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RAPID COMMUNICATION

Deletion of heterogeneous nuclear ribonucleoprotein K in satellite cells leads to inhibited skeletal muscle regeneration in mice

Heterogeneous nuclear ribonucleoprotein K (hnRNPK) is a predominantly nuclear RNA-binding protein that can bind to DNA or RNA through three KH domains and interact with multiple proteins by interactive region. These binding activities enable hnRNPK to link the function in a wide array of diverse cellular processes, such as chromatin remodeling, gene transcription, RNA metabolism, protein translation, DNA repair, and cell signal transduction, thereby playing crucial roles in many biological processes, including development, axonal regeneration, spermatogenesis, cell cycle, apoptosis, differentiation, and carcinogenesis.[1] Lack of hnRNPK in C2C12 myoblasts results in the decreased proliferation rate of myoblasts and an inhibitory effect on muscle differentiation. Homozygous *Hnrnpk* knockout (*Hnrnpk*$^{-/-}$) mice are embryonic lethal, suggestive of its decisive role in development and neonatal survival.[2] In addition, mutation of *HNRNPK* in humans causes Au-Kline syndrome which is a rare neurodevelopmental multiple congenital malformation syndrome associated with global developmental delay, characteristic facies, congenital heart defects, skeletal abnormalities, and muscle weakness.[3] These findings demonstrate that hnRNPK plays a critical role in skeletal muscle development and myogenesis. However, the molecular mechanism of hnRNPK in skeletal muscle development has not been convincingly demonstrated, especially in animal models. Here, we constructed an hnRNPK-inducible skeletal muscle satellite cell-specific knockout mouse model and found that hnRNPK depletion in mice inhibited muscle regeneration, emphasizing the importance of hnRNPK in myogenesis. Further research demonstrated that hnRNPK may feature in skeletal muscle regeneration via binding *Cdnk1a* 3′UTR to modulate *Cdnk1a* mRNA stability. These findings suggested that hnRNPK is required for muscle regeneration and might be a potential novel target for the treatment of muscle disorders.

To examine the expression pattern of *Hnrnpk* in mouse muscle satellite cells (MuSCs), we re-analyzed the previous transcriptomic examination dataset (GSE113631) of MuSC quiescence and activation generated by Lu Yue,[4] and scRNA-seq dataset (GSE189088) of E14 skeletal muscle published by Ruochen Guo.[5] We found that the expression abundance of *Hnrnpk* gene was significantly higher in satellite cells. Moreover, *Hnrnpk* was especially higher in activated satellite cells (Fig. S1A). Interestingly, the t-distributed stochastic neighbor embedding plots revealed *Hnrnpk* was highly expressed in MuSCs and mature skeletal muscle cells using skeletal muscle cell marker genes, such as *Pax7*, *Myog*, *Myod1*, and *Cdnk1a*, which make up the myogenic lineage (Fig. S1B). In addition, further immunostaining for hnRNPK on MuSCs also showed hnRNPK was widely expressed during MuSC proliferation and differentiation, and hnRNPK was mainly located in the nucleus (Fig. S1C). Together, these results demonstrated that hnRNPK plays a crucial role in both the resting and activation of skeletal muscle satellite cells, especially in the activation of satellite cells.

To further investigate the functions of hnRNPK *in vivo*, we generated *Hnrnpk*$^{LoxP/LoxP}$; *Pax7*^{CreER} mice (*Hnrnpk* pKO) by crossing Pax7^{CreER} mice driving Cre-recombinase from the *Pax7* locus with mice bearing floxed *Hnrnpk* alleles (Fig. S2A, B). As *Hnrnpk* in the pKO mouse model was only knockout in the MuSCs and *Hnrnpk* was still expressed in other non-MuSCs, we isolated MuSCs to detect the knockdown efficiency of *Hnrnpk*. Immunofluorescence staining of hnRNPK in MuSCs revealed that the proportion of hnRNPK^{+} satellite cells in the experimental group was

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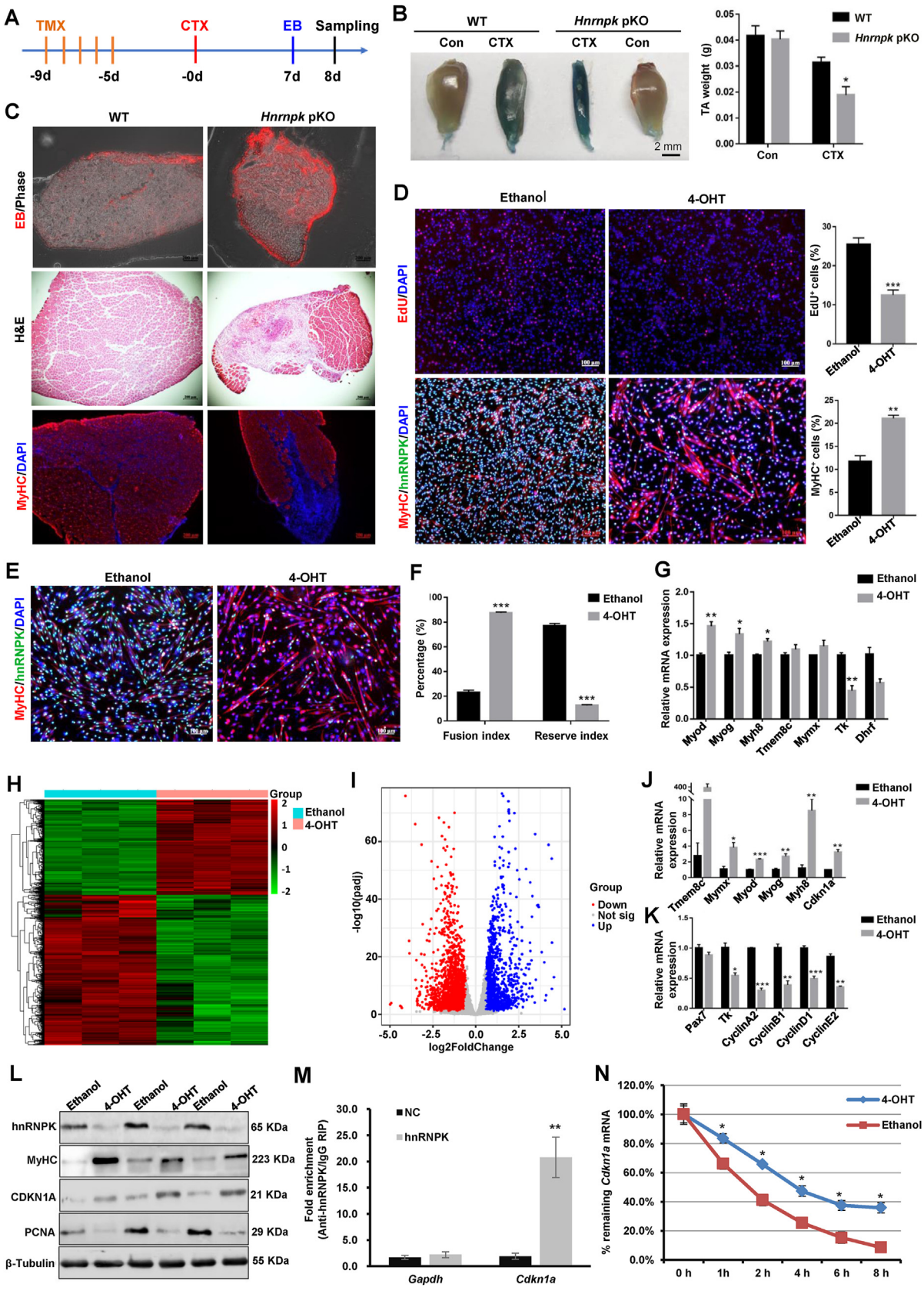

Figure 1 hnRNPK is essential for muscle regeneration in mice. **(A)** Schematic of animal experimental design. The 7 d represents the sample collection time for IF and H&E staining, while 8 d represents the sample collection time for EB staining. **(B)** The representative images of whole tibialis anterior (TA) muscles at day 8 after CTX injection showing decreased muscle mass in the *Hnrnpk* pKO mice. The blue color indicates EB uptake. The right histogram represents the comparison results of TA tissue weight

decreased by about 50% (Fig. S2C). Western blot analysis of proteins extracted from the MuSCs supported that hnRNPK expression in the *Hnrnpk* pKO MuSCs was reduced sharply (Fig. S2D). This knockout efficiency is calculated as a "meaningful" deletion, proving that the knockout mouse model was successfully constructed. Next, deletion of *Hnrnpk* was induced by tamoxifen intraperitoneal injection on postnatal days 1−5, and then a muscle regeneration model in which tibialis anterior muscle was injured with cardiotoxin (CTX) determined the role of *Hnrnpk* during skeletal muscle regeneration *in vivo*, and muscles were examined day 7 after injury (Fig. 1A). Tissue immunofluorescence staining of hnRNPK in mice at day 7 after injury showed that hnRNPK was barely detectable in the MuSCs of the *Hnrnpk* pKO mice (Fig. S3A). Evans blue (EB) signal in the CTX-treated *Hnrnpk* pKO muscles was much more immensely than in the control muscles (Fig. 1B, C; Fig. S3B), indicating the inhibition of muscle regeneration. Conformably, hematoxylin and eosin staining demonstrated that the *Hnrnpk* pKO TA muscles on day 7 after injury had more sparsely organized and many degenerated myofibers, and contained extensive empty space, while the WT muscles were satisfactorily regenerated with uniformly formed centrally nucleated regenerating fibers and little interstitial space at the point of maximal injury (Fig. 1C; Fig. S3C). In addition, using immunofluorescence staining, we observed neonatally regenerated myosin heavy chain (MyHC) positive myofibers were enriched in the WT muscles near the center of injury, while the MyHC positive myofibers were rarely in the *Hnrnpk* pKO muscles (Fig. 1C; Fig. S3D). Taken together, these results revealed that hnRNPK depletion in MuSCs led to impaired muscle regeneration, further emphasizing the importance of hnRNPK in myogenesis.

Given muscle regeneration depending on the self-renewal and differentiation of MuSCs, we further analyzed primary myoblasts isolated from *Hnrnpk* pKO mice and WT littermates. The results of 5-Ethynyl-2′-deoxyuridine (EdU) staining, immunofluorescence, and qRT-PCR revealed that hnRNPK deletion significantly inhibited myoblast proliferation and promoted differentiation (Fig. 1D−G). Moreover, after hnRNPK-induced knockout, MuSCs showed a tendency to differentiate even without the addition of an inducing differentiation medium (Fig. 1D, G). Subsequently, to uncover the underlying molecular mechanisms of hnRNPK in regulating skeletal muscle satellite cells, RNA-Seq was performed to assess the gene expression profile in hnRNPK knockout and WT MuSCs. Approximately 3846 differentially expressed genes (DEGs) (Q-value < 0.05, fold change ≥ 1.5) were screened between the two groups (Fig. 1H, I and Table S1). We selected 15 genes (8 genes associated with myogenic differentiation and 7 genes associated with cell cycle) representative of the expression profiles in accordance with RNA-Seq results, and examined their expression levels via reverse transcription quantitative real-time PCR (RT-qPCR) in triplicates (Fig. 1J, K), sustaining the reliability of RNA-Seq results. To achieve a profound understanding of the regulatory pathways perturbed by hnRNPK depletion, we carried out an analysis of the biological functions selectively enriched among the DEGs using Metascape software. Interestingly, hnRNPK knockout in MuSCs had significant enrichment in a number of pathways, including regulation of cell cycle process, cell division, muscle structure development, regulation of kinase activity, and skeletal muscle cell differentiation (Table S2). Western blot results demonstrated that hnRNPK knockout significantly inhibited MuSC proliferation and improved differentiation (Fig. 1L). The interactions between different pathways showed that the pathway was centered on cell cycle process (Fig. S4). More importantly, a subset of mRNAs dysregulated in hnRNPK-knockout myoblasts compared with control repeatedly arose the cyclin-dependent kinase inhibitor (Cdkn1a), which increased sharply after hnRNPK induction deletion. Furthermore, in human endothelial cells, hnRNPK can specifically bind to the cell cycle inhibitor *CDKN1A* mRNA, and silence of *HNRNPK* resulted in increases of *CDKN1A* mRNA expression, suggesting that hnRNPK may normally be targeting it for degradation in progenitor cells to prevent differentiation and cell cycle exit. More importantly, *Cdnk1a* is indispensable for the regulation of cell cycle exit and differentiation in myogenic cells, and the deletion of *Cdnk1a* obviously

between the control group and the CTX group ($n = 3$). **(C)** The representative TA muscle sections showing EB fluorescence (in red), hematoxylin and eosin (H&E) staining, and MyHC immunohistochemical staining. **(D)** hnRNPK knockout inhibits proliferation and induces differentiation of MuSCs. In EdU staining analysis for cell proliferation in the Ethanol and 4-hydroxytamoxifen (4-OHT) groups, nuclei were stained with DAPI ($n = 3$; scale bar $= 100$ μm). In co-immunofluorescence staining analysis with hnRNPK (in green) and MyHC (in red) for cell differentiation in the Ethanol and 4-OHT groups, nuclei were stained with DAPI ($n = 3$; bar $= 100$ μm). **(E−G)** MuSCs were differentiated for 3 days in the Ethanol and 4-OHT groups. Myotube morphology (showed by MyHC staining, in red), fusion index (% nuclei in myotubes), reserve cell index (% nuclei that are MyHC), and relative mRNA levels of *Myod*, *Myog*, *Myh8*, *Tmem8c*, *Mymx*, *Tk*, and *Dhrf* are shown. **(H)** Heatmap of normalized expression values of DEGs in MuSCs of Ethanol and 4-OHT groups. **(I)** Volcano plot of the DEGs in MuSCs of the 4-OHT group compared with the Ethanol control. The red dots and green dots represent the significantly down-regulated and up-regulated genes, respectively (*Q*-value < 0.05, fold change of RPKM > 1.5, and baseMean > 50), and the gray dots represent unchanged genes. **(J, K)** qRT-PCR validation of differentially expressed genes including the myogenesis-related genes (*Tmem8c*, *Mymx*, *Myod*, *Myog*, *Myh8*, and *Cdkn1a*) (J) and the proliferation-related genes (*Pax7*, *Tk*, *cyclin A2*, *cyclin B1*, *cyclin D1*, and *cyclin E2*) (K). **(L)** Western blot analysis of hnRNPK, MyHC, CDKN1A, and PCNA expression levels in MuSCs treated with Ethanol or 4-OHT ($n = 3$). The expression level of β-Tubulin was used as the internal control. **(M)** RIP RT-qPCR assay was carried out to verify the interaction between *Cdkn1a* mRNA and hnRNPK in MuSCs ($n = 3$). **(N)** hnRNPK depletion increases the stability of endogenous *Cdkn1a* mRNA. The MuSCs of the Ethanol (rad squares) or 4-OHT (blue squares) groups were treated with the transcription inhibitor ActD for 8 h. All results were expressed as mean ± SEM and a paired two-tailed student's *t*-test was used for statistical analysis. $^{**}P < 0.01$, $^{*}P < 0.05$.

impaired muscle regeneration. So, we speculated that the hnRNPK might target *Cdkn1a* and make a key role in myogenesis. Accordingly, hnRNPK-*Cdkn1a* interaction was further confirmed using hnRNPK antibody RNA immunoprecipitation RT-qPCR in MuSCs (Fig. 1M). In addition, the expression pattern changes of *Cdkn1a* were opposite with hnRNPK during MuSC differentiation (Fig. 1L). To further assess the regulatory relationship between hnRNPK and *Cdkn1a*, we subsequently carried out the transcription inhibitor actinomycin D (ActD) treatment experiments. Upon hnRNPK-depletion, the *Cdkn1a* mRNA degradation rate was powerfully affected and its half-life was strongly increased (Fig. 1N), which indicates hnRNPK could participate in the control of endogenous *Cdkn1a* mRNA stability. Collectively, these data confirm that hnRNPK can target *Cdkn1a* mRNA in MuSCs, and modulate a pathway referred to *Cdkn1a* expression controls by RNA-protein physical interaction.

In conclusion, we used conditional knockout approaches to highlight the direct involvement of hnRNPK in a novel pathway that could modulate *Cdkn1a* levels to control myogenesis. HnRNPK absence could increase the expression of *Cdnk1a*, leading to premature cell cycle exit of MuSCs and causing a remarkably reduced number of MuSCs available for skeletal muscle formation and regeneration. Therefore, perturbations of hnRNP have a strong impact on skeletal muscle regeneration and myogenesis. It is crucial to understand the implication of hnRNPK in skeletal muscle regeneration and the pleiotropic functions of hnRNPK during myogenesis in future research.

Ethics declaration

All animal procedures were carried out by the Ethics Committee of Xinyang Normal University (protocol code XYEC-2021-011; approval time: January 1, 2021).

Author contributions

Yognjie Xu, Pengpeng Zhang, and Cencen Li designed the study and revised the manuscript. Yognjie Xu, Haixia Xu, and Xiaofang Cheng wrote the manuscript. Haixia Xu and Yaling Wang generated conditional knockout mice, performed the investigation, and validated the results. Nuo Chen, Yaling Wang, and Yueru Huang carried out molecular and cellular experiments; Jiahua Guo and Cencen Li performed the single-cell RNA-seq analysis and RNA-seq analysis; Jiahua Guo, Yuqian Zheng, Mengjia Zhang, and Chunyu Du helped with cell culture, cell transfection, qPCR, EdU-staining, immunofluorescence, RIP, and Western blot. Cunzhen Zhao helped revise the manuscript. All authors agreed to publish this manuscript.

Conflict of interests

The authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.06.031.

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