (Sun et al., 1993). Interestingly, it has been shown that IGF-1 stimulates a transient phosphorylation of IRS1, followed by proteasomal degradation of IRS1 protein in breast cancer cells (Lee et al., 2000). Despite the vital role of IRS1 in tumor development, the precise mechanisms by which LPIN1 modulated IGF-1 signaling network pathway in epithelial cell transformation and tumorigenesis in the breast have not yet been well explored. Here, we report that LPIN1 directly interact with IRS1 to inhibit its poly-ubiquitination. Specifically, we found that LPIN1 block the IGF-1-mediated IRS1 phosphorylation at Ser307, which lead to its ubiquitin-proteasome-mediated degradation (Sun et al., 1999). Ubiquitinated cytosolic proteins undergo degradation via the proteasome or the lysosome, which comprise the two

major intracellular proteolytic systems in mammalian cells routes (Kirkin et al., 2009). The acid hydrolases in lysosomes degrade most membrane and extracellular proteins taken up by endocytosis, as well as cytoplasmic proteins and organelles through autophagy (Scott et al., 1999). Our results demonstrate that IRS1 can be regulated by lysosomal degradation in SK-BR3 and MDA-MB231 cells, a process that is inhibited by LPIN1. In addition, LPIN1 suppressed the degradation of IRS1 through autophagy pathway activated by LC-3 overexpression or starvation. Interestingly, the increased endogenous IRS1 levels by LPIN1 were augmented by synergistic inhibition of proteasome and lysosome pathway, suggesting these two proteolytic processes for IRS1 are negatively regulated by LPIN1. These studies, however, have not resolved precisely how IRS1 degraded by autophagic/lysosomal pathway or the consequences of reduced expression of IRS1. Possibly the increased phosphorylation of IRS1 at serine 307 per se might to facilitate its ubiquitin-mediated recognition by selective autophagy. Indeed, the expression of IRS1 was reduced by overexpression of LC-3, which had been proposed to be critical in the activation of autophagy. Such results may provide evidence for a future theory to explain the autophagic/lysosomal dependent IRS1 degradation.

In addition to providing the molecular mechanisms for LPIN1-mediated regulation of IRS1 stability, we demonstrated that overexpression of LPIN1 enhances the ability of IRS1 to activate the RAF-1mediated MAP kinase-signaling cascade,

leading to induction of epithelial cell transformation and proliferation. The data presented in this report show that the elevated IRS1 stability by LPIN1 enhanced the pathway from RAF1 through MEK1/2 to ERK1/2 and p90RSK. The silencing of LPIN1, and also the inhibition of LPIN1 activity by propranolol, markedly suppressed IGF-1-induced RAF1-MEKs-ERKs-p90RSK signaling pathway in SK-BR3 and MDA-MB231 cells. The phosphorylation of the ternary complex factor by the Ras-RAF1-MEKs-ERKs signal cascade might promote c-Fos expression and thereby stabilize the c-Fos/c-Jun heterodimer leading to expression of AP-1-regulated genesv (Sachsenmaier et al., 1994). Overexpression of the Fos and Jun proteins was found to correlate with a positive effect on neoplastic cell transformation (Clayton et al., 2000). The importance of c-Fos in tumor development has been supported in vivo, as the progression of chemically induced papillomas to invasive squamous cell carcinomas is impaired in c-Fos-deficient mice (Saez et al., 1995). In our results, LPIN1 silencing significantly inhibited *c-fos* and AP-1 transcriptional activity in SK-BR3 and MDA-MB231 cells. Also, inhibition of LPIN1 and IRS1 significantly suppressed the *c-fos* and AP-1 promoter activity induced by IGF-1 in both cells. These findings, couple with recently published work showing that LPIN1 controls main cellular processes involved in cancer progression (Brohee et al., 2015), indicate that LPIN1 may have an essential role in IGF-1-medicated tumor development in the breast. Interestingly, IRS1 levels were positively correlated with LPIN1 expression in breast cancer tissues.

These results define an LPIN1/IRS1 signaling axis that performs an oncogenic signal to promote aggressiveness in human breast cancer through the induction of RAF1 activity. Consistent with these observations, our results also showed that inhibition LPIN1 suppressed the mammary gland tumor development in BALB/c mice, a phenotype that correlates with the inhibition of the IRS1. These results implicate LPIN1 as an upstream regulatory protein for the IRS1 signaling pathway and further suggest that overexpression of LPIN1 may contribute to the elevated IRS1 function for mediating mitogenic effects found in breast cancer. Consistent with this hypothesis, we found that in a panel of breast cancer patients, there is a positive correlation between LPIN1 overexpresssion and the elevated expression of IRS1.

The findings that LPIN1 enhances IRS1 function offer an exciting mechanism to manipulate IRS1 activity in human diseases. To this end, it has been shown that reducing LPIN1 expression or inhibiting IRS1 function in cancer cells effectively suppresses tumorigenesis (Brohee et al., 2015; Tanaka et al., 1996). Our results indicate that oncogenic LPIN1 may be an important enzyme, which can physiological enhance IRS1 function. In addition to IRS1, we also identified that LPIN1 activates IGF-1-induced signaling pathway, leading to AP-1 activation and tumor progression in breast cancer. This indicates that LPIN1 might potentially regulate the proliferation of various cancer cells, allowing LPIN1 to a greater range of signaling pathways. However, many more studies are essential to gain a better understanding of the exact

role of LPIN1 to develop more specific therapeutic strategies for treating cancer. Nonetheless, these results taken together make LPIN1 an attractive therapeutic target, inhibition of which could potentially inhibit the IRS1 activity and its downstream to retard the growth of human breast cancer cells.

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ABSTRACT

Insight into the roles of interleukin-33 and LPIN1 in epithelial cell transformation and mammary tumorigenesis

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The tumor microenvironment is now recognized as an important participant of tumor progression and response to treatment. There is increasing interest in developing novel therapies targeting the microenvironment, particularly as it relates to invasive and metastatic progression. The changes of the tumor microenvironment have been closely correlated to cancer-mediated inflammation. In addition, obesity can result in an inflammatory environment that can contribute to tumorigenesis. It is now clear that breast cancer cells in the tumor microenvironment play an important role in cancer development. However, the role of the inflammatory cytokine interleukin-33 and LPIN1 in tumor growth and development of breast cancer remains unclear. In this paper, we propose that interleukin-33 and LPIN1 has a crucial role to

play in controlling tumorigenesis and epithelial cell proliferation in the breast. Cytokines of the interleukin-1 (IL-1) family, such as IL-1α/β and IL-18, have pleiotropic activities in innate and adaptive immune responses in host defense and diseases. Insight into their biological functions helped develop novel therapeutic approaches to treat human inflammatory diseases. IL-33 is an important member of the IL-1 family of cytokines and is a ligand of the ST2 receptor, a member of the IL-1 receptor family. However, the role of the IL-33/ST2 axis in tumor growth and metastasis of breast cancer remains unclear. Here, we demonstrate that is a critical tumor promoter during epithelial cell proliferation and tumorigenesis in the breast. IL-33 dose- and time-dependently increased Cancer Osaka Thyroid (COT) phosphorylation via ST2-COT interaction in normal epithelial and breast cancer cells. The IL-33/ST2/COT cascade induced the activation of the MEK-ERK, JNK-cJun, and STAT3 signaling pathways, followed by increased AP-1 and *stat3* transcriptional activity. When small interfering RNAs of ST2 and COT were introduced into cells, IL-33-induced AP-1 and *stat3* activity were significantly decreased, unlike that in the control cells. The inhibition of COT activity resulted in decreased IL-33-induced epithelial cell transformation, and knockdown of IL-33, ST2 and COT in breast cancer cells attenuated tumorigenicity of breast cancer cells. Consistent with these observations, ST2 levels were positively correlated with COT expression in human breast cancer. Here, we demonstrate that LPIN1 promotes epithelial cell

transformation and tumorigenesis in breast and LPIN1 colocalizes and interacts with IRS1. In addition, LPIN1 overexpression in SK-BR3 and MDA-MB231 cells increased endogenous IRS1 levels by IGF-1. In contrast, silencing of LPIN1 attenuated the IRS1 levels increased by IGF-1. IRS1 mRNA level was not affected by knockdown of LPIN1. In contrast, overexpression of LPIN1 increases the half-life of IRS1 by cycloheximide. These results indicate that LPIN1 might be associated with IRS1 stability. Therefore, LPIN1 overexpression inhibited the ubiquitination of IRS1 that LPIN1 promotes IRS1 stability via inhibition of its ubiquitination. In addition, the lysosomal inhibition by chloroquine substantially enhanced the elevated IRS1 levels by LPIN1 overexpression. These results indicate that LPIN1 inhibits IRS1 degradation by the proteasomal and lysosomal pathways. Moreovere, LPIN1 contribute to the regulation of RAF1 and MAPK signaling cascade. To determine the regulatory role of LPIN1 on the phosphorylation of RAF1 and its downstream kinases, FLAG-LPIN1 overexpressing SK-BR3 and MDA-MB231 cells were exposed to IGF-1. The levels of RAF1, MEK1/2, ERK1/2, and p90RSK phosphorylation in LPIN1 overexpressing cells were enhanced compared with the control cells. In contrast, we observed the downregulated RAF1, MEK, ERK, p90RSK signaling cascade in human LPIN1-silenced cells. Propranolol and NT157 decreased the IGF-1-induced phosphorylation of RAF1 as well as its downstream kinases. In addition, propranolol and NT157 significantly inhibited the IGF-1-promoted *c-fos* and AP-1 promoter

activity. Moreover, demonstrated that a profound reduction in the weight and volume of tumors was mediated by treatment of propranolol and NT157, compared with only IGF-1-promoted tumor. Thus, LPIN1 promotes breast tumorigenesis via upregulation of IRS1 stability. In conclusion this study, we demonstrate that role of the interleukin-33 and LPIN1 in supporting cancer-associated in the tumor microenvironment.