The histone code reader Spin1 controls skeletal muscle development

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While several studies correlated increased expression of the histone code reader Spin1 with tumor formation or growth, little is known about physiological functions of the protein. We generated Spin1⁴⁵ mice with ablation of Spin1 in myoblast precursors using the Myf5-Cre deleter strain. Most Spin1⁴⁵ mice die shortly after birth displaying severe sarcomere disorganization and necrosis. Surviving Spin1⁴⁵ mice are growth-retarded and exhibit the most prominent defects in soleus, tibialis anterior, and diaphragm muscle. Transcriptome analyses of limb muscle at embryonic day (E) 15.5, E16.5, and at three weeks of age provided evidence for aberrant fetal myogenesis and identified deregulated skeletal muscle (SkM) functional networks. Determination of genome-wide chromatin occupancy in primary myoblast revealed direct Spin1 target genes and suggested that deregulated basic helix-loop-helix transcription factor networks account for developmental defects in Spin1⁴⁵ fetuses. Furthermore, correlating histological and transcriptome analyses, we show that aberrant expression of titin-associated proteins, abnormal glycolgen metabolism, and neuromuscular junction defects contribute to SkM pathology in Spin1⁴⁵ mice. Together, we describe the first example of a histone code reader controlling SkM development in mice, which hints at Spin1 as a potential player in human SkM disease.

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SkM fiber formation in mice comprises three successive phases, an embryonic wave from around embryonic day (E) 10.5 to E12.5, a fetal wave from around E14.5 to E17.5, and a postnatal period during which adult fibers are established.¹⁴,¹⁶-¹⁸ Adult myofibers exhibit distinct contractile properties (slow- or fast-twitch), patterns of innervation, and metabolic activities (oxidative or glycolytic), which correlate with the expression of specific myosin heavy chain (MHC) isoforms.¹⁹,²⁰ Limb muscle of adult mice is composed of type I (slow, oxidative), type IIa (fast, oxidative), type IIX (fast, glycolytic), and type IIb (fast, glycolytic) fibers.¹⁹,²⁰ SkM mass and functions become compromised in disease and numerous gene mutations causing myopathies or muscular dystrophies have been documented.²¹-²⁸ Interestingly, selected fiber or muscle types preferentially degenerate in certain disease states.²⁹,³⁰ In this study, we crossed mice harboring conditional Spin1 alleles (Spin¹⁵⁶) with the Myf5-Cre deleter strain to ablate Spin1 in myogenic precursors. Most homozygous Spin¹⁵⁶ Myf5-Cre mice (hereafter termed Spin¹⁵⁶) die shortly after birth, while surviving mice display severe growth retardation. Histological, transcriptome, and cistrome analyses provide evidence for aberrant fetal myogenesis and deregulated basic bHLH transcription factor networks around the onset of SkM defects. Furthermore, our observations suggest that altered expression of titin-associated proteins, aberrant glycogen...
Spin1 controls skeletal muscle development

H Greschik et al.

Cell Death and Disease
metabolism, and defective NMJs contribute to SkM pathology in Spin1M5 mice. In summary, our data reveal a severe developmental defect caused by ablation of a histone code reader in SkM and hint at Spin1 as a potential player in human SkM disease.

Results

Loss of Spin1 in SkM results in postnatal lethality. To investigate physiological functions of Spin1 in vivo, we generated ubiquitous knockout (Spin1R26) mice by crossing Spin1R26 mice with the Rosa26-Cre deleter strain (Supplementary Figure 1a). Spin1R26 mice were born, but died within one day after birth (Supplementary Figure 1b), which is in agreement with observations by others.12 Of note, at E18.5 Spin1R26 mice displayed dropping forelimbs (Figure 1a) indicating a neuromuscular defect.33

To address potential functions of Spin1 in SkM, we analyzed Spin1 protein expression in hind limb sections of control mice at E15 and after birth (P0) by immunofluorescence. At both time points, we observed intense Spin1 staining in Pax7-positive myoblast precursors (Figure 1b (arrowheads)) and weaker staining in nuclei of myofibers (Figure 1c (arrowheads)). Of note, in newborn mice, Spin1 expression was undetectable in some nuclei of myofibers (Figure 1c, bottom row (arrows)).

Next, we deleted Spin1 in myoblast precursors by crossing Spin1R26 mice with the Myf5-Cre deleter strain resulting in Spin1M5 mice. Immunostaining confirmed the absence of Spin1 protein in nuclei of Pax7-positive myoblast precursors (Figure 1d (arrowheads)); Supplementary Figure 1e (Supplementary Figure 1d) and myofibers of Spin1M5 fetuses (Figure 1e (arrowheads)); Supplementary Figure 1d). Remaining Spin1 staining is due to expression in non-mygogenic cells such as Tcf4-positive fibroblasts (Supplementary Figure 1e (arrowheads)); see Materials and Methods and Supplementary Figure 1f, g for further characterization).

Homozygous Spin1M5 mice were obtained at the expected Mendelian ratio at birth (Supplementary Figure 1h). However, about 80% of Spin1M5 mice died within one day after birth. Newborn Spin1M5 mice could typically be distinguished from control littermates by an abnormal posture and the absence of milk in the stomach (Figure 1f). Moreover, at E16.5, we observed dropping forelimbs for Spin1M5 fetuses (Figure 1g). Together, our data show that ablation of Spin1 in SkM causes early postnatal death of the majority of mice.

SkM of Spin1M5 mice is characterized by necrosis and structural defects in non-necrotic fibers. To characterize SkM defects in Spin1M5 mice, we inspected hematoxylin & eosin (H&E)-stained hind limb sections at different stages of development. Compared with control littermates, we observed in newborn Spin1M5 mice loss of fibers (Figure 2a, top row (black asterisks)) and numerous immature or degenerating fibers lacking contractile material (dashed circles). In Spin1M5 fetuses at E16.5, we also noted fibers with irregular H&E staining (Figure 2a, middle row (dashed circles)) and at E15 enlarged fibers, which were less abundant in control samples (Figure 2a, bottom row (white asterisks)).

Electron microscopy analyses of hind limb sections revealed for newborn Spin1M5 mice degenerating, necrotic fibers (Figure 2b, columns I–II (demarcated by dashed lines)), defective mitochondria (Figure 2b, column III (arrows)), and abnormal glycogen accumulation (Figure 2b, column IV (asterisks)). Similar defects were detected for Spin1M5 fetuses at E16.5 (Supplementary Figure 2). Furthermore, in non-necrotic fibers of Spin1M5 fetuses, we observed structural defects including a low density of contractile material and the lack of a clear M-line (Supplementary Figure 2, column III (triangles)). Together, our H&E and electron microscopy analyses uncovered necrotic and structurally defective fibers in Spin1M5 mice. In addition, the data suggested an onset of SkM defects before E16.5.

Transcriptome and histological analyses provide evidence for aberrant fetal myogenesis in Spin1M5 mice. To investigate alterations of the transcriptome in SkM of Spin1M5 fetuses, we performed RNA sequencing (RNA-seq) analyses using RNA isolated from limb muscle. At E15.5, we observed only 17 differentially expressed genes (DEGs) (P ≤ 1e–3; fold change ≥ 1.5) (Figure 3a; Supplementary Table 1a). In comparison, at E16.5 RNA-seq detected 193 DEGs, of which seven (Ankrd1, Ankrd2, Pmaip1, Scn4b (upregulated); Myf5, Msc, Nos1 (downregulated)) overlapped with the E15.5 DEGs (Figure 3a; Supplementary Table 1b). The strong increase in DEGs from E15.5 to E16.5 provided evidence for E15.5 as the approximate onset of SkM defects in Spin1M5 fetuses.

The common E15.5/E16.5 DEGs Ankrd1 (CARP) and Ankrd2 (ARPP) encode titin-associated regulators of sarcomere function, whose expression is often deregulated in SkM disease.35 We therefore investigated Ankrd1 and Ankrd2 protein levels in hind limb sections of E16.5 fetuses by immunofluorescence (Figures 3b and c). Most prominently, in control fetuses, expression of both proteins was restricted to the inner (prospective oxidative) part of the tibialis anterior (TA) neighboring the tibia (Figure 3c (demarcated by dashed lines)), whereas in Spin1M5 fetuses staining was also present in the outer (prospective glycolytic) part of the TA (Figure 3c (asterisks)). In comparison, at E15 Ankrd1 and Ankrd2 staining of hind limb muscle of Spin1M5 and control mice was similar (Supplementary Figures 3a and b). Thus,
deregulation of Ankrd1 and Ankrd2 transcripts at E15.5 and E16.5 and aberrant protein expression at E16.5 are early markers of SkM defects in Spin1M5 fetuses.

To analyze which proportion of developmentally regulated genes is affected by the absence of Spin1, we compared the E16.5 DEGs with gene expression changes in control fetuses from E15.5 to E16.5. In control fetuses, we observed 2042 genes differentially expressed between E15.5 and E16.5 (Figure 3d; Supplementary Table 1c). Phenotype and pathway analyses for these 2042 DEGs using WebGestalt confirmed relevance of the identified genes for SkM development or function (Supplementary Figures 3c and d). Intersection with the 193 E16.5 DEGs revealed an overlap of 125 genes (Figure 3d). Therefore, the absence of Spin1 prevents adequate expression of a subset of genes regulated during the fetal phase of myogenesis in control mice.

To corroborate aberrant fetal myogenesis in Spin1M5 mice, we compared the E16.5 DEGs with transcripts previously observed to be differentially expressed in embryonic or fetal myotubes. Of the 27 transcripts more highly expressed in

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**Figure 2**  SkM of Spin1M5 mice is characterized by necrosis and structural defects in non-necrotic fibers. (a) Hematoxylin and eosin (H&E) staining of transversal tibialis anterior sections of Spin1M5 and control mice at P0, E16.5, and E15. Fiber loss in Spin1M5 mice is indicated by black asterisks (top row), fibers with irregular H&E staining are encircled, and unusually large fibers more frequently observed in Spin1M5 than in control fetuses at E15 are marked with white asterisks (bottom row). (b) Electron microscopy (EM) images of SkM samples of newborn (P0) Spin1M5 and control mice. Dashed lines demarcate degenerating, necrotic fibers (I–II, bottom), arrowheads mark normal mitochondria (III, top), arrows point at defective mitochondria (III, bottom), and asterisks indicate abnormal glycogen accumulation (IV, bottom).
embryonic (compared with fetal) myotubes, only one (Tnnc1) overlapped with the E16.5 DEGs (Figure 3e). Importantly, eight out of thirteen transcripts more highly expressed in fetal myotubes (Myh8, Tnni2, Tnnt3, Atp2a1, Casq1, Pvalb, Ckm, and Eno3) were downregulated DEGs in Spin1M5 mice at E16.5 (Figure 3e). Together, our data provided evidence for an impaired progression of fetal myogenesis in Spin1M5 mice.

Identification of deregulated SkM functional networks in Spin1M5 fetuses. To identify genes accounting for SkM defects in Spin1M5 fetuses, we performed phenotype and pathway analyses for the E16.5 DEGs using WebGestalt36 (Figures 4a and b). These analyses revealed terms related to SkM function, metabolism, and pathology. We grouped the genes associated with the phenotype and pathway terms according to functional networks previously proposed in systems biology analyses for SkM38,39 (Figure 4c). The deregulated SkM networks comprise genes encoding transcription factors and signaling molecules, proteins important for sarcomere function, titin (Tnn)-associated factors, metabolic enzymes, and proteins involved in the neuromuscular junction (NMJ) and excitation-contraction coupling (ECC).
**Spin1 controls skeletal muscle development**  
H Greschik et al

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**a** Phenotype analysis for set of 193 DEGs at E16.5
- Abnormal muscle physiology n=28
- Abnormal muscle contractility n=15
- Abnormal muscle morphology n=21
- Muscle fatigue n=4
- Increased triglyceride level n=10

**b** Pathway analysis for set of 193 DEGs at E16.5
- Metabolic pathways n=21
- Insulin signaling pathway n=8
- Adipocytokine signaling pathway n=6
- Calcium signaling pathway n=8
- Staphylococcus aureus infection n=5

**c** Transcription & selected E16.5 DEGs

| Transcription & signaling | Selected E16.5 DEGs | Fatty acid & lipid |
|--------------------------|---------------------|-----------------|
| **Glucose & glycogen**   |                     |                 |
| Eno3 (1.5x)              | Actn3 (1.5x)         | Atp2a1 (1.6x)   |
| Fbp2 (1.6x)              | Mybph (1.7x)         | Casq1 (1.6x)    |
| Hk2 (1.7x)               | Myoz1 (1.7x)         | Clcn1 (1.8x)    |
| Phkg1 (2.3x)             | Mysbp2 (3.3x)        | Kcnq1 (1.7x)    |
| Ppp3r1a (1.7x)           | Myoz3 (1.3x)         | Pvalb (3.0x)    |
| Pygm (1.7x)              | Tnmd4 (1.6x)         | Ryr3 (1.7x)     |
| Slc2a4 (1.6x)            | Tnnc1 (1.7x)         | Scn4b (2.0x)    |
| **Sarcomere**            | Tnni2 (1.5x)         | Syp1d2 (1.7x)   |
| **Tn-associated**        | Tnn2 (1.5x)          | Cyp4b1 (1.9x)   |
| **NMJ**                  | Tnt2 (1.5x)          | Crat (1.7x)     |
| **ECM**                  | Tnt3 (1.5x)          | Lpin1 (1.7x)    |
| **ECC**                  |                     | Lpin4 (4.2x)    |
| **ECM**                  |                     | Lpin5 (2.5x)    |

**Figure 4** Identification of deregulated SkM functional networks in Spin1<sup>MS</sup> fetuses. (a,b) Phenotype (a) and pathway (b) analysis for the set of 193 DEGs observed in SkM of Spin1<sup>MS</sup> fetuses at E16.5. The number of genes in each category is indicated. For the phenotype analysis, the top 5 non-redundant terms are depicted. (c) Assignment of selected E16.5 DEGs identified by phenotype and pathway analyses to functional SkM networks. Upregulated DEGs are depicted in red, downregulated in blue color (fold change in brackets).

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Deregulated basic helix-loop-helix transcription factor networks account for SkM defects in Spin1<sup>MS</sup> fetuses. In addition to genes required for SkM structure and function, we found the myogenic bHLH transcription factor-encoding genes *Myf5, Myog, Msc* (musculin, MyoR), and *Hes6* among the E16.5 DEGs (Figure 4c (network ‘transcription and signaling’)). Therefore, deregulated bHLH transcription factor-controlled gene expression may be an early event accounting for aberrant myogenesis in Spin1<sup>MS</sup> fetuses. In support of this idea, *Myf5* and *Msc* were also among the E15.5 DEGs (Supplementary Table 1a).

To correlate gene expression with Spin1 chromatin occupancy, we performed chromatin immunoprecipitation followed by massive parallel sequencing (ChiP-seq) using primary myoblasts. ChiP-seq with Spin1-specific antibody revealed 17106 peaks, of which the majority (61.2%) was located at the promoters (transcription start site (TSS) ± 1 kb) of 12438 genes (Figure 5a; Supplementary Figure 4a). Next, ChiP-seq analysis for H3K4me3 detected 37% of the peaks at the promoters of 14832 genes (Figure 5a; Supplementary Figure 4a). Further analyses confirmed the presence of H3K4me3 at almost all gene promoters occupied by Spin1 (Figure 5a) as well as overlapping intensity profiles (Figure 5b). Thus, in primary myoblasts Spin1 and H3K4me3 occupy a large portion of cellular gene promoters.

Despite occupying thousands of gene promoters, Spin1 apparently only regulates a subset of target genes during fetal myogenesis. To further investigate Spin1-mediated gene regulation, we intersected the E16.5 DEGs with the cistrome observed in primary myoblasts. This analysis revealed the presence of Spin1 and H3K4me3 at the promoter of 88 E16.5 DEGs (Figure 5a). A transcription factor target analysis for these 88 genes using WebGestalt<sup>36</sup> revealed a significant enrichment of potential Myod1 target genes followed by E12 (Tcf3) and Mef2 targets (Figure 5c).

To validate Myod1 promoter occupancy of Spin1 target genes, we analyzed a previously deposited Myod1 ChiP-seq data set for C2C12 myoblasts (GEO data set GSE36024), which uncovered Myod1 peaks at the promoters of 14612 genes (Figure 5d). Intersection with Spin1-occupied genes showed a large overlap of 11695 direct targets of Spin1 and Myod1 (Figure 5d). Comparison with the E16.5 DEGs identified 82 direct Spin1/Myod1 targets (Figure 5d). Inspection of ChiP-seq tracks confirmed the presence of Spin1, H3K4me3, and Myod1 at promoters of SkM functional, metabolic, and regulatory genes (Supplementary Figure 4b) as well as the promoters of *Myf5, Myod1, Myog, Msc*, and *Hes6* (Figure 5e). Together, our data suggest that deregulated bHLH transcription factors affect Spin1/Myod1-dependent target gene transcription thereby accounting, at least in part, for SkM defects in Spin1<sup>MS</sup> fetuses.

**Surviving Spin1<sup>MS</sup> mice exhibit major defects in soleus, tibialis anterior, and diaphragm.** While the majority of Spin1<sup>MS</sup> mice died shortly after birth, about 20% survived and reached adulthood. We next investigated the consequences of aberrant fetal myogenesis for SkM in surviving Spin1<sup>MS</sup> mice between 15 and 30 weeks of age, the latter representing the oldest cohort obtained. Adult Spin1<sup>MS</sup> mice exhibit a severe scoliosis, cachexia, and weigh only around 60% of control littermates (Figures 6a and b).

Inspection of hind limb muscles revealed as the most prominent feature an abnormally thin and pale soleus (SOL) muscle in Spin1<sup>MS</sup> mice (Figure 6c). Other hind limb muscles (gastrocnemius (GC), plantaris (PL), TA, extensor digitorum...
longus (EDL), and quadriceps (QC)) also displayed a reduced mass relative to controls (Figure 6d; Supplementary Figure 5a). The muscle mass reduction was more pronounced (about 60%) for TA than for gastrocnemius-plantaris, EDL, or quadriceps (about 30%) (Supplementary Figure 5a). Counting showed that the number of TA fibers was reduced by about 50%, but did not uncover a significant difference for the EDL of Spin1<sup>MS</sup> compared with control mice (Supplementary Figure 5b).

H&E staining confirmed degeneration of the soleus as evidenced by rounded fibers, enormous differences in fiber diameters, and the presence of inflammatory cells (Figure 6e). Major defects were also observed in the TA, while gastrocnemius and EDL appeared largely normal (Figure 6e). Also, the diaphragm (DP) of Spin1<sup>MS</sup> mice displayed severe muscle fiber degeneration (Supplementary Figure 5c). Gomori staining detected abnormal collagen deposition indicating fibrosis mainly in the soleus and TA muscle of Spin1<sup>MS</sup> mice.
Spin1 controls skeletal muscle development

H Greschik et al

Cell Death and Disease
Spin1 controls skeletal muscle development
H Greschik et al

Figure 6 Surviving Spin1<sup>MS</sup> mice exhibit major defects in soleus, tibialis anterior, and diaphragm. (a,b) Appearance (a) and average body weight (b) of Spin1<sup>MS</sup> and control mice (<i>n</i> = 5 females in each category) at 30 weeks of age. Error bars represent ±S.D., **<i>P</i> < 0.01. (c) Degeneration of the soleus (SOL) muscle in adult Spin1<sup>MS</sup> compared with control mice exemplified at 16 weeks of age. (d) Hind limb muscles (gastrocnemius (GC), plantaris (PL), soleus, tibialis anterior (TA), extensor digitorum longus (EDL), and quadriceps (QC)) of Spin1<sup>MS</sup> and control mice at 30 weeks of age. Arrows point at the soleus embedded in gastrocnemius and plantaris, which is visible in control but degenerated control mice exemplified at 16 weeks of age. (e) Hematoxylin & eosin (H&E) staining of gastrocnemius, soleus, TA, and EDL muscle of Spin1<sup>MS</sup> and control mice at 30 weeks of age. (f) Fiber types in glycolytic (white) or oxidative (red) parts of the TA of Spin1<sup>MS</sup> and control mice at 15 weeks of age (top and middle rows) observed by immunofluorescence (IF) staining. Tissue sections were stained with selective antibody directed against MHC-I (purple), MHC-IIb (cyan), MHC-IIa (red), and MHC-IIx (green). For comparison, NADH staining was included (bottom row). Fibers with abnormal NADH staining are marked with arrows. Corresponding fibers in each column of images are squared.

Identification of deregulated SKM functional networks in surviving Spin1<sup>MS</sup> mice. We next analyzed the transcriptomes of TA of Spin1<sup>MS</sup> and control mice at three weeks of age (P21), which revealed 1040 DEGs (<i>P</i> < 1e−5; fold change ≥1.5; Figure 7a; Supplementary Table 1d). Intersection with the E16.5 DEGs detected a highly significant overlap of 50 genes (Figure 7a) suggesting that defects in surviving Spin1<sup>MS</sup> mice are related to differential gene expression at early stages of disease.

Phenotype and pathway analyses for the P21 DEGs uncovered terms related to muscle function and disease, as well as metabolism (Figures 7b and c). In addition, we noted terms such as ‘focal adhesion’ and ‘regulation of actin cytoskeleton’, which were not identified at E16.5 (Figures 4a and b). Grouping of the P21 DEGs according to systems biology classifications<sup>28</sup> revealed the same deregulated SKM networks (Figure 7d) as for the E16.5 DEGs (Figure 4c). At P21, however, the networks contained more DEGs than at E16.5, and we identified additional networks (‘hypertrophy & atrophy pathways’ and ‘cytoskeleton’). Of note, the strong increase in deregulated genes including collagen isoforms in the network ‘extracellular matrix (ECM)’ (Figure 7d) correlates with fibrosis detected in the TA of adult Spin1<sup>MS</sup> mice (Supplementary Figure 5d). Together, this analysis provided a comprehensive disease signature for the TA of surviving Spin1<sup>MS</sup> mice.

Differential gene expression in surviving Spin1<sup>MS</sup> mice correlates with SkM disease patterns. In the final set of experiments, we aimed to correlate differential gene expression in surviving Spin1<sup>MS</sup> mice with SkM disease patterns. Since several P21 DEGs encode titin-associated proteins<sup>21,22</sup> (Figure 7d, network ‘Tit-associated’), we hypothesized similarity with diseases caused by Tit mutations such as tibial muscular dystrophy (TMD).<sup>44,45</sup> Muscular dystrophy with myositis (<i>mdm</i>) mice (serving as a model of TMD) express a titin mutant lacking the N2A region, which binds Ankrd1, Ankrd2, and Ankrd23. Accordingly, defective Ankrd1 signaling has been implicated in TMD in <i>mdm</i> mice.<sup>46</sup> Intersection of the P21 DEGs with 75 DEGs previously reported in <i>mdm</i> mice<sup>46</sup> revealed a significant overlap of 26 genes (Figure 8a). Given that Ankrd1 and Ankrd2 are already deregulated in Spin1<sup>MS</sup> fetuses at E16.5 (Figures 3c and 4c), aberrant expression of titin-associated proteins may account for SkM defects in Spin1<sup>MS</sup> mice.

Next, we observed downregulation of genes involved in glycogen metabolism including <i>Pygm</i>, <i>Pfk</i>, <i>Phk1</i>, <i>Phk1g</i>, <i>Pkha2</i>, and <i>Prkag3</i> (Figure 7d, networks ‘glucose & glycogen’ metabolism and ‘hypertrophy & atrophy pathways’). Mutations of these genes are known to occur in glycogen storage diseases (glycogenoses).<sup>28</sup> Periodic acid–Schiff (PAS) staining revealed abnormal glycogen deposits in TA and soleus fibers of Spin1<sup>MS</sup> mice (Figure 8b, black triangles). Although these deposits are limited to individual fibers, which differs from typical glycogenoses, our observation suggests that defective glycogen metabolism contributes to SkM disease in adult Spin1<sup>MS</sup> mice.

Finally, we noted strong deregulation of acetylcholine receptor subunits (<i>Chrna1</i>, <i>Chrnd</i>, <i>Chrne</i>, <i>Chrng</i>) and several genes involved in excitation-contraction coupling (Figure 7d, networks ‘NMJ’ and ‘ECC’) hinting at defective neuromuscular junctions and/or excitation-contraction coupling. Therefore, we analyzed neuromuscular junctions in the diaphragm of newborn and adult Spin1<sup>MS</sup> mice by electron microscopy (Figure 8c). At both time points, the analysis revealed defects of the synaptic membrane (white triangles) and an abnormal appearance as well as a reduced number of synaptic vesicles (arrows) in Spin1<sup>MS</sup> mice (Figure 8c). Furthermore, we observed the presence of vacuoles at nerve terminals of adult Spin1<sup>MS</sup> mice (Figure 8c, right columns (asterisks)). These data provide evidence for neuromuscular junction damage and abnormal excitation-contraction coupling in Spin1<sup>MS</sup> mice.
Spin1 controls skeletal muscle development
H. Greschik et al

Figure 7 Identification of deregulated SkM functional networks in surviving Spin1<sup>MG</sup> mice. (a) Intersection of DEGs observed for TA of Spin1<sup>MG</sup> mice at P21 (blue) and SkM at E16.5 (red). (R: enrichment factor; p: P-value calculated for the intersection.) (b,c) Phenotype (b) and pathway (c) analysis for the set of 1040 DEGs observed in the TA of Spin1<sup>MG</sup> mice at P21. For the phenotype analysis, the 'top 5' non-redundant terms are depicted. (d) Assignment of selected P21 DEGs identified by phenotype and pathway analyses to functional SkM networks. Upregulated DEGs are depicted in red, downregulated in blue color (fold change in brackets)
Discussion

In this study, we ablated the H3K4me3 reader Spin1 in myoblast precursors resulting in abnormal fetal myogenesis, early postnatal death, or severe SkM defects in few surviving Spin1M5 mice. Our analyses suggest that numerous E16.5 DEGs are direct target genes of Spin1 and Myod1. Furthermore, aberrant expression of Myf5, Myog, Msc, and Hes6 in Spin1M5 fetuses may indirectly affect Myod1-dependent gene regulation. Msc, for example, can repress transcription and antagonize the action of Myod1 in undifferentiated myoblasts by heterodimerization with E-proteins.\(^{40}\) Hes6 was reported to inhibit expression of Msc thereby enhancing myoblast differentiation.\(^{43}\) Therefore, deregulated bHLH transcription factor networks appear to determine, at least in part, aberrant gene expression in Spin1M5 fetuses.

The preferential degeneration of soleus, TA, and diaphragm in adult Spin1M5 mice raised the question whether this is caused by fiber type- or muscle type-selective mechanisms. Currently, we favor the latter hypothesis. While degeneration of type I and type II fibers might account for soleus defects, it would not convincingly explain the severe TA damage since this muscle contains only about 1 and 10% of these fiber types, respectively.\(^{47}\) Furthermore, at E15.5 or E16.5, we did not identify DEGs involved in the regulation of fiber type identity or plasticity (e.g., Six1, Six4, Eya1, Sox6).\(^{30}\)

One possible determinant of muscle type-selective degeneration in Spin1M5 mice could be the deregulation of titin-associated proteins such as Ankrd1, which has been linked...
with TMD in mdm mice.46 However, despite some similarity with TMD, the SkM phenotype of Spin1M5 mice is apparently more complex. We exemplarily correlated the P21 DEGs with abnormal glycogen accumulation in individual TA and soleus fibers as well as neuromuscular junction defects in Spin1M5 mice. Due to the high number of DEGs observed in the TA of Spin1M5 mice at P21, these examples only provide an initial characterization of deregulated SkM networks.

Compared with transcription factors, signaling molecules, SkM structural proteins, or metabolic enzymes, the roles of epigenetic regulators in SkM physiology and disease have only more recently become a focus of research. However, while epigenetic writers, erasers, and non-coding RNAs have received considerable attention,48,49 little is known about potential functions of histone code readers in SkM. Few readers (Brd4, Ing2, Sfbm1, Dpif3) have been implicated in myogenesis using the C2C12 cell culture model50 or in zebrafish.51 However, in these cases either knockout mouse models have not been reported, or in mice no apparent SkM phenotype was observed.55–57 Thus, to the best of our knowledge, Spin1 is the first histone code reader, for which ablation in SkM has fatal consequences in mice. Together, our histological and transcriptome analyses provide insight into severe SkM defects in Spin1M5 fetuses and surviving adult mice, hinting at Spin1 as a potential player in human SkM disease.

Materials and Methods

Mouse studies. All mice were housed in the pathogen-free barrier facility of the University Medical Center Freiburg in accordance with institutional guidelines and approved by the regional board. Mice were maintained under temperature- and humidity-controlled conditions with a 12-h light/dark cycle, free access to water, and a standard rodent chow (3807, Provimi Kliba). Animals were killed by cervical dislocation and tissues immediately processed for further analyses.

Generation and validation of Spin1R26 and Spin1M5 mice. The targeting strategy for generation of a conditional Spin1 allele is outlined in Supplementary Figure 1a. Details are available upon request. The targeting construct was electroporated into C57BL/6 N Tac embryonic stem (ES) cells (Taconic), and neomycin-and puromycin-resistant clones were expanded. Selected ES cells were injected into blastocysts of C57BL/6 N mice. Resulting mice were bred to Rosa26-Flp mice to remove NeoR and PuroR selection markers. Offspring carrying the conditional allele, in which exon 4 of Spin1 was flanked by loxP sites. C57Bl6N mice homozygous for the conditional allele (Spin1R26) were crossed with mice harboring a green fluorescent protein/nuclear lacZ (GNZ) construct was electroporated into C57BL/6 N Tac embryonic stem (ES) cells (Taconic), and neomycin-and puromycin-resistant clones were expanded. Selected ES cells were injected into blastocysts of C57BL/6 N mice. Resulting mice were bred to Rosa26-Flp mice to remove NeoR and PuroR selection markers. Offspring carrying the conditional allele, in which exon 4 of Spin1 was flanked by loxP sites. C57Bl6N mice homozygous for the conditional allele (Spin1R26) were crossed with mice harboring a green fluorescent protein/nuclear lacZ (GNZ) reporter.55 In mice harboring the reporter, GNZ expression is Cre dependent. GNZ-positive myogenic cells in Spin1M5 mice did not express Spin1, whereas Spin1-positive cells were GNZ-negative and therefore non-myogenic (Supplementary Figure 1f). Part of these Spin1-expressing, non-myogenic cells were Tcf4-positive (Supplementary Figure 1e). Reduction of Spin1 mRNA in hind limb muscle of newborn Spin1M5 mice was confirmed by quantitative RT-PCR (Supplementary Figure 1g).

Quantitative RT-PCR and RNA sequencing. SkM tissue was homogenized in TRIzol reagent (Life Technologies, Darmstadt, Germany) using a Minilys personal homogenizer (Berlin, Montigny, France) and 0.5 or 2.0 ml CK14 lysing kits (Preccells, Montigny, France). RNA was isolated using a standard phenol/chloroform extraction protocol. cDNA was prepared by reverse transcription of total RNA using SuperScript II (Life Technologies) and oligo(dT) primer according to the supplier’s protocol. Quantitative RT-PCR was performed with a Lightcycler 480 II (Roche, Mannheim, Germany) using Absolute SYBR green ROX Mix (Thermo Scientific, Schirnbeck, Germany) and the following primers: Spin1 (exon 4, forward) 5’-CAGTGTCGCTGTGAARTCTCTC-3’, Spin1 (exon 5, reverse) 5’-ACATGTTGC TACACTGTGTT-3’, Tbp (forward) 5’-CCCCCTTACCTCTCACCAR-3’, Tbp (reverse) GAAGCTCGCGTGACATTCCAG-3’, Hprt (forward) 5’-GTTRAGCG AGTACAGCCTCAAAA-3’, Hprt (reverse) 5’-AGGGCATATCCCAACAAACATT-3’, Potr2a (forward) 5’-CACCCACGTCTTCTCCAAAT-3’, and Potr2a (reverse) 5’-AGTATGCTGGGAGAGTGTGA-3’. Data were analyzed using the 2(ΔΔC-T) method.59

RNA-seq analyses for E15.5 and E16.5 were performed with SkM dissected from hind and front limb of three Spin1M5 and four control fetuses. RNA-seq analysis of mice at three weeks of age (P21), TA isolated from four Spin1M5 and four control mice was used. RNA was isolated as described above, except that minced tissue in TRIzol was further homogenized with QIA shredder spin columns (Qiagen, Hilden, Germany). RNA quality was determined using the RNA 6000 Nano Kit (Agilent, Waldbronn, Germany) on an Agilent 2100 Bioanalyzer. RNA with a RIN above 8.0 was sequenced at the DKFZ core facility (Heidelberg, Germany) or the Deep Sequencing Unit (Max-Planck-Institute of Immunology and Epigenetics, Freiburg) using the standard Illumina protocol. Paired-end reads were mapped to Ensemble annotation NCBI_m38/mm10 with TopHat56 using default parameters. The aligned reads were counted with HOMER software57 and DEGs calculated with edgeR.62 Overrepresentation analyses for the identified DEGs (P ≤ 0.01 < 0.05 and E15.5, P ≤ 0.01 < 0.05 (P21); fold change > 1.5; > 50 reads in all Spin1M5 or all control samples) were performed using WebGestalt63 with ‘genome’ as reference set.

Of note, none of the RNA-seq analyses identified Spin1 as differentially expressed gene. Examination of the normalized reads at exons 1 to 6 of the Spin1 gene explained this observation (Supplementary Table 1e). Reads were significantly decreased at exon 4 (which is excised in a Myf5-Cre-dependent manner in myogenic cells), but not at other exons in Spin1M5 relative to control mice. Thus, in myogenic cells of Spin1M5 mice a truncated transcript (lacking exon 4) is expressed, which results in translation of a truncated Spin1 peptide (containing 56 N-terminal amino acids lacking any known functional domain) due to the presence of a premature STOP codon in exon 5. Normal Spin1 transcript (containing exon 4) present in SkM of Spin1M5 mice is most likely produced by non-myogenic cells (Supplementary Figures 1e and 1f). Since mapped reads at all exons contribute to the overall count, differential expression of Spin1 is not detected in RNA-seq analyses.

Antibodies. The generation and validation of anti-SPIN1 antibodies 5865 and 5867 was described previously. Antibodies Spin1(5865) and H9K4mes3 (Diagnoscope, Oxford, UK, C1541003) were used for ChIP. For immunofluorescence staining the following primary antibodies were used: SPIN1(5865) 1 μg/ml; Pax7 (PAx7, DSHB, batch 7/2/12) 2 μg/ml; Tcf4 (6H5-3, Millipore, Darmstadt, Germany, 05-511, batch 2155406) 10 μg/ml; Ank1d1 (Proteintech Group, Manchester, UK, 11427-1-AP, batch 1951) 1:100; Ankr2d (Proteintech Group, 11821-1-AP, batch 7649) 1:100; dystrophin (Abcam, Cambridge, UK, ab15277, batch GR26876-1) 1:500; Mhc-I (NOQ7.54D, Sigma, Munich, Germany, M8421, batch 035M4792V) 1:2000; Mhc-ll-a (SC-71, DSHB, batch 4/7/16) 1:100; Mhc-ll-b (6H1, DSHB, batch 3/3/16) 1:10; Mhc-ll-b (BF-F3, DSHB, batch 5/12/16) 1:20; MCH, skeletal, fast (MY32, Sigma, M4267, batch 083M7490V) 1:1000; GNZ (anti-SGP, Abcam, ab13970, batch GR268651) 1:500; normal rabbit IgG (Santa Cruz, Heidelberg, Germany, sc-2027). The following secondary antibodies were used: Alexa Fluor 488 (goat anti-mouse IgM, Molecular Probes, Karlsruhe, Germany, sc-2048, batch 1306597) 1:100; Alexa Fluor 488 (goat anti-mouse IgG, Molecular Probes, A21424, batch 1306597) 1:600; Alexa Fluor 546 (goat anti-mouse IgG, Molecular Probes, A21424, batch 1306597) 1:600; Alexa Fluor 594 (goat anti-chicken IgG, Molecular Probes, A21424, batch 762712) 1:600; donkey anti-rabbit IgG-HRP (Santa Cruz, sc-2033) 1:150.

Cell Death and Disease

Spin1 controls skeletal muscle development

H Greschik et al
Immunofluorescence staining. SkM tissues were either fixed in 4% paraformaldehyde/PBS or flash-frozen in 2-methylbutane (Sigma). Paraffin sections (5 μm) were deparaffinized, heated in antigen retrieval solution (20 mM Tris (pH 9.0)) for 20 min in a pressure cooker, and blocked for 1 h at room temperature in 3% skim milk powder/PBS containing 0.1% Tween (PBST). Cryosections (10 μm) were blocked with 5% fetal calf serum/PBST. Sections were incubated overnight with primary antibody at 4 °C, washed with PBST, incubated with secondary antibody for 1 h at room temperature, and then washed with PBST. For Spin1 staining, signal amplification using the TSA fluorescence system (Perkin Elmer, NEL701A001KT) was applied according to the manufacturer's instructions. Briefly, tissue sections were blocked in 0.5% TBN blocking buffer, labeled with primary and secondary antibody, and then incubated with TSA reagent for 15 minutes at room temperature followed by two washing steps with PBST. Finally, nuclei were stained with DAPI (1 μg/ml) followed by two washing steps with PBST, and sections were mounted using Fluoromount-G (SouthernBiotech). Images were recorded with a confocal microscope (Leica TCS SP2 AOBS). Counting was done with ImageJ, followed by visual validation of the results. Pax7-positive nuclei or nuclei of myofibers (expressing Spin1 or being Spin1-negative) were quantified using confocal images with dimensions of 187.5 μm × 187.5 μm or 375 μm × 375 μm, respectively.

Isolation of primary myoblasts and ChIP sequencing. Primary myoblasts were isolated from 10- day-old C57BL/6 N mice using an established preplating protocol. Briefly, limb muscles were collected from front and hind leg, minced, digested for 1 h in 0.2% collagenase type I (Sigma, CO130), and filtered through 100 μm cell strainers (Falcon, 352360). Cells were collected by centrifugation, resuspended in DMEM medium (Gibco, Schwerte, Germany, 11995-065) supplemented with 10% FCS, 10% horse serum, and 1.25% chicken embryo extract (Seralab, CE-650-J), and cultivated for 1 h on 6 cm tissue culture dishes coated with collagen (Gibco, A10483-01). Myoblasts were cultivated for 3 to 4 days until a confluency of about 70% was reached. Cells were fixed with 1% formaldehyde for 5 minutes, quenched in glycine (1.25 M), washed with PBS buffer, collected, and snap-frozen in liquid nitrogen.

Chromatin was prepared using the NEXSON procedure. Briefly, nuclei were extracted by sonication with a Covaris E220 sonicator (75 W peak power, 2% duty factor, 200 cycles/burst). Chromatin was diluted 1:2 in buffer H (Diagenode auto histone ChIP-seq kit buffer, and sonicated for 12 min (140 W peak power, 5% duty factor, 200 cycles/burst, 60 s). Nuclei were pelleted, resuspended in 1 ml of shearing buffer, and sonicated for 12 min (140 W peak power, 5% duty factor, 200 cycles/burst). Supernatants were transferred to 6 cm dishes, again incubated for 1 hour, and then transferred to 10 cm tissue culture dishes coated with collagen (Gibco, A10483-01). Myoblasts were cultivated for 3 to 4 days until a confluency of about 70% was reached. Cells were fixed with 1% formaldehyde for 5 minutes, quenched in glycine (1.25 M), washed with PBS buffer, collected, and snap-frozen in liquid nitrogen.

Electron microscopy. Muscle samples were fixed by immersion in 2.5% glutaraldehyde and 2.5% paraformaldehyde in cacodylate buffer (0.1 M, pH 7.4) and then washed in cacodylate buffer for further 30 min. The samples were post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at 4 °C, and dehydrated in an ascending ethanol gradient (50, 70, 90, and 100%) and propylene oxide for 30 min each. Samples were embedded in Epon 812 substitute (Sigma-Aldrich). Semi-thin sections cut at 2 μm and ultra-thin sections cut at 70 nm were contrasted with uranyl acetate and lead citrate and examined at 70 kV with a Morgagni 268D electron microscope. Images were digitally captured by Mega View III camera (Soft Imaging System).

Hematoxylin & eosin staining. Deparaffinized and rehydrated flash-frozen tissue sections (5 or 10 μm, respectively) were stained according to a standard protocol with hematoxylin (Gill No. 3, Sigma, GH5332) and eosin Y solution (Sigma, HT110332) and mounted using Roti-Histokit (Roth).

Gomori trichrome staining. Deparaffinized and rehydrated tissue sections (5 μm) were stained using Bouin's solution (Sigma, HT10132), hematoxylin-Weigert's iron kit (Dianova, HWI-2), and trichrome stain (blue) solution (Dianova, TGB500) according to the supplier's instructions and mounted using Roti-Histokit (Roth).

NADH staining. Cryosections (10 μm) were incubated with staining solution (0.2 M Tris (pH 7.4), 1.5 mM NADH (Roche, 10128015001), 1.5 mM nitroblue tetrazolium (Sigma, N-6676)), dehydrated in an ascending ethanol gradient, incubated twice with xylene, and mounted using Roti-Histokit (Roth).

Periodic acid–Schiff staining. Deparaffinized and rehydrated tissue sections (5 μm) were treated with 0.5% periodic acid solution (Sigma, 3951), stained with Schiff's reagent (Sigma, 395216), dehydrated, and mounted using Roti-Histokit (Roth).

Statistics. Transcriptome and cistrome data were analyzed as described above. Statistical significance of gene set intersections was evaluated by a hypergeometric test using the program 'R' (http://www.R-project.org) [phyper (N12-1, N1, N-N1, N2, lower.tail = FALSE) with N1 (genes in set 1), N2 (genes in set 2), N12 (genes in intersection), and N (genome size)]. The enrichment factor (R) was calculated according to $R = (N \times N12) / (N1 \times N2)$. Other data are presented as the mean value or percentage change ±S.D. Comparisons between two data sets were made using the two-tailed Student's t-test for parametric data and the Wilcoxon signed-rank test for nonparametric data. A P-value of less than 0.05 was considered statistically significant. Statistical significance is indicated as follows: * P<0.05, ** P<0.01, *** P<0.001.

Conflict of Interest
The authors declare no conflict of interest.

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