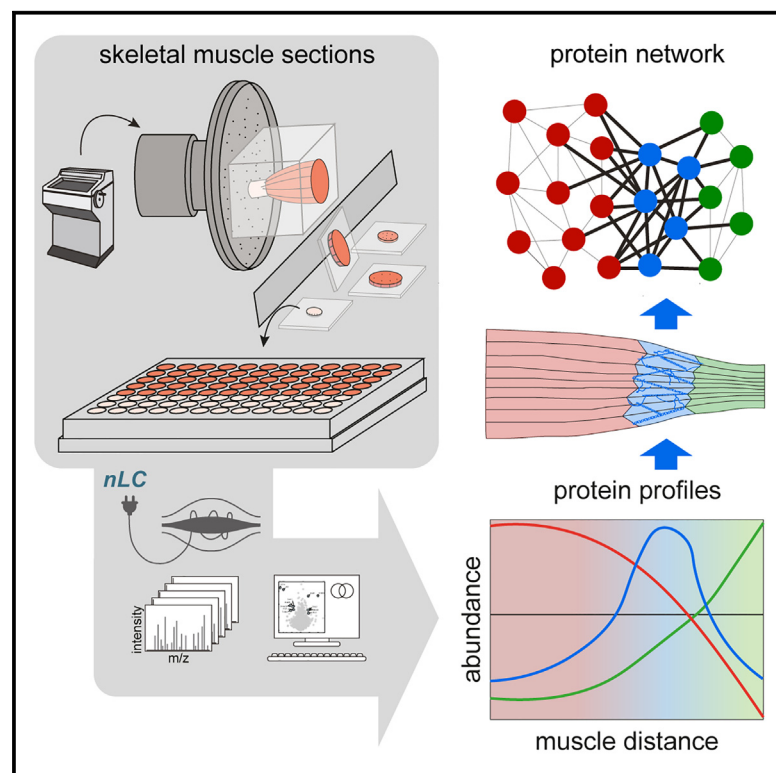


# Spatial proteomics of skeletal muscle using thin cryosections reveals metabolic adaptation at the muscle-tendon transition zone

## Graphical abstract



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## In brief

Schmidt et al. generated high-resolution protein profiles of skeletal muscles using thin cryosections and sensitive mass spectrometry to identify the molecular architecture of the muscle-tendon transition zone. This analysis reveals that the myotendinous junction is a highly complex connected area, including metabolic activities related to fatty acid synthesis and TGF- $\beta$  signaling.

## Highlights

- High-resolution spatial proteomic profiles (3,000+) localized muscle-tendon junction zone
- Distance-based networks reveal that myotendinous junction is complex and highly connected
- Longitudinal profiles highlight *de novo* fatty acid synthesis and renin-angiotensin system
- This indicates modulation of matrix formation in the muscle-tendon junction and tendon



## Resource

# Spatial proteomics of skeletal muscle using thin cryosections reveals metabolic adaptation at the muscle-tendon transition zone

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## SUMMARY

Morphological studies of skeletal muscle tissue provide insights into the architecture of muscle fibers, the surrounding cells, and the extracellular matrix (ECM). However, a spatial proteomics analysis of the skeletal muscle including the muscle-tendon transition zone is lacking. Here, we prepare cryotome muscle sections of the mouse soleus muscle and measure each slice using short liquid chromatography-mass spectrometry (LC-MS) gradients. We generate 3,000 high-resolution protein profiles that serve as the basis for a network analysis to reveal the complex architecture of the muscle-tendon junction. Among the protein profiles that increase from muscle to tendon, we find proteins related to neuronal activity, fatty acid biosynthesis, and the renin-angiotensin system (RAS). Blocking the RAS in cultured mouse tenocytes using losartan reduces the ECM synthesis. Overall, our analysis of thin cryotome sections provides a spatial proteome of skeletal muscle and reveals that the RAS acts as an additional regulator of the matrix within muscle-tendon junctions.

## INTRODUCTION

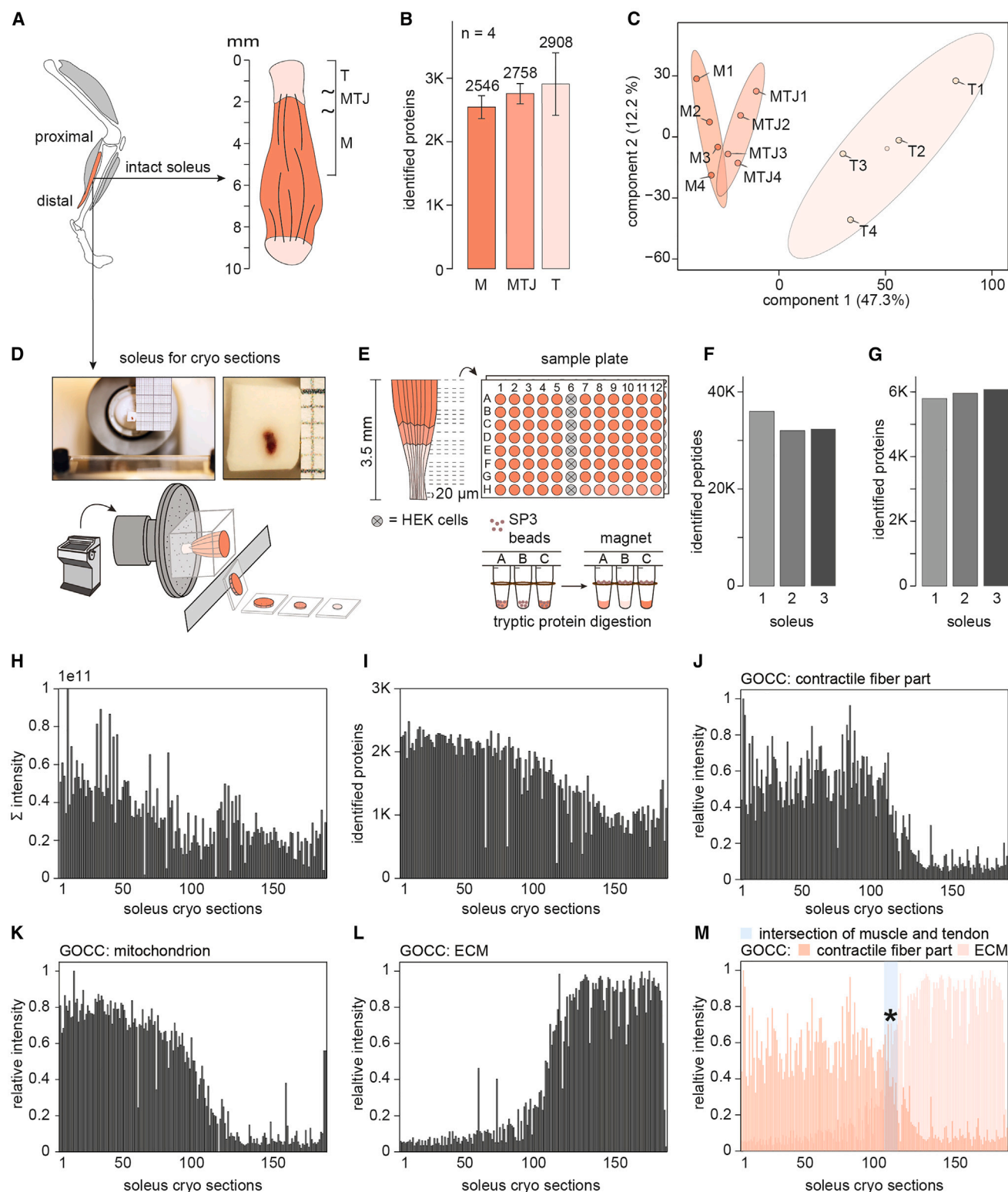
The mouse is one of the most widely used animal models to study musculoskeletal systems under normal and disease-related conditions. To handle the high tensile forces and loads, skeletal muscles have developed a specialized extracellular matrix (ECM) that surrounds and stabilizes contractile muscle fibers.<sup>1</sup> The myotendinous junction (MTJ), a junction area between the tips of muscle fibers and tendons that transmits muscle force to the bone, is another spatially defined region of skeletal muscle.<sup>2</sup> Earlier anatomical studies showed that the MTJ consists of interacting tendon fibers and terminal myocytes that form finger-like structures, resulting in strong bonds between tendons and muscle fibers. However, the MTJ is the weakest part of the muscle-tendon-bone axis and a high muscle load often leads to injuries of the MTJ in humans.<sup>3</sup> Another interesting feature of the MTJ is its neuronal innervation and the presence of pain receptors. Neuronal sensory units within the MTJ,

such as the Golgi tendon organs, may also play a role in muscle and tendon regeneration processes.<sup>4</sup>

At the molecular level, the MTJ exhibits specific protein expression patterns compared to neighboring muscle and tendon areas. For example, some proteins—including xinn-actin-binding repeat protein 2 (XIRP2), the collagen type XXII (COL22), and tenascin-C (TNC)—are enriched at the MTJ.<sup>5–7</sup>

So far, several quantitative proteomics studies have demonstrated a specific protein expression pattern of the MTJ in mouse,<sup>8</sup> horse,<sup>9</sup> and human samples.<sup>5,10</sup> In particular, analysis of human semitendinosus muscle-tendon samples revealed approximately 112 proteins are enriched in the MTJ, including COL22, tetraspanin-24 (CD151), and the cartilage intermediate layer protein 1 (CILP1).<sup>5</sup> The separation of muscle, MTJ, and tendon regions based on morphological features has provided a comprehensive overview of protein expression; however, unbiased spatial mapping of skeletal muscle fibers and associated tendons has not yet been conducted.





**Figure 1. Distinct protein profiles along the longitudinal muscle-tendon axis**

(A) Overview of the soleus muscles and the position in the hindlimb with indicated areas of muscle, MTJ, and tendon.

(B) Average number of proteins  $\pm$  SEM identified from manually dissected skeletal muscle, MTJ, and tendon areas of the soleus ( $n = 4$ ).

(C) PCA of protein expression patterns from manually dissected muscle, MTJ, and tendon samples.

(legend continued on next page)

Since the sensitivity of mass spectrometers has improved significantly in recent years, it is now possible to analyze proteins in samples with low protein concentrations. For example, sensitive mass spectrometers identified more than 1,000 proteins from individual muscle fibers of humans<sup>11</sup> and mice.<sup>12,13</sup>

In this study, we aimed to develop a method for high-resolution mapping of proteins in skeletal muscle fibers, the MTJ, and tendons. We isolated the soleus muscle from the mouse hind leg and analyzed single thin cryosections with a thickness of 20  $\mu\text{m}$  using short liquid chromatography-mass spectrometry (LC-MS) gradients to generate a spatial protein profile along the longitudinal muscle fiber-tendon axis. On average, we detected  $\sim 2,000$  proteins per cryo slice by LC-MS and identified more than 7,200 proteins in the whole dataset. Based on the 3,000 spatial protein profiles generated, we created a complex protein network and demonstrated increased network connectivity within the MTJ transition zone. We discovered that the expression of the renin-angiotensin system (RAS), which is involved in blood pressure regulation and modulation of the extracellular matrix, is significantly elevated in the MTJ and adjacent tendon areas compared to muscle. This is an unbiased approach to study the protein patterns within skeletal muscle and we demonstrate the high sensitivity of our workflow by detecting multiple transcription factors, signaling molecules, and matrix proteins.

## RESULTS

### Manual dissection of soleus skeletal muscle based on morphological features only partially identifies the MTJ

First, we isolated soleus muscles ( $n = 4$ ) from the hind leg of mice and dissected the muscle based on macroscopic features into three parts: central muscle, muscle-tendon, and tendon (Figures 1A and S1A–S1C). In total, we identified 4,120 protein groups with a false discovery rate (FDR) below 1% at the protein and peptide level. Comparison of all three parts revealed 3,164 common proteins and 252 proteins with restricted protein expression in tendons (Figures 1B and S1D; Table S1). Principal-component analysis (PCA) of the soleus showed clear separation between the tendon and muscle samples, whereas the MTJ and muscle samples were barely separated (Figure 1C). Hierarchical clustering and Gene Ontology (GO) analysis of 896 significant proteins (ANOVA) also revealed enhanced expression of matrix proteins in the tendon compared to the muscle and MTJ samples (Figures S1E and S1F). In total, we identified 1,260 significantly altered proteins between the MTJ and tendon (Figures S1G–S1I; Table S1). However, a volcano plot showed no significant outliers between the MTJ and muscle samples (Figure S1J). Thus, we conclude that manual dissection of muscle, MTJ, and tendon areas is helpful but does not enable clear delineation of the MTJ and muscle tissue. Therefore, there is a need

for an unbiased method to spatially analyze the muscle-tendon transition.

### Study design, quality control, and optimization of protein identification rates from thin cryotome sections using short LC-MS gradients

To map protein expression along the longitudinal muscle-tendon area, we cryo-embedded soleus muscles and prepared consecutive cross-sections from the central to distal areas using a cryotome (Figure 1D). The distal (tendon) slices have an area of less than  $\sim 0.2 \text{ mm}^2$  and yield less than 1  $\mu\text{g}$  of protein (Figures S2A–S2C); therefore, we first tested comparable amounts of protein from muscle lysates using different lengths of LC-MS gradients combined with a 15-cm reverse-phase column. Approximately 1,900 protein hits were achieved with 100-ng input and 30-min LC-MS gradients (Figures S2D–S2G). We also used 100 ng of HeLa cell lysates as control and identified  $\sim 5,000$  proteins (Figures S2H–S2K). Finally, we measured central and distal muscle slices with thicknesses of 10–30  $\mu\text{m}$  and detected an average of  $\sim 2,000$  proteins per 20- $\mu\text{m}$ -thick slice (Figures S2L and S2M; Table S5).

### Protein normalization and implementation of missing values to generate complete protein profiles

We prepared approximately 175 20- $\mu\text{m}$ -thick cryotome slices, representing a total length of around 3.5 mm of tissue, covering the area between the central muscle and the proximal tendon areas (Figure S1A, upper part). Single cryotome slices were transferred to 96-well plates and subjected to tryptic digestion according to the SP3 protocol (Figure 1E).<sup>14</sup> To ensure high data completeness, we chose a data-independent acquisition (DIA) approach and identified an average of  $\sim 6,000$  proteins per muscle profile ( $n = 3$  muscles; Figures 1F and 1G). As a control, we used HEK293T cell lysates and identified  $\sim 4,000$  proteins, demonstrating constant instrument performance and high technical reproducibility during the MS analysis period of  $\sim 11$  days (Figures 1E column 6 and S3A). In addition, we observed virtually no carry-over of contractile myosin heavy chain (MYH) peptides in blank runs (Figure S3B) and the peptide retention times were monitored by adding non-naturally occurring peptides (Figures S3C and S3D).<sup>15</sup> To obtain reliable protein profiles, we filtered the generated datasets for proteins that were identified based on at least two peptides and protein intensities  $> 10^4$  and each protein had to be detected in at least two out of the three biological replicates (Figure S3E).

As the muscle and tendon slices exhibited different protein intensities (Figures 1H and 1I), we normalized the data by dividing the individual protein intensities by the sum of the protein intensity per slice and performed Z score normalization. As an example, we plotted the intensities of TNC, alcohol dehydrogenase 1 (ADH1), and MYH2 before and after normalization

(D and E) Overview of the experimental workflow.

(F and G) (F) Identified precursors and (G) proteins in the three profiling experiments ( $n = 3$  muscles).

(H–M) Bar plots for the sectioned soleus samples, starting from the left side section 1 (central muscle area) to right side section 175 (tendon area), showing (H) sum intensity of identified proteins, (I) number of proteins identified per section, (J) relative intensity of GO terms enriched in contractile fiber part, (K) mitochondria, and (L) ECM and (M) an overlay of the relative intensity of the contractile fiber part and ECM GO terms. GOCC, GO cellular component; M, muscle; SP3, single-pot solid-phase sample preparation; T, tendon. Blue line indicates the muscle-tendon transition.

(Figures S4A–S4C). Although the DIA approach leads to a high degree of data completeness, we observed missing intensities for proteins with low abundances and for proteins that have restricted expression in muscle or tendon. Hence, to generate a complete protein profile, we used sliding windows covering adjacent sections to fill in the missing protein intensities. When we detected a missing protein intensity on a section, we selected the protein intensities of the same protein from the 10 sections before and 10 sections after the missing value and used a linear model to impute the protein intensity for that section (Figure S4D). For proteins that were only found in the muscle and not in the tendon, the protein intensities of the lowest 5% of all protein intensities in the dataset were used to replace the missing values in the tendon sections (Figure S4E). To include proteins with local expression in our profiling dataset, we accepted profiles that had at least 70% data completeness in a range of 200  $\mu\text{m}$  from the total length of 3,500  $\mu\text{m}$  (Table S2).

We observed a slight profile shift between the three muscles, indicating different starting points for the sections within the central muscle areas or a slight variation in muscle size. To obtain congruent protein profiles across the three solei, we first calculated the probability distribution for every protein in each experiment using a bootstrap analysis. Based on this analysis, each protein profile was correlated to the same randomized sample set to exclude any bias, variances, and predicted errors. Every protein that had a probability distribution with a d-score higher than 5 was selected as a protein with a significantly increasing or decreasing intensity profile from muscle to distal (tendon) end.<sup>16</sup> Using these proteins, we then calculated the offset and performed a shift for all other profiles (Figure S4F). Finally, all profiles were combined using locally estimated scatter smoothing (LOESS) to obtain a representative profile for each individual protein (Figure S4G). In total, we generated ~3,000 protein profiles from three independent soleus muscles (Data S1 and Table S2).

### Protein profiles of sarcomeric and matrix proteins reflect the position of the MTJ in the muscle

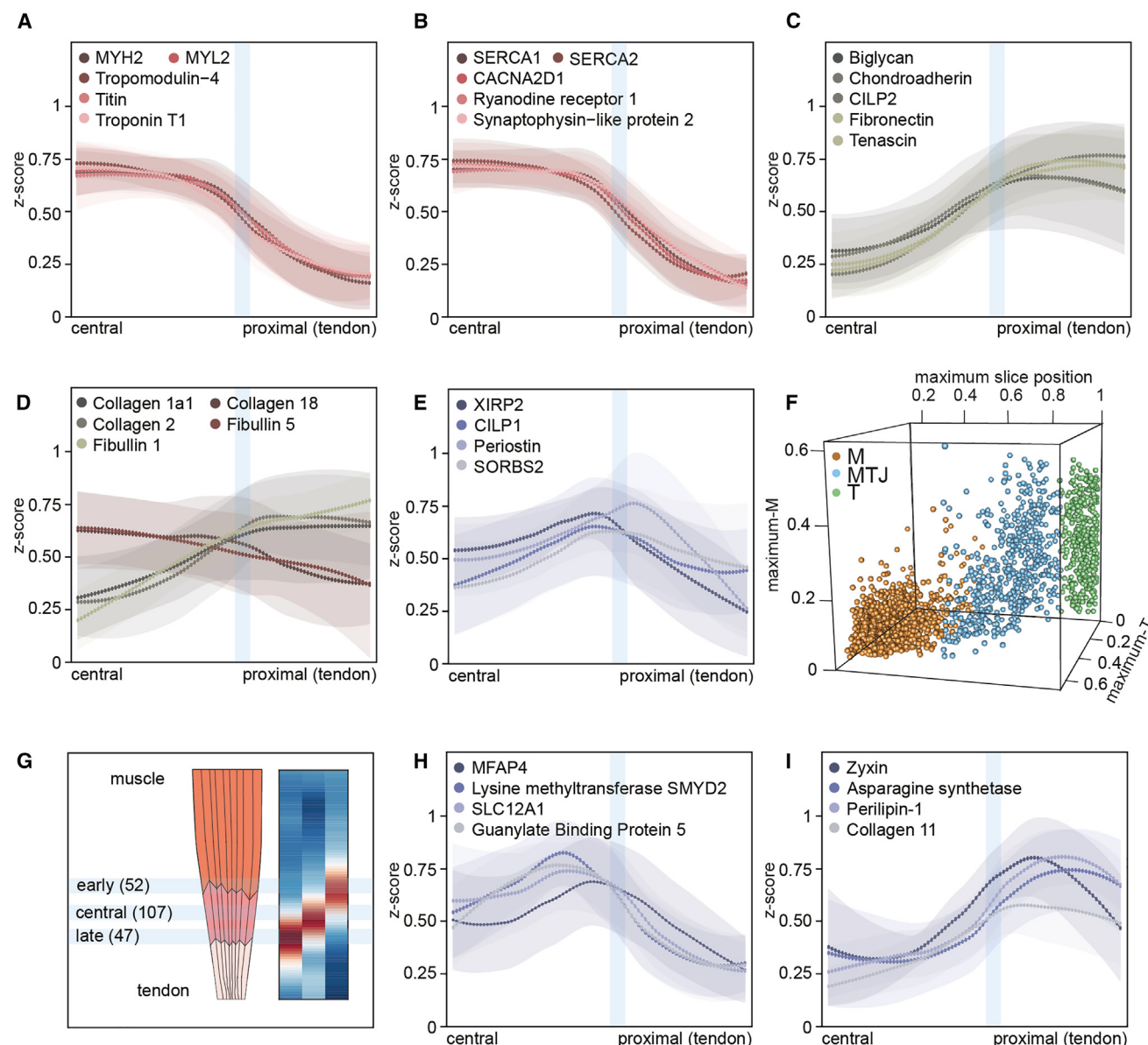
The protein profiles were assessed using systematic GO term analysis. As expected, we mainly observed GO terms associated with components of contractile fibers and mitochondria in the central muscle areas (Figures 1J and 1K), while the tendon area showed enrichment of terms connected to ECM organization (Figure 1L). Since we observed an intersection between contractile and matrix proteins at the junction between the muscle and tendon, we suggest that this intersection may represent the MTJ (Figure 1M, blue line). A heatmap of the enriched GO terms also substantiated the distinct biological functions of the muscle and tendon (Figures S5A–S5D). To demonstrate how protein expression profiles change along the muscle-tendon axis, we plotted the Z score normalized protein profiles of sarcomeric (Figure 2A),  $\text{Ca}^{2+}$  handling (Figure 2B), and mitochondrial proteins (Figure S6A). These proteins show high expression in central muscle areas and steadily decrease in the transition zone between muscle and tendon, indicating the end of the muscle fiber unit. Conversely, the most abundant matrix proteins, including collagen 1 (COL1A1), chondroadherin (CHAD), and TNC, showed a marked increase in expression toward the proximal end of the skeletal muscle, reflecting the start of the tendon area (Figures 2C and 2D).

In addition to the change in the muscle fiber-matrix ratios along the longitudinal axis, we also observed intracellular proteins with increasing profiles from muscle to tendon, possibly representing specific cell populations such as tenocytes, tendon-derived stem cells, or other fibroblasts.<sup>17</sup> For example, the detection of different isoforms of creatine kinase (CK) and filamins confirmed these cellular changes; we also observed high intensities of the muscle- and mitochondria-specific (CKM, CKMT2) isoforms in skeletal muscle, while the brain isoform (CKB) exhibited a reciprocal pattern (Figure S6B). Similarly, the filamin c (FLNC) isoform showed a similar decreasing profile from muscle to tendon, whereas filamin B (FLNB) increased toward the MTJ. Together, the isoform profiles of CK and filamins demonstrate the presence of different cell populations in the muscle and tendon areas.<sup>18–20</sup> Of note, the intersections of the CK and filamin isoform profiles with the GO terms for sarcomere and matrix proteins are congruent, supporting the suggested position of the MTJ.

Selected protein profiles with constant protein abundances along the muscle-tendon axis, including collagen 4, are shown in Figure S6C as a control. Systematic k-means clustering revealed 10 clusters with 143 constant, 646 ascending, and 1,850 descending protein profiles (Figures S7A–S7J). The large number of proteins with descending profiles reflects the high number of proteins in skeletal muscle fibers.

Next, we investigated the profiles of previously described MTJ proteins, including XIRP2, periostin (POSTN), CILP1, and SORBS2.<sup>8,21</sup> These MTJ candidates exhibited maximum intensities at the interface between muscle and matrix proteins (blue line), which further substantiates the position of the MTJ (Figure 2E). For example, the profiles of XIRP2 and CILP1 showed increased intensities in ~13 slices, corresponding to a distance of ~260  $\mu\text{m}$ . To systematically identify potential MTJ proteins in our dataset, we determined the profile maximum for each protein and subtracted the average intensity of the same protein in muscle and tendon slices. Proteins with a  $\geq 1.5$ -fold increase in their maximum intensity in the MTJ compared to the average intensity in muscle and tendon were categorized as potential MTJ proteins. Three-dimensional plotting of all protein maxima and slice positions illustrated the distribution of the muscle, MTJ, and tendon proteins (Figure 2F). The separation of the different muscle areas was additionally visualized by PCA of three muscle profiles (Figures S8A–S8C). Using this approach, we identified 206 proteins with maximum intensities around the central MTJ (Table S2), including 52 protein profiles with early maxima, 107 protein profiles with central maxima, and 47 protein profiles with late maxima at the MTJ (Figure 2G).

Comparison of the 206 MTJ protein profiles generated from analysis of the soleus sections and our analysis of the manually dissected M, MTJ, and T samples (Figure 1) revealed a congruent pattern for six proteins (Figure S10A). We detected an overlap on either the muscle or tendon side for 93 proteins, and 107 proteins were only partially or not detectable in the intact muscle samples compared to our protein profiling. This demonstrates the high sensitivity of our profiling approach and significantly expands the catalog of potential MTJ proteins. One example of a potential early MTJ protein is extracellular



**Figure 2. Proteomic profiling of soleus muscles reveals additional MTJ marker proteins**

(A–E) Protein expression profiles along the longitudinal muscle-tendon axis of (A) selected sarcomeric proteins, (B) selected calcium housekeeping proteins, (C) and (D) selected ECM proteins, and (E) selected MTJ proteins.

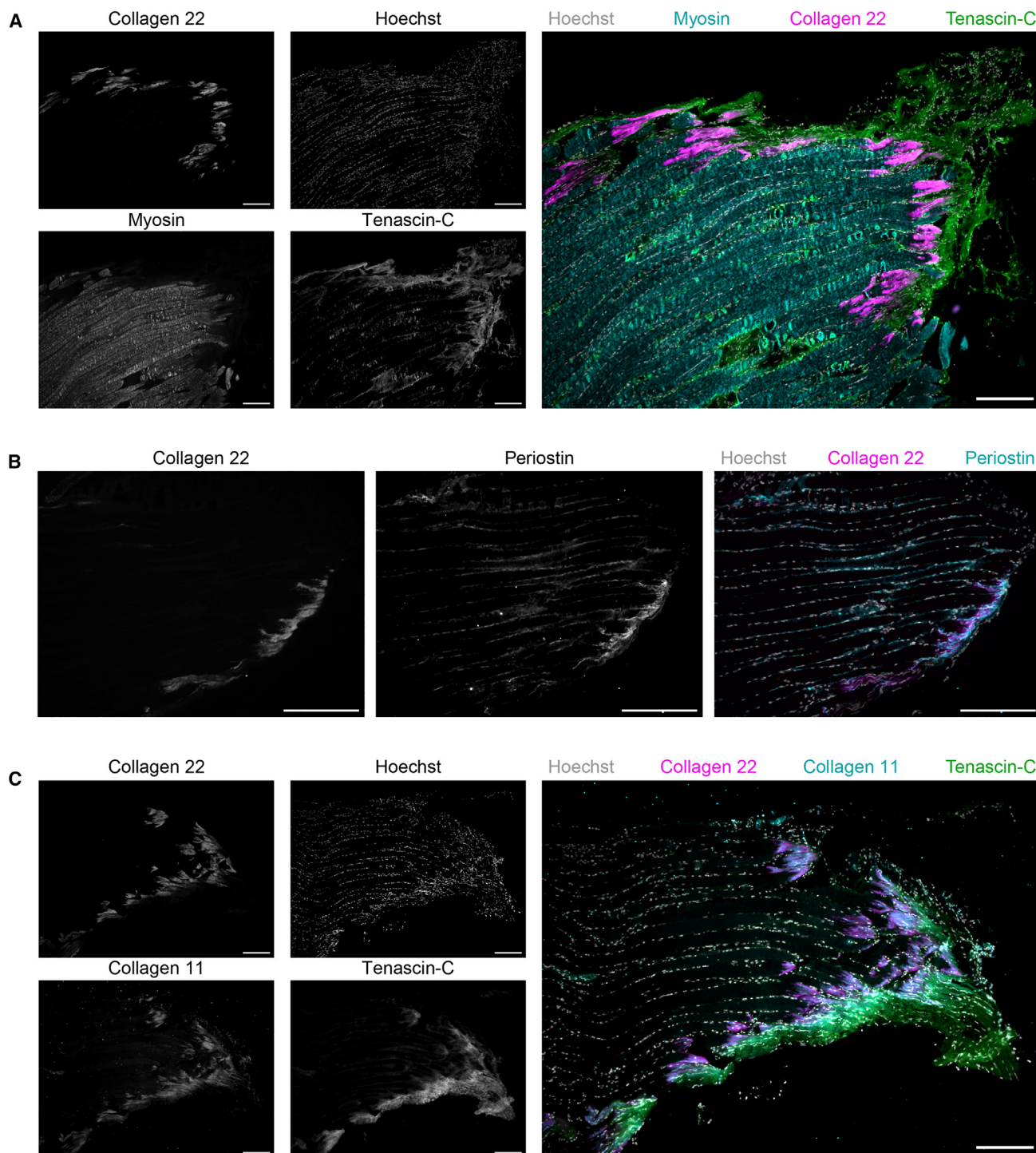
(F) Three-dimensional plot of proteins localized at muscle, MTJ, and tendon areas. Each dot represents the maximum of one protein profile. The x, y, and z axes show the spatial orientation of the maxima of the protein profiles in the muscle (orange), MTJ (blue), and tendon (green) area, and the differences in protein abundance between the maxima and the muscle and tendon.

(G) Summary of identified proteins with early, central, and late maxima in the MTJ area.

(H and I) (H) Selected protein expression profiles with early and (I) late maxima in the MTJ area. Blue line indicates the muscle-tendon transition.

microfibril-associated glycoprotein 4 (MFAP4), which binds to collagen in elastic fiber-rich regions (Figure 2H). A study of protein expression profiles in human muscle across the lifespan found that MFAP4 is one of the most underrepresented proteins during aging.<sup>22</sup> Therefore, the expression of MFAP4 within the MTJ might be involved in the correct arrangement of elastic fibers and stabilize the transition zone. Another protein with an early maximum is the lysine methyltransferase SMYD2, which

methylates chaperones outside the nucleus and interacts with myosin heavy chains and titin.<sup>23,24</sup> Whether the increased expression of SMYD2 at the MTJ also contributes to the stability of the compound by methylating chaperones and/or sarcomere-associated proteins remains unclear and needs to be investigated in future experiments. MTJ protein profiles with late maxima include collagen 11 and the focal adhesion protein zyxin (Figure 2I).



**Figure 3. Microscope images and immunostaining of potential MTJ candidates**

Immunostaining of collagen 22, MYH, TNC (A), periostin (B), and collagen 11 (C) in longitudinal mouse soleus sections. Muscle is stained using MYH and tendon is stained using TNC. Collagen 22 is used as an MTJ marker. Periostin and collagen 11 staining overlap with collagen 22 staining. Two biological replicates were performed for each staining. Scale bar, 200  $\mu$ m.

Next, we performed immunostaining of longitudinal sections of mouse soleus muscles with anti-MYH pan, anti-collagen 22, and anti-TNC antibodies, which substantiated the location of

the muscle, MTJ, and tendon area, respectively (Figure 3A). Similarly, to COL22, the MTJ marker protein periostin also localized at the MTJ (Figure 3B). The spatially restricted expression of

COL11 within the muscle-tendon transition zone is shown in Figure 3C. Other proteins with transient maxima at the MTJ that have not previously been described include immunoglobulin superfamily member 8 (IGSF8), myosin light-chain kinase, and smooth muscle (MYLK) (Figure 4A). Furthermore, we detected several proteins with ascending protein expression profiles from the muscle to tendon, including the clock gene *BMAL2* and the secreted protein connective tissue growth factor (CTGF, also known as *CCN2*) (Figure 4B).<sup>25</sup>

Integrins are cell adhesion receptors that bind extracellular matrix proteins, such as collagen, laminin, and fibronectin. We found enhanced levels of integrin *ITGα11* and *ITGβ4* in the tendon area (Figure S6D). We also observed constant or descending profiles for the detected laminin isoforms (*laminin α2*, *α4*, *α5*, *β1*, *β2*) and basal cell adhesion molecule (*BCAM*) from muscle to tendon (Figures S6E and S6F–S6H).

Interestingly, neuronal marker proteins such as neurofilament light polypeptide (*NEFL*) and myelin proteins (*MBP*, *MPZ*, *MAG*) showed biphasic profiles, with high levels in the central muscle sections and second maxima at the MTJ (Figure 4C). The dyslexia-associated *KIAA0319*-like protein also exhibited a similar profile to the neuronal marker proteins. *KIAA0319*-like protein is known to be involved in brain development and neuronal migration.<sup>27,28</sup> Overall, our protein profiling demonstrates a heterogeneous profile of membrane, adhesion, and matrix proteins along the longitudinal muscle-tendon axis<sup>4,21</sup> and highlights the distinct cellular composition along the longitudinal muscle-tendon axis.

### The MTJ and tendon areas show increased abundances of enzymes related to *de novo* fatty acid synthesis and the RAS

Since skeletal muscle fibers contain high numbers of mitochondria, we also observed descending profiles for most mitochondrial proteins starting at the MTJ, reflecting the end of the skeletal muscle fiber unit (Figure S6A). Accordingly, similarly to mitochondrial proteins, we also detected descending profiles for 11 members of the solute carrier family 25 member (*SLC25*), including *SLC25A11* and *SLC25A12* (Figure 4D). *SLC25A* transport molecules across mitochondrial membranes and are involved in a variety of cellular processes.<sup>29</sup> Interestingly, however, we observed increasing profiles from muscle to tendon for some mitochondrial proteins, such as the tricarboxylate transporter *SLC25A1* (also known as citrate transporter protein [CTP]) (Figure 4D). This suggests a higher cytoplasmic content of citrate, which is used as the main carbon source for the biosynthesis of fatty acids, in the distal muscle-tendon area. It is important to note that a number of enzymes involved in the conversion of citrate to acetyl-coenzyme A (CoA) and fatty acids, such as ATP citrate lyase (*ACLY*), acetyl-CoA carboxylase (*ACC*), and fatty acid synthase (*FASN*), were also upregulated in the MTJ and tendon compared to muscle areas (Figures 4E and 4F).

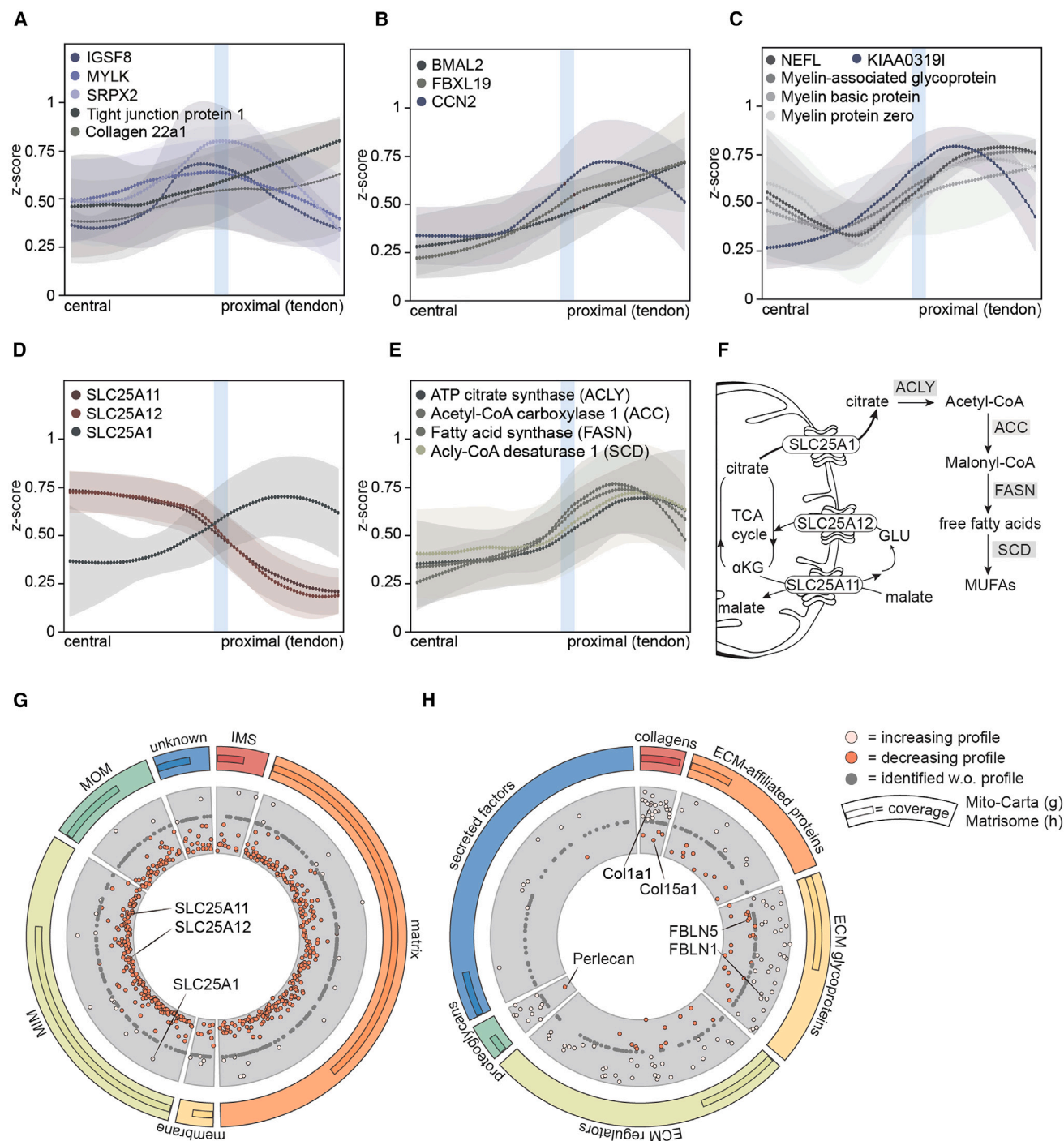
The solar protein expression profile plot of mitochondrial proteins highlights the enhanced abundance of *SLC25A1* in tendons. In contrast, most mitochondrial proteins, including members of the *SLC25A* family, showed the opposite trend, suggesting that other cell populations, such as tenocytes, express this transporter more abundantly (Figure 4G). We also

plotted and observed increased expression of annotated ECM proteins in the matrix compared to the muscle. Extracellular proteins that exhibited the opposite trend, such as *Col15*, *Col18*, fibulin 5, and perlecan, are shown in Figures 4H and S6G.

The RAS is closely linked to blood pressure regulation. The angiotensin-converting enzyme (ACE) and serine proteinase chymase 1 (*CMA1*) cleave the inactive peptide angiotensin I into active angiotensin II. Both of these enzymes showed strong ascending profiles from muscle to tendon (Figure 5A). Similarly, other RAS proteins, including the aminopeptidase N (*ANPEP*) and glutamyl aminopeptidase (*ENPEP*), also exhibited similar ascending profiles from muscle to tendon areas. Although we did not detect the transforming growth factor beta (*TGFβ*) receptor and its downstream factors suppressor of mothers against decapentaplegic (*SMAD*) 2/3, we observed elevated levels of *EGFR*, the *TGFβ1*-induced transcript 1 protein (*TGFβ1I1*), and CTGF in the tendon area compared to skeletal muscle (Figure 5B). In addition, comparison of intact muscle and tendon samples revealed increased expression of angiotensinogen (*AGT*), type-1 angiotensin II receptor-associated protein (*AGTRAP*), *TGFβ1*, and *TGFβR3* in tendons (Figures 5C and 5D). LC-MS analysis of immortalized mouse tenocyte cell lines substantiated the presence of several members of the RAS (Figure 5E). To examine the effect of a commonly used RAS inhibitor on tenocytes, we treated immortalized tenocytes with losartan for 1–4 days. We observed significant downregulation of ECM proteins after 4 days of treatment with low (10  $\mu$ M) and higher doses (50  $\mu$ M) of the inhibitor (Figures 5F and S9A; Table S3). To further elucidate activation of the RAS signaling pathways, we cultivated tenocytes with 10  $\mu$ M losartan for 4 h. Phosphopeptide enrichment revealed altered phosphorylation of known *TGFβ* pathway members, including *CDK2* (pY15) and *TGFβ1I1* (Figures S9B and S9E–S9G). Similarly, GO analysis showed decreased phosphorylation of terms such as cellular response to growth and *TGFβ* stimulus (Figures S9C and S9D). This indicates that treatment with losartan inhibits the *TGFβ* and RAS signaling pathways in tenocytes and thereby alters the matrix.

### Network analysis of protein profiles along the muscle-tendon axis reveals the complex connectivity of the muscle-tendon junction

Since we identified ~3,000 protein profiles within the soleus, we aimed to correlate the protein expression patterns to develop a soleus protein-protein connection network to better visualize the different areas of the soleus. We used the generated protein profiles to perform a delay analysis.<sup>30,31</sup> Delay analysis was initially designed to identify the time delay between time-series profiles (i.e., the amount of time it takes for one feature to respond to the other). By shifting the time index of one of the features and repeating the correlation analysis, the time delay is then defined as the required shift in time to reach a maximum correlation. In our analysis, we replaced the time factor with the spatial arrangement of the slices to illustrate the distance within the muscle profile. Pairs of protein profiles were correlated, and the spatial delay to reach the maxima was identified (Figures 6A–C). Those pairs with a delay of less than 100  $\mu$ m were then subjected to a data processing inequality approach to remove indirect connections (i.e., links that occur as a result of sequential protein correlations). The resulting links between proteins were visualized using



**Figure 4. Protein expression profiles identify different pathways related to metabolic and neuronal activity along the longitudinal muscle-tendon axis**

(A and B) Protein expression profiles of proteins not previously described as MTJ markers and showing maximal protein intensities in the MTJ area.

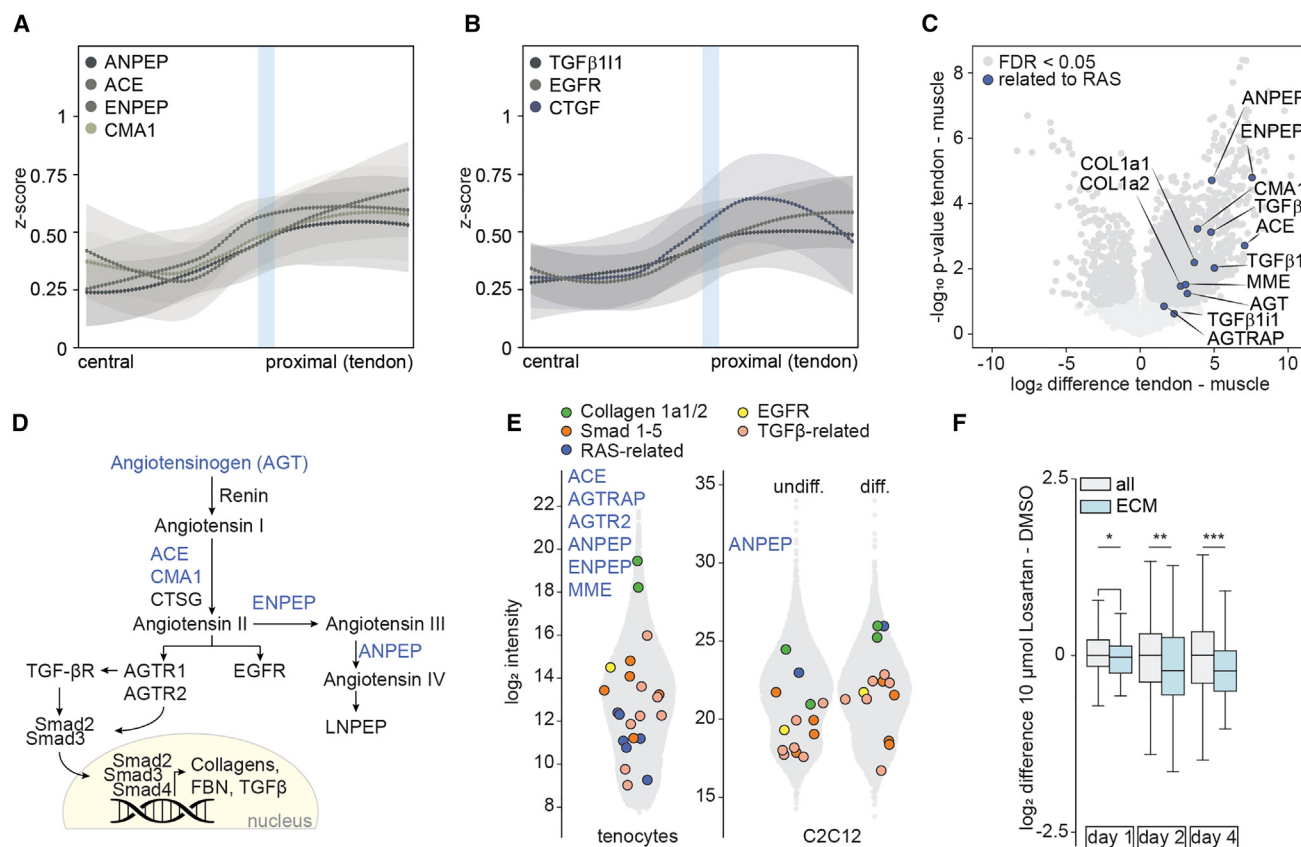
(C) Selected protein expression profiles of neuronal factors showing biphasic profiles with maxima at the central muscle and distal MTJ-tendon areas.

(D) Mitochondrial solute carrier proteins of the SLC25A family exhibit descending (SLC25A11 and SLC25A12) or ascending (SLC25A1) profiles.

(E) Protein expression profiles of selected members of the fatty acid synthesis pathway.

(F) Mitochondrial TCA cycle and fatty acid synthesis pathway.

(G and H) Hamilton plots of mitochondrial proteins and ECM proteins based on Mitocarta entries and the mouse matrisome.<sup>26</sup> Blue line indicates the muscle-tendon transition.



**Figure 5. AGTR1 inhibitor reduces ECM proteins in isolated tenocytes**

(A and B) Protein expression profiles along the longitudinal muscle-tendon axis of ACE, ANPEP, CMA1, and ENPEP (A) and CTGF, EGFR, and TGFβ111 (B). (C) Volcano plot of intact muscle and tendon samples reveals higher expression of the renin-angiotensin system (RAS)-related proteins in tendon. Differentially abundant proteins are highlighted in dark gray (Student's t test: FDR < 0.05, s0 > 0.1) and RAS-related proteins in blue. (D) Schematic overview of the RAS. Identified proteins are labeled in blue. (E) Jitter plot of log<sub>2</sub> intensities of identified proteins in immortalized tenocytes and undifferentiated and differentiated C2C12 cells/myotubes. Identified proteins related to the RAS are labeled in blue. (F) Boxplot of immortalized tenocytes treated with 10 μM losartan, showing a decrease in ECM proteins. Data are represented as mean ± SEM. Asterisks denote significance (t test: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001). ANPEP, alanyl aminopeptidase; CMA1, chymase 1; CTGF, connective tissue growth factor; EGFR, epidermal growth factor receptor. Blue line indicates the muscle-tendon transition.

Cytoscape and arranged in a spring-embedded layout. Mapping of known muscle (orange) and tendon proteins (green) enabled us to identify specific regions within the network that highlight the spatial organization of proteins (Figure 6D, Data S2, and interactive online version <https://medex.uber.space/networks/index.html#/>). Known MTJ proteins (n = 10) and MTJ proteins identified in this study (n = 196) localized to a specific region of the network (blue). GO term analysis of the MTJ network proteins for molecular function and cellular localization indicated enrichment of proteins associated with the terms ECM structural constituent, ECM structure organization, cell-substrate junction, and anchoring junction (Figure 6E). In addition, we observed a significantly higher betweenness centrality score for MTJ proteins compared to the muscle and tendon proteins, suggesting MTJ proteins are more interconnected than the proteins in the other areas (Figure 6F). As an example, we selected collagen 1 and observed biglycan and fibronectin 1 as direct neighbors with a correlation distance of less than 100 μm. Moreover, we observed several MTJ proteins

with late maxima, including elastin, in proximity with collagen 1 (Figure 6G).

## DISCUSSION

Our comprehensive longitudinal protein profiling based on short LC-MS runs of single muscle sections allowed us to exactly localize the transition area between muscle and tendons in an unbiased manner and revealed 206 proteins have a local maximum at the MTJ. In addition, our protein network analysis shows that the MTJ has a higher level of connections compared with the muscle and tendon areas, supporting the function of the MTJ as a link between muscle fiber tips and tendon. We also observed that several members of the fatty acid synthesis pathway and RAS exhibit ascending profiles from muscle to tendons. In validation of these observations, treatment of isolated tenocytes with losartan reduced ECM protein secretion, which suggests that the RAS contributes to matrix formation within the MTJ and tendon.

In addition to the complex protein expression pattern of the MTJ, the anatomical arrangement of skeletal muscle fibers and tendons is also an important factor in muscle stability. Skeletal muscles with a bipennate shape, such as the soleus, resemble a feather-like structure, with the fibers on either side of the tendon arranged at a specific angle. Manual dissection or separation of certain muscle areas such as the transition between muscle and tendon is therefore almost impossible. Our approach enabled us to determine the transition zone based on (1) the intersection of GO terms for skeletal muscle and matrix proteins, (2) the profiles of specific isoforms, and (3) observation of maximal protein expression of several known MTJ marker proteins, including XIRP2 and CILP1, at the intersection. Overall, the generated protein profiles clearly indicate the area of the MTJ and highlight the complex spatial architecture of the muscle and tendon regions.

### **Protein profiles of ECM proteins indicate tissue-specific adaptations of the matrix along the longitudinal muscle and tendon axis**

Increasing profiles from muscle to tendon were observed for about 10% of all proteins identified in our dataset, and the identified proteins consist mainly of matrix proteins such as collagens, tenomodulin, and proteoglycans. Conversely, we also observed that extracellular proteins exhibited the opposite trend, showing increased protein expression in muscle areas compared to tendons. For example, the basement laminin proteins LAMA2, LAMA4, and LAMA5, as well as the heparan sulfate proteoglycans perlecan and nidogen 1, were expressed at higher levels in central muscle areas compared with tendons. This reflects their function as basal lamina proteins that surround skeletal muscle fibers and interact with collagen type IV (Figure S6F). In addition, higher signals for fibrillar collagen 11 were detected in the MTJ than in muscle and tendon; this finding has not been described previously. We suggest that the expression of collagen 11 at the MTJ could induce enhance fibrillogenesis and thus regulate the formation of tendon fibrils and mechanical stability at the MTJ.<sup>32,33</sup>

Members of the fibulin (FBN) family are known interactors of collagens and are also involved in the organization, assembly, and stabilization of the ECM.<sup>34</sup> Similarly to several collagens, we observed an ascending profile from muscle to tendon for FBN1. In contrast, FBN5 was more abundant in muscle areas compared to tendon; thus, we hypothesize that FBN5 might be involved in cell adhesion and anchoring muscle fibers to the ECM through its interaction with other ECM proteins and cell surface receptors. There is also evidence to suggest that FBN5 may be involved in certain muscular dystrophies and muscle pathologies such as muscle fibrosis, a condition characterized by excessive accumulation of fibrous connective tissue in muscle.

Overall, our profiling demonstrates that muscle fibers and tendons are surrounded by specific matrix structures that provide contractility support to the muscle fibers.

### **Comparison of transcriptomics and proteomics datasets shows similar enrichment of known MTJ marker proteins**

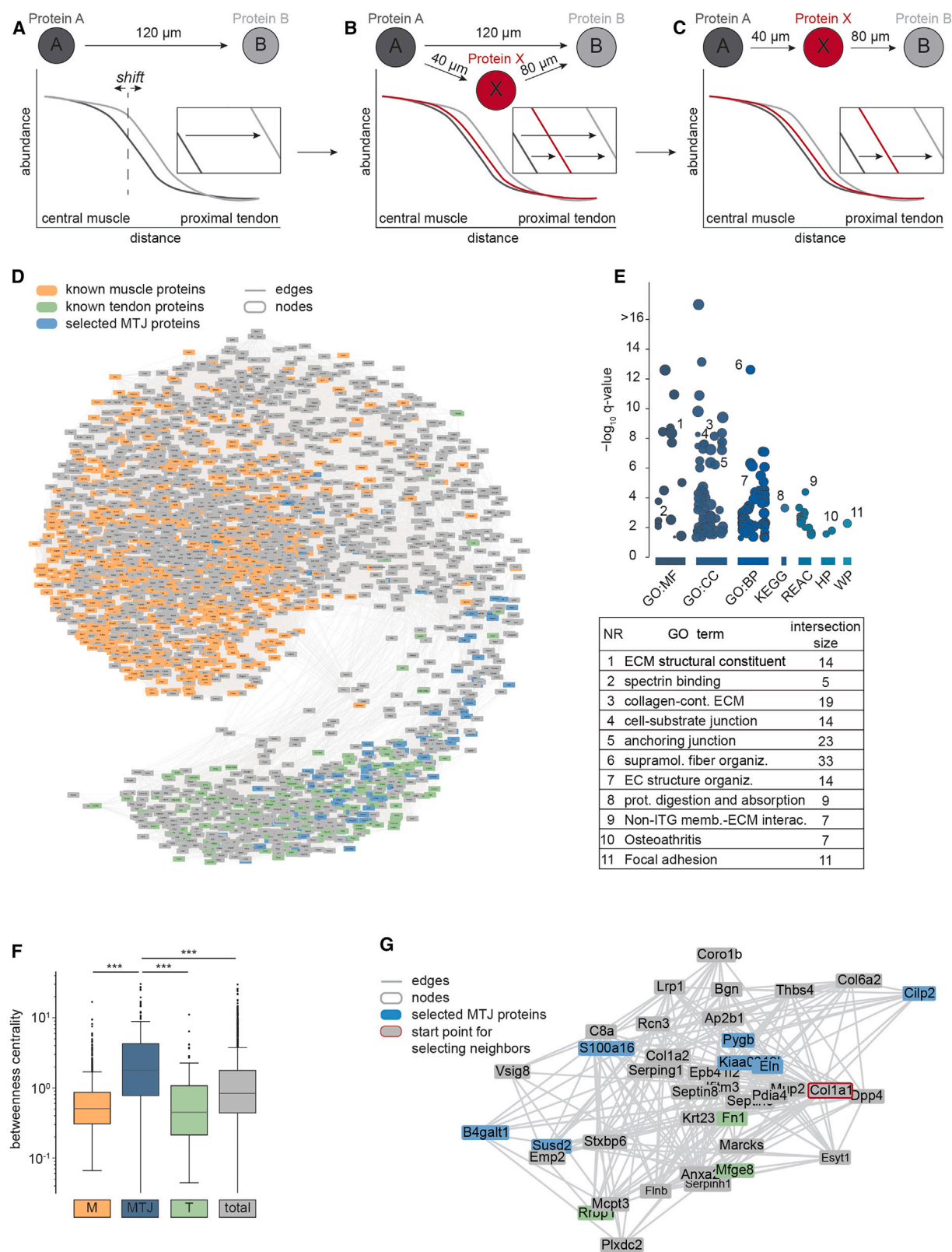
Comparison of three previous omics studies in mouse,<sup>8</sup> horse,<sup>35</sup> and human<sup>10</sup> muscles with our proteomics data demonstrated corresponding enrichment of 10 of 29 proteins at the MTJ,

including XIRP2, SORBS2, periostin, and CILP1 (Figure 2E; Table S2). For the other 19 proteins that were previously described to be enriched in the MTJ, we observed increasing protein profiles from muscle to tendon, which possibly indicates these proteins function within the MTJ and adjacent tendon areas. One reason for the low extent of overlap between these datasets could be that the correlation between mRNA and protein expression is poor due to variations in the half-lives of proteins and post-transcriptional regulation. Moreover, accumulation of proteins in the extracellular matrix cannot be detected at the mRNA level. As indicated earlier, skeletal muscles undergo various morphological and functional adaptations, depending on their location in the body. The isolation of different skeletal muscles from mice, horses, and humans could be another possible explanation for the high variation in the MTJ candidates identified to date (Figures S10B and S10C). In addition, recent RNA sequencing (RNA-seq) profiling of single myonuclei identified 291 genes that were enriched in MTJ myonuclei. Comparison with our proteomics data revealed an overlap of 115 proteins, including seven proteins that we defined as MTJ marker proteins.<sup>36</sup> To determine the spatial and cellular localization of these MTJ candidates more precisely, immunostaining based on co-detection by indexing (CODEX) could be used in future experiments.<sup>37,38</sup>

### **The biphasic profiles of several neuronal factors indicate neuronal innervation of distal muscle tissue**

We also found several proteins related to neuronal activity with biphasic maxima at the central muscle area and MTJ-tendon transition zone (Figure 4C). For example, the maxima of NEFL and myelin proteins (MBP, MAG, MPZ) in the central muscle area are probably due to neuronal innervation of the muscle fibers and indicate the presence of motor endplates. Although the MTJ and tendon are primarily responsible for connecting muscles and bones, they are also innervated by neurons and contain several mechanoreceptors (Figures S10D–S10F).<sup>4</sup> These neurons convert mechanical energy into electrical signals that are then used by the central nervous system to control body movements. Studies on the skeletal muscles of fish, mice, and horses confirmed the presence of sensory nerve endings and neuronal marker proteins, proving that the MTJ acts as a hub for mechanosensing and pain reception.<sup>21,35</sup> Since more than 4% of the 1,000 most abundant tendon proteins are neuronal factors (Figures S10G and 10H), we suggest that the influence of neuronal innervation and mechanoreceptors at the muscle-tendon junction has a greater impact than previously assumed.

A neuronal protein with a biphasic profile is the dyslexia-associated protein KIAA0319-like protein (Figure 4C). KIAA0319 might be involved in neuronal migration and is thought to influence the formation of neural circuits involved in language processing. KIAA0319 also interacts with reticulon-4 receptor (RTN4R), also known as nogo receptor 1 (NGR1), which is an axon guidance receptor.<sup>39</sup> A protein with a similar function is neuronal cell adhesion molecule (NCAM), which also promotes neuritogenesis and neuronal migration.<sup>40</sup> Furthermore, NCAM has recently been identified as MTJ protein.<sup>21</sup> It is tempting to speculate that the interaction between RTN4R and KIAA0319 may be related to axon guidance in the MTJ region. Thus, our



(legend on next page)

spatial proteomic analysis is a useful tool to determine the extent of neuronal innervation in different areas of the skeletal muscle and could be used to study the alterations in neuronal structures under stress or disease-related conditions.

### Network analysis of our protein profiles showed higher connectivity within the MTJ than the muscle or tendon areas

Our network analysis revealed significantly higher betweenness centrality at the MTJ compared to the other skeletal muscle or tendon sections and therefore suggests more cellular connections occur at the junction between muscle fiber tips and matrix proteins than the centrally located muscle fibers (Figure 6). Betweenness centrality is also often used to find nodes in a network that serve as a bridge from one part of the graph to another. As muscle and tendon are connected by the MTJ, it is reasonable to assume that the higher connectivity observed for this area in our analysis highlights this bridging property of the MTJ. In addition, the high connectivity is supported by the identification of more than 200 protein groups with maxima at the MTJ and demonstrates the high complexity of the MTJ. Other proteins that are closely positioned near the identified MTJ area within the network may represent further MTJ candidates and require additional validation.

Compared to earlier studies, we identified similar biological processes, including neuronal activity, immune regulation, and cell adhesion, and various metabolic pathways are enriched at the MTJ. Although the MTJ proteins identified vary compared to the studies in other species and muscles, in many cases we identified similar isoforms of the same protein family. For example, we observed a maximum at the MTJ for the immunoglobulin superfamily 8 (IGSF8) member (Figure 4A), whereas the MTJ of the human hamstring muscle showed enrichment of the family member IGFS1.<sup>21</sup> Both proteins are cell surface proteins related to cellular signaling and the immune system. IGSF8 (also known as prostaglandin regulatory-like protein [PGRL]) is associated with various cellular processes related to motility, fusion, and neuronal growth. In addition, IGSF8 interacts with the tetraspanin molecules CD9 and CD81.<sup>41,42</sup> Accordingly, we also observed increasing protein abundance of the cell membrane proteins CD9 and CD81 from muscle to tendon, supporting interactions between these proteins and IGFS8. Another CD9- and CD81-interacting protein with a similar expression profile is CD9P-1, also known as prostaglandin F2 receptor negative regulator (PTGFRN) (Figure S6I). Genetic ablation of CD9 and CD81 reduces muscle regeneration, and both tetraspanins are expressed by centrally

nucleated myofibers in response to injury. Since centrally positioned myonuclei are also detectable in myofibers near the MTJ in humans,<sup>35</sup> we hypothesize that increased expression of cell surface proteins such as tetraspanins and members of the immunoglobulin superfamily might be involved in regeneration, fusion, and adhesion processes within the MTJ and surrounding areas. Further study of the network, particularly the area we identify as the MTJ, may further reveal the true complexity of this important region and establish a deeper understanding of its molecular state. This could lead to further targets relevant to pathophysiology that could not be easily identified by more traditional statistical approaches.

### Fatty acid metabolism and the RAS are involved in maintenance of the tendon matrix

Although most mitochondrial SLC transporters were more abundant in muscle tissue, we observed the opposite trend for SLC25A1, also known as CTP. SLC25A1 exports citrate or isocitrate from the mitochondria into the cytosol in exchange for malate. Cytosolic citrate functions as a source of acetyl-CoA for fatty acid and sterol biosynthesis and as an allosteric regulator of enzymes that control glycolysis, lipogenesis, and gluconeogenesis.<sup>43</sup> In addition, citrate is an essential intermediate molecule in the tricarboxylic acid (TCA) cycle and is central to energy production, which is ultimately essential to muscle contraction.

Since tendons are mainly composed of tenocytes, fibroblasts, and myofibroblasts, we suggest that these cell populations express high levels of citrate transporters to increase the cytosolic concentration of citrate for fatty acid synthesis. Mass spectrometric analysis confirmed that citrate transporter expression was higher in isolated mouse tenocytes compared to myoblasts (Figure S9H). In addition, the transport of citrate and acetyl-CoA from mitochondria to the nucleus is also used by histone acetyltransferases (HATs) to transfer the acetyl group to histone residues for histone acetylation, which in turn regulates gene activity.<sup>44</sup> For example, the capacity of mesenchymal stem cells to express osteogenic genes is tightly regulated by the concentration of citrate and histone acetylation.<sup>45</sup> Hence, a high concentration of citrate transporters in tendon cells or fibroblasts may not only regulate the synthesis of fatty acids but also influence the expression of secretory proteins and matrix formation via histone acetylation. A recent study in cancer-associated fibroblasts demonstrated that increased levels of acetyl-CoA in fibroblasts induced H3K27 hyperacetylation and transcriptional activation of collagens, suggesting that acetyl-CoA is a key epigenetic regulator.<sup>46</sup> Overall, increased concentrations of acetyl-CoA in the cytosol of tenocytes could affect histone acetylation and

### Figure 6. Spatial network analysis of the soleus muscle reveals higher connectivity at the MTJ area

(A–C) Schematic workflow for creating a distance-based network using delay analysis. (A) The correlation between two protein profiles was calculated using distance as a variable. (B) All correlations between two proteins were selected and added as connections. (C) Connections with a distance shorter than 100  $\mu$ m were used for visualization.

(D) Distance-based network generated using the protein expression profiles. Known muscle (orange), tendon (green), and potential MTJ (blue) proteins are marked.

(E) GO term analysis of MTJ proteins revealed enrichment of terms related to organization, binding, and junction.

(F) The betweenness score was significantly higher for the MTJ compared to muscle and tendon areas, indicating more connections between MTJ proteins than muscle and tendon proteins. Data are represented as mean  $\pm$  SEM. Asterisks denote significance (t test: \*\*\* $p$  < 0.001).

(G) Direct protein connections in the network analysis using collagen 1 (COL1a1) as a starting point.

thus modulate the expression of the matrix proteins responsible for matrix formation.

Another metabolic pathway closely linked to blood pressure regulation is the RAS. The ACE cleaves the inactive peptide angiotensin I to produce the active peptide angiotensin II. We demonstrated ascending profiles for ACE and related enzymes from muscle to tendon (Figures 5A–5D), suggesting enhanced levels of angiotensin II in tendons. Earlier studies showed that the RAS activates TGF $\beta$  and SMAD2/3 signaling, which also play critical roles in the regulation of ECM gene expression and fibrosis.<sup>47</sup> In addition, administration of several medications, including agents of the RAS, may increase the risk of Achilles tendon injury.<sup>48,49</sup>

Therefore, increased expression of ACE in the MTJ and tendons may help to activate the TGF $\beta$ -SMAD signaling pathway, induce the expression of CTGF, and promote the production of collagen and other ECM components. Previous studies showed that inhibition of the RAS by losartan reduces collagen formation and CTGF secretion in various experimental models of fibrosis.<sup>47,50</sup> We hypothesize that the RAS also influences the activity of the TGF $\beta$ -SMAD pathway in tenocytes and thus affects matrix formation at the MTJ and tendons. As further evidence of enhanced activation of the TGF $\beta$ -SMAD pathway, we also detected an ascending profile for DAB adaptor protein 2 (DAB2), which is required for internalization of TGF $\beta$ R and subsequent activation of the SMAD pathway (Figure S6G).<sup>51</sup>

Excessive production and accumulation of ECM components in skeletal muscle induced by chronic kidney disease and Duchenne muscular dystrophy lead to contractile dysfunction, stiffness, and muscle weakness. Although the administration of ACE inhibitors prevents fibrosis of skeletal muscle and has a positive effect on muscle function, the possible adverse effects on the MTJ are completely unclear. Therefore, a detailed analysis of the RAS signaling pathways that are activated in the context of matrix formation after muscle injury could provide detailed insight into whether ACE and ACE receptor inhibitors significantly affect the maintenance and regeneration of the muscle-tendon transition.

In conclusion, we provide a comprehensive spatial expression profile from the central to distal areas of a skeletal muscle. In addition, our protein expression profiling approach could be used to screen any tissue, such as heart or brain, to achieve a comprehensive spatial proteome. Furthermore, our results demonstrate a number of physiological differences between the muscle fibers, MTJ, and tendon areas, and the quantitative proteomic analysis highlights several proteins with associations to the MTJ.

### Limitations of the study

We provide a longitudinal profile of a mouse skeletal muscle, paving the way for future spatial proteomics studies. Although we profiled over 3,000 proteins from the central muscle to tendon areas, our dataset does not have a cellular resolution. Complementary approaches such as CYTOF, CODEX, and laser capture dissection microscopy could be used to assign expression profiles to specific fiber types and cell populations. In addition, our protein profiling dataset does not include complete profiles of transcription factors expressed at low levels; in

the future, targeted mass spectrometry approaches and ultra-sensitive single-cell proteomics could be used to obtain profiles of low-abundance proteins. To further increase the resolution of our protein profiles, thinner cryosections of 10  $\mu$ m could be generated. The increased measurement time could be compensated by even shorter LC-MS gradients. Finally, this analysis was limited by the fact that we only measured one type of skeletal muscle. Other muscles, and muscles from other species, could provide further insights into the molecular architecture of muscle-tendon junctions. Last, further experiments using mouse models with muscle weakness and impaired regeneration are necessary to characterize the spatial proteome of the skeletal muscle. Overall, our unbiased spatial proteomic profiling of the soleus muscle can serve as a reference for the study of specific muscle structures such as the neuromuscular and MTJ and provides a valuable resource for the skeletal muscle community.

### STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2024.114374>.

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## AUTHOR CONTRIBUTIONS

Conceptualization, L.S. and M. Krüger; methodology, L.S. and M. Krüger; software, L.S. and P.A.; validation, L.S., M.S., and C.H.; formal analysis, L.S. and P.A.; investigation, L.S.; resources, M. Krüger, M. Kjaer, A.M., J.-W.L., A.S., S.M., B.B., and M. Koch; writing – original draft, L.S., M. Krüger, and P.A.; writing – review & editing, L.S., M. Krüger, A.M., P.A., J.-W.L., B.B., and M. Koch; visualization, L.S. and C.H.; supervision and project administration, M. Krüger; funding acquisition, M. Krüger, B.B., and M. Koch.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE                                                                                                                               | SOURCE                                                                                                                                                         | IDENTIFIER                                                                                                                                                        |
|---------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Antibodies</b>                                                                                                                                 |                                                                                                                                                                |                                                                                                                                                                   |
| Tenascin-C                                                                                                                                        | Leica Biosystems                                                                                                                                               | Cat# NCL-TENAS-C; RRID: AB_564026                                                                                                                                 |
| Myosin pan                                                                                                                                        | DSHB                                                                                                                                                           | Cat# A4.1025; RRID: AB_528356                                                                                                                                     |
| Collagen 11a1                                                                                                                                     | Invitrogen (Zymed Custom Ab Lab),<br><a href="https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3121453/">https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3121453/</a> | N/A                                                                                                                                                               |
| Collagen 22                                                                                                                                       | Koch, <a href="https://pubmed.ncbi.nlm.nih.gov/15016833/">https://pubmed.ncbi.nlm.nih.gov/15016833/</a>                                                        | N/A                                                                                                                                                               |
| Periostin                                                                                                                                         | Abcam                                                                                                                                                          | Cat# ab14041; RRID: AB_2299859                                                                                                                                    |
| Hoechst                                                                                                                                           | Invitrogen                                                                                                                                                     | Cat# H1399                                                                                                                                                        |
| AF568-conjugated anti-rabbit                                                                                                                      | Molecular Probes                                                                                                                                               | Cat# A-11036; RRID: AB_10563566                                                                                                                                   |
| AF488-conjugated anti-rabbit                                                                                                                      | Thermo Fisher Scientific                                                                                                                                       | Cat# A-11034; RRID: AB_2576217                                                                                                                                    |
| AF568-conjugated anti-guinea pig                                                                                                                  | Thermo Fisher Scientific                                                                                                                                       | Cat# A-11075; RRID: AB_2534119                                                                                                                                    |
| AF488-conjugated anti-mouse IgG2b                                                                                                                 | Thermo Fisher Scientific                                                                                                                                       | Cat# A-21141; RRID: AB_2535778                                                                                                                                    |
| AF680-conjugated anti-mouse IgG2a                                                                                                                 | Thermo Fisher Scientific                                                                                                                                       | Cat# A31563; RRID: AB_2536177                                                                                                                                     |
| <b>Chemicals, peptides, and recombinant proteins</b>                                                                                              |                                                                                                                                                                |                                                                                                                                                                   |
| Lysyl Endopeptidase                                                                                                                               | Wako                                                                                                                                                           | Cat# 129-02541                                                                                                                                                    |
| Sequencing Grade Modified Trypsin                                                                                                                 | Serva                                                                                                                                                          | Cat# 37286.03                                                                                                                                                     |
| Losartan                                                                                                                                          | Merck                                                                                                                                                          | Cat# SML3317-10MG                                                                                                                                                 |
| iRT peptides                                                                                                                                      | GeneCust                                                                                                                                                       | N/A                                                                                                                                                               |
| <b>Deposited data</b>                                                                                                                             |                                                                                                                                                                |                                                                                                                                                                   |
| C57BL/6 untargeted proteomics<br>(3 tissues, 4 mice, males; tissue<br>slices, 3 mice, males; tenocytes,<br>C2C12, 4 replicates, losartan treated) | This paper                                                                                                                                                     | ProteomeXchange<br>( <a href="http://www.proteomexchange.org">http://www.proteomexchange.org</a> )<br>PXD046596                                                   |
| Code of the spatial analysis<br>(Sliding window for filter,<br>imputation, k-means clustering,<br>distance-delay analysis)                        | This paper                                                                                                                                                     | Zenodo ( <a href="https://zenodo.org/">https://zenodo.org/</a> )<br><a href="https://doi.org/10.5281/zenodo.10678005">https://doi.org/10.5281/zenodo.10678005</a> |
| <b>Experimental models: Cell lines</b>                                                                                                            |                                                                                                                                                                |                                                                                                                                                                   |
| Tenocytes                                                                                                                                         | <a href="https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9884217/">https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9884217/</a>                                      | N/A                                                                                                                                                               |
| C2C12                                                                                                                                             |                                                                                                                                                                | RRID:CVCL_0188                                                                                                                                                    |
| <b>Experimental models: Organisms/strains</b>                                                                                                     |                                                                                                                                                                |                                                                                                                                                                   |
| Mice: C57BL/6                                                                                                                                     | The Jackson Laboratory                                                                                                                                         | Cat#000664                                                                                                                                                        |
| <b>Software and algorithms</b>                                                                                                                    |                                                                                                                                                                |                                                                                                                                                                   |
| R (V 4.2.2)                                                                                                                                       | The R Project                                                                                                                                                  | <a href="https://www.r-project.org/">https://www.r-project.org/</a> ;<br>RRID: SCR_001905                                                                         |
| Rstudio                                                                                                                                           | Rstudio                                                                                                                                                        | <a href="https://www.rstudio.com/">https://www.rstudio.com/</a> ;<br>RRID: SCR_000432                                                                             |
| ProteoWizard (V 3.0.21218)                                                                                                                        | ProteoWizard                                                                                                                                                   | <a href="http://proteowizard.sourceforge.net/">http://proteowizard.sourceforge.net/</a> ;<br>RRID: SCR_012056                                                     |
| Freestyle software suite                                                                                                                          | Thermo Fisher Scientific                                                                                                                                       | RRID: SCR_022877                                                                                                                                                  |
| Perseus                                                                                                                                           | Tyanova and Cox<br><a href="https://pubmed.ncbi.nlm.nih.gov/29344888/">https://pubmed.ncbi.nlm.nih.gov/29344888/</a>                                           | <a href="https://www.maxquant.org/perseus/">https://www.maxquant.org/perseus/</a> ;<br>RRID: SCR_015753                                                           |
| InstantClue                                                                                                                                       | Nolte <a href="https://www.nature.com/articles/s41598-018-31154-6">https://www.nature.com/articles/s41598-018-31154-6</a>                                      | <a href="http://www.instantclue.uni-koeln.de/">http://www.instantclue.uni-koeln.de/</a> ;<br>RRID: SCR_024673                                                     |

(Continued on next page)

**Continued**

| REAGENT or RESOURCE      | SOURCE                                                                                                                     | IDENTIFIER                                                                                             |
|--------------------------|----------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------|
| DIA-NN                   | Demichev <a href="https://www.nature.com/articles/s41592-019-0638-x">https://www.nature.com/articles/s41592-019-0638-x</a> | <a href="https://github.com/vdemichev/DiaNN">https://github.com/vdemichev/DiaNN</a> ; RRID: SCR_022865 |
| Spectronaut              | Biognosys <a href="https://pubmed.ncbi.nlm.nih.gov/25724911/">https://pubmed.ncbi.nlm.nih.gov/25724911/</a>                | N/A                                                                                                    |
| Cytoscape                | Cytoscape Consortium                                                                                                       | <a href="http://cytoscape.org">http://cytoscape.org</a> ; RRID: SCR_003032                             |
| ImageJ (V 1.54d)         | ImageJ                                                                                                                     | <a href="https://imagej.net">https://imagej.net</a> ; RRID: SCR_003070                                 |
| <b>Other</b>             |                                                                                                                            |                                                                                                        |
| Orbitrap Eclipse Tribid  | Thermo Fisher                                                                                                              | RRID: SCR_023618                                                                                       |
| Faims Pro                | Thermo Fisher                                                                                                              | N/A                                                                                                    |
| timsTOF Pro 2            | Bruker Daltonics                                                                                                           | N/A                                                                                                    |
| Ultimate 3000            | Thermo Fisher                                                                                                              | N/A                                                                                                    |
| EvoSep One               | EvoSep                                                                                                                     | RRID: SCR_024590                                                                                       |
| Easy nLC1200             | Thermo Fisher                                                                                                              | N/A                                                                                                    |
| Olympus BX51 microscope  | Olympus                                                                                                                    | RRID: SCR_018949                                                                                       |
| DMi8A inverse microscope | Leica                                                                                                                      | N/A                                                                                                    |

**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Marcus Krüger ([marcus.krueger@uni-koeln.de](mailto:marcus.krueger@uni-koeln.de)).

**Materials availability**

This study did not generate new unique reagents.

**Data and code availability**

- Proteomic data have been deposited at ProteomeXchange and are publicly available as of the date of publication. Accession numbers are listed in the [key resources table](#).
- All original code has been deposited at Zenodo and is publicly available as of the date of publication. DOIs are listed in the [key resources table](#).
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request

**EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**

**Experimental model**

All animal experiments were performed in accordance with national and institutional guidelines. Three- to four-month-old male wild-type C57BL/6 mice were maintained at  $22 \pm 2^\circ$ ,  $55 \pm 10\%$  humidity, and an air exchange rate of 15 times per hour under a 12:12 h light-dark cycle and had free access to standard chow diet.

All mouse experiments were performed in accordance with the regulations stated in European, national, and institutional guidelines and were approved by local government authority Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) of North Rhine-Westphalia, Germany.

**Cell lines**

Immortalized murine tenocytes were kindly provided by Gil Lola Oreff (Vienna) and cultivated in minimal essential medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ .

**METHOD DETAILS**

**Intact tissue sample processing, protein digestion, and desalting**

Isolated soleus skeletal muscles were manually dissected into three parts: the central part of the muscle, the MTJ, and the tendon ( $n = 4$ ). Samples were snap-frozen in liquid nitrogen, ground using a mortar and pestle, resuspended in 4% SDS in PBS, homogenized by heating for 5 min at  $95^\circ\text{C}$ , and sonicated with a Bioraptor sonicator; the sonication settings were  $21^\circ\text{C}$  water with 10 cycles of 30 on and 30 off. Lysates were cleared by centrifugation for 10 min at 16,000 g and protein concentrations were determined using the

Pierce Protein Assay (Thermo Scientific), then 25  $\mu$ g protein extract was reduced and alkylated for 45 min at 45°C using 5 mM TCEP and 15 mM CAA. After acetone precipitation, the protein pellet was reconstituted in 3M GuHCl in 50 mM HEPES, 2.5 mM CaCl<sub>2</sub>. Proteins were digested with the protease LysC (substrate:enzyme ratio 50:1) for 3 h at room temperature followed by dilution of the GuHCl with 50 mM HEPES, 2.5 mM CaCl<sub>2</sub> to a final concentration of 0.5 M.

Further protein digestion was performed with trypsin (substrate:enzyme ratio 100:1) overnight at room temperature. Samples were acidified to 1% formic acid (FA) and cleaned-up using house-made SDB-RPS tips.

### Sample preparation for cryo-embedding and sectioning with a cryostat

Soleus muscles were fixed on both sides using insect needles on cork matrix, embedded in optimal cutting temperature (OCT) resin, and stored at –80°C until further use. OCT embedded solei were cut into 10–30- $\mu$ m-thick slices at –25°C using a Reichert Jung cryostat (Leica, Nussloch, Germany) and each slice was placed individually into a well of a 96-well-plate. As controls, one column on each 96-well plate was loaded with a HEK293T protein lysate. The slices placed in the wells were mixed with 40  $\mu$ L of 4% SDS in PBS containing 5 mM TCEP and 10 mM CAA. The 96-well-plates were incubated at 95°C for 10 min followed by Bioraptor sonication at 20°C with 10 cycles of 30 on and 30 off. Samples were digested following the standard SP3 protocol.<sup>14</sup> Briefly, 20  $\mu$ g of washed SP3 beads were added to each well, followed by one sample volume of acetonitrile (ACN). After incubation for 8 min and a further 2 min on a magnet, the supernatant was discarded, and the magnetic beads were washed twice with 70% EtOH and once with 100% ACN. Samples were digested with 10  $\mu$ L of 20 ng LysC and 40 ng trypsin in 50 mM ammonium bicarbonate buffer at 37°C on a rotating wheel (750 rpm) overnight, acidified by addition of 100  $\mu$ L 0.1% FA, followed by clean-up with house-made SDB-RPS tips. Peptides were reconstituted in 2% ACN, 5% FA, with indexed retention time (iRT) peptides (Table S5 for peptide sequences).

### ACE inhibitor incubation and MS analysis

Immortalized murine tenocytes were provided by Gil Lola Oreff (Vienna) cultivated in minimal essential medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin at 37°C and 5% CO<sub>2</sub> until the desired number of cells were obtained. Cells were seeded at a density of 0.4x10<sup>5</sup> in 24-well-plates in culture medium containing 10, 50, or 100  $\mu$ M Losartan (Sigma Aldrich) or DMSO as control and cultured for 1–4 days. The media were changed daily until harvesting and all experiments were carried out in biological triplicate ( $n = 3$ ). Cells were washed with PBS, lysed with a PBS buffer containing 4% SDS, and 8  $\mu$ g of protein lysate was used for digestion as described above, followed by purification with in-house prepared SDB-RPS tips.

For phosphopeptide enrichment, cells were seeded in culture medium with 1% FBS. The media was changed daily until harvesting and all experiments were carried out in biological triplicate ( $n = 3$ ). Cells were washed with PBS, lysed with PBS buffer containing 4% SDS, and 8  $\mu$ g of protein lysate was used for LC-MS analysis. Magnetic Ti-NTA beads (Cube BioTech) were used for phosphopeptide enrichment following the manufacturer's instructions. In brief, 250  $\mu$ g of desalted peptides were reconstituted in 250  $\mu$ L binding buffer (80% ACN, 5% TFA, 0.1% glycolic acid). Peptides were incubated with magnetic beads in a 1:10 ratio for 20 min at 1300 rpm. After binding, the beads were washed twice with wash buffer 1 (80% ACN, 5% TFA) and wash buffer 2 (10% ACN, 0.2% TFA). Peptides were eluted with 1% ammonium hydroxide and acidified with 10  $\mu$ L FA.

### LC-MS analysis

For peptide separation, an in-house made fused silica emitter (75  $\mu$ m diameter, length 15–30 cm) was packed with 5  $\mu$ m C18 Poros-hell resin (Agilent, USA) and applied to an Easy nLC1200 (Thermo-Fisher Scientific, Waltham, USA) with the column temperature maintained at 50°C using an integrated column oven. Peptides were separated using a binary buffer system of buffer A (0.1% FA) and buffer B (80% ACN, 0.1% FA) on a 30 cm in-house-packed analytical column filled with 1.9  $\mu$ m C18-AQ Reprosil Pur beads (Dr. Maisch, Germany). A 90 min segmented gradient of 4–32% Solvent B over 72 min, 32–55% Solvent B over 13 min and 55–95% over 2 min at a flow rate of 250 nL/min was applied to elute peptides and the peptides were measured with an Orbitrap Eclipse Tribrid mass spectrometer equipped with an FAIMS-Pro interface (Thermo-Fisher Scientific, Waltham, USA). A data-independent (DIA) method was used for peptide acquisition and each acquisition cycle consisted of four scans, two full scans, and two DIA scans at CVs of –50 and –70 V, respectively, employing 25  $\times$  22 Da windows from 350 to 1600  $m/z$  with an overlap of 1 Da on each side. All scans were acquired in positive ionization mode at 15k resolution and DIA scans were acquired after peptide fragmentation at a normalized collision energy (NCE) of 30.

MS analysis of tissue sections was performed with a single CV of –50 V and a staggered-window DIA method, using a duty cycle of two sets shifted by ½ isolation window from 400 to 900  $m/z$ . The MS methods and duty cycle are described in Table S5 and Method overview.

Samples from cell culture experiments were processed as described before and digested peptides were loaded on EvoTips. Pure and measured using a 44-min LC-MS gradient on an EvoSep One system coupled to a 15 cm PepSep column (EvoSep, Denmark). The mobile phases were compromised of 0.1% FA as solvent A and 0.1% FA in ACN as solvent B. The HPLC system was coupled to a timsTOF pro 2 using a CaptiveSpray source (both Bruker). Samples were measured in dia-PASEF mode with daily ion mobility calibration using three ions in the Agilent ESI-Low Tuning Mix following the vendor's specifications. The DIA-PASEF window ranged in

dimension from 1/k0 0.7–1.35, with 24 x 25 Th windows and in dimension from 350 to 1250 *m/z* and 1/k0 0.7–1.45, with 2 x 16 Th windows and in dimension from 400 to 1400 *m/z* for proteomics and phosphoproteomics analysis, respectively.

### Bioinformatic analysis

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD046596.<sup>52</sup> For the analysis of intact skeletal muscle samples, spectra from different CV settings were separated manually using the Freestyle software suite (Thermo-Fisher Scientific) and analyzed with DIA-NN 1.8 using library free search against the UniProt mouse database (Sep. 2017) complemented with protein sequences from myosin heavy chain variants and collagens. Mass ranges were set according to the settings of the mass spectrometer; mass deviation was automatically determined from the first data file. Data was further processed using R (V 4.2.2), with the tidyverse, diann, data.table, magrittr, FactoMineR, factoextra, ggplot2, and gprofiler libraries. An in-house modified R-script based on the version by V. Demichev was used to calculate MaxLFQ values.<sup>53,54</sup> To show identified peptides we used the term “precursor”, which refers to an ion analyzed by the MS instrument. The precursor ion is fragmented into product ions and contain information about the sequence, modification status, and the charge state. Data input was filtered for unique precursor, *q*-value < 0.01, Lib.Q.Value < 0.01, PG.Q.Value < 0.01, Global.Q.Value < 0.01, Quantity.Quality > 0.7, Fragment.count ≥ 4; previously separated data from different compensation voltages were combined for each protein entry to the maximum value. Statistical analysis was performed with R (V 4.2.2) and Perseus (V.1.6.5.0) and visualized with Instant clue (V 0.10.10.20210315). For the analysis of the cell culture experiments and identification of phosphopeptides we used Spectronaut software (version 1.8).<sup>55</sup>

For the analysis of sectioned tissue, RAW data were first deconvoluted using ProteoWizard (V 3.0.21218). Files were pre-processed as described before. Further calculations were performed within R (version 4.2.2) using the diann, tidyverse, data.table, samr, vsn, ggplot2, and gprofiler libraries. All protein intensities smaller than 10<sup>4</sup> were replaced as missing values.

The protein intensities in each slice were normalized to the total protein intensity in each slice. Slices were arranged spatially and proteins were filtered in a distance related manner; a sliding window was generated that covered an overall a distance of 200 μm, i.e., five sections back and five sections forward. Proteins with less than 70% coverage over one sliding window were excluded. For imputation, a pseudo-slice ID was established to capture sections for which measurement completely failed and to ensure that the sliding window always contained 10 sections before and 10 sections after the current slice. Sections close to the edge of the sample were truncated but required a minimum of three samples to be present for imputation. For windows that fulfilled the 70% limit, missing values in the windows were imputed with a normal linear imputation using 200 μm distances. The remaining missing values were imputed with random values 1.6 points below the mean of the lower 5% of the total expression values across all proteins.

Samples from different biological replicates had to be adjusted to obtain congruent protein profiles. We normalized each muscle section by dividing each section by the length of the whole muscle. Then, each experiment was analyzed with a bootstrap approach based on the Significance analysis of Microarrays (SAM) method<sup>16,56</sup> to detect gradual increases or decreases over distance for specific proteins in a linear and non-linear relationship as local minima and maxima. The reported distribution statistics (*d*-statistics) were further used as readout for significantly up- or downregulated proteins. The average score across all samples was calculated and proteins with a *d*-statistic > 5 were selected to shift the profiles required to achieve improved matching of minima and maxima within the profiles. Z-scores were calculated row-wise and the average protein profile was smoothed using a local regression function, called LOESS, in which the 95% confidence intervals were calculated as representative profiles of each protein. For ease of visualization, the representative profiles were normalized to range from 0 to 1 for all data.

### Development of a network-based representation of the muscle tendon regions

The representative profile of each protein was used as input for a time impact analysis, replacing time with distance. This comprehensive technique is used to analyze each delay event individually in chronological order to calculate its impact and to quantify each delay based on the schedule immediately before and after the delay event took place. The delay is calculated by the highest correlation of two protein profiles. Correlation was calculated by shifting two protein profiles in both directions by up to 20% of the total distance. The reported delays were filtered for correlation scores higher than 0.7 and for distances less than 100 μm. The interactions were visualized in Cytoscape and arranged using the Spring Embedded Layout of the delay, leading to a hierarchical arrangement of the proteins over distance.

An interactive online version of the network is available using following link: <https://medex.uber.space/networks/index.html#/>. Wilcoxon *t*-tests of the betweenness scores and Gotermin enrichment analysis were performed and visualized using R (V 4.2.2) using the packages gprofiler2 and ggplot2.

### Histochemistry and immunostaining

Mouse solei were collected, embedded as described previously, cut longitudinally and transversely in 3–10 μm sections, collected on glass slides (VWR) and stored at –80°C until further use. Slides were removed from the freezer and dried at room temperature. Two biological replicates were performed for each staining. Longitudinal sections were fixed with Histofix (Histolab) for 10 min at RT. Primary antibodies against Tenascin-C (Leica Biosystems Cat# NCL-TENAS-C; RRID:AB\_564026), Myosin pan (DSHB Cat# A4.1025; RRID:AB\_528356), Collagen 11a1 (Invitrogen/Zymed Custom Ab Lab),<sup>57</sup> Collagen 22 (provided from Manuel Koch (Cologne)<sup>7</sup>), Periostin (Abcam Cat# ab14041; RRID:AB\_2299859) were diluted 1:100 in 1% BSA in TBS, except Collagen 22 was used 1:500, and

applied overnight at 4°C; the following day, secondary antibodies were diluted 1:200 and Hoechst (Invitrogen, #H1399) 1:100 in TBS and applied for 60 min at RT. As secondary antibodies were used (AF568-conjugated anti-rabbit (Molecular Probes Cat# A-11036 (also A11036); RRID:AB\_10563566), AF488-conjugated anti-rabbit (Thermo Fisher Scientific Cat# A-11034 (also A11034); RRID:AB\_2576217), AF568-conjugated anti-guinea pig (Thermo Fisher Scientific Cat# A-11075; RRID:AB\_2534119), AF488-conjugated anti-mouse IgG2b (Thermo Fisher Scientific Cat# A-21141; RRID:AB\_2535778), AF680-conjugated anti-mouse IgG2a (Thermo Fisher Scientific Cat# A31563; RRID:AB\_2536177)). Samples were washed twice for 10 min in TBS between all steps in the protocol. Finally, slides were mounted with coverslips using Prolong Gold Antifade Reagent (Thermo Fisher Scientific). For widefield fluorescence microscopy, images were captured using two different objectives (10x/0.3NA; 20x/0.5NA) on an Olympus BX51 microscope with a 0.5x camera (Olympus DP71, Olympus Deutschland GmbH, Hamburg, Germany) at the ISMC using the software Olympus cellSens Software (RRID:SCR\_014551). Transverse sections were used for Masson Goldner Trichrome staining (Morphisto) and imaged using a Dmi8A inverse microscope (Leica). Images were further processed using ImageJ (version 1.54d; RRID:SCR\_003070) and colorblind-friendly pseudo colors were applied to the composite images.

### QUANTIFICATION AND STATISTICAL ANALYSIS

All experiments were carried out in at least triplicates as outlined in the methods details. One-way analysis of variance (ANOVA) was calculated within Perseus. The Permutation-based FDR and Number of randomizations was set to 0.05 and 500, respectively. For intact muscle tissue and cell culture proteome analysis Student's *T* test was used to calculate significantly changed proteins. A protein was classified as significant with an FDR <0.05 and  $s_0 > 0.1$ . For phosphoproteome analysis the Welch's *T* test analysis was performed and a phosphosite was classified as significant with a *p* value <0.05 and  $\log_2$  difference > |1|. GOterms and phosphosites on a protein were compared using a *T* test analysis and significance are indicated using asterisks (\* adjusted *p* value <0.05. \*\* adjusted *p* value <0.01, \*\*\* adjusted *p* value <0.001). Statistical details can also be found in the according figure legends.