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A Doctoral Thesis

Ubiquitylation of Src promotes secretion of Src via small extracellular vesicles to suppress its oncogenic potential

(Src のユビキチン化はその発がん能を抑制するために細胞外小胞による分泌を促進する)

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Contents

Abstract

Upregulation of Src tyrosine kinase has been implicated in the progression of cancer malignancy. To suppress the oncogenic potential, Src has multiple negative-regulation systems including degradation via the ubiquitin-proteasome pathway. Here, we show that ubiquitylation of Src is also involved in promoting secretion of Src via small extracellular vesicles (sEV) to suppress its oncogenic potential. In MDCK cells, activated Src was transported from plasma membrane to late endosomes/lysosomes and was eventually secreted via sEV by promoting that secretion. Inhibition of global ubiquitylation and ablation of E3 ligases for Src attenuated its secretion, indicating that ubiquitylation of Src is involved in these processes. Activated Src was ubiquitylated at multiple sites, among which Lys429 was identified as a critical site required for promotion of sEV secretion. A point mutant of Src was resistant to ubiquitylation and had a lower ability to promote sEV secretion, although it had a kinase activity comparable with that of wild type. Activated mutant was transported to late endosomes/lysosomes like wild type, while the efficiency of its incorporation into intraluminal vesicles was significantly reduced. Furthermore, activation of the mutant potentiated the Src-induced invasive phenotypes, i.e., invadopodia formation, production of matrix metalloproteases, and in vitro invasive activity. Together, these findings suggest that ubiquitylation of activated Src at Lys429 promotes secretion of Src via sEV, providing a new avenue to suppress the oncogenic potential of upregulated Src.

General introduction

SRC was identified in viral genome of Rous sarcoma virus as *v-src*, and the cellular counterpart *c-SRC* was discovered as first proto-oncogene in vertebrates. The gene encodes membrane-anchored tyrosine kinase that plays pivotal roles in coordinating a variety of cellular processes upon extracellular cues.

Src is a member of Src family kinases that exhibit conserved domain structure, which consists of fatty acid modified N-terminal region, followed by Src homology 3 (SH3), SH2 and tyrosine kinase domain [1]. The N-terminal glycine residue is modified with myristoylation, which facilitates the anchoring to the membrane; SH3 domain controls protein interactions through recognizing proline rich motif; SH2 domain recognizes phosphotyrosine. Src activity is regulated by intramolecular interactions via phosphorylations at Tyr418 in kinase domain (in mouse) and Tyr529 in C-terminal tail, whose modification is performed by C-terminal Src kinase Csk [2]-[4]. While Src is on inactive status, phosphorylated Tyr529 binds to its SH2 domain, and the linker between SH2 domain and kinase domain also binds to SH3 domain, resulting in closed conformation. Conversely, when dephosphorylated by phosphatases, the removal of these interactions allows Src to catalyze the autophosphorylation at Tyr418 in the activation loop, which keeps the catalytic pocket open up and leads to interactions with the substrates.

Other mechanisms of Src regulation include ubiquitin proteasome pathway. Martin lab. and Howley lab. have shown that active Src is less stable and is stabilized by proteasome inhibitors, and poly-ubiquitinated forms are detected [5][6]. In part, Cbl E3 ligase is responsible for the modification, and Cbl itself is also ubiquitinated, leading to the degradation of the complex by the proteasome [7].

Src deficient mice mostly die within the first weeks, and their survival exhibit the osteopetrosis, a bone remodeling disease in which excess bone accumulates as a result of defective activity of osteoclasts, which fail to form ruffled border, the area where they contact and resorb the bone [8][9]. Though Src protein levels are the highest in platelets, deficient mice have no bleeding symptoms and the normal number of platelets. In contrast, a gain-of-function mutation E527K substitution (in human) causes thrombocytopenia, myelofibrosis, bleeding and bone pathologies [10].

In a number of human cancers, including gastrointestinal, breast, ovarian and lung

cancers, the upregulation and activation of Src have been reported although genetic alteration is not predominant mechanism. In colon cancers, genetic abnormalities are sequentially accumulated during neoplastic progression from adjacent normal mucosa, to malignant polyps or adenomas, and to invasive carcinomas. The elevation of Src expression is correlated with the progression of colon cancer. The increases in Src activity are observed in malignant polyps and the highest level in metastases relative to the corresponding primary tumors [1][11]. Under the appropriate circumstances, Src plays pivotal roles in coordinating a variety of cellular processes such as motility, proliferation, survival and immune response. Therefore, the disregulation in cancer cells, activating the downstream pathways constitutively, enhances the tumorigenesis and invasive or metastatic potential. According to the extended findings, Src is considered as therapeutic target, and Src inhibitors have been developed and undergoing clinical trials [12][13]. Dasatinib, bosutinib and ponatinib are FDA-approved for CML, stands for chronic myeloid leukemia, and Ph+ALL, philadelphia positive acute lymphoblastic leukemia, and though so far the efficacies of single agent have been limited in solid tumors, combination strategies have been conducted given the cooperative roles of Src.

Exosomes are one of the extracellular vesicles, approximately 30 - 150 nm in diameter, mediators of intercellular communications, and released as a consequence of the fusion of multivesicular bodies with the plasma membrane, corresponding to intraluminal vesicles.

 The term exosome was defined as vesicles secreted from cultured cells and containing the ectoenzyme nucleotidase [14]. Subsequently, the term was adopted to refer to vesicles released by reticulocytes during maturation to eliminate transferrin receptor [15]. Matured endosomes multivesicular bodies (MVB) contain intraluminal vesicles, formed by inward budding of endosomal membrane. Upon the fusion of MVB with the plasma membrane, intraluminal vesicles are secreted into extracellular environment and came to be called exosomes. This study firstly reported that exosomes are involved in this vesicular trafficking. As they described, exosomes were thought to be as a means to discard unnecessary molecules, however, it was suggested that exosomes function as mediators of intercellular communications. Raposo et al. showed that the exosomes derived from B lymphocytes are able to stimulate CD4^{+} T cell proliferation by presenting MHC class II-antigen complexes in vitro [16]; Zitvogel et al. showed that the

exosomes derived from dendritic cells induce antitumoral immune responses in vivo [17]. Nowadays, it has been considered that exosomes are secreted from various cells, playing a physiological and pathological role, and circulate in any body fluids, such as blood, urine, saliva, breast milk and semen [18].

Though preserving the same topological orientation as the donor cells, exosomes are not mere imitations, nor have different features and compositions. Exosomes bear lipids, nucleic acids and proteins. Lipid bilayer of exosomes consists of the enrichment of phosphatidylserine, ceramide, cholesterol, sphingomyelin and ganglioside GM3 comparable to whole cell membranes [19][20]. Of note, phosphatidylserines are assumed to be exposed in the outer leaflet of exosomes, whereas the existence in the inner leaflet of the plasma membrane is observed in live cells [21][22]. Exosomes contain mRNA and small noncoding RNA, such as micro RNA, vault RNA and Y RNA, and transfer them to other cells. This intercellular transfer was reported in the study showing that exosomes, secreted from mast cells, contain mRNA and miRNA, and are transferable to other mast cells, in which transferred mRNAs are translated [23]. Following this first description, numerous studies have devoted to demonstrations of functional RNA transfer among distant cells.

The process of exosome biogenesis is roughly divided into three phases: intraluminal vesicle formation, the transport of MVB and the fusion between MVB and the plasma membrane. Intraluminal vesicles are generated on the circumference of endosomes through inward budding and subsequent fission by some machinery. Endosomal sorting complex required for transport (ESCRT) is composed of four complexes, known as ESCRT-0, -I, -II and -III, and involved in membrane rearrangement. ESCRT-0 and -I complexes recognize and segregate ubiquitinated cargoes on endosomes, to which ESCRT-III is recruited via ESCRT-II, and ESCRT-III drives budding and fission, finally, Vps4 dissociates the complexes, thus sequestering cargoes in intraluminal vesicles [24][25]. The sphingolipid ceramide triggers budding of intraluminal vesicles into MVB [26]. Ceramide is hydrolysed to sphingomyelin and phophocholine by neutral sphingomyelinase 2 (nSMase2) [27], and incudes the merging of microdomains into larger domains, in which promotes the budding [28]. In addition, sphingosin 1 phosphate, motabolized from ceramide, activates its receptor S1PR that mediates the maturation of MVB [29]. Tetraspanin family, including CD9, CD63, CD81 and CD82, regulates intraluminal vesicle formation [30]-[31]. Tetraspanins gather together with

tetraspanins themselves and selected transmembrane proteins, such as integrins and immunoglobulin superfamily receptors, to form tetraspanin-enriched microdomains, where budding is induced [32]. Second, intracellular transport of organelles is associated with the cytoskeleton, motors and small GTPases [33]. Both Rab27a and Rab27b isoforms were highlighted in Rabs screening on exosome secretion [34]. Silencing Rab27b results in CD63 positive endosomes clustering in perinuclear area, suggesting that Rab27b mediates MVB transfer from microtubules to actin-rich cortex, and scilencing Rab27a induces the increases in MVB size due to the lack of motor linkage. Besides, Rab35 was identified in GAPs overexpression based screening [35]. At final step, tethering and docking processes might be provided by SNAREs, intertwining opposing membranes to fuse together [36]–[39]. Though it should be thoroughly examined whether SNAREs are associated with exosome secretion [40], the uptake of calcium triggers to promote exosome secretion by activating calcium sensors, subunits of SNARE complex [38][43].

A group of neurodegenerative diseases classified in transmissible spongiform encephalopathy is caused by the aggregation of specific misfolded proteins. The pathogenic aggregation-prone proteins of Alzheimer's disease, Creutzfeldt-Jakob disease and Parkinson's disease are contained in exosomes, circulating in cerebrospinal fluid [42]–[45]. Given that the transmission is associated with exosomes, age-related impairment of protein quality control promotes or otherwise suppresses the transcellular flux, and exosomes provide the environment with infectious property [46]–[48].

Exosomes are involved in the cancer, which affecting in either promotive or suppressive manner [18][49]. Exosomes secreted from tumors deliver tumor antigen presenting molecules, MHC class I or heat shock proteins, to dendritic cells for cross presentation to cytotoxic T lymphocytes [50]. HSP70 exposed on exosomal surface stimulates lytic activity of natural killer cells through the release of granzyme B [51], however, NKG2D, an activating receptor for NK cells and CTL, is downregulated by exosomes expressing its ligand and TGF beta, resulting in the attenuation of lymphocytic killing function [52]. Aside from immune system, exosomes secreted from brain metastatic breast cancer trigger the destruction of blood brain barrier via activating actin reorganizer cofilin by miRNA in endothelial cells [53]. Exosomes are associated with metastatic organotropism, for instance, exosomes from liver-tropic tumor cells are preferentially uptaken by Kupffer cells in predictably destined organ,

wherein prepare the premetastatic niche through inducing proinflammatory S100 expression [54].

Thus, exosomes have numerous potential for clinical application. It is attempted to bioengineering use of exosomes as vectors for delivery of compounds, interfering RNA and proteins, with advantages of exosomal properties: the compatibility with immune responses and the tissue directivity. In another emerging trial, exosomes are used as biomarkers for assessing risk of tumor progression and neurodegenerative diseases [55]. Exosomes have high potential for therapeutic and diagnostic field though, it remains the lack of understanding in depth, and further investigations are required.

Introduction

Src is a non-receptor tyrosine kinase that was identified as the first proto-oncogene product [1]. In normal cells, Src functions as a molecular signaling switch, playing a pivotal role in the regulation of cell proliferation, differentiation, adhesion, and migration [2]. Src is ordinarily present as an inactive form that is phosphorylated at the C-terminal negative regulatory site by another tyrosine kinase Csk [3-6]. Upon stimulation with extracellular signals, such as growth factors and extracellular matrices, Src is activated via interaction with several activating adaptor proteins, such as FAK and Cas [7, 8], resulting in the activation of downstream signaling pathways. In cancer cells, the expression and activity of Src is frequently upregulated; e.g., Src is upregulated in more than one-fourth of colorectal cancers (COSMIC database). Increased Src activity causes constitutive activation of downstream signals implicated in malignant progression, i.e., cancer invasion/metastasis and relapse [9]. However, unlike Ras, that is frequently mutated in cancer cells and acts as a cancer driver, no significant somatic mutations of Src is detected in any type of human tumors [10, 11]. This intriguing fact raises the possibility that Src mutated cells might be rather eliminated by strong suppressive pressures, although the underlying mechanisms are not thoroughly understood.

The activity and function of Src is also regulated via its intracellular localization. Previous studies showed that inactive Src is localized to peri-nuclear region and activated Src is translocated to the plasma membrane [12], and that Src is transported via the endosomal system, particularly recycling endosomes, in a manner dependent on a specific Rho GTPase [13, 14]. Furthermore, Src transport is regulated by late endosomes and lysosomes (macropinosomes) [15, 16], and Src translocation from the plasma membrane to lysosomes is regulated by the ESCRT complex, which is required for transport of endocytic cargo to multivesicular bodies (MVB) [17, 18]. From these findings, it is hypothesized that activated Src is transported from the plasma membrane to MVB and finally to lysosomes for degradation in an ESCRT-dependent manner [19]. On the other hand, it is also known that activated Src is ubiquitylated and subjected to degradation via the proteasome pathway [20-22]. Since the ESCRT complex recognizes ubiquitylated cargoes [23], it is likely that activated Src is downregulated through degradation by either lysosomes or proteasomes, although the functional difference

between these two Src suppressive pathways remains elusive.

Recently, it was found that Src is incorporated into exosomes that are secreted from cells via MVB [24-26], and also that activation of Src promotes exosome secretion and contribute to cell-cell communication and tumor progression [27-29]. These observations suggest that the trafficking of Src from endosomes to exosomes via MVB has crucial roles in the regulation of oncogenic ability of Src, but the underlying mechanisms and physiological relevance are yet to be elucidated.

In this study, we addressed the role of endosomal trafficking of Src in the regulation of its oncogenic potential by employing normal epithelial Madin-Darby Canine Kidney (MDCK) cells expressing Src-MER construct that can be activated by hydroxytamoxifen (4OHT). We here show that ubiquitylation of a specific site of activated Src is required for promoting exosome secretion, and the excretion of activated Src via exosomes has a suppressive role for the oncogenic potential of Src. These findings provide a missing link between Src ubiquitylation and exosome secretion, and suggest a tumor suppressive role for exosome secretion.

Results

Activated Src is transported from plasma membrane to late endosomes/lysosomes

To investigate the intracellular behavior of activated Src, we used normal epithelial MDCK cells expressing Src-MER, a Src protein fused to a modified estrogen receptor, that can be activated by treatment with 4OHT [30]. The treatment of MDCK cells with 4OHT induced activation of Src-MER as indicated by increase in 4G10, anti-phosphotyrosine, and p-Src (Y418) signals (Fig. 1). Immunofluorescence analysis for Src-MER in the cells revealed that inactive Src was localized to small granules at perinuclear region, while, upon activation, Src was translocated to the plasma membrane at the early stages and then returned to perinuclear organelles at the later stages (Fig. 2). To identify the organelles to which activated Src was transported, we examined the colocalization between activated Src and organelle markers such as Sec61b (endoplasmic reticulum), Cox4 (mitochondria), TGN38 (trans Golgi body), Rab7 (late endosome), and Lamp2 (lysosome). Results showed that activated Src was accumulated at the circumference of the late endosomes/lysosomes (Fig. 2). These results were consistent with the previous observations of the intracellular localization of Src [12-16], indicating that our MDCK-Src-MER system is useful for further analysis of the fate of activated Src.

Activation of Src promotes sEV secretion

The accumulation of activated Src in the late endosomes/lysosomes suggests that the activated Src is processed via the endosomal system. Recent studies showed that activated Src is secreted from cells as a cargo of exosomes [24-29]. Thus, we examined if this is indeed the case even under our conditions. To this end, small extracellular vesicles (sEV) were collected by ultracentrifugation from the culture media of Src activated cells. Western blot analysis of EV revealed that Src was concentrated in EV in a manner dependent on the Src activity (Fig. 3). Nanoparticle Tracking Analysis (NTA) also showed that the number of particles with diameter around 100 nm was increased in parallel with the Src activity (Fig. 3). To determine if EV are indeed exosomes, we performed an immunoelectron microscopy analysis. It was observed that Src was incorporated in intraluminal vesicles (ILV). Furthermore, secretion of Src-containing EV was robustly suppressed by the ablation of Rab27b, a critical factor for exosome

secretion [31], and by the treatment with an inhibitor of nSMase2 that promotes exosome secretion [32] (Fig. 4). These observations corroborate that activation of Src promotes secretion of exosome-like vesicles that contain Src itself.

Ubiquitylation of Src is required for secretion of Src via sEV

Although it became evident that activated Src is secreted via sEV, the molecular mechanism underlying how activated Src is sorted into sEV remains elusive. A number of studies showed that various post-translational modifications, including ubiquitylation, phosphorylation, acetylation and glycosylation, are involved in protein sorting to ILV on the surface of the MVB [33]. Since it is established that activated Src is ubiquitylated [20-22], we first examined the roles of ubiquitylation in secretion of Src. Inhibition of E1 activating enzyme with PYR41 suppressed the secretion of Src-containing sEV (Fig. 5). Genetic ablation of E3 ligases that act on Src, including c-Cbl [34], Lnx1 [35] and Stub1 [36], also attenuated the secretion of Src (Fig. 6). These findings suggest that ubiquitylation of Src contributes to its secretion. We then detected ubiquitylation of Src by coexpressing HA-tagged ubiquitin in 293T cells (Fig. 7B). Immunoprecipitation assay revealed that activation of Src promoted poly- and mono-ubiquitylation of Src proteins. LC-MS/MS-based diGly analysis of ubiquitylated Src identified eight Lys residues as potential ubiquitylation sites (Fig. 7A). Thus, we generated mutant Src having a Lys to Arg substitution at the ubiquitylation site, and examined their ubiquitylation status (Fig. 7B) and effects on their secretion (Fig. 7C). Results showed that the substitution at Lys429 to Arg decreased the level of ubiquitylation to that of mutant Src having substitutions at all the potential sites (8R), and that reversion of Lys429 to 8R (7R) restored the level of ubiquitylation. Consistent with the levels of ubiquitylation, 8R and R429 mutants failed to promote their secretion. These findings suggest that Lys429 is one of the major ubiquitylation sites in activated Src, and that ubiquitylation at Lys429 is required for promotion of Src secretion.

Ubiquitylation at Lys429 is required for secretion of Src and promoting sEV secretion

To further evaluate the role of ubiquitylation at Lys429 in sEV secretion, we examined the effects of R429 mutation on the kinase activity of Src. Western blot analysis using anti-4G10 showed that R429 mutant has almost equivalent activity and substrate

specificity to those of wide-type Src (Fig. 8), although R429 mutation suppressed the secretion of Src and sEV secretion (Fig. 8). However, we showed that activation of Src was important for its secretion (Fig. 3). These seemingly conflicting observations could be explained by the fact that the activation and subsequent autophosphorylation of Src triggers ubiquitylation of itself by the specific E3 ligases [34]. Therefore, it is likely that ubiquitylation of Src as a consequence of its autoactivation is critical for promoting its secretion.

  To elucidate the role of Lys429 ubiquitylation in the processes of Src secretion, we dissected the intracellular localization of R429 mutant. Immunofluorescent analysis showed that activated R429 was accumulated at the circumference of the late endosomes/lysosomes in a manner similar to wild-type Src (Fig. 9A), indicating that R429 can be normally transported through the endosomal system up to the late endosomes/lysosomes. Therefore, we hypothesized that R429 mutation might affect the loading into ILV, which is mediated by ESCRT proteins that recognize ubiquitylated cargoes [23]. To verify this possibility, we performed an immunoelectron microscopy analysis of MVB (Fig. 9B). Although R429 mutation seemed to somehow attenuate the incorporation of Src into ILV, it was difficult to evaluate the results quantitatively. It is known that overexpression of constitutively active Rab5 prevents early/late endosomes from segregating, resulting in formation of enlarged endosomes that contain ILV [37]. Thus, we used this system to assess the ability of incorporation. In cells expressing wild-type Src, Rab5-positive ILV in enlarged endosomes are frequently colocalized with Src, while the efficiency of colocalization was significantly decreased in R429 mutant expressing cells (Fig. 10). These results suggest that ubiquitylation at Lys429 is involved in sorting of Src.

Impaired Src secretion promotes Src-induced invasive phenotypes
Finally, to address the pathophysiological relevance of Src secretion via sEV, we examined the effects of R429 mutation on Src-induced invasive phenotypes. Src activation induces formation of invadopodia/podosomes, which is an actin-rich membrane protrusion implicated in cell invasion [30]. Immunofluorescent analysis for actin assembly and cortactin mediator showed that formation of invadopodia/ podosomes was significantly enhanced in cells expressing R429 mutant (Fig. 11). Gene expression of matrix metalloproteases (MMP) is also upregulated by Src activation.

qPCR analysis revealed that activation of R429 mutant selectively enhanced the induction of MMP2/9/13 (Fig. 12). Consistent with these cellular events, activation of R429 mutant more strongly promoted in vitro invasive activity, compared with wild-type Src (Fig. 13). These results demonstrate that R429 mutant can more potently induce invasive phenotypes than wild-type Src, and in turn suggest that excretion of activated Src via sEV might function to suppress the oncogenic ability of activated Src.

Discussion

In this study, we showed that ubiquitylation of activated Src at Lys429 promoted secretion of Src via sEV, and that the mutation at Lys429 enhanced Src-induced invasive phenotypes. These findings suggest that the ubiquitylation-mediated excretion of activated Src might function to suppress oncogenic potential of Src.

Previous study showed that activated Src is ubiquitylated by E3 ligase such as c-Cbl, and degraded by proteasomes in some cell types [20, 21]. However, in MDCK cells used in this study, ubiquitylated Src was not accumulated by the inhibition of proteasomes with MG132, indicating that ubiquitylated Src was not efficiently degraded by proteasomes. Thus, it is possible that ubiquitylation of Src preferentially promotes secretion of Src via sEV to downregulate activated Src in this cell type. Although the mechanisms by which these differential fates of ubiquitylated Src are determined remain unknown, it is likely that Src is regulated in a cell context-dependent manner. On the other hand, Src has been identified in exosomes from various normal and cancer cells, such as colorectal cancer [26], prostate cancer and breast cancer [27,29], indicating that secretion of Src via exosomes may be used to regulate Src in a wide array of cell types.

This is the first report for the identification of ubiquitylation site on Src protein. We identified Lys429, which is located near autophosphorylation site Tyr418 in the kinase domain, as a critical ubiquitylation site required for promotion of sEV secretion. Based on the molecular sizes of ubiquitylated Src, Lys429 appeared to be both mono- and poly-ubiquitylated, although it remains to be determined which type of ubiquitylation is functional in this case. Genetic ablation of E3 ligases for Src attenuated secretion of Src. Among them, c-Cbl ablation showed most prominent effects, suggesting that c-Cbl is a responsible E3 ligase for promotion of Src secretion. We found that sEV secretion was dependent on the kinase activity of Src, while R429 mutant, which has kinase activity almost comparable with that of wild-type Src, was much less effective for promoting sEV secretion. This indicates that tyrosine kinase activity may not be directly involved in the promotion of sEV secretion, but plays a triggering role for ubiquitylation by providing autophosphorylated tyrosine residue Tyr418 that is recognized by SH2 domain of E3 ligase such as c-Cbl [34]. Therefore, it is likely that Src-induced promotion of sEV secretion is dependent on ubiquitylation status, which is determined

by autophosphorylation activity of Src.

We observed that R429 mutant was normally transported to the circumference of the late endosomes/lysosomes, but it showed defects in incorporation into ILV. This suggests that ubiquitylated Src is recognized by some components of ESCRT complex to facilitate formation of ILV. Since R429 mutant can be targeted to the late endosomes/lysosomes, it is possible that ubiquitylated Src is specifically recognized by the components of ESCRT-I on the surface of MVB, which have ubiqutin-binding domain and are incorporated into ILV, such as Tsg101 and Vps28 [23]. To elucidate the sorting mechanisms of ubiquitylated Src on the surface of MVB, identification of the binding partners of ubiquitylated Src is now under progress.

Upregulation of Src induces cell transformation in various cell types [2, 9]. Particularly, activation of downstream pathways of Src promotes cytoskeletal remodeling, invadopodia formation, expression of MMPs, resulting in activation of invasive and metastatic properties even in non-cancerous MDCK cells [30]. In this study, we found that activation of R429 mutant, which is defective for its secretion, enhanced Src-induced formation of invadopodia, MMP production and in vitro invasive activity. These results indicate that the function of Src is potentiated when the secretion via sEV is repressed, and in other words that excretion of activated Src via sEV has a suppressive role for Src-induced cell transformation. However, no apparent difference was detected in the activity and protein level of wild-type and R429 when both types of Src are fully activated. This suggests that the termination of initial activation of Src by ubiquitylation may be delayed in R429 expressing cells, thereby persisting the transforming signals. Alternatively, it is also possible that R429-induced suppression of ILV formation may affect the trafficking of late endosomes/lysosomes which is involved in integrin-mediated cell migration and invasion [38]. Further analysis of R429 expressing cells will be necessary to exemplify these possibilities.

In conclusion, we show that ubiquitylation of Src at Lys429 promotes excretion of activated Src via sEV to suppress its oncogenic potential. Our finding may provide new insights into the molecular mechanisms for suppressing oncogenic function of Src that is frequently upregulated in various human cancers.

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Materials and Methods

Cell culture and gene transfer

Cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) FBS (Gibco), 1.5g/l sodium bicarbonate, 4mM glutamine, 100U/ml penicillin and 100ug/ml streptomycin in 5% CO_2 atmosphere. Src-MER was constructed by fusing modified oestrogen receptor (MER, amino acids 281–599) to its C-terminus and subcloned into the pCX4 retroviral plasmid (generously donated by Dr. Akagi) [39] as described previously [30]. Src-MER mutants, including 8R, 7R, R429, were generated by PCR using Src-MER as the template. CD63-GFP, mCherry-SEC61B, COX4-mCherry and TGOLN2-mCherry were generated by subcloning each cDNA into pEGFP-N1, pmCherry-C1, pmCherry-N1, pmCherry-N1 plasmid (Clontech) respectively. HA-octameric ubiquitin was subcloned into pBlueScript SK(-). All constructs were confirmed by sequencing. Gene transfer was carried out by retroviral infection, and cells stably expressing the constructs at appropriate levels were cloned. Retroviral production and infection were performed as described previously [40]. Transient transfection into HEK293T cells was performed using PEI max (Polyscience).

Antibodies and inhibitors

The following primary antibodies were used in this study: anti-phosphotyrosine (4G10, 05-1050, Millipore), anti-ERα (MC-20, sc-542, Santa Cruz Biotechnology), anti-GAPDH (6C5, sc-32233, Santa Cruz Biotechnology), anti-p-Src (Y418) (44-655G, Thermo Fisher Scientific), anti-Histone (MAB052, Chemicon), anti-Rab7 (D95F2, 9367s, CST), anti-Lamp2 (AC17, MA5-16561, Thermo Fisher Scientific), anti-Src (Ab-1, OP07, Millipore), anti-GFP (G10362, Thermo Fisher Scientific), anti-TSG101 (4A10, GTX70255, GeneTex), anti-HA-tag (C29-F4, 3724s, CST), anti-cortactin (4F11, 05-180, Millipore), anti-ubiquitin (P4D1, sc-8017, Santa Cruz Biotechnology). The following inhibitors were used in this study: GW4869 was purchased from Sigma-Aldrich, and PYR41 was from RandD, and PR619 was from LifeSensor, MG132 was from ChemScene.

Western blotting

Cells were lysed with lysis buffer (20mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA,

1% NP40, 5% glycerol, 2% n-octyl-beta-D-glucoside, 20mM sodium fluoride, 1mM sodium orthovanadate and protease inhibitor cocktail) and cleared from nuclei by centrifugation at 15,000 xg for 10 min. Protein extracts were separated on SDS-PAGE and blotted on Immobilon PVDF membrane (Merck). HRP-conjugated secondary antibodies were revealed with Chemei-Lumi One (nacalai tesque) using WSE6200H LuminoGraph 2 (ATTO). In immunoprecipitation assay, cell lysate, prepared as described above, was incubated with antibody and protein G-conjugated sepharose beads under rotation. The mixture was centrifuged at 3,000 xg to remove supernatant, and rinsed with Tris buffered saline repeatedly. The precipitant was eluted in SDS buffer. For ubiquitylation assay, 30ug/ml deubiquitylation inhibitor PR619 was added in lysis buffer.

CRISPR/Cas9-based gene knockout

Target-gRNA containing the pSilencer1.0-U6 plasmid, Cas9 and the eGFP co-expressing plasmid (pMJ920; Addgene) were transfected into MDCK cells with MDCK Cell Avalanche Transfection Reagent according to the manufacturer's protocol (EZ Bioscience). GFP-positive single cells were isolated with a FACSAria III Sorter (BD Biosciences). Knockout of the genes was confirmed by immunoblotting or genomic sequencing. The sequence of the gRNA and the genomic sequence are listed in **Table S1.**

Quantitative real-time PCR

RNAs were isolated from cells with Sepasol-RNA I Super G (nacalai tesque) and were reverse transcribed with ReverTra Ace qPCR RT Master Mix (TOYOBO). PCR was performed with SYBR Green Realtime PCR Master Mix (TOYOBO) on ABI QuantStudio5 (Thermo Fisher). The primers used in this analysis are listed in **Table S2**.

Immunofluorescent microscopy

Cells were seeded on glass coverslips, fixed in 4% paraformaldehyde, permeabilized in PBS with 0.05% triton-X100 and incubated with primary antibodies. Sequentially incubated with alexa-conjugated secondary antibodies and immersed in Prolong Gold antifade mountant (Invitrogen). Subjected to observation using laser scanning confocal microscope Olympus FluoView FV1000.

Immunoelectron microscopy

Cells were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PB (pH 7.4) and 1% osmium tetraoxide and embedded in epoxy resin mixture as in conventional TEM. Ultrathin sections were cut from epoxy-embedded cells, mounted on nickel grids, and air-dried. Ultrathin sections were etched briefly with saturated sodium metaperiodate in distilled water, followed by exposure to 0.1 N HCl. The sections were then washed in filtered, deionized water and incubated in 1% BSA in PBS. After incubation with anti-ERa (MC-20, Santa cruz) primary antibody, sections were washed several times in Tris-HCl buffer containing 0.5% Tween 20 and incubated with biotin-conjugated anti-rabbit secondary antibody (ab6720). After incubation with 10-nm, gold-conjugated streptavidin (CRL-1121-00), grids were washed briefly stained with uracil acetate and lead citrate by standard methods. All samples were observed by TEM (Hitachi).

Invasion assay

BioCoat Matrigel Invasion Chambers (BD Biosciences) were used for the invasion assay. Cells (1×10^5) were seeded on inserts and moved into chambers containing DMEM containing 10% FBS. After incubation at 37°C for 24 h, invaded cells were fixed with 4% paraformaldehyde and then stained with 1% crystal violet. Invaded cells were counted on micrographs. Same experiments were repeated at least three times.

sEV isolation

Cell culture conditioned media were cleared by centrifugation at 10,000 xg for 30 min and filtered through 0.22um filters. Clarified media were centrifuged at 100,000 xg for 70 min and the precipitates were rinsed with PBS by centrifugation in the same condition. The resulting sEV pellets resuspended in PBS. The size distribution and concentration were determined by nanoparticle tracking analysis on NanoSight LM10.

Statistics and reproducibility

For data analyses, unpaired two-tailed *t*-tests were used to determine the *P*-values. A *P*-value less than 0.05 was considered to be significant. All data and statistics were derived from at least three independent experiments.

4OHT - 1 3 6 12 24   - 1 3 6 12 24 (hr)

(kDa)

SrcMER —

SFK —

119

61

48

37

4G10   p-Src (Y418)

ERα   GAPDH
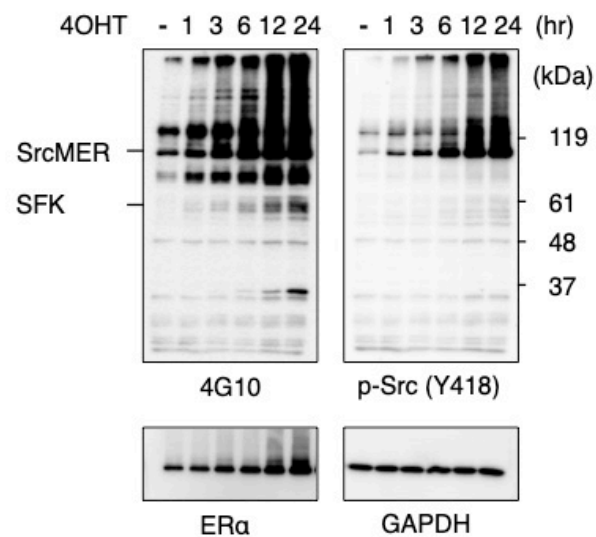
Figure 1
Time course of Src-MER activation by 4OHT.
MDCK cells expressing Src-MER were incubated
with 4OHT for the indicated time, and total cell
lysates (TCL) were analyzed by immunoblotting
with the indicated antibodies.

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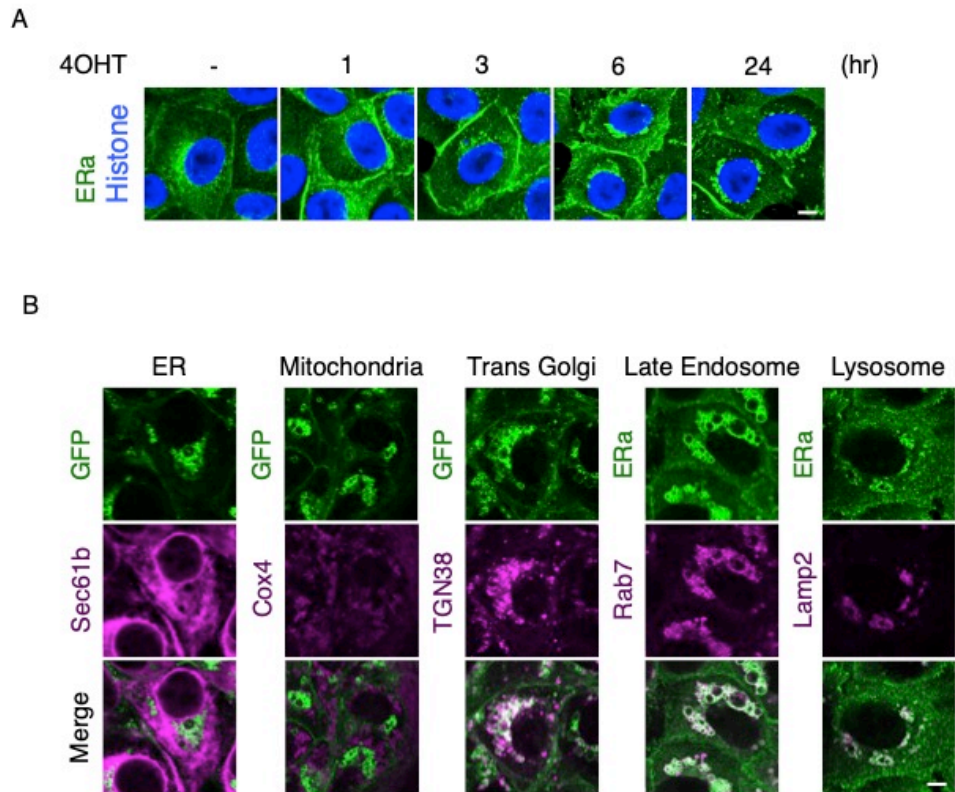
Figure 2

(A) Changes in intracellular localization of activated Src-MER. MDCK cells expressing Src-MER were treated for the indicated time and stained with anti-ERa for Src-MER and anti-Histone. Merged images are shown. Scale bars, 5um.

(B) Accumulation of activated Src-MER at late endosomes/lysosomes. For ER, mitochondria and trans golgi, MDCK cells co-expressing Src-MER-GFP and the indicated organelle markers fused to mRFP or mCherry were stimulated with 4OHT for 24 hr, and fluorescence images are shown. For late endosomes/lysosomes, MDCK cells expressing Src-MER were stimulated with 4OHT for 24 hr, and analyzed by immunofluorescence staining with the indicated antibodies. Scale bars, 5um.
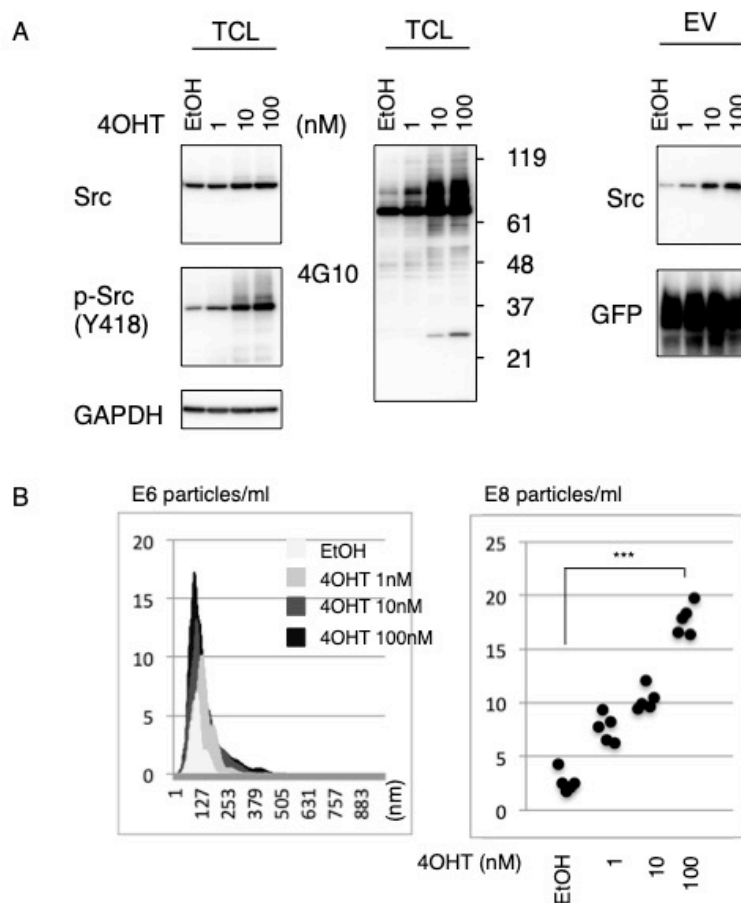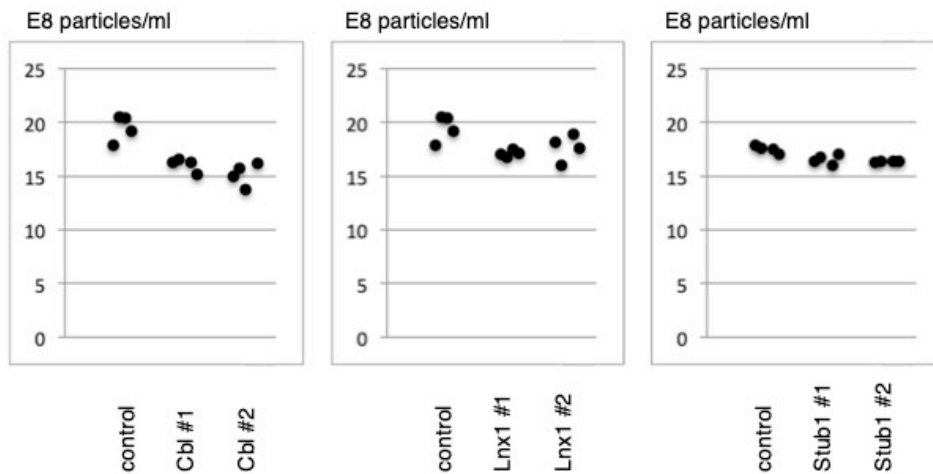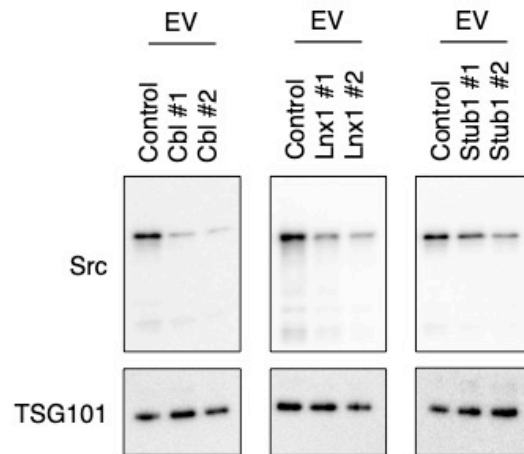
Figure 3
(A) Incorporation of Src in EV by activation of Src-MER with 4OHT. MDCK cells expressing Src-MER and CD63-GFP, a marker of exosome, were treated with 4OHT at the indicated concentrations, and the total cell lysates (TCL) were analyzed by immunoblotting with the indicated antibodies (left and middle panels). EV were collected by ultracentrifugation from the culture media and analyzed by immunoblotting with the indicated antibodies.
(B) Size and the number of EV secreted by Src activation. EV collected as above were analyzed by NTA (left panel). Quantitative data are also shown (right panel). ***P<0.005
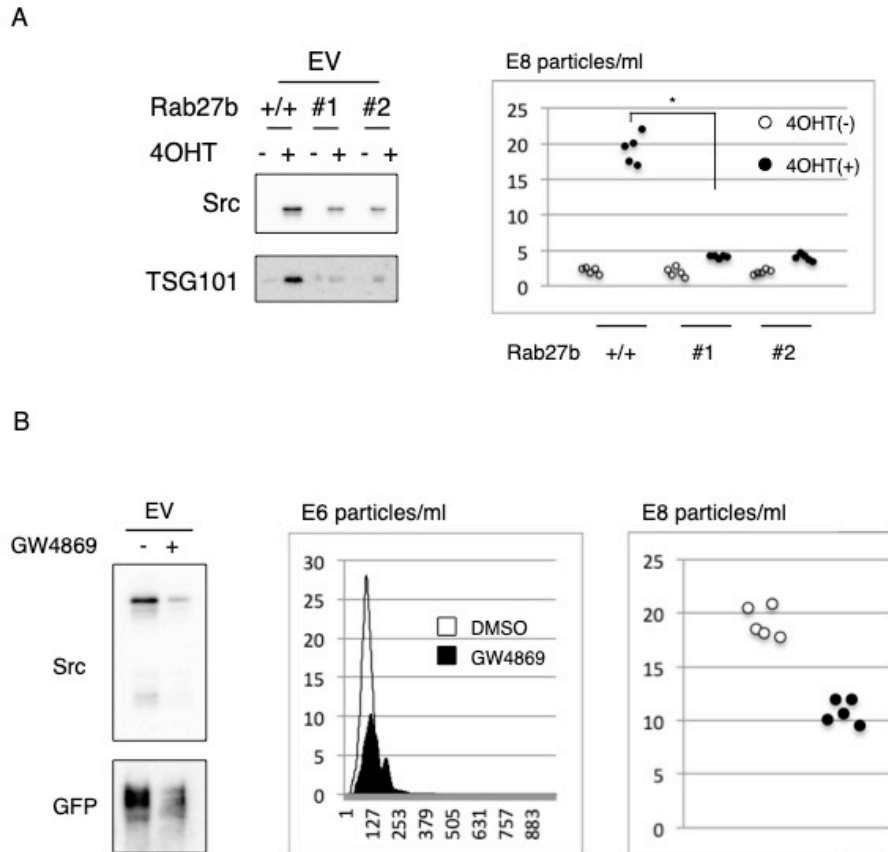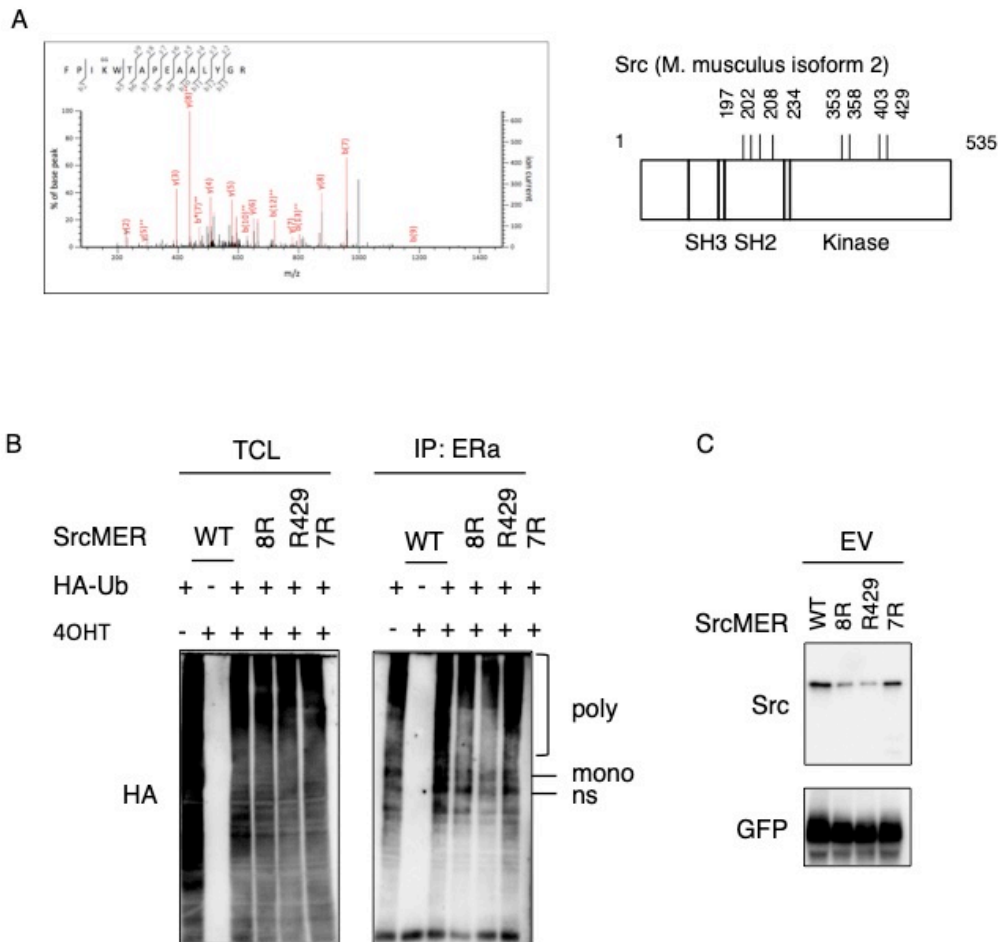
Figure 4

(A) Src-induced EV secretion is dependent on Rab27b. MDCK Rab27b$^{+/+}$ or Rab27b$^{-/-}$ cells expressing Src-MER were treated with or without 4OHT, and their EV were collected and analyzed by immunoblotting with the indicated antibodies (left panels). The numbers of EV particles were analyzed by NTA (right panel). *P<0.05

(B) EV were collected from cultures of MDCK cells expressing Src-MER and CD63-GFP in the presence or absence of GW4869, an inhibitor of nSMase2, and analyzed by immunoblotting with the indicated antibodies (left panels). The numbers of EV particles were analyzed by NTA (right panels).
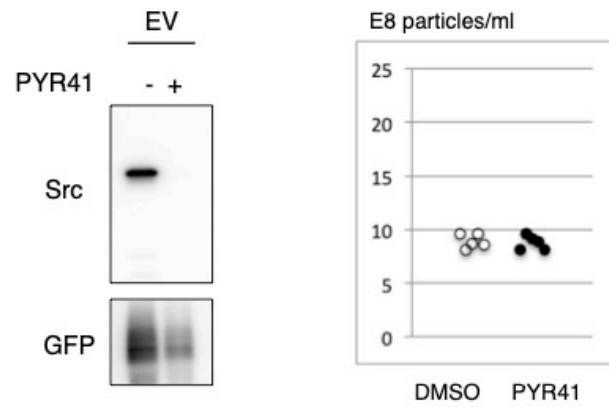
Figure 5
Inhibition of global ubiquitylation suppresses
secretion of Src via EV. MDCK cells expressing Src-
MER and CD63-GFP were treated with or without
50uM PYR41 for 8 hr in the presence of 4OHT, and
EV collected were analyzed by immunoblotting with
the indicated antibodies (left panels). The numbers
of EV particles were analyzed by NTA (right panel).

Figure 6
Ablation of E3 ligases for Src suppresses secretion of Src via sEV. MDCK cell clones expressing Src-MER, in which c-Cbl, Lnx1 or Stub1 were knocked out, were treated with 4OHT, and EV collected were analyzed by immunoblotting with the indicated antibodies (top panels). The numbers of EV particles were analyzed by NTA (bottom panels).

Figure 7

(A) A representative data for diGly MS analysis (left panel). Identified potential ubiquitylation sites are indicated in schematic structure of Src (right).

(B) Src is ubiquitylated at the specific sites. HEK293T cells transfected with HA-Ub and/ or Src-MER or its mutant having a Lys to Arg substitution(s) at all the ubiquitylated sites (8R), Lys429 (R429) or seven sites except for Lys429 (7R) were treated with or without 4OHT, and TCL and immunoprecipitates with anti-ERa (IP) were analyzed by immunoblotting with anti-HA. poly, poly-ubiquitylated Src-MER; mono, mono-ubiquitylated Src-MER; ns, nonspecific band.

(C) Ubiquitylation at Lys429 is required for secretion of Src via EV. MDCK cells expressing CD63-GFP and Src-MER or the indicated mutant were treated with 4OHT, and EV collected were analyzed by immunoblotting with the indicated antibodies.
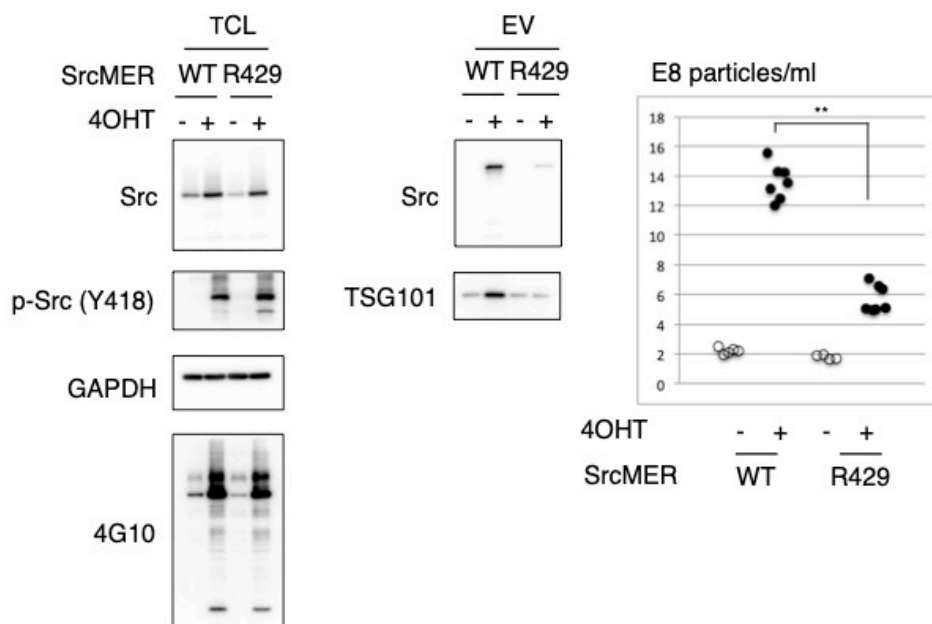
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Figure 8

R429 mutant has kinase activity comparable with that of wild-type Src. MDCK cells expressing Src-MER or R429 were treated with or without 4OHT, and TCL were analyzed by immunoblotting with the indicated antibodies (left panels). R429 mutation suppresses secretion of Src on EV. EV were collected from above cultures and analyzed by immunoblotting with the indicated antibodies (center panels). The numbers of EV particles were analyzed by NTA (right panel). **P<0.01

A

ERa   Lamp2   Merge

WT

R429

B   WT   R429
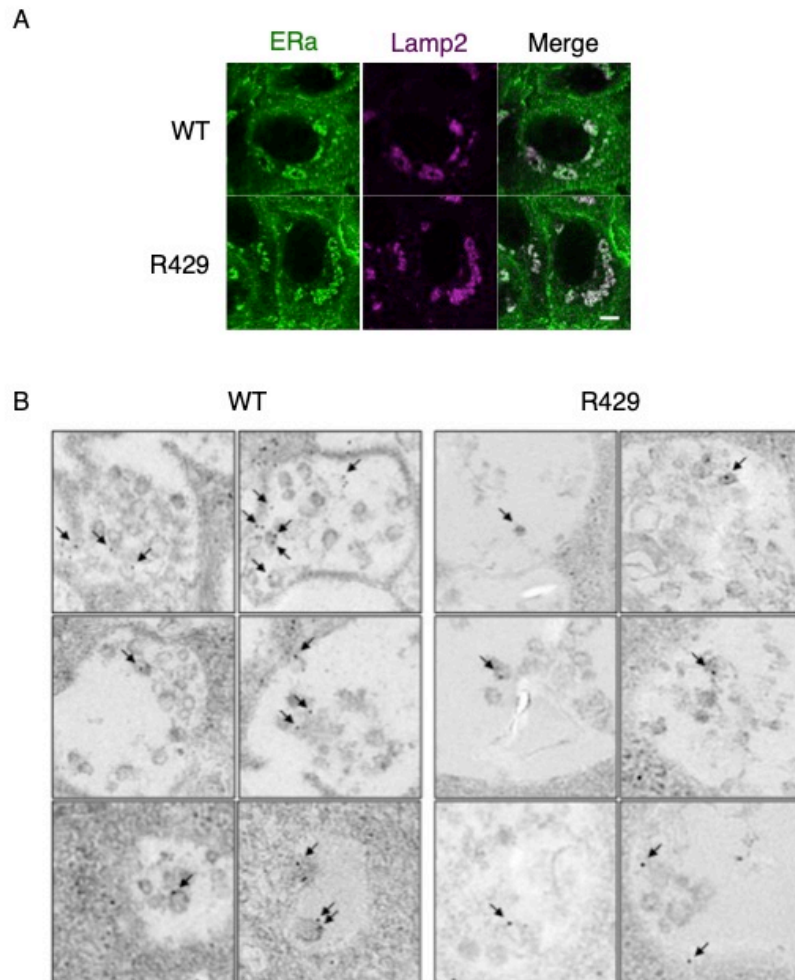
Figure 9
(A) R429 mutant are accumulated at the late endosomes/lysosomes like wild-type Src. Immunofluorescence staining was performed using the indicated antibodies. Scale bars, 5um.
(B) MDCK cells expressing Src-MER or R429 were treated with 4OHT and localization of Src-MER was analyzed by immunoelectron microscopy with anti-ERa. Arrows indicate immunoreactive gold particles.
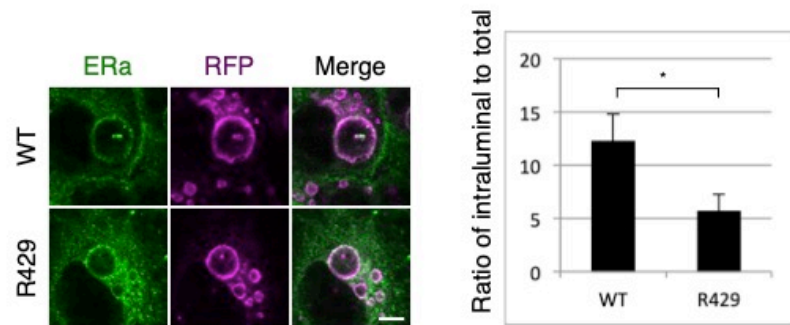
Figure 10
Defective incorporation of R429 mutant into ILV. Fluorescence images of Src-MER and R429 mutant in 4OHT-stimulated MDCK cells expressing RFP-Rab5a CA are shown. Scale bars, 5um. The right graph represents ratios of Src in ILV to that on endosomes. Values are indicated as mean ±SE. *P<0.05
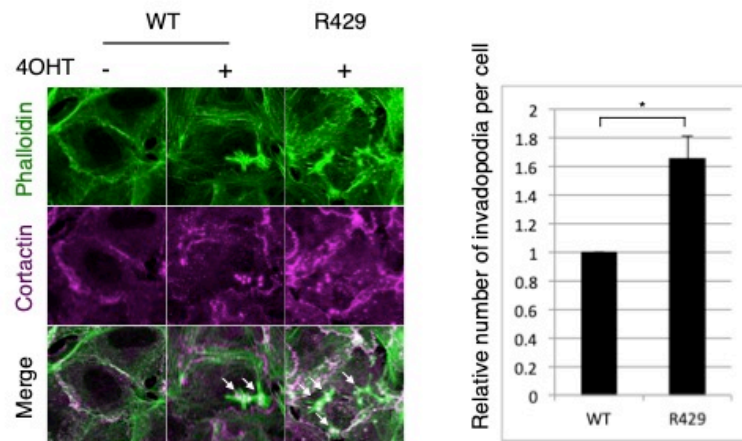
Figure 11

Src-induced invadopodia formation is promoted in R429 expressing cells. MDCK cells expressing Src-MER or R429 were stimulated with 4OHT, and invadopodia were visualized by immunofluorescence staining for F-actin (phalloidin) and cortactin (left panels). White arrows indicate invadopodia. Scale bars, 5um. Relative numbers of invadopodia per cell were counted (right panel). Values are mean±SE. *P<0.05
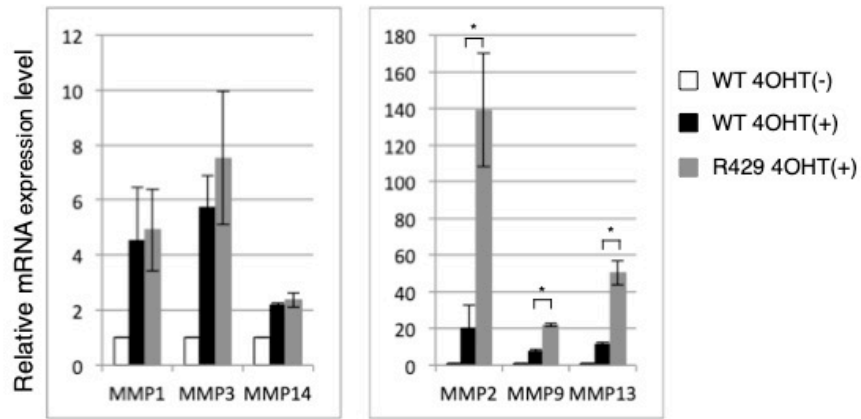
Figure 12
Src-induced expression of a set of MMPs is promoted by R429 mutation.
Gene expressions of the indicated MMPs are assessed by qPCR analysis in
MDCK cells used in above experiments. Cycle thresholds were normalized
to that of GAPDH. Values are mean±SE. *P<0.05
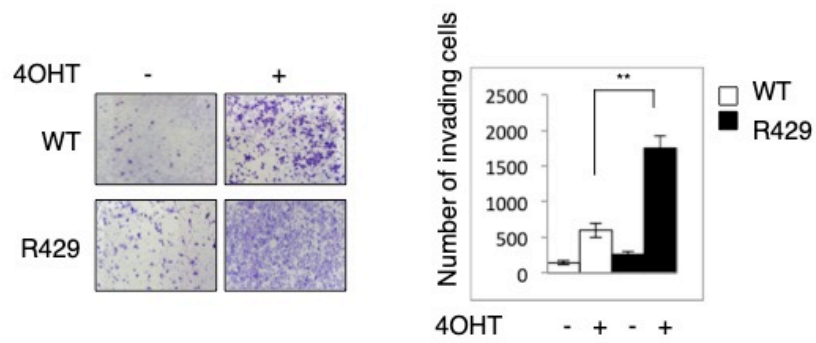
Figure 13

Src-induced in vitro invasive activity is promoted by R429 mutation. In vitro invasive activity of MDCK cells expressing Src-MER or R429 was measured using matrigel invasion chambers. Representative pictures of invaded cells are shown (left panels). The numbers of invaded cells are quantified (right panel). Values are mean ± SE. **P<0.01

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