Communication between distant epithelial cells by filopodia-like protrusions during embryonic development

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ABSTRACT
Long-range intercellular communication is essential for the regulation of embryonic development. Apart from simple diffusion, various modes of signal transfer have been described in the literature. Here, we describe a novel type of cellular extensions found in epithelial cells of the somites in chicken embryos. These filopodia-like protrusions span the subectoderms, providing a dorsal surface of the somites and contact the ectoderm. We show that these protrusions, according to the role of actin based cellular extensions in mediating signaling events between distant epithelial cells, are actin- and tubulin-positive and require Rac1 for their formation. This mechanism of transfer of paracrine signaling molecules such as Wnts between ectoderm and somites remains unknown. Here, we show that, in chicken embryos, the subectodermal gap is bridged by a special kind of cytoplasmic extensions formed by the epithelial cells of the dermomyotome. These filopodia-like protrusions are actin- and tubulin-based, contain cytoskeletal regulatory proteins, such as fascin and coflin, and require Rac1 for their formation. Immunohistochemistry reveals the presence of microtubule motors, including kinesin and dynein. Characterizing their functions, we found that they contain glycocephalaminositol-anchored proteins (GPI-APs) and retrogradely transport the Wnt-receptor Frizzled-7 (FZ7). Therefore, we propose a role of dermomyotomal filopodia-like protrusions in long-range molecular signaling during somite development.

INTRODUCTION
The formation of a complex multicellular organism is a highly orchestrated process with a tight spatial and temporal control of gene expression during development. The regulation of embryogenesis depends on molecular signaling events between different cells. Various mechanisms of signal transport from the signaling to the receiving cell have been described, including simple diffusion, transcytosis, and migrating cells (Crick, 1970; Entchev et al., 2000; Serralbo and Marcell, 2014). Recent studies have also implicated a role of actin-based cellular extensions in transducing signals from one cell to another, namely, cytonemes in the wing imaginal disks of Drosophila and the recently discovered specialized filopodia of mesenchymal cells in chicken limb buds (Ramirez-Weber and Kornberg, 1999; Sanders et al., 2013). Somites are metameric mesodermal structures that originate from the paraxial mesoderm. Initially, a somite resembles an epithelial sphere, but soon the ventral half undergoes epithelial-to-mesenchymal transition (EMT), forming the sclerotome, which eventually gives rise to the axial skeleton. The dorsal part remains mesenchymal, forming the dermomyotome, to form embryonic muscle fibers (Gros et al., 2004). All these events in somite maturation are known to depend on molecular signals from the neighboring embryonic structures.

In chick and mouse embryos, it has been shown that signals from the dorsal surface of the somite regulate the formation of epithelial somites and the patterning of the dermomyotome (Correia and Conlon, 2000; Fan and Tessier-Lavigne, 1994). Intriguingly, dermomyotomal development requires contact-dependent molecular signals from the overlying ectoderm, notably Wnts, despite the fact that somites and ectoderm are physically separated by the somite basal lamina and the subectodermal space containing extracellular matrix proteins (Fan and Tessier-Lavigne, 1994; Geetha-Loganathan et al., 2006; Rifas and Thorsteinssottir, 2012; Schmidt et al., 2004). The mechanism of transfer of paracrine signaling molecules such as Wnts between ectoderm and somites remains unknown. Here, we show that, in chicken embryos, the subectodermal gap is bridged by a special kind of cytoplasmic extensions formed by the epithelial cells of the dermomyotome. These filopodia-like protrusions are actin- and tubulin-based, contain cytoskeletal regulatory proteins, such as fascin and coflin, and require Rac1 for their formation. Immunohistochemistry reveals the presence of microtubule motors, including kinesin and dynein. Characterizing their functions, we found that they contain glycocephalaminositol-anchored proteins (GPI-APs) and retrogradely transport the Wnt-receptor Frizzled-7 (FZ7). Therefore, we propose a role of dermomyotomal filopodia-like protrusions in long-range molecular signaling during somite development.

RESULTS AND DISCUSSION
Dermomyotomal cells possess filopodia-like protrusions
Electroporation of a construct encoding a membrane-bound form of green fluorescent protein (mGFP) into the dorsal part of the newly formed epithelial somites revealed cellular extensions in the subectodermal space formed by the dermomyotomal cells, which we named filopodia-like protrusions (FiLiPs) (Fig. 1A,B). In contrast to specialized filopodia in chicken limb buds (Sanders et al., 2013), FiLiPs can be preserved by paraformaldehyde (PFA) and can be further subjected to regular histological procedures. Three-dimensional (3D) reconstruction of z-stack confocal images of cultured transverse slices of the trunk region of Hamburger–Hamilton stage 16 (HH16) embryos stained with CellMask orange plasma membrane stain revealed that an average of 82% (±s.e.m. ± 2.3, n=8, 3D reconstructions) of the extensions are in stable contact with the overlying surface ectoderm (Fig. 1C,D). Time-lapse experiments showed no change in their length for at least 10 min (Fig. 1E; see supplementary material Movie 1). Therefore, we categorized these protrusions as ‘stable FiLiPs’. A subset of extensions is not in stable contact with the ectoderm. These extensions have a highly dynamic nature, extending in all directions. We categorized these as ‘dynamic FiLiPs’. We calculated the average extending and retracting velocities of the
dynamic FiLiPs using kymographic images and found them to be 53.76 nm/s (s.e.m.±1.73, n=10 protrusions) and 68.80 nm/s (s.e.m.±1.54, n=10 protrusions), respectively (Fig. 1F-I; see supplementary material Movie 2). Both stable and dynamic FiLiPs showed considerable variability in their thickness, varying between 200 and 500 nm (n=50 protrusions; Fig. 1J). Each dermomyotomal cell was found to form 2-11 protrusions (mean=3.8 protrusions per cell, s.e.m.±1.0, n=50 cells; Fig. 1K) and the overall length of dermomyotomal FiLiPs was limited by the overlying surface ectoderm (≤20 µm).
Molecular characterization of dermomyotomal FiLiPs
Filopodial structures have been shown to contain cross-linked actin filaments (Small and Celis, 1978). Accordingly, phalloidin staining revealed the presence of actin in the dense network of FiLiPs within the subectodermal space (Fig. 2A,A' and supplementary material Movie 3). In order to further characterize the molecular composition of dermomyotomal FiLiPs, we performed somite electroporation experiments with constructs containing fascin, coflin and α-tubulin fused with GFP. In accordance with other filopodia-like structures described for various cell types (Bernstein and Bamburg, 2010; Kureishy et al., 2002), FiLiPs contain fascin and coflin (Fig. 2B-C'). FiLiPs can be visualized by overexpression of the cytoplasmic form of GFP in dermomyotomal cells (Fig. 2D,D'), which is in line with the observation of filopodia in somite cells by Linker et al. (2005). Surprisingly, dermomyotomal FiLiPs were also positive for tubulin, distinguishing them from previously described protrusions, such as cytonemes and specialized filopodia of limb bud mesenchymal cells (Fig. 2E,E') (Ramirez-Weber and Kornberg, 1999; Sanders et al., 2013). Time-lapse imaging of dermomyotomal cells co-electroporated with tubulin-GFP and the red fluorescent tag pmKate2, as well as immunohistochemistry against α-tubulin, revealed that the entire population of FiLiPs is microtubule-based (Fig. 2F,G and supplementary material Movie 4). Moreover, dermomyotomal FiLiPs were also found to contain kinesins and dyneins, which are motor proteins moving along the microtubule tracks to carry cellular cargo. This indicates the potential role of dermomyotomal FiLiPs in vesicular transport (Fig. 2H,I).

Rac1 inactivation leads to complete inhibition of FiLiPs
Rho family GTPases are known to have profound effects on cytoskeleton architecture and cell migration (Burridge and Wennerberg, 2004). It has been shown that Rac1, a member of the Rho family of GTPases, regulates the formation of long cellular protrusions via actin organization and microtubule stability (Bosco et al., 2009). We co-electroporated a dominant-negative form of Rac1 (dnRac1-1RES-GFP) and pCAG-DsRed into somites I-IV of HH16 embryos to analyze the effect of Rac1 inactivation on the formation of FiLiPs. Intriguingly, dermomyotomal cells expressing dnRac1 exhibited complete loss of FiLiPs (Fig. 2J'). A complete loss of cellular protrusions was also observed in cultured dermomyotomal cells electroporated with dnRac1 (Fig. 2K,K'). Together, this demonstrates that Rac1 is required for FiLiPs formation.

Dermomyotomal FiLiPs exist at different somite levels and embryonic stages, and surrounding tissues are not required for their formation
As the dermomyotome is a structure of mature somites, we tested whether FiLiPs are also exhibited by earlier stages, notably by presomitic mesodermal cells and by newly formed epithelial somites. Actin staining of sagittal sections of HH16 embryos confirmed the presence of FiLiPs in the subectodermal space overlying the cranial presomitic mesoderm (PSM) and the epithelial somites (Fig. 3A-C). However, the number of FiLiPs formed by the PSM cells was considerably lower compared with that visible in epithelial somites and in the dermomyotome (Fig. 3D). The earliest developmental stage studied was HH10, with FiLiPs already present in the subectodermal space of somite I, whereas the latest stage studied was HH22, with FiLiPs in the dermomyotome of somite stage VIII just prior to EMT (Fig. 3E,F). To investigate whether ectoderm is required for the formation of dermomyotomal FiLiPs, we surgically removed the surface ectoderm overlying the electroporated somites and incubated the operated embryos for 3 h before time-lapse imaging. Despite the absence of overlying ectoderm, dermomyotomal cells were found to form FiLiPs (Fig. 3G,H and supplementary material Movie 5). However, such protrusions were longer and lost their orientation towards the ectoderm. We also dissociated and cultured the electroporated dermomyotomal cells in vitro, and observed protrusions emanating from the cultured dermomyotomal cells without any evident contact with other cell types (Fig. 3G, inset). These results suggest that the ability to form FiLiPs is an intrinsic property of dermomyotomal cells.

Dermomyotomal FiLiPs show retrograde membrane transport and contain GPI-APs
We further hypothesized a role of FiLiPs in signal transduction through membrane trafficking. Our hypothesis was supported by the observation of ‘membrane puncta’ along the FiLiPs of the mGFP-electroporated dermomyotomal cells (Fig. 1E). Live-imaging of transverse slices of mGFP electroporated somites revealed a retrograde transport of these membrane dots along the FiLiPs (Fig. 4A,B and supplementary material Movie 6). Using kymographic analysis, the average retrograde transport velocity of such puncta was found to be 56.25 nm/s (s.e.m.±2.78, n=5 puncta) (Fig. 4C). We also tested whether FiLiPs contain glycosphingolipidinositol-anchored proteins (GPI-APs). GPI anchors facilitate the attachment of proteins to the outer leaflet of the plasma membrane, and GPI-APs have been shown to be organized in special microdomains rich in cholesterol and sphingolipids, called lipid rafts (Mayor and Riezman, 2004; Varma and Mayor, 1998). Various studies have implicated the role of lipid rafts in signal transduction (Simons and Toomre, 2000). To determine the presence of GPI-APs in dermomyotomal FiLiPs, we electroporated GFP-GPI into the somites and analyzed their localization in the protrusions. Our results showed the presence of GPI-APs puncta along the actin filaments in the dermomyotomal FiLiPs (Fig. 4D). To exclude the possibility of artificial results concerning the subcellular localization of overexpressed proteins, the mitochondria-specific aconitase fused with GFP (mAcn-GFP) was electroporated into the somites of HH17-18 embryos at a high concentration (7 µg/µl). According to its mitochondrial destination, mAcn-GFP should be solely present in the mitochondrial compartment and should be distinct from the pattern obtained with any of the other overexpressed fusion proteins, thus serving as control. Our results show specifically labeled mitochondria in the dermomyotomal cells and no labeling in the FiLiPs, thereby confirming the reliability of the experimental approach used to characterize the dermomyotomal FiLiPs (Fig. 4F,G).

Wnt receptor Frizzled-7 is retrogradely transported along dermomyotomal FiLiPs
As FiLiPs connect two different cell types, ectodermal and somitic epithelia, we investigated their potential functional role in mediating signaling events during dermomyotome differentiation. The role of Wnt signaling during the various stages of somite patterning and differentiation has been well studied. It has been shown that three G protein-coupled transmembrane receptors for Wnt signaling, namely Frizzled-1, Frizzled-2 and Frizzled-7, are expressed in epithelial somites and in the dermomyotome, whereas Wnt6, which regulates dermomyotomal development, is expressed by the overlying surface ectoderm (Geetha-Loganathan et al., 2006; Linker et al., 2003; Schmidt et al., 2004). As Wnts are lipid-modified proteins, which makes them hydrophobic and barely diffusible (Nusse, 2003), we tested whether dermomyotomal FiLiPs
Fig. 2. Molecular machinery required for the formation and dynamics of dermomyotomal FiLiPs. (A) Phalloidin-labeled F-actin (red) in FiLiPs (transverse section, HH16, somite IV). (B-E) Dermomyotomal cells electroporated with (B) fascin-GFP (HH18, somite VI), (C) cofilin-EGFP (HH17, somite VII), (D) cytoplasmic GFP (HH18, somite VIII) and (E) tubulin-GFP (HH18, somite VII) reveal dermomyotomal FiLiPs spanning the subectodermal space. (A’-E’) Corresponding images merged with DAPI (blue) and DIC. (F) Representative time-lapse images of a dermomyotomal cell (HH18, somite VIII) electroporated with tubulin-GFP and pmKate2 showing that all FiLiPs are microtubule-based. (G-I) Antibody staining against α-tubulin, dynein and kinesin on frozen sections (HH17, interlimb-level somites). Dermomyotomal FiLiPs contain microtubules and the motor proteins dynein and kinesin. Nuclei are stained with DAPI (blue). Phalloidin staining labels F-actin (green) in G. (J) A representative transverse section of somite VII of a HH18 embryo co-electroporated with a dominant-negative form of Rac1 (dnRac1-IRES-GFP) and pCAG-DsRed. Merged image (dnRac1, green; and DsRed, red) showing the inhibition of FiLiPs. (J’) Image merged with DAPI (blue) to visualize the ectoderm. (K) A representative image of a cell taken from the primary culture of dermomyotomal cells electroporated with dnRac1-IRES-GFP. Cells were stained with phalloidin (red) and DAPI (blue). No protrusions are visible. (K’) pCAG-DsRed-electroporated cells served as control. Green represents F-actin and blue represents nuclei in the composite images. Scale bars: 10 µm; 5 µm in F.
Average of 96.7% (s.e.m.±3.3, n=20 protrusions) of the stable FiLiPs were found to transport Frizzled-7 along them (Fig. 4M). Further analysis revealed that an average of 28.5% (s.e.m.±4.11, n=20 protrusions) of the stable FiLiPs suggest their general role in signal transportation. Taken together, our results add new support to the recent concept that dynamic FiLiPs did not seem to be involved in receptor trafficking. An average of 96.7% (s.e.m.±3.3, n=20 protrusions) of these puncta were transported along the stable FiLiPs (Fig. 4L), whereas the dynamic FiLiPs did not seem to be involved in receptor trafficking. An average of 28.5% (s.e.m.±4.11, n=20 protrusions) of the stable FiLiPs were found to transport Frizzled-7 along them (Fig. 4M). These results suggest a role for dermomyotomal FiLiPs in transducing Wnt signaling events during somite differentiation.

CONCLUSIONS
Here, we describe a novel type of filopodia-like cellular protrusions in epithelial cells, which we called FiLiPs, and provide evidence that these protrusions play a role in long-range signaling events during somite development. Intriguingly, they are formed from the basal part of the epithelial somites, thus penetrating the basement membrane of the somitic epithelium, and are not only actin-based structures but also contain microtubules. Partly dynamic and partly stable, they bridge the subectodermal gap and connect the somites to the ectoderm. We provide evidence that these filopodia-like protrusions are involved in ectodermal-dermomyotomal signaling, as the Wnt receptor Frizzled-7 is retrogradely transported in punctate form along the FiLiPs. In addition, the presence of GFP-anchored proteins and retrograde transport of mGFP-labeled membrane puncta along FiLiPs suggest their general role in signal transportation. Taken together, our results add new support to the recent concept that intercellular signaling through cytoplasmic projections is an important principle during embryonic development.

MATERIALS AND METHODS
Expression constructs and in ovo electroporation
Constructs used for electroporation (both previously published and designed by us) are listed in the supplementary material methods. Embryos at HH16 were electroporated in modified form as described previously (Scaal et al., 2004). Fertilized chicken eggs (Gallus gallus domesticus, White Leghorn) were incubated at 38°C and staged. Embryos at HH16 were electroporated as described previously (Scaal et al., 2004). Eggs were fenestrated and the vitelline membrane over somites I-V was carefully removed. DNA constructs (final concentration 5-7 µg/µl) were mixed with Fast Green (Sigma-Aldrich, 1% final concentration) and injected into the somitocoel of somites I-V. To achieve mosaic and distinct single-cell pattern of the electroporated cells wherever required, we reduced the concentration of expression constructs to 0.5-1 µg/µl. For further details, see supplementary material methods.
Immunohistochemistry
To label actin filaments in the protrusions, the free-floating sections were incubated with Alexa Fluor 488/Rhodamine Phalloidin (Life Technologies) diluted to a final concentration of 0.2 µM in PBS containing 1% BSA for 1 h. Immunohistochemistry was performed on 20 µm-thick cryosections. The sections were washed three times in PBS containing 0.1% Triton X-100 (PBT) and incubated overnight at 4°C with desired primary antibodies (final concentration 5 µg/ml), which were diluted in 0.1% PBT solution containing 10% serum. The sections were rinsed, washed frequently for 1 h in 0.1% PBT and incubated with the respective biotinylated secondary antibodies (1:500, 10% serum/0.1% PBT) overnight at 4°C. The sections were again washed frequently for 1 h in 0.1% PBT, incubated with Cy3-conjugated streptavidin (1:500, 0.1% PBT) overnight at 4°C and mounted with DAPI Fluoromount-G (Southern Biotechnology) after frequent washes in 0.1% PBT.

Fig. 4. Dermomyotomal FiLiPs contain GPI-anchored proteins and transport Wnt receptor Frizzled-7. (A) Time-lapse images of a representative FiLiP visible through the mGFP electroporation of a HH17 embryo showing the retrograde transport of a membrane punctum (white arrowheads). (B) Top, representative kymograph showing the mGFP punctum transport along the dermomyotomal FiLiP shown in A. Bottom, instantaneous retrograde transport velocities of the same punctum calculated using kymographic analysis. (C) The average retrograde transport velocity of mGFP puncta (n=5). (D) Left, a GFP-GPI-electroporated dermomyotome (somite VII, HH17) reveals that GPI-APs are present in punctate form along the FiLiPs (arrowheads), actin filaments labeled using phalloidin (red). Right, higher magnification image showing GPI-AP (green, white arrowheads) distribution along the FiLiPs containing actin filaments (red, phalloidin staining) in a representative protrusion. (E) A deconvoluted image from a transverse section of somite VI of HH18 embryo electroporated with FZ7-GFP shows the presence of Frizzled-7 (white arrowheads) along a representative FiLiP stained for actin (red). (F) Electroporation of a construct expressing mitochondria-specific aconitase (mAcn) fused with GFP specifically labels mitochondria (HH18, somite VII). (G) Composite image of mAcn (green) and DAPI (blue) with DIC. (H) Time-lapse images of a FZ7-GFP-electroporated dermomyotome (HH17, somite VIII) showing retrograde transport of two Frizzled-7 puncta (white and red arrowheads) along a representative FiLiP. White line marks a stable FiLiP with no Frizzled-7 punctum. (I) Top, a representative kymograph showing the transport of Frizzled-7-GFP punctum along the dermomyotomal FiLiP shown in H (white arrowhead). Bottom, graph quantifying the instantaneous retrograde transport velocities of the same Frizzled-7-GFP punctum calculated using kymographic analysis. (J) Instantaneous retrograde velocities of Frizzled-7 puncta from five different embryos (HH17-18, interlimb-level somites). Velocities were calculated using kymographs. (K) Average retrograde transport velocity of Frizzled-7-GFP puncta (n=5). (L) Graph quantifying the distribution of Frizzled-7 puncta on stable and dynamic FiLiPs. 96.67% of the Frizzled-7 puncta were seen on stable FiLiPs (n=20). (M) The percentage of stable FiLiPs positive for Frizzled-7 puncta. 28.5% of the stable FiLiPs transport Frizzled-7 along them (n=20). Scale bars: 10 µm in D (left), E-G; 2 µm in A,H; 1 µm in D (right).
Embryo slice preparation for live imaging
Successfully electroporated embryos were immediately embedded in 2% low-melting agarose (Sigma-Aldrich) and excised in blocks, which were mounted on vibratome wells for cutting. Vibratome wells were surrounded by ice and filled with ice-cold PBS. 200 μm-thick trunk-level transverse sections were collected in ice-cold PBS, placed on 35 mm-high glass-bottom dishes (Ibidi, Germany) that were coated with 0.1% poly-L-lysine (Sigma-Aldrich) and containing slice culture media (neurobasal medium, B27 supplement and penicillin-streptomycin). The sections were processed for time-lapse confocal imaging as described below. All media reagents were purchased from Life Technologies.

Confocal microscopy, image processing and statistical analysis
Glass-bottom dishes containing the electroporated chick embryo slices were imaged in real-time using a Carl Zeiss LSM 510 laser-scanning confocal microscope. Images were processed using Imaris software (Bitplane), and time-lapse movies were created using ImageJ (NIH). Fixed sections were imaged using a Leica TCS SP8 confocal laser-scanning microscope. Images were acquired, processed and analyzed using Leica application suite – advance fluorescence (LAS-AF) software. Fig. 4E was deconvoluted using the Huygens professional deconvolution software package (Scientific Volume Imaging). Statistical analyses were performed using Prism software (GraphPad). Error bars represent s.e.m. For further details, see supplementary material methods.

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Competing interests
The authors declare no competing financial interests.

Author contributions
M.S. and S. designed the experiments; S., F.P. and C.W. performed the experiments; S. analyzed the data, and S, F.P. and M.S. wrote the manuscript.

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