Syntenin mediates SRC function in exosomal cell-to-cell communication

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The cytoplasmic tyrosine kinase SRC controls cell growth, proliferation, adhesion, and motility. The current view is that SRC acts primarily downstream of cell-surface receptors to control intracellular signaling cascades. Here we reveal that SRC functions in cell-to-cell communication by controlling the biogenesis and the activity of exosomes. Exosomes are viral-like particles from endosomal origin that can reprogram recipient cells. By gain- and loss-of-function studies, we establish that SRC stimulates the secretion of exosomes having promigratory activity on endothelial cells and that syntenin is mandatory for SRC exosomal function. Mechanistically, SRC impacts on syndecan endocytosis and on syntenin–syndecan endosomal budding, upstream of ARF6 small GTPase and its effector phospholipase D2, directly phosphorylating the conserved juxtamembrane DEGSY motif of the syndecan cytosolic domain and syntenin tyrosine 46. Our study uncovers a function of SRC in cell–cell communication, supported by syntenin exosomes, which is likely to contribute to tumor–host interactions.

exosome | SRC | syndecan | syntenin | ARF6

The proto-oncogene SRC plays an important role in cell proliferation, invasion, motility, and signal transduction induced by a variety of external stimuli such as growth factors and integrins (1, 2). SRC displays a highly conserved modular structure that includes a lipophilic N terminus, followed by the regulatory SH3 and SH2 domains, a linker sequence, the tyrosine kinase domain, and the C-terminal regulatory tail. In the inactive conformation, the SRC C-terminal regulatory tail is phosphorylated at tyrosine 527 (in chicken or 530 in human), which mediates an intramolecular interaction with the SH2 domain and keeps the protein in closed conformation. When SRC is activated by extracellular stimuli, the C-terminal tyrosine is dephosphorylated by protein tyrosine phosphatases, opening the protein and ultimately resulting in the autophosphorylation of tyrosine 416 (in chicken and 419 in human) in the kinase domain (3, 4).

Although SRC has been envisioned for decades as a molecule controlling signaling in a cell-autonomous manner, recent studies challenged this concept. Mineo et al. illustrated that inhibition of SRC kinase activity, using SRC inhibitors, reduces the secretion of exosomes by chronic myeloid leukemia cells and reduces the ability of these exosomes to stimulate HUVEC cell migration and tube formation (5). Exosomes are a subset of secretory vesicles, with a size ranging from 40 to 100 nm in diameter, originating from endosomal compartments. They contain membrane-anchored receptors, adhesion molecules, signaling proteins, active oncogenes, and nucleic acids as cargo. By transferring their cargo to recipient cells, they can alter the behavior of these cells and are now envisioned as key players in intercellular communication (6, 7). Cancer cells, for example, appear to exploit exosomal pathways to promote tumor progression and to control premetastatic niche formation (8). We previously established that syntenin is implicated in the biogenesis of a subset of exosomes (9). Syntenin is a cytosolic adaptor that binds to the intracellular domain (ICD) of syndecans, a family of proteins that by virtue of their extracellular heparan sulfate chains interact with a plethora of signaling and adhesion molecules (10, 11). Syntenin also binds to ALIX, via $LYPX_nL$ motifs that resemble the late domain structures used by some viruses to egress from cells by budding (12). ALIX in turn connects the syntenin–syndecan complexes to the ESCRT machinery, playing a role in membrane budding and scission at the endosome and generating intraluminal vesicles (ILVs) that will be released as exosomes when multivesicular endosomes will fuse with the plasma membrane (9, 12–14). Known regulators of this process include, on the cytosolic side, the small GTPase ARF6 and the lipid-modifying enzyme PLD2 (13) and, on the luminal side, the endoglycosidase heparanase, trimming the syndecan heparan sulfate chains (14).

In a recent phosphoproteomic study on a colorectal model of SRC oncogenic activity, Sirvent et al. (15) showed that SRC gain-of-function is accompanied by an increase in the tyrosine phosphorylation of syntenin. Here, we show that SRC is a regulator of exosomal communication. SRC acts on syndecan–syntenin endosomal trafficking by virtue of its kinase activity, upstream of ARF6 and PLD2. Moreover, SRC controls the promigratory activity of exosomes in a way that strictly depends on syntenin.

Significance

Viral-like nanovesicles of endosomal origin, or "exosomes," are newly recognized vehicles of signals that cells use to communicate, in various systemic diseases, including cancer. Yet the molecular mechanisms that regulate the biogenesis and activity of exosomes remain obscure. Here, we establish that the oncogenic protein SRC stimulates the secretion of exosomes loaded with syntenin and syndecans, known co-receptors for a plethora of signaling and adhesion molecules. SRC phosphorylates conserved tyrosine residues in the syndecans and syntenin and stimulates their endosomal budding. Moreover, SRC-dependent exosomes have a promigratory activity that strictly depends on syntenin expression. This work sheds light on a function of SRC in cell-to-cell communication and mechanisms of exosome biogenesis and activity, with potential broad impact for physiopathology.

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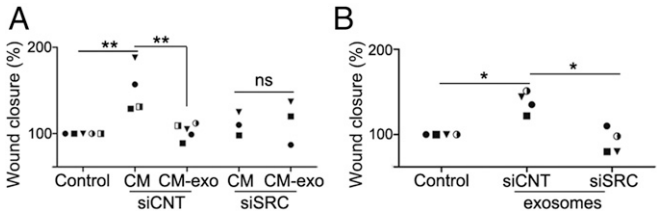

Fig. 1. SRC levels in MCF-7 donor cells determine the impact of exosomes on the migration of recipient HUVEC cells. (*A*) Conditioned media (CM) were collected from MCF-7 cells treated with nontargeting control RNAi (siCNT) or SRC RNAi (siSRC), grown in equal numbers for equal lengths of time. CM and corresponding CM that were depleted of exosomes by ultracentrifugation at 100,000 × *g* (CM-exo) were used to stimulate wound closures in monolayers of HUVEC cells. Closures are expressed as percentages, relative to the closure measured in the presence of DMEM (Control, taken as 100%). (*B*) Equivalent amounts of exosomes (50 μg as measured by Bradford assay) isolated from CM were used to stimulate wound closures. Exosomes were collected by ultracentrifugation and were resuspended in PBS. Cells were either depleted of SRC (siSRC) or treated with nontargeting RNAi (siCNT). Closures are expressed as percentages, relative to the closure measured in the presence of PBS (Control, taken as 100%) (ns, nonsignificant; $*P < 0.05$; $**P < 0.01$).

Results

SRC Supports the Promigratory Activity of Exosomes. Culture medium conditioned by MCF-7 human breast cancer cells stimulates the migration of HUVEC cells in wound-healing assays. Interestingly, this promigratory activity is lost upon exosome depletion or upon SRC silencing (Fig. 1*A*). Moreover, exosomes isolated from conditioned media by ultracentrifugation are able to stimulate the HUVEC wound closure, and SRC depletion abolishes their effect (Fig. 1*B*). Further purification of the exosomes by size exclusion chromatography (SEC) confirmed that exosomes, and not contaminating proteins, are supporting cell migration (Fig. S1 *A–F*). Of note, MCF-7 exosomes appear to stimulate migratory speed (Fig. S1*G*). These data clearly suggest a role for SRC in exosome activity. The syndecan–syntenin pathway accounts for a major fraction of the exosomes produced by MCF-7 cells (9). We therefore further investigated possible effects of SRC on this specific pathway.

SRC Regulates Syntenin Exosomes. Next, we tested the effect of SRC on the secretion of exosomal cargo. Compared with exosomes secreted by control cells, exosomes derived from SRC-depleted cells show a significant decrease in syntenin, ALIX, syndecan-1 C-terminal fragment (SDC1 CTF), and CD63 (Fig. 2*A* and Fig. S2*A*). However, not all exosomal markers are decreased, as exemplified by CD9 (Fig. 2*A*). Similar observations were obtained with other cell lines (Fig. S2*B*). The effects of SRC silencing were prevented by transfecting a vector encoding an SRC RNA resistant to the SRC siRNA (Fig. S2*C*), confirming the specificity of the siRNA effects. Nanoparticle tracking analyses (NTAs) reveal that SRC depletion decreases the total number of particles that is secreted (Fig. 2*B*). The size of the particles, in contrast, is not affected (Fig. S2*D*). Finally, SRC depletion also decreased the loading of exosomes with bona fide SDC-binding adhesion and signaling molecules (16, 17), such as EGFR, β1-integrin, and fibronectin, a deficit that is clearly apparent when loading equivalent numbers of exosomes from siCNT- and siSRC-treated cells (Fig. 2*C*).

To test the effects of SRC gain-of-function on exosome production, we overexpressed wild-type SRC, or a constitutively active SRC mutant, SRC Y527F. Both constructs induced an increase of the cellular levels of active SRC, as detected by

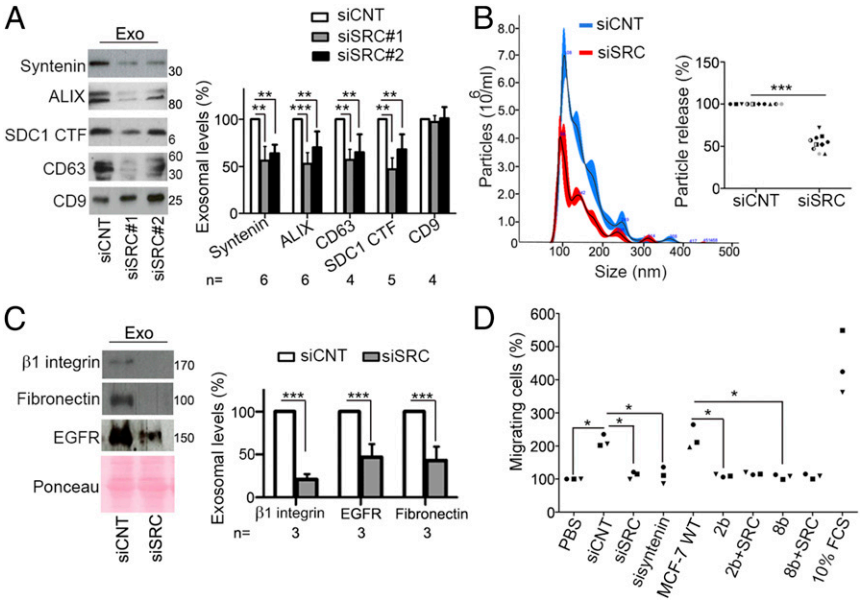

Fig. 2. SRC regulates exosome number and cargo, and syntenin is mandatory for the promigratory activity of SRC-dependent exosomes. (*A–C*) SRC effects on exosomes. Exosomes, isolated by ultracentrifugation, derived from control (siCNT) and SRC-depleted (siSRC) cells were analyzed by Western blot (*A* and *C*) using antibodies for several different markers, as indicated, or by NTA (*B*). Histograms represent signal intensities, mean ± SD, relative to signals measured in control samples (white bars), taken as 100% ($**P < 0.01$, $***P < 0.001$). The dot plot represents the total number of particles, relative to the number measured in control samples, taken as 100%, with each independent experiment being represented by a different symbol ($***P < 0.005$). (*D*) SRC exosome promigratory effects require syntenin. Equivalent amounts of exosomes (40 μg measured by Bradford assay) isolated (by ultracentrifugation) from media conditioned by MCF-7 cells were used to stimulate the migration of HUVEC cells across the membrane of a Boyden chamber. MCF-7 cells were either "wild-type" or "syntenin-null" (clones 2b and 8b, obtained by gene inactivation, using CRISPR/Cas9) and were treated with various RNAi or transfected to overexpress SRC, as indicated. Migration is expressed as a percentage, relative to the number of cells migrating when the lower chamber is filled with DMEM/F12 medium not supplemented with exosomes but with PBS (taken as 100%), and is also compared with the migration measured when the lower chamber is filled with media containing 10% of FCS (not depleted of exosomes) ($*P < 0.05$).

Western blotting with anti–phosphoTyr418-SRC (p-SRC) antibodies (Fig. S2 *E* and *F*). Gain of SRC in cells leads to an increase in the exosomal levels of proteins associated with the syntenin pathway (i.e., syntenin, ALIX, and SDC-CTF) but only for cells grown in serum-deprived conditions (Fig. S2 *E* and *F*). This suggests that SRC effects on exosomes might be "saturated" in cells grown in the presence of serum. As expected, SRC gain of function increases the total number, and not the size, of secreted particles in serum-deprived conditions (Fig. S2 *G* and *H*). Noteworthy, exosomes purified from MCF-7 cells cultured in the absence of serum and overexpressing wild-type SRC significantly increase the migration of HUVEC cells (Fig. S3 *A* and *B*).

Finally, we also tested the importance of syntenin for the biological effects of SRC exosomes. For that we used a Boyden chamber assay, stimulating HUVEC cells with exosomes derived from MCF-7 cells (Fig. S3*C*). In this assay, exosomes purified from MCF-7 cells (grown in serum-free media) that overexpress SRC significantly increase the migration of HUVEC cells, in a dose-dependent manner (Fig. S3*D*). This result is consistent with the wound-healing data (Fig. 1). Exosomes originating from SRC-depleted or syntenin-depleted MCF-7 cells (grown in the presence of serum) are significantly less promigratory than exosomes from control cells treated with nontargeting siRNA (Fig. 2*D*). Most importantly, exosomes originating from cells with (CRISPR/Cas9-mediated) cellular knockout of syntenin are depleted of syntenin cargo (Fig. S3*E*) and fail to stimulate the migration of HUVEC cells (Fig. 2*D*), even when these cells are overexpressing SRC (Fig. 2*D* and Fig. S3*E*), indicating that syntenin is mandatory for the SRC effects. We conclude SRC works on exosomes specifically through the syntenin pathway.

SRC Acts on SDC–Syntenin Endosomal Trafficking Upstream of ARF6–PLD2. We further clarified how SRC impacts on the biogenesis of exosomes along the endocytic pathway. Consistent with the effect of SRC on the internalization of various receptors (18), reversible cell-surface biotinylation experiments indicated that SRC knockdown decreases the net internalization of SDC1 and SDC4, the two SDCs expressed in MCF-7 cells (Fig. 3*A*). However, the internalization of CD63, another syntenin cargo and a bona fide exosomal marker, was not affected by SRC depletion (Fig. S4 *A* and *B*). Nevertheless, the late endosomal trafficking of CD63 is altered in SRC-depleted cells, as CD63 is observed to accumulate with late endosomal/lysosomal markers like Lamp1 and Lamp2 (Fig. S4 *C* and *D*). We also tested whether SRC has an impact on the endosomal budding of syntenin, a key process in the generation of (syntenin) exosomes (6, 12, 13, 19). For that, we took advantage of the RAB5(Q79L) mutant, which induces the formation of enlarged endosomes containing a large number of ILVs (20). Therefore, Cerulean (Ce)-RAB5(Q79L) was expressed along with monomeric Cherry (mCh)-syntenin, and we tested for the effect of SRC silencing on the accumulation of mCh inside endosomes outlined by Ce. Confocal microscopy revealed that the accumulation of mCh-syntenin inside the lumen of Ce-RAB5(Q79L) endosomes is strongly decreased in SRC-depleted cells (Fig. 3*B*). Consistently, in cells cultured in the absence of serum, overexpression of SRC, WT, or SRC Y527F significantly increases the filling of endosomes with mCh-syntenin (Fig. S4*E*), suggesting that SRC might support the budding process at endosomal membranes. We have recently shown that the small GTPase ARF6 and its effector phospholipase D2 (PLD2) control budding of syntenin into multivesicular endosomes and the biogenesis of exosomes (13). We therefore also investigated the effect of SRC gain of function in PLD2- and ARF6-deficient cells. Strikingly, overexpression of SRC Y527F was unable to rescue syntenin endosomal budding in PLD2-depleted cells (Fig. 3*C*) and also failed to rescue the exosomal levels of syntenin and ALIX in ARF6-depleted cells (Fig. S4*F*). On the contrary, the ARF6 fast cycling mutant (ARF6T157N) significantly increases the exosomal release of syntenin and ALIX in SRC-depleted cells (Fig. 3*D*). Consistently, we found that the ARF6 guanine nucleotide exchange factor (GEF) ARNO, which

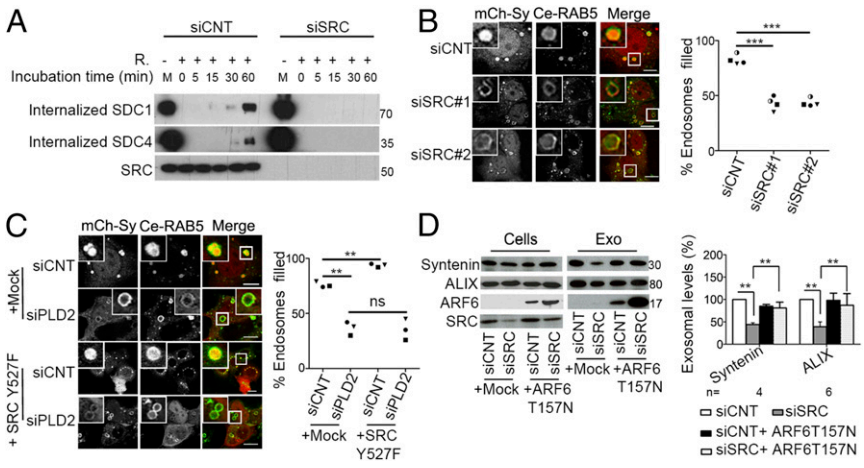

Fig. 3. SRC controls SDC endocytosis and syntenin endosomal budding upstream of ARF6–PLD2. (*A*) SDC internalization was monitored in reversible (by reduction, R) cell-surface biotinylation experiments. Western blots illustrate full-length SDC1 and SDC4 internalized over time and SRC depletion. M represents the membrane pool before internalization. Blots are representative of four independent SDC1 and two independent SDC4 experiments. (*B*) Confocal micrographs (*Left*) showing the accumulation of mCh-syntenin (mCh-Sy) inside the lumen of enlarged endosomes outlined by Ce-RAB5(Q79L) (Ce-RAB5) in control and SRC-depleted cells. (Scale bar, 10 μm.) Corresponding dot plot (*Right*) indicates the percentage of Ce-RAB5(Q79L) endosomes that is filled with mCh-syntenin, in the different conditions. Each quantification was performed considering at least 40–50 RAB5(Q79L) endosomes, in each experiment. (*C*) Confocal micrographs (*Left*) of MCF-7 cells that were treated with siCNT or PLD2 RNAi for 48 h and then transfected with expression vectors encoding SRC Y527F, Cerulean-RAB5(Q79L), and mCh-syntenin for 24 h. (Scale bar, 10 μm.) The dot plot (*Right*) represents the percentage of RAB5(Q79L) endosomes that is filled with mCh-syntenin, in the different conditions. (*D*) MCF-7 cells were treated with either SRC siRNA (siSRC) or nontargeting siRNA (siCNT) for 48 h and then transfected with an expression vector encoding the fast cycling mutant of ARF6 (ARF6-T157N) or mock-transfected with an empty vector. The cells and the exosomes produced by these cells were analyzed by Western blotting, using antibodies for several different markers, as indicated. Corresponding histograms (*Right*) represent signal intensities, mean ± SD, measured in exosomes, relative to signals measured in exosomes derived from the control cells (siCNT, followed by mock-transfection) (taken as 100%, white bars). All data were compiled from at least three independent experiments (ns, nonsignificant; $^{**}P < 0.01$; $^{***}P < 0.005$).

was described as a potential important GEF for ARF6 exosomal effects (13), also rescues the exosomal levels of syntenin and ALIX (Fig. S4*G*) and restores syntenin endosomal budding in SRC-depleted cells (Fig. S4*H*). These data indicate that SRC acts in exosome biogenesis upstream of ARF6/PLD2, controlling SDC internalization and syntenin endosomal budding.

Molecular Mechanisms Supporting SRC Effects on Exosomes. Further investigating the molecular mechanisms responsible, we found that SRC effects on exosomes depend on the kinase activity but are independent of the scaffolding function of SRC. Treatment of the cells with SRC inhibitor-1 significantly decreases the exosomal release of syntenin, ALIX, and SDC1-CTF and reduces exosome numbers (Fig. 4 *A* and *B*), without notable effects on the cellular levels of these marker proteins (Fig. S5*A*). SRC inhibitors also decrease the percentage of RAB5(Q79L) endosomes filled with mCh-syntenin (Fig. S5*B*). In contrast to WT SRC, a kinase-dead SRC fails to increase exosomal proteins (Fig. S5*C*) and fails to stimulate mCh-syntenin budding in endosomes (Fig. S5*D*). Unlike the kinase-dead mutation, mutations of the SRC SH2 and/or SH3 domain that abolish SRC binding to proteins containing phosphotyrosine and PXXP motifs, respectively, do not affect the capacity of SRC to increase the exosomal levels of syntenin or ALIX (Fig. S5*E*). Taken together, these results indicate that SRC kinase activity stimulates the biogenesis of syntenin-dependent exosomes.

We next searched for tyrosines in SDCs that would support SRC effects. When mutating each of the three conserved tyrosine residues in the ICD of SDC1 and SDC4, we found that only the Y-to-F mutations in the membrane-proximal DEGSY motifs (Y286F for SDC1 and Y180F for SDC4) significantly reduce the endosomal budding of mCh-syntenin and SDC ICD (Fig. 4*C* and Fig. S6 *A* and *B*). Conversely, SDCs with phosphomimetic forms of the DEGSY motif, SDC1 Y286E and SDC4 Y180E, lead to increased endosomal budding of SDC ICD and of mCh-syntenin (Fig. 4*D* and Fig. S6 *C* and *D*). Consistently, in vitro phosphorylation assays with recombinant proteins indicate that the tyrosine in the SDC cytoplasmic DEGSY motif is the major site of SRC phosphorylation (Fig. S6*E*). Taken together, these data indicate that SRC can impact on exosome production by phosphorylating the tyrosine in the SDC DEGSY motif. The dominant-negative effect of the Y-to-F mutation even suggests that phosphorylation of SDC in that motif represents a requirement for exosome formation.

We also performed label-free quantitative phosphoproteomics (Fig. S7 *A* and *B*) and observed that syntenin is phosphorylated on tyrosine 46 in MCF-7 cells, in an SRC-dependent manner (Fig. S7*C*). Moreover, we established that recombinant active SRC directly phosphorylates recombinant GST-syntenin (Fig. S7*D*). We thus tested the importance of this SRC phosphorylation site for syntenin activity and observed that introduction of a Y46F phosphodeficient mutation reduces syntenin endosomal budding, whereas the phosphomimetic Y46E mutation increases this budding (Fig. 4*E* and Fig. S7*E*). Consistently, when overexpressed, and compared with wild-type syntenin, syntenin Y46E significantly increases the exosomal release of syntenin, ALIX, and SDC1 CTF (Fig. S7*F*).

To assess to what extent SDC and/or syntenin phosphorylations by SRC support SRC exosomal function, we also performed endosomal budding experiments in the presence of the SRC inhibitor. The SRC inhibitor decreased mCherry-syntenin endosomal budding when either SDC or syntenin or when both SDC and syntenin were wild-type but not when both SDC and syntenin were

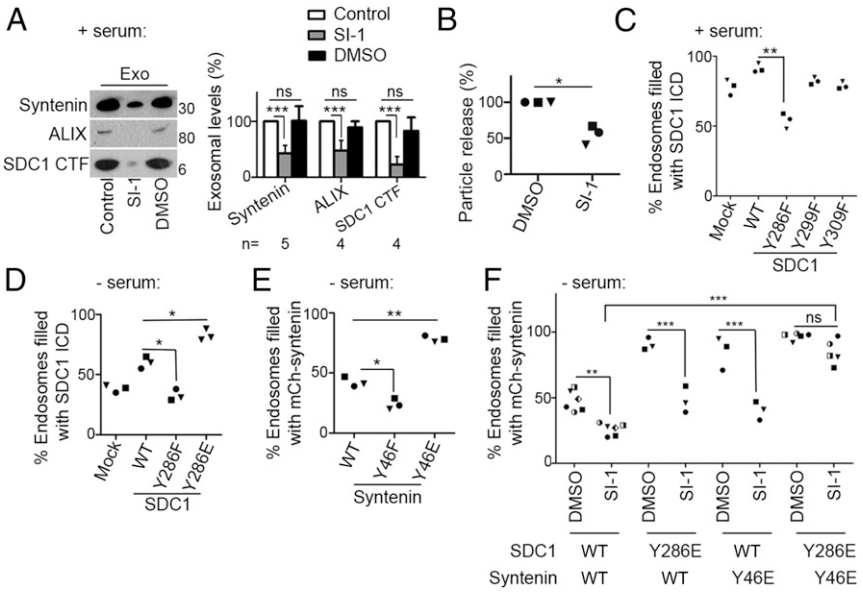

Fig. 4. SRC kinase activity and the tyrosine phosphorylations of both SDC and syntenin mediate SRC controls on endosomal budding and exosome release. (*A*) Equal numbers of MCF-7 cells, grown in the presence of serum, were left untreated (control) or treated with SRC inhibitor-1 (SI-1) or vehicle (DMSO). Proteins in exosomes (prepared by ultracentrifugation) were analyzed by Western blotting and quantified by densitometry, taking signals measured in exosomes derived from control MCF-7 cells as 100%. (*B*) Dot plot illustrating the total numbers of particles present in exosomal preparations from cells treated with SRC inhibitor-1, relative to the values obtained for preparations originating from cells treated with DMSO (taken as 100%), as determined by NTA. (*C* and *D*) Dot plot indicating the percentage of Ce-RAB5(Q79L) endosomes filled with SDC1 ICD, in MCF-7 cells transfected with empty expression vector (Mock), and in cells overexpressing WT or various tyrosine mutant forms of SDC1. Note that cells are either grown in serum (*C*) or starved for 24 h (*D*), before fixation. (*E*) Dot plot indicating the percentage of Ce-RAB5(Q79L) endosomes filled with mCh-syntenin, in MCF-7 cells overexpressing WT or various tyrosine mutant forms of mCh-syntenin, and starved for 24 h before fixation. (*F*) Dot plot indicating the percentage of Ce-RAB5(Q79L) endosomes filled with mCh-syntenin, in MCF-7 cells expressing either wild-type or phosphomimetic forms of SDC1 and mCh-syntenin, and treated with SRC inhibitor or vehicle (DMSO) in the combinations indicated. Cells were starved for 24 h before fixation. Each quantification (*D*–*F*) was performed considering at least 40–50 RAB5(Q79L) endosomes, in each experiment (ns, non-significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.005$).

phosphomimetic (Fig. 4*F* and Fig. S8*A*). This suggests that the phosphorylations of both SDC and syntenin by SRC are necessary and sufficient to support endosomal budding. Consistently, only the combination of SDC and syntenin phosphomimetics rescues the release of exosomal markers and the exosomal numbers in the presence of an SRC inhibitor (Fig. S8 *B* and *C*). Taken together, these data indicate that SRC controls exosome biogenesis by directly phosphorylating syntenin, at tyrosine 46, and SDC, at the tyrosine in the juxtamembrane-conserved DEGSY motif.

Discussion

In this present study, we identified a function of the oncogene SRC kinase as a regulator of exosome biogenesis and function. In addition, we provide insight into the events regulated by SRC at different levels of the endocytic pathway and identify the tyrosine phosphorylations of both SDC and syntenin as mediating these SRC effects (Fig. 5).

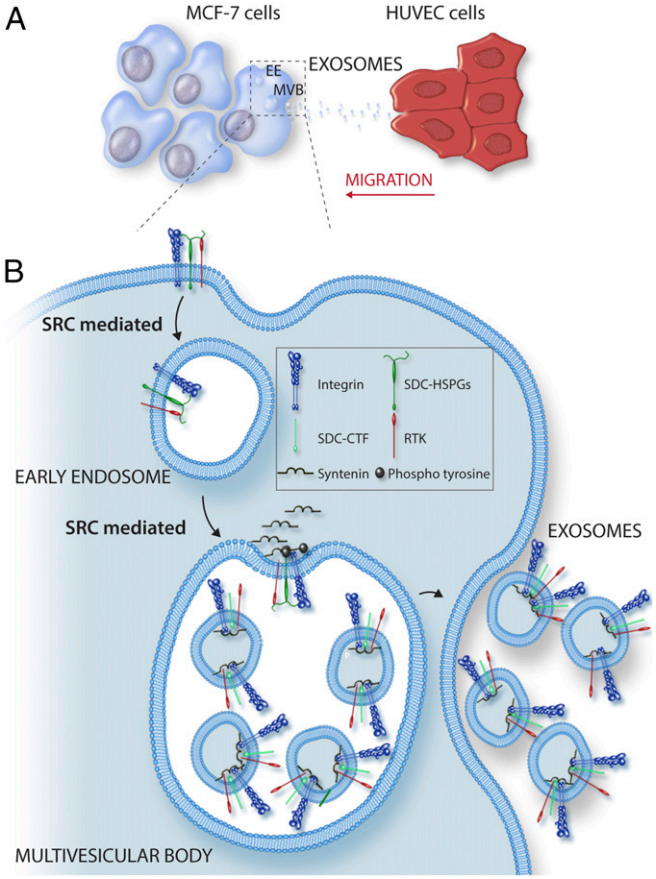

Fig. 5. Model recapitulating the relation between SRC kinase and the SDC–syntenin exosomal pathway, as revealed in the present study. (*A*) The conditioned medium of MCF-7 cells has promigratory effects on HUVEC cells. This activity is mediated by exosomes and is lost upon SRC depletion and increased upon SRC gain of function in exosome donor cells. In MCF-7 cells, syntenin is mandatory for SRC promigratory effects on HUVEC cells. (*B*) Processes under the control of SRC. SRC acts by favoring SDC internalization and SDC–syntenin endosomal budding (upstream of ARF6/PLD2). SRC is directly phosphorylating the conserved tyrosine in the membrane proximal DEGSY motif of SDC and thereby increases the recruitment and binding of syntenin at the endosomal membrane. However, the phosphorylation of syntenin by SRC, on the tyrosine at position 46, contributes to endosomal budding and exosome release. SRC thereby influences the number of exosomes that is released and controls the loading of exosomes with cargo such as integrins and receptor tyrosine kinases, known partners of SDCs.

We show that SRC is required for the internalization of the syndecans (Fig. 3*A*). This result is consistent with data from Chen and Williams obtained using a chimeric protein consisting of the ectodomain of the IgG Fc receptor and the transmembrane and cytoplasmic domain of SDC1 (21). Reduced internalization of SDCs observed upon SRC depletion might be directly linked to a lack of SDC tyrosine phosphorylation in the DEGSY motif, of all three cytosolic tyrosines the most important target of SRC kinase in vitro (Fig. S6*E*). Single tyrosine point mutations in SDC1 and SDC4, including phosphomimetic mutations, show that this tyrosine is crucial in endocytic processes, while the two other tyrosines seem to have no contribution (Fig. 4 *C* and *D* and Fig. S6 *A*–*D*). Noteworthy, our previous work suggests that SDCs recruit syntenin on endosomes (22). By supporting SDC internalization, SRC might thus promote the recruitment of syntenin on endosomes, a prerequisite for syntenin function in ILV budding and exosome secretion (12). SRC depletion, compromising SDC internalization, might, on the contrary, result in reduced syntenin levels at endosomes/late endosomes, possibly explaining the late endosomal accumulation of CD63 (Fig. S4 *C* and *D*), an exosomal tetraspanin directly interacting with syntenin (23).

SRC kinase might also function in exosome biogenesis downstream of SDC internalization, by stimulating the SDC–syntenin interaction on endosomes. Indeed, Morgan et al. (24) documented that SRC phosphorylation of the syndecan-4 DEGSY motif enhances SDC binding to syntenin, an observation confirmed in the present study (Fig. S9*A*). However, SRC effects on SDC internalization and/or SDC–syntenin interaction do not suffice to fully explain the effects of SRC on endosomal budding and exosomal release, as syntenin phosphorylation is also required (Fig. 4*F* and Fig. S8). SRC phosphorylates syntenin Y46 (Fig. S7 *A*–*D*). Phosphodeficient mutants, Y46F for syntenin and DEGSY/F for syndecan, display impaired endosomal budding, while phosphomimetics (Y46E and DEGSY/E) undergo significantly more budding (Fig. 4 *D* and *E* and Figs. S6 *C* and *D* and S7*E*). Favoring the formation of endosomal syndecan–syntenin complexes that in turn can interact with ALIX would support endosomal budding and consequently exosome biogenesis (12). It is nevertheless not clear at this stage how syntenin Y46 phosphorylation supports endosomal budding and exosome biogenesis/secretion. One possible explanation could be that SRC phosphorylation of syntenin Y46 helps in maintaining the protein in an open/active conformation as it has been found for Y56E (25), but BIAcore data are not supporting this possibility. Indeed, the syntenin Y46E phosphomimetic mutation does not improve the direct interaction of syntenin with ALIX (Fig. S9*B*). Noteworthy also, ALIX is documented as an SRC substrate (15, 26). Relieving an autoinhibitory intramolecular interaction in ALIX has been described as a critical step for ALIX to interact with its partner proteins and to participate in retroviral budding (27), a process reminiscent of syntenin exosomal budding (12). One could therefore conceive that SRC also brings ALIX in an "open/active" conformation to support SDC–syntenin ILV budding and exosome biogenesis, but we did not find evidence for ALIX phosphorylation by SRC in our phosphoproteomic analysis (Fig. S7 *B* and *C*). Moreover, SDC–syntenin phosphorylation seems to be sufficient to explain SRC effects (Fig. 4*F* and Fig. S8).

Interestingly, syntenin has been proposed to interact with SRC and to stimulate its activity (28, 29), suggesting that SRC–syntenin might function in a positive feedback loop. Even though SRC directly phosphorylates recombinant syntenin in our assays (Fig. S7*D*), we failed to obtain the evidence for a robust direct interaction of SRC with syntenin. Moreover, forms of SRC with mutations of the SRC SH2 and/or SH3 domains are as potent as wild-type SRC in stimulating exosomal releases of syntenin and ALIX (Fig. S5*D*), indicating direct interactions might be of little if any contribution.

Our data clearly indicate that, in endosomal budding and exosome biogenesis, SRC acts upstream of ARNO, ARF6, and

PLD2 (Fig. 3 *C* and *D* and Fig. S4 *F* and *H*). We have proposed that syntenin endosomal budding is dependent on direct interaction with phosphatidic acid (PA), the product of PLD2 (13). If SRC controls the efficiency of syntenin and ALIX function by favoring the open conformation and thereby ESCRT function in membrane budding, the observation that SRC functions upstream of PLD2 might indicate that PA is required for late rather than early stages of ILV formation.

From a functional point of view, our data show that SRC confers promigratory activity to breast tumor cell-derived exosomes (Figs. 1 and 2*D* and Figs. S1 *F* and *G* and S3 *A*–*D*). This is in line with a previous study (5) and suggests that the ability of SRC to confer promigratory activity to exosomes might be generic. The surprising observation is that the promigratory activity of "SRC exosomes" is strictly dependent on the presence of syntenin in donor cells (Fig. 2*D*). The question then is: What could be the nature of the cargo responsible for the promigratory effect? Exosomes originating from syntenin-null cells are not promigratory irrespective of the SRC levels and the presence of some SRC in exosomes (Fig. 2*D* and Fig. S3*E*), implying exosomal SRC by itself is not responsible for the promigratory effect. The active exosomal cargo could obviously be syntenin itself, as the protein has been described to control cell migration in many studies (30, 31). Cargo components directly connected to the syntenin–SDC exosome sorting machinery would also be obvious candidates. Among them are plausible candidates such as extracellular matrix protein, fibronectin, and integrins (32) (Fig. 2*C*). Knowing the functional role of exosomes that are loaded with fibronectin for directional cell migration (33) and that of exosomes loaded with specific integrins for tissue-specific metastasis (34), it would be interesting to verify whether the SRC–syntenin exosomal pathway contributes to these processes.

Exosomes appear to be important for many aspects of cancer progression, including tumor growth, metastasis, angiogenesis, and immune surveillance (8). SRC but now also syntenin are emerging as important players in these processes (35, 36). Importantly, the present study clearly indicates that syntenin is mandatory for SRC effects. While the present study certainly supports the use of SRC inhibitors in exosomal diseases including metastatic cancer, it also supports the idea that syntenin inhibitors would be valuable and possibly more specific pharmacological tools. Indeed, they would act downstream of SRC in exosome biogenesis and possibly have less side effects.

Materials and Methods

Detailed information is provided in *SI Materials and Methods*.

Data were analyzed using Student's *t* test or, in case of more than two experimental groups, by one- or two-way ANOVA, followed by post hoc multiple-comparison tests (Bonferroni's) using GraphPad Prism.

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