Tropomyosin Tm5NM1 Spatially Restricts Src Kinase Activity through Perturbation of Rab11 Vesicle Trafficking

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In order for cells to stop moving, they must synchronously stabilize actin filaments and their associated focal adhesions. How these two structures are coordinated in time and space is not known. We show here that the actin association protein Tm5NM1, which induces stable actin filaments, concurrently suppresses the trafficking of focal-adhesion-regulatory molecules. Using combinations of fluorescent biosensors and fluorescence recovery after photobleaching (FRAP), we demonstrate that Tm5NM1 reduces the level of delivery of Src kinase to focal adhesions, resulting in reduced phosphorylation of adhesion-resident Src substrates. Live imaging of Rab11-positive recycling endosomes that carry Src to focal adhesions reveals disruption of this pathway. We propose that tropomyosin synchronizes adhesion dynamics with the cytoskeleton by regulating actin-dependent trafficking of essential focal-adhesion molecules.

Cell migration is essential for normal embryonic development, wound healing, and immune response, and migration dysregulation underpins an array of pathological conditions. Much focus is given to the initiation and speed of migration; however, of equal importance are the mechanisms by which migration is arrested. Integrin-based focal adhesions, which are bound on the external surface to the extracellular matrix and internally to the actin cytoskeleton, are key structural elements of the machinery that drives cell migration (1). While actin filament stabilization inhibits cell migration (2–6), little is known about how the associated focal adhesions are synchronously stabilized along with actin filaments. This is critical, because events that trigger focal adhesion disassembly correspondingly break the adhesion-cytoskeleton linkage (7–9), facilitating new cycles of membrane protrusion and forward movement (10). Thus, in order to stop migrating, cells must have mechanisms to synchronize actin and adhesion stability.

The tropomyosins are a multi-isoform family of actin-binding proteins that display spatially and temporally restricted association with discrete actin filament populations (11). Dimers of tropomyosin associate head to tail, forming a coiled polymer that lies in the major groove of the actin filament. The association of discrete tropomyosin isoforms is thought to regulate the dynamic state of actin filaments by affecting the association of other actin-regulatory proteins (12). Thus, cells with altered tropomyosin expression profiles display differential actin filament stability (2, 4, 13). Moreover, tropomyosin isoform expression is correlated with different focal-adhesion morphology (4, 14) and isoform-specific effects on cell migration (2, 4, 14–16). The tropomyosin isoform Tm5NM1 is ubiquitously expressed (17), and elevated expression inhibits both 2-dimensional (2D) (2, 4) and 3D (16) cell migration. In line with these migration effects, Tm5NM1 induces actin filament stability (13) coupled with highly stabilized focal adhesions (4).

Tm5NM1-mediated actin filament stabilization is accompanied by reduced activation of the nonreceptor kinase Src (16). Src is a major regulator of focal-adhesion disassembly (18, 19), and depletion of Src family kinase activity reduces cell motility (20) and causes enlarged and stabilized focal adhesions (18, 21). Src is delivered to the focal adhesions via actin-dependent translocation from the perinuclear region in Rab11-positive recycling endosomes (22). The Src protein consists of an N-terminal myristoylation site and a series of basic residues that together mediate membrane targeting. This is followed by a unique domain, then by Src homology 3 (SH3) and SH2 domains and the kinase domain, and finally by tyrosine residue Y527 in the Src C terminus. When phosphorylated, Y527 interacts with the Src SH2 domain, holding the molecule in a closed and inactive conformation. While neither the myristoylation sequence nor Src kinase activity is required for targeting to focal adhesions, targeting requires dephosphorylation of the inhibitory Y527 residue and the presence of the Src SH2 or SH3 domain to direct Src to the focal adhesions (18, 23, 24). Although the microtubules are known to play a key role in protein trafficking, the role of the actin cytoskeleton in directing protein trafficking via endocytic vesicles toward the plasma membrane is increasingly appreciated (25). Endosome movement is slowed following actin stabilization (26). Actin may determine endosome movement through direct association between the actin polymerization machinery and the endocytic vesicle to provide propulsive forward movement (22, 26–28) in a recently described mechanism of long-range transport along actin filaments oriented toward the cell periphery (29), or by an as yet unidentified mechanism. In the present study, we have investigated whether the coordination of actin filament and focal-adhesion stability in-
duced by elevated Tm5NM1 expression may be mediated by reduced Rab11-positive vesicle trafficking of Src kinase to focal adhesions.

MATERIALS AND METHODS

Cell lines and culture. The B35 cell clone overexpressing Tm5NM1 and TmBr3 and the matched control cell line have been characterized previously (2, 14). Src/Yes/Fyn-deficient mouse embryonic fibroblasts (SYF-/- MEFs) (20) were purchased from the ATCC (Manassas, VA). All cells were maintained under 5% CO2 at 37°C in high-glucose Dulbecco’s modified Eagle medium (DMEM) (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine (both from Gibco, Invitrogen).

Antibodies, plasmids, and reagents. Primary antibodies were sourced as follows: paxillin and Rab11 from BD Transduction Laboratories, phosho-Y118-paxillin and phoshypo-Y418-Src from Invitrogen, and Src (clone GD11) from Millipore. The sheep polyclonal anti-γ9d antibody used to detect Tm5NM1 (17) was kindly provided by Peter Gunning. Secondary antibodies for immunofluorescence, including Alexa 488-, Cy3-, and Cy5-conjugated donkey anti-mouse and donkey anti-rabbit antibodies, were purchased from Jackson Immunoresearch. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies for Western blot analysis were purchased from GE Healthcare. Tetramethyl rhodamine isocyanate (TRITC)- and Cy5-conjugated phallolidin and 4',6-diamidino-2-phenylindole (DAP1) were from Sigma-Aldrich. The following expression plasmids, all of which have been described previously, were generously provided by our colleagues as follows: plasmids YFP.Tm5NM1 and GFP.Tm5NM1 by Peter Gunning (30), plasmids GFP.Src and GFP.Y527FSrc by Margaret Frame (22), and Src Förster resonance energy transfer (FRET) reporters by Shu Chien (31). YFP.Rab11, GFP.RasB, and mCherry.VAMP3 plasmids were provided by Rachael Murray, and GFP-paxillin and mCherry-paxillin were provided by Katharina Gaus. B35 cells were transfected with Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions, and MEFs were transfected using a Nucleofector kit (Amaxa) and an MEF2 Nucleofector kit (Lonza). In vitro Src kinase assays were performed using a Src kinase assay kit (Millipore).

Immunoprecipitation, Western blotting, and immunofluorescence. Proteins for Src and Tm5NM1 analysis were extracted with 1% SDS-radioimmunoprecipitation assay (RIPA) lysis buffer (1% Nonidet P-40, 0.1% SDS, 1% Cu2+H2PO4Na2O4 [sodium deoxycholate], 50 mM Tris [pH 8.0], 150 mM NaCl, 5 mM EDTA [pH 8.0]) supplemented with inhibitors (1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 µg/ml aprotinin, 1 µg/ml leupeptin). The extract was sheared by shaking in a 26-gauge needle. For all other proteins, extraction was performed using PBT lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% [vol/vol] Triton X-100, 50 mM NaF, 10 mM Na2PO4, 10H2O, distilled water) supplemented with inhibitors (1 mM Na3VO4, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin) (32). Src immunoprecipitation was performed with protein G-Sepharose (50% slurry; Sigma) mixed with anti-Src antibodies or an equal quantity of control mouse IgG (Invitrogen). The conditions for Western blot analysis have been reported previously (4). Cells for immunofluorescence analysis were plated onto either 35-mm glass-bottom dishes (MatTek) or glass coverslips (Menzel-Gläser/Lomb) that were precoated with fibronectin and laminin. Subsequent fixation and staining were performed as described previously (4). Stained coverslips were mounted with FluorSave mounting reagent (Merck Chemicals) except for cells expressing cyan fluorescence, which were mounted with SlowFade antifade reagent (Invitrogen) to preserve the cyan fluorescence. Epifluorescence imaging was performed using an ORCA ERG cooled charge-coupled device (CCD) camera (Hamamatsu) mounted on an Olympus IX81 inverted microscope equipped with a heater and a chamber to maintain a 37°C condition, a 63× (numerical aperture [NA], 1.3) oil objective or a 40× (NA, 0.65) oil objective and fluorescence filters that have been described previously (4).

FRET. An emission wavelength scan with a 5-nm step size over the range of 470 to 600 nm with a constant excitation wavelength of 433 nm was performed using a Leica SP2 confocal microscope with a 63×, 1.32-NA oil immersion objective to confirm the emission range of the FRET reporters. Cyan (donor) fluorescence images were obtained in the emission wavelength range of 470 to 500 nm, and yellow (acceptor) fluorescence images were obtained simultaneously in the emission wavelength range of 510 to 540 nm. Laser power, detector gain, and image thresholding after image capture were consistent for control and sample cells. Cyan-to-yellow ratio images were prepared as described previously (4), and ratios of cyan fluorescence intensities to matched yellow fluorescence intensities were calculated.

FRAP. Fluorescence recovery after photobleaching (FRAP) analysis was performed on cells plated in coated glass-bottom dishes in CO2-independent medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. Imaging was performed with an Olympus FV1000 confocal inverted microscope equipped with a 37°C heater and a 63×, 1.3-NA water objective. Different time scales of molecule dynamics necessitated the use of different imaging time intervals: GFP-Src was imaged every 0.065 s, while GFP-paxillin and GFP-Y527FSrc were imaged every 1.1 s. Following the capture of multiple reference images, green fluorescent protein (GFP) fluorescence at focal adhesions (in the case of GFP-Src localization, which was determined with reference to the mCherry.paxillin localization) was bleached using a 473-nm excitation laser beam for as long as 2 s, and multiple images were captured postbleaching. Fluorescence recovery was quantified using Olympus Fluoview FV10-ASW software. Following background subtraction, intensities were corrected for any nonspecific changes in fluorescence intensity at matching non-bleached adhesions. These values were then normalized to the prebleaching intensity from the reference images. At least 40 individual focal adhesions from 2 independent experiments were captured for each construct. The rate constant, mobile fraction, and half-time of recovery were calculated by fitting a one-phase exponential association curve using GraphPad Prism.

Endosome time-lapse imaging. Prior to imaging, transfected cells were placed in CO2-independent medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. Time-lapse images were acquired with the Olympus IX81 inverted microscope as described above. Cells were viewed with an Olympus 100×, 1.25-NA oil objective, and images were captured every second for a total of 5 min. Large endosomes were masked in Metamorph, version 7.7, by applying high-pass filtering to maximum z-projections of confocal sections of Rab11 vesicles, followed by image smoothing to highlight edges, autothresholding for light objects, and selection of elements of <100 pixels and <550 pixels. Visual representation of endosome movements was achieved by first filtering each time point of the epifluorescence series (using a high-pass filter as described above) and then creating a maximum projection of the time series (t projection). The first and last images of each series were pseudocolored green and blue, respectively, and the intervening t projection was colored red.

Image analysis. Images were pseudocolored and overlaid using Metamorph software, version 7.7 (Molecular Devices). Final micrograph images and grayscale level adjustments were prepared in Adobe Photoshop. Image quantification and measurement procedures were carried out using Metamorph software, version 7.7. Filtering of epifluorescence images to enhance the detection of focal adhesions, ratio imaging, analysis of paxillin phosphorylation at focal adhesions, and fluorescence line scans were all performed as described previously (4).

Statistical analysis. All error bars on histograms show the standard errors of the means (SEM). Statistical comparison of two means was performed using a Student t test.

RESULTS

Src activity is reduced at focal adhesions. Cells expressing high-level exogenous Tm5NM1 (Fig. 1A) are larger than B35 control cells, with arced peripheral membranes (Fig. 1B) and have en-
larged focal adhesions (Fig. 1C). In B35 control cells, Src exhibits the expected punctate, perinuclear staining pattern (Fig. 1D). However, in Tm5NM1-overexpressing cells, the punctate Src staining is more dispersed and does not adopt the typical tight perinuclear staining pattern. Active Src is seen as punctate staining throughout the cytoplasm in both B35 and Tm5NM1-overexpressing cells (Fig. 1D). In the B35 cells, phosphorylated Src (p-Src) also localizes to the membrane periphery (Fig. 1D, arrowheads). This membrane localization is lost in the Tm5NM1 cells.

We next investigated the effect of other tropomyosin isoforms on the intracellular distribution of Src. For this purpose, we chose to analyze the effects of the tropomyosin isoform TmBr3. In contrast with Tm5NM1, TmBr3 leads to smaller focal adhesions (Fig. 1C) and has no effect on Src expression (14). Examination of the distribution of Src and p-Src in TmBr3 cells reveals the same intracellular distribution as that seen in B35 cells (Fig. 1D). Therefore, TmBr3 does not change the localization of Src, and altered Src distribution is thus not a property universal to all tropomyosin isoforms.

Finally, we asked whether Tm5NM1 expression altered the distribution of other membrane-associated focal-adhesion enzymes. We analyzed focal adhesion kinase (FAK), since FAK localization and autophosphorylation are upstream of Src (33); thus, this choice of enzyme avoided potentially confounding effects due to decreased Src activity in Tm5NM1 cells. FAK is robustly associated with focal adhesions in both B35 and Tm5NM1 cells (Fig. 1E). Moreover, there is strong concordance between FAK distribution and FAK autophosphorylated on tyrosine 397 (p-FAK), indicating that FAK targeting and activation are unaffected in Tm5NM1 cells (Fig. 1E).

We next confirmed that the Tm5NM1-overexpressing cells have reduced levels of p-Src, as we have shown previously (Fig. 2A) (16). The punctate p-Src staining at the membrane periphery in B35 cells (Fig. 1D) is reminiscent of a focal-complex/focal-adhesion staining pattern. Coimmunostaining with paxillin to mark the adhesions confirmed the presence of small paxillin-positive focal complexes at the membrane peripheries of B35 cells rich in p-Src (Fig. 2B). We have demonstrated previously that the level of focal-complex formation is reduced in Tm5NM1 cells (4), and in line with this, there is no evidence of paxillin- or p-Src-positive focal complexes in these cells (Fig. 2B). Rather, in Tm5NM1 cells, p-Src staining is dispersed throughout the cytoplasm, with some colocalized paxillin and p-Src in the large paxillin-positive adhesions. Together, these data suggested an altered spatial organization of active Src in Tm5NM1 cells. Next, we compared Src activation and distribution in the absence of Tm5NM1 expression using mouse embryo fibroblasts (MEFs) derived from mice that are genetically null for Tm5NM1 (Tm5NM1−/−) (34). As we have reported previously, the loss of Tm5NM1 expression induced increased formation of focal complexes, contrasting with the loss of focal complexes in cells with high-level Tm5NM1 expression (Fig. 2C) (4, 34). Assessment of Src kinase activity by an in vitro kinase activity assay reveals ~2-fold-higher activity in Tm5NM1−/− cells than in wild-type cells (Fig. 2D). Moreover, active, phosphorylated Src is evident at both focal complexes and focal adhesions in Tm5NM1−/− MEFs (Fig. 2E).

To directly measure the spatial distribution of Src kinase activity in Tm5NM1 cells, we used previously described CFP/YFP-Src Förster resonance energy transfer (FRET) reporters (31). In the absence of Src activity, FRET occurs between the proteins of the cyan fluorescent protein (CFP)-yellow fluorescent protein (YFP) pair. Src phosphorylation of the substrate domain bridging CFP and YFP causes a conformational change and consequently decreased FRET, detected as an increase in the ratio of the CFP signal emission to the YFP signal emission (31). A nonphosphorylatable form of the reporter (Y662,4F) served as a positive control for reporter function (Fig. 3A). Ratio imaging of cells transfected with the cytoplasmic Src FRET reporter suggested high-level cytoplasmic Src activity in both B35 and Tm5NM1 cells (Fig. 3A), agreeing...
with the high levels of p-Src seen in the cytoplasm by immunofluorescence (Fig. 1). Quantification of the Src activity (CFP/YFP ratio) revealed no significant difference in cytoplasmic Src activity between B35 and Tm5NM1 cells (Fig. 3B). Because our goal was to specifically measure Src activity at focal adhesions, we employed the membrane-targeted version of the Src FRET reporter in cells that were coimmunostained with antipaxillin antibodies in order to create a mask to identify focal adhesions (Fig. 3C). This approach revealed a significant decrease in Src activity at focal adhesions in Tm5NM1 cells (Fig. 3D).

Increased convergence of Rab-11-positive recycling endosomes. Since Src translocation to focal adhesions has been demonstrated to be mediated by Rab11-positive recycling endosomes (22), we next assessed Rab11 distribution. Rab11 is distributed in punctate perinuclear vesicles in B35 cells (Fig. 4A). In contrast, it shows a more dispersed pattern in Tm5NM1 cells (Fig. 4A). Rab11 and Src are colocalized in the perinuclear region, but Src is absent from Rab11 vesicles that are distant from the perinuclear area in B35 cells. In Tm5NM1 cells, Rab11- and Src-costained vesicles can instead be seen spread throughout the cytoplasm. Comparison with the distribution of another endosome protein, Rab5, indicates that the Tm5NM1 effects are specific to Rab11-positive vesicles, since B35 and Tm5NM1 cells show highly similar patterns of Rab5 vesicle localization and size (Fig. 4B). The greater dispersion of the Rab11 vesicles in the Tm5NM1 cells was confirmed by quantification of the total cell area encompassing the vesicles (Fig. 4C). Strikingly, many of the Rab11 vesicles in the Tm5NM1 cells were enlarged compared with the B35 Rab11 vesicles (Fig. 4C).

Since vesicles traffic along both microtubules and actin filaments, cells transfected with YFP-tagged Rab11 were costained for tubulin and actin microfilament systems in order to determine whether the dispersion of Rab11 vesicles in Tm5NM1 cells re-
periphery rich in both microtubules and actin filaments were strongly expressed in the B35 cells, as evidenced by Tm5NM1 cells (Fig. 4D, arrowheads). To determine whether the Rab11 vesicles are more tightly clustered in the perinuclear region of Tm5NM1 cells, we performed time-lapse imaging of Tm5NM1 cells expressing YFP-tagged Rab11. First, we noted that the Rab11 vesicles were more tightly clustered in the perinuclear region of Tm5NM1 cells than in B35 cells and that although the vesicles were similarly motile, their movements were restricted to the perinuclear region (see Movie S1 in the supplemental material). The chief differences between the vesicle dynamics of B35 and Tm5NM1 cells were that the vesicles were smaller in the B35 cells and that although the vesicles were similarly motile, their movements were restricted to the perinuclear region (see Movie S1 in the supplemental material). In contrast, the behavior of Rab5 vesicles was similar in B35 and Tm5NM1 cells (see Movie S2 in the supplemental material). In both cell lines, there was less movement of the Rab5 vesicles in the cell periphery, and the greatest movement and largest vesicles were seen in the perinuclear region. Therefore, we focused on the behavior of the Rab11 vesicles in the Tm5NM1 cells that were dispersed in the cytoplasm. Examples of a large and a small vesicle that traveled extended distances in Tm5NM1 cells are shown in Fig. 5A. Analysis of the time-lapse movies revealed that the Rab11 vesicles resulted from the convergence of multiple vesicles. In the example shown, three individual Rab11 vesicles converged into one large vesicle after 39 s (Fig. 5B). The vesicles converged and stayed attached for considerable periods; however, they did not appear to fuse, since the individual vesicle shapes remained discernible. This behavior of the Rab11 vesicles was independently confirmed using a second recycling endosome marker, VAMP3/cellubrevin. Time-lapse imaging of cells transfected with mCherry-VAMP3 confirmed that the distal VAMP3-positive vesicles were more motile (Fig. 5C), and they exhibited the same vesicle convergence properties as the Rab11-positive vesicles (Fig. 5D). Finally, the same vesicle convergence behavior was observed in time-lapse images of GFP-Src puncta (Fig. 5E). In summary, Tm5NM1 induces dispersal of Rab11 vesicles throughout the cytoplasm and the formation of enlarged vesicles through vesicle convergence.

**Src dynamics are reduced at focal adhesions.** We hypothesized that reduced Src activity at focal adhesions may be due to slower dynamic exchange of Src at the focal adhesions due to the perturbations in Rab11 trafficking. To test this, we performed fluorescence recovery after photobleaching (FRAP) analysis of GFP-tagged Src kinase at focal adhesions. We first confirmed the efficacy of the GFP-tagged Src constructs: wild-type Src and Src carrying a Y527F mutation that results in constitutive targeting to focal adhesions (28). The exogenously expressed Src constructs were active, as determined by immunoblot analysis for p-Src (Fig. 6A). Notably, both exogenous wild-type Src and Y527F Src were efficiently phosphorylated in Tm5NM1 cells; thus, the reduced levels of Src activity in these cells is not due to an intrinsic inability to activate Src.

In order to measure the exchange specifically at the focal adhesions, cells were cotransfected with mCherry-tagged paxillin...
mCherry.paxillin-positive focal adhesions were then used to identify regions of interest for photobleaching in the GFP channel (Fig. 6C). Using this approach, recovery of GFP-Src at the focal adhesion regions appeared slower in Tm5NM1 cells than in B35 cells (Fig. 6D). Analysis of the normalized percentage of fluorescence recovery confirmed this observation (Fig. 6E). Quantification revealed that the GFP-Src fluorescence recovery rate ($k$) was significantly lower (Fig. 6F) and the mobile fraction was significantly smaller (Fig. 6G) in Tm5NM1 cells.

Src has been reported to exist in at least two populations, one in the perinuclear region that is targeted to the focal adhesions and a second, rapidly activated population in the plasma membrane (35). Thus, a caveat to the analysis with GFP-Src presented above is that it does not distinguish Src that is directly targeted to the focal adhesions from the diffusion of membrane-targeted Src. Since Y527F.Src is prominently targeted to focal adhesions (Fig. 6B), we took advantage of this mutant to examine precisely the dynamics of focal-adhesion-targeted Src kinase. Parenthetically, the dynamics of GFP-Y527FSrc (measured in tens of seconds) were considerably slower than those of GFP-Src (measured in seconds), in line with earlier suggestions about the mobilities of the different populations of Src (35) (compare Fig. 6B with Fig. 6E). Importantly, the recovery of GFP-Y527FSrc at focal adhesions was strikingly reduced in Tm5NM1 cells (Fig. 7A and B). Quantification of the GFP-Y527FSrc fluorescence recovery rate and mobile fraction confirmed a significant slowing of the kinetics of this molecule in Tm5NM1 cells (Fig. 7C and D). Approximately 50% of the total GFP-Y527FSrc at focal adhesions in these cells is immobile over the time frame of the FRAP analysis (up to 70 s). Although we have shown previously that focal adhesion turnover rates are significantly decreased in Tm5NM1 cells (4), this cannot explain the dramatic decrease in GFP-Y527FSrc recovery, given the much longer time scales of focal adhesion turnover rates (measured in tens of minutes) compared to the shorter time scale of molecular exchange at focal adhesions.

We asked whether the exchange of other focal adhesion molecules is affected in Tm5NM1 cells. Analysis of GFP-tagged paxillin at focal adhesions (Fig. 7E) revealed that paxillin exchange is identical for the two cell lines (Fig. 7F to H). The similarity of the recovery curves for the cell lines renders them indistinguishable on the graph (Fig. 7F), a finding mirrored by the absence of any significant differences between the calculated rates of recovery (Fig. 7G) or mobile fractions (Fig. 7H). Again, the lack of difference in paxillin dynamics, despite the large difference in focal

**FIG 5** Large vesicles arise through vesicle convergence. (A) Maximum projection of a time-lapse series (150 s total) of Tm5NM1 cells expressing YFP-Rab11. The first image (t0) is false-colored green; the last image (t150) is shown in blue; and the intervening time points are shown in red. Areas boxed in white are shown magnified on the right (a’ and a”), where arrows point to the position of the vesicle at the start of the time-lapse series and arrowheads show the position at the end. (B) Time-lapse images showing Rab11 vesicle convergence. Magnified images of vesicles in the region boxed in white in the main image are shown below at the indicated time points. The arrowheads point to two individual vesicles at time zero that converge 1 s later; 26 s later, these converged vesicles travel toward a third, larger vesicle. At 39 s, all three have converged. The rightmost image is a time projection with each time point colored as in the images to the left. (C) Maximum projection of a time-lapse series (150 s total) of images of Tm5NM1 cells expressing mCherry.VAMP3. (D) Time-lapse series showing VAMP3-positive vesicle convergence. (E) Time-lapse series showing convergence of GFP-Src-positive vesicles.
adhesion turnover rates (4), indicates that the altered exchange rates for Y527FSrc are not caused by focal adhesion stabilization. Collectively, these data suggest that Tm5NM1 causes a specific inhibition of Src dynamics at focal adhesions.

Tm5NM1 reduces the level of substrate phosphorylation by Y527FSrc. Next, we asked whether the reduced rate of Y527FSrc delivery to focal adhesions results in decreased phosphorylation of focal-adhesion-localized substrate molecules. We have shown previously that levels of phosphorylated paxillin are inversely correlated with Tm5NM1 expression (4, 34). To explicitly assess the relationship between Tm5NM1 expression and Src-mediated phosphorylation of substrates at focal adhesions, Src/Yes/Fyn−/− MEFs were reconstituted with exogenous GFP-tagged Src together with CFP-tagged Tm5NM1. Total paxillin phosphorylation was increased in cells transfected with wild-type Src and was further increased by the expression of Y527FSrc (Fig. 8A). However, based on the Src FRET reporter data showing that Src activity is specifically reduced at focal adhesions, we anticipated that Tm5NM1 should only affect Src activity specifically at focal adhesions. Indeed, Tm5NM1 coexpression had no effect on total paxillin phosphorylation levels. Instead, the level of paxillin phosphorylation was reduced relative to the total paxillin levels specifically at the focal adhesions in cells coexpressing CFP-Tm5NM1 (Fig. 8D). Thus, Tm5NM1 appears to inhibit Y527FSrc-mediated phosphorylation of paxillin at focal adhesions.

DISCUSSION

Coordinated regulation of focal adhesion dynamics with actin filament dynamics underpins cell movement, and correspondingly,
that Tm5NM1-mediated actin filament stabilization is coordinated with the suppression of the delivery of Rab11 vesicles.

The spatial restriction and organization of Src kinase represents an important control mechanism for determining kinase activity (22, 35). Using a membrane-targeted FRET reporter for Src activity, we show reduced levels of Src activity specifically at the focal adhesions in cells overexpressing Tm5NM1. Corresponding with this finding, we show a dramatic reduction in the dynamic exchange of the focal-adhesion-targeted Y527F Src mutant in cells with high-level Tm5NM1. Moreover, the presence of exogenous Tm5NM1 significantly inhibited Y527F-mediated phosphorylation of paxillin at focal adhesions in SYF−/− cells reconstituted with Y527FSrc. Together, the data indicate that Tm5NM1 reduces the delivery of Src to focal adhesions. The suppression of Y527FSrc dynamics in Tm5NM1 cells was considerably stronger than that seen with the wild-type Src molecule. It has been suggested that there are two Src kinase populations, one associated with the plasma membrane and one associated with the perinuclear pool that is translocated to the focal adhesions (35). Therefore, the dynamic exchange of wild-type GFP-Src at focal adhesions may be a combination of exchange with Src in the plasma membrane and exchange with Src that is trafficked from the perinuclear pool. In contrast, the Y527F mutant is dominantly trafficked through the perinuclear translocation mechanism. Thus, the greater effects seen with this mutant protein suggest that Tm5NM1 specifically affects the focal-adhesion-targeted pool.

Complete disassembly of the actin cytoskeleton with cytochalasin D inhibited the activation of the focal-adhesion-targeted Src pool (35), and the expression of dominant negative Scar1, which interferes with actin polymerization, similarly inhibits this kinase (22). In contrast, in the present study we show that Tm5NM1 specifically affects actin filament dynamics. Tm5NM1 reduces the delivery of Src to focal adhesions. The suppression of Y527FSrc dynamics in Tm5NM1 cells was considerably stronger than that seen with the wild-type Src molecule. It has been suggested that there are two Src kinase populations, one associated with the plasma membrane and one associated with the perinuclear pool that is translocated to the focal adhesions (35). Therefore, the dynamic exchange of wild-type GFP-Src at focal adhesions may be a combination of exchange with Src in the plasma membrane and exchange with Src that is trafficked from the perinuclear pool. In contrast, the Y527F mutant is dominantly trafficked through the perinuclear translocation mechanism. Thus, the greater effects seen with this mutant protein suggest that Tm5NM1 specifically affects the focal-adhesion-targeted pool.

There is debate about the specific role that actin plays in vesicle trafficking, and while it is generally thought that actin filaments mediate only short-range transport, recent studies have suggested that there may be a role for actin filaments in longer-range trafficking from the cell interior to the plasma membrane (29). The precise contribution of actin to the movement of Src-containing vesicles is not known; moreover, Src activity itself has been reported to control the actin-dependent movement of vesicles to the cell membrane (22). Under certain conditions, Src aligns with stress fibers, suggesting that it might be directly trafficking along stress fibers (37), in agreement with other studies that have shown alignment of recycling endosomes along actin filaments (38). Alternatively, other studies have noted Src-positive recycling endosomes displaying a “cloud” of polymerizing actin, suggested to drive the motion of the vesicle (22). We could not detect any actin clouds associated with the Rab11 vesicles in our study. On the other hand, Rab11 vesicles were observed aligned with actin stress fibers in the Tm5NM1 cells, in agreement with other studies.
There is a precedent for tropomyosins playing a role in vesicle transport. In the fission yeast *Schizosaccharomyces pombe*, actin filament decoration with the single *S. pombe* tropomyosin gene product regulates movements of specific myosin motors that move cargo along actin filaments (39–41). Moreover, *Drosophila* oocytes with mutant tropomyosin display mislocalized transport of mRNA to the anterior pole (42). Notably, Tm5NM1 expression induces the association of myosin II motors with the enlarged actin stress fibers (2), and one function of tropomyosin might be to regulate the association of actin filaments with different myosin motors (12). In contrast with other studies showing that changes to actin dynamics slowed the speed of vesicle motility (22,38), there was little evidence of a change in speed in the Rab11 vesicles in Tm5NM1 cells. Instead the most striking finding was the enlarged Rab11 vesicles. The enlarged and bright vesicles remained visible for consider-

**FIG 8** Tm5NM1 expression reduces the level of paxillin phosphorylation at focal adhesions. (A) SYF−/− MEFs transfected with the indicated CFP (CFP, CFP-Tm5NM1) and GFP (GFP, GFP-Src, and GFP-Y527FSrc) expression constructs were lysed and analyzed for protein expression as shown. (B) Transfected SYF−/− MEFs were plated onto fibronectin- and laminin-coated dishes, fixed, costained for paxillin (Cy3-tagged secondary antibodies) and phosphopaxillin (Cy3-tagged secondary antibodies), and imaged along with the GFP and CFP channels. An overlay of paxillin (red) and phosphorylated paxillin (p-pax [phospho-Y118-paxillin]) (blue) is shown in the second column from the right (arrows point to the regions that are shown magnified in panel C). The rightmost column shows paxillin phosphorylation represented by ratio imaging. High levels of paxillin phosphorylation relative to total paxillin levels are indicated in red hues. Insets show magnified regions of focal adhesions. (C) Line scans showing fluorescence intensities of focal adhesions. Paxillin intensities are shown in red and phosphopaxillin intensities in blue. (D) Distribution of the ratios of phosphorylated paxillin at focal adhesions (>50 cells were analyzed per condition). Each data point shows the average value for all focal adhesions in each cell, and horizontal bars indicate the mean for each population. Asterisks indicate significant differences (***, *P* < 0.001) by Student’s *t* test.
able periods and continued to be motile. Two types of Rab11 directional movement along actin filaments have been described: convergence of neighboring vesicles traveling along actin filaments that connect them, followed by movement of vesicles toward the plasma membrane (29). The greater convergence of Rab11 vesicles in Tm5NM1 cells may reflect either increased actin filament connections between vesicles, a loss of direction causing increased convergence, inability to escape the actin filament, or failure to move from the converged vesicle to the plasma membrane. Interestingly, similar enlarged Rab11 vesicles were seen following expression of the Rab11 family of interacting proteins (Rab11-FIP) lacking the C2 phospholipid-binding domain (43). Notably, the C2 domain is required to target the vesicles to the plasma membrane, and the larger vesicles generated by the Rab11-FIP mutants were attributed to trapping of the vesicles in aggregates. Thus, we propose that the enlarged vesicles may represent a suppression of the final step of delivery to the plasma membrane. Clearly, this is not completely abrogated, since Src recovery is still observed at the focal adhesions; however, there appears to be a hold-up in the final step of the delivery system.

In summary, we suggest that tropomyosins may concurrently regulate actin and adhesion dynamics by regulating the transport of focal-adhesion regulators, in addition to their established role in regulating actin filament dynamics. There are more than 40 mammalian tropomyosin isoforms that are spatially and temporally restricted in their expression (11). Expression of alternative tropomyosin isoforms has been shown to change the size and signaling of focal adhesions (4, 14). Changed expression levels of the different tropomyosin isoforms may therefore coordinate actin and adhesion dynamics to generate discrete migration outcomes. The synchronizing effects of Tm5NM1 on focal adhesion and actin filament stability are in line with the in vivo role we have described for Tm5NM1 during skin wound healing (34). Wound healing is a precisely orchestrated series of timed migration events that are necessary for efficient wound closure. Lack of Tm5NM1 induces an inappropriately early migration of cells into the wound (34). Correspondingly, under normal conditions, Tm5NM1 expression is induced by wounding, in agreement with a role for this molecule in putting a brake on cell migration. The large repertoire of tropomyosin isoforms may thus provide opportunities for fine-tuning the initiation and arrest of cell migration under both normal and pathological conditions.

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