SPECC1L-deficient primary mouse embryonic palatal mesenchyme cells show speed and directionality defects

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Cleft lip and/or palate (CL/P) are common anomalies occurring in 1/800 live-births. Pathogenic SPECC1L variants have been identified in patients with CL/P, which signifies a primary role for SPECC1L in craniofacial development. Specc1l mutant mouse embryos exhibit delayed palatal shelf elevation accompanied by epithelial defects. We now posit that the process of palate elevation is itself abnormal in Specc1l mutants, due to defective remodeling of palatal mesenchyme. To characterize the underlying cellular defect, we studied the movement of primary mouse embryonic palatal mesenchyme (MEPM) cells using live-imaging of wound-repair assays. SPECC1L-deficient MEPM cells exhibited delayed wound-repair, however, reduced cell speed only partially accounted for this delay. Interestingly, mutant MEPM cells were also defective in coordinated cell movement. Therefore, we used open-field 2D cultures of wildtype MEPM cells to show that they indeed formed cell streams at high density, which is an important attribute of collective movement. Furthermore, activation of the PI3K-AKT pathway rescued both cell speed and guidance defects in Specc1l mutant MEPM cells. Thus, we show that live-imaging of primary MEPM cells can be used to assess mesenchymal remodeling defects during palatal shelf elevation, and identify a novel role for SPECC1L in collective movement through modulation of PI3K-AKT signaling.

Development of secondary palate involves coordinated growth and movement of palatal shelves in conjunction with surrounding craniofacial structures1–3. In mice, the palatal shelves originate as a pair of vertical outgrowths from the maxillary processes at embryonic-day 11.5 (E11.5) and extend downward adjacent to the tongue until E13.5. By E14.5, the palatal shelves elevate to position themselves horizontally above the tongue and extend toward the midline. By E15.5, the shelves have adhered and fused at the midline to form the secondary palate. Defects in palatal shelf outgrowth, elevation, or fusion can lead to cleft palate, one of the most common human birth defects1,4. Among these steps, elevation is the most poorly understood. Histological studies of palate elevation have shown that the mechanism differs along the anteroposterior axis1,5,6. The anterior palate exhibits a “flipping-up” motion where the distal ends of the shelves rise toward the midline. In contrast, the middle and posterior sections of the palate undergo more extensive remodeling, wherein the medial walls of the shelves extend horizontally as the distal ends of the shelves retract, creating a “bulge”5–7. This latter process can be referred to as mesenchymal remodeling5. Interestingly, the “bulging” appears to occur first, indicating it may be the driving event during elevation5.

Several cellular mechanisms for mesenchymal remodeling during palate elevation have been proposed1,2,8,9. Cell proliferation is required, but not sufficient, for elevation to occur10–12. Organ culture of palatal shelf explants have shown that palatal mesenchyme also has migratory properties, potentially guided by WNT5A and FGF10 chemotactic gradients13. Thus, coordinated movement of the palatal mesenchyme may contribute to palate remodeling, however, collective migration attributes have not been investigated in palate mesenchymal cells.

SPECC1L is a cytoskeletal protein that associates with both filamentous actin and microtubules14. Mutations in SPECC1L have been identified in multiple patients with craniofacial malformations, including cleft palate15–17.

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Thus far, these mutations have been autosomal dominant nonsynonymous variants that cluster in the second coiled-coil (CCD2) and C-terminal calponin homology (CHD) domains\(^\text{15}\). Mouse embryos homozygous for a null Specc1l gene-trap allele die between E9.5 and E10.5 with open neural folds and defects in cranial neural crest cell delamination\(^\text{19}\). In cultured U2OS osteosarcoma cells, loss of SPECC1L results in poor cell migration in wound-repair assays\(^\text{14}\), increased filamentous actin staining\(^\text{14,18}\), and at high cell-density, cell-shape change and abnormal staining of adherens junction markers β-catenin and E-cadherin\(^\text{18}\). We have recently reported generation of a new gene-trap allele (Specc1l\(^\text{fG}^\text{T}\)), and a Specc1l-truncation allele lacking 510 C-terminal amino acids (Specc1l\(^\text{fG}^\text{C510}\)) that removes the CHD. Homozygous mutants for Specc1l\(^\text{fG}^\text{T}\) are embryonic lethal, while those for Specc1l\(^\text{fG}^\text{C510}\) are perinatal lethal\(^\text{19}\). Crossing these alleles resulted in Specc1l\(^\text{fG}^\text{T}\)/Specc1l\(^\text{fG}^\text{C510}\) compound heterozygous mutant embryos that were also perinatal-lethal and exhibited a delay in palate elevation as well as oral epithelial defects\(^\text{19}\). In particular, we reported the presence of transient oral epithelial adhesions between palatal shelf and mutant embryos that were also perinatal-lethal and exhibited a delay in palate elevation as well as oral epithelial defects\(^\text{19}\). In particular, we reported the presence of transient oral epithelial adhesions between palatal shelf and mutant embryos that were also perinatal-lethal and exhibited a delay in palate elevation as well as oral epithelial defects\(^\text{19}\). In particular, we reported the presence of transient oral epithelial adhesions between palatal shelf and mutant embryos that were also perinatal-lethal and exhibited a delay in palate elevation as well as oral epithelial defects\(^\text{19}\). In particular, we reported the presence of transient oral epithelial adhesions between palatal shelf and mutant embryos that were also perinatal-lethal and exhibited a delay in palate elevation as well as oral epithelial defects\(^\text{19}\).

In this study we used quantitative analyses of motility to show that primary mouse embryonic palatal mesenchyme (MEPM) cells exhibit stream formation, an attribute of collective migration, and showed that this behavior is impaired in Specc1l-mutant MEPM cells. We also performed wound-repair experiments using primary MEPM cells from Specc1l\(^{\text{fG}^\text{T}}/\text{fG}^\text{C510}\) mutant embryos to show defects in both cell speed and directionality. Importantly, we show that pharmacological activation of the PI3K-AKT pathway, which is reduced in Specc1l mutants, is sufficient to rescue speed and directionality defects in these cells. Together, these data show a novel role for SPECC1L in collective cell movement through regulation of PI3K-AKT signaling pathway, as well as establish MEPM cells as a proxy model to study mesenchymal remodeling defects during palate elevation.

### Materials and methods

#### MEPM isolation and cell culture.

Specc1l\(^{\text{fG}^\text{T}}/\text{fG}^\text{C510}\) × Specc1l\(^{\text{fG}^\text{T}}/\text{fG}^\text{T}\) mouse mating-pairs were placed together overnight, and the gestational stage was defined as E0.5 at noon on the day plug was identified. Females were euthanized at E13.5, and embryos were harvested in 1× PBS. Palatal shelves were dissected away from the oral cavity under aseptic conditions, placed in a 1.5 mL tube with 0.5 mL of 0.25% Trypsin (ThermoFisher, 25200-056), and incubated at 37 °C for 10 min. Occasional pipetting was performed to accelerate mechanical dissociation. The trypsinized MEPM cells were mixed with 5 mL of high-glucose DMEM (HyClone, SH30243.01) containing 10% FBS (Corning, 35-010-CV) in a conical tube. The tube was centrifuged at 200rcf for 5 min to pellet the cells. MEPM cells were resuspended in fresh DMEM containing 10% FBS, plated on tissue-culture treated plastic dishes, and incubated at 37 °C. Following expansion, cells were cryopreserved, and only passaged up to five times for use in experiments. All experiments involving animals were carried out with a protocol approved by the KUMC Institutional Animal Care and Use Committee, in accordance with their guidelines and regulations.

#### U2OS cell culture.

Control and SPECC1L-kd U2OS cells were generated previously from U2OS osteosarcoma cells (ATCC HTB-96) as reported\(^\text{14}\). U2OS cells were cultivated in DMEM (HyClone, SH30243.01) containing 10% FBS (Corning, 35-010-CV) as shown previously\(^\text{18}\).

#### PI3K-AKT pathway activator treatment.

For PI3K-AKT pathway small-molecule activation experiments, 100 µg/mL 740Y-P (ApexBio, B5246) was added 24 h prior to imaging. The activator containing medium was refreshed at the onset of imaging, and it remained throughout the duration of the experiment (48 h). An equal proportion of water was added as vehicle to control wells.

#### Live-cell imaging.

Imaging was performed using the EVOS FL Auto Imaging System (ThermoFisher, AMAFD1000) with the EVOS Onstage Incubator (ThermoFisher, AMC1000). Cell cultures were kept in a humidified, 5% CO₂ environment at 37 °C over a period of 48–72 h. Phase contrast images were collected at ×4 or ×10 magnification, every 10 or 20 min.

#### Image processing.

To detect cell-occupied area, a global threshold was applied to the local standard deviation of image brightness following the procedures outlined in Neufeld, et al.\(^\text{20}\). The code is available at http://github.com/aczirok/cellconfluency. Cell motility was extracted using our particle image velocimetry (PIV) algorithm, with an initial window size of 50 µm\(^2\), resulting in a velocity field \(v(x,t)\) for each frame \(t\) and image location \(x\). The average speed of cell motility was extracted from \(v(x,t)\) as a spatial average over the cell-occupied area.

#### Wound-repair assay.

For wound-repair assays, MEPM and U2OS cells were seeded at superconfluent densities of 1400/mm\(^2\) and 2300 cells/mm\(^2\) (respectively) into silicone inserts (Ibidi, 81176) in 35 mm diameter tissue culture dishes. These silicone inserts provide 500 µm wide cell-free gaps. Live-imaging started at the removal of the silicone insert, 48 h after cell attachment (56 h after seeding). For each wound, 4–5 non-overlapping fields were recorded every 10 min at ×10 objective magnification. Image sequences were evaluated by compiling confluency data \(A(t)\)—the size of cell-occupied area normalized to the field of view, as a function of time. The speed of wound-closure was established as \(V = (\frac{w}{2})(\frac{dA}{dt})\), where \(w\) denotes the width of the field.

#### Spontaneous collective motility assay.

For the analysis of general stream formation and collective motility of MEPM cells, we first estimated the effective density of cells migrating into the wound. This density of cells migrating into the wound along the cell-front after 14-h of migration was approximately 600 cells/mm\(^2\).
Therefore, we started by seeding cells at a lower density of 300 cells/mm², allowed them to grow for 20 h, and then live-imaged these cells for 30 additional hours. This time-frame allowed us to capture the desired “high” density of 600 cells/mm² that we found in the migratory cell-front of wound assays. We then seeded cells at densities of 100 cells/mm² (“medium”) and 30 cells/mm² (“low” or individual cells) for comparison. MEPM cells were seeded at low (30/mm²), medium (100/mm²), and high (300/mm²) densities into 3D-printed 6 mm diameter rings on 35 mm tissue culture dishes²³. Following overnight growth, cultures were live-imaged every 10 min with a 4× phase contrast objective for an additional 30 h.

Local spatial correlations of cell movements were characterized by the average flow field that surrounds moving-cells as described previously²⁴,²⁵. Briefly, a reference system was aligned to each vector $v(x,t)$ and their “neighbors” registered in the appropriate bin (front, rear, etc.). The average velocity vector ($\bar{v}$) in each bin is indicative of spatial correlation: the average of random vectors is close to zero, while it is non-zero when velocities share a common component. The calculated $\bar{v}(x)$ flow field was fitted with an exponential function $\bar{v}(x) = a \exp\left(-\frac{x}{x_0}\right) + \bar{v}_0$, where $x_0$ is the correlation length: the characteristic distance at which local velocity–velocity correlations disappear.

**Nuclear orientation assay.** To quantitatively characterize local orientational ordering of mesenchyme nuclei, we performed the following image analysis sequence: (1) DAPI-labeled frozen sections were imaged with ×20 objective magnification. (2) After loading the images into ImageJ²⁶, the palatal shelf area was delineated, then nuclei were segmented by brightness-based global thresholding. (3) Touching nuclei in the binary segmented image were resolved by a watershed transformation. (4) Segmented clusters larger than 10 pixels were identified and fitted with an ellipse. (5) Nuclei with an unambiguous orientation (the ratio of the minor and major ellipse axes being less than 0.8) were assigned into cells of a spatial grid with a cell size of 50 um. (6) In each
cell, we calculated the scalar 2D nematic order parameter $S_2 = \langle \cos2\theta \rangle^2 + \langle \sin2\theta \rangle^2$, where theta ($\theta$) is the angle between the long axis of the ellipse and a reference direction, and the averages $\langle .. \rangle$ are calculated for each nucleus within the grid cell. The order parameter $S = 1$ indicates a configuration in which nuclei are completely parallel, while $S = 0$ indicates a completely random set. Intermediate numbers $0 < S < 1$ indicate various degrees of partial ordering. Using the same data, we also calculated the direction of the local prevailing order.

Analysis of individual cell-trajectories. Individual cells were tracked manually on consecutive images (github/donnagreta/cm_track) yielding positions $P(i,t)$ of cell $i$ at time $t$. Motivated by a similar analysis performed by Biggs et al., trajectories were characterized by the total path-length $T(i,t) = \sum_{t'=0}^{t} |P(i,t' + 1) - P(i,t')|$, net displacement into the wound $D(i,t) = |X(i,t) - X(i,0)|$, where $X$ is the projection of $P$ in the direction perpendicular to the wound. Guidance efficiency was calculated as $D(i,t)/T(i,t)$ for each cell $i$ and timepoint $t$. Cultures were characterized by the population average of these single cell measures, evaluated at a suitable timepoint.

Statistical analysis. To establish statistical significance, we calculated the quantitative measure for each independent sample (time-lapse recording, physical section of an embryo). The sets, containing at least 4 independent values, were compared by two-tailed Welch t-tests, which does not assume equal variance or paired data. For the calculations we used the scipy.stats.ttest_ind function of the python programming language.

Results

$\textit{Speccl}^{\text{EGT/AC510}}$ mutant embryos show abnormal palatogenesis. $\textit{Speccl}^{\text{EGT/AC510}}$ compound heterozygotes are perinatal lethal and show a delay in palate elevation at E14.5. However, palatal shelves in most mutants recover and fuse by E15.5. Analysis of E14.5 sections at mid-palate region indicated that the tongue in the mutant is usually adequately depressed, but the palatal shelves are acutely angled and do not immediately move inwards to occupy the space above the tongue. Importantly, palate elevation in these mutants follows an abnormal sequence where posterior palate advances prior to middle and anterior palates, as in wildtype (WT) embryos. Given the delayed and abnormal palate elevation in $\textit{Speccl}^{\text{EGT/AC510}}$ mutants, we hypothesized that (a) this delay is due to poor mesenchymal remodeling during elevation, and (b) coordinated movement of palatal mesenchyme cells is required for efficient remodeling. To determine the role of SPECC1L in the mesenchymal remodeling during palate elevation, we decided to perform in vitro motility assays using primary mouse embryonic palatal mesenchyme cells (MEPM) from WT and $\textit{Speccl}^{\text{EGT/AC510}}$ compound heterozygous embryos.
Specc1l-deficient mouse embryonic palatal mesenchyme (MEPM) cells show migration defects during wound-closure. We previously reported that SPECC1L-deficient U2OS osteosarcoma cells showed poor migration in wound-repair assays. To determine if MEPM cells from Specc1l cGT/ΔC510 compound heterozygous embryos showed similar defects, we live-imaged wound-repair assays at superconfluent cell densities (Suppl. Movie 1). As representative images of time-lapse recordings indicate (Fig. 3a–f), mutant MEPM cells (Fig. 3d–f) take longer to close the wound than WT cells (Fig. 3a–c). This effect was quantified by image-analysis measures of confluency (Fig. 3g), from which a 33% reduction in the average speed of wound-fronts, from 6 to 4 µm/h, could be established (Fig. 3i). We used PIV analysis to quantify overall cell-motility within the entire recorded time period of 40 h (p < 2.2 × 10⁻⁵, Welch’s t-test). Error bars represent SEM.
The analysis indicated that MEPM cells remained motile even after closure of the wound (Fig. 3h), and the overall motile activity of Specc1 GT/ΔC510 mutant MEPM cells was reduced by 20%, from 4.4 to 3.5 µm/h (Fig. 3i). To identify motility defects at the level of individual cells, 40 WT and 40 Specc1 GT/ΔC510 mutant MEPM cells were tracked manually as they moved into the wound. Cell trajectories of Specc1 GT/ΔC510 mutant MEPM cells (Fig. 4a,b) indicated a larger deviation perpendicular to the direction of wound-closure, hence a less-efficient guidance into the wound. Each trajectory was characterized at various timepoints during wound-closure by: (1) the length of total distance migrated (Fig. 4c, solid lines), (2) the net displacement into the wound (Fig. 4c, dashed lines), and (3) the ratio of these two distances as a measure of guidance efficiency (Fig. 4d). While these quantities did not indicate a sudden change in cell behavior, the guidance efficiency of WT MEPM cells improved by ~50% during the first 10 h of the wound-closure process (Fig. 4d). Compared to these WT measures, the Specc1 GT/ΔC510 mutant MEPM cells exhibited a 25% reduction in total distance migrated, and a larger 30% reduction in the net displacements into the wound (Fig. 4c). The larger reduction in directed motility is associated with consistently poor guidance efficiency of mutant MEPM cells (Fig. 4d). Distribution of the three motility measures within the tracked-cell population is quite broad (Fig. 4e). The most conspicuous difference between WT and Specc1 GT/ΔC510 mutant cultures is the presence of an unguided cell population within the latter (Fig. 4e, green circle). Thus, population-level measures as well as analysis of individual trajectories indicate a substantial role of SPECC1L in both promoting MEPM migration and responding to guidance cues.

Figure 4. Specc1 GT/ΔC510 MEPM cells exhibit defects in directional migration. (a, b) Representative paths of individual cells moving near the wound edge in WT (a) and mutant (b) cultures. Trajectories were shifted so that each originate at the origin. The WT cells exhibited a more directed movement to the open area compared to the mutant cells, as evidenced by the clustering of trajectories along the horizontal axis. Markers indicate the spread of trajectories: 90% of the trajectories pass through the drawn segments. (c) Individual cell trajectories were statistically characterized by their mean total path lengths (solid lines) and net displacements directed into the wound area (dashed lines). The inset demonstrates these concepts with an example trajectory (black). Both measures were plotted as a function of time elapsed, and show a reduction of motile activity in Specc1 GT-mutant MEPM cells. Lines represent an average of 40 cell trajectories, shaded areas indicate SEM. (d) The average ratio of the directed (net) displacement and total path length of the 40 cell trajectories, as a function of time. The typically lower values obtained for mutant MEPM cells indicate a guidance defect. (e) Statistical analysis of individual cell trajectories using the measures of total displacement, directed displacement and their ratio. The bee swarm plots show these quantities for each tracked cell, evaluated at 10 h culture time. The unguided population delineated by green oval was present only in cultures of Specc1 GT-mutant MEPM cells. The significance of differences was established by pairwise Welch's t-tests, yielding p-values of 0.01, 0.05 and 0.03 for the total path length, directed displacement and ratio data sets, respectively.
MEPM cells display attributes of collective movement. Since our analysis of wound-repair live-imaging studies indicated that Specc11GT/ΔC510 mutant cells had defects in speed and guidance, we asked a more fundamental question: do WT MEPM cells display attributes of collective movement? To test this, we recorded WT and Specc11GT/ΔC510 mutant MEPM cells in a simple two-dimensional tissue-culture environment (Fig. 5a–c, WT cells; Suppl. Movie 2). We use the cell-front density in the wound of ~ 300 cells/mm² as reference (“high”), and included two lower cell densities of 100 cells/mm² (“medium”) and 30 cells/mm² (“low” or individual cells) for comparison. Interestingly, only when cultured at high density, MEPM cells created ordered and long-range oriented domains reaching up to 1 mm (Fig. 5c). While we could not perform live-imaging at such resolution in embryos, we looked at E13.5 embryonic tissue sections (Fig. 2a,b) for evidence of alignment of palatal shelf mesenchymal cells. We used nuclear shape and orientation as a proxy marker for orientation of the spindle-shaped mesenchymal cells (Fig. 6a,b), and found that palatal shelf mesenchymal cells tend to be aligned with their neighbors (Fig. 6c), consistent with our findings of stream-formation in cultured MEPM cells. In particular, we found that the prevailing orientation of the nuclei is parallel with the vertical axis of the E13.5 palatal shelf (Fig. 6d). The buccal region, however, exhibits a significant orientation toward the tongue at E13.5, just prior to the elevation of the palatal shelves (Fig. 6d). Thus, cultured MEPMs at high density appear ordered similarly to the in vivo palatal mesenchyme.

Analysis of spontaneous MEPM cell motility by PIV analysis, revealed a sustained motile behavior despite the high cell density (Fig. 5d). The average motile speed was not significantly different between WT and mutant cultures (Fig. 5e, ~ 4 µm/h), in contrast to PIV cell speeds observed in wound-repair experiments (Fig. 3i). Trajectories of individually tracked cells revealed a cell-density-dependent ordering (Suppl. Movie 3): cell trajectories of adjacent cells in low-density cultures appeared uncorrelated (Fig. 5f, WT cells), while those in high-density cultures were largely parallel (Fig. 5g, WT cells).

To quantify this apparent collective motion of MEPM cells at high density, we adopted a spatial cross-correlation measure, which determined the average co-movement speed of cells at various locations (e.g., front, rear, left, right) relative to a moving cell (Fig. 7a–d). Correlation between cell movements was higher when cells were in close vicinity and gradually tapered off as distance increased beyond 300 µm (Fig. 7e,f). This effect
was quantified by fitting the co-movement speed profiles with an exponential function, yielding the correlation length as a fitted parameter. Correlation length values indicated a 33% reduction, only at high density, in both the parallel and perpendicular correlation lengths of *Specc1LcGT/ΔC510* mutant MEPM cells when compared with WT MEPM cells. This reduction in correlated movement at high cell-densities (Fig. 7g) suggests an impaired ability of mutant cells to form streams.

Collective movement, especially in mesenchymal cells, requires active cell–cell communication involving cell-adhesion molecules, as well as dynamic reorganization of the cytoskeleton to direct movement in response to signals from neighboring cells\(^{24,25,30}\). The more pronounced correlation in movement seen in high-density MEPM cultures is consistent with this contact-dependent mechanism. To further support this contact-dependent mechanism, we looked at both expression of adhesion molecules that are involved in collective migration of mesenchymal cells\(^{32}\) and reorganization of the actin cytoskeleton during migration. Using a previously published primary MEPM RNAseq dataset\(^{33}\), we were able to identify robust expression of all major adhesion molecules identified to be involved in collective migration of mesenchymal cells\(^{32}\). These molecules include N-cadherin, RhoA, Rac1, Cdc42, as well as components of Robo/Slit and Eph/EphR signaling.
We also confirmed the presence of cadherins in primary MEPM cells at the protein level, using a pan-Cadherin antibody (Suppl. Fig. S1). Similar to other cell types, primary MEPM cells during migration also organized their actin filaments perpendicular to the direction of migration (Suppl. Fig. S2). Consistent with our previous U2OS data, fewer mutant cells reorganized their actin filaments adequately (Suppl. Fig. S2). Thus, our data indicate that cultured MEPM cells have an innate ability to move collectively.

Upregulation of PI3K-AKT signaling rescues Specc1l-deficient migration defects. We previously reported that loss of SPECC1L results in decreased PI3K-AKT signaling along with defects in cell-adhesion and cell-shape68. Pharmacological activation of the pathway was sufficient to rescue these phenotypes68. To test if PI3K-AKT pathway activation could also rescue wound-closure defects in mutant MEPM cells, we repeated the wound-closure experiments reported in Fig. 2 in the presence and absence of 740Y-P (100 µg/mL), a peptide activator of PI3K (Fig. 8). We found that activation of PI3K-AKT pathway did indeed rescue the wound-closure defect in Specc1l<sup>GT/ΔCS10</sup> mutant MEPM cultures (Fig. 8a). Specifically, 740Y-P treatment increased the rate of wound-closure in both WT and Specc1l<sup>GT/ΔCS10</sup> mutant MEPM cultures by almost twofold (Fig. 8c), even beyond the rate observed in untreated WT MEPM cultures. Interestingly, PIV analysis of 740Y-P treatment did not show an increase in overall WT cell motility, and only a 20% increase in Specc1l<sup>GT/ΔCS10</sup> mutant cell motility (Fig. 8b,c). Therefore, we hypothesized that improved cell-guidance into the wound contributed to the improved wound-closure in mutant cultures treated with 740Y-P.
Figure 8. Upregulation of PI3K-AKT signaling rescues both speed and directionality of *Speccll*^C510^-mutant cells in wound-closure assays. (a) Confluency analysis of wound-closure recordings indicated a substantially increased speed of the process as a response of 100 µg/mL 740Y-P, a PI3K activator. Orange and blue colors indicate data obtained from wild-type (WT) and *Speccll*-mutant cultures, while lighter and darker colors indicate vehicle-treated and 740Y-P treated cultures, respectively. Solid lines represent the average of 13 independent microscopic fields, recorded in 4 independent experiments. Shaded areas indicate SEM. (b) Average cell motility speed, calculated by PIV analysis of the wound-closure recordings analyzed in (a). The activator 740Y-P induced an increased motile activity, persistent for at least a day, in *Speccll*-mutant cells. (c) Statistical analysis of data presented in (a,b) indicated that the motility defect of mutant MEPM cells was rescued by the PI3K-AKT activator 740Y-P. Left: average speed of front propagation, calculated within the 1–15 h time interval. The speed-up in wound-closure is significant (p < 5 × 10^{-7} for WT and p < 3 × 10^{-10} for mutant, Welch’s t-test). Right: average speed of cell movements within the entire recorded time period of 24 h. The increase of speed was significant in *Speccll*-mutant cells (p < 4 × 10^{-8}, Welch’s t-test). Error bars represent SEM. (d) Trajectories of individual, 740Y-P treated *Speccll*-mutant MEPM cells (dark blue) was compared with that of untreated MEPM cells (gray). The PI3K-AKT activator enhanced the guidance of cell movements into the wound area. (e-h) Statistical analysis of individual cell trajectories. Total path length (e), net directed displacement into the wound (f) and their ratio (g) indicated a sustained improvement in the performance of *Speccll*-mutant MEPM cells. (h) Bar charts indicating the cell trajectory measures evaluated at 10 h after the onset of migration. The significance of rescue was established by pairwise Welch’s t-tests, yielding p-values of 2 × 10^{-3}, 2 × 10^{-5}, and 4 × 10^{-7} for the total path length, net directed displacement and ratio data sets, respectively. The significance values for the WT-mutant comparison were reported in Fig. 2.
To determine if cell-guidance was improved in the rescue experiments, we compared the trajectories of individual MEPM cells from treated and untreated cultures (Fig. 8d). The dispersion of Specc1l<sup>GfT/AC510</sup> mutant cells perpendicular to the direction of wound-closure was visibly reduced by PI3K activation, indicating improved guidance into the wound. Statistical evaluation of individual cell-trajectories (Fig. 8e-h) revealed a 40% increase in the total distance migrated (Fig. 8e,h), a twofold increase in the net displacement directed into the wound (Fig. 8f,h), and a 66% increase in guidance efficiency (Fig. 8g,h) in 740Y-P treated Specc1l<sup>GfT/AC510</sup> mutant MEPM cells. Taken together, the data indicate that PI3K activation can rescue SPECC1L deficiency-related motility defects by improving both cell motility speed and guidance efficiency.

**SPECC1L-dependent collective migration in other cell types.** SPECC1L deficiency has been previously shown to result in defects in cell migration in cultured U2OS cells<sup>14,18</sup>. To support a primary role of SPECC1L in collective cell migration, we analyzed time-lapse imaging of control and SPECC1L-kd U2OS cells in wound-repair assays, similarly to the MEPM data. We were able to show again in U2OS cells that SPECC1L deficiency resulted in slower wound-closure coupled with poor stream-formation and directional movement (Suppl. Fig. S3). We also confirmed that activation of the PI3K-AKT pathway rescued the cell speed and guidance defects in SPECC1L-kd U2OS cells (Suppl. Fig. S4). Interestingly, in contrast to WT MEPM cells, control U2OS cells did not exhibit a significant effect following PI3K-AKT pathway activation. This lack of a marked response likely reflects that U2OS is a cancer cell line with an intrinsically higher than normal level of PI3K-AKT signaling<sup>33–35</sup>. Taken together, these findings point to a common role of SPECC1L in the regulation of cell motility and guidance.

**Discussion**

Studies of palatogenesis have demonstrated that remodeling from vertical to horizontal direction is a primary feature of the elevation process<sup>5,6,36</sup>. This remodeling is likely initiated by the medial edge epithelium of the palatal shelf, but driven by the palate mesenchyme. While primary MEPM cells have been used for testing the effect of various inhibitory compounds, growth factors, and gene-expression changes<sup>31,37–42</sup>, only a few studies have investigated the migration of MEPM cells<sup>39,43,44</sup>, and none, to our knowledge, have examined if they can migrate collectively. We identify the presence of stream formation in MEPM cells, a behavior generally considered to be a feature of collective movement<sup>24,25,45</sup>. We also show that SPECC1L deficiency impaired the ability of palatal mesenchyme cells to move collectively—a result that we posit partially explains the observed delayed and abnormal palate elevation in Specc1l<sup>GfT/AC510</sup> mutants (Fig. 1)<sup>19</sup>. The defect in collective migration was also validated in U2OS cells, an independent cell line, which strongly supports a primary role for SPECC1L in collective movement.

We previously showed that SPECC1L deficiency leads to reduced PI3K-AKT signaling, and that PI3K-AKT activation was able to rescue cell-shape and cell-adhesion phenotypes in SPECC1L-kd U2OS cells<sup>14</sup>. Our analyses now show that activation of PI3K-AKT signaling rescued both cell speed and directionality defects during wound-closure of Specc1l<sup>GfT/AC510</sup> mutant MEPM cells, as well as in SPECC1L-kd U2OS cells. We have shown previously that SPECC1L deficiency leads to a reduction in total AKT levels, rather than just activated phospho-AKT<sup>14</sup>. This reduction in total AKT is at the protein level as RNA levels do not change<sup>18</sup>. Several processes have been identified that play a role in AKT stability, including ubiquitin proteasome-dependent pathway, caspase-mediated cleavage, and caspase-dependent ubiquitination<sup>46</sup>. AKT is known to require translocation to the cell membrane for full activation<sup>47–49</sup>. Thus, it is possible that SPECC1L deficiency affects one or more of these processes involved in AKT stability, resulting in increased AKT degradation. Taken together, our results are consistent with a pro-migratory role for AKT<sup>50</sup> and suggest that SPECC1L regulates cell migration through PI3K-AKT signaling.

This study represents the first depiction of collective movement characteristics in cultured MEPM cells, and supports the hypothesis that palate elevation involves collective movement of the mesenchyme. In addition to our Specc1l mutants, there remain several mouse mutants with elevation defects for which the underlying pathogenic mechanisms have not been fully elucidated<sup>13,31</sup>. Future analysis of these mutants may benefit from using primary MEPM cell migration as a proxy for mesenchymal remodeling during palatal shelf elevation.

**Data availability**

The live-imaging data that support the findings of this study are available from the corresponding authors upon reasonable request.

Received: 12 August 2020; Accepted: 4 January 2021
Published online: 14 January 2021

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Acknowledgements
This project was supported in part by the National Institutes of Health Grants DE026172 (I.S.), GM102801 (A.C.), and F31 DE027284 (E.H.). I.S. was also supported in part by the Center of Biomedical Research Excellence (COBRE) Grant (National Institute of General Medical Sciences P20 GM104936), Kansas IDeA Network for Biomedical Research Excellence grant (National Institute of General Medical Sciences P20 GM103418), and Kansas Intellectual and Developmental Disabilities Research Center (KIDDRC) Grant (U54 Eunice Kennedy Shriver National Institute of Child Health and Human Development, HD090216). The Confocal Imaging Facility, the Integrated Imaging Core, and the Transgenic and Gene Targeting Institutional Facility at the University of Kansas Medical Center are supported, in part, by NIH/NIGMS COBRE Grant P30GM122731 and by NIH/ NICHD KIDDRC Grant U54HD090216.

Author contributions
J.P.G., D.G.I., E.G.H., N.R.W., A.C. and I.S. conceived and designed the experiments. J.P.G., E.G.H., E.K., N.R.W. and L.W.W. performed the experiments. D.G.I. and A.C. performed the time-lapse imaging analysis. J.P.G., Z.U. and A.Y. performed the manual cell-tracking. E.G.H., A.C. and I.S. wrote the paper. J.P.G., D.G.I., N.R.W. and L.W.W. edited the manuscript. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-81123-9.
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