Endosomal-sorting complexes required for transport (ESCRT) pathway-dependent endosomal traffic regulates the localization of active Src at focal adhesions

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Active Src localization at focal adhesions (FAs) is essential for cell migration. How this pool is linked mechanistically to the large pool of Src at late endosomes (LEs)/lysosomes (LY) is not well understood. Here, we used inducible *Tsg101* gene deletion, TSG101 knockdown, and dominant-negative VPS4 expression to demonstrate that the localization of activated cellular Src and viral Src at FAs requires the endosomal-sorting complexes required for transport (ESCRT) pathway. *Tsg101* deletion also led to impaired Src-dependent activation of STAT3 and focal adhesion kinase and reduced cell migration. Impairment of the ESCRT pathway or Rab7 function led to the accumulation of active Src at aberrant LE/LY compartments followed by its loss. Analyses using fluorescence recovery after photo-bleaching show that dynamic mobility of Src in endosomes is ESCRT pathway-dependent. These results reveal a critical role for an ESCRT pathway-dependent LE/LY trafficking step in Src function by promoting localization of active Src to FAs.

cell migration | endosomal sorting complexes required for transport | focal adhesion | late endosomal trafficking

The nonreceptor tyrosine kinase cellular Src (c-Src) is critical for signal transduction downstream of growth factor and cell matrix attachment receptors (reviewed in ref. 1). All eight mammalian Src family kinases (SFKs) have a conserved N-terminal lipid modification and an Src homology 2 (SH2), an SH3, and a tyrosine kinase domain. Intramolecular binding of the SH2 domain to a phosphorylated C-terminal inhibitory tyrosine keeps c-Src in an inactive conformation (2). Absence of the C-terminal tail, including the inhibitory tyrosine, makes viral Src (v-Src) constitutively active and transforming (3). Notably, aberrantly elevated activity of c-Src correlates with the development and progression of multiple types of human cancers (reviewed in ref. 4).

Src activity is closely associated with its subcellular localization. The unique N-terminal lipid modification localizes Src to multiple membranous compartments including the plasma membrane, the late endosomes (LEs) and focal adhesions (FAs); the active pool, a small fraction of the total Src, is localized mostly to FAs and plasma membrane (5, 6) but is essential for Src-mediated cell migration and proliferation (7). Recent studies indicate that Src exchanges dynamically between endosomal compartments and FAs (6, 8, 9). Specifically, the delivery of activated Src to the cell periphery was shown to require an LE-localized Rho family protein, RhoB (6), but not the highly homologous early endosome-localized RhoD (9). In contrast, palmitoylated SFKs such as Fyn, which do not localize to LEs/late lysosomes (LYs), require RhoD for their translocation to the plasma membrane (9). Although the pool of Src localized within the intracellular compartments is mostly inactive, the unique localization of Src to the LE/LY compartment correlates with higher transforming ability (9–11). How the polarized distribution of active Src is maintained and how the late endosomal localization plays a positive role in Src function has remained unclear.

Endocytic trafficking has emerged as a critical mechanism for controlling the duration and strength of signaling via sorting of internalized receptors and their associated signaling complexes for recycling or lysosomal degradation. Ubiquitinated receptors are targeted to LYs for degradation in a process executed by a group of highly conserved protein complexes known as "endosomal-sorting complex required for transport" (ESCRT) proteins (reviewed in ref. 12). In the LE/multivesicular body (MVB) compartment, ubiquitinated proteins are sorted for delivery to the intraluminal vesicles for final degradation within the LYs. Tsg101, an ortholog of yeast Vps23 and a key component of mammalian ESCRT-I, helps deliver ubiquitinated proteins to other ESCRT complexes (13). The AAA ATPase Vps4 catalyzes the disassembly and return of ESCRT complexes from LE/MVB membranes to the cytoplasm, ensuring continuity of the process (14–16). Recently, additional roles of ESCRT proteins at endosomes have been identified. For example, depletion of TSG101 (17) or overexpression of ESCRT-III component charged MVB protein 6 (CHMP6) (18) or dominant-negative form of VPS4(dnVPS4) (16) halt the normal recycling of transferrin receptor, suggesting that the endosomal functions of the ESCRT pathway are not limited to targeting ubiquitinated receptors to internal vesicles of MVBs.

We used conditional deletion of a floxed *Tsg101* gene, siRNA-mediated TSG101 depletion, and expression of dnVPS4 to demonstrate the importance of ESCRT-dependent endosomal transport in active Src translocation to its critical sites of cellular function in FAs. Furthermore, we show that Tsg101 is required for Src-mediated cell migration and downstream cellular signaling. These data reveal a role of the ESCRT machinery in regulating proper subcellular localization and function of active Src.

Results

To address the role of Tsg101 in Src function, we used immortal mouse embryonic fibroblasts (MEFs) derived from $Tsg101^{fl/fl}$ mouse embryos (19). Infection of $Tsg101^{fl/fl}$ MEFs with a Cre-expressing adenovirus eliminated Tsg101 expression within 2 to 3 d. Immunoblotting with anti-pY416-Src, which detects all active Src (20),

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showed that deletion of *Tsg101* elevated the level of the active form of Src (Fig. 1*A*). Elevated levels of active Src also were observed during the first 2 d of Cre infection of v-Src–expressing *Tsg101*$^{fl/fl}$ MEFs; later, as Tsg101 was fully depleted, the active Src level decreased (Fig. 1*B*). Strong active Src signals were observed in aberrant (lysosomal-associated membrane protein 1, LAMP-1$^+$) LE/LY vesicles of *Tsg101*-deleted v-Src MEFs (Fig. 1*C*), perhaps reflecting sustained activity of growth factor receptors or reduced degradation of active Src in LYs (13, 21). In contrast, little or no colocalization between active Src and LAMP-1 was seen in control v-Src MEFs (Fig. 1*C*). Because Src activity is critical for cell migration (7, 22), we assessed the impact of *Tsg101* deletion on cell migration. Surprisingly, even at early time points when the overall level of active Src was increased, Cre-mediated deletion of *Tsg101* in both *Tsg101*$^{fl/fl}$ (Fig. 1*D*) and v-Src-*Tsg101*$^{fl/fl}$ MEFs (Fig. 1*E*) reduced the cell migration in a wound-healing assay.

Because active Src localization to FAs is critical for cell migration (7), we evaluated the effect of *Tsg101* depletion on the localization of active Src using confocal microscopy. Active Src was detected predominantly in FAs (located at the bottom of cells and mostly at cell periphery) and costained with paxillin and vinculin in control v-Src *Tsg101*$^{fl/fl}$ MEFs. However, active Src signals at these locations diminished drastically 3 d after *Tsg101* deletion (Fig. 2*A* and *SI Appendix*, Fig. S1), as demonstrated clearly by a significant reduction in the ratio of the fluorescence intensity of pY416-Src/paxillin in *Tsg101*-deleted v-Src *Tsg101*$^{fl/fl}$ MEFs vs. the ratio in control cells (Fig. 2*A*). Additionally, the number of invadopodia (large actin-rich protrusions at the ventral side of cells and mostly near the center of v-Src–transformed cells) and staining of invadopodia-associated active Src were reduced by deletion of *Tsg101* (*SI Appendix*, Fig. S1*C*). In v-Src *Tsg101*$^{fl/fl}$ MEFs infected with GFP-Cre for 2 d, a significant fraction of active Src (42.5 ± 24%) could be detected at confocal planes above the cell bottom; in contrast, most active Src (86.4 ± 4%) was concentrated at the bottom of control v-Src MEFs where FAs and invadopodia reside (Fig. 2*B*). To exclude the possibility that the effect of *Tsg101* deletion was peculiar to MEFs, we depleted TSG101 in HeLa cells using a previously validated siRNA (23). TSG101-depleted HeLa cells showed a slightly higher level of active Src than control siRNA-transfected cells (Fig. 2*C*). In control cells, active Src concentrated at FAs located at the termini of stress fibers near the cell bottom; in contrast, in TSG101-depleted HeLa cells active Src staining signal was reduced at FAs and was increased at higher confocal planes (Fig. 2*D*). These data suggest that Tsg101 is required for proper localization of active Src at FAs in fibroblasts and epithelial cells.

In a complementary approach, ectopic expression of mutant VPS4A^{K173Q}-GFP (24) (dnVPS4) reduced the active Src signals at the cell bottom to less than 30% of those in nontransfected cells. In some dnVPS4-expressing cells, active Src was detected at large, aberrant LAMP-1$^+$ vesicles (Fig. 3*A*). As in previous studies (25, 26), *Tsg101* deletion or dnVPS4 expression altered the morphology of LAMP-1$^+$ vesicles from compact to aberrantly enlarged vacuolar structures (*SI Appendix*, Fig. S2*A*). This effect was specific to LEs/LYs, because Tsg101 deficiency had little effect on the morphology, size, or distribution of early endosome-antigen-1$^+$ early endosomes, which did not colocalize with c-Src-GFP (*SI Appendix*, Fig. S2*B*).

These results prompted us to explore further the function of the LE/LY compartments in the subcellular distribution of active Src. As expected, the expression of Rab7^{T22N}-GFP, which perturbs the function of LEs/LYs by blocking membrane-fusion events (27), altered the morphology of LAMP-1$^+$ vesicles (*SI Appendix*, Fig. S3). Similar to dnVPS4, overexpressed Rab7^{T22N}-GFP reduced the localization of active Src at the bottom (where FAs and invadopodia reside) of v-Src MEFs to about 36% of that of nontransfected control cells (Fig. 3*B* and *SI Appendix*, Figs. S3 and S4). Occasionally, we observed some Rab7^{T22N}-GFP–expressing v-Src MEFs with increased intracellular and reduced peripheral active Src staining (Fig. 3*B*). In contrast, Rab11^{S25N}-GFP, which interferes with the function of recycling endosomes, did not affect the peripheral localization of active Src (*SI Appendix*, Fig. S4*B*). Because Rab7 regulates the function of LEs/LYs through a mechanism distinct from that of ESCRT proteins, these results suggest that normal function of the LE/LY compartment is required for FA localization of active Src.

Next, we investigated whether subcellular localization and dynamics of total Src protein also were altered when the ESCRT pathway was perturbed using c-Src-GFP, which has been established as a faithful reporter of endogenous c-Src dynamics (28). Transfected c-Src-GFP localized at the plasma membrane, in the cytoplasm, and on LAMP-1$^+$ vesicles as described previously (Fig. 4*A*). Notably, the colocalization of c-Src-GFP with LAMP-1 was increased dramatically in *Tsg101*-deleted or dnVPS4-expressing MEFs (Fig. 4*A*).

To determine if the accumulation of active Src or c-Src-GFP at LE/LYs reflects altered dynamics of Src trafficking, we used fluorescence recovery after photo-bleaching (FRAP) analysis (29) to estimate the mobility of c-Src-GFP in live cells. Consistent with a previous report (8), the mobility and intensity of c-Src-GFP was highest in the perinuclear region and declined gradually toward the

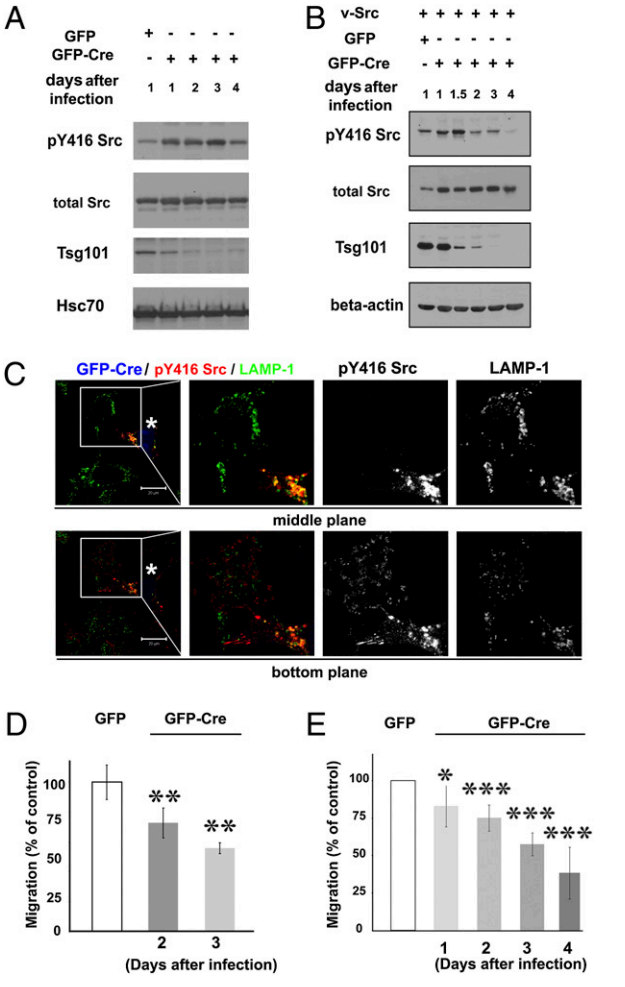

Fig. 1. Deletion of *Tsg101* impairs cell motility. (*A* and *B*) GFP or GFP-Cre adenovirus-infected *Tsg101*$^{fl/fl}$ MEFs (*A*) or v-Src *Tsg101*$^{fl/fl}$ MEFs (*B*) were analyzed by immunoblotting. (*C*) Noninfected and 2-d GFP-Cre–infected (*) v-Src V$^{fl/fl}$ MEFs were cultured together and stained with pY416Src and LAMP-1 antibodies. Serial Z-section confocal images show staining at bottom (*Lower row*) and middle (*Upper row*) planes. (Scale bars: 20 μm.) (*D* and *E*) Migratory ability of GFP- or GFP-Cre–infected *Tsg101*$^{fl/fl}$ MEFs (*D*) and v-Src *Tsg101*$^{fl/fl}$ MEFs (*E*) were assayed with the wound-healing assay. Cell motility of GFP-infected cells was set as 100% (means ± SD). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

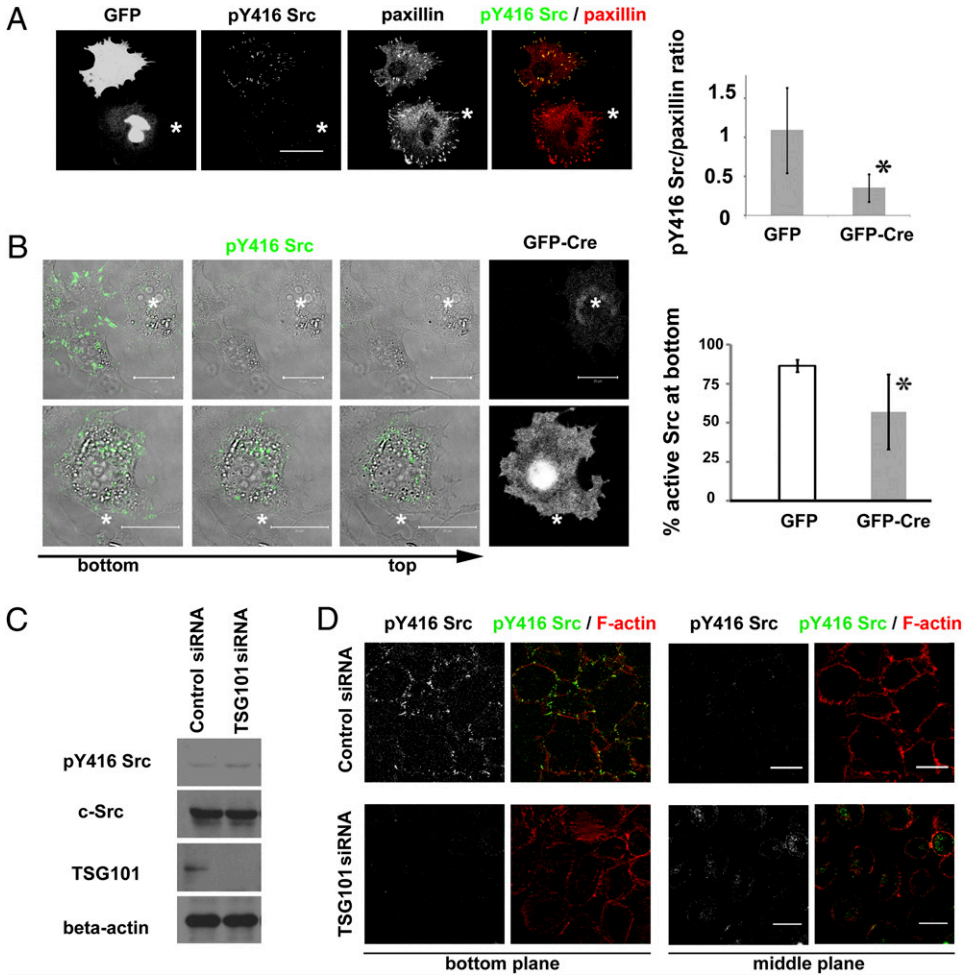

Fig. 2. Tsg101 regulates subcellular localization of active Src. (*A*) v-Src *Tsg101*$^{fl/fl}$ MEFs infected with GFP and GFP-Cre (*) for 3 d were cocultured and stained with pY416Src and paxillin antibodies. The ratio of the fluorescent intensity of pY416Src and paxillin near the bottom of cells was calculated from GFP-infected (n = 31) and GFP-Cre–infected (n = 30) v-Src *Tsg101*$^{fl/fl}$ MEFs (means ± SD; *P < 0.001). (*B*) Serial Z-section confocal images show the distribution of active Src in noninfected and 3-d GFP-Cre–infected v-Src *Tsg101*$^{fl/fl}$ MEFs (*Upper row*), or 2-d GFP-Cre–infected v-Src *Tsg101*$^{fl/fl}$ MEFs (*Lower row*). GFP-Cre–infected v-Src *Tsg101*$^{fl/fl}$ MEFs are marked by an asteriak. The intensity of active Src located at the bottom plane versus that of the entire Z-section was calculated for non-infected (n = 86) and 2-d GFP-Cre–infected (n = 25) cells (means ± SD; *P < 0.001). (*C* and *D*) HeLa cells treated with control or TSG101-targeting siRNAs for 48 h were analyzed by immunoblotting (*C*) or confocal microscopy (*D*). Serial Z-section confocal images show staining of active Src and F-actin in control and TSG101-depleted HeLa cells. (Scale bars: 20 μm.)

cell periphery (*SI Appendix*, Fig. S5*A*). In control v-Src MEFs, c-Src-GFP fluorescence in endosomal vesicles recovered rapidly after photo-bleaching, with a recovery $t_{1/2}$ of about 18.1 ± 6.2 s (Fig. 4*C*, *SI Appendix*, Fig. S5*B*, and Movie S1). Strikingly, the *Tsg101*-deleted or myc-VPS4A^{E228Q}–expressing v-Src MEFs showed a dramatically reduced recovery with essentially flat recovery curves (Fig. 4*C* and Movies S2 and S3), preventing accurate measurement of the recovery $t_{1/2}$. Compared with nearly 65 ± 21.6% recovery of endosomal c-Src-GFP in control cells, the mobile fraction constituted less than 25% in *Tsg101*-deleted or dnVPS4-expressing cells (Fig. 4*D*). The effect was specific, because Tsg101 deficiency had no effect on the trafficking dynamics of GFP, which diffused freely in the cytoplasm (*SI Appendix*, Fig. S5*C*). These data indicate that ESCRT function is required for dynamic movement of c-Src at endosomal membranes and that inhibition of this process leads to an arrest of c-Src at LEs and inability of Src to traffic to peripheral locations such as FAs. Consistent with the role of Tsg101 and Vps4 in facilitating Src trafficking at late endosomes, transiently transfected c-Src-DsRed also colocalized with TSG101-GFP or VPS4^{K173Q}-GFP in vesicular structures inside the cells (Fig. 4*D*).

Western blot analysis demonstrated that the steady-state level of active Src was reduced when *Tsg101*$^{fl/fl}$ and v-Src *Tsg101*$^{fl/fl}$ MEFs were analyzed 3 to 4 d after *Tsg101* deletion (Figs. 1*A* and 5*A*). Focal adhesion kinase (FAK) and STAT3 are downstream effectors of Src signaling and are critical for Src-dependent migration and oncogenic transformation (30, 31). Deletion of *Tsg101* led to reduction in the levels of activated STAT3 and loss of v-Src–dependent phosphorylation of FAK on Y925 (Fig. 5*A*). In contrast, autophosphorylation of FAK at Y397 and the level of phospho-ERK1/2, which did not increase as a result of v-Src expression, was minimally altered by *Tsg101* deletion (Fig. 5*A*). Overexpression of dnVPS4-GFP and to a lesser extent the wild-type VPS4-GFP, reported to have a small inhibitory effect on ESCRT function when overexpressed (32), mildly reduced the level of active STAT3 when cotransfected with v-Src in HEK 293 cells (Fig. 5*B*).

We found that activation of endogenous c-Src upon cell adhesion to fibronectin-coated substratum or addition of serum, stimuli known to activate Src (6), was intact in *Tsg101*-deleted cells (*SI Appendix*, Fig. S6). Because v-Src activity is independent of growth factors and adhesion, the reduced steady-state level of active

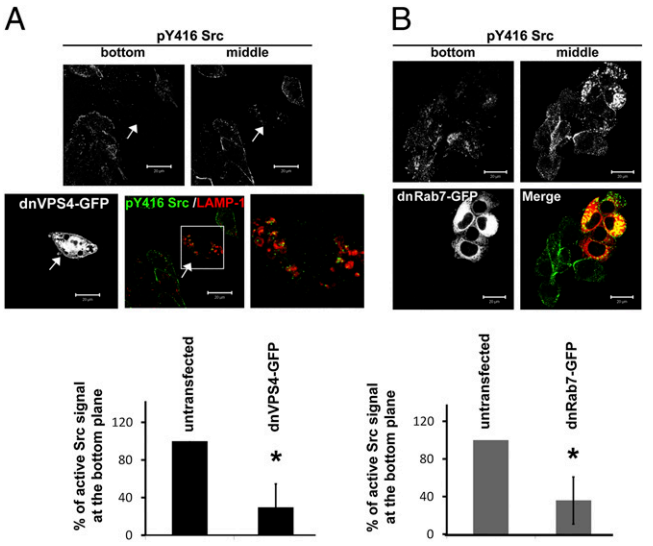

Fig. 3. Vps4 and Rab7 regulate the peripheral localization of active Src. (*A*) Serial Z-section confocal images show distribution of active Src in nontransfected or VPS4A^{K173Q}-GFP–expressing v-Src MEFs (arrows). High-magnification image shows colocalization of active Src in LAMP-1 vacuoles. The fluorescence intensity of active Src at the bottom of VPS4A^{K173Q}-GFP–expressing v-Src MEFs ($n = 13$) was compared with that of nontransfected cells ($n = 31$) (means ± SD; *$P < 0.001$). (*B*) Serial Z-section confocal images show the distribution of active Src in nontransfected and Rab7^{T22N}-GFP–expressing v-Src MEFs. The fluorescence intensity of active Src at the bottom of Rab7^{T22N}-GFP–expressing v-Src MEFs ($n = 18$) was compared with that of nontransfected cells ($n = 44$) (means ± SD; *$P < 0.001$). (Scale bars: 20 μm.)

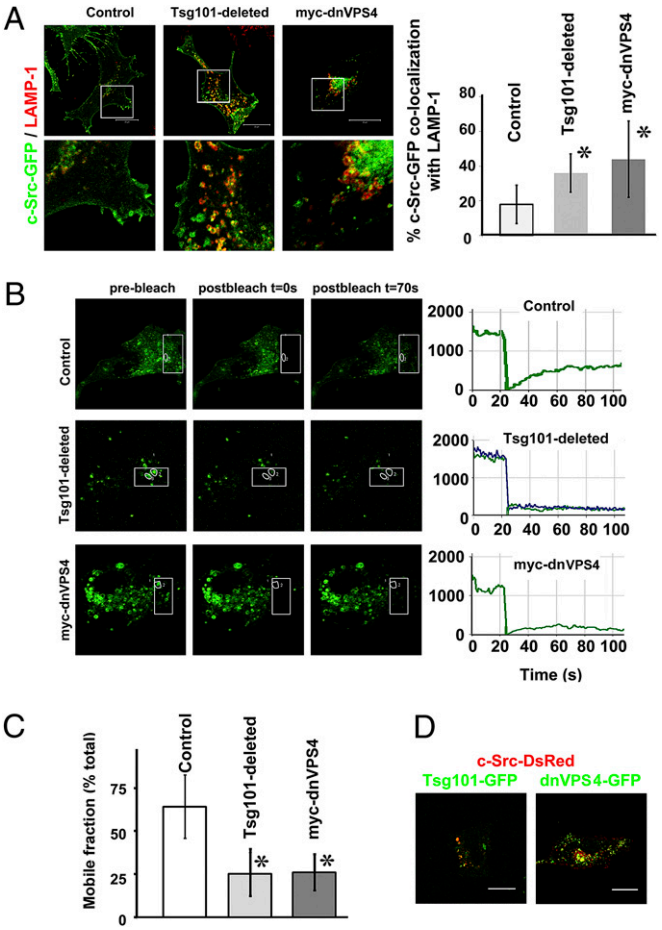

Fig. 4. Tsg101 and Vps4 regulate dynamic trafficking of Src at endosomes. (*A*) Localization of LAMP-1 and transfected c-Src-GFP in control, *Tsg101*-deleted (infected with adeno-Cre for 3 d), and mycVPS4A^{E228Q}-expressing v-Src *Tsg101*$^{fl/fl}$ MEFs. High-magnification images of the boxed areas are shown in the lower row. (Scale bars: 20 μm.) The percentage of c-Src-GFP pixels positive for LAMP-1 was quantified in Src-GFP–transfected control ($n = 15$), Tsg101-deficient ($n = 10$), or mycVPS4A^{E228Q}-expressing ($n = 11$) v-Src MEFs. *, statistically significant changes ($P < 0.001$). (*B*) Representative c-Src-GFP FRAP results from control, *Tsg101*-deleted (infected with adeno-Cre for 3 d), and mycVPS4A^{E228Q}-expressing v-Src *Tsg101*$^{fl/fl}$ MEFs. Rectangles indicate bleached regions. Recovery curves for circled c-Src-GFP vesicles were shown on the right. (*C*) Mobile fractions of c-Src-GFP at vesicular structures are calculated for control ($n = 22$), *Tsg101*-deleted (adeno-Cre for 3 d) ($n = 12$), and mycVPS4A^{E228Q}-expressing ($n = 6$) v-Src MEFs. *, statistically significant changes ($P < 0.001$). (*D*) v-Src MEFs were cotransfected with c-Src-DsRed and GFP-TSG101 (*Left*) or VPS4A^{K173Q}–GFP (*Right*). (Scale bars: 10 μm.)

Src upon *Tsg101* depletion is unlikely to be caused by a diminution of c-Src activation in response to these stimuli.

Discussion

Although the importance of Src in signal transduction and oncogenesis is well established, precise control of the spatial aspects of its function has begun to receive attention only recently (reviewed in ref. 33). Here, we demonstrate that the function of an LE/LY compartment regulated by the ESCRT pathway is important for the localization of active Src at FAs and for Src-dependent signaling events and cell migration.

The overall conclusion that the LE/LY compartment has a critical role in active Src localization at FAs is based on multiple lines of evidence. We demonstrate that disruption of LE function by overexpression of dnVPS4, by inducible knockout of the *Tsg101* gene in MEFs, or by siRNA knockdown of TSG101 in HeLa epithelial cells led to depletion of active Src at FAs. FRAP studies further demonstrated that the fast and dynamic mobility of Src (8) is dramatically reduced by *Tsg101* deletion as well as by dnVPS4 expression, resulting in the accumulation of active Src and total Src at LE/LY. Importantly, direct disruption of membrane fusion between the LE and LY compartments using dnRab7 had a similar effect. Consistent with our conclusion, previous studies have demonstrated that myristoylation and absence of palmitoylation, which localize Src to LE/LY (8, 34), are required for transforming activity of SFKs (10, 11). Notably, loss of active Src was not a result of impaired activation of endogenous Src (*SI Appendix*, Fig. S6): In fact, at early time points after induced deletion of *Tsg101*, the overall level of activated Src was elevated (Fig. 1 *A* and *B*). Furthermore, impaired translocation of active Src upon *Tsg101* deletion could not be attributed to structural changes in FAs (Fig. 2*A*) or to the lack of FAK Y397 phosphorylation (Fig. 5*A*) which recruits active Src to FAs (35). Although our results invoke the importance of the ESCRT pathway in active Src translocation to FAs, a role for Rab11-regulated recycling endosome was suggested previously (6). Our negative results using a dominant-negative Rab11 may reflect the use of a different mutant (S25N here vs. N124I previously) or different experimental conditions (v-Src here vs. c-Src previously), but the disparity points to the need for future studies to assess the relative contributions of Rab11- and ESCRT-dependent endocytic compartments in active Src localization.

Recent studies have shown that, in addition to targeting ubiquitinated receptors to the inner vesicles of MVBs, ESCRT proteins also are essential for trafficking to other membrane compartments such as the Golgi apparatus and the plasma membrane. For instance, dynamic recycling of the ESCRT complex components themselves (36), a Rho family GTPase Rnd2 (37), transferrin receptor, and low-density lipoprotein receptor (16, 38) all require functional VPS4. Moreover, depletion of TSG101 blocks transferrin receptor recycling (17) and amphiregulin-induced EGF receptor recycling (39). Thus, our results and those of others support

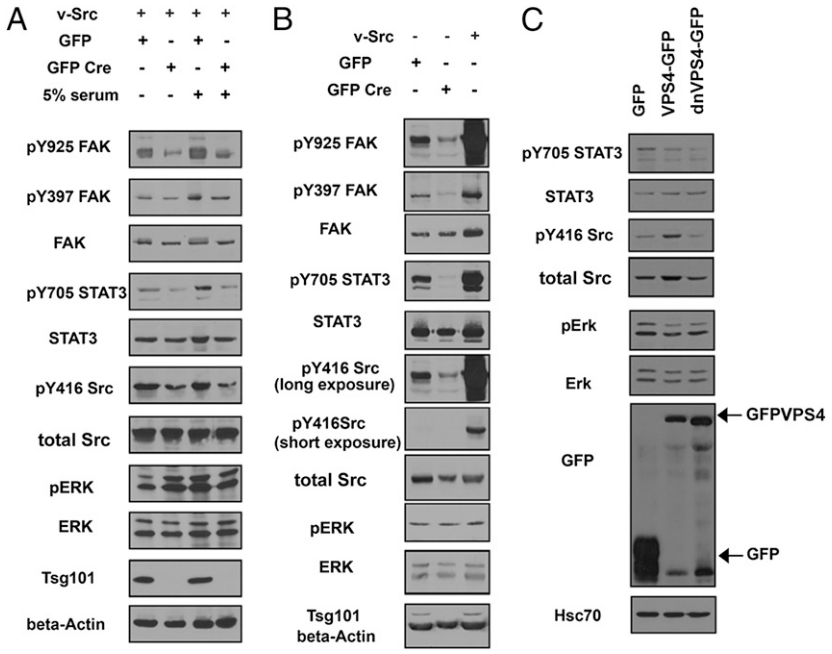

Fig. 5. Prolonged deletion of *Tsg101* reduces c-Src and v-Src activity. (*A*) v-Src *Tsg101*$^{fl/fl}$ MEFs infected with GFP or GFP-Cre for 2 d were serum-starved overnight or kept in medium containing 5% FCS for another 24 h before immunoblot analyses. (*B*) v-Src *Tsg101*$^{fl/fl}$ MEFs and *Tsg101*$^{fl/fl}$ MEFs infected with GFP or GFP-Cre for 3 d were analyzed by immunoblotting. (*C*) HEK 293 cells cotransfected with v-Src and GFP, VPS4A-GFP, or VPS4A^{K173Q}-GFP were analyzed by immunoblotting.

the notion that ESCRT proteins are important for dynamic trafficking of endosomal cargos to destinations other than lysosomes.

Currently, ESCRT proteins, including Tsg101, are viewed as suppressors of signaling pathways that facilitate the lysosomal degradation of ubiquitinated receptors including receptor tyrosine kinases. However, the expression of Tsg101 in mice and mammalian cells is essential for cell viability, proliferation, and growth (19, 40–42). A recent study indicates that Tsg101 is required for the formation of central supermolecular activation cluster, a signature of T cell activation (43). Our study, which connects ESCRT-dependent endocytic traffic to Src-dependent function, provides a potentially important mechanism for these positive roles of the ESCRT pathway.

An important question raised by our findings is how active Src molecules in the LE/LY compartments may be recognized by the ESCRT machinery for delivery to the periphery of the cell. The well-studied Cbl-dependent direct ubiquitination of active Src and other SFKs, which serves to regulate SFK function negatively by promoting their proteasomal or lysosomal degradation (44, 45), is unlikely to provide a basis for their recognition by ESCRT proteins for delivery to the cell periphery, although this mechanism may account for the overall accumulation of active Src we observed upon loss of Tsg101 expression or dnVPS4 expression. However, further studies will be needed to rule out this mechanism formally. It is more likely that ESCRT complexes either recognize ubiquitin modification of Src-associated proteins or recognize Src or its complexes with other proteins via ubiquitin-independent interactions. For example, both Src and Tsg101 are known to associate with Tom1l1/Srcasm, a recently described endosome-associated Src regulator (46, 47). Src also is known to interact with and phosphorylate the ESCRT pathway protein Alix (48), which in turn associates with Tsg101. Finally, an ubiquitin-independent direct Tsg101–Src interaction is possible, because purified SH3 domain of the SFK Hck can pull down Tsg101 from cell lysates by binding to its proline-rich region (49). It also is possible that ESCRT proteins control the traffic of a modulator of Src activity, such as an activating kinase, a phosphatase, or another cofactor. For example, in addition to Tom1l1/Srcasm (47, 50), a putative protein tyrosine phosphatase, His-domain–containing protein tyrosine phosphatase (HD-PTP) (51, 52) is known to regulate Src activity and to interact with ESCRT machinery. Further investigations, including a reconstitution approach-based structure–function analysis of Tsg101, should help explain the mechanism by which the ESCRT pathway helps target active Src to its sites of action.

Taken together, our data demonstrate that translocation of active Src to FAs, a process critical for Src-mediated functions, requires an ESCRT-dependent LE/LY compartment. Future studies should help elucidate how this pathway is linked to Src and ESCRT biology during normal cellular function and oncogenesis.

Materials and Methods

Antibodies and DNA Constructs. Reagents used in this study are described in *SI Appendix*.

General Methods. Cell culture, wound-healing assay, confocal immunofluorescence microscopy, live cell imaging, FRAP analysis, siRNA-mediated Tsg101 depletion, immunoblotting, and statistical analysis are described in *SI Appendix*. *Tsg*101$^{fl/fl}$ MEFs or v-Src *Tsg*101$^{fl/fl}$ MEFs were incubated with GFP adenovirus or GFP-Cre adenovirus separately. Infected cells were either used for immunoblotting or cocultured together before being processed for confocal immunofluorescence microscopy. For transient transfection, v-Src MEFs were seeded on coverslips and transfected with plasmid-lipid complex (Lipofectamine 2000). Cells were analyzed 24 or 48 hrs later. For siRNA-mediated depletion of TSG101, HeLa cells were transfected with 20 nM control or TSG101-targeting siRNA and analyzed 48 hrs later.

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