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Terminal Schwann Cells Regulate Presynaptic Vesicle Homeostasis but Not Neuromuscular Junction Integrity in Mice

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eLife Assessment

This **useful** study builds on the previously identified terminal Schwann cell (tSC) marker *Col20a1* to generate a novel *Col20a1*-CreERT2 knock-in mouse line that enables the selective genetic labelling and ablation of tSCs at the neuromuscular junction. This model represents a new tool for distinguishing tSCs from myelinating Schwann cells. The authors conclude that loss of tSCs does not cause major alterations in the overall structure of the neuromuscular junction, but that tSC ablation intrinsically compromises the presynaptic reserve pool. However, the study remains **incomplete** because the evidence provided does not adequately support these conclusions.

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Abstract

Terminal Schwann cells (tSCs) are specialized glial cells persistently residing at the neuromuscular junction (NMJ), where they are widely postulated to participate in synaptic transmission and maintenance. However, elucidating their precise *in vivo* roles has been hindered by a lack of tSC-specific genetic models, as conventional tools simultaneously targeted axonal Schwann cells. Here, we identify *Col20a1* as a highly specific marker for tSCs through single-cell transcriptomic analysis and establish a novel *Col20a1*-CreERT2 knock-in mouse model. Utilizing this model, we achieved highly specific systemic labeling and ablation of tSCs during the postnatal maturation window. Surprisingly, systemic tSC loss disrupted neither gross NMJ architecture nor overall motor behavior. Instead, electrophysiological and ultrastructural analyses revealed profound pre-synaptic defects, characterized by increased spontaneous miniature endplate potentials (mEPPs), accelerated synaptic depression during high-frequency stimulation, and significant physical depletion of pre-synaptic vesicles. Furthermore, long-term analysis revealed a robust glial repopulation that sustained prolonged neuromuscular integrity. Together, our findings establish *Col20a1* as a definitive genetic handle for tSC research, delineating that while tSCs are dispensable for macroscopic synaptic structure, they serve a precise and critical role in regulating pre-synaptic vesicle homeostasis.

Introduction

The neuromuscular junction (NMJ) is a highly specialized peripheral synapse that transmits motor signals from neurons to skeletal muscles. Because of this pivotal role, impairment in NMJ integrity or function is linked to various neuromuscular disorders, such as myasthenia gravis (Darabid et al., 2018 [DOI](#); Engel et al., 2003 [DOI](#); Plomp et al., 2015 [DOI](#)), amyotrophic lateral sclerosis (ALS) (De Winter et al., 2006 [DOI](#); Dupuis & Loeffler, 2009 [DOI](#); Khabibrakhmanov et al., 2025 [DOI](#)), and spinal muscular atrophy (SMA) (Kariya et al., 2008 [DOI](#); Kong et al., 2009 [DOI](#)). Thus, investigating the precise

mechanisms underlying NMJ function is of critical importance. Traditionally viewed as a bipartite structure, the NMJ is now recognized as a tripartite synapse, consisting of the pre-synaptic motor nerve terminal, the post-synaptic muscle fiber, and the specialized glial cells known as terminal Schwann cells (tSCs) (Araque et al., 2014 [DOI](#); Araque et al., 1999 [DOI](#); Barik et al., 2016 [DOI](#); Kettenmann & Ransom, 2012 [DOI](#); Ko & Robitaille, 2015 [DOI](#); Sanes & Lichtman, 2001 [DOI](#); Santosa et al., 2018 [DOI](#); Todd et al., 2007 [DOI](#); Volterra et al., 2002 [DOI](#); Wu et al., 2010 [DOI](#)). While the neuronal and muscular components have been extensively characterized, tSCs remain relatively understudied due to lack of tSC-specific genetic models (Hastings & Valdez, 2024 [DOI](#); Jablonka-Shariff et al., 2021 [DOI](#); Santosa et al., 2018 [DOI](#)), despite an increasing recognition of their essential contributions to the synapse (Barik et al., 2016 [DOI](#); Darabid et al., 2018 [DOI](#); Fuertes-Alvarez & Izeta, 2021 [DOI](#); Hastings & Valdez, 2024 [DOI](#); Petrov et al., 2021 [DOI](#)).

In mice, while the nascent NMJ structure forms before birth, the extensive NMJ maturation occurs within the first three postnatal weeks (Kariya et al., 2014 [DOI](#); Medina-Moreno & Henríquez, 2022 [DOI](#); Sanes & Lichtman, 1999 [DOI](#), 2001 [DOI](#)). NMJs transition from polyneuronal to mononeuronal innervation through competitive synapse elimination (Balice-Gordon & Lichtman, 1993 [DOI](#); Brown et al., 1976 [DOI](#); Sanes & Lichtman, 1999 [DOI](#)), and post-synaptic acetylcholine receptor (AChR) clusters mature from plaque-like structure into complex pretzel-like structure, accompanied by the extensive formation of junctional folds (Marques et al., 2000 [DOI](#); Matthews-Bellinger & Salpeter, 1983 [DOI](#); Shi et al., 2012 [DOI](#)). During this developmental window, tSCs support nerve pruning process by phagocytosing axosomes (Bishop et al., 2004 [DOI](#); Darabid et al., 2013 [DOI](#); Lee et al., 2016 [DOI](#); Riley, 1981 [DOI](#); Smith et al., 2013 [DOI](#)) and decode synaptic activity through Ca^{2+} and purinergic signaling (Darabid et al., 2013 [DOI](#); Darabid et al., 2018 [DOI](#); Heredia et al., 2018 [DOI](#); Jahromi et al., 1992 [DOI](#); Reist & Smith, 1992 [DOI](#); Robitaille, 1998 [DOI](#)). The fundamental necessity of Schwann cells has been further evidenced by studies using ErbB2 or ErbB3 mutant mice, where the failure of Schwann cell formation and migration led to severe nerve retraction (Lin et al., 2000 [DOI](#); Morris et al., 1999 [DOI](#); Riethmacher et al., 1997 [DOI](#); Woldeyesus et al., 1999 [DOI](#)). Furthermore, ablating tSCs in frog NMJ by antibody and complement system reduced nerve growth and induced nerve retraction, suggesting that tSCs are vital for long-term maintenance of NMJ (Feng & Ko, 2008 [DOI](#); Koirala et al., 2003 [DOI](#)).

Although tSCs have been suggested as dynamic regulators of synaptic efficacy and plasticity, deciphering their exclusive contributions in mammals has faced ongoing challenges. Because of the absence of tSC-specific markers, many genetic studies have relied on markers expressed across the entire Schwann cell lineage, which necessitates careful interpretation to distinguish tSC-specific effects from those of axonal Schwann cells (aSCs) (Barik et al., 2016 [DOI](#)). While localized observations have characterized micro-scale tSC dynamics, there remains a need for systemic models that can evaluate the macroscopic, organism-level consequences of tSC manipulation. Recently, Castro et al. (2020) [DOI](#) reported that S100b-GFP/NG2-dsRed double-positive cells can effectively label tSCs in skeletal muscles. A more refined genetic manipulation would provide a powerful means to elucidate the physiological role of tSCs.

Here, we have identified *Col20a1* as a unique and specific marker for tSCs, distinct from other aSC lineages such as myelinating Schwann cells (mSCs) and Remak non-myelinating Schwann cells (nmSCs). By developing a novel *Col20a1*-CreERT2 mouse model, we achieved highly specific *in vivo* labeling and systemic ablation of tSCs, but not aSCs. Furthermore, we induced targeted ablation of tSCs at postnatal day 10 (P10), a critical period when the NMJ undergoes active structural refinement to establish adult synaptic homeostasis. This approach allowed us to investigate the systemic impact of tSC loss during this period on both fine synaptic physiology and gross motor behavior. Surprisingly, systemic tSC ablation did not induce an overt collapse of gross NMJ morphology and overall functionality as judged by behavioral tests. However, careful electrophysiological examinations revealed an indispensable requirement for tSCs in maintaining pre-synaptic vesicle homeostasis. Our study thus establishes a new genetic platform for studying tSC function *in vivo* and redefines its specialized functional contributions in NMJ.

Results

Identification of *Col20a1* as a specific marker for tSCs

To develop a tSC-specific genetic model, we needed to identify a reliable tSC-specific marker. To this end, we analyzed gene expression profiles of muscle resident cells to find genes highly and specifically expressed in tSCs. To investigate the transcriptomic profile of Schwann cell populations residing in skeletal muscle, we analyzed a publicly available single-cell RNA sequencing (scRNA-seq) dataset of skeletal muscle-resident cells (Giordani et al., 2019). We first generated a UMAP plot of the entire muscle cell population (Figure 1A) and identified the Schwann cell cluster based on the expression of canonical glial markers *Plp1* and *S100b* (Figure 1B; Figure 1—figure supplement 1). Subsequent isolation and sub-clustering of this Schwann cell population revealed three distinct clusters (Figure 1C). To annotate the identity of each sub-cluster, we evaluated the expression of well-known marker genes for Schwann cell subtypes. Cluster 1 and Cluster 2 exhibited high expression of myelinating Schwann cell (mSC) and Remak non-myelinating Schwann cell (nmSC) markers, respectively. Notably, Cluster 3 displayed significant enrichment for genes previously described to be expressed in tSCs (Castro et al., 2020), indicating that Cluster 3 represents the tSC population (Figure 1D; Figure 1—figure supplement 2). Clusters 1 and 2 likely correspond to the glial populations residing within intramuscular nerve bundles, representing mSCs and nmSCs that envelope the axons. The mSC population (Cluster 1) is also presumed to include myelinating axonal Schwann cells (aSCs) that wrap the motor axons leading to the NMJ. The anatomical distribution of these defined Schwann cell subtypes within skeletal muscle is schematized in Figure 1E.

Next, we sought to identify a highly specific marker gene for tSCs by performing rigorous differentially expression gene (DEG) analysis using two distinct criteria. First, to distinguish tSCs from other Schwann cell subtypes, we compared Cluster 3 against Clusters 1 and 2. We focused on candidates showing widespread expression within tSCs, selecting only those expressed in at least 50% of Cluster 3 cells with at least a twofold increase in expression relative to other Schwann cell populations, yielding 15 candidates. Second, to ensure specificity within the entire skeletal muscle tissue, we compared Cluster 3 against all other non-Schwann muscle cells. To guarantee that the markers are highly restricted to tSCs, we filtered for genes expressed in less than 1% of other cell populations, while also exhibiting at least a twofold increase in expression, which resulted in 81 candidates. The intersection of these two criteria identified only three candidate genes: *Col20a1*, *Acsbg1*, and *Fabp7* (Figure 1F, G; Figure 1—figure supplement 3).

To minimize potential off-target effects in systemic applications or genetic targeting models, we further scrutinized systemic expression profiles of these three candidate genes using the CELLxGENE database (Figure 1H) (Program et al., 2025). We noted that *Acsbg1* and *Fabp7* showed considerable expression in the brain and central nervous system. Since our objective is to investigate tSC function at the NMJ, we prioritized a marker with minimal expression in adjacent neuromuscular components to avoid artefacts in subsequent genetic manipulation models. *Col20a1* showed relatively minimal expression in these regions. While slight expression was observed in mucosal tissues, its overall restricted expression pattern makes *Col20a1* a favorable candidate for investigating tSC functions. Furthermore, re-analysis of independent bulk RNA-seq datasets (Castro et al., 2020; Ham et al., 2020) confirmed that *Col20a1* has the highest expression level and most significant fold-change among the three candidates in *S100b*⁺/*NG2*⁺ double-positive cells and NMJ-enriched regions (Figure 1—figure supplement 4). Taken together, *Col20a1* was highly prioritized in various criteria for an ideal tSC-specific marker in skeletal muscle, leading us to select it for the generation of tSC-specific genetic model.

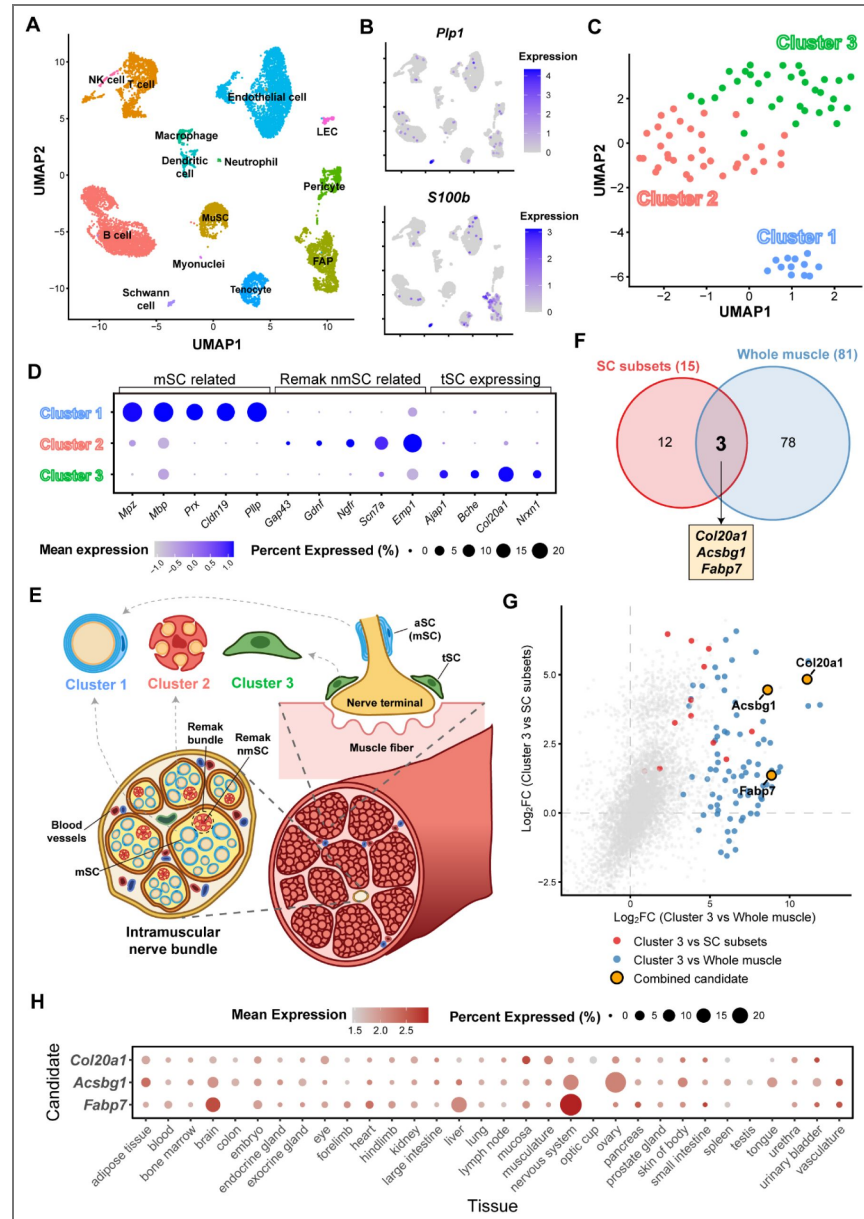


Figure 1. Identification of *Col20a1* as a specific marker for terminal Schwann cells (tSCs) in skeletal muscle.

(A) UMAP plot of the entire skeletal muscle-resident cell populations from a public scRNA-seq dataset (Giordani et al., 2019). (B) Feature plots showing the expression of canonical Schwann cell markers, *Plp1* and *S100b*. (C) Sub-clustering of the isolated Schwann cell population reveals three distinct sub-populations (Clusters 1–3). (D) Dot plot displaying the expression profiles of established marker genes for myelinating Schwann cells (mSC, Cluster 1), Remak non-myelinating Schwann cells (Remak nmSC, Cluster 2), and tSC-expressing genes (Cluster 3). Node size indicates the percentage of cells expressing the gene, and color intensity represents the mean expression level. (E) Schematic illustration depicting the anatomical distribution of the three defined Schwann cell subtypes within the skeletal muscle and neuromuscular junction. (F) Venn diagram illustrating the intersection of two differential gene expression screening criteria: genes highly expressed in Cluster 3 compared to other SC subsets (15 genes; $\log_2FC > 1$, adjusted p -value < 0.05 , pct.1 > 0.5), and genes specifically expressed in Cluster 3 compared to all other muscle cells (81 candidates; $\log_2FC > 1$, adjusted p -value < 0.05 , pct.2 < 0.01). Three shared candidate genes (*Col20a1*, *Acsbg1*, and *Fabp7*) were identified. (G) Scatter plot comparing the Log2 fold-change (FC) of gene expression for Cluster 3 vs SC subsets (y-axis) and Cluster 3 vs whole muscle cells (x-axis). Genes that passed each screening criteria is depicted by red and blue dots, respectively. The intersected candidate genes are highlighted in orange circle. (H) Dot plot from the CELLxGENE database (Program et al., 2025) showing the cross-tissue expression profiles of the three candidate genes, highlighting the minimal expression of *Col20a1* in the central nervous system compared to *Acsbg1* and *Fabp7*.

Generation of *Col20a1*-CreERT2 mouse model and validation of its universal expression in tSCs

Building upon our identification of *Col20a1* as a highly specific tSC marker, we generated a *Col20a1*-CreERT2 knock-in mouse model to selectively target tSCs *in vivo*. P2A-CreERT2 cassette was inserted at the C-terminus of the endogenous *Col20a1* gene (Figure 2—figure supplement 1A [↗](#)). We crossed these mice with a *Rosa26*-LSL-tdTomato (*Rosa*-tdT) reporter line to generate *Col20a1*-CreERT2; *Rosa*-tdT (*Col20a1*-tdT) mice. To induce recombination, adult mice were administered tamoxifen via oral gavage (160 mg/kg body weight) for five consecutive days, and the extensor digitorum longus (EDL) muscles were analyzed one week after the first dose. Immunofluorescence analysis of the neuromuscular junctions revealed robust tdTomato expression specifically within the tSC region in *Col20a1*-tdT mice, whereas control *Rosa*-tdT mice lacking the CreERT2 allele showed no detectable signal (Figure 2A [↗](#)). High-magnification imaging further confirmed that the tdTomato signal co-localized tightly with the S100B glial marker and accurately enveloped the alpha-bungarotoxin (Btx)-labeled AChR region. DAPI staining confirmed the distinct cellular morphology of these labeled tSCs (Figure 2B [↗](#)).

To quantitatively validate the cell-type specificity of our model, we performed fluorescence-activated cell sorting (FACS) on whole muscle mononuclear cells. Cells were segregated into Lineage-positive (Lin^+ ; CD31^+ , CD45^+ , or Ter119^+), muscle stem cells (MuSCs; $\text{Lin}^-/\text{VCAM1}^+/\text{Sca-1}^-$), fibro-adipogenic progenitors (FAPs; $\text{Lin}^-/\text{VCAM1}^-/\text{Sca-1}^+$), and double-negative cells (DN; $\text{Lin}^-/\text{VCAM1}^-/\text{Sca-1}^-$), which harbor the Schwann cell population (Figure 2—figure supplement 1B [↗](#)). Flow cytometry analysis revealed that the vast majority of tdTomato-positive cells (~92%) were located exclusively within the DN population, consistent with the expected identity of tSCs (Figure 2—figure supplement 1C-E [↗](#)). Finally, to confirm successful genetic recombination, we extracted genomic DNA from the sorted cell populations and performed PCR. The recombined tdTomato amplicon was strictly detected in the tdT+ fraction of the DN population, confirming that Cre-mediated recombination occurred specifically in the intended target cells (Figure 2—figure supplement 1F [↗](#)). These structural and genomic validations suggest that the *Col20a1*-CreERT2 mouse is a highly specific and robust tool for targeting tSCs in skeletal muscle.

Having confirmed the specificity, we next characterized the spatial distribution of the reporter expression across various skeletal muscles to establish the model as a universally reliable tool. We analyzed representative cranial (masseter, sternomastoid), axial (diaphragm, psoas), and appendicular (EDL, soleus) muscles, covering diverse anatomical locations and fiber type compositions. Immunofluorescence analysis revealed consistent and robust tdTomato labeling within tSCs across all examined muscle groups (Figure 2C [↗](#)). This uniform pattern confirms that the *Col20a1*-CreERT2 model can target tSCs globally throughout the body, regardless of their anatomical niche.

Temporal mapping of *Col20a1*-CreERT2 expression during postnatal maturation

While the *Col20a1*-CreERT2 model showed high specificity in adult muscles, its application for other developmental stages requires a precise understanding of its expression dynamics. Thus, we investigated the temporal expression profile of this model during the critical window of NMJ maturation. *Col20a1*-tdT pups were administered a single intraperitoneal (IP) injection of tamoxifen (160 mg/kg body weight) at postnatal day 4 (P4), P7, P10, or P14, and analyzed at 4 weeks of age (Figure 3A [↗](#)). Interestingly, early induction at P4 and P7 resulted in tdTomato expression not only in tSCs but also partially in upstream axonal Schwann cells (aSCs) (Figure 3B [↗](#), white arrowheads). However, this broader labeling became highly restricted as development progressed; induction at P10 and P14 yielded strictly tSC-specific labeling, with no observable aSC expression. These findings indicate that while *Col20a1* is broadly expressed in both tSCs and aSCs during early postnatal development, its expression becomes restricted to tSCs by P10. Consequently, we established P10 or later as the standard induction time point for selective tSC targeting.

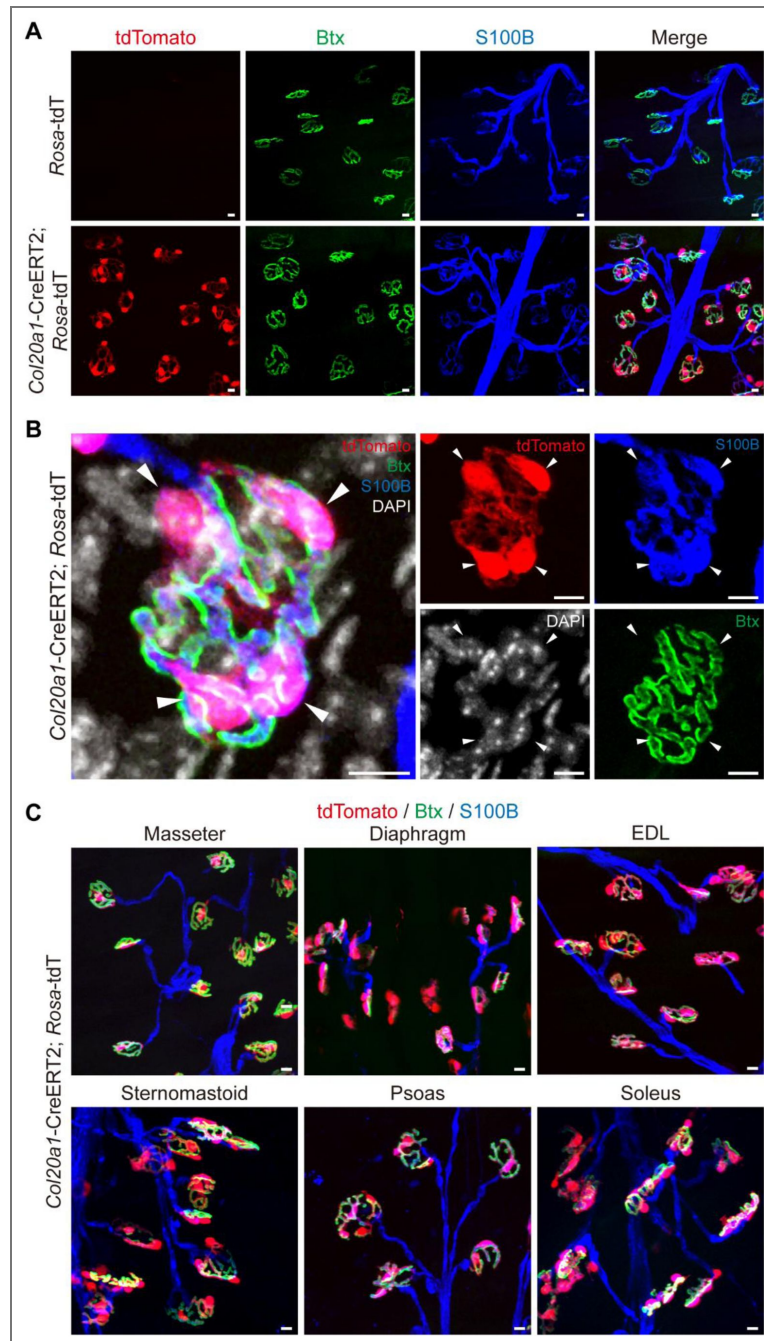


Figure 2. Generation of the *Col20a1-CreERT2* mouse model and validation of its highly specific, system-wide expression in tSCs.

Adult mice (8–12 weeks old) were treated with tamoxifen (TMX) via oral gavage (160 mg/kg body weight) for five consecutive days and analyzed one week after the initial dose. (A) Representative immunofluorescence images of EDL muscle neuromuscular junctions (NMJs) from control (*Rosa-tdT*) and *Col20a1-CreERT2; Rosa-tdT* mice. The tdTomato reporter signal (red) is robustly and exclusively detected at the NMJs of *Col20a1-CreERT2; Rosa-tdT* mice. Post-synaptic acetylcholine receptors (AChRs) are labeled with α -bungarotoxin (Btx, green), and glial cells are labeled with S100B (blue). (B) High-magnification confocal images highlighting the precise cellular localization of the tdTomato reporter. White arrowheads indicate the tight co-localization of tdTomato (red) with the tSC marker S100B (blue), which effectively envelops the post-synaptic AChR clusters (green). DAPI (white) staining indicates nuclei. (C) Representative immunofluorescence images showing the spatial distribution of the reporter across diverse anatomical locations, including cranial (masseter, sternomastoid), axial (diaphragm, psoas), and appendicular (EDL, soleus) muscles. The *Col20a1-CreERT2; Rosa-tdT* model reliably targets tSCs across all examined muscle types. Scale bars: 10 μ m in all images.

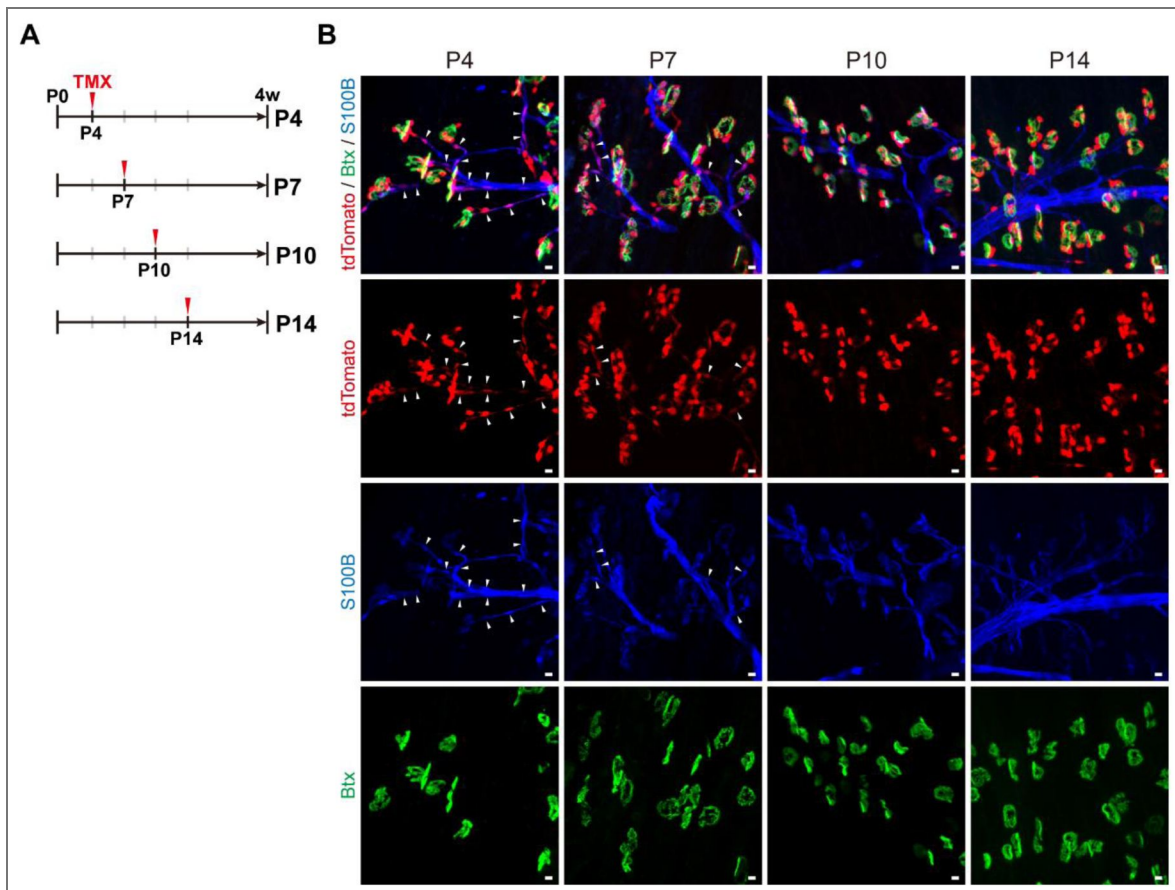


Figure 3. Temporal mapping establishes the postnatal specificity window of the *Col20a1*-CreERT2 model.

(A) Experimental timeline for investigating the temporal dynamics of *Col20a1*-CreERT2 model. A single dose of tamoxifen (TMX, 160 mg/kg body weight) was administered in *Col20a1*-tdT mice via intraperitoneal injection at postnatal day 4 (P4), P7, P10, or P14, followed by analysis at 4 weeks (4w) of age. (B) Representative immunofluorescence images of NMJs corresponding to the temporal induction timeline. Early TMX induction at P4 and P7 results in tdTomato labeling (red) of both tSCs and portions of upstream axonal Schwann cells (aSCs, indicated by white arrowheads). In contrast, induction at P10 and P14 yields strictly tSC-restricted expression without off-target aSC labeling. S100B (blue); Btx (green). Scale bars: 10 μ m.

tSC ablation during the postnatal maturation window does not disrupt gross NMJ structure

Having established P10 as time point for tSC-specific targeting (Figure 3 [↗](#)), we sought to elucidate the functional requirement of tSCs during the postnatal NMJ maturation. We generated a genetic ablation model, *Col20a1*-CreERT2; *Rosa*-tdT/DTA (*Col20a1*-tdT/DTA), in which tamoxifen induction simultaneously drives the expression of diphtheria toxin subunit A (DTA) for targeted cell death and tdTomato to label any surviving “escaper” cells. Tamoxifen was administered at P10, and NMJs were analyzed at 4 weeks of age.

Immunofluorescence analysis of EDL muscle revealed a striking reduction in tdTomato+ (tdT⁺) tSCs in the *Col20a1*-tdT/DTA mice compared to *Col20a1*-tdT controls (Figure 4A [↗](#)). Quantification of the tdTomato-positive NMJ ratio showed an ablation efficiency of approximately 80%, with only ~20% of NMJs retaining escaper tSCs (Figure 4C [↗](#), yellow arrowheads in 4A). High-magnification views of these regions (indicated by white dashed boxes in Figure 4A [↗](#)) confirmed that the lack of tdTomato correlated with the absence of S100B⁺ Schwann cells (Figure 4B [↗](#)).

Unexpectedly, despite the profound loss of tSCs during this dynamic developmental window, the overall architecture of both the pre- and post-synaptic compartments remained largely intact. During early postnatal development, NMJs transition from polyneuronal to single motor axon innervation which is completed by 2 weeks of age (Balice-Gordon & Lichtman, 1993 [↗](#); Brown et al., 1976 [↗](#); Sanes & Lichtman, 1999 [↗](#)). In our ablated model, nerve terminals successfully achieved single innervation without overt signs of denervation or axon terminal pathology (Figure 4B [↗](#)). Furthermore, post-synaptic AChR clusters did not exhibit severe fragmentation or morphological immaturity. Collectively, these data suggest that while tSCs are efficiently ablated by *Col20a1*-tdT/DTA model, their physical presence is surprisingly dispensable for the fundamental structural maintenance and normal developmental pruning of the NMJ at this stage.

Systemic ablation of tSCs does not impair overall growth or gross motor behavior

As our newly developed model offers the advantage of system-wide tSC targeting, it enables the assessment of whole-body physiological impacts. Thus, we next investigated whether widespread tSC loss translates to macroscopic physiological or behavioral deficits. We first monitored general somatic growth of tSC-ablated mice by measuring body weight at the time of tamoxifen induction (P10) and post-ablation at 4 weeks of age. We observed no significant differences in body weight between *Col20a1*-tdT controls and *Col20a1*-tdT/DTA mice at either time point, indicating normal growth trajectories (Figure 5A, B [↗](#)). To assess overall motor coordination and muscle function, we performed a beam walking test (Lukong & Richard, 2008 [↗](#); Luong et al., 2011 [↗](#); Quinn et al., 2007 [↗](#)). The latency to cross the beam was comparable between the ablated mice and controls, indicating normal balance and gross motor coordination (Figure 5C [↗](#)). Furthermore, we conducted a gait analysis to evaluate fine motor control, a metric that is often altered in classical neuromuscular disorders (Maricelli et al., 2016 [↗](#); Preisig et al., 2016 [↗](#); Sheppard et al., 2020 [↗](#); Wooley et al., 2005 [↗](#)). Consistent with the beam walking results, systemic tSC ablation did not significantly alter either stride length or stride width (Figure 5D, E [↗](#)). Taken together with our morphological data (Figure 4 [↗](#)), these behavioral results indicate that tSCs are dispensable for overall somatic growth and basic motor behavior during the postnatal maturation period. This lack of a severe macroscopic phenotype suggests that tSCs do not govern broad neuromuscular integrity.

tSC ablation impairs pre-synaptic vesicle homeostasis and high-frequency synaptic transmission

To determine if tSCs are required for fine-tuned synaptic function despite normal morphology, we performed electrophysiological recordings. We first measured miniature endplate potentials (mEPPs) (Figure 6A [↗](#)) and found that mutant NMJs exhibited a significant increase in mEPP

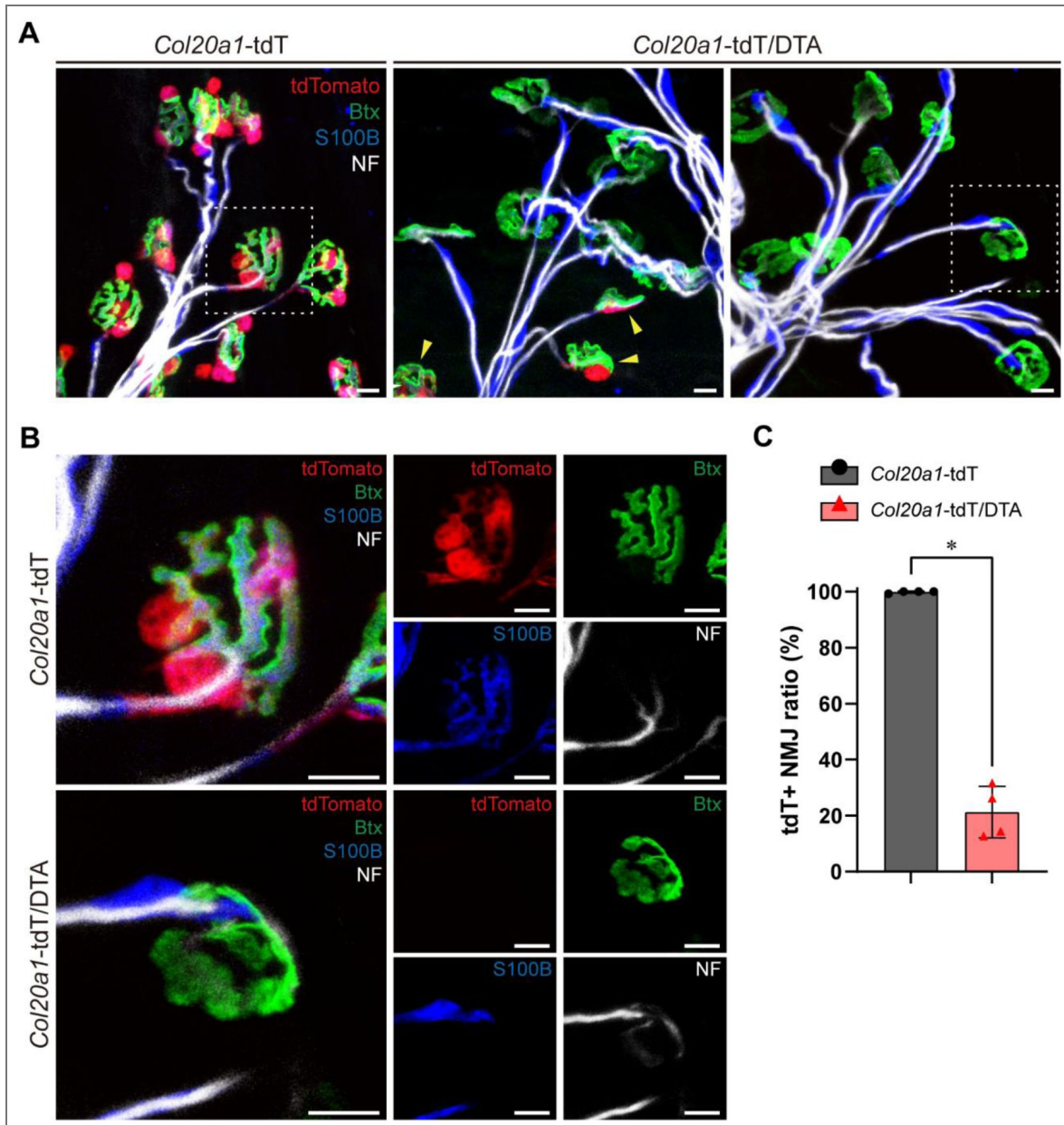


Figure 4. Targeted tSC ablation during the postnatal maturation window efficiently depletes glial cells without disrupting gross NMJ architecture.

To induce tSC ablation, tamoxifen (TMX, 160 mg/kg body weight) was administered at postnatal day 10 (P10) via intraperitoneal injection, and NMJs were analyzed at 4 weeks of age. (A) Representative low-magnification immunofluorescence images of EDL muscle NMJs from *Col20a1-tdT* control (left) and *Col20a1-tdT/DTA* mice (middle and right). Compared to controls, *Col20a1-tdT/DTA* mice exhibit a profound loss of the tdTomato reporter signal (red). Yellow arrowheads indicate rare surviving “escaper” tSCs, and white dashed boxes outline the regions shown at higher magnification in B. Scale bars: 10 μ m. (B) High-magnification confocal images of individual NMJs from *Col20a1-tdT* control (top) and tSC-ablated *Col20a1-tdT/DTA* mice (bottom). In *Col20a1-tdT/DTA* mice, the lack of tdTomato strictly correlates with the absence of the S100B glial marker (blue). Despite the profound loss of tSCs, pre-synaptic motor axons labeled by Neurofilament (NF, white) establish normal single innervation, and post-synaptic AChR clusters labeled by α -bungarotoxin (Btx, green) remain structurally intact. Scale bars: 10 μ m. (C) Quantification of the tdTomato-positive (tdT⁺) NMJ ratio. The data confirm an approximately 80% ablation efficiency in *Col20a1-tdT/DTA* mice compared to controls. Data are presented as mean $\pm$ SD (n = 4 mice per experimental group). Statistical significance was determined using the Mann-Whitney test; **p* < 0.05.

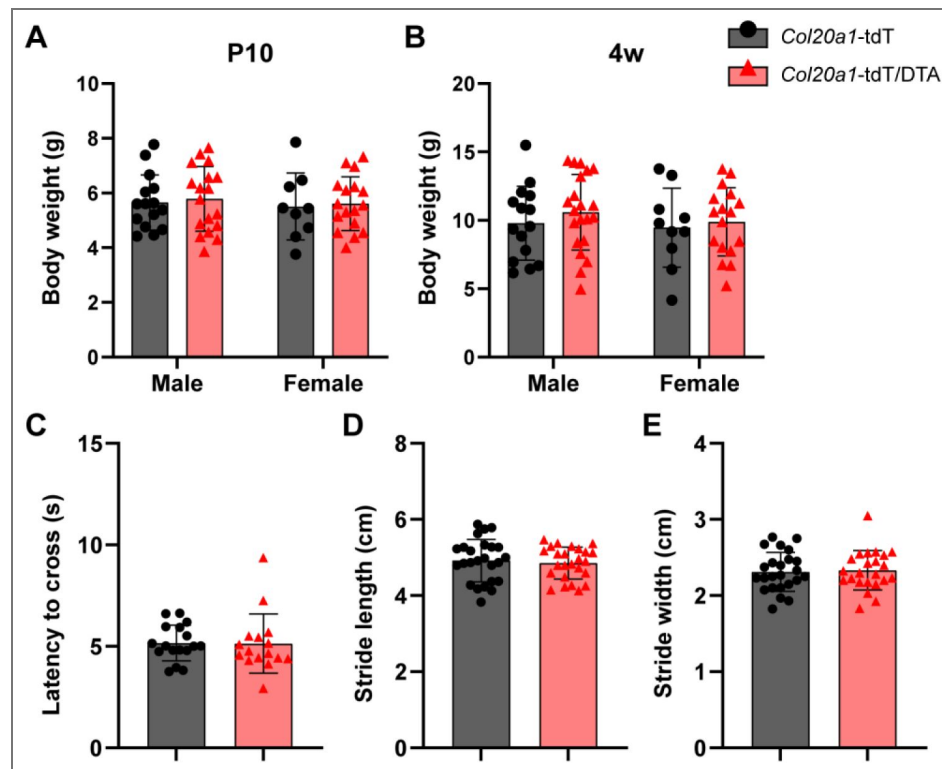


Figure 5. Systemic ablation of tSCs does not impair overall somatic growth or gross motor behavior.

(A, B) Body weight analysis of male and female control (*Col20a1-tdT*) and tSC-ablated (*Col20a1-tdT/DTA*) mice at (A) postnatal day 10 (P10, time of induction; control: $n = 15$ males, 9 females; ablated: $n = 18$ males, 17 females) and (B) 4 weeks of age (4w, post-ablation; control: $n = 15$ males, 10 females; ablated: $n = 21$ males, 17 females). Systemic loss of tSCs does not significantly alter normal growth trajectories in either sex. (C) Assessment of gross motor coordination using the beam walking test. The latency to cross the beam is comparable between the ablated mice and controls (control, $n = 17$ mice; ablated, $n = 16$ mice). (D, E) Quantitative gait analysis evaluating fine motor control. Systemic tSC ablation does not significantly affect (D) stride length or (E) stride width at 4 weeks of age ($n = 25$ mice per group). Data are presented as mean $\pm$ SD. Statistical significance was determined using one-way ANOVA with Bonferroni's post hoc test for A and B, and Student's t-test for C, D, and E.

frequency compared to controls (Figure 6D, E [↗](#)), while mEPP amplitude remained unchanged (Figure 6B, C [↗](#)). This selectively increased mEPP frequency indicates an elevation in spontaneous presynaptic neurotransmitter release in the absence of tSCs. Post-synaptic receptor sensitivity, however, remains intact.

Analysis of single evoked endplate potentials (eEPPs) (Figure 6F [↗](#)) revealed slightly increased amplitudes and accelerated rise times in mutant NMJs (Figure 6G, H [↗](#)), while decay time and half-width were normal (Figure 6I, J [↗](#)). The unaltered decay time and half-width indicate that post-synaptic AChR kinetics and neurotransmitter clearance mechanisms remain intact. The paired-pulse ratio (PPR) was comparable between groups across all intervals, suggesting that the initial release probability of neurotransmitter and the fundamental machinery for evoked release are largely preserved (Figure 6K–M [↗](#)).

Despite the preservation of basal transmission properties, the functional endurance of mutant NMJs was compromised under high-demand conditions. Specifically, under repetitive 20 Hz stimulation (eEPP-train), mutant NMJs exhibited a progressively severe depression of normalized eEPP amplitudes compared to controls (Figure 6N–O [↗](#)). Interestingly, estimation of the readily releasable pool (RRP) size via back-extrapolation of cumulative amplitude revealed no significant difference between the two groups (Figure 6P, Q [↗](#)). This suggests that the fatigability does not result from an absolute quantitative deficit of available vesicles, but rather from the depletion of the reserve pool caused by the chronic resting vesicle leakage. Finally, CMAP amplitude remained normal (Figure 6R, S [↗](#)), consistent with the lack of gross behavioral deficits. Collectively, these results reveal that tSCs are essential for maintaining pre-synaptic vesicle homeostasis and sustaining high-frequency synaptic transmission.

Ultrastructural analysis reveals pre-synaptic vesicle depletion following tSC ablation

To determine whether the electrophysiological defects observed in tSC-ablated mice manifest at the ultrastructural level, we performed electron microscopy (EM) on NMJs (Figure 7A [↗](#)). We categorized the analyzed NMJs into three groups: control (*Col20a1*-tdT), mutant NMJs where tSCs were retained (*Col20a1*-tdT/DTA; tSC-retained), and mutant NMJs with successful tSC ablation (*Col20a1*-tdT/DTA; tSC-ablated).

Consistent with the overall preservation of gross NMJ morphology, normal motor behavior, and intact post-synaptic receptor kinetics observed in our previous analyses, there were no significant differences in synaptic cleft width (Figure 7B [↗](#)) or junctional fold density (Figure 7C [↗](#)) across any of the experimental groups. These results confirmed that the overall physical integrity of the synapse is well-maintained and that post-synaptic structural maturation proceeds normally without signs of degeneration despite the loss of tSCs.

In contrast to the preserved post-synaptic architecture, synaptic vesicle density was significantly reduced exclusively in the tSC-ablated NMJs compared to both control and tSC-retained NMJs (Figure 7D [↗](#)). This physical depletion of synaptic vesicles strongly correlates with the accelerated eEPP-train depression and the increased spontaneous leaky release observed previously, providing a structural basis for the loss of pre-synaptic homeostasis. Taken together, these findings suggest that rather than broadly governing macroscopic synaptic architecture or post-synaptic structural development, tSCs specifically function to sustain pre-synaptic vesicle homeostasis and preserve the reserve pool required for continuous neurotransmission.

Long-term analysis reveals Schwann cell repopulation and sustained gross neuromuscular function

At 4 weeks post-ablation, we confirmed a profound loss of tSCs that induced specific pre-synaptic defects while leaving gross structural and behavioral phenotypes intact. To determine whether this deficient yet stable state persists long-term, or if it triggers compensatory glial restoration as reported in previous studies (Hastings et al., 2020 [↗](#)), we extended our analysis to 12 weeks post-ablation. Immunofluorescence analysis of the NMJs at 12 weeks revealed a recovery of S100B glial

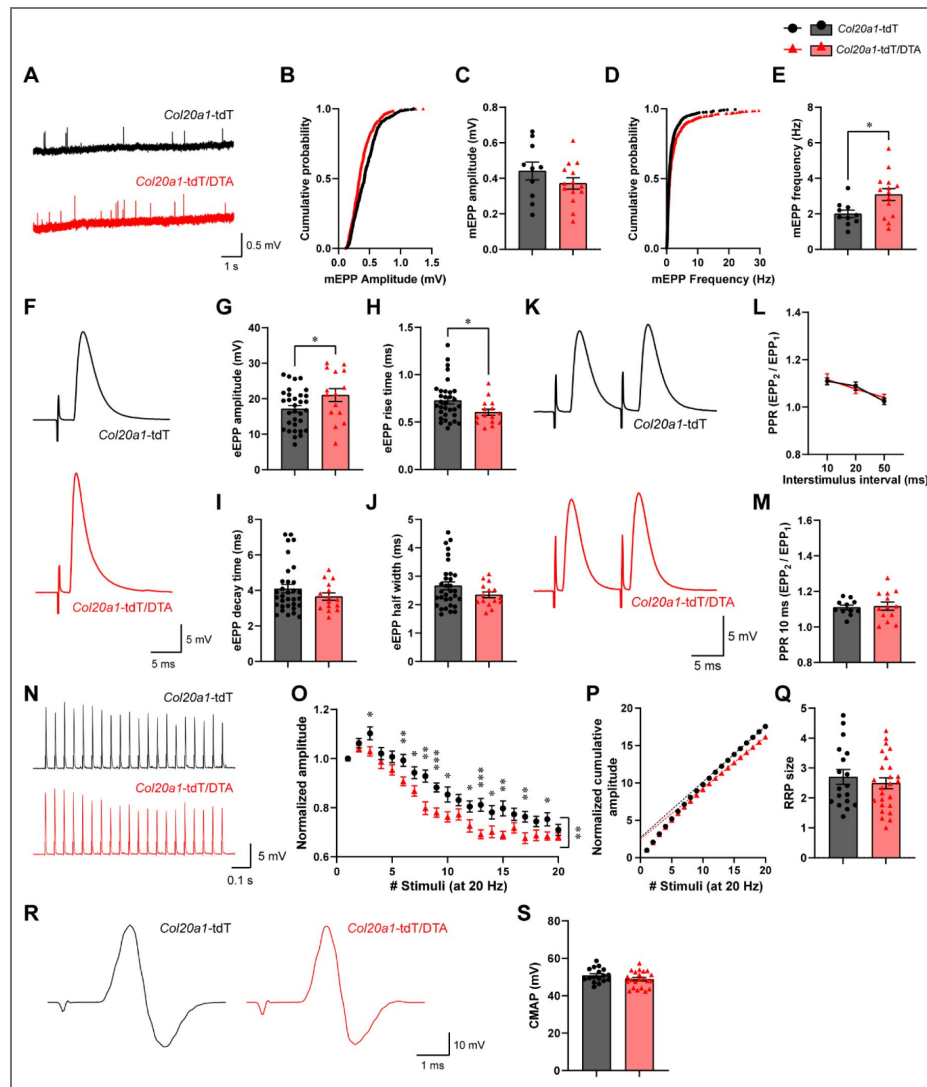


Figure 6. Targeted tSC ablation impairs pre-synaptic vesicle homeostasis and compromises high-frequency synaptic transmission.

(A) Representative electrophysiological traces of miniature endplate potentials (mEPPs) recorded from isolated EDL muscles of control (*Col20a1*-tdT, top) and tSC-ablated (*Col20a1*-tdT/DTA, bottom) mice at 4 weeks of age. (B–E) Cumulative probability distributions and summary quantification of mEPP parameters. While mEPP amplitude (B, C) remains unchanged, mutant NMJs display a significant increase in mEPP frequency (D, E) compared to controls, indicating a “leaky” pre-synaptic spontaneous release (control, $n = 10$ NMJs from 3 mice; tSC-ablated, $n = 14$ NMJs from 5 mice). (F) Representative traces of single evoked endplate potentials (eEPPs). (G–J) Quantification of eEPP kinetics. Mutant NMJs exhibit significantly increased eEPP amplitudes (G) and accelerated rise times (H). There are no significant differences in decay time (I) or half-width (J), indicating intact post-synaptic receptor kinetics (control, $n = 32$ NMJs from 7 mice; tSC-ablated, $n = 15$ NMJs from 6 mice). (K) Representative traces of paired-pulse responses at an inter-stimulus interval of 10 ms. (L, M) Analysis of the paired-pulse ratio (PPR, EPP_2/EPP_1) across varying inter-stimulus intervals (L) and specifically at 10 ms (M). The PPR is comparable between groups, indicating a preserved basal release probability in the absence of tSCs (control, $n = 11$ NMJs from 3 mice; tSC-ablated, $n = 13$ NMJs from 5 mice). (N) Representative eEPP trains elicited by repetitive 20 Hz stimulation (20 pulses). (O) Normalized eEPP amplitudes during the 20 Hz stimulation train. Mutant NMJs exhibit a progressively severe synaptic depression compared to controls. (P, Q) Cumulative amplitude plot (P) and estimation of the readily releasable pool (RRP) size (Q) calculated via back-extrapolation of the cumulative eEPP amplitude. Despite the severe fatigue, absolute RRP size is not significantly altered (control, $n = 18$ NMJs from 4 mice; tSC-ablated, $n = 25$ NMJs from 5 mice). (R) Representative traces of Compound Muscle Action Potential (CMAP). (S) Quantification of CMAP amplitude, indicating normal macroscopic physiological function in mutant mice, consistent with behavioral data. Data are presented as mean $\pm$ SEM. Statistical significance was determined using Student’s t-test for C, E, G–J, M, Q, S, two-way ANOVA for L, and Repeated Measures ANOVA with Bonferroni’s multiple comparisons for O; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

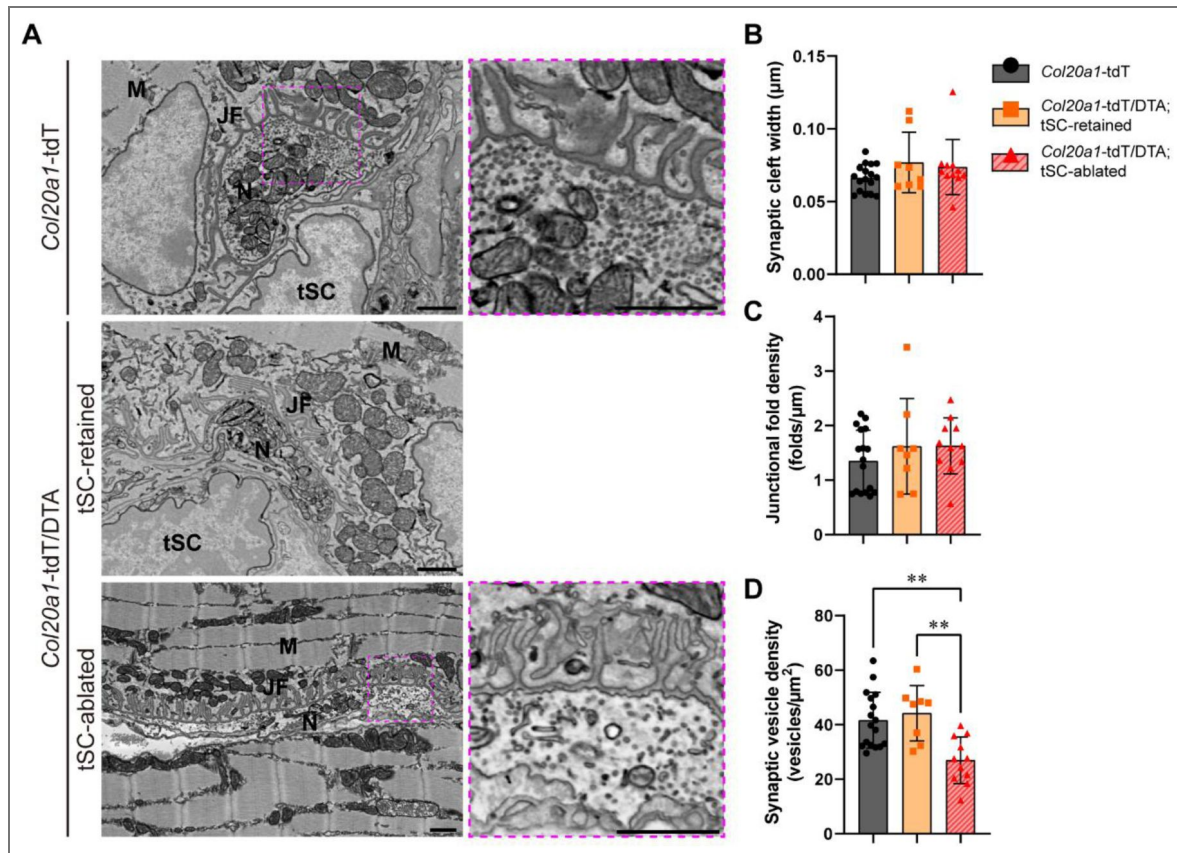


Figure 7. Ultrastructural analysis reveals pre-synaptic vesicle depletion following targeted tSC ablation. (A) Representative backscattered scanning electron microscopy (BSE-SEM) micrographs of ultra-thin sections of NMJs from control (*Col20a1*-tdT), mutant NMJs with retained tSCs (*Col20a1*-tdT/DTA; tSC-retained), and mutant NMJs with ablated tSCs (*Col20a1*-tdT/DTA; tSC-ablated). High-magnification views of the regions indicated by pink dashed boxes are shown on the right, highlighting the ultrastructural details of synaptic vesicles, junctional folds, and the synaptic cleft. M, muscle; N, nerve terminal; JF, junctional folds; tSC, terminal Schwann cell. Scale bars: 1 μ m. (B, C) Quantitative analysis of (B) synaptic cleft width and (C) junctional fold density. Both parameters remain unchanged across all experimental groups, indicating that overall synaptic physical integrity and post-synaptic architecture are preserved despite tSC loss. (D) Quantification of synaptic vesicle density. The tSC-ablated NMJs exhibit a significant reduction in vesicle density compared to both control and tSC-retained groups, providing ultrastructural evidence for the loss of pre-synaptic homeostasis. Data are presented as mean $\pm$ SD (control, n = 17 NMJs from 5 mice; mutant NMJs with retained tSCs, n = 8 NMJs from 4 mice; mutant NMJs with ablated tSCs, n = 11 NMJs from 3 mice). Statistical significance was determined using one-way ANOVA with Bonferroni's post hoc test; ** $p < 0.01$.

signals at the synapse in *Col20a1*-tdT/DTA mice, with the persistent absence of the tdTomato reporter (Figure 8A, B). This indicates that the ablated tSCs were successfully replaced by unlabeled, non-recombined Schwann cells, presumably migrating from the upstream aSC pool.

Furthermore, we assessed various functional and behavioral metrics to confirm that this repopulation successfully supports long-term neuromuscular health. Similar to our observations at 4 weeks, we found no significant differences in CMAP amplitudes (Figure 8C, D). Regarding gross physiological indicators, while a slight reduction in body weight was observed in tSC-ablated males at 12 weeks (Figure 8E), this did not translate into any macroscopic motor impairment. Indeed, all motor behavioral assessments, including grip strength (Figure 8F), rotarod motor coordination (Figure 8G), and endurance running capacity (Figure 8H, I), remained comparable to controls. Together, these data suggest that the neuromuscular system possesses a robust compensatory mechanism to replace lost tSCs, thereby maintaining overall synaptic stability and sustaining fundamental motor function.

Discussion

In this study, we identified *Col20a1* as a highly specific marker for tSCs and established a novel *Col20a1*-CreERT2 mouse model to investigate their functional roles *in vivo*. By inducing targeted tSC ablation during the critical postnatal maturation window, we found that while tSCs are surprisingly dispensable for the maintenance of gross NMJ structure and behavioral phenotype, they are essential for preserving pre-synaptic vesicle homeostasis. Furthermore, our long-term analysis revealed a robust compensatory mechanism of the neuromuscular system through Schwann cell repopulation, underscoring the dynamic plasticity of the tripartite synapse.

A long-standing challenge in tSC biology has been the lack of a genetic model that specifically targets these cells without affecting other Schwann cell lineages (Hastings & Valdez, 2024; Jablonka-Shariff et al., 2021; Santosa et al., 2018). Previous research has largely relied on pan-Schwann cell markers, such as *Plp1* and *S100b* (Barik et al., 2016; Zuo et al., 2004), or indirect physiological observations through calcium imaging or microscopy (Darabid et al., 2013; Reist & Smith, 1992; Smith et al., 2013). While pan-glial models provide foundational insights, isolating tSC-specific roles can be challenging due to the concurrent manipulation of aSCs (Barik et al., 2016; Doerflinger et al., 2003). Furthermore, although acute physiological methods offer high-resolution detail, they are inherently designed for localized observations rather than long-term, organism-wide assessments. Our *Col20a1*-CreERT2 model overcomes these limitations by enabling tSC-specific manipulation within the muscle, effectively sparing the upstream aSCs. This allowed us to isolate the “pure” physiological role of tSCs and, for the first time, investigate how tSC-level alterations translate into macroscopic behavioral and physiological phenotypes.

Conventional wisdom, supported by various experimental models, has suggested that tSCs are indispensable for the structural maintenance of the NMJ. For instance, antibody-mediated ablation of tSCs in frog NMJs led to nerve retraction within a week (Koirala et al., 2003), and mouse models utilizing *Plp1*-CreERT2 observed both pre- and post-synaptic structural collapses (Barik et al., 2016). Furthermore, acute treatments with patient-derived sera or antibodies on isolated muscles reported structural abnormalities within an hour (Jablonka-Shariff et al., 2023). These findings collectively established the view that tSC loss triggers fundamental structural failure. However, our findings surprisingly show that systemic tSC ablation induced at P10 does not lead to overt structural disintegration, such as denervation or AChR fragmentation. This discrepancy likely stems from the differences in experimental contexts and methodologies. Previous models may have induced accessory effects due to the inherent broad expression of pan-glial promoters (Barik et al., 2016), or by exerting off-target collateral damage to motor nerve terminals via antibody-mediated complement activation (Halstead et al., 2004). In contrast, the *Col20a1*-CreERT2 model induces DTA-mediated apoptosis specifically within tSCs. By minimizing off-target collateral damage and preserving aSC integrity, our study reveals that the mammalian NMJ possesses a higher degree of structural resilience than previously anticipated.

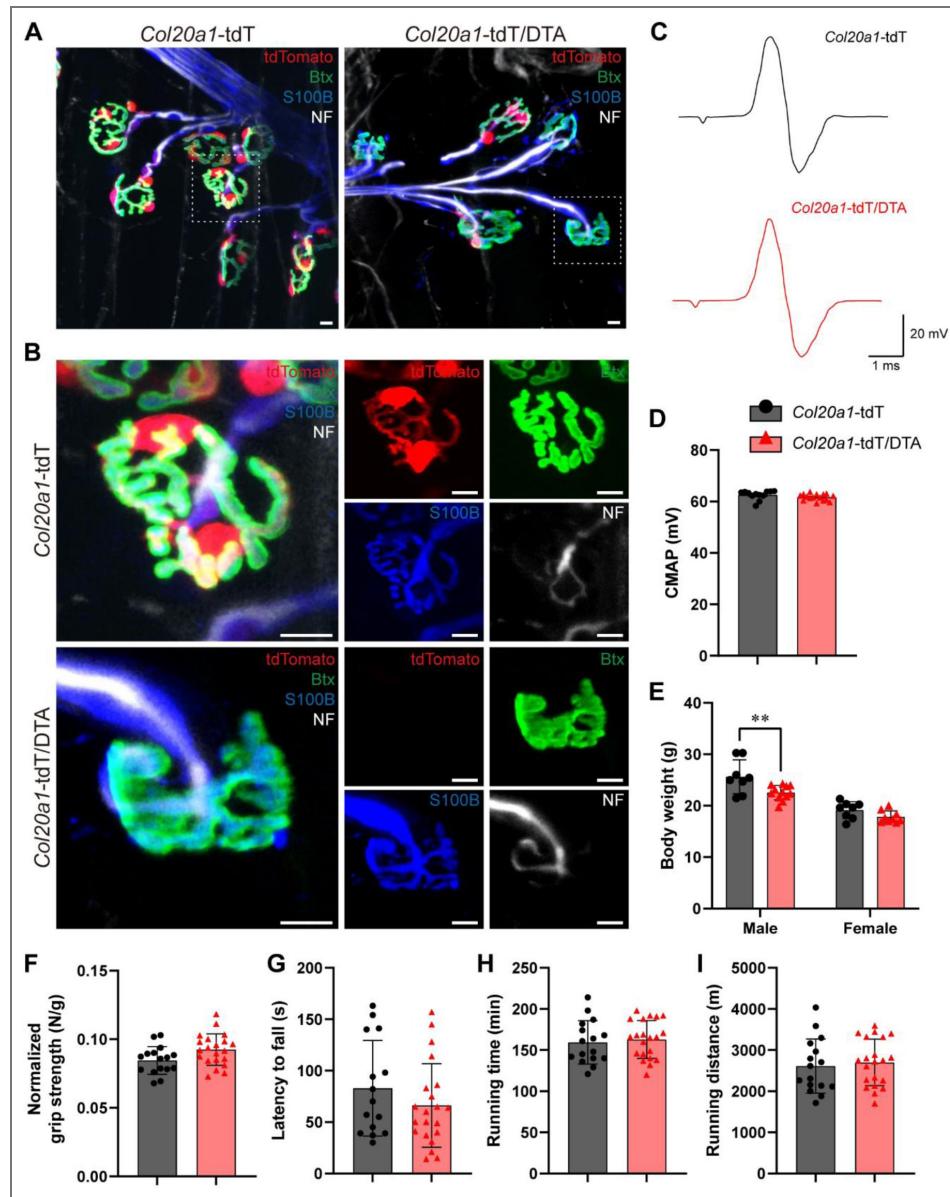


Figure 8. Long-term repopulation of Schwann cells maintains structural integrity and gross neuromuscular function.

To assess long-term recovery, tamoxifen (TMX, 160 mg/kg body weight) was administered at postnatal day 10 (P10) by intraperitoneal injection, and mice were analyzed at 12 weeks of age. (A) Representative immunofluorescence images of muscle NMJs from *Col20a1-tdT* control (left) and tSC-ablated *Col20a1-tdT/DTA* mice (right) at 12 weeks post-induction. White dashed boxes indicate regions magnified in B. Scale bars: 10 μ m. (B) High-magnification confocal images of individual NMJs from *Col20a1-tdT* (top) and *Col20a1-tdT/DTA* (bottom) mice. In the mutant NMJs, S100B⁺ glial cells (blue) successfully repopulate the synapse despite the persistent absence of the tdTomato reporter (red). The gross architecture of pre-synaptic motor axons (NF, white) and post-synaptic AChRs (Btx, green) is sustained following repopulation. Scale bars: 10 μ m. (C) Representative traces of Compound Muscle Action Potential (CMAP). (D) Quantification of CMAP amplitude, indicating sustained physiological function in tSC-ablated mice (control, n = 12 mice; ablated, n = 16 mice). Data are presented as mean $\pm$ SEM. (E) Body weight analysis of male and female mice at 12 weeks of age (control: n = 8 males, 8 females; ablated: n = 12 males, 9 females). Data are presented as mean $\pm$ SD. (F–I) Macroscopic motor behavioral assessments showing normal long-term function in mutant mice, including (F) normalized grip strength (control, n = 16 mice; ablated, n = 21 mice), (G) motor coordination assessed by latency to fall in the rotarod test (control, n = 15 mice; ablated, n = 21 mice), and endurance running capacity measured by (H) running time and (I) running distance (control, n = 16 mice; ablated, n = 21 mice). Data are presented as mean $\pm$ SD. Statistical significance was determined using one-way ANOVA with Bonferroni's post hoc test for E and Student's t-test for D and F–I; ***p* < 0.01.

Beyond the maintenance of existing structures, our results also show that the fundamental hallmarks of postnatal NMJ maturation—notably the transition from polyneuronal to single-axon innervation (Balice-Gordon & Lichtman, 1993 [↗](#); Brown et al., 1976 [↗](#); Sanes & Lichtman, 1999 [↗](#)), formation of “pretzel-like” AChR morphologies, and junctional fold maturation (Marques et al., 2000 [↗](#); Matthews-Bellinger & Salpeter, 1983 [↗](#); Shi et al., 2012 [↗](#))—proceeded normally despite the loss of tSCs from P10. While tSCs has been shown to actively coordinate the withdrawal of redundant axons during early postnatal development (Lee et al., 2016 [↗](#)), our results suggest a distinct temporal boundary for this requirement. The physical elimination of redundant axons takes place within the first two postnatal weeks (Duxson, 1982 [↗](#); Favero et al., 2015 [↗](#); Hirata et al., 1997 [↗](#); Lanuza et al., 2002 [↗](#)), but it is possible that the molecular commitment or the signaling cues required for this process are already established prior to P10. Consequently, tSCs might play a more indispensable role in the earlier stages of NMJ maturation, whereas their contribution to the later stages of structural refinement may be redundant or secondary to neuron-intrinsic programs. While this suggests a temporal limitation in assessing early structural assembly, our model remains effective for evaluating the functional maturation that occurs during and after this period.

Despite the preservation of macroscopic structure, our electrophysiological and ultrastructural analyses revealed profound defects in pre-synaptic homeostasis. Increased spontaneous leaky release and the accelerated synaptic fatigue during high-frequency stimulation, along with decreased synaptic vesicle density revealed by EM, indicate that tSCs play an indispensable role in maintaining the integrity of the pre-synaptic vesicle pool. This suggests that tSCs function as active metabolic or signaling modulators of the nerve terminal, rather than as passive bystanders. Several mechanisms may underlie this functional regulation. For example, given that tSCs are known to sense nerve terminal activity via purinergic or calcium signaling (Darabid et al., 2013 [↗](#); Darabid et al., 2018 [↗](#); Heredia et al., 2018 [↗](#); Jahromi et al., 1992 [↗](#); Robitaille, 1998 [↗](#)), they may provide a feedback loop that stabilizes the reserve pool. Alternatively, tSCs may regulate the perisynaptic ionic environment by buffering excess potassium or calcium (Heredia et al., 2018 [↗](#); Mi et al., 1996 [↗](#)); thus, their absence could lead to a chronic hyperexcitable state of the nerve terminal and subsequent vesicle exhaustion. Further studies are required to decipher the exact molecular cross-talk responsible for this tSC-mediated vesicle homeostasis.

It is noteworthy that the profound electrophysiological alterations did not translate into overt motor deficits. This disconnect is likely attributable to the inherent limits in the sensitivity of currently available behavioral assays for juvenile mice. While we employed gait analysis and beam walking as the most suitable measures of early motor coordination, these macroscopic readouts likely lack the granularity required to detect subtle, localized synaptic inefficiencies. The vesicle depletion and accelerated synaptic depression we observed represent a fine-tuned homeostatic failure under high demand, rather than a gross structural collapse. Therefore, the absence of an overt behavioral phenotype does not preclude the existence of functional impairments; rather, it highlights a resolution mismatch between microscopic synaptic physiology and macroscopic behavioral tools. Unmasking the precise functional consequences of tSC loss on fine-motor coordination will ultimately require the development of higher-sensitivity behavioral paradigms.

Our long-term analysis at 12 weeks post-induction highlighted the remarkable plasticity of the NMJ niche, as evidenced by the repopulation of tSCs. This aligns with previous report showing tSC repopulation after ablation using *Plp1*-DTR model (Hastings et al., 2020 [↗](#)), suggesting that the NMJ can sense the absence of glial coverage and trigger the migration and differentiation of neighboring aSCs to restore the niche. While gross motor functions remained stable long-term, we observed a subtle but significant reduction in body weight specifically in tSC-ablated males. We interpret this as a cumulative effect of the subtle synaptic inefficiencies observed at 4 weeks. Given that male mice undergo more rapid somatic growth, the persistent, albeit minor, homeostatic defects may impose a cumulative metabolic penalty that manifests as reduced body weight over time.

In conclusion, our study establishes *Col20a1* as a definitive genetic handle for tSC research and redefines the role of tSCs from a passive structural scaffold to an active functional buffer essential for presynaptic homeostasis. By establishing a high-specificity genetic platform, we have shown that while the mammalian NMJ structure is remarkably resilient to glial loss, its physiological efficiency is critically dependent on tSC-mediated regulation. These insights provide a new framework for understanding glial-neuronal interactions and their contributions to neuromuscular health and disease.

Materials and Methods

Experimental Animals

The *Col20a1*^{CreERT2} knock-in mice were generated on a C57BL/6N genetic background at the Lee Gil Ya Cancer and Diabetes Institute Center of Animal Care and Use, Gachon University (Incheon, Republic of Korea). The wildtype C57BL/6J (RRID:ISMR_JAX:000664 [link](#)) mice, the *Rosa26*^{LSL-tdTomato} (*Rosa*-tdT) (RRID:ISMR_JAX:007914 [link](#)) and the *Rosa26*^{LSL-DTA} (*Rosa*-DTA) (RRID:ISMR_JAX:009669 [link](#)) reporter lines were acquired from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained on a C57BL/6J genetic background. To generate *Col20a1*^{CreERT2}; *Rosa26*^{LSL-tdTomato} (*Col20a1*-tdT) mice, *Col20a1*^{CreERT2/+} mice were crossed with *Rosa26*^{LSL-tdTomato/+}, and both *Col20a1*^{+/+}; *Rosa26*^{LSL-tdTomato/+} and *Col20a1*^{CreERT2/+}; *Rosa26*^{LSL-tdTomato/+} mice were used in experiments. To generate *Col20a1*^{CreERT2}; *Rosa26*^{LSL-tdTomato/LSL-DTA} mice (*Col20a1*-tdT/DTA), *Col20a1*^{CreERT2/+}; *Rosa26*^{LSL-tdTomato/+} mice were crossed with *Rosa26*^{LSL-DTA/+} mice, generating both *Col20a1*^{CreERT2/+}; *Rosa26*^{LSL-tdTomato/+} mice and *Col20a1*^{CreERT2/+}; *Rosa26*^{LSL-tdTomato/LSL-DTA} mice as littermates. In experiments using *Col20a1*^{CreERT2/+}; *Rosa26*^{LSL-tdTomato/LSL-DTA} mice, *Col20a1*^{CreERT2/+}; *Rosa26*^{LSL-tdTomato/+} mice were used as controls. All animal experiments were conducted in accordance with the ethical guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (Approval No. SNU-250324-2-1). Mice were housed in a specific pathogen-free (SPF) facility under a 12-hour light/dark cycle with *ad libitum* access to standard chow and water. Both male and female mice were used in this study.

Generation of *Col20a1*-CreERT2 Knock-in Mice

To generate the terminal Schwann cell (tSC)-specific *Col20a1*-CreERT2 knock-in mouse line, we employed a microinjection-free, CRISPR/Cas9-mediated homology-directed repair (HDR) strategy termed CRISPR-READI (CRISPR RNP electroporation and AAV donor infection) (Chen et al., 2019 [link](#)) (Chen et al., 2019 [link](#)). A targeting donor vector was designed to insert a cassette containing a P2A self-cleaving peptide, the CreERT2 recombinase sequence, and an SV40 polyadenylation (pA) signal immediately upstream of the endogenous STOP codon in exon 35 of the *Col20a1* gene. To achieve high-efficiency integration, the HDR donor sequence was flanked by AAV2 inverted terminal repeats (ITRs) and packaged into recombinant AAV virions. Briefly, fertilized mouse zygotes were harvested and infected with the recombinant AAV harboring the HDR donor template. Subsequently, pre-assembled Cas9/single-guide RNA (sgRNA) ribonucleoproteins (RNPs) were introduced into the AAV-transduced zygotes via electroporation to induce DNA double-strand breaks and stimulate homologous recombination. Following successful homologous recombination, the targeted allele (*Col20a1*^{CreERT2}) allows the co-translation of the endogenous COL20A1 protein and the CreERT2 recombinase, separated by the P2A peptide. Founder mice were identified by PCR genotyping and subsequently backcrossed with C57BL/6J mice to establish a stable germline-transmitted colony.

Tamoxifen Administration for Inducible Labeling and Ablation

To induce Cre-mediated recombination, tamoxifen (Sigma-Aldrich) was dissolved in corn oil at a concentration of 20 mg/mL. For adult mice (8–12 weeks old), tamoxifen was administered via oral gavage at a dose of 160 mg/kg body weight for five consecutive days. For postnatal induction (e.g.,

P10), tamoxifen was administered via a single intraperitoneal (IP) injection at the same weight-adjusted dose (160 mg/kg). All mice including control mice lacking the CreERT2 allele received the identical tamoxifen regimen.

Genotyping

Routine genotyping of the transgenic and mutant mice was performed by PCR using genomic DNA extracted from tail biopsies or ear snips. The tissues were lysed in DirectPCR Lysis Reagent (Viagen Biotech) supplemented with Proteinase K at a final concentration of 0.2 µg/µL. The lysis mixture was incubated at 65°C for 30 minutes, followed by a heat-inactivation step at 95°C for 1 hour.

The following primers were used: Col20a1_common_F: 5' – ATC AGC CAC ACA AGC AAC CC – 3', Col20a1_WT_R: 5' – ATT GTT CAG CAG AGC TCC CC – 3', Col20a1_Tg_R: 5' – ACC GGC AAA CGG ACA GAA GC – 3', Rosa-tdT_WT_F: 5' – AAG GGA GCT GCA GTG GAG TA – 3', Rosa-tdT_WT_R: 5' – CCG AAA ATC TGT GGG AAG TC – 3', Rosa-tdT_Tg_F: 5' – GGC ATT AAA GCA GCG TAT CC – 3', Rosa-tdT_Tg_R: 5' – CTG TTC CTG TAC GGC ATG G – 3', DTA_F: 5' – GAC TGA CGA AGG TTC TCG CA – 3', DTA_R: 5' – GCT TAA CGC TTT CGC CTG TT – 3'.

PCR amplification was carried out using EmeraldAmp GT PCR Master Mix (Takara Bio) in a SimpliAmp Thermal Cycler (Applied Biosystems). For the *Col20a1* and *Rosa26*-tdTomato loci, the respective WT and Tg primer sets were combined in single multiplex PCR reactions. The general thermal cycling conditions are as follows: an initial denaturation at 95°C for 5 minutes, followed by 33 cycles of denaturation at 95°C for 15 seconds, annealing for 20 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 7 minutes. The annealing temperatures were individually optimized for each target: 65°C for the *Col20a1*-CreERT2 multiplex, 61°C for the *Rosa26*-tdTomato multiplex, and 57°C for the DTA amplification. Finally, the amplicons were resolved on 1.5% agarose gels, stained with EcoDye (Biofact), and visualized using a Davinch Gel Imaging System (Davinch-K).

Single-cell RNA Sequencing Data Analysis

Data Acquisition and Quality Control

To characterize the transcriptomic profile tSCs and other skeletal muscle-resident populations, we utilized a publicly available single-cell RNA sequencing (scRNA-seq) dataset generated from adult wild-type mouse hindlimb muscles (Giordani et al., 2019), accessible via the NCBI Gene Expression Omnibus (GEO) under accession number GSE110878. All computational and statistical analyses were performed using R version 4.5.3 and the Seurat package version 5.4.0 (Hao et al., 2024). The datasets of two mice were read and further processed independently. Initial quality control was performed to filter out cells with fewer than 500 or more than 4,000 expressed genes, fewer than 500 or more than 9,000 total UMI counts, or greater than 10% mitochondrial gene expression.

Doublet Detection and Filtering

To facilitate doublet detection, standard preprocessing—including normalization, scaling, principal component analysis (PCA), and clustering—was initially performed on each sample independently. Specifically, the gene expression matrices were normalized using global-scaling normalization (“LogNormalize”) with a scale factor of 10,000. To identify highly variable features, the “vst” method was applied to select the top 2,000 highly variable genes. The data were then linearly scaled and centered. PCA was performed on the scaled data using the identified highly variable genes. To cluster the cells, a K-nearest neighbor (KNN) graph was constructed based on the Euclidean distance in PCA space, using the first 30 principal components (PCs). Cell clustering was performed using the Louvain algorithm with a resolution of 0.5. The DoubletFinder package was then applied to each dataset. The expected doublet rate was set to 4.9%. The optimal pK values were empirically determined as 0.06 and 0.005 for Sample 1 and Sample 2, respectively, utilizing a pN value of 0.25 and the first 30 PCs. The proportion of homotypic doublets was estimated based on the initial clustering to adjust the final doublet expectations. Only cells classified as “Singlet” were retained for subsequent analyses.

Data Integration and Dimensionality Reduction

The singlet-filtered objects from both samples were merged into a single dataset. To normalize the data, stabilize variance, and mitigate the confounding effects of mitochondrial gene expression, we applied the SCTransform method, explicitly regressing out the mitochondrial percentage (percent.mt). PCA was performed on the SCTransform-normalized data. Cell clustering was conducted using a KNN graph constructed on the first 30 PCs and the Louvain algorithm with a resolution of 0.5. Uniform Manifold Approximation and Projection (UMAP) was generated using the same top 30 PCs for non-linear dimensional reduction and visualization.

Schwann Cell Subsetting and Sub-clustering

The pan-Schwann cell population was initially annotated based on the enrichment of previously established marker genes, including *Plp1* and *S100b*, and subsequently subsetting for downstream analysis. The subsetting Seurat object was re-processed by normalizing with global-scaling (“LogNormalize”), scaling, and re-identifying variable features. PCA was re-calculated on the top 2,000 highly variable genes of this subpopulation. Sub-clustering was performed using the first 10 PCs and a KNN graph, followed by the Louvain algorithm with a resolution of 1.0. UMAP was generated using the same 10 PCs for visualization, which yielded distinct Schwann cell sub-clusters. The tSC population was annotated based on the enrichment of previously established marker genes (Castro et al., 2020), explicitly separating them from myelinating Schwann cells (mSCs) and Remak non-myelinating Schwann cells (nmSCs).

Differential Expression and Marker Gene Identification

To identify highly specific marker genes for putative tSCs, differential expression analysis was conducted using the FindMarkers function in Seurat, utilizing the non-parametric Wilcoxon rank-sum test. A two-tier intersection strategy was employed to ensure the identification of both highly representative and exclusively specific markers. First, differentially expressed genes (DEGs) were calculated by comparing the putative tSC cluster with the other Schwann cell sub-clusters. Significant DEGs for this lineage-specific comparison were filtered based on a log2 fold change > 1, an adjusted *p*-value < 0.05, and min.pct = 0.5. Secondly, a subsequent comparison was conducted between the putative tSC cluster and all the other cell populations in the whole-muscle dataset. For this tissue-wide comparison, alongside the same fold change and significance thresholds, significant DEGs were strictly filtered based on minimal expression in background cells (pct.2 < 0.01). The final tSC marker gene candidates were established by intersecting the significant DEGs derived from these two independent criteria.

Tissue-wide Expression Profiling

To assess the tissue-wide expression profiles and cellular specificity of the candidate genes across diverse organ systems, we utilized the Chan Zuckerberg CELLxGENE Discover platform (Program et al., 2025). Single-cell transcriptomic datasets spanning various adult mouse tissues and organs were acquired to evaluate the expression patterns of the selected candidate genes, including *Col20a1*, *Acsbg1*, and *Fabp7*. Cell-type-specific enrichment patterns were analyzed and visualized as dot plots in R.

Analysis of RNA-seq Datasets

To cross-validate and robustly identify tSC-specific marker genes, we performed a meta-analysis utilizing publicly available RNA-sequencing (RNA-seq) datasets from previous studies. Transcriptomic data profiling isolated synaptic Schwann cells (Castro et al., 2020) and skeletal muscle/NMJ tissues (Ham et al., 2020) were retrieved from the NCBI Gene Expression Omnibus (GEO) database. The datasets are accessible under the GEO accession numbers GSE152774 and GSE139209, respectively. Raw count matrices were downloaded for downstream computational analysis. The normalized read counts, expressed as Counts Per Million (CPM) or Transcripts Per Million (TPM), for each candidate genes were visualized using GraphPad Prism software (version 9, GraphPad Software). The DEG analysis for bulk RNA-seq data were performed within R using DESeq2 package version 1.50.2. For the DESeq2 workflow, raw counts were normalized using the

median of ratios method to account for sequencing depth variations, and gene-wise dispersion estimates were modeled based on a negative binomial distribution. To identify DEGs, the Wald test was applied, and *p*-values were adjusted for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) procedure. Genes were defined as significantly differentially expressed if they met the strict criteria of an adjusted *p*-value (FDR) < 0.05 and a log₂ fold change > 1. Volcano plots for DEG visualization were generated using ggplot2 packages in R.

Isolation and Sorting of Muscle-Resident Cells

To isolate tdTomato labelled (tdT⁺) cells and categorize distinct populations of muscle-resident cells from tamoxifen-treated *Col20a1*-tdT mice, fluorescence-activated cell sorting (FACS) was performed based on a previously described protocol (Liu et al., 2015) with minor optimizations. Briefly, hindlimb muscles were harvested, finely minced using surgical scissors, and rinsed in Dulbecco's Modified Eagle Medium (DMEM; HyClone) supplemented with 10% horse serum (Gibco). For enzymatic tissue digestion, the minced samples were incubated in DMEM containing 10% horse serum, 800 U/mL Collagenase II (Worthington Biochemical), and 1.1 U/mL Dispase II (Gibco) for 40 minutes at 37°C with gentle agitation. Subsequent mechanical dissociation was achieved by triturating the suspension through a 20-gauge needle ten times. The resulting homogenate was passed through a 40-μm cell strainer to obtain a mononuclear single-cell suspension. For immunophenotyping, the cells were incubated with the following primary antibodies (all diluted at 1:100 in PBS containing 10% horse serum): APC-conjugated anti-mouse CD31, CD45, and Ter119 (Biolegend); biotin-conjugated anti-mouse VCAM1 (BioLegend); and FITC-conjugated anti-mouse Sca-1 (BD Pharmingen). To detect the biotinylated VCAM1 antibody, cells were secondarily stained with PE/Cy7-conjugated streptavidin (1:100 in PBS containing 10% horse serum). Cell viability was assessed by adding 7-aminoactinomycin D (7-AAD; Sigma-Aldrich) to exclude dead cells. Following the staining process, the cells were resuspended in a FACS buffer consisting of 2% horse serum in PBS immediately prior to analysis.

Cell sorting was executed using a BD FACS Aria III cell sorter (BD Biosciences) controlled by BD FACS Diva software for data acquisition. Live (7-AAD⁻) cells were gated and sorted into specific sub-populations using the following strategies: muscle stem cells (MuSCs; Lin⁻/VCAM1⁺/Sca-1⁻), fibro-adipogenic progenitors (FAPs; Lin⁻/VCAM1⁻/Sca-1⁺), lineage-positive cells (Lin⁺; CD31⁺, CD45⁺ or Ter119⁺), and double-negative cells (DN; Lin⁻/Vcam⁻/Sca-1⁻). Within each gated population, tdT⁺ cells were quantified. Finally, tdT⁺ and tdT⁻ fractions from the DN population were independently sorted to evaluate genomic DNA recombination via PCR. Post-sorting data analysis and structural visualization were performed using FlowJo software (Tree Star).

Genomic DNA Extraction and Recombination Analysis

To verify the genomic recombination of the *Rosa26*-LSL-tdTomato allele in specific muscle-resident cell populations, FACS-sorted cells were subjected to genomic DNA (gDNA) extraction using a standard phenol-chloroform method. Briefly, the sorted cells were lysed and homogenized in 100 μL of Tris-EDTA (TE) buffer containing 1% SDS, followed by overnight incubation at 50°C. For complete protein digestion, Proteinase K was added to a final concentration of 0.1 μg/μL, and the lysates were incubated at 37°C for 1 hour. The samples were adjusted to a volume of 250 μL with TE buffer and extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, Invitrogen). Following centrifugation at 20,000 × *g* for 3 minutes at 4°C, the upper aqueous phase was carefully collected. The gDNA was precipitated by adding 0.1 volumes of sodium acetate (2.5 M, pH 5.2) and 2 volumes of absolute ethanol, followed by incubation at -80°C for at least 1 hour. The DNA pellets were recovered by centrifugation (20,000 × *g* for 15 minutes at 4°C), washed twice with 70% ethanol, completely air-dried, and resuspended in TE buffer at 60°C.

To detect the tdTomato recombination event, PCR was performed using the purified gDNA as a template. The specific primer sequences used for the amplification were as follows: CAG_tdTomato_F, 5'-GCAACGTGCTGGTTATTGTG-3' and CAG_tdTomato_R, 5'-CGCATGAACCTCTTTGATGACC-3'.

The PCR amplification was carried out using EmeraldAmp GT PCR Master Mix (Takara Bio) in a SimpliAmp Thermal Cycler (Applied Biosystems). The thermal cycling conditions consisted of an initial denaturation at 95°C for 5 minutes, followed by 33 cycles of denaturation at 95°C for 15 seconds, annealing at 56°C for 20 seconds, and extension at 72°C for 30 seconds. This was followed by a final extension step at 72°C for 7 minutes. The expected size of the recombined amplicon was exactly 280 bp. Finally, the PCR products were electrophoretically separated on a 1.5% agarose gel, stained with EcoDye nucleic acid staining solution (Biofact), and visualized using a Davinch Gel Imaging System (Davinch-K).

Whole-Mount Immunofluorescence

Tissue Preparation and Whole-Mount Staining

For the majority of histological analysis, the extensor digitorum longus (EDL) muscle was primarily used. For the comparative analysis across diverse muscle types (Figure 2C), additional skeletal muscles (masseter, sternomastoid, diaphragm, psoas, and soleus) were also harvested and processed in the same manner. All freshly isolated muscles were immediately fixed in 4% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature. Fixed muscles were quenched in 0.1M glycine in PBS for 1 hour at room temperature. After rinsing with PBS, muscle bundles were teased into smaller fibers using fine forceps under a dissecting microscope. The teased muscle fibers were incubated in a blocking buffer containing 5% bovine serum albumin and 2% Triton X-100 in PBS for 2 hours at room temperature. Tissues were then incubated with primary antibodies diluted in the blocking buffer for 3 days at 4°C. The primary antibodies used in this study include: rabbit anti-S100 (Ready-to-use formulation further diluted at 1:5; Agilent, IR50461-2) and mouse anti-neurofilament (1:1000; DSHB, 2H3). After washing three times with washing buffer (2% Triton X-100 in PBS) for 1 hour each, the tissues were incubated with a mixture of secondary antibodies and specific fluorescent probes diluted in blocking buffer for 3 days at 4°C. To visualize targeted proteins and postsynaptic acetylcholine receptors (AChRs), the following secondary antibodies and probes were used as dictated by the experimental design: Alexa Fluor 647-conjugated goat anti-rabbit IgG (1:500; Invitrogen, A32733), Alexa Fluor 405-conjugated goat anti-mouse IgG (1:500; Invitrogen, A31553), Alexa Fluor 488-conjugated α -bungarotoxin (α -Btx, 1:500; Invitrogen, B13422), and/or Hoechst 33342 (1:2000; Thermo Fisher, 62249). Following three 1-hour washes in washing buffer, the stained muscle fibers were whole-mounted on glass slides using Vectashield without DAPI (Vector Laboratories, H-1000).

Confocal Image Acquisition

Fluorescence images of the whole-mount stained muscles were acquired using a Leica TCS SP8 confocal laser scanning microscope equipped with 40 $\times$ and 63 $\times$ water-immersion objective lenses. Z-stack images were collected encompassing the entire depth of the motor endplate band. The laser power, gain, and pinhole settings were kept consistent across all biological replicates to ensure comparability. Image processing was performed using LAS X software (Leica Microsystems). Z-stack confocal images were collapsed into maximum intensity projections (MIPs). The structural integrity of the NMJs, including the degree of postsynaptic AChR cluster fragmentation and the status of motor axon innervation, was assessed via visual inspection of the MIPs. Multiple fields of view from at least three independent biological replicates were evaluated to determine the predominant morphological phenotypes.

Electrophysiology

The extensor digitorum longus (EDL) muscles, together with the attached peroneal nerve, were carefully dissected from mice and pinned to a Sylgard-coated recording chamber. Intracellular recordings were performed using 3 M KCl-filled glass microelectrodes submerged in oxygenated Ringer's solution (138.8 mM NaCl, 4 mM KCl, 12 mM NaHCO₃, 1 mM KH₂PO₄, 1 mM MgCl₂ and 2 mM CaCl₂ with a pH of 7.4). During the initial dissection process, Ca²⁺ free Ringer's solution was utilized to prevent muscle contraction and maintain structural stability. The evoked endplate potentials (eEPs) were elicited using bipolar stimulation of the peroneal nerve. Prior to recording, muscle action potentials were completely blocked by incubating the preparation with 2.5 μ M μ -

conotoxin GIIIB (Alomone, Jerusalem, Israel) for 10 minutes. Synaptic transmission efficacy was evaluated using paired-pulse stimulation (with 10, 20, and 50 ms inter-stimulus intervals) and high-frequency train stimulation (20 pulses at 20 Hz). Data were acquired and analyzed using an Axoclamp 900A amplifier, a Digidata 1550B digitizer, and Clampfit 10.7 software.

For compound muscle action potential (CMAP) measurement, Mice were fully anesthetized via an intraperitoneal injection of Avertin (250 mg/kg; Sigma-Aldrich). The sciatic nerve was percutaneously stimulated at the sciatic notch using an Isolated Pulse Stimulator Model 2100 (A-M Systems). Supramaximal square-wave stimuli (~70 mA) were delivered at a frequency of 1 Hz with a pulse duration of 0.1 ms. For signal acquisition, a recording electrode was carefully inserted subdermally over the belly of the GA muscle, ensuring that the underlying muscle fibers were not physically punctured. Reference and ground electrodes were positioned near the Achilles tendon and on the tail, respectively. Electromyographic signals were captured using an IX-RA-834 Data Recorder (iWorx). The CMAP amplitude was quantified by calculating the absolute voltage difference between the maximum positive and negative peaks. For robust statistical analysis, four consecutive CMAP traces were elicited from each GA muscle, and the mean amplitude of these measurements was utilized as the representative value for each animal.

Electron Microscopy

Tissue Processing and OTO Staining

EDL muscles were dissected from mice and immediately fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer overnight at 4°C. To enhance the electron contrast of lipid membranes and synaptic vesicles, the tissues were processed using an OTO (osmium-thiocarbohydrazide-osmium) staining protocol. Samples were post-fixed in a solution of 1% osmium tetroxide and 1.5% potassium ferrocyanide for 1 hour at room temperature. Then, samples were incubated in a 1% thiocarbohydrazide (TCH) solution for 20 minutes, followed by incubation in a 2% osmium tetroxide solution for 30 minutes at room temperature. Following *en bloc* staining with 0.5% uranyl acetate overnight at 4°C, samples were further stained with 20 mM lead aspartate in a 60°C oven, and subsequently dehydrated through a graded ethanol series (30%, 50%, 70%, 80%, 90%, and 100% three times, 20 minutes each at room temperature). Following dehydration, samples were cleared in 100% propylene oxide (PO) for 15 minutes twice. For resin infiltration, the samples were incubated in a 1:1 mixture of PO and Spurr's resin (Electron Microscopy Sciences) for 1.5 hours, followed by a 1:2 mixture of PO and Spurr's resin for an additional 1.5 hours. Finally, the samples were immersed in 100% pure Spurr's resin and incubated overnight at room temperature for complete infiltration. The 100% Spurr's resin was changed the next day, and the samples were incubated for an additional 3 hours. Finally, the samples were embedded in molds with fresh resin and polymerized in an oven at 70°C.

Ultramicrotome Section and BSE-SEM Imaging

Embedded resin blocks were trimmed, and ultrathin sections with a thickness of 70 nm were cut using an ultramicrotome (Leica EM UC7). The ultrathin sections were carefully collected onto conductive silicon wafers. High-resolution imaging of the presynaptic terminals was performed using a SIGMA 360 Scanning Electron Microscope (SEM; Carl Zeiss). Images were acquired utilizing a backscattered electron detector (BSD). The microscope was operated at an accelerating voltage of 3.0 kV with a working distance of 6.5 mm.

Quantitative Morphometric Analysis of the NMJ

Quantitative morphometric analysis of the neuromuscular junctions (NMJs) was performed using ImageJ/Fiji software (NIH) by an investigator blinded to the genotypes. For each analyzed NMJ, the cross-sectional area of the presynaptic motor terminal was delineated by manually tracing the presynaptic membrane in the SEM-BSD images. All clearly distinguishable synaptic vesicles within this defined presynaptic profile were counted. The synaptic vesicle density was calculated by dividing the total number of counted vesicles by the cross-sectional area of the presynaptic terminal, expressed as vesicles per square micrometer (vesicles/ μm^2). The synaptic cleft width was determined by measuring the distance between the pre- and post-synaptic membranes at 7 to 10

distinct, regularly spaced points per NMJ and averaging the values. The number of junctional folds was counted and normalized to the synaptic apposition length. In total, at least 8 NMJ profiles from at least 3 mice per experimental group were analyzed.

Behavioral Assays

General Behavioral Testing Procedures

All behavioral assays were performed during the light cycle, at a fixed time of the day (3 PM). To minimize stress, mice were transferred to the behavioral testing room and allowed to acclimatize for at least 30 minutes prior to the onset of any experiments. To ensure objectivity, all behavioral tests and subsequent data analyses were conducted by an investigator strictly blinded to the genotypes of the mice. Both male and female mice were used in the experiments, and no sex difference was observed in all behavioral tests.

Gait Analysis

To evaluate the walking pattern and locomotor coordination of 4-week-old mice, gait analysis was performed. The forepaws and hindpaws of the mice were coated with non-toxic ink of different colors. The mice were then allowed to walk along a paper-lined runway (50 cm long, 6 cm wide) into a dark goal box. From the recorded footprints, the stride length and stride width (base of support) were measured to assess motor coordination.

Beam Walking Test

Prior to the actual testing, 4-week-old mice underwent a pre-training phase to familiarize themselves with the apparatus. Mice were trained to cross an elevated cylindrical wooden beam (50 cm long, 3 cm diameter) elevated 30 cm above a padded surface, leading to an enclosed safety box. After completing three consecutive training trials, the mice were transitioned to a narrower testing beam (50 cm long, 1 cm diameter) for further adaptation. The actual test was conducted on the following day, using the testing beam. Each mouse was subjected to three independent trials, and the latency to successfully cross the beam was recorded for each trial. The values from the three individual trials were averaged to determine the representative crossing latency for each animal.

Grip Strength Test

Whole-body muscle strength was evaluated using a digital grip strength meter (BIO-GS3; Bioseb). 12-week-old mice were placed on the apparatus and allowed to firmly grasp the metal grid with all four paws. The base of the tail was gently and steadily pulled backward parallel to the grid until the animal released its grip. The maximum peak force exerted was recorded in Newtons (N). Each testing session consisted of five consecutive pulls. The three highest peak force values out of the five trials were averaged to determine the score for that session. Each animal underwent a total of three independent testing sessions, separated by a resting interval of at least 30 minutes. The overall absolute grip strength for each mouse was calculated by averaging the scores from these three sessions. The absolute peak force values were normalized to the individual body weight of each mouse to account for potential body size differences among the cohorts.

Rotarod Test

Motor coordination, balance, and physical endurance were assessed using an accelerating rotarod apparatus (LE8500, Panlab/Harvard Apparatus). Following a brief habituation phase to the testing room, mice were carefully placed on the rotating rod. The apparatus was programmed to accelerate linearly from an initial speed of 4 rpm to a maximum speed of 40 rpm over a 5-minute period. The trial was terminated when the mouse fell off the rod, and the latency to fall (in seconds) was automatically recorded. Each mouse was subjected to three independent trials with a minimum resting interval of 15 minutes. The final representative latency to fall for each mouse was determined by averaging the values obtained from the three trials.

Treadmill Endurance Running

Exercise capacity and endurance limits were assessed using a motorized rodent treadmill (DJ2-242 Dual Treadmill; Daejong Instrument) equipped with a mild electrical shock grid to provide running motivation. Prior to the actual testing, mice were acclimated to the treadmill apparatus for three consecutive days. Each daily training session consisted of a 5-minute stationary exploration period (0 m/min), followed by running at 5 m/min, 10 m/min, and 15 m/min for 5 minutes at each speed. On the actual test day, the treadmill was set at a 0° incline. The initial belt speed was set to 10 m/min and was progressively increased by 2 m/min every 20 minutes until the animals reached exhaustion. Exhaustion was strictly defined as the point at which a mouse remained on the shock grid for more than 10 consecutive seconds without attempting to resume running. Both the total running time and the total distance covered were recorded for each animal.

Statistical Analysis

Statistical analyses for the histological and behavioral assays were performed using GraphPad Prism software (version 9, GraphPad Software). For electrophysiological experiments, statistical analysis was performed using SPSS Statistics software (version 29.0.1.0; IBM). All quantitative data were visualized using GraphPad Prism version 9 and presented as the mean $\pm$ standard deviation (SD), except for the electrophysiological data, which were expressed as the mean $\pm$ standard error of the mean (SEM). Prior to statistical comparisons, the normality of the data distribution was assessed using the Shapiro-Wilk test, and the homogeneity of variances was evaluated using the F-test or Brown-Forsythe test. For comparisons between two independent experimental groups, a two-tailed unpaired Student's t-test was utilized for normally distributed data with equal variances. If the assumption of equal variances was violated, Welch's t-test was applied. If the data did not meet the assumption of normality, a nonparametric Mann-Whitney U test was applied. For comparisons involving three or more groups, a one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test was performed. For the analysis of train data involving repeated measurements, a repeated measures two-way ANOVA followed by Bonferroni's multiple comparisons test was performed. Statistical significance was defined as a *p*-value of less than 0.05. In all graphical representations, significance levels are denoted as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001.

Figure supplements

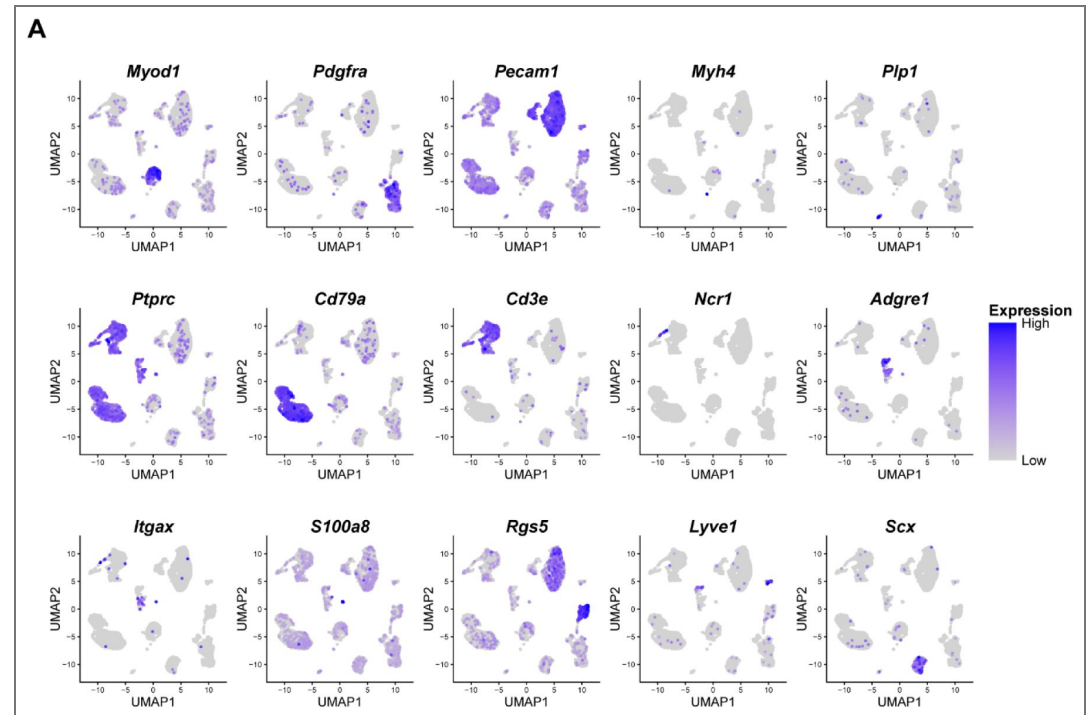


Figure 1—figure supplement 1. Expression profiling of canonical cell-type markers in the skeletal muscle-resident cell population. (A) UMAP feature plots showing the distribution and expression levels of established marker genes for diverse cell types within the whole skeletal muscle scRNA-seq dataset.

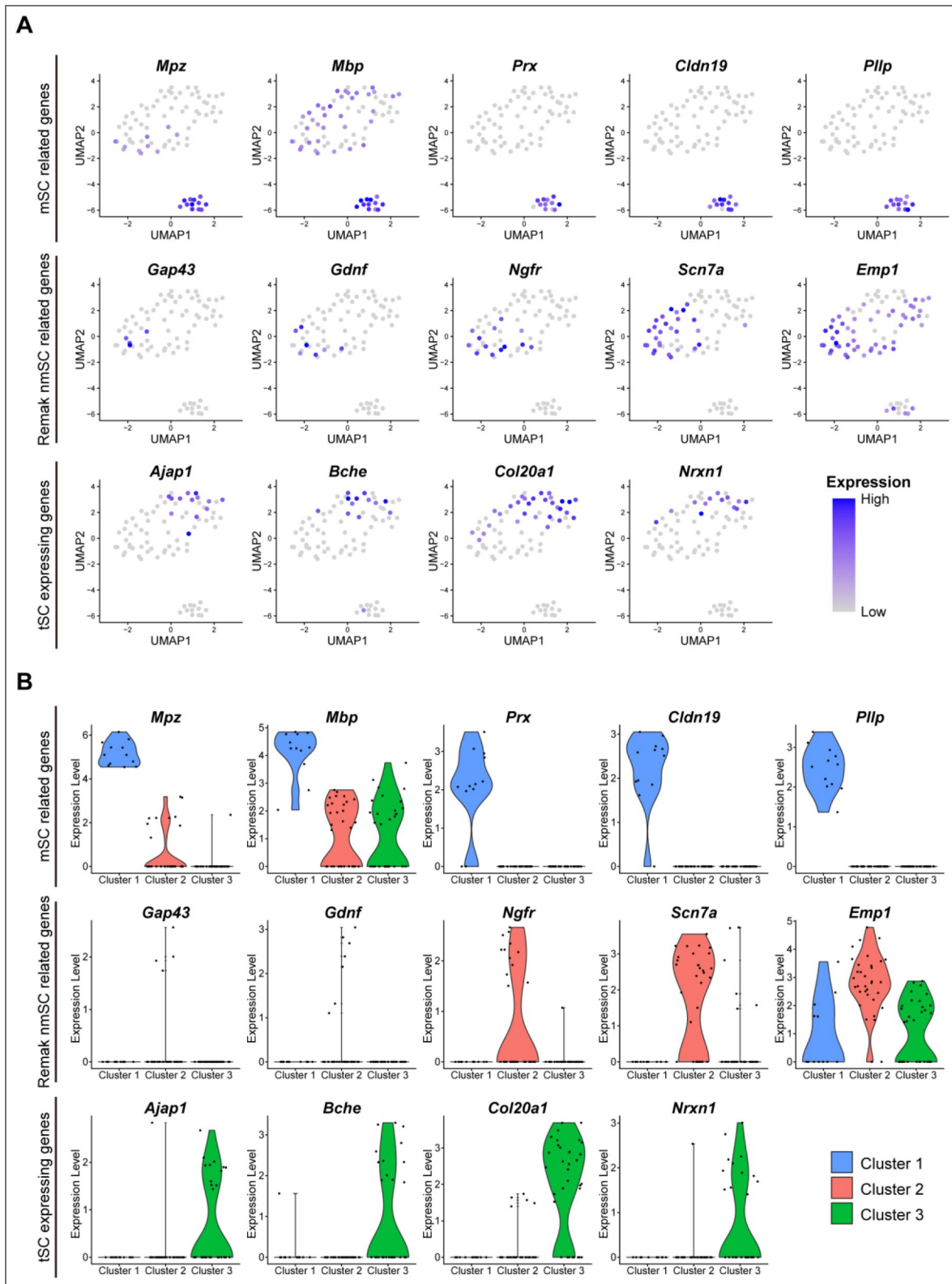


Figure 1—figure supplement 2. Validation of Schwann cell sub-cluster identities using established subtype-specific marker genes.

(A) UMAP feature plots and (B) Violin plots illustrating the distinct expression patterns of known Schwann cell subtype markers across the three isolated sub-clusters.

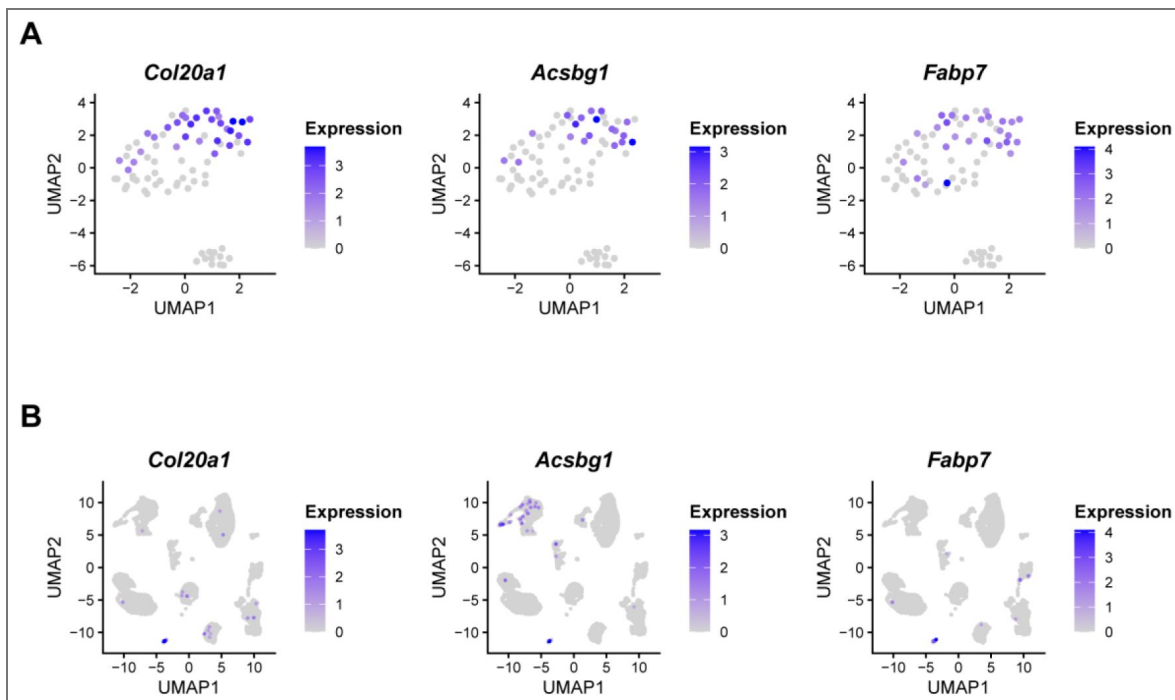


Figure 1—figure supplement 3. Highly restricted expression of the three final candidate genes within the tSC cluster.

UMAP feature plots illustrating the localized expression patterns of the three selected candidate genes (*Col20a1*, *Acsbg1*, and *Fabp7*). The plots highlight their highly specific enrichment in the tSC population when mapped across (A) the isolated Schwann cell sub-clusters and (B) the entire skeletal muscle-resident cell population.

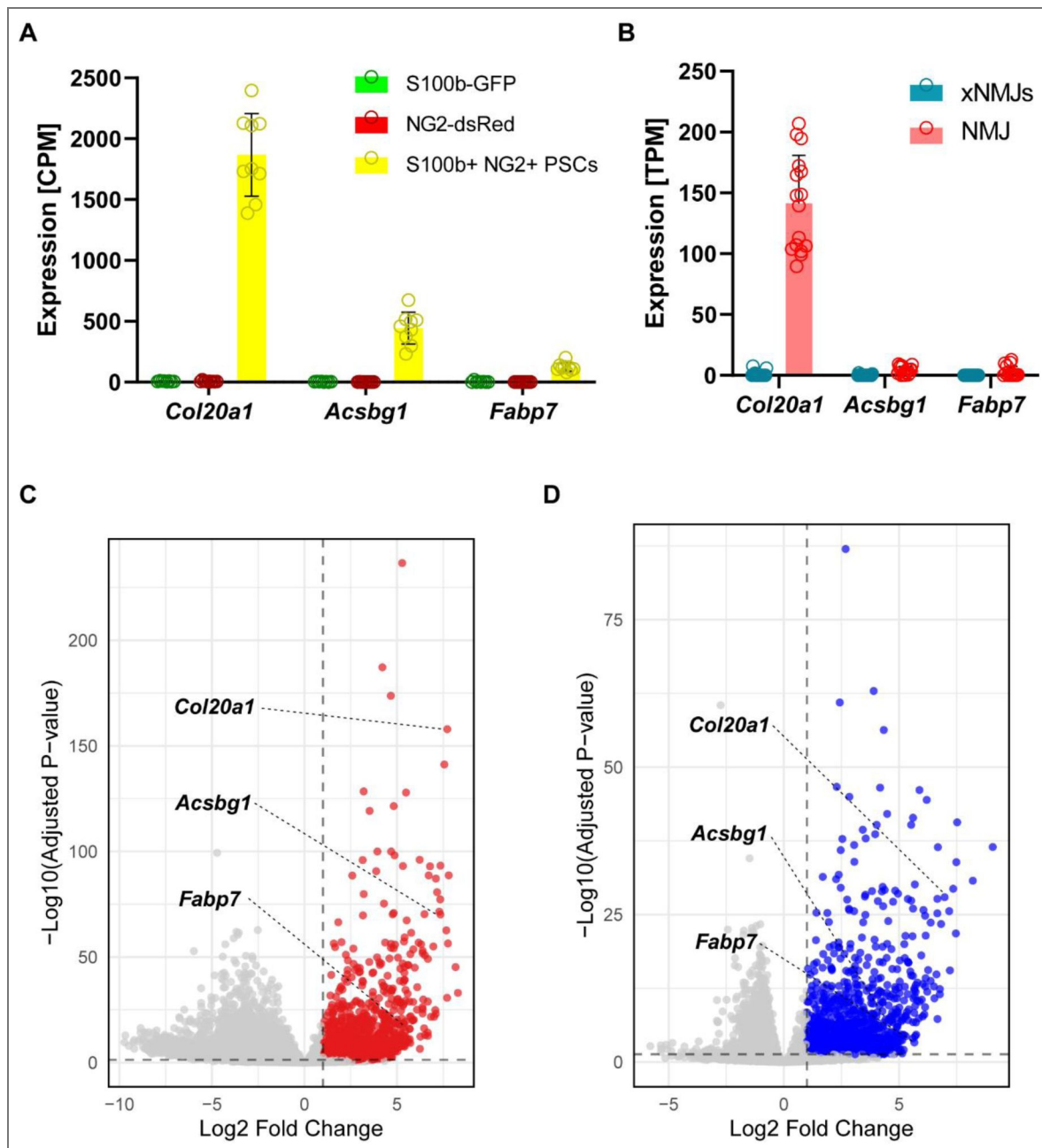


Figure 1—figure supplement 4. Independent bulk RNA-seq analyses confirm *Col20a1* as the most robust tSC-specific marker.

Re-analysis of publicly available bulk RNA-seq datasets validates the enrichment of the candidate genes in tSCs. (A) Bar graphs showing expression levels (CPM) of the three candidate genes, *Col20a1*, *Acsbg1*, and *Fabp7*, in FACS-sorted S100b-GFP/NG2-dsRed double-positive tSCs compared to S100b-GFP or NG2-dsRed single positive cells (Castro et al., 2020). (B) Bar graphs showing expression levels (TPM) of the three candidate genes in micro-dissected NMJ regions (NMJ) compared to non-NMJ regions (xNMJ) (Ham et al., 2020). (C, D) Volcano plots indicating the statistical significance ($-\text{Log}_{10}$ adjusted p -value) and Log_2FC of the candidate genes derived from the datasets in (A) and (B), respectively. For dataset (A), the volcano plot in (C) displays the differential expression between double-positive tSCs and the combined single-positive cell populations. *Col20a1* exhibits the highest expression level and the most significant fold-change among the candidates, prioritizing it for the genetic model generation.

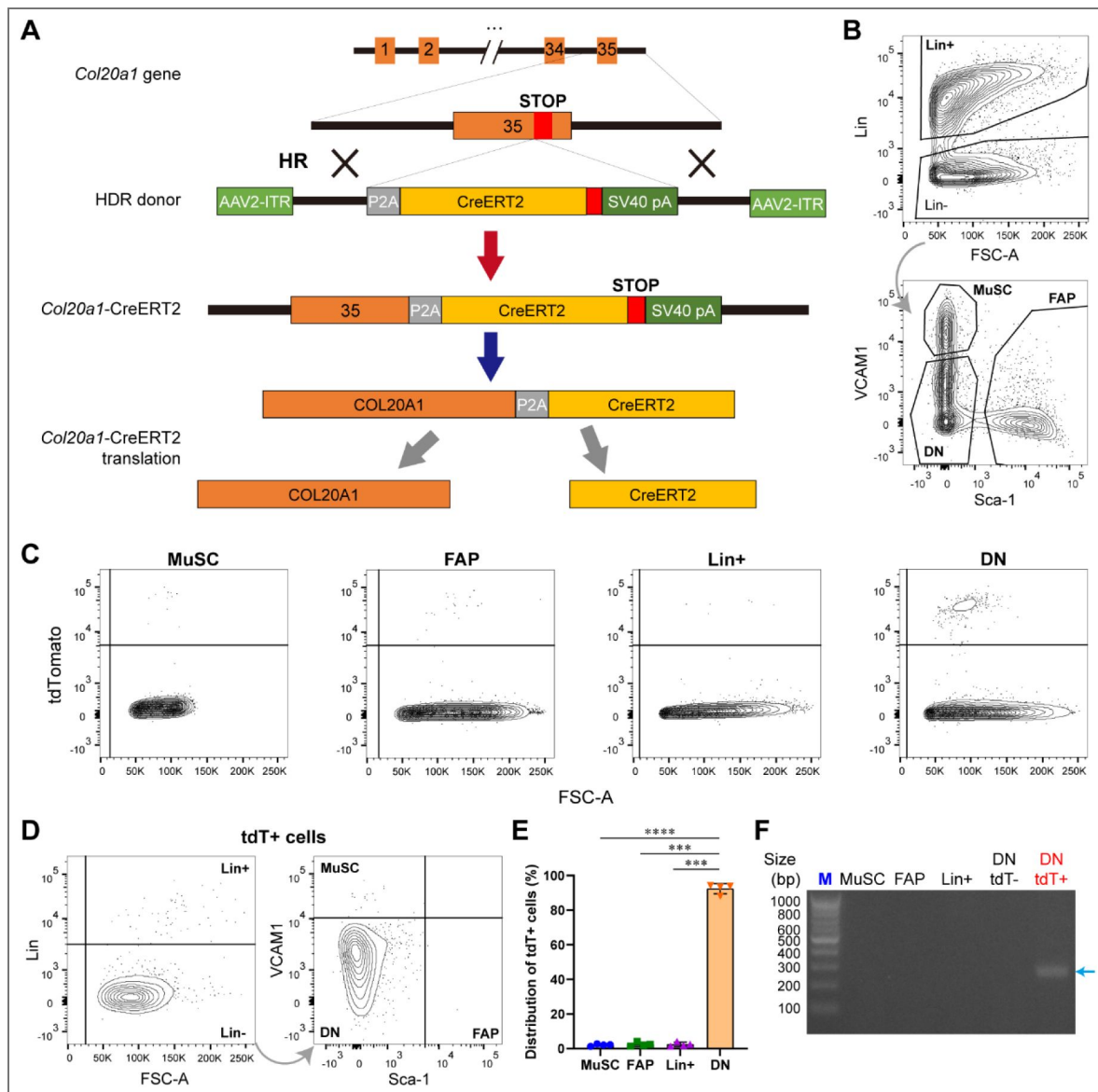


Figure 2—figure supplement 1. Generation strategy and quantitative flow cytometry validation of the *Col20a1*-CreERT2 mouse model.

(A) Schematic diagram illustrating the design of the *Col20a1*-CreERT2 knock-in allele. A P2A-CreERT2 cassette was inserted at the C-terminus of the endogenous *Col20a1* gene via homologous recombination (HR). (B) Flow cytometry gating strategy for isolating mononuclear cells from whole skeletal muscle. Cells were separated into Lineage-positive (Lin⁺; CD31⁺, CD45⁺ or Ter119⁺), muscle stem cells (MuSC; Lin⁻/VCAM1⁺/Sca-1⁺), fibro-adipogenic progenitors (FAP; Lin⁻/VCAM1⁺/Sca-1⁺), and double-negative cells (DN; Lin⁻/VCAM1⁻/Sca-1⁻), the latter of which harbors the Schwann cell population. (C) Representative flow cytometry plots displaying tdTomato fluorescence across the four isolated cell populations. (D, E) Flow cytometry analysis (D) and quantification (E) of the tdTomato-positive (tdT⁺) cell distribution. The vast majority of tdT⁺ cells (~92%) are highly restricted to the DN population. Data are presented as mean $\pm$ SD (n = 4). Statistical significance was determined using Repeated Measures ANOVA with Bonferroni's post hoc test; ***p < 0.001, ****p < 0.0001. (F) PCR validation of genomic DNA extracted from the sorted cell populations. The recombinant tdTomato amplicon (blue arrow) is exclusively detected in the tdT⁺ fraction of the DN population. M: DNA size marker.

Data availability

The publicly available transcriptomic datasets analyzed in this study can be accessed in the NCBI Gene Expression Omnibus (GEO) under accession numbers GSE110878, GSE152774, GSE139209, and the CZ CELLxGENE Discover platform. All other data generated or analyzed during this study (including behavioral, electrophysiological, and morphological data) are included in the manuscript and its supporting files. Source data files will be provided for all quantitative figures upon request or prior to publication.

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Additional information

Author Contributions

H.K., K.Y., and Y.-Y.K. designed research; K.Y. discovered the marker gene; H.K. performed *in vivo* experiments, morphological, and behavioral research; S.-Y.K. performed electrophysiological research; S.-Y.C. contributed new reagents/analytic tools; H.K., S.-Y.K., K.Y., S.-Y.C., and Y.-Y.K. analyzed data; and H.K. and Y.-Y.K. wrote the paper.

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Peer reviews

Reviewer #1 (Public review):

Summary:

This manuscript's major strength is the identification of Col20a1 as a novel marker for terminal Schwann cells (tSCs) and the generation of the Col20a1-CreERT2 knock-in mouse line, which represents a valuable new genetic tool for studying tSC biology at the neuromuscular junction. However, the central conclusion that terminal Schwann cells regulate presynaptic vesicle homeostasis is not sufficiently supported because the electrophysiological and ultrastructural analyses are based on limited sample sizes. Increasing the number of animals and NMJs analyzed would substantially strengthen the conclusions. In addition, further validation of Col20a1 expression using RNAscope or immunostaining, together with comparisons to established tSC markers such as Kir4.1 and NG2, would help establish its specificity. Finally, the developmental appearance of Col20a1-positive axonal Schwann cells is intriguing and warrants further investigation to determine whether these cells migrate and differentiate into terminal Schwann cells during postnatal development.

Strengths:

The authors identified Col20a1 as a specific marker of terminal Schwann cells (tSCs) at the neuromuscular junction (NMJ) in mice and generated a Col20a1-CreERT2 knock-in mouse line for in vivo labeling of tSCs. This represents a novel and significant technical advance for the NMJ field, providing a valuable genetic tool for studying the development, maintenance, and function of terminal Schwann cells in vivo.

Weaknesses:

The major weakness of this manuscript is that the sample sizes are too small to support the authors' conclusion that "Terminal Schwann Cells Regulate Presynaptic Vesicle Homeostasis but Not Neuromuscular Junction Integrity in Mice."

Although the authors state that "Quantification of the tdTomato-positive NMJ ratio showed an ablation efficiency of approximately 80%, with only ~20% of NMJs retaining escaper tSCs" (page 9), they do not provide the sample size or sufficient quantitative information for the Col20a1-tdTomato/DTA ablation experiment shown in Figure 4. This information is essential for evaluating the robustness and reproducibility of the ablation strategy.

The electrophysiological analyses are also based on very limited sample sizes. According to Figure 6, mEPP recordings were obtained from 10 NMJs from 3 control mice and 14 NMJs from 5 tSC-ablated mice. The EPP recordings were based on similarly small numbers (control, $n = 10$ NMJs from 3 mice; tSC-ablated, $n = 14$ NMJs from 5 mice). Thus, only approximately 2-3 NMJs were analyzed per tSC-ablated mouse on average. Given the inherent variability among individual NMJs and animals, these sample sizes are insufficient to support broad conclusions regarding the effects of terminal Schwann cell ablation on synaptic transmission.

In addition, the authors describe the phenotype as "leaky" presynaptic spontaneous release, but this terminology is not defined and lacks mechanistic explanation. It is therefore unclear what specific physiological alteration the authors intend to describe.

The sample sizes for the electron microscopy analyses (Figure 7) also appear to be limited, making it difficult to determine whether the reported changes in synaptic vesicle distribution are representative or statistically robust. Because the central conclusion relies heavily on these electrophysiological and ultrastructural data, the evidence presented is not sufficient to support the claim that terminal Schwann cells regulate presynaptic vesicle homeostasis. At present, this conclusion is overly broad and not adequately supported by the available data.

Minor comments:

(1) While several figures contain high-quality NMJ images (e.g., Figures 1B and 4), the image quality in other figures should be improved. For example, the S100B immunostaining appears overexposed in some panels, making it difficult to distinguish individual Schwann cells or visualize the boundaries between adjacent cells.

(2) Some neuromuscular junctions shown in Figure 2C appear to be partially denervated. The authors should clarify whether these represent normal variability, effects of the experimental manipulation, or imaging artifacts.

(3) The authors should specify the muscle preparation used in Figure 3, as this information is necessary for interpreting the results and comparing them with previous studies.

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Reviewer #2 (Public review):

Summary:

Two types of Schwann cells (SCs) ensheath motor axons - myelinating SCs along the axonal length and terminal SCs (tSCs) that cover nerve terminals at the neuromuscular junction (NMJ). Therefore, the NMJ is, like other synapses, tripartite, with specialized presynaptic, postsynaptic, and glial cells. Many studies have shown that tSCs play roles in the development and function of the NMJ, but for some of these, interpretation is difficult because it is hard to manipulate tSCs without also manipulating myelinating SCs. To circumvent this problem, Kong et al. make use of a gene selectively expressed in tSCs, Col20a1 (Figure 1), to generate a knock-in mouse line, Col20a1-CreER, that gives them genetic access to tSCs. They cross this to Cre-dependent lines that mark tSCs with a red fluorescent protein (Figures 2 and 3) or ablate them by expression of diphtheria toxin along with the fluorescent protein (Figure 4). They show that ablation at postnatal day (P) 10 does not affect the overall structure or function of

the NMJ (Figures 4 and 5). It does, however, affect some aspects of neuromuscular transmission over the following few weeks (Figures 6 and 7). Long-term effects cannot be studied by this method, however, because terminal SCs are replaced, presumably from the preterminal population (Figure 8).

Strengths:

The work is done to a high technical standard, including detailed characterization of the knock-in model. Results are presented clearly and illustrated beautifully. The finding that some early reports of synaptic alterations may result from concurrent loss of axonal SCs is important in rethinking the role of tSCs.

Weaknesses:

(1) The authors claim that tSCs are dispensable for some aspects of NMJ maturation, including synapse elimination (called pruning here), formation of "pretzel-like" postsynaptic topology, and generation of junctional folds in the postsynaptic membrane (lines 223 and 363). However, this conclusion is based on injection of tamoxifen to initiate tSC ablation at P10, which is necessary because Col20a1 is expressed in some preterminal SCs at earlier times. It presumably takes a few days for CreER to translocate to the nucleus and activate the toxin transgene, and some more time for the toxin to be generated and act. This is problematic because synapse elimination and other aspects of maturation mentioned occur during the first two postnatal weeks and are largely complete by P14. Therefore, one cannot conclude that these aspects "proceeded normally despite the loss of tSCs....".

(2) Effects on synaptic transmission are modest at best, being significant at a level of $p < 0.05$ but not $p < 0.01$ (Figure 6E, G, H and most of L). Effects on vesicle density are more robust (Figure 7).

(3) The authors use red fluorescent protein from the Col20a1 to label tSCs, and antibodies to S100b to label all SCs. This is appropriate in normal muscle and soon after tSC ablation. At later times, however, the NMJ is repopulated by S100+ Col20a1- SCs (Figure 8B). It is therefore important to show when this repopulation begins, because a modest recovery of SC coverage could have a big effect. For example, Figure 4C quantifies loss of NMJs with residual RFP+ cells but not S110+ cells; both should be quantified at this and slightly later stages.

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Reviewer #3 (Public review):

Summary:

This manuscript reports a novel genetic model, Col20a1-CreERT2 knock-in mouse, to target terminal Schwann cells (tSCs) at mouse neuromuscular junctions in a cell-type-specific and temporal manner. The authors analyzed multiple publicly available single-cell transcriptome databases to identify Col20a1 as the tSC marker. The authors crossed Col20a1-CreERT2 and Rosa26-LSL-tdTomato to label tSCs successfully. In addition, the authors generated Col20a1-CreERT2; Rosa-tdT/ diphtheria toxin subunit A (DTA) to specifically ablate tSCs and analyze the role of tSCs in motor behavior, neuromuscular junction electrophysiological function, and the histology and ultrastructure of neuromuscular junctions.

Strengths:

The Col20a1-CreERT2 x Rosa26-LSL-tdTomato mice successfully labeled the tSCs at NMJs and reported the developmental distribution of the Col20a1-positive cell population. The Col20a1-CreERT2; Rosa-tdT/DTA mice successfully ablated tSCs, which did not cause changes in gross neuromuscular junction architecture, neuromuscular synapse physiology, or motor behavior.

Weaknesses:

The conclusion of this manuscript will be strengthened by additional analysis showing time-course data of tSC ablation and replacement by non-recombined Schwann Cells. Currently, it is not clear when and how long the tSCs are ablated, which makes it difficult to interpret the data and phenotype. Detailed review comments are provided to the authors in the "recommendations for the authors" section.

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