c-Src regulates formation and secretion of exosome by direct interaction with Alix

(c-Src は Alix との直接の相互作用を介して exosome の形成・分泌を制御する)

Doctoral Thesis

by

Atsushi Kuwahara

Graduate School of Science, Osaka University
Department of Oncogene Research, Research Institute for Microbial Diseases,
Osaka University

February 2023

Table of Contents

Abstract.....4

General introduction.....6

Introduction.....20

Results.....22

Discussion.....28

Legends for figures.....32

Materials and methods.....35

References.....41

Figures.....55

Abstract

The non-receptor tyrosine kinase c-Src plays a pivotal role in cancer progression and is strictly regulated under physiological conditions. In addition to the well-established mechanisms of phosphorylation and conformational changes, c-Src is also controlled by its subcellular localization.

In this study, we found that only activated c-Src is recruited to multivesicular endosomes (MVEs), which form intraluminal vesicles (ILVs) in the lumen. The active form of c-Src is then incorporated into ILVs and finally secreted as exosomes, which are nano-sized extracellular vesicles. Moreover, c-Src enhanced the secretion of exosomes containing activated c-Src. In addition to kinase activity, experiments utilizing c-Src mutants indicated that interaction with the SH2 and SH3 domains of c-Src is also essential in the process of exosome regulation. Based on these results, exosomal c-Src-binding proteins were identified, and ALG-2-interacting protein X (Alix) was identified as a mediator of exosome control.

From the analysis of Alix mutants, it was found that Alix interacts with the c-Src SH3 domain through the N-terminal proline-rich domain (PRD) and that binding was not mediated by the interaction between c-Src SH2 and the phosphorylated Alix Bro1 domain, which was previously reported. Exosome secretion induced by c-Src was suppressed by knockdown of Alix, and this decrease was rescued by re-expression of wild-type Alix, but not by the Alix mutant lacking the PRD, indicating the importance of the interaction between Alix and c-Src in exosome regulation.

Since Alix interacts with ESCRT proteins that perform invagination and budding of the endosomal limiting membrane to form ILV/exosomes, the upregulated secretion was possibly the result of an increase in vesicle formation. To confirm this possibility, an *in vitro* MVE reconstitution assay was performed. Quantification of MVE formed in the solution reconstituted by isolated endosomes and cytoplasm showed that c-Src augments vesicle formation depending on its kinase activity. Corresponding to the result that the interaction between c-Src SH3 and Alix PRD domains is essential for exosome secretion from c-Src-activated cells, the Alix deletion mutant that

lacks the PRD failed to enhance vesicle formation *in vitro*.

These findings indicated that activated c-Src is recruited to MVEs. Then, the c-Src protein is incorporated into ILV depending on its kinase activity and interaction with Alix PRD via the c-Src SH3 domain. The loading of c-Src into ILVs results in an increase in vesicle formation and exosome secretion. These results also indicate a mechanism by which c-Src regulates exosome formation and, simultaneously, c-Src is shed from cells by exosomes.

General Introduction

1. Structure and regulation of c-Src

c-Src, a non-receptor tyrosine kinase localized at the plasma membrane, is a proto-oncogene identified as a homolog in normal cells of v-Src, an oncogene derived from the Raus Sarcoma virus [1]. c-Src affects cell survival, proliferation, adhesion, and motility, and its increased activity has been observed in many cancers [2]. In normal cells, c-Src is tightly regulated not only by regulation of gene expression [3] but also by post-transcriptional regulation [4], degradation by ubiquitination [5, 6], subcellular localization [7, 8], and conformational regulation by tyrosine phosphorylation [9].

The second glycine from the N-terminus of c-Src is myristoylated, thereby localizing c-Src to the membrane, although it has no transmembrane domain [10]. The protein structure of c-Src can be divided into an N-terminal lipid modification site, a unique domain (SH4), SH3 and SH2 domains responsible for protein-protein interactions, a kinase (SH1) domain, and a C-terminal regulatory tail. This basic structure is common to eight Src family kinases (SFKs) [11]. The most significant difference between v-Src, capable of transforming expressed cells, and c-Src, expressed in normal cells, is the regulatory tail at the C-terminus, which is missing and replaced by a different sequence in v-Src [12].

The regulatory tail of c-Src contains a tyrosine residue (Tyr529 in humans) that is phosphorylated by Csk, a C-terminal Src kinase. The phosphorylated regulatory tail is recognized by the SH2 domain of c-Src and binds intramolecularly. This binding also causes the SH3 domain to bind to the polyproline type-II helix in the linker region between SH2 and SH1 (kinase). These two intramolecular bindings result in a "closed" conformation of c-Src and suppression of kinase activity. In the absence of C-terminal phosphorylation, c-Src is in an "open" conformation, and tyrosine in the activation loop of the kinase domain (Tyr418 in humans) is intermolecularly autophosphorylated to activate c-Src [11].

Csk, which inhibits c-Src by phosphorylation, is also a protein consisting of an SH2/3 domain and a tyrosine kinase domain. However, while c-Src

phosphorylates a wide variety of proteins, Csk is highly substrate-selective and has no known substrates other than SFKs. Furthermore, since Csk is not modified to localize to the membrane, efficient inhibitory phosphorylation of c-Src requires Cbp/PAG1 [7], which mediates the interaction of both proteins as an adapter protein.

Cbp/PAG1 is a single transmembrane protein that undergoes palmitoylation at two cysteine residues close to the membrane and localizes to the cholesterol- and sphingolipid-rich region of the cell membrane, lipid raft [13]. Nine tyrosine residues in the intracellular region of Cbp are phosphorylated by SFKs. Four of these are motifs to which the Src SH2 domain binds, and one is a binding motif for the Csk SH2 domain. This SH2 domain-mediated binding to Cbp results in the formation of the Src-Cbp-Csk complex on the lipid raft and efficient inhibitory phosphorylation of c-Src by Csk.

Mechanism to dephosphorylate the C-terminus of the c-Src is less clear than the suppression mechanism by the Csk. The cytoplasmic phosphatases, PTP1B, SHP1, and SHP2, and transmembrane phosphatases, CD45, PTPα, PTPε, and PTPγ, exhibit dephosphorylation activity at the C-terminus of c-Src. However, since the phenotype is observed in the absence of either molecule, it seems that these phosphatases dephosphorylate the c-Src C-terminus in a tissue- and environment-dependent manner.

In addition to the C-terminal inhibitory phosphorylation and activative autophosphorylation mechanisms described above, SFKs are also activated by the interaction of the SH2/3 domain with other molecules. Even if the C-terminus is phosphorylated and in an inactive conformation, SFKs, including c-Src, are activated [14-18] when peptides with high affinity to the SH2 domain bind [17, 19] or proteins bind to the SH3 domain [20-22]. A cooperative binding mode that binds both SH2/3 has also been reported [23-26]. In such cases, it seems that the bound c-Src phosphorylates the binding protein to generate a new SH2 binding site, stabilizing the complex and causing further binding of proteins that possess SH2 domains. Phosphorylation can be decreased or increased when proteins phosphorylated by SFKs lack [25, 26] or are artificially added [27] the SH2/3

domain-binding motif. Thus, the interactions through the SH2/3 domain and, phosphorylation, and activation of SFKs are interdependent and inseparable. The search for proteins that interact with c-Src is essential for studying its regulatory mechanism of c-Src.

2. General introduction of exosome

Definition, biogenesis, and components of exosomes

Exosomes are extracellular vesicles composed of a lipid bilayer membrane approximately 50-200 nm in diameter. Exosomes originate from multivesicular endosomes (MVEs), which are part of late endosomes and contain vesicles within their lumen. The fusion of the MVE with the plasma membrane results in the release of intraluminal vesicles (ILVs) into the extracellular space, which then becomes extracellular vesicles and exosomes [28].

Proteins that deform and invaginate the limiting membrane of the endosome are a group of proteins called the endosomal sorting complex required for transport (ESCRT) machinery. ESCRT proteins are classified into four subgroups according to their functional stage: 0, I, II, and III. ESCRT-0 binds to and targets the membrane, ESCRT-I and -II invaginate and deform the membrane, and ESCRT-III accumulates at the neck of the invaginated membrane to execute fission. The Vps4 complex, which disassembles and recycles the remaining ESCRT-III oligomers on the membrane after vesicle formation, is essential for the function of the ESCRT machinery. ESCRT-0, -I, and -II also contain components with ubiquitin-binding domains that simultaneously load ubiquitinated cargo into vesicles [29, 30].

In addition, ESCRT machinery-independent mechanisms of MVE formation have been observed [31-33]. In a study in which four ESCRT-0 to -III proteins were simultaneously knocked down by siRNA, EGFR incorporation into ILVs was suppressed, while the formation of MVEs was confirmed using electron microscopy [34]. Lipid-mediated MVE formation has been proposed as the mechanism for ESCRT-independent MVE formation.

In a study on the trafficking of proteolipid protein (PLP) to MVE in oligodendroglial cells, PLP transport to the MVE was blocked by inhibition of ceramide synthase neutral sphingomyelinase 2 (nSMase2) and was ESCRT-independent [35]. This report also showed that when sphingomyelinase was added to sphingomyelin-containing giant unilamellar

vesicles (GUVs), vesicles were formed in the lumen of the GUVs along with ceramide. These results support the concept that membrane lipids form microdomains that drive membrane invagination and vesicle formation. A recent report showed that different populations of extracellular vesicles are released from the apical and basolateral sides of MDCK cells in an ESCRT-related protein Alix and a ceramide-dependent manner [36]. The heterogeneity of exosomes has become a problem in the research of exosome function, and it is necessary to analyze and classify the mechanisms by which exosomes of interest are formed and regulated.

The lipids that comprise the exosomal membrane have a different composition than that of the cell membrane and are composed of lipids such as cholesterol and sphingomyelin, which are abundant in the lipid raft, as well as ceramide [37, 38].

In addition to the accumulation of these lipid raft-localized proteins [39], exosomes also contain a large amount of the transmembrane protein, tetraspanins. The tetraspanin-enriched microdomain, in which tetraspanins bind to each other, contributes to protein sorting into exosomes and ILV formation [32, 33]. Therefore, exosome-enriched tetraspanins (CD9, CD63, and CD81) have been used as marker proteins for exosomes [40].

Exosomes contain not only characteristic lipids and proteins but also nucleic acids, especially mRNAs and microRNAs. These RNAs are not only shed extracellularly but are also taken up by the surrounding cells and function within incorporated cells [41]. RNAs in exosomes do not merely mirror the intracellular RNA constitution but exhibit a unique composition [41-44]. The RNA in exosomes is predominantly composed of relatively short sequences of 200 nt or less [45]. However, RNAs longer than 5 kb have also been identified, and there are significant differences in their composition depending on the sample origin and cell type [43, 44, 46]. These results indicate a mechanism by which RNA is selectively loaded into exosomes. Although this mechanism remains largely unexplored, several proteins are responsible for RNA loading into exosomes.

According to Shurtleff et al., Y-box protein 1 (YBX1) is an RNA-binding protein that transports Y RNA and a group of short non-coding RNAs,

especially miR-223 and tRNAs, into the exosomes [44, 47]. The group also reported that the Lupas La protein binds to miR-122 for loading into exosomes as a mechanism distinct from YBX1 and that UGGA is a sequence motif in miRNAs for loading into exosomes [48]. Another study analyzed the RNA sequences of exosomes secreted from T cells and identified the miRNA sequence motifs that determine their localization to exosomes or cells. The GGAG sequence on the 3' side of the miRNA and the position-independent CCCU sequence are motif sequences responsible for the trafficking of the miRNA to the exosome. SUMOylated hnRNPA2B1 binds to this sequence and loads miR-198 into exosomes in both a SUMOylation and RNA motif-dependent manner [49]. Similarly, SYNCRIP, also known as hnRNPQ or NASP1, binds to the hEXO sequence (GGCU/A) and loads miRNAs into exosomes [50, 51].

Considering the diversity of RNAs in exosomes, the proteins and RNA sequences reported thus far are only a part of the mechanism controlling RNAs in exosomes, and exosomal RNAs are expected to be regulated through many other proteins. The mechanisms of the transfer and loading of proteins, lipids, and RNA into exosomes have not been fully elucidated, and further research is required.

3. Physiological functions of exosome

One of the greatest difficulties in exosome research is the lack of *in vivo* models. The lack of a specific method to inhibit secretion is a major limitation in elucidating the physiological significance of exosomes. Therefore, considering the number of studies that have used culture supernatants of cancer cells or patients' body fluids, the roles that exosomes play under healthy conditions are still largely unknown.

Regardless of the technical restrictions mentioned above, Wnt signaling in *Drosophila* is one of the few examples in which the role of exosomes *in vivo* has been documented. Wg, the major Wnt protein in *Drosophila*, is a soluble signaling factor that is secreted extracellularly and travels long distances. However, Wg is highly hydrophobic owing to the addition of fatty chains in post-translational modifications, and the factors that provide the solubility and diffusivity of Wg have been a subject of debate [52, 53]. In addition, the multiple transmembrane protein “evenness interrupted” (Evi), also known as Wls, which binds to Wg and transports it intracellularly, is also secreted from the cells at the neuromuscular junction (NMJ), showing that Wg is transported across synapses in an Evi-dependent manner [54]. As not only hydrophobic Wg but also transmembrane proteins were secreted, it appeared that secretion was mediated by vesicular transport, that is, by exosomes. The same group showed that Wnt signaling in NMJ is mediated by exosomes [54, 55].

Interestingly, not all secreted Wg are exosomal. In the case of Wg-expressing Drosophila S2 cells, only about 12% of the total Wg protein in the culture supernatant or about 23% of the signaling activity was exosomal [56]. Similarly, in mouse fibroblast L-Wnt3a cells, the percentage of secreted Wnt3a in exosomes was approximately 40% [57]. Exosomal transmission of Wg has also been observed in the wing imaginal discs of *Drosophila*. In addition, knockdown of the ESCRT machinery and Ykt6, a newly discovered factor involved in exosome secretion, inhibits Wnt3a secretion in human cell lines, suggesting that this mechanism is conserved across species [57].

The physiological function of exosomes in vertebrates is largely unexplored, but a part of it has recently been demonstrated in an elegant experimental

model. In experiments of heterochronic blood exchange by heterochronic parabiosis, when young and old mice were surgically joined to share blood flow, the old mice exposed to the blood flow from the young mice showed improved skeletal muscle, hepatocyte, and nervous/cognitive function; in other words, they were rejuvenated in some aspects [58-61]. GDF11 [60] and Creb [61] are improving factors, and CCL11 [59] is an impairing factor affecting neurogenesis and cognitive function. However, the factors involved in skeletal muscle rejuvenation have been unknown since the first report in 2005 [58].

Recently, Sahu et al. [62] demonstrated through heterochronic blood exchange experiments that circulating extracellular vesicles (EVs = $CD81^{high}$ exosomes) from young mice contained Klotho mRNA and that these EVs regenerated the skeletal muscle by supplying Klotho mRNA to elder mice. The amount of Klotho mRNA decreased in the blood EVs of old mice, and a similar tendency was observed in human plasma samples. These results suggest that circulating EVs maintain skeletal muscle homeostasis and that this function is lost with aging. Further, this report demonstrated that EVs play a physiological role as humoral factors that transport mRNA throughout the body, even across individuals. Interestingly, the majority of Klotho-containing exosomes do not appear to be derived from the kidney, the primary expression site of Klotho, and the origin of exosomes remains unknown.

Not only multicellular organisms but also unicellular organisms secrete and utilize exosome-like vesicles. In particular, the parasitic protozoan *Leishmania* exploits exosomes during its propagation. *Leishmania* secretes exosomes when they reside in the midgut of the vector sand fly, and when the sand fly bites, the exosomes and protozoa are ejected together cutaneously. Exosomes inoculated with protozoa enhance infection, promote protozoa growth at the site of infection, and exacerbate pathology [63, 64].

The yeast *Saccharomyces cerevisiae*, which made a major contribution to the discovery and classification of the ESCRT machinery [65], also secretes exosome-like vesicles that are dependent on the ESCRT machinery. These vesicles function in cell wall synthesis [66].

Species that secrete ESCRT-containing vesicles can be traced back to some archaea [67]. The EVs secreted by the archaeon *Sulfolobus* sp. were approximately 100 nm in size, showed a lipid composition different from that of the cell membrane, and contained several proteins in common with the mammalian exosomes. Electron microscopy revealed that the vesicle surface was covered with a protein layer that appeared to be an S-layer, similar to the plasma membrane, and that the vesicles showed a smaller buoyant density (<0.7 g/ml) than eukaryotic exosomes. Considering that the endosome system is not developed in this archaeon, it seems that these vesicles bud directly from the plasma membrane [68].

The Asgard superphylum, the group of archaea most closely related to eukaryotes, was recently identified by genome analysis of aquatic sediments sampled from all over the world. The genes of the ESCRT machinery and ubiquitination apparatus were present as gene clusters in the genomes of these archaea and have attracted much attention [69, 70]. Currently, only one species of Asgard archaea has been successfully established in culture [71], and further research is needed to determine if these ESCRT and ubiquitination systems are functional for vesicle formation. However, the ESCRT machinery proteins encoded in Asgard archaea genomes have been experimentally confirmed to interact in a manner similar to that found in eukaryotes [72]. The prototype of the extracellular vesicle formation and secretion mechanism observed in eukaryotes may have existed at the time of the last eukaryotic common ancestor.

4. Exosomes in cancers

Since it was first demonstrated that exosomes contain RNA and that their contents can be transferred from cell to cell [41], great attention has been paid to the relationship between exosomes and cancer. This is because exosomes can function in the crucial steps of cancer development, proliferation, invasion, and metastasis, such as interaction with the cancer microenvironment and escape from immune surveillance. In fact, in the past 10+ years of research, a large number of reports have been published regarding the function of exosomes secreted by cancer cells and surrounding cells in both cancer progression and suppression. Their major functions are described below:

4-1. Exosomes in the tumor microenvironment

In cancer tissues, not only cancer cells but also a wide variety of surrounding cells form an environment that should be regarded as an ecosystem, supporting the maintenance and progression of cancer. The environment surrounding cancer cells is called the tumor microenvironment, which is a complex interplay of extracellular matrix (ECM), vascular cells, fibroblasts (cancer-associated fibroblasts, CAFs), macrophages (tumor-associated macrophages, TAMs), and a wide variety of infiltrated immune cells [73, 74].

The ECM is likely the first object that exosomes encounter when they are secreted from cancer cells. Cancer cell-derived exosomes contain ECM-modulating proteases and can form tumor-supportive niches [75]. A study on squamous cell carcinoma cells showed that exosomes secreted from invadopodia improve cell invasiveness by stabilizing invadopodia and ECM degradation [76]. Similar *in vivo* results have shown that the exosomes secreted from cells stabilize protrusion and adhesion and are required for the directional migration of cells in tissues [77]. There are also indirect examples of ECM modifications. Pancreatic ductal carcinoma (PDAC)-derived exosomes induce TGF-ß production from Kupffer cells via macrophage migration inhibitory factor (MIF) and induce the production of fibronectin in stellate cells. This alteration of the ECM promotes metastasis of PDAC cells

to the liver [78].

The exosomes secreted by cancer cells can also induce angiogenesis. Head and neck squamous cell carcinoma (HNSCC) cell lines and HNSCC patient sera-derived exosomes have been experimentally reported to cause angiogenesis both *in vitro* and *in vivo* [79]. In another study using epithelial ovarian cancer cells, angiogenesis was induced in a mouse transplantation model via the long non-coding RNA MALAT 1, which is loaded in exosomes [80]. Furthermore, exosomes increase vascular leakiness by affecting the endothelial cells of blood vessels, which promotes the metastasis of cancer cells [81-83]. These results indicated that exosomes not only induce angiogenesis, which is necessary for the survival and growth of cancer cells, but also mediate cancer progression and metastasis.

4-2. Tumor-associated macrophages (TAMs)

Macrophages infiltrating tumors, called tumor-associated macrophages (TAMs), are associated with poor prognosis and chemotherapy resistance in many types of cancers [84]. The interaction between TAMs and cancer cells via exosomes has also been investigated intensively. miR-223, present in exosomes secreted by TAMs, enhances the invasiveness of breast cancer cells [85]. In addition, a diverse range of functions has been reported for TAM-derived exosomes, including the enhancement of cancer cell proliferation and chemotherapy resistance [86]. Conversely, the effect of exosomes secreted by cancer cells on TAMs has been reported. In a study using lung cancer cell lines, exosomes induced pro-metastatic inflammatory responses *in vivo* by suppressing TLR-8 of TAMs via exosomes containing miR-21 and miR-29 [87]. In addition to the pro-tumorigenic effects described above, anti-tumorigenic effects were observed in the relationship between TAMs and exosomes. For example, triple-negative breast cancer (TNBC) cell-derived exosomes induce proinflammatory macrophages and consequently T-cell infiltration, leading to a better prognosis [88]. Non-metastatic melanoma cell line-derived exosomes also induce the differentiation of anti-metastatic macrophages and suppress metastasis [89]. Overall, exosomes function reciprocally in a balance between cancer cells and

TAMs, which can function either as suppressive or supportive.

4-3. Cancer-associated fibroblasts (CAFs)

Fibroblasts found in cancer tissues have been identified as cancer-associated fibroblasts (CAFs). Despite the absence of mutations in their genome, they play a multifaceted role in supporting cancer, including ECM construction, growth factor secretion, angiogenesis, and restricted drug access [90]. The roles of exosomes transferred between CAFs and cancer cells have been analyzed.

Wnt proteins are transported extracellularly on exosomes, as described above, and this mechanism has also been observed in cancer cells. Remarkably, when mouse fibroblast-derived exosomes are taken up by breast cancer cells, Wnt11 is loaded onto these exosomes and re-secreted from recipient cells. Wnt11-loaded exosomes act in an autocrine manner to activate Wnt-PCP signaling in breast cancer cells, resulting in increased motility and metastasis [91].

Conversely, experiments using prostate cancer cell lines have shown that TGF-β-bearing exosomes induce fibroblast differentiation into myofibroblast-like cells to promote angiogenesis and tumor growth [92, 93]. In addition, CAF-derived exosomes affect cancer cell metabolism to support tumor growth [94] and escape from ER+ breast cancer cell dormancy [95].

4-4. Immune surveillance and cancer exosome

Escape from immune surveillance is one of the most critical processes in the establishment, proliferation, and metastasis of cancer [96, 97]. The exosome is one of the factors involved in this process. Numerous reports describing the functions of exosomes in cancer and immune cells illustrate the intense competition between these two populations.

Immune-suppressive exosomes

The simplest mechanism for suppressing antitumor immunity is to suppress cytotoxic immune cells. Cancer cell-derived exosomes can inhibit $CD8^+$ effector T cells [98] and NK cells[99, 100]. Cancer cell-derived

exosomes can also exert immunosuppressive effects by inducing Treg cells [98] and myeloid-derived suppressor cells (MDSC) [101]. Its effects on immune checkpoints have also been reported. Functional PD-L1 is present on the surface of exosomes secreted by metastatic melanoma cells, and these exosomes promote tumor growth in an *in vivo* model by inhibiting the infiltration and proliferation of $CD8^+$ T cells. The anti-PD-1 antibody inhibits this effect [102]. This report also showed that the fold change of exosomal PD-L1 in patients' circulation after PD-1 antibody treatment stratified responders and non-responders to treatment. A higher fold change in exosomal PD-L1 after treatment was associated with the response to treatment and a better prognosis. A study of chronic lymphocytic leukemia (CLL) revealed that CLL-derived exosomes contained non-coding Y RNA hY4, and the exosomal Y RNA induced PD-L1 expression in monocytes, resulting in immune escape [103].

Immune-activating exosomes.

Because exosomes contain proteins of cellular origin, they can be recognized as antigens by immune cells. Wolfers et al. showed that when cancer cell-derived exosomes are taken up by dendritic cells, the proteins in the exosomes are recognized as antigens and activate antigen-specific $CD8^+$ T cells to suppress tumors in an *in vivo* mouse model [104]. Moreover, the exosomes purified from melanoma patients' ascites could induce an antigen-specific, in this case, melanoma MART-1-specific, $CD8^+$ T cell response when incorporated into monocyte-derived dendritic cells (MD-DCs) obtained from the blood of patients [105].

Some cancer cells secrete immune-activating exosomes to improve their immunogenicity against tumors. For example, in studies using Muc1-expressing mouse colon cancer cells, the cells secrete exosomes that contain more Hsp70 upon heat stimulation and induce a stronger immune response than unstimulated exosomes [106]. In a mouse glioblastoma cell model, prior "vaccination" of mice with glioblastoma-derived exosomes induced immunity against the cancer cells, and the mouse rejected transplantation of the cells [107]. Notably, in this study, "vaccination" with

exosomes after transplantation did not cause rejection.

In the case of tumor-suppressive macrophages described in the TAMs section, cancer cell-derived exosomes also induce differentiation of anti-metastatic macrophages and partially suppress metastasis via NK cells [89]. Similarly, exosomes derived from triple-negative breast cancer cells ultimately induce T and NK cell infiltration [88]. Thus, these exosomes also have immune-activating effects.

It is surprising that the exosomes secreted by some cancer cells not only have a tumor-promoting effect but can also induce antitumor immunity, as described above, even though the cells have once established cancer. At present, it is not understood how cells acquire exosomal function during cancer development or how they lose the exosome-mediated functions that they may originally possess to prevent cancer. We do not know whether these functions are acquired accidentally or whether they are aberrations in the cells' innate functions. Therefore, it is necessary to develop better methodologies and biological models to elucidate the significance of exosomes *in vivo*.

Introduction

c-Src is a non-receptor-type tyrosine kinase that is involved in broad physiological processes and contributes to tumor malignancy [2]. In response to extracellular stimuli, including growth factors and adhesion signals, c-Src is activated and, in turn, phosphorylates a broad group of proteins to transduce signals for proliferation, migration, and angiogenesis [108]. Because of these functions, c-Src is strictly regulated by multiple mechanisms in cells, including inhibitory phosphorylation by Csk [9], lipid raft and Cbp-mediated control with Csk [7, 8, 109], translational regulation by microRNAs [4], and degradation via ubiquitination [5, 6].

In addition, c-Src is regulated by its subcellular localization. Since c-Src undergoes lipid modification at the N-terminus [10], it localizes to membranous structures in the cells [110]. In addition to plasma membrane localization, which transduces signaling linked to extracellular stimuli, endosomal trafficking of c-Src is also important for proper signaling [111, 112].

Endosomes include those that perform intracellular trafficking and multivesicular endosomes (MVEs) responsible for the degradation and secretion of proteins localized at the membrane. On the surface of the MVE, a part of the limiting membrane is invaginated and budded into the lumen, forming intraluminal vesicles (ILVs). When MVEs fuse with the plasma membrane, ILVs are secreted extracellularly. These secreted vesicles, called exosomes, are exchanged between cells in the tumor micorenvironment [113] or sometimes with remote tissues [62, 114] and contribute to cancer promotion by various mechanisms [28, 115].

c-Src is found in exosomes [116-119], but the significance of exosomal c-Src and the process by which c-Src is recruited into exosomes are not well understood.

Considering the lack of understanding of c-Src in exosomes, this study analyzed the mechanism of loading c-Src into exosomes using cells transformed by c-Src and exosomes secreted from the cells. The analysis revealed that c-Src is recruited to ILV/MVE depending on its kinase activity and interaction via SH2 and SH3 domains. c-Src also upregulates exosome

formation and secretion by promoting vesicle formation through direct interaction with Alix. These findings indicate that transformed cells sequester c-Src protein into ILV/exosomes and finally expel it by secretion as exosomes.

Results

c-Src activity regulates exosome secretion

First, the amount of exosomes secreted from model cells transformed by c-Src (Fig.1a-c) was investigated. Parallel to c-Src activation (Fig.1b, left pY418), the amount of exosome particles increased in c-Src-induced $Csk^{-/-}$ cells, and the enhanced secretion was reversed by re-expressing Csk, a kinase that negatively regulates c-Src (Fig.1a, b). Corresponding to the increase in particle counts measured using nanoparticle tracking analysis (NTA), exosome marker proteins, including Alix, Tsg101, CHMP4C, and Flot-1, also showed a significant increase in exosomal lysates (Fig.1c). These results indicate that c-Src activity, but not c-Src protein amount controls exosome secretion. To confirm that exosome release depends on c-Src activity, we examined HT29 cells treated with the Src family kinase (SFK) inhibitor dasatinib (Fig.1d, e). Even in a human colon adenocarcinoma cell line, c-Src inhibition induced a decrease in exosome secretion, supporting the results of the model cell system. In addition to the upregulation of exosomes, another remarkable result was that the c-Src protein level was increased in exosomes (Fig.1c, right). As shown in the pY418 blot, c-Src in the exosomes held an active conformation. Based on these observations, we speculated that activated c-Src was specifically recruited into exosomes and enhanced exosome secretion.

c-Src localization in endosome/MVB

As shown in Fig.1, c-Src is secreted as exosomal "cargo". Cnsistent with this result, c-Src is localized in late endosomes, especially in the MVE/exosome marker protein-positive compartment (Fig.2a, Rab7: pan-late endosome marker, CD9, and CD63: MVE/exosome marker). c-Src is a non-receptor tyrosine kinase linked to the cell membrane via N-terminal lipid modification [10]. c-Src that mutated N-terminal myristoylation site (Fig.2b, G2A), which is not myristoylated, could not enhance exosome secretion and localization, suggesting the importance of c-Src recruitment into exosomes.

Not only kinase activity but also interaction via SH2 and SH3 domains were essential for exosome regulation

To elucidate the underlying molecular mechanisms, we analyzed three other mutants that had a deficiency in the function of each of the three domains. Corresponding to the reversible suppression of exosome secretion by Csk, the K295M [120] kinase-inactive mutant failed to upregulate exosome secretion (Fig.3a, b). In addition, the two other mutants that lacked the binding ability of the SH2 [121] or SH3 [122] domain also failed to increase secretion (Fig.3a, b). The fact that the latter two mutants retain kinase activity but fail to increase exosome secretion suggests that a protein interacts with c-Src via the SH2/3 domain determines exosome regulation.

c-Src regulates exosome by direct binding to Alix C-terminal proline-rich region

The results described above indicate the existence of a protein that interacts with c-Src via the SH2/3 domain and controls exosome secretion. To identify the exosome-regulating protein(s), we analyzed c-Src-binding proteins in exosomes using immunoprecipitation and identified Alix as a mediator.

Alix is a protein involved in MVE/exosome vesicle formation cooperatively with the ESCRT machinery and promotes exosome secretion [123]. Alix interacts with ESCRT-I, -III, and other factors as a scaffold protein; however, the regulatory mechanism of Alix in exosome formation is poorly understood. Schmidt et al. reported that c-Src phosphorylates Alix Tyr319, and the c-Src SH2 domain binds to the phosphorylated region [124]. The R175L SH2 domain-deficient mutation impedes binding to Alix (Fig.4a). However, the Alix 4YF mutant, which replaced its tyrosine residues with phenylalanine at Tyr319 and newly identified three other tyrosine phosphorylation sites as illustrated in Fig.4b, showed no effect on the interaction with c-Src (Fig.4c, 4YF).

Instead of the interaction between phospho-tyrosine and the SH2 domain, the immunoprecipitation results suggested the importance of interaction via the SH3 domain, since even the W118A SH3 domain mutant lost function to

upregulate exosome secretion (Fig.2) and binding to Alix (Fig.4a).

The SH3 domain recognizes proline-rich sequences, in the case of c-Src SH3, the-P-x-x-P- motif (P: Proline, x: arbitrary residue) [125]. Alix contains a proline-rich domain (PRD) that starts from the 717th amino acid and stretches to the C-terminal end (Fig.4b). In fact, deletion of the PRD abolished binding to c-Src (Fig.4c). Reflecting the binding between c-Src and Alix, the wild type and 4YF mutant rescued the decreased exosome secretion induced by shRNA targeting Alix (Fig.4d). In contrast, the Δ717 mutant, which lacked the PRD, failed to rescue exosome secretion (Fig.4d), supporting the concept that direct interaction between the c-Src SH3 domain and Alix PRD is critical for c-Src-dependent exosome regulation.

c-Src/Alix complex regulates exosome at vesicle formation step

When exosome secretion increases, there are two possible explanations for the increase in exosome secretion. This is due to an enhancement in the intraluminal vesicle (ILV) formation step or an increased frequency of MVB fusion with the plasma membrane. Given that Alix interacts with the ESCRT machinery, c-Src seems to promote vesicle formation directly by binding to Alix. To investigate this possibility, we utilized an *in vitro* reconstitution approach. As illustrated in Fig.5a, cells were fractionated into the endosomal membrane and cytoplasm by moderate mechanical disruption of the plasma membrane, followed by stepwise centrifugation. c-Src was fused with luciferase and regarded as cargo in this assay. The membrane and cytoplasm fractions were mixed with luciferase substrate and ATP and incubated at 30°C for 20 min to allow ILV/exosome formation. As ILV/exosomes are formed, luciferase-fused c-Src is incorporated into ILVs with an outer solution containing a luciferase substrate. After the reaction, the endosome/MVE membranes were treated with trypsin. Since incorporated c-Src-luciferase is sequestered doubly by ILV and the endosome membrane, trypsin is not accessible to the internalized c-Src-luciferase. In contrast, c-Src-luciferase that is not loaded into ILV is exposed to the outer solution and is quenched by trypsin digestion. Additionally, c-Src-luciferase contained in ILV/MVE before the reaction is also protected from trypsin;

however, the pre-existing vesicles do not emit luminescence because the luciferase substrate cannot access the inside of the ILVs.

Using this strategy, we defined the luciferase activity protected from trypsin as the ILV/MVE formation. As shown in Fig.5b, we confirmed that *in vitro* MVE formation progressed only when all components were mixed. The reaction was also inhibited by the anti-CHMP4C antibody (Fig.5b), suggesting that the assay depends on a CHMP4C-mediated reaction. In the last step of vesicle budding, CHMP4 proteins polymerize and form a spiral structure at the budding "neck" to offer a physical force for membrane scission [126]. The inhibition caused by the antibody neutralizing one of the ESCRT-III proteins indicated that this assay truly reflects ILV/MVE formation executed by the ESCRT machinery.

To assess the impact of Csk on exosome formation *in vitro*, Csk overexpression or mock vector-infected $Csk^{-/-}$/c-Src cell cytoplasm was used in the assay, and it was confirmed that Csk suppressed c-Src-dependent vesicle formation (Fig.5c).

Similarly, we applied cytoplasm derived from shAlix and its rescue cells in the assay. Wild-type Alix expression increased MVE/ILV formation *in vitro*, but the AlixΔ717 mutant did not show such an increase (Fig.5d), corresponding to the results of the exosome amount rescued by re-expression of the Alix mutants (Fig.4d). These *in vitro* assay results indicate that activated c-Src is recruited to endosomes that form ILVs and promotes vesicle formation via direct interaction with the Alix C-terminal proline-rich domain. c-Src is internalized during this process, resulting in increased exosome secretion.

Dysfunction at activated c-Src loading to ILV/exosome leads to disruption of CD63-positive endosome structure

We next observed the localization of c-Src on inhibiting Alix-mediated c-Src incorporation into ILVs/exosomes.

To estimate the effect of the presence or absence of Alix and Csk at the endogenous level, we used $Csk^{flox/flox}$ fibroblasts [127].

First, $Csk^{flox/flox}$ fibroblasts which optimally expresses Src-EGFP was cloned,

and Alix-knockout cells were generated from the clone using the CRISPR/Cas9 system. To generate double knockout cells with Csk, Cre recombinase was transiently introduced into the AlixKO cells. Alix and Csk double knockout cells were then cloned. Simultaneously, Cre recombinase was transiently introduced into the Src-EGFP-expressing cells as a control. The lysate of each cell line was analyzed using western blotting (Fig.6g).

Cells generated by the process described above were stained with antibodies against CD63, a marker of exosomes (and their origin, MVE), or LAMP1, a pan-late endosome marker protein, and examined for localization with Src-EGFP.

In cells where Csk was endogenously expressed, puncta staining of CD63 was observed independently of AlixKO (Fig.6c, d red). Similar CD63 puncta were observed in CskKO cells transfected with Src-EGFP (Fig.6a, b top panels). However, in Csk and Alix double-knockout (DKO) cells, CD63 staining was extremely weak, and almost no structure was visible (Fig.6 a, b, middle and bottom panels). In the lysates, there was no significant change in the amount of CD63 protein (Fig.6g), suggesting that the MVE/late endosomes where CD63 was localized may be fragmented into smaller pieces and no longer be observed as puncta. Another possibility is that the endosomes in which CD63 was localized were not properly formed, and CD63 was expressed in a scattered distribution. In contrast, staining for LAMP1, another late endosomal protein, showed no significant change in the structure of endosomes stained by LAMP1, even when Csk and Alix were simultaneously knocked out (Fig.6e, f). Therefore, it seems that only CD63-positive late endosomes are affected when c-Src is not incorporated into ILVs/exosomes by AlixKO, whereas the majority of LAMP1-localized late endosomes are unaffected.

In summary, the results are dependent on c-Src activation, as this abnormal CD63 staining image was not observed with AlixKO alone but only when Csk was knocked out, and c-Src activation was accompanied by the absence of Alix. Even if the c-Src activity was high, CD63-positive endosomes showed a normal structure as long as Alix was expressed normally. In contrast, even if Alix was knocked out, and c-Src activity was suppressed,

CD63-positive endosomes showed a normal structure.

Therefore, these results suggest that when the process of incorporating activated c-Src into ILVs/exosomes is inhibited by AlixKO, the structure of CD63-positive endosomes, which form ILVs/exosomes, becomes aberrant.

Discussion

In this study, the molecular mechanisms of exosome regulation by c-Src activity and its interaction with Alix were revealed. When c-Src is activated, it takes an "open conformation" that provides accessibility to the SH2 and SH3 domains [11]. Phosphorylation at Y527 (in chickens) by Csk[9] and subsequent intramolecular interactions make c-Src a "closed" conformation [128, 129]. Our findings clearly showed that only the activated open conformation was recognized and recruited to the exosome (Fig.1c), depending on the interaction via the SH2/3 domain and kinase activity (Fig.3a, b). c-Src recruitment and localization to multivesicular endosomes enhanced ILV/exosome formation by directly binding to Alix (Fig.4, 5), leading to robust secretion of exosomes. Disruption of this process resulted in the aberrant formation of CD63-positive endosomes (Fig.6).

Research on ESCRT machinery starting from vps defect mutants in yeast [130, 131] identified four classes of proteins (ESCRT-0 to III) and the Vps4 complex, which is involved in the disassembly of the ESCRT-III oligomer [65]. The ESCRT machinery is assembled on the surface of late endosomes in an orchestrated manner. During machinery construction, they deform the membrane to invaginate, form a constricting neck, and finally accomplish the scission of a vesicle into the lumen. ESCRT components recognize ubiquitinated proteins as "cargo" and determine cargo sorting. Interestingly, deubiquitinases are also recruited by ESCRT and its accessory protein [132-135]. Deubiquitination possibly occurs before vesicle scission, and ubiquitin escapes packaging into intraluminal vesicles.

Our results indicate the importance of interaction via the SH2/3 domain; however, these observations do not exclude the possibility of ubiquitin-dependent recruitment of c-Src to the MVB. Rather, considering that c-Src is ubiquitinated for degradation, especially when c-Src adopts an open conformation [5, 136-138], active c-Src might be ubiquitinated depending on its kinase activity and accessibility to the SH2/3 domain and internalized into the ILV where deubiquitination occurs before vesicle closure. Even in this case, the interaction between c-Src and Alix is still critical for exosome control. If ILV formation is enhanced just because the

number of ubiquitinated cargos increases, overexpression of any other protein that can be ubiquitinated should induce exosome upregulation. However, a limited number of proteins upregulate exosome secretion [139]. Only a specific subset of proteins are sorted into exosomes. These facts indicate that the exosome is not a redundant garbage box for discarding unnecessary proteins.

Given the strict selectivity for cargo sorting, our finding that Alix binds to c-Src by interaction via the PRD and SH3 domains and that the interaction was critical for exosome regulation suggests another model. Similar to c-Src, Alix has open and closed conformations [140]. In the steady state, Alix PRD forms intramolecular binding to the Bro1 domain and conceals its Bro1 and V domains. Sun et al. showed that there are several mechanisms to unlatch Alix autoinhibition, including phosphorylation at Ser718-721 [141] and Ca^{2+}-dependent ALG-2 binding to Alix PRD [142]. In this context, active c-Src reduces the binding of Alix to CIN85/SETA, EGFR, and Pyk2 [124], indicating that c-Src modulates the binding affinity of Alix, although the report also showed that an interaction between SH2 and phosphorylated Tyr319 at the Bro1 domain was essential in their experiment.

Based on these reports and our findings, another hypothetical model is that active c-Src induces a conformational change in Alix by directly binding to PRD. Alix then presents a binding site for CHMP4 and Tsg101, which are ESCRT-I/-III components, to enhance the construction of the machinery on the membrane. The involvement of kinase activity and the SH2 domain in this mechanism is still unclear.

Regarding conformational changes in Alix, it has been reported that detergents contained in the buffer affect the structure of Alix [140]. Thus, immunoprecipitation performed with cell lysis may not completely reflect protein status under physiological conditions. We found that the Alix4YF mutant, which contains one of the mutations at the previously reported c-Src SH2 binding site Tyr319, did not affect the interaction between c-Src and exosome secretion (Fig.4). In contrast, our analysis showed that kinase activity and the SH2 domain are essential for exosome regulation (Fig.2 and 4). This discrepancy may be due to technical problems related to

immunoprecipitation.

Furthermore, the existence of a cofactor should also be considered. Our observations were all made in cell lysates but not in recombinant proteins. Plausibly, c-Src and Alix form a complex with another protein via phosphorylation and the SH2 domain, and the interaction with the cofactor could stabilize the whole complex or contribute to unlatching the Alix structure. Previously reported results in yeast two-hybrid experiments [123], which analyzed the binding between Alix and Syntenin, also support this view. Since tyrosine kinases are absent in yeast [143], tyrosine kinase-related regulatory mechanisms must also be absent. In yeast, no interaction between Alix and Syntenin was observed when a full-length sequence was used; however, truncated mutants lacking the Bro1 domain interacted with Syntenin via the Alix V domain [123]. These observations also support the notion that tyrosine kinase/SH2-dependent mechanisms induce conformational changes in Alix; however, further research is required.

In addition to Alix, the activation mechanism of the ESCRT machinery is still unclear. Alix interacts with Syntenin-Syndecan and upregulates exosome secretion [123]. Trimming the heparan sulfate modification at Syndecan enhances the amount of endosomal syndecan, leading to an increase in Syndecan-Syntenin-Alix-dependent exosome secretion [144]. These results proved that the Alix complex in the endosome regulates exosome formation. However, the mechanism by which the ESCRT machinery is activated remains unknown. c-Src appears to be a potent candidate for bridging the Alix complex to ESCRT activation. Accumulated c-Src with Alix in the limiting membrane may activate the ESCRT machinery by direct phosphorylation, and the phosphorylated protein initiates invagination and/or vesicle closure. Accompanied by phosphorylation, binding of the SH2 domain to phosphorylated protein(s) may also contribute to the activation of the machinery. Further analysis is required to elucidate the link between cargo and ESCRT activation.

Our findings provide new insights into c-Src regulation. Only activated c-Src is recruited to exosomes, indicating that activated c-Src is selectively

packaged into ILVs and finally expelled to the extracellular space. If c-Src is incorporated into the ILVs, substrates in the cytoplasm are inaccessible to c-Src, and phosphorylated proteins in the ILVs do not translocate to the cytoplasm; in other words, c-Src and the downstream phosphorylated proteins are sequestered from the cytoplasm. To maintain cell homeostasis, the spatiotemporal control of c-Src is critical. Aberrant activation of c-Src is linked to the transformation and malignant growth of cells [108]. However, it also induces exclusion from the epithelia through cell competition [145]. To prevent uncontrolled activation, cells develop multiple suppression mechanisms, including Csk phosphorylation at Y527 [9, 128, 129], proteasomal degradation by ubiquitination [5, 136-138], lipid raft/Cbp-dependent downregulation [8], and autophagy [6]. Exosomal packaging of c-Src can now be regarded as an acute downregulation mechanism that does not require a change in gene expression or large membrane remodeling.

The fact that Alix and Csk double-knockout cells showed abnormal CD63-positive endosome morphology also supports this view. If activated c-Src is transported to endosomes and cannot be loaded into vesicles, it will continue to be exposed to the cytoplasm and keep transmitting signals. The accumulation of c-Src at the surface of the limiting membrane, which cannot be loaded into the ILV, and the resulting excessive signaling may disrupt the endosome structure in double-knockout cells.

Legends for Figures

Fig.1. c-Src activity upregulates exosome secretion, and c-Src protein is localized in the exosome.

(a-c) Comparison of the amount of exosomes secreted from cells transformed by c-Src and control cells using nanoparticle tracking analysis (NTA) (a, b) and western blotting (WB) (c). The culture started with an equal number of cells, and no growth difference was observed among the compared cells. Exosomes derived from an equal number of cells were subjected to WB, reflecting the difference in exosome amounts. The levels of each protein in the whole-cell lysate are also shown.
(d, e) Effect of inhibiting SFKs by dasatinib on exosome secretion in HT29 cells analyzed using NTA (d) and WB (e). Exosome amount is shown as mean ± SE, **P<0.01.

Fig.2. Observation of c-Src localized in endosome/MVE.

(a) Co-localization of EGFP-fused c-Src with late endosome (Rab7) and MVE/exosome (CD9 and CD63) marker protein in $Csk^{-/-}$ cells. (b)Effect of membrane unbound mutation (G2A, a myristylation defect mutant) on c-Src localization and exosomal secretion. Exosome amount is shown as mean ± SE, **P<0.01.

Fig.3. c-Src null of function in each domain mutants failed to upregulate exosome release and localize in exosome.

The amount of exosomes secreted from cells transfected with wild-type c-Src and its domain-deficient mutants was quantified using NTA (a) and WB (c). The whole-cell lysate is also shown in (b). The culture started with an equal number of cells, and no growth difference was observed among the compared cells. Exosome amount is shown as mean ± SE, **P<0.01.

Fig.4. Alix directly binds to c-Src and regulates c-Src-dependent exosome secretion.

Molecular analysis of the binding between c-Src and Alix and its contribution to exosome regulation. (a) Effect of domain-deficient mutagenesis on c-Src-Alix interaction. (b) Schematic view of the structure of Alix. Locations of the mutated tyrosine residues are indicated. PRD: proline-rich domain. (c) Immunoprecipitation to analyze the binding ability of Alix mutants to wild-type c-Src. (d) Effect of shAlix on the exosome and rescue by mutants. Exosome amount is shown as mean ± SE, **P<0.01, ns: not significant.

Fig.5. Src/Alix enhances multivesicular endosome formation *in vitro*.

Results of *in vitro* MVE formation assay (a) Schematic of the assay procedure (b) Positive and negative controls of the assay (Left) and inhibitory effect of anti-CHMP4C as a neutralizing antibody for MVE formation (right). The results were normalized to those of the complete reaction or IgG control. (c) c-Src activity-dependent *in vitro* MVE formation. $Csk^{-/-}$/c-Src cytoplasm was set as the standard. (d) Alix-dependent MVE formation and the effect of Δ717 deletion in Alix. The results were normalized to those of the shRNA controls.

All the data were shown as mean ± SD, *P<0.05,**P<0.01.

Fig.6. Overactivation of c-Src in the absence of Alix disrupts CD63-stained but not LAMP1-stained endosome structure.

Immunocytochemistry of AlixKO cells staining endosome marker protein CD63 and LAMP1.

(a, b) Src-EGFP-expressing CskKO and Csk and Alix double knockout (DKO) cells were co-stained with anti-CD63 and anti-GFP antibodies. (b) shows the corresponding zoomed images of (a).

(c, d) Src-EGFP-expressing Csk unknocked out ($Csk^{flox/flox}$) and Alix knockout cells were co-stained with anti-CD63 and anti-GFP antibodies. (d) shows the corresponding zoomed images of (c).

(e, f) Src-EGFP-expressing CskKO and Csk and Alix double-knockout (DKO)

cells were co-stained with anti-LAMP1 and anti-GFP antibodies. (f) shows the corresponding zoomed images of (e).
(g) All cells stained in (a-f) were lysed and subjected to western blotting to confirm successful gene knockout and analyze the effect on protein amount and phosphorylation.

Materials and Methods

Cell culture

Csk knockout and other related cell lines of mouse embryonic fibroblasts [146] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), as described previously [147]. $Csk^{flox/flox}$ skin fibroblasts were established from K5-Cre $Csk^{flox/flox}$ mice[127] and cultured under the same conditions. HT29 cells were cultured in McCoy's 5a medium supplemented with 10% FBS. Cells were grown at 37°C in a humidified atmosphere containing 5% CO2.

For exosome collection culture, FBS was centrifuged at 100,000 × g overnight to remove FBS-derived contaminants, including bovine exosomes and other sediments. This FBS was named "exosome-depleted FBS."

Immunoblotting and Immunoprecipitation

Cell lysates were prepared in 2x SDS sample buffer (0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, and 20% (w/v) glycerol). The protein concentration was determined using the BCA assay (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The total protein concentration of the lysates was adjusted to 1 mg/ml with 0.01% (w/v) bromophenol blue (BPB) and 5%(v/v) β-mercaptoethanol containing 2x SDS sample buffer according to the results of the BCA assay. The lysates were then boiled at 95°C for 10 min and, if not used immediately, stored at -20°C until use. Equal amounts of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose or polyvinylidene difluoride (PVDF) membranes. The membranes were blocked and incubated with primary antibodies, followed by incubation with HRP-conjugated secondary antibodies. Signals from immunopositive bands were visualized using X-ray film and CCD imagers (LAS-4000 mini imaging analyzer; GE HealthCare, Piscataway, NJ, USA and WSE-6200 LuminoGraph; ATTO CORPORATION, Tokyo, Japan).

Immunoprecipitation was performed as previously described [148]. Briefly,

cells were lysed in ODG buffer (50 mM Tris-HCl, pH 8.0, 1 mm EDTA, 0.25 mM NaCl, 20 mM NaF, 1 mM Na3VO4, 1% Nonidet P-40, 2% octyl-d-glucoside, 5% glycerol and protease inhibitor cocktail; Nacalai Tesque, Inc., Kyoto, Japan), and the protein concentration was determined using the BCA assay. The control IgG or antibody was added to the lysate, adjusted to 1 mg/ml, and incubated with rotation for 1 h or overnight at 4°C. Protein G Sepharose (GE HealthCare) was then added to pull-down antibody-protein complexes, and finally, binding proteins were lysed in a 2x SDS sample buffer.

Retrovirus-mediated gene transfer

All gene transfers to achieve stable expression in this study were performed using a retrovirus-based method [149] as described previously [147].

Gene silencing by small-hairpin RNAs

Mouse Pdcd6ip/Alix shRNA vectors (TRCN0000119362, TRCN0000119363), control vectors, and lentiviral packaging mix were purchased from Sigma-Aldrich (St. Louis, MO, USA). These vectors were transfected into Lenti-X 293T cells (Takara Bio USA, Inc. Mountain View, CA, USA), and culture supernatants containing lentiviral particles were produced. The produced viral particles were concentrated using a Lenti-X concentrator (Takara Bio USA, Inc.) and used for infection. Knockdown efficacy was confirmed using immunoblotting.

Csk knockout by Cre induction to $Csk^{flox/flox}$ fibroblast

The pCre-Pac plasmid [150] was induced in $Csk^{flox/flox}$ fibroblasts using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. After lipofection for 24 h, cells were immediately treated with puromycine (4 μg/ml) for 24 h, and surviving cells were picked up to obtain single-cell clones. A successful knockout was confirmed using immunoblotting.

CRISPR/Cas9 genome editing

A pSpCas9(BB)-2A-GFP (PX458) plasmid [151] (Addgene plasmid # 48138) was used to clone sgRNAs targeting mouse Alix. Two sgRNA sequences targeting mouse Alix exon1 were designed using CRISPR direct software [152] (https://crispr.dbcls.jp/). The resultant targeting sequence pairs are shown below (from 5' to 3'). These pairs of nucleotides were annealed and ligated into a BbsI-digested plasmid. The EGFP sequence in the plasmid was replaced by mCherry as the subject cells expressed Src-EGFP.

sgAlix1 Fw: CACCGATCCAGCAGACGTACCCGAG
sgAlix1 Rv: AAACCTCGGGTACGTCTGCTGGATC
sgAlix2 Fw: CACCGCAGCAGACGTACCCGAGCGG
sgAlix2 Rv: AAACCCGCTCGGGTACGTCTGCTGC

The constructed plasmid was transiently induced in Src-EGFP-expressing $Csk^{flox/flox}$ cells using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. One day after lipofection, EGFP/mCherry double-positive cells were sorted into 96-well dishes in a single cell/well ratio using flow cytometry (BD FACSAria™II; BD, Franklin Lakes, NJ, USA). These clones were allowed to expand, and the knockout was confirmed using immunoblotting.

Reagents

Anti-GAPDH (6C5), anti-Tsg101 (C-1), anti-Csk (C-20), and anti-LAMP1 (1D4B) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-pY416 was from Cell Signaling Technology (Danvers, MA, USA). Anti-v-Src (Ab-1) antibody was obtained from Merck-Millipore (Burlington, MA, USA). Anti-Flotillin-1 and anti-AIP1/Alix antibodies were obtained from BD. Anti-GFP (A6455) and Alexa conjugated 2nd antibodies for immunocytochemistry were purchased from Thermo Fisher Scientific. Secondary antibodies conjugated to horseradish peroxidase (HRP) for immunoblotting were purchased from Fortis Life Sciences (Waltham, MA, USA).

Immunocytochemistry

Cells (1.5×10^4) were cultured for a day on a 15 mm round cover glass coated with fibronectin and then fixed with 4% paraformaldehyde. After permeabilization with a digitonin/PBS (0.05 mg/ml) solution, cells were blocked with Blocking One (Nacalai Tesque) for 1 h at room temperature. Primary antibodies were diluted 500-fold with a 1:1 solution of digitonin/PBS (0.05 mg/ml) and Can Get Signal solution 1(TOYOBO, Osaka, Japan), and cells were treated with the primary Ab solution for 1 h at room temperature. Alexa488 or 594 conjugated secondary antibodies were diluted 1000-fold with a 1:1 solution of digitonin/PBS (0.05 mg/ml) and Can Get Signal solution 2 (TOYOBO). After incubation with the primary Ab, the cells were washed and incubated with the secondary Ab solution for 30 min at room temperature. The cover glass was mounted on a glass slide with a drop of ProLong Gold Antifade (Thermo Fisher Scientific).
Observation and image acquisition was performed using confocal microscopy FV-3000 (Olympus, Tokyo, Japan).

Exosome purification

Exosome-producing culture started from 4.5×10^6 cells with 1% exosome-depleted FBS. After 24 h of culture, the culture supernatant was collected, and when comparing exosome secretion activity, the final cell number was measured using a Coulter Counter (Beckman Coulter, Brea, CA, USA) to confirm no growth difference among different conditions or cells. The supernatant was centrifuged at 1,000 × g and then applied to 0.22 μm filtration to remove cells and debris. After the stepwise large particle exclusion, the supernatant was centrifuged by 100,000 × g (SW41Ti rotor, Beckman Coulter, 24100 RPM) for 70 min at 4°C to produce exosome sediment. The sediment was suspended in 11 ml of HEPES buffer (20 mM HEPES-KOH pH 7.4) and centrifuged under the same conditions as for washing. Finally, the exosomal sediment was suspended in a 200 μl HEPES buffer. For western blotting, exosomes were lysed by directly adding 2x SDS sample buffer to the pellet.

Nanoparticle tracking analysis (NTA)

The concentration and size distribution of exosomes were measured using a Nanosight LM10V-HS (Malvern Panalytical, Malvern, UK). The exosome suspension was diluted to 50-100 folds to achieve a range between 10 and 100 particles per frame. One measurement consisted of five repetitions of 30 s movie acquisition with camera level 14, and the temperature was recorded at the end of each repeat. The recorded particle movement was tracked using Nanosight NTA 3.2 software with a detection threshold of 10.

In vitro multivesicular endosome formation assay

The assay was based on the method developed by Shurtleff et al. [47] except for the use of Oplophorus gracilirostris luciferase, that is, NanoLuc (Promega, Madison, WI, USA).

Cell Homogenization: The cells expressing the C-terminal NanoLuc-Src fusion protein were collected and suspended in DMEM containing 10% FBS, and the cell number was counted. For the assay, 1×10^7 cells were centrifuged by 500 × g for 5 min at 4°C, resuspended in 1x PBS for washing, and centrifuged again under the same conditions. The cell sediment formed by centrifugation was dissolved in 400 μl homogenization buffer. The cells were then passed through a 27-gauge needle 15 times, and the disrupted cell suspension was subjected to centrifugation at 1,500 × g for 10 min at 4°C to obtain the cytoplasm/endosome-containing supernatant. The collected supernatant was centrifuged at 15,000 × g for 10 min at 4°C to separate the cytoplasm from the endosomal membrane, which was produced as a pellet.

Membrane preparation: The membranous pellet described above was resuspended in 300 μl homogenization buffer with 150 μl of 1M LiCl for a wash. After centrifugation at 15,000 × g for 10 min at 4°C, the supernatant was removed, and the membrane solution was obtained by suspending the pellet in 300 μl of homogenization buffer.

Cytoplasm preparation: For further purification, the crude cytoplasmic supernatant produced after the homogenization was then applied to ultracentrifugation at 200,000 × g for 70 min at 4°C (TLA100.3 rotor, 69,000 RPM; Beckman Coulter), and the supernatant was collected as cytoplasm

solution.

***In vitro* reconstitution:** The complete reaction consists of 15 µl of membrane sol, 12 µl 5x incorporation buffer, 0.6 µl 100x Nluc substrate (Promega), 6 µl of 10 mM ATP, 25 mM Tris-HCl, pH 7.4, and 26.4 µl cytoplasm sol. The mixture was incubated at 30°C for 20 min to promote MVE formation. After the incubation, the mixture was centrifuged at 15,000 × g for 10 min at 4°C, and the supernatant was removed. To inactivate luciferase, which was not internalized into MVE, the pellet was suspended in a 0.5 mg/ml Trypsin solution and digested for 1 h at 4°C. The remaining luciferase activity was measured as a result of MVE formation.

Statistical Analysis

The exosome amount is shown as the mean ± SE. Other quantitative data are shown as mean ± SD. The Student's t-test and one-way ANOVA were performed using SPSS (IBM, Armonk, NY, USA). A single asterisk indicates significance at $P<0.05$, and a double asterisk indicates significance at $P<0.01$.

References

1. Jove, R. and H. Hanafusa, *Cell transformation by the viral src oncogene.* Annu Rev Cell Biol, 1987. **3**: p. 31-56.
2. Irby, R.B. and T.J. Yeatman, *Role of Src expression and activation in human cancer.* Oncogene, 2000. **19**(49): p. 5636-5642.
3. Ritchie, S., et al., *Transcription of the Human c-Src Promoter Is Dependent on Sp1, a Novel Pyrimidine Binding Factor SPy, and Can Be Inhibited by Triplex-forming Oligonucleotides.* Journal of Biological Chemistry, 2000. **275**(2): p. 847-854.
4. Oneyama, C. and M. Okada, *MicroRNAs as the fine-tuners of Src oncogenic signalling.* Journal of Biochemistry, 2015. **157**(6): p. 431-438.
5. Hakak, Y. and G.S. Martin, *Ubiquitin-dependent degradation of active Src.* Curr Biol, 1999. **9**(18): p. 1039-42.
6. Sandilands, E., et al., *Autophagic targeting of Src promotes cancer cell survival following reduced FAK signalling.* Nat Cell Biol, 2012. **14**(1): p. 51-60.
7. Kawabuchi, M., et al., *Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases.* 2000.
8. Oneyama, C., et al., *The lipid raft-anchored adaptor protein Cbp controls the oncogenic potential of c-Src.* Mol Cell, 2008. **30**(4): p. 426-36.
9. Nada, S., et al., *Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60c-src.* Nature, 1991. **351**(6321): p. 69-72.
10. Resh, M.D., *Myristylation and palmitylation of Src family members: the fats of the matter.* Cell, 1994. **76**(3): p. 411-3.
11. Okada, M., *Regulation of the SRC family kinases by Csk.* Int J Biol Sci, 2012. **8**(10): p. 1385-97.
12. Martin, G.S., *The hunting of the Src.* Nature Reviews Molecular Cell Biology, 2001. **2**(6): p. 467-475.
13. Sezgin, E., et al., *The mystery of membrane organization: composition,*

regulation and roles of lipid rafts. Nature Reviews Molecular Cell Biology, 2017. **18**(6): p. 361-374.

14. Arias-Salgado, E.G., et al., *Src kinase activation by direct interaction with the integrin β cytoplasmic domain.* Proceedings of the National Academy of Sciences, 2003. **100**(23): p. 13298-13302.
15. Xiao, R., et al., *Structural framework of c-Src activation by integrin β3.* Blood, 2013. **121**(4): p. 700-706.
16. Adolph, D.r., et al., *Deciphering the Cross Talk between hnRNP K and c-Src: the c-Src Activation Domain in hnRNP K Is Distinct from a Second Interaction Site.* Molecular and Cellular Biology, 2007. **27**(5): p. 1758-1770.
17. Moarefi, I., et al., *Activation of the Sire-family tyrosine kinase Hck by SH3 domain displacement.* Nature, 1997. **385**(6617): p. 650-653.
18. Trible, R.P., L. Emert-Sedlak, and T.E. Smithgall, *HIV-1 Nef Selectively Activates Src Family Kinases Hck, Lyn, and c-Src through Direct SH3 Domain Interaction*.* Journal of Biological Chemistry, 2006. **281**(37): p. 27029-27038.
19. Porter, M., et al., *Reciprocal Regulation of Hck Activity by Phosphorylation of Tyr527 and Tyr416.* Journal of Biological Chemistry, 2000. **275**(4): p. 2721-2726.
20. Saksela, K., G. Cheng, and D. Baltimore, *Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4.* The EMBO Journal, 1995. **14**(3): p. 484-491.
21. Ostareck-Lederer, A., et al., *c-Src-Mediated Phosphorylation of hnRNP K Drives Translational Activation of Specifically Silenced mRNAs.* Molecular and Cellular Biology, 2002. **22**(13): p. 4535-4543.
22. Lerner, E.C. and T.E. Smithgall, *SH3-dependent stimulation of Src-family kinase autophosphorylation without tail release from the SH2 domain in vivo.* Nature Structural Biology, 2002.
23. Thomas, J.W., et al., *SH2- and SH3-mediated Interactions between Focal Adhesion Kinase and Src.* Journal of Biological Chemistry, 1998. **273**(1): p. 577-583.

24. Alexandropoulos, K. and D. Baltimore, *Coordinate activation of c-Src by SH3- and SH2-binding sites on a novel p130Cas-related protein, Sin.* Genes & Development, 1996. **10**(11): p. 1341-1355.
25. Guappone, A.C. and D.C. Flynn, *The integrity of the SH3 binding motif of AFAP-110 is required to facilitate tyrosine phosphorylation by, and stable complex formation with, Src.* Molecular and Cellular Biochemistry, 1997. **175**(1): p. 243-252.
26. Nakamoto, T., et al., *Direct Binding of C-terminal Region of p130 to SH2 and SH3 Domains of Src Kinase (∗).* Journal of Biological Chemistry, 1996. **271**(15): p. 8959-8965.
27. Pellicena, P., K.R. Stowell, and W.T. Miller, *Enhanced Phosphorylation of Src Family Kinase Substrates Containing SH2 Domain Binding Sites*.* Journal of Biological Chemistry, 1998. **273**(25): p. 15325-15328.
28. Kalluri, R. and V.S. LeBleu, *The biology,function, and biomedical applications of exosomes.* Science, 2020. **367**(6478).
29. Shields, S.B. and R.C. Piper, *How Ubiquitin Functions with ESCRTs.* Traffic, 2011. **12**(10): p. 1306-1317.
30. Vietri, M., M. Radulovic, and H. Stenmark, *The many functions of ESCRTs.* Nature Reviews Molecular Cell Biology, 2019. **21**(1): p. 25-42.
31. Kajimoto, T., et al., *Ongoing activation of sphingosine 1-phosphate receptors mediates maturation of exosomal multivesicular endosomes.* Nature Communications, 2013. **4**(1).
32. van Niel, G., et al., *The Tetraspanin CD63 Regulates ESCRT-Independent and -Dependent Endosomal Sorting during Melanogenesis.* Developmental Cell, 2011. **21**(4): p. 708-721.
33. Chairoungdua, A., et al., *Exosome release of β-catenin: a novel mechanism that antagonizes Wnt signaling.* Journal of Cell Biology, 2010. **190**(6): p. 1079-1091.
34. Stuffers, S., et al., *Multivesicular Endosome Biogenesis in the Absence of ESCRTs.* Traffic, 2009. **10**(7): p. 925-937.
35. Trajkovic, K., et al., *Ceramide triggers budding of exosome vesicles*

into multivesicular endosomes. Science, 2008. **319**(5867): p. 1244-7.

36. Matsui, T., et al., *ALIX and ceramide differentially control polarized small extracellular vesicle release from epithelial cells.* EMBO reports, 2021. **22**(5).
37. Llorente, A., et al., *Molecular lipidomics of exosomes released by PC-3 prostate cancer cells.* Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 2013. **1831**(7): p. 1302-1309.
38. Skotland, T., K. Sandvig, and A. Llorente, *Lipids in exosomes: Current knowledge and the way forward.* Prog Lipid Res, 2017. **66**: p. 30-41.
39. de Gassart, A., et al., *Lipid raft-associated protein sorting in exosomes.* Blood, 2003. **102**(13): p. 4336-4344.
40. Jeppesen, D.K., et al., *Reassessment of Exosome Composition.* Cell, 2019. **177**(2): p. 428-445.e18.
41. Valadi, H., et al., *Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells.* Nature Cell Biology, 2007. **9**(6): p. 654-659.
42. Ekström, K., et al., *Characterization of mRNA and microRNA in human mast cell-derived exosomes and their transfer to other mast cells and blood CD34 progenitor cells.* Journal of Extracellular Vesicles, 2012. **1**(1): p. 18389.
43. Sork, H., et al., *Heterogeneity and interplay of the extracellular vesicle small RNA transcriptome and proteome.* Scientific Reports, 2018. **8**(1).
44. Shurtleff, M.J., et al., *Broad role for YBX1 in defining the small noncoding RNA composition of exosomes.* Proceedings of the National Academy of Sciences, 2017. **114**(43).
45. O'Brien, K., et al., *RNA delivery by extracellular vesicles in mammalian cells and its applications.* Nature Reviews Molecular Cell Biology, 2020. **21**(10): p. 585-606.
46. Huang, X., et al., *Characterization of human plasma-derived exosomal RNAs by deep sequencing.* BMC Genomics, 2013. **14**(1): p. 319.
47. Shurtleff, M.J., et al., *Y-box protein 1 is required to sort microRNAs into exosomes in cells and in a cell-free reaction.* eLife, 2016. **5**.

48. Temoche-Diaz, M.M., et al., *Distinct mechanisms of microRNA sorting into cancer cell-derived extracellular vesicle subtypes.* eLife, 2019. **8**.
49. Villarroya-Beltri, C., et al., *Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs.* Nature Communications, 2013. **4**(1).
50. Santangelo, L., et al., *The RNA-Binding Protein SYNCRIP Is a Component of the Hepatocyte Exosomal Machinery Controlling MicroRNA Sorting.* Cell Reports, 2016. **17**(3): p. 799-808.
51. Hobor, F., et al., *A cryptic RNA-binding domain mediates Syncrip recognition and exosomal partitioning of miRNA targets.* Nature Communications, 2018. **9**(1).
52. Zhang, L. and J.L. Wrana, *The emerging role of exosomes in Wnt secretion and transport.* Current Opinion in Genetics & Development, 2014. **27**: p. 14-19.
53. McGough, I.J. and J.-P. Vincent, *Exosomes in developmental signalling.* Development, 2016. **143**(14): p. 2482-2493.
54. Korkut, C., et al., *Trans-Synaptic Transmission of Vesicular Wnt Signals through Evi/Wntless.* Cell, 2009. **139**(2): p. 393-404.
55. Koles, K., et al., *Mechanism of Evenness Interrupted (Evi)-Exosome Release at Synaptic Boutons.* Journal of Biological Chemistry, 2012. **287**(20): p. 16820-16834.
56. Beckett, K., et al., *Drosophila S2 Cells Secrete Wingless on Exosome-Like Vesicles but the Wingless Gradient Forms Independently of Exosomes.* Traffic, 2013. **14**(1): p. 82-96.
57. Gross, J.C., et al., *Active Wnt proteins are secreted on exosomes.* Nature Cell Biology, 2012. **14**(10): p. 1036-1045.
58. Conboy, I.M., et al., *Rejuvenation of aged progenitor cells by exposure to a young systemic environment.* Nature, 2005. **433**(7027): p. 760-764.
59. Villeda, S.A., et al., *The ageing systemic milieu negatively regulates neurogenesis and cognitive function.* Nature, 2011. **477**(7362): p. 90-94.
60. Katsimpardi, L., et al., *Vascular and Neurogenic Rejuvenation of the*

Aging Mouse Brain by Young Systemic Factors. Science, 2014. **344**(6184): p. 630-634.

61. Villeda, S.A., et al., *Young blood reverses age-related impairments in cognitive function and synaptic plasticity in mice.* Nature Medicine, 2014. **20**(6): p. 659-663.
62. Sahu, A., et al., *Regulation of aged skeletal muscle regeneration by circulating extracellular vesicles.* Nature Aging, 2021. **1**(12): p. 1148-1161.
63. Atayde, Vanessa D., et al., *Exosome Secretion by the Parasitic Protozoan Leishmania within the Sand Fly Midgut.* Cell Reports, 2015. **13**(5): p. 957-967.
64. da Silva Lira Filho, A., et al., *Leishmania Exosomes/Extracellular Vesicles Containing GP63 Are Essential for Enhance Cutaneous Leishmaniasis Development Upon Co-Inoculation of Leishmania amazonensis and Its Exosomes.* Frontiers in Cellular and Infection Microbiology, 2022. **11**.
65. Henne, W.M., N.J. Buchkovich, and S.D. Emr, *The ESCRT pathway.* Dev Cell, 2011. **21**(1): p. 77-91.
66. Zhao, K., et al., *Extracellular vesicles secreted by Saccharomyces cerevisiae are involved in cell wall remodelling.* Communications Biology, 2019. **2**(1).
67. Ellen, A.F., et al., *Proteomic analysis of secreted membrane vesicles of archaeal Sulfolobus species reveals the presence of endosome sorting complex components.* Extremophiles, 2008. **13**(1): p. 67-79.
68. Makarova, K.S., et al., *Evolution of diverse cell division and vesicle formation systems in Archaea.* Nature Reviews Microbiology, 2010. **8**(10): p. 731-741.
69. Spang, A., et al., *Complex archaea that bridge the gap between prokaryotes and eukaryotes.* Nature, 2015. **521**(7551): p. 173-179.
70. Zaremba-Niedzwiedzka, K., et al., *Asgard archaea illuminate the origin of eukaryotic cellular complexity.* Nature, 2017. **541**(7637): p. 353-358.
71. Imachi, H., et al., *Isolation of an archaeon at the prokaryote–*

eukaryote interface. Nature, 2020. **577**(7791): p. 519-525.

72. Hatano, T., et al., *Asgard archaea shed light on the evolutionary origins of the eukaryotic ubiquitin-ESCRT machinery.* Nature Communications, 2022. **13**(1).
73. Whiteside, T.L., *The tumor microenvironment and its role in promoting tumor growth.* Oncogene, 2008. **27**(45): p. 5904-12.
74. Quail, D.F. and J.A. Joyce, *Microenvironmental regulation of tumor progression and metastasis.* Nat Med, 2013. **19**(11): p. 1423-37.
75. Mu, W., S. Rana, and M. Zöller, *Host Matrix Modulation by Tumor Exosomes Promotes Motility and Invasiveness.* Neoplasia, 2013. **15**(8): p. 875-IN4.
76. Hoshino, D., et al., *Exosome Secretion Is Enhanced by Invadopodia and Drives Invasive Behavior.* Cell Reports, 2013. **5**(5): p. 1159-1168.
77. Sung, B.H., et al., *Directional cell movement through tissues is controlled by exosome secretion.* Nat Commun, 2015. **6**: p. 7164.
78. Costa-Silva, B., et al., *Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver.* Nature Cell Biology, 2015. **17**(6): p. 816-826.
79. Ludwig, N., et al., *Exosomes from HNSCC Promote Angiogenesis through Reprogramming of Endothelial Cells.* Molecular Cancer Research, 2018. **16**(11): p. 1798-1808.
80. Qiu, J.-J., et al., *Exosomal Metastasis-Associated Lung Adenocarcinoma Transcript 1 Promotes Angiogenesis and Predicts Poor Prognosis in Epithelial Ovarian Cancer.* International Journal of Biological Sciences, 2018. **14**(14): p. 1960-1973.
81. Zhou, W., et al., *Cancer-Secreted miR-105 Destroys Vascular Endothelial Barriers to Promote Metastasis.* Cancer Cell, 2014. **25**(4): p. 501-515.
82. Tominaga, N., et al., *Brain metastatic cancer cells release microRNA-181c-containing extracellular vesicles capable of destructing blood–brain barrier.* Nature Communications, 2015. **6**(1).
83. Zeng, Z., et al., *Cancer-derived exosomal miR-25-3p promotes pre-metastatic niche formation by inducing vascular permeability and*

angiogenesis. Nature Communications, 2018. **9**(1).

84. Cassetta, L. and J.W. Pollard, *Targeting macrophages: therapeutic approaches in cancer.* Nature Reviews Drug Discovery, 2018. **17**(12): p. 887-904.
85. Yang, M., et al., *Microvesicles secreted by macrophages shuttle invasion-potentiating microRNAs into breast cancer cells.* Molecular Cancer, 2011. **10**(1): p. 117.
86. Chen, Q., et al., *Exosome-Mediated Crosstalk Between Tumor and Tumor-Associated Macrophages.* Frontiers in Molecular Biosciences, 2021. **8**.
87. Fabbri, M., et al., *MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response.* Proceedings of the National Academy of Sciences, 2012. **109**(31): p. E2110-E2116.
88. Tkach, M., et al., *Extracellular vesicles from triple negative breast cancer promote pro-inflammatory macrophages associated with better clinical outcome.* Proceedings of the National Academy of Sciences, 2022. **119**(17).
89. Plebanek, M.P., et al., *Pre-metastatic cancer exosomes induce immune surveillance by patrolling monocytes at the metastatic niche.* Nature Communications, 2017. **8**(1).
90. Sahai, E., et al., *A framework for advancing our understanding of cancer-associated fibroblasts.* Nature Reviews Cancer, 2020. **20**(3): p. 174-186.
91. Luga, V., et al., *Exosomes Mediate Stromal Mobilization of Autocrine Wnt-PCP Signaling in Breast Cancer Cell Migration.* Cell, 2012. **151**(7): p. 1542-1556.
92. Webber, J., et al., *Cancer Exosomes Trigger Fibroblast to Myofibroblast Differentiation.* Cancer Research, 2010. **70**(23): p. 9621-9630.
93. Webber, J.P., et al., *Differentiation of tumour-promoting stromal myofibroblasts by cancer exosomes.* Oncogene, 2014. **34**(3): p. 290-302.
94. Zhao, H., et al., *Tumor microenvironment derived exosomes pleiotropically modulate cancer cell metabolism.* eLife, 2016. **5**.

95. Sansone, P., et al., *Packaging and transfer of mitochondrial DNA via exosomes regulate escape from dormancy in hormonal therapy-resistant breast cancer.* Proceedings of the National Academy of Sciences, 2017. **114**(43).
96. Chen, D.S. and I. Mellman, *Elements of cancer immunity and the cancer–immune set point.* Nature, 2017. **541**(7637): p. 321-330.
97. Hou, J., M. Karin, and B. Sun, *Targeting cancer-promoting inflammation — have anti-inflammatory therapies come of age?* Nature Reviews Clinical Oncology, 2021. **18**(5): p. 261-279.
98. Wieckowski, E.U., et al., *Tumor-Derived Microvesicles Promote Regulatory T Cell Expansion and Induce Apoptosis in Tumor-Reactive Activated CD8+T Lymphocytes.* The Journal of Immunology, 2009. **183**(6): p. 3720-3730.
99. Clayton, A., et al., *Human Tumor-Derived Exosomes Down-Modulate NKG2D Expression.* The Journal of Immunology, 2008. **180**(11): p. 7249-7258.
100. Szczepanski, M.J., et al., *Blast-derived microvesicles in sera from patients with acute myeloid leukemia suppress natural killer cell function via membrane-associated transforming growth factor- 1.* Haematologica, 2011. **96**(9): p. 1302-1309.
101. Xiang, X., et al., *Induction of myeloid-derived suppressor cells by tumor exosomes.* International Journal of Cancer, 2009. **124**(11): p. 2621-2633.
102. Chen, G., et al., *Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response.* Nature, 2018. **560**(7718): p. 382-386.
103. Haderk, F., et al., *Tumor-derived exosomes modulate PD-L1 expression in monocytes.* Science Immunology, 2017. **2**(13): p. eaah5509.
104. Wolfers, J., et al., *Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming.* Nature Medicine, 2001. **7**(3): p. 297-303.
105. Andre, F., et al., *Malignant effusions and immunogenic*

tumour-derived exosomes. The Lancet, 2002. **360**(9329): p. 295-305.

106. Cho, J.-a., et al., *MHC independent anti-tumor immune responses induced by Hsp70-enriched exosomes generate tumor regression in murine models.* Cancer Letters, 2009. **275**(2): p. 256-265.
107. Graner, M.W., et al., *Proteomic and immunologic analyses of brain tumor exosomes.* The FASEB Journal, 2008. **23**(5): p. 1541-1557.
108. Yeatman, T.J., *A renaissance for SRC.* Nat Rev Cancer, 2004. **4**(6): p. 470-80.
109. Oneyama, C., et al., *Transforming Potential of Src Family Kinases Is Limited by the Cholesterol-Enriched Membrane Microdomain.* Molecular and Cellular Biology, 2009. **29**(24): p. 6462-6472.
110. Sandilands, E. and M.C. Frame, *Endosomal trafficking of Src tyrosine kinase.* Trends Cell Biol, 2008. **18**(7): p. 322-9.
111. Reinecke, J. and S. Caplan, *Endocytosis and the Src family of non-receptor tyrosine kinases.* Biomolecular Concepts, 2014. **5**(2): p. 143-155.
112. Tu, C., et al., *Endosomal-sorting complexes required for transport (ESCRT) pathway-dependent endosomal traffic regulates the localization of active Src at focal adhesions.* Proc Natl Acad Sci U S A, 2010. **107**(37): p. 16107-12.
113. Wortzel, I., et al., *Exosome-Mediated Metastasis: Communication from a Distance.* Developmental Cell, 2019. **49**(3): p. 347-360.
114. Hoshino, A., et al., *Tumour exosome integrins determine organotropic metastasis.* Nature, 2015. **527**(7578): p. 329-335.
115. Barros, F.M., et al., *Exosomes and Immune Response in Cancer: Friends or Foes?* Frontiers in Immunology, 2018. **9**.
116. Di Noto, G., et al., *C-src enriched serum microvesicles are generated in malignant plasma cell dyscrasia.* PLoS One, 2013. **8**(8): p. e70811.
117. Arscott, W.T., et al., *Ionizing Radiation and Glioblastoma Exosomes: Implications in Tumor Biology and Cell Migration.* Translational Oncology, 2013. **6**(6): p. 638-IN6.
118. Ji, H., et al., *Proteome profiling of exosomes derived from human primary and metastatic colorectal cancer cells reveal differential*

expression of key metastatic factors and signal transduction components. Proteomics, 2013. **13**(10-11): p. 1672-86.

119. Clark, D.J., et al., *Triple SILAC quantitative proteomic analysis reveals differential abundance of cell signaling proteins between normal and lung cancer-derived exosomes.* J Proteomics, 2016. **133**: p. 161-9.
120. Kamps, M.P., S.S. Taylor, and B.M. Sefton, *Direct evidence that oncogenic tyrosine kinases and cyclic AMP-dependent protein kinase have homologous ATP-binding sites.* Nature, 1984. **310**(5978): p. 589-592.
121. Bibbins, K.B., H. Boeuf, and H.E. Varmus, *Binding of the Src SH2 domain to phosphopeptides is determined by residues in both the SH2 domain and the phosphopeptides.* Mol Cell Biol, 1993. **13**(12): p. 7278-87.
122. Erpel, T., G. Superti-Furga, and S.A. Courtneidge, *Mutational analysis of the Src SH3 domain: the same residues of the ligand binding surface are important for intra- and intermolecular interactions.* EMBO J, 1995. **14**(5): p. 963-75.
123. Baietti, M.F., et al., *Syndecan-syntenin-ALIX regulates the biogenesis of exosomes.* Nat Cell Biol, 2012. **14**(7): p. 677-85.
124. Schmidt, M.H., I. Dikic, and O. Bogler, *Src phosphorylation of Alix/AIP1 modulates its interaction with binding partners and antagonizes its activities.* J Biol Chem, 2005. **280**(5): p. 3414-25.
125. Alexandropoulos, K., G. Cheng, and D. Baltimore, *Proline-rich sequences that bind to Src homology 3 domains with individual specificities.* Proceedings of the National Academy of Sciences, 1995. **92**(8): p. 3110-3114.
126. Hurley, J.H., *ESCRTs are everywhere.* EMBO J, 2015.
127. Yagi, R., et al., *C-terminal Src kinase controls development and maintenance of mouse squamous epithelia.* The EMBO Journal, 2007. **26**(5): p. 1234-1244.
128. Xu, W., S.C. Harrison, and M.J. Eck, *Three-dimensional structure of the tyrosine kinase c-Src.* Nature, 1997. **385**(6617): p. 595-602.

129. Young, M.A., et al., *Dynamic coupling between the SH2 and SH3 domains of c-Src and Hck underlies their inactivation by C-terminal tyrosine phosphorylation.* Cell, 2001. **105**(1): p. 115-26.
130. Rothman, J.H. and T.H. Stevens, *Protein sorting in yeast: mutants defective in vacuole biogenesis mislocalize vacuolar proteins into the late secretory pathway.* Cell, 1986. **47**(6): p. 1041-51.
131. Banta, L.M., et al., *Organelle assembly in yeast: characterization of yeast mutants defective in vacuolar biogenesis and protein sorting.* J Cell Biol, 1988. **107**(4): p. 1369-83.
132. Amerik, A.Y., et al., *The Doa4 deubiquitinating enzyme is functionally linked to the vacuolar protein-sorting and endocytic pathways.* Mol Biol Cell, 2000. **11**(10): p. 3365-80.
133. McCullough, J., et al., *Activation of the endosome-associated ubiquitin isopeptidase AMSH by STAM, a component of the multivesicular body-sorting machinery.* Curr Biol, 2006. **16**(2): p. 160-5.
134. Kyuuma, M., et al., *AMSH, an ESCRT-III associated enzyme, deubiquitinates cargo on MVB/late endosomes.* Cell Struct Funct, 2007. **31**(2): p. 159-72.
135. Row, P.E., et al., *The ubiquitin isopeptidase UBPY regulates endosomal ubiquitin dynamics and is essential for receptor down-regulation.* J Biol Chem, 2006. **281**(18): p. 12618-24.
136. Yokouchi, M., et al., *Src-catalyzed phosphorylation of c-Cbl leads to the interdependent ubiquitination of both proteins.* J Biol Chem, 2001. **276**(37): p. 35185-93.
137. Kim, M., et al., *Cbl-c suppresses v-Src-induced transformation through ubiquitin-dependent protein degradation.* Oncogene, 2004. **23**(9): p. 1645-55.
138. Laszlo, G.S. and J.A. Cooper, *Restriction of Src activity by Cullin-5.* Curr Biol, 2009. **19**(2): p. 157-62.
139. Hessvik, N.P. and A. Llorente, *Current knowledge on exosome biogenesis and release.* Cell Mol Life Sci, 2017.
140. Zhou, X., et al., *The CHMP4b- and Src-docking sites in the Bro1 domain are autoinhibited in the native state of Alix.* Biochem J, 2009.

418(2): p. 277-84.

141. Sun, S., et al., *Phosphorylation-Dependent Activation of the ESCRT Function of ALIX in Cytokinetic Abscission and Retroviral Budding.* Dev Cell, 2016. **36**(3): p. 331-43.
142. Sun, S., et al., *Erratum: ALG-2 activates the MVB sorting function of ALIX through relieving its intramolecular interaction.* Cell Discov, 2016. **2**: p. 16023.
143. Miller, W.T., *Tyrosine kinase signaling and the emergence of multicellularity.* Biochim Biophys Acta, 2012. **1823**(6): p. 1053-7.
144. Roucourt, B., et al., *Heparanase activates the syndecan-syntenin-ALIX exosome pathway.* Cell Res, 2015. **25**(4): p. 412-28.
145. Kajita, M., et al., *Interaction with surrounding normal epithelial cells influences signalling pathways and behaviour of Src-transformed cells.* J Cell Sci, 2010. **123**(Pt 2): p. 171-80.
146. Thomas, S.M., P. Soriano, and A. Imamoto, *Specific and redundant roles of Src and Fyn in organizing the cytoskeleton.* Nature, 1995. **376**(6537): p. 267-271.
147. Oneyama, C., et al., *Functional dissection of transformation by c-Src and v-Src.* Genes Cells, 2008. **13**(1): p. 1-12.
148. Rengifo-Cam, W., et al., *Csk defines the ability of integrin-mediated cell adhesion and migration in human colon cancer cells: implication for a potential role in cancer metastasis.* Oncogene, 2004. **23**(1): p. 289-297.
149. Akagi, T., K. Sasai, and H. Hanafusa, *Refractory nature of normal human diploid fibroblasts with respect to oncogene-mediated transformation.* Proceedings of the National Academy of Sciences, 2003. **100**(23): p. 13567-13572.
150. Taniguchi, M., et al., *Efficient production of Cre-mediated site-directed recombinants through the utilization of the puromycin resistance gene, pac: A transient gene-integration marker for ES cells.* Nucleic Acids Research, 1998. **26**(2): p. 679-680.
151. Ran, F.A., et al., *Genome engineering using the CRISPR-Cas9 system.*

Nature Protocols, 2013. **8**(11): p. 2281-2308.

152. Naito, Y., et al., *CRISPRdirect: software for designing CRISPR/Cas guide RNA with reduced off-target sites.* Bioinformatics, 2015. **31**(7): p. 1120-1123.

Figures

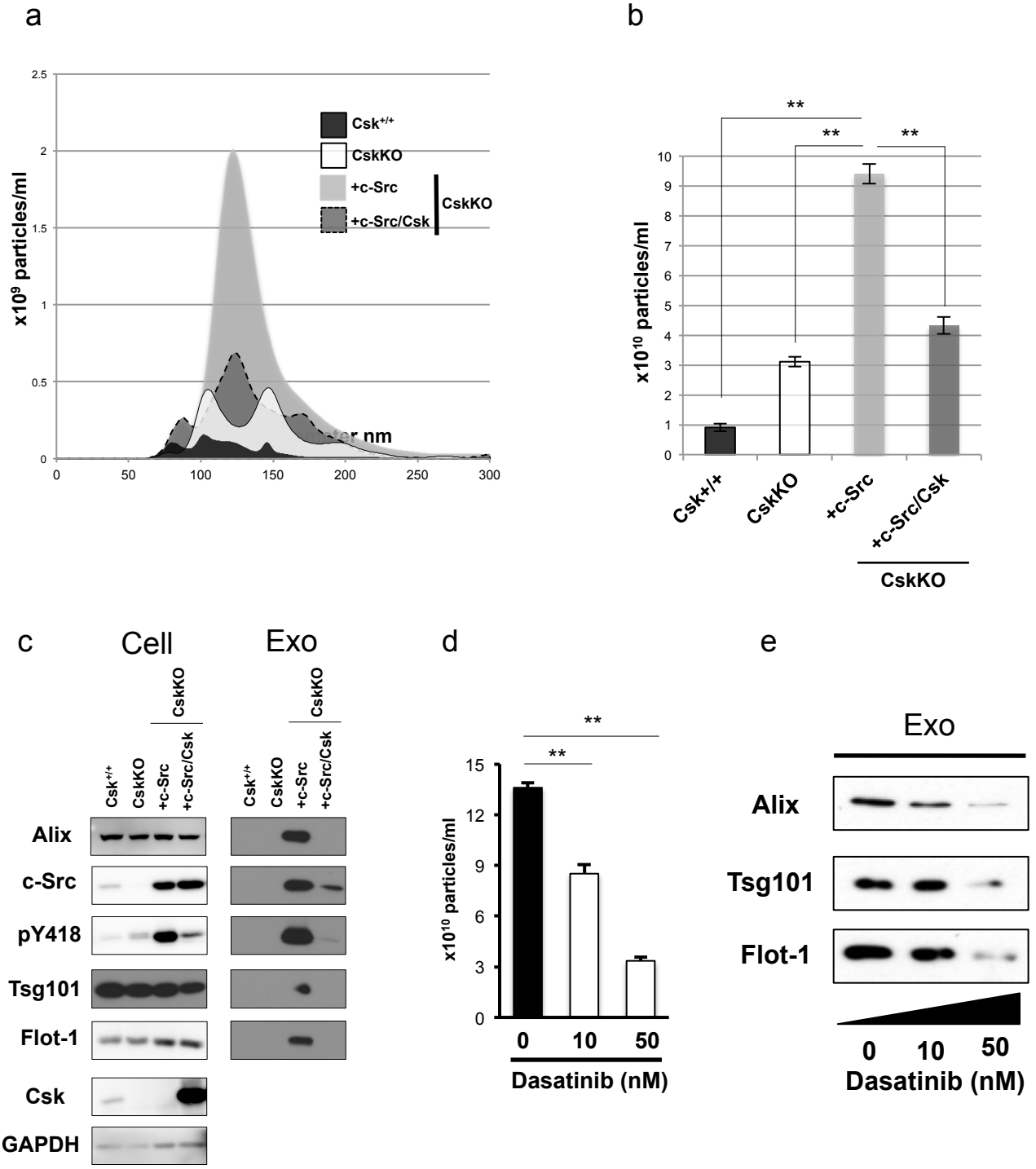


Fig.1. c-Src activity up-regulates exosome secretion and c-Src protein is localized in the exosome.

a

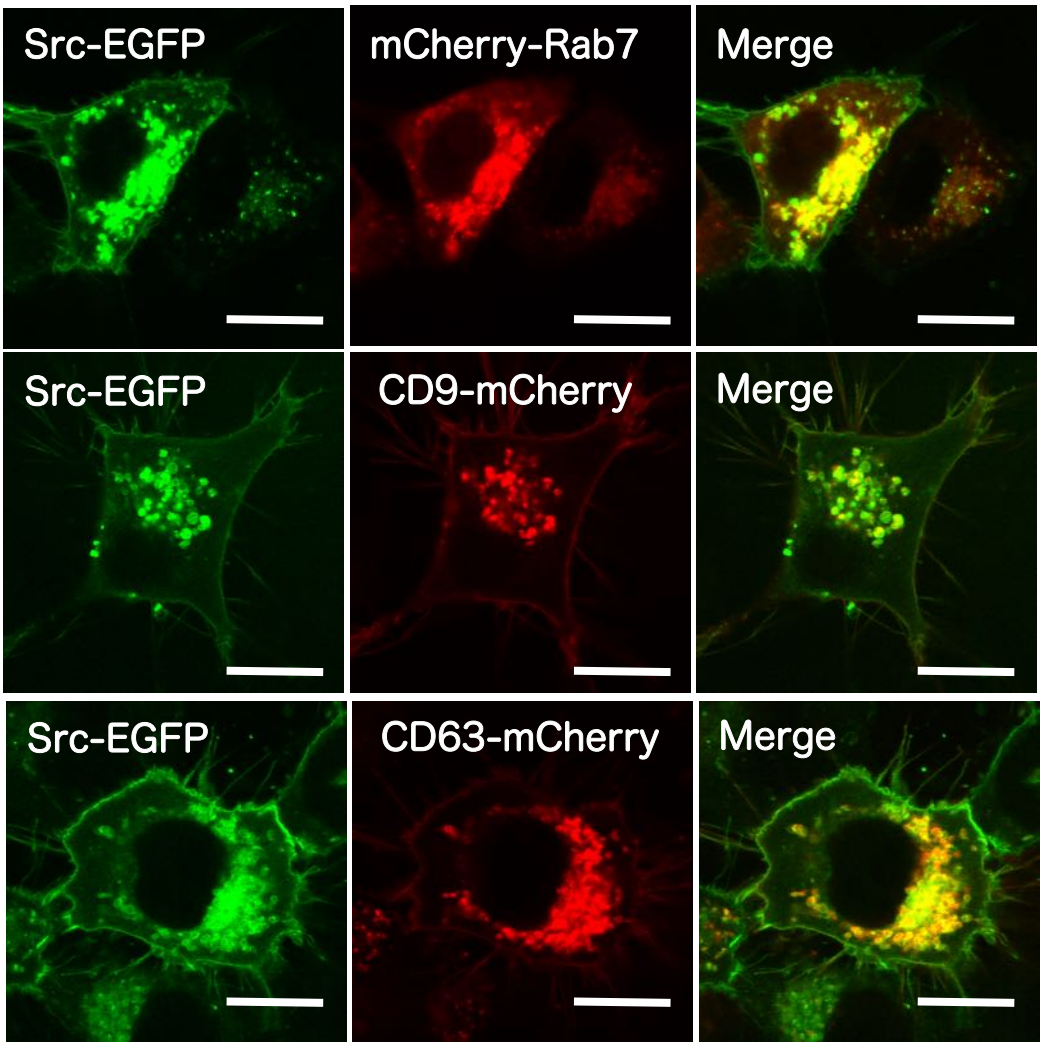
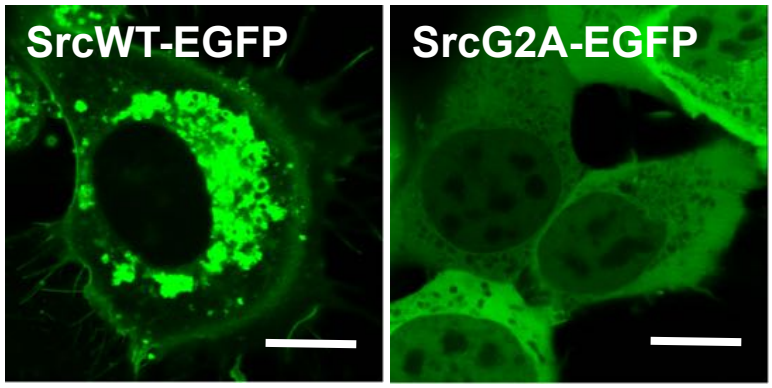
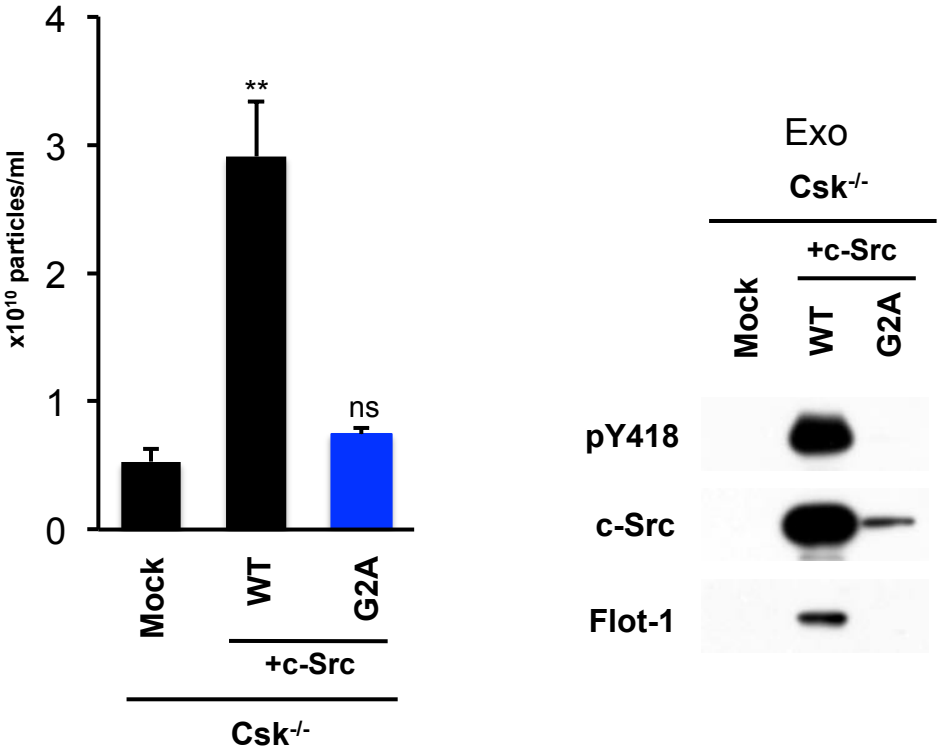


Fig.2. Observation of c-Src localizing in endosome/MVB.

b





(Fig.2. continued from the previous page)

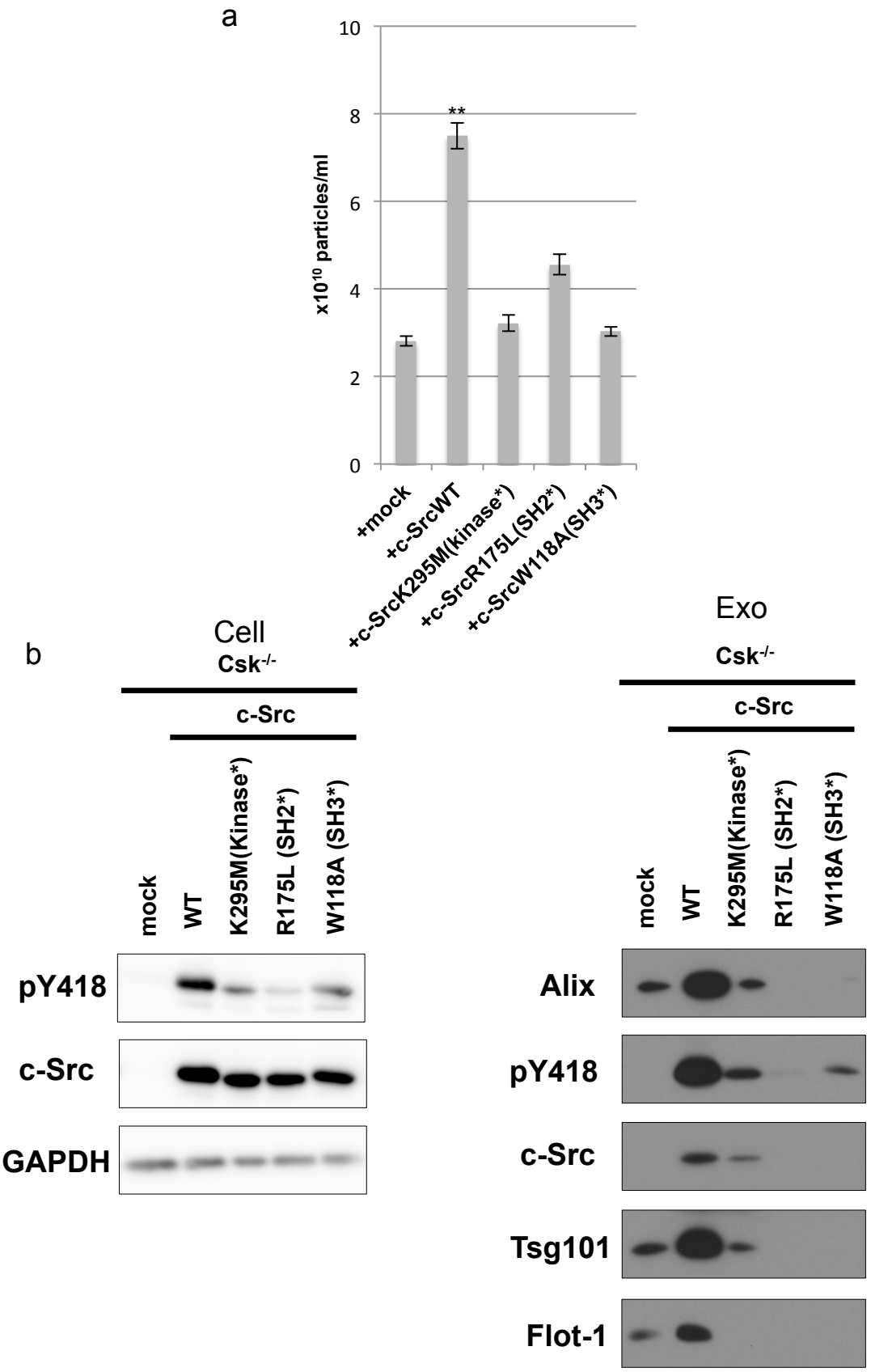


Fig.3. c-Src Null of function in each domain mutants failed to up-regulate exosome release and to be localized in exosome.

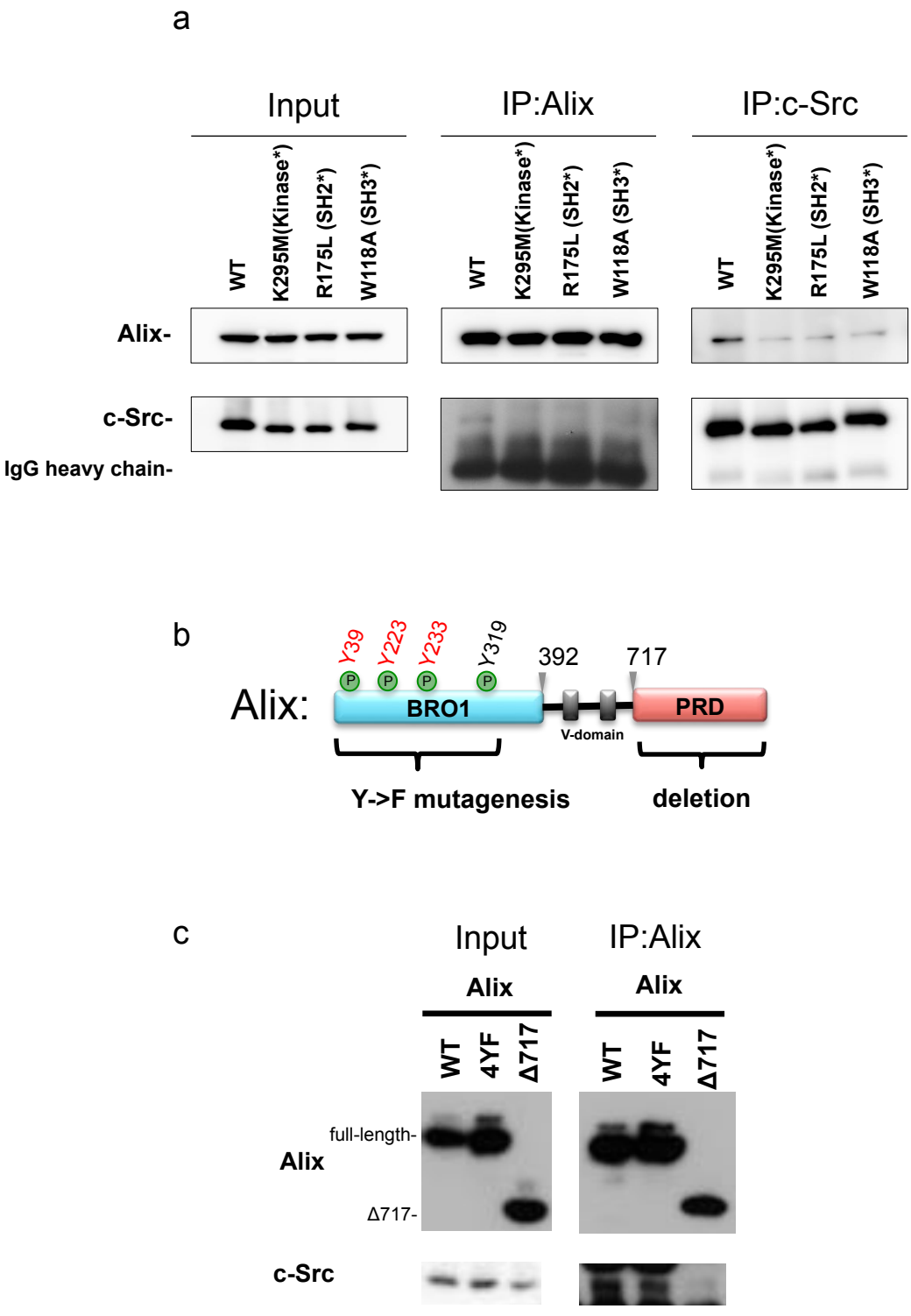
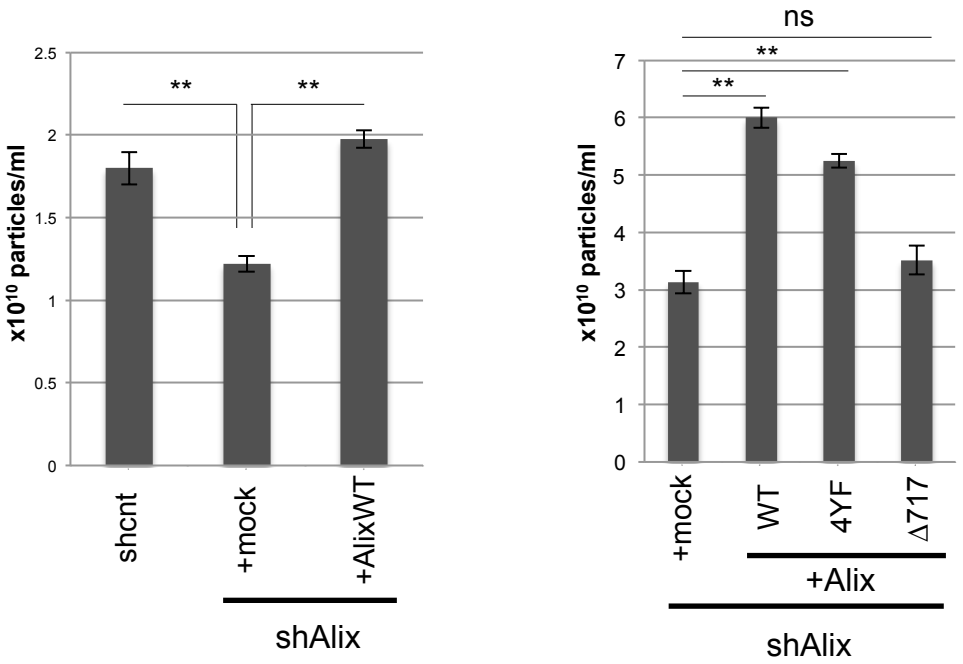
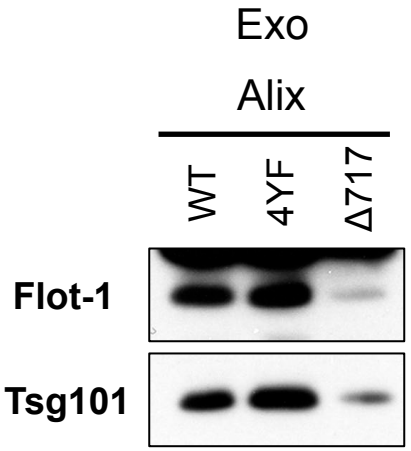


Fig.4. Alix directly binds to c-Src and regulates c-Src dependent exosome secretion.

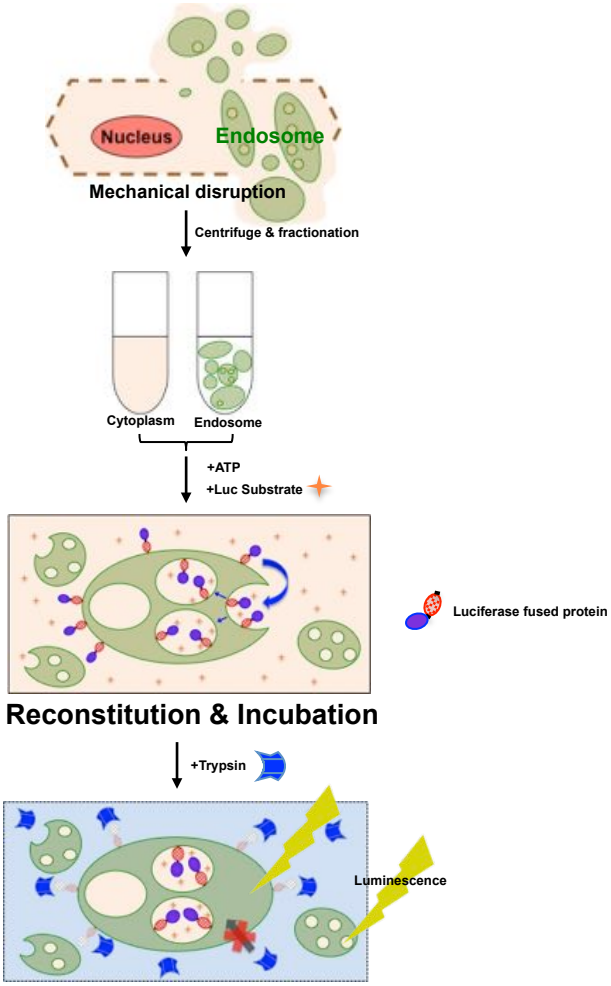
d





(Fig.4. continued from the previous page)

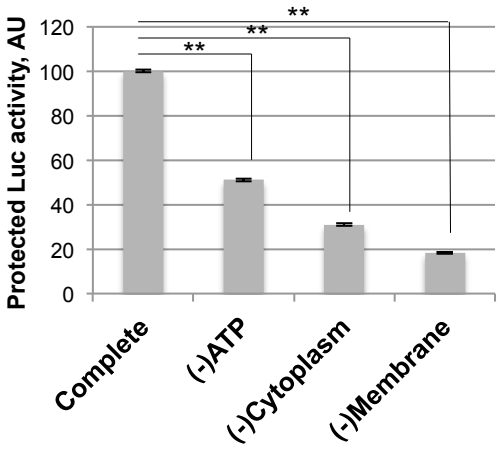
a

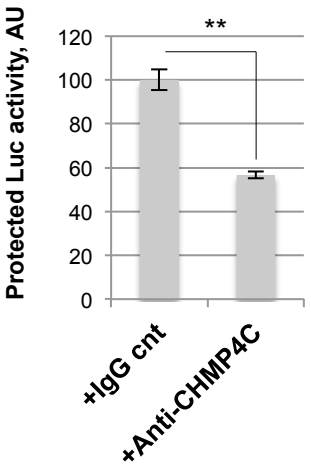


Protected Luciferasse activity = MVB formation

Fig.5. Src/Alix enhances multivesicular body formation *in vitro*.

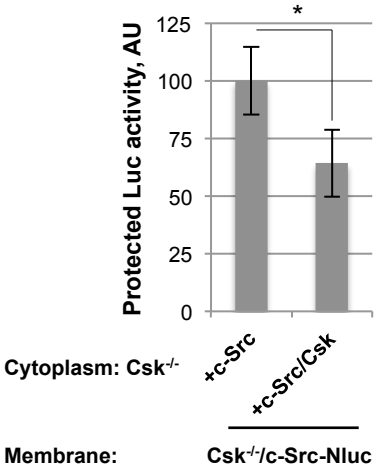
b



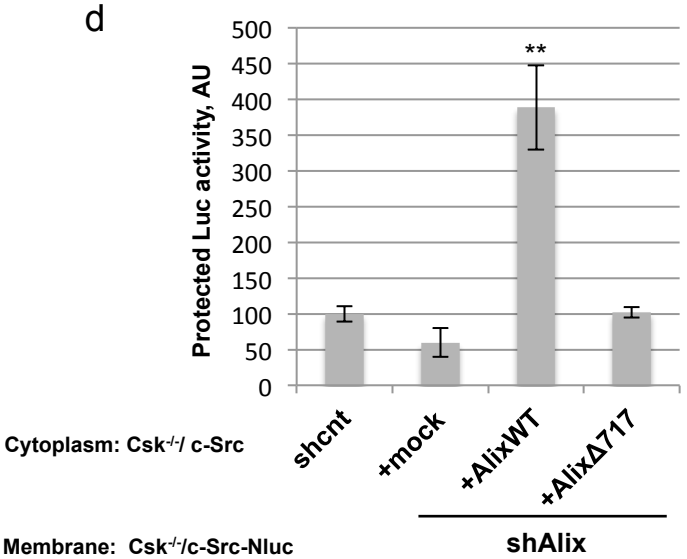


	Complete	(-)ATP	(-) Cytoplasm	(-)Membrane
Membrane	+	+	+	-
Cytoplasm	+	+	-	+
ATP	+	-	+	+

c



d



(Fig.5. continued from the previous page)

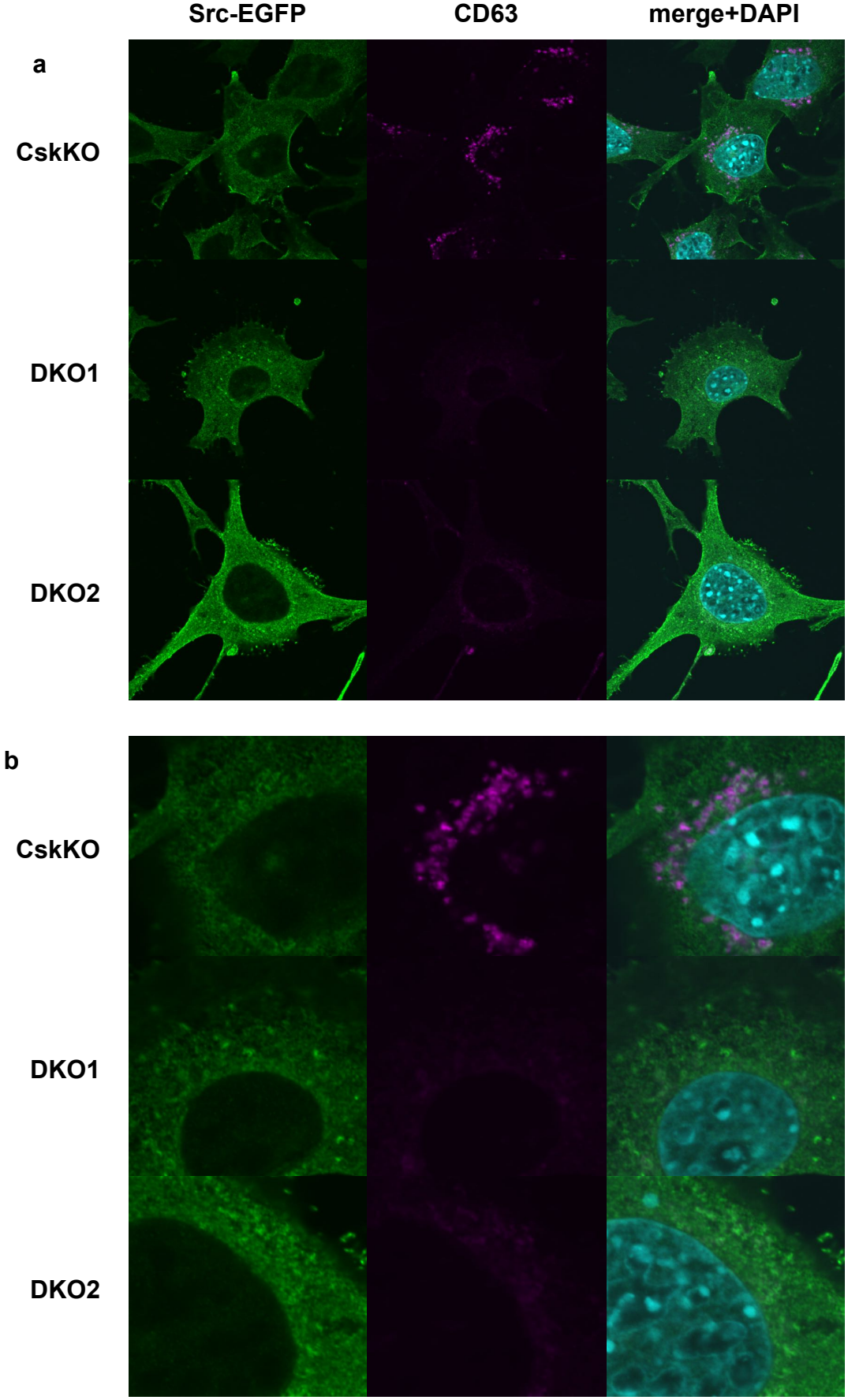
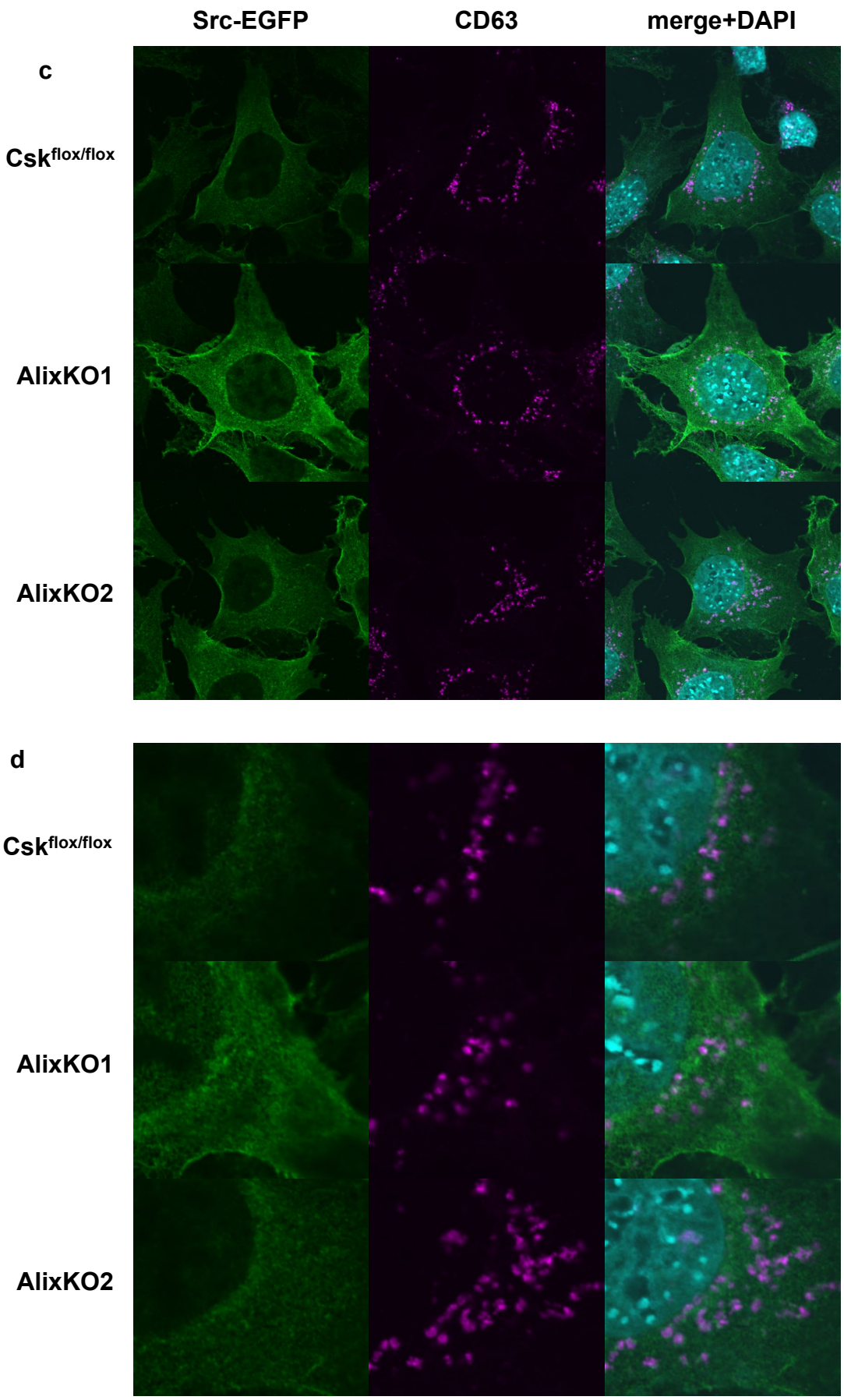
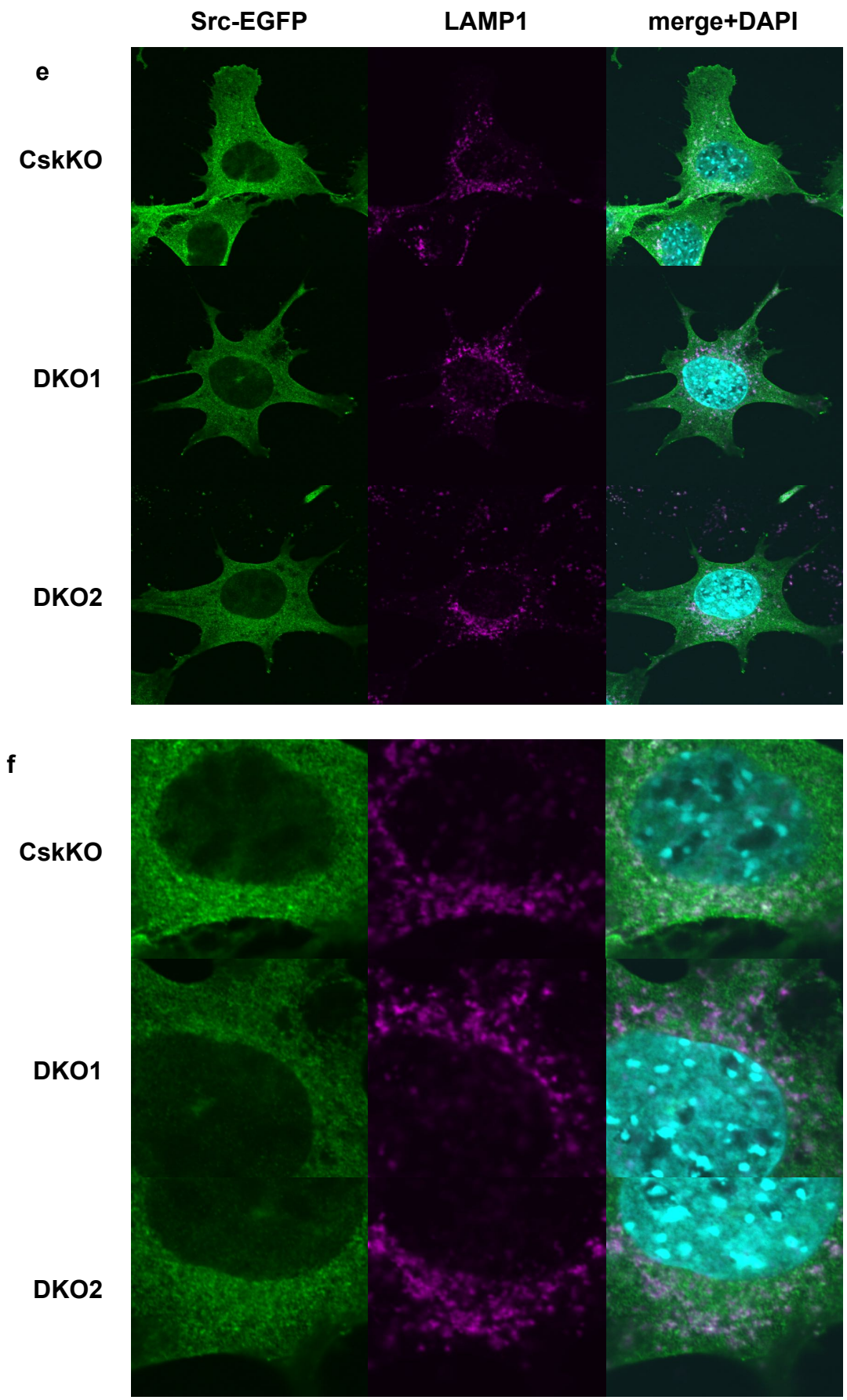


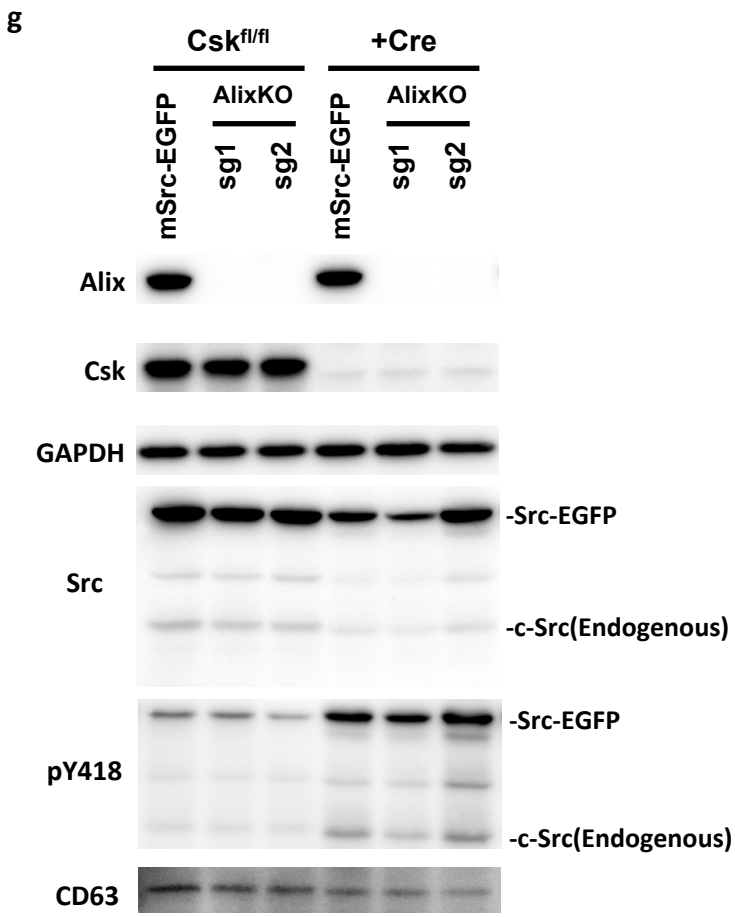
Fig.6. Over activation of c-Src in the absense of Alix disrupts CD63 stained, but not LAMP1 stained endosome structure.



(Fig.6. continued)



(Fig.6. continued)



(Fig.6. continued)