Satellite cell-specific ablation of Cdon impairs integrin activation, FGF signalling, and muscle regeneration

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Abstract

Background  Perturbation in cell adhesion and growth factor signalling in satellite cells results in decreased muscle regenerative capacity. Cdon (also called Cdo) is a component of cell adhesion complexes implicated in myogenic differentiation, but its role in muscle regeneration remains to be determined.

Methods  We generated inducible satellite cell-specific Cdon ablation in mice by utilizing a conditional Cdon allele and Pax7CreERT2. To induce Cdon ablation, mice were intraperitoneally injected with tamoxifen (tmx). Using cardiotoxin-induced muscle injury, the effect of Cdon depletion on satellite cell function was examined by histochemistry, immunostaining, and 5-ethynyl-2’-deoxyuridine (EdU) incorporation assay. Isolated myofibers or myoblasts were utilized to determine stem cell function and senescence. To determine pathways related to Cdon deletion, injured muscles were subjected to RNA sequencing analysis.

Results  Satellite cell-specific Cdon ablation causes impaired muscle regeneration with fibrosis, likely attributable to decreased proliferation, and senescence, of satellite cells. Cultured Cdon-depleted myofibers exhibited 32 ± 9.6% of EdU-positive satellite cells compared with 58 ± 4.4% satellite cells in control myofibers (P < 0.05). About 32.5 ± 3.7% Cdon-ablated myoblasts were positive for senescence-associated β-galactosidase (SA-β-gal) while only 3.6 ± 0.5% of control satellite cells were positive (P < 0.001). Transcriptome analysis of muscles at post-injury Day 4 revealed alterations in genes related to mitogen-activated protein kinase signalling (P < 8.29 e-5) and extracellular matrix (P < 2.65 e-24). Consistent with this, Cdon-depleted tibialis anterior muscles had reduced phosphorylated extracellular signal-regulated kinase (p-ERK) protein levels and expression of ERK targets, such as Fos (0.23-fold) and Egr1 (0.31-fold), relative to mock-treated control muscles (P < 0.001). Cdon-depleted muscles exhibited impaired ERK activation in response to basic fibroblast growth factor. Cdon ablation resulted in decreased and/or mislocalized integrin β1 activation in satellite cells (weak or mislocalized integrin 1 in tmx = 38.7 ± 1.9%, mock = 21.5 ± 6%, P < 0.05), previously linked with reduced fibroblast growth factor (FGF) responsiveness in aged satellite cells. In mechanistic studies, Cdon interacted with and regulated cell surface localization of FGFR1 and FGFR4, likely contributing to FGF responsiveness of satellite cells. Satellite cells from a progeria model, Zmpste24-/− myofibers, showed decreased Cdon levels (Cdon-positive cells in Zmpste24-/− = 63.3 ± 11%, wild type = 90 ± 7.7%, P < 0.05) and integrin β1 activation (weak or mislocalized integrin 1 in Zmpste24-/− = 64 ± 6.9%, wild type = 17.4 ± 5.9%, P < 0.01).

Conclusions  Cdon deficiency in satellite cells causes impaired proliferation of satellite cells and muscle regeneration via aberrant integrin and FGF signalling.

Keywords  Satellite cell; Muscle regeneration; Cdon; Cellular senescence; FGFR; Growth factor signalling

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Introduction

Skeletal muscle has a resilient regenerative capacity primarily governed by stem cells, called satellite cells. Normally, satellite cells are kept in a quiescent state and capable of self-renewal and differentiation upon injury-stimulated activation. Quiescent satellite cells express Pax7 and when activated, express myogenic transcription factors, such as Myf5 and MyoD. This is followed by expansion and differentiation of progenitor cells, coinciding with decreased Pax7 and increased Myogenin expression. The ability of satellite cells to proliferate and return to quiescence is critical for the maintenance of the stem cell population. Various muscle wasting conditions related to aging or dystrophy have been associated with a decline in the number and proliferative capacity of satellite cells. Satellite cells are remarkably resistant to cellular stress but progressively lose their ability to proliferate and differentiate with age. Satellite cell aging and dysfunction have been linked with cellular senescence driven by the accumulation of DNA damage and irreversible cell cycle arrest. Senescent cells have characteristic features such as flat appearance, senescence-associated-β-galactosidase (SA-β-gal) activity, and upregulation of cyclin-dependent kinase inhibitors p16Ink4a (p16) and p21Waf1 (p21). In addition, senescent cells produce the senescence-associated secretory phenotype (SASP), producing inflammatory cytokines and insulin-like growth factor binding proteins. Although the dysregulation of cell cycle inhibitors appears to be the critical mechanism underlying cellular senescence, the molecular mechanisms for satellite cell senescence are still largely unknown.

Several niche and cell-intrinsic factors have been linked to satellite cell dysfunction. Secreted factors like cytokines and growth factors, including fibroblast growth factor (FGF) family members, have been shown to play essential roles in satellite cell activation and proliferation upon injury. Accumulating evidence suggests that perturbed growth factor signalling is associated with satellite cell dysfunction, leading to muscle loss and weakness related to aging. In addition, the interaction of satellite cells with their niche, through adhesion to myofibers via N-cadherin and M-cadherin or through adhesion to the basal lamina via integrins plays essential roles in satellite cell function. Removal of N-cadherin and M-cadherin in satellite cells results in activation of quiescent satellite cells without injury, whereas deletion of integrin β1 or decreased fibronectin has been linked with satellite cell aging and decreased FGF response, resulting in impaired self-renewal and proliferation capacity. Furthermore, alteration in polarized localization of active integrin β1 (Itgb1*) is associated with impaired responsiveness of aged satellite cells to FGF, contributing to reduced regenerative capacity. However, the underlying regulatory mechanism of this polarized activation of integrin signalling is currently unclear.

The cell surface protein Cdon (also called Cdo) forms complexes with N-cadherin at contact sites between skeletal myoblasts, and this interaction is critical for Cdon’s promyogenic function. Consistent with its promyogenic role, Cdon-null mice exhibit delayed skeletal muscle development, and Cdon-deficient myoblasts differentiate less efficiently with decreased levels of muscle-specific proteins. In addition, the promyogenic function of Cdon appears to be dependent on integrin/focal adhesion kinase (FAK) signalling. Based on these observations, it is conceivable that Cdon plays an important role in adult muscle regeneration. In this study, we set out to elucidate the in vivo function of Cdon in adult satellite cells. Here, we report that inducible ablation of Cdon in satellite cells results in impaired muscle regeneration with fibrosis in a repetitive cardiotoxin (CTX)-triggered injury model. Cdon deletion reduces the proliferative capacity of satellite cells. Furthermore, Cdon depletion in satellite cells or myoblasts elicits cellular senescence with enhanced expression of cell cycle inhibitors (p16 and p21), SASP, and phosphorlated-histone2AX (pH2AX), a marker for DNA double strand breaks. RNA sequencing analysis of muscles at post-injury Day 4 revealed that Cdon ablation alters gene expression profiles related to the cellular response to cytokines and the MAPK signalling pathway without any significant change in the expression of myogenic regulators, such as Pax7, Myf5, MyoD, and Myogenin. Consistent with this, Cdon deletion elicits a decrease in FGF responsiveness and ERK activation, coinciding with reduction in the expression of immediate early genes. Interestingly, Cdon-depleted satellite cells exhibit perturbed Itgb1* levels and localization. Furthermore, Cdon and Itgb1* levels were either decreased or mislocalized in satellite cells on myofibers isolated from Zmpste24−/− mice, a model of progeria. Mechanistic analyses demonstrate that Cdon forms complexes with FGFR1 and FGFR4, and Cdon depletion reduces cell surface levels of FGFRs, likely contributing to reduced FGF responsiveness. Taken together, these data argue that Cdon regulates satellite cell proliferation via modulation of integrin activation and FGFR-mediated signalling.

Methods

Animal experiments

To generate Cdon conditional knockout mice, three embryonic stem (ES) cells lines with conditional knockout potential
for Cdon were obtained from the European Conditional Mouse Mutagenesis Program. Southern blotting analysis was performed by standard protocols to confirm the conditional Cdon allele (Cdon\textsuperscript{m30} in Supporting Information, Figure S1) in two of the three ES cell lines (C03 and H02). Mouse lines were generated from these ES lines by the Mouse Genetics and Gene Targeting Core of Icahn School of Medicine at Mount Sinai. Both lines were similar, and line H02 was used for further analysis. The probes for the Southern blots were derived by PCR with the following probes: NsiI forward, AATGGACTGGCGTTGTGTACTGG; NsiI reverse, GTTTGTGTGTGTG; NheI reverse, AAAGCCCTATTCCAGCCCAGC. See the legend for Figure S1 for further details.

Cdon\textsuperscript{m30} mice were crossed with Pax\textsuperscript{CreERT2} mice (obtained from the Jackson laboratory). The primers used for genotyping are listed in Table S1. Tamoxifen (tmx) (Sigma-Aldrich, 1.5 mM) was administered intraperitoneally every 2 days for five times prior to muscle injury. To induce the skeletal muscle regeneration, mice were anaesthetized with a 1–2% isoflurane followed by CTX (Sigma-Aldrich, 10 μM) injection into tibialis anterior (TA) muscle in a volume of 25 μl. Zmpste24\textsuperscript{-/-} mice (kindly gifted by Dr. Zhongjun Zhou, University of Hong Kong) and wild type (WT) control mice were sacrificed to analyze satellite cells. All animal experiments were approved by the Institutional Animal Care and Research Advisory Committee at Sungkyunkwan University School of Medicine Laboratory Animal Research Center and complied with the regulations by the institutional Ethics Committee.

Cell culture and single myofiber isolation

C2C12 myoblasts and 293 T cells were cultured as previously described.\textsuperscript{27} To determine the response to basic FGF (bFGF; Sigma-Aldrich), C2C12 myoblasts were changed from Dulbecco’s Modified Eagle Medium (DMEM) containing 15% foetal bovine serum (FBS) to DMEM containing 0.2% bovine serum albumin for overnight, followed by 5 ng/mL bFGF treatment as indicated. For adhesive culture, C2C12 cells were cultured either on matrigel (Corning) or poly-L-lysine (PLL) (Sigma-Aldrich) coated petri dishes for 2 days. Primary myoblasts were isolated from hindlimbs of Cdon\textsuperscript{+/+}, Cdon\textsuperscript{-/-}, and Cdon\textsuperscript{m30}/Pax\textsuperscript{CreERT2} mice as previously described.\textsuperscript{28}

For the Cre recombinase-mediated in vitro deletion of Cdon, 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich, 1 μM) were treated for 4 days after amplification of isolated Cdon\textsuperscript{m30}/Pax\textsuperscript{CreERT2} primary myoblasts in F10 medium (Gibco) containing bFGF (2.5 ng/mL) and 20% FBS. Single myofiber isolation was performed as previously described.\textsuperscript{27} Briefly, extensor digitorum longus (EDL) muscles were digested with collagenase 2 ( Worthington, 400 unit/mL), followed by seeding on matrigel-coated plates in DMEM medium containing 20% FBS. Lentivirus-expressing shRNA Cdon (shCdon) was generated with a modified lentiviral vector derived from pLVX.1 (Sigma-Aldrich) in HEK 293 T cells using helper plasmids pCMV-VSV-G and pCMV DR8.9. The shRNA sequences for Cdon used in this study are as previously reported.\textsuperscript{28}

Tissue preparation, histology, and immunostaining

For histology and immunostaining of muscles, snap freezing and cryosections were carried out as previously described.\textsuperscript{29,30} To assess the structure and fibrosis, fixed cryosections were stained with Mayer’s haematoxylin and eosin (BBC Biomedical) and Picro-Sirius red (Abcam) according to the manufacturer’s protocol. Immunostaining was performed as previously described.\textsuperscript{29,30} Cultured cells, single myofibers, and frozen muscle sections were fixed, permeabilized and processed for incubation with primary antibodies against Cdon, Pax7, Laminin, eMyHC, activated-integrin β1 (\*integrinβ1), cleaved Caspase3, pYH2AX, and secondary antibodies (Thermo Fisher). SA-β-gal staining was conducted by using SA-β-gal kit (Cell Signaling Technology) as previously described.\textsuperscript{31} SA-β-gal staining for muscle tissue was performed as previously described.\textsuperscript{32} Briefly, freshly frozen sections were fixed, followed by incubating X-gal mixture (pH 5.5) for 2 days at 37°C. 5-Ethynyl-2’-deoxyuridine, a nucleoside analog of thymidine (EdU) incorporation assay was performed by adding EdU with a final concentration of 10 μM for 2 h before fixation and then analyzed using the Click-IT EdU kit (Invitrogen) according to the manufacturer’s protocol. Images were acquired under LSM-710 Meta confocal microscope or Nikon ECLIPSE TE2000-U microscope.

Real-time polymerase chain reaction analysis and RNA sequencing

Quantitative real-time PCR analysis was examined as previously described.\textsuperscript{33} cDNA was obtained from total RNA using a PrimeScript RT reagent kit (TAKARA) and analyzed by quantitative real-time PCR using SYBR Premix Ex Tag\textsuperscript{TM} (TAKARA). The primers sequences are listed in Table S1, and each transcript levels were normalized with ribosomal protein L32. RNA sequencing analysis was performed with the Illumina NextSeq500 (ebiogen, Korea). All gene set enrichment analysis (GSEA) plots including GSEA enrichment plot and map were generated with the GSEA software (Broad Institute; software.broadinstitute.org/gsea/).

Protein analysis

Immunoblot and immunoprecipitation analyses were carried out as previously described.\textsuperscript{30} Briefly, tissues and cells were lysed in radioimmunoprecipitation assay (Thermo) buffer

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with complete protease inhibitor cocktail (Roche) and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. For immunoprecipitation, precleared cell extracts were incubated with primary antibodies overnight at 4°C, followed by incubation with protein A-agarose or G-agarose beads (Roche Diagnostics) for 1 h and washing three times with cell extraction buffer. Precipitates were analyzed by western blotting. Surface biotinylation was performed as previously explained. Briefly, biotinylated surface proteins were precipitated with streptavidine-agarose beads (Pierce) and analyzed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Primary antibodies are listed in Table S2.

**Statistical analysis**

All values were analyzed using GraphPad Prism 7 or Excel spreadsheet. To assess the statistical difference, Student’s t-test with two-tailed paired comparison was performed. In case of comparison groups as indicated in the figure legend, we determined the statistical significance by one-way analysis of variance with Tukey post hoc test. Statistical significance was indicated as P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***)]. Error bar were represented by mean ± standard deviation (mean ± SD).

**Results**

*Cdon ablation in satellite cells impairs muscle regeneration*

To investigate the role of Cdon in muscle regeneration, we have analyzed the expression of Cdon in satellite cells by immunostaining of myofibers isolated from EDL muscles. Cdon is expressed in Pax7-positive satellite cells (Figure 1A), preferentially at the basal membrane (Figure S2). To define the *in vivo* function of Cdon in muscle regeneration, we generated mice carrying a floxed allele of *Cdon* (Figure S1). These mice were crossed with *Pax3CreERT2* mice, which express tmx-inducible Cre recombinase in satellite cells but not other cells in skeletal muscle tissue. 3-month-old *Cdon*^{fl/fl};*Pax3CreERT2* mice were injected five times with saline (mock) or tmx starting 8 days prior to the first CTX injury of TA muscles; 21 days after the first injury, a second injury was initiated (Figure 1B). Immunoblotting of TA muscles revealed that Cdon protein was depleted in tmx-treated muscles 7 days after the second injury, compared with the mock saline-treated muscles (Figure S3A and S3B). In addition, quantitative RT-PCR analysis of isolated myoblasts and fibroblasts treated with mock or 4-hydroxytamoxifen (4-OHT) showed that Cdon transcription was specifically decreased in myoblasts (Figure S3C). Cdon ablation in satellite cells mildly impaired muscle regeneration as assessed by myofiber cross-sectional area 21 days after the first injury (Figure S4A and S4B). After the second injury, a more severe impairment in muscle regeneration was observed (Figure 1C). Control muscles displayed myofibers with centrally localized nuclei, characteristic of regenerating muscles 7 days after the second CTX injury (PID7), while Cdon-depleted muscles accumulated single cells in interstitial areas between regenerating myofibers. In addition, immunostaining for laminin and embryonic myosin heavy chain (eMyHC, a marker for immature myofibers) demonstrated that more myofibers in Cdon-depleted muscles at PID7 were positive for eMyHC, compared with control muscles (Figure 1C and 1D). Consistent with this, eMyHC transcript levels were significantly increased in Cdon-depleted muscles, relative to control muscles (Figure 1E). The myofiber size in Cdon-depleted muscles 21 days after the second injury (PID21) was greatly reduced, compared with the control muscles (Figure 1C and 1F). Impaired muscle regeneration processes are frequently linked with aberrant extracellular matrix (ECM) remodelling and fibrosis.

In agreement with this notion, Sirius red staining showed that Cdon depletion in satellite cells led to an exacerbated fibrotic response at PID7 and PID21 (Figure 1G and 1H). Similarly, transcript levels of Collagen 1α1 (Col1α1) and Col1α3 were mildly but significantly increased in Cdon-depleted muscles (Figure 1I). The weights of Cdon-depleted muscles at PID7 or PID21 were also significantly decreased, compared with control muscles (Figure S5). Taken together, these data demonstrate that Cdon ablation impairs satellite cell function.

*Cdon ablation reduces proliferative and self-renewal capacity of satellite cells*

To investigate the detailed mechanism underlying the impaired regenerative capacity of Cdon-ablated satellite cells, we first analyzed their proliferative capacity. To do so, TA muscles of mock or tmx-treated mice were injected and injected with bromodeoxyuridine (BrdU) 2 days after a single injury to label proliferating cells, followed by immunostaining analysis 24 h later (Figure 2A). Forty per cent of cells in control regenerating muscles were BrdU-positive, while ~20% of cells in the tmx-treated muscles were BrdU-positive (Figure 2B and 2C). In addition, TA muscles of mock or tmx-treated mice at 4 days after CTX injury were immunostained for Pax7 and Ki67 (Figure S6). In the control muscles, about 27% of cells were double positive for Pax7 and Ki67, while ~13% of cells in tmx-treated muscles were double positive. To further examine this, single myofibers from EDL muscles were isolated from mock or tmx-treated mice and cultured for 48 h in the presence of bFGF followed by 5-ethyl-2′-deoxyuridine (EdU) incorporation assay (Figure 2D and 2E). In agreement with the results from *in vivo* BrdU

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Figure 1  The ablation of Cdon in satellite cells impairs muscle regeneration. (A) Cdon (red) and Pax7 (green) immunostaining of satellite cell on single myofiber isolated from extensor digitorum longus (EDL) muscles. DAPI labels nucleus (blue). (B) The experimental scheme for repetitive muscle injury. Cdonfl/fl::Pax7CreERT2 mice were injected intraperitoneally with tamoxifen (tmx) five times prior to cardiotoxin injection in the tibialis anterior (TA) muscle. (C) Histological analysis (haematoxylin and eosin, H&E) and immunostaining for laminin and embryonic myosin heavy chain (eMyHC) with mock or tmx-treated TA muscles from 7 (PID7) or 21 days post the second injury. (D) Quantification of eMyHC-positive myofibers in the injured area of PID7 muscles (n = 3, **P < 0.01). (E) Quantitative RT-PCR for eMyHC with control and tmx-treated muscles at PID7. L32 served as an internal control. The value of control muscles was set to 1. (n = 3, **P < 0.01). (F) Quantification of myofiber cross-sectional area (CSA) of PID21 muscles. (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001). (G) Sirius red staining to assess fibrosis. (H) The relative fibrotic area was assessed by using Image J software. (n = 4, ***P < 0.001, Student’s t-test). (I) Quantitative RT-PCR for Col1a1 and Col1a3 with control and tmx-treated muscles at PID7. The value of control muscles is set to 1. (n = 3, **P < 0.01, ***P < 0.001).
incorporation, EdU incorporation was significantly lower in satellite cells on myofibers from tmx-treated mice, compared with control myofibers. Furthermore, Cdon−/− primary myoblasts exhibited significantly reduced BrdU incorporation relative to that of Cdon+/+ myoblasts (Figure 2F and 2G).

However, Cdon-deficient myoblasts showed no overt signs of cell death, as measured by cleaved Caspase 3 immunostaining (Figure S7). Self-renewal of satellite cells is critical for muscle regeneration and maintenance.7,9 Mock or tmx-treated TA muscle sections from PID21 were therefore
immunostained for Pax7 and laminin, and the number of Pax7-positive cells was quantified (Figure 2H and 2I). In control muscle sections, there were about 12 Pax7-positive cells per 100 regenerating myofibers 21 days after the first injury, which decreased to ~10 such cells after the second injury. Cdon-depleted muscles had a significantly decreased number, 5–6 Pax7-positive cells per 100 regenerating myofibers 21 days after each injury. Taken together, these data reveal that Cdon depletion causes reduced proliferative and self-renewal capacity of satellite cells.

**Cdon depletion in satellite cells elicits cellular senescence**

We next analyzed muscles at PID21 for production of Pax7 and pγH2AX (Figure 3A and 3B). Cells double positive for Pax7 and pγH2AX or positive for pγH2AX only were quantified. Cdon depletion resulted in an increased proportion of pγH2AX-positive cells in both Pax7-positive and Pax7-negative populations, compared with controls. To address whether impaired self-renewal and proliferative capacity in Cdon-depleted satellite cells are associated with cellular senescence, mock and tmx-treated muscle sections from PID21 were subjected to SA-β-gal staining (Figure 3C and 3D). SA-β-gal-positive cells were rarely found in the control muscles, while Cdon-depleted muscles averaged 11 SA-β-gal-positive cells per field. To confirm the effect of Cdon depletion on cellular senescence, primary myoblasts isolated from Cdon<sup>+/−</sup>; Pax7<sup>CreERT2</sup> mice were treated in vitro with dimethyl sulfoxide or 4-OHT for 4 days in the presence of bFGF and subjected to SA-β-gal staining (Figure 3E and 3F). More than 30% of Cdon-depleted myoblasts were positive for SA-β-gal staining, while <5% of dimethyl sulfoxide-treated control myoblasts were SA-β-gal-positive cells. Additionally, Cdon<sup>+/−</sup> primary myoblasts exhibited significantly increased pγH2AX-positive cells relative to that of Cdon<sup>+/+</sup> myoblasts (Figure S8). These data indicate that Cdon depletion resulted in cellular senescence and suggested it produces defective responsiveness to bFGF.

Similar to Cdon-deficient satellite cells, C2C12 cells infected with a Cdon shRNA lentivirus expressed higher levels of p21 and p16 proteins, compared with control lentivirus-infected cells (Figure 3G). Cdon-depleted C2C12 cells also proliferated less efficiently than control cells (Figure 3H). SA-β-gal staining and pγH2AX immunostaining revealed that Cdon depletion significantly elevated SA-β-gal-positive cells and pγH2AX-foci, compared with control (Figure 3I–3K). To further examine this, qRT-PCR analysis for SASP genes (Igfbp5 and interleukin-6) and E2F target genes was performed in control or Cdon-depleted C2C12 cells (Figure 3L). Similar to the SA-β-gal staining, the expression of Igfbp5 and interleukin-6 was significantly increased in Cdon-depleted cells. In contrast, the E2F target genes Cyclin E1, Lamin B1, Mcm3, and Cdc6 were mildly but significantly decreased in Cdon-depleted cells, compared with the control cells. Taken together, these data suggest that Cdon depletion in satellite cells and myoblasts results in cellular senescence and impaired stem cell function, likely contributing to decreased muscle regeneration.

**Cdon depletion in satellite cells alters global gene expression profiles induced by muscle injury**

To determine the regulatory pathways underlying regeneration defects caused by Cdon deficiency, we performed RNA sequencing analysis with muscles of either mock-injected or tmx-injected mice 4 days after a single injury (Figure 4A). To identify groups of genes associated with observed phenotypes in Cdon-deficient satellite cells, GSEA, as an unbiased approach, was performed (Figure 4B–4E). GSEA analysis revealed that gene sets implicated in endogenous stimulus, external stimulus, and negative regulation of response to stimulus were significantly altered (dashed lines indicate nominal P = 0.05 and false discovery rate q = 0.05) (Figure 4B and Figure S9). Moreover, KEGG pathway mapping showed that genes associated with arginine and proline metabolism, ECM-receptor interaction, pathways in cancer, and MAPK signalling pathway were altered in Cdon-depleted muscles. Enrichment Map (Figure 4C), a network-based method for gene set enrichment visualization, revealed that the key seven gene sets are tightly associated with each other, implying that genes of those seven pathways underpin the phenotypes of Cdon-deficient satellite cells. Additionally, the gene expression patterns of those gene sets (e.g. KEGG MAPK signalling pathway and GO Response to external stimulus) seemed to be divided in half (Figure 4D and 4E). Furthermore, genes involved in inflammatory response, ECM, negative regulation of cell population proliferation were perturbed in Cdon-depleted muscles (Figure S10). Interestingly, the expression of myogenic regulatory factors, including Myf5, Myogenin, Pax7, Pax3, Myod1, and Mef2c were not significantly altered between control and Cdon-deleted muscles (Figure S11A), suggesting satellite cell activation initiated normally.

**Cdon ablation in satellite cells impairs extracellular signal-regulated kinase activation and fibroblast growth factor response**

To further examine whether decreased proliferation of Cdon-depleted satellite cells is because of perturbed MAPK signalling, qRT-PCR analysis of genes regulated by the ERK was performed. Expression of FosB, Fos, and Egr2, immediate early response genes induced by ERK, was significantly decreased, while expression of Oas1a and Oas2, which is inhibited by ERK, was elevated (Figure 5A). Furthermore, Cdon-deficient
primary myoblasts cultured in the presence of bFGF and Cdon-depleted C2C12 cells in growth medium exhibited significantly decreased Egr1 expression (Figure 5B). The RNA sequencing data showed no significant alteration in expression of growth factors (various FGFs and HGF) or FGF receptors that are related to ERK activation (Figure S11B). Similarly, Cdon+/+ and Cdon−/− myoblasts had similar levels of FGFR1 and FGFR4 proteins (Figure 5C). To explore further, control and Cdon-depleted C2C12 cells were serum starved for 16 h and then treated with bFGF (5 ng/mL) for 5, 10, or 30 min and analyzed for expression of Fos and Egr1 by qRT-PCR (Figure 5D). In line with the results shown in Figure 5B, Cdon-depleted C2C12 cells showed significantly delayed and reduced Fos and Egr1 expression, compared with the control cells. Treatment with bFGF for 2 min greatly elevated active, phosphorylated ERK1/2 (p-ERK1/2) levels in control cells, and this was further increased at 5 min of treatment (Figure 5E). In contrast, Cdon-depleted cells exhibited starkly attenuated

Figure 3  Cdon ablation in satellite cells induces cellular senescence of myoblasts. (A) Immunostaining of phospho-γH2AX (pγH2AX) and Pax7 on muscle sections at PID21 after the second injury. (B) Quantification of pγH2AX-positive cells in the Pax7-negative or Pax7-positive populations. (n = 4; mock, 170–276 nuclei per sample; tmx, 278–340 nuclei per sample, *P < 0.05, ***P < 0.001). (C, D) SA-β-gal staining of TA muscles at PID21 after the second injury (147–270 myofibers per sample were counted. n = 3, *P < 0.05). (E, F) SA-β-gal staining of isolated primary myoblasts from Cdon+/LPLF;Pax7CreERT2/+ mice. Isolated cells were treated with 1 μM 4-hydroxytamoxifen (4-OHT) for 4 days. (n = 3, ***P < 0.001). (G) Immunoblot analysis for cell cycle inhibitors p21 and p16 in control or shCdon-expressing lentivirus infected C2C12 myoblasts. (H) Growth curves of control and Cdon-depleted C2C12 myoblasts. (n = 3, *P < 0.05, **P < 0.01). (I) SA-β-gal staining and pγH2AX immunostaining in control lentivirus-expressing and shCdon-expressing lentivirus-infected C2C12 myoblasts. (J) Quantification for SA-β-gal-positive cells in Panel I (n = 3, **P < 0.001). (K) Quantification for pγH2AX foci of C2C12 cells in Panel I (n = 3, **P < 0.01). (L) Quantitative RT-PCR for SASP and E2F target genes in control and Cdon-depleted C2C12 myoblasts (n = 3, *P < 0.05, ***P < 0.001).
ERK activation in response to bFGF. To assess whether this decrease in ERK activation also occurred in regenerating Cdon-depleted muscles, mock and tmx-treated muscles at PID4 were subjected to immunoblotting. The levels of p-ERK1/2 were substantially reduced in Cdon-depleted muscles, compared with control muscles (Figure 5F and 5G). Collectively, these data demonstrate that the ablation of Cdon in satellite cells impairs ERK activation in response to FGF signalling, likely contributing to defective proliferation and regeneration.

To investigate the potential mechanism leading to the impaired FGF responsiveness in Cdon-deficient satellite cells, we analyzed a potential interaction between FGFR and Cdon in C2C12 cells by coimmunoprecipitation with control IgG or Cdon antibodies (Figure 5H). FGFR1 and FGFR4 proteins were coprecipitated with Cdon in C2C12 cells. Next, we assessed whether Cdon regulates the surface localization of FGFRs by surface biotinylation (Figure 5I). Interestingly, the surface levels of FGFR1 and FGFR4 decreased in Cdon-depleted cells while the surface cadherin level was largely unchanged, relative to the control cells. In a previous study, we demonstrated that Cdon levels were decreased in C2C12 myoblasts cultured on a non-specific cell adhesion promoting substratum, PLL. Cdon depletion by siRNA or by culture on PLL decreased integrin/FAK/ERK signalling.

To further explore this observation, C2C12 cells were cultured on PLL-coated or Matrigel-coated petri dishes to promote integrin signalling in presence of serum. The levels of Cdon, p-ERK, and pFAK were greatly decreased in cells cultured on PLL while p21 levels were strongly enhanced (Figure 5J). Cells cultured on PLL exhibited starkly blunted proliferation, evident by significantly decreased BrdU incorporation and a concurrent...
Figure 5  Cdon depletion causes reduced responsiveness to bFGF treatment and Cdon regulates FGFR1/4 surface expression. (A) qRT-PCR analysis for the expression of ERK target genes (Fos, Fosb, Egr1, Oas1a, and sOas2) and Cdon in Cdon-depleted muscles. (n = 3, *p < 0.05, **p < 0.01, ***p < 0.001). (B) qRT-PCR analysis for Egr1 expression in primary myoblasts with indicated genotypes and control or Cdon-depleted C2C12 cells. (n = 3, *p < 0.05, **p < 0.01). (C) Immunoblot analysis for FGFR1 and FGFR4 in primary myoblasts from Cdon−/− or Cdon−/− mice. β-tubulin served as loading control. (D) qRT-PCR analysis for Fos and Egr1 in C2C12 myoblasts. Cells were serum starved overnight followed by 5 ng/mL bFGF treatment as indicated (n = 3, **p < 0.01, ***p < 0.001). (E) Immunoblot analysis for ERK activation in control and Cdon-depleted C2C12 cells in response to bFGF treatment. (F, G) Immunoblot analysis for ERK and p-ERK1/2 in TA muscles at PID4. The relative signal intensity of p-ERK was plotted, and the value from the mock control was set to 1 (n = 3, *p < 0.05). (H) Coimmunoprecipitation of endogenous Cdon with FGFR1 and FGFR4 in C2C12 cells. (I) Control and Cdon-depleted C2C12 cells were subjected to biotinylation and pull down with streptavidin bead followed by immunoblotting for FGFR1, FGFR4, and cadherin as control. (J) C2C12 cells cultured on normal plastic and poly-L-lysine-coated plates were subjected to biotinylation analysis and pull down with streptavidin bead followed by immunoblotting for Cdon, FGFR1, FGFR4, and cadherin.
increase in SA-β-gal expression, compared with cells cultured on Matrigel (Figure S1C and S1D). Similar to Cdon-depleted cells, C2C12 cells cultured on PLL had diminished surface levels of FGFR1, FGFR4, and Cdon while cadherin levels were largely unchanged (Figure S1J). Collectively, these data suggest that Cdon regulates surface localization of FGFR1 and FGFR4, thereby regulating FGF responsiveness of myoblasts.

Cdon ablation in satellite cells perturbs integrin 1 activation

A previous study suggested that defective integrin signalling is linked with impaired FGF responsiveness, thereby contributing to reduced proliferation of aged muscle stem cells.20 We therefore examined integrin activation in satellite cells on freshly isolated myofibers from vehicle and tmx-treated mice by immunostaining with antibodies to active integrin β1 (Itgb1*) and Cdon (Figure 6A and 6B). About 76% of control Pax7-positive satellite cells had polarized localization of Itgb1* at the basal lamina contact site of satellite cells. In contrast, only ~40% of Cdon-depleted satellite cells had nonpolarized Itgb1* proteins, and they frequently had weaker overall Itgb1* signals. This result resembled the phenotype observed in aged satellite cells.20 The level of Itgb1* protein was assessed in control and Cdon-deficient primary myoblasts (Figure 6C and 6D). Itgb1* levels decreased in Cdon-deficient myoblasts, relative to the control myoblasts while total Itgb1 proteins were unchanged. Additionally, we did not observe any coimmunoprecipitation between Cdon and Itgb1 when overexpressed in 293T cells nor did Cdon depletion reduce cell surface levels of Itgb1 in C2C12 cells (Figure S13). A reduction in expression of the integrin ligand fibronectin has been implicated in aged satellite cell function.22 However, the RNA sequencing data revealed that Fibronectin expression was not altered in Cdon-depleted muscles whereas distinct collagen types (Col1a1, Col5a1, Col3a1, and Col14a1) were significantly altered, likely reflecting the enhanced fibrosis in Cdon-depleted muscles (Figure S10).

To further understand the role of Cdon in muscle aging, we examined expression of Cdon and Itgb1* in satellite cells from mice with the Zmpste24 protease deficiency, of a model for progeria.39 These mice exhibit, among other aging-related phenotypes, premature muscle loss associated with elevated cellular senescence and reduced proliferation of muscle stem cells.40–42 Myofibers isolated from EDL muscles of 4-month-old wild type or Zmpste24−/− mice were immunostained for Cdon, Itgb1*, and Pax7 (Figure 6E–6G). About 90% of Pax7-positive satellite cells from control myofibers were positive for Cdon expression, while about 63% of Pax7-positive cells from Zmpste24-deficient myofibers were positive for Cdon. Similarly to Cdon-deficient satellite cells, more Zmpste24-deficient satellite cells exhibited either weaker or nonpolarized Itgb1* signal, compared with the wild type cells. These data collectively suggest that Cdon and integrin signalling are perturbed in aging satellite cells. Furthermore, SA-β-gal-positive cells were easily observed in Zmpste24-deficient muscles while wild type muscles had only very few cells positive for SA-β-gal-positive cells (Figure 6H and 6I). Additionally, Zmpste24-deficient muscles also exhibited greatly elevated immunostaining for pγH2AX in both Pax7-positive and negative cells compared with wild type muscles (Figure 6J and 6K). These results suggested that Cdon depletion is correlated with satellite cell aging. In summary, our study demonstrates a key role of Cdon in the maintenance of satellite cell self-renewal and proliferation through FGFR trafficking and signalling.

Discussion

In the current study, we determined the role of Cdon in satellite cells by using inducible Pax7-CreERT2-mediated ablation in mice. Cdon is expressed in Pax7-positive satellite cells and more frequently localized basally, similar to Itgb1* proteins. Satellite cell-specific deletion of Cdon led to impaired, fibrotic muscle regeneration, likely attributable to diminished proliferation and cellular senescence of satellite cells; 4 days after CTX injury, we did not observe differences in expression of myogenic regulators, including Myf5, MyoD, and Mef2c, between control and Cdon-deficient satellite cells, suggesting no significant defects in satellite cell activation. In contrast, we detected major alterations in genes related to proliferation, inflammation, and extracellular matrices in Cdon-deficient muscles. This alteration in the transcriptome is well aligned with the decreased proliferation of muscle progenitor cells, impaired muscle regeneration, and increased fibrosis observed in Cdon-depleted muscles.

Regulation and strength of cell adhesion signalling, including integrin and cadherin signalling, is essential to control of quiescence and proliferation of satellite cells.20,21 Loss of N-cadherin and M-cadherin in satellite cells leads to a state of partial activation and long-term expansion of a regeneration-proficient satellite cell pool.21 Alteration in integrin signalling causes reduced responsiveness to FGF, thereby reducing the proliferative capacity of satellite cells.20 Considering the relationship of Cdon with cell adhesion signalling, especially the physical interaction of Cdon with N-cadherin and M-cadherin,23 we predicted a function for Cdon similar to cadherins in the control of satellite cell quiescence and activation. Therefore, the difference in their phenotypes was surprising. One possible mechanism might be distinct cellular localizations in satellite cells. Cadherins are localized at the myofiber contact side of satellite cells, while Cdon is found more often at the basal lamina contact side, similarly to Itgb1*. This localization appears to be important because
Loss of Cdon results in impaired integrin β1 signalling. (A) Immunostaining for Cdon, activated Itgb1 (Itgb1*) and Pax7 in freshly isolated EDL myofiber. (B) Quantification of Pax7-positive cells, which have weak or mislocalized Itgb1* (n = 3, each group; 8–12 myofibers per animal, *P < 0.05) (C, D) Immunoblot analysis for total Itgb1 and Itgb1* in Cdon−/− or Cdon+/+ primary myoblasts. (n = 3, *P < 0.05). (E) Immunostaining for Cdon, activated Itgb1 (Itgb1*) and Pax7 in freshly isolated EDL myofiber of wild type (WT) or Zmpste24−/− mice. (F) Quantification of double positive cells for Cdon and Pax7. (n = 3, each group; 8–12 myofibers per animal, *P < 0.05) (G) Quantification of Pax7+ cells with decreased or mislocalized Itgb1* (n = 3, each group; 8–12 myofibers per animal, **P < 0.01). (H) SA-β-gal staining of TA muscles from Zmpste24−/− mice. (I) Quantification of SA-β-gal-positive cells in the Pax7-negative or Pax7-positive populations. (n = 3; WT, 214–231 nuclei per sample; Zmpste24−/−, 192–240 nuclei per sample, **P < 0.05).
Cdon-depleted satellite cells showed aberrant regulation of Itgb1*. Consistent with this, satellite cells from Zmpste24-deficient progeric mice also exhibited aberrant Itgb1* localization and Cdon expression. It was shown previously that Zmpste24-deficiency causes cellular senescence and decreased proliferation of muscle progenitor cells,39,41,42 resembling the phenotype of Cdon deficiency. Therefore, aberrant integrin β1 signalling may contribute to the impaired FGF responsiveness of Cdon-depleted satellite cells. Another mechanism for impaired responsiveness to bFGF is likely to be reduction in cell surface FGFR1 and four levels in Cdon-depleted myoblasts. The physical interaction of FGFR1/4 and Cdon resembles the interaction of FGFR with other immunoglobulin superfamily members like N-CAM and L1 in the control of neurite outgrowth.43 This interaction is mediated through a cell adhesion molecule-homology domain of these molecules.43 Although Cdon shares ectodomain structural homology with N-CAM and L1, their sequences are not well-conserved at the amino acid level.44,45

The deletion of Itgb1 in satellite cells caused impaired muscle regeneration via defects in self-renewal and proliferation of satellite cells.20 Furthermore, aged satellite cells exhibited aberrant Itgb1* localization with abrogated FGF responsiveness.20 Cdon ablation caused defects similar to those seen in Itgb1-ablated satellite cells, and aberrant Itgb1 activation was observed in Cdon-ablated cells without affecting Itgb1 cell surface levels. Although Cdon and Itgb1 colocalized at the ECM contact site, we failed to detect a physical interaction between Cdon and Itgb1. It is conceivable that Cdon regulates the surface localization of FGFR in the vicinity of Itgb1 at the ECM contact site, leading to efficient FGFR signalling. Similar to Cdon-depleted myoblasts, C2C12 cells cultured on non-integrin-binding PLL matrix had impaired proliferation and senescence. Thus, it is likely that crosstalk between Cdon and integrin signalling is important for the proliferation of myoblasts, and Cdon deficiency likely causes impaired proliferation of satellite cells via aberrant integrin and FGFR signalling.

Interestingly, Cdon depletion in satellite cells did not cause overt cell death while it elevated cellular stress and senescence, as seen by the expression of p21, SASPs, and p16IP1. Because the above mentioned pathways are not directly implicated in cellular senescence, Cdon likely regulates other pathways also, and further study will be required to identify the direct trigger for cellular senescence in Cdon-depleted cells. In summary, we have demonstrated that Cdon is a critical factor in regulation of integrin and FGFR signalling and thereby the proliferative capacity of satellite cells.

Author contributions

J. H. B., M. H., H. J. J., H. B. K., R. S. K., and J. S. K. developed the study concept and design. J. H. B. M. H., H. J. J., H. B. K., S. J. L., M. H., Y. S. L., and S. C. J. performed experiments and collected data. J. H. B., H. J. J., D. R. R., Y. S. L., and J. S. K. interpreted data, prepared the figures. J. H. B., H. J. J., G. U. B., R. S. K., and J. S. K. wrote the manuscript.

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. (A) Scheme for derivation of a conditional Cdon knockout allele. Cdon<sup>tm3a</sup> is a targeted trap allele with a lacZ reporter-tagged insertion. Cdon<sup>tm8b</sup> is a lacZ-tagged knockout allele obtained after crossing Cdon<sup>tm3a</sup> mice with mice expressing Cre recombinase. Exon 9 is deleted leading to a truncated Cdon-lacZ fusion protein. Cdon<sup>tm8c</sup> is a conditional allele with loxP sites flanking Exon 9, obtained by crossing Cdon<sup>tm3a</sup> mice with mice expressing Flp recombinase. For this study, Cdon<sup>tm8c</sup> mice were generated by crossing Cdon<sup>tm3a</sup> mice with ROSA-Flpo mice (kindly provided by Phil Soriano). Cdon<sup>tm3d</sup> is a knockout allele obtained by crossing Cdon<sup>tm3c</sup> mice with mice expressing Cre recombinase, and is referred to in this manuscript as Cdon<sup>-/-</sup>. For this study, Cdon<sup>tm8c</sup> mice were crossed with Pax<sup>CreERT2</sup> mice, to generate the Cdon<sup>tm3d</sup> genotype specifically in satellite cells. Bold black lines indicate probes used on Southern blots. The predicted Cdon protein from each allele is in green, with or without lacZ (in blue). Yellow boxes represent exons, except the floxed exon 9, which is in orange. Arrows represent primers for screening and genotyping. Primers are: Cdon5: TAGCTTCCCAGAGGGTGTG AGAGC; Cdon3: ATGCTGACATTAGGAGCATAATGCG; LAR3: CAACGGGTTCTCTCTGATGCCCC; CdonF: CCTGGTGATGTTGAG ACATTGTC; LoxR: TGAACGATGGCCAGCTACAGACC. (B) Southern blot analysis. Genomic DNA from the ES cells was digested with NsiI for detection of the recombined 3’ arm and NheI for detection of the recombined 3’ arm. Wt and mutant bands are indicated by arrows. (C) PCR genotyping of each allele. Primers used for each PCR and product size: Cdon<sup>tm3a</sup> b: Cdon5, Cdon3, and LAR3 (wt band is 517 bp, DOI: 10.1002/jcsm.12563
Cdon<sup>tm3b</sup> band is 330 bp; Cdon<sup>tm3c</sup>: Cdon5 and Cdon3 (wt band is 494 bp, Cdon<sup>tm2</sup> band is 603 bp); Cdon<sup>tm3d</sup>: Cdon5, Cdon3, and loxP (wt band is 517 bp, Cdon<sup>tm3d</sup> band is 266 bp). (D) Western blot analysis of the conditional knockout allele, Cdon<sup>tm3d</sup>. Cdon<sup>tm3c</sup> was crossed to Meox2cre mice (which express Cre in the epiblast; kindly provided by Phil Soriano) to generate Cdon<sup>tm3d</sup> mice. Western blot of two wt and ten Cdon<sup>tm3d</sup> embryos. Whole embryo lysates were prepared from four different litters harvested at E10.5 were probed with antibodies to Cdon (R&D Systems) and as a control-actin (Abcam).

**Figure S2.** Cdon (red) and Pax7 (green) immunostaining of satellite cells on single myofibers isolated from EDL muscles. DAPI labels nuclei (blue). Cdon localization in individual Pax7 + cells was quantified and shown in the pie chart as no signal or the site of predominant localization in the whole membrane, basal membrane, or apical membrane. 8-12 myofibers per EDL muscle from three 4-month-old mice were used for immunostaining and total 66 Pax7-positive cells were quantified.

**Figure S3.** (A) Immunoblot of Cdon depletion (B) Quantification of Cdon protein levels in regenerating muscles after Cdon ablation by tamoxifen treatment. (n = 3, *p < 0.05) (C) Quantitative RT-PCR for Cdon. Satellite cells and fibroblasts were isolated from hindlimbs of Cdon<sup>Fop/Fop</sup>;Pax7<sup>CreERT2</sup> mice. (n = 3, ***p < 0.001).

**Figure S4.** (A) Histological analysis (hematoxylin and eosi, H&E) of mock or tmx-treated TA muscles from 21 days post the first injury. (B) Quantification of myofiber size. (n = 3, **p < 0.01, ***p < 0.001).

**Figure S5.** Weights of TA muscles of mock- or tmx-treated Cdon<sup>Fop/Fop</sup>;Pax7<sup>CreERT2</sup> mice at PID7 or PID21. (n = 3, *p < 0.05).

**Figure S6.** (A, B) TA muscles at 4 days post the first injury were immunostained for Pax7 (green) and Ki67 (red). Nuclei were visualized by DAPI staining (blue). Quantification of Pax7 and Ki67- double positive cells and the values presented as percentile relative to total Pax7-positive cells. Total Pax7-positive cells counted were 693 for mock and 579 for tux muscles. (n = 3, *p < 0.05).

**Figure S7.** Immunostaining for cleaved-Caspase 3 in Cdon<sup>+/+</sup> and Cdon<sup>−/−</sup> myoblasts. As a control, cell death was induced by treatment with 1 M staurosporin for 3 hours, n = 5.

**Figure S8.** Immunostaining for pyH2AX in Cdon<sup>+/+</sup> and Cdon<sup>−/−</sup> myoblasts. (n = 5, ***p < 0.001).

**Figure S9.** Top 10 list for enriched GO terms based on biological function (A), KEGG pathway (B) or GO terms on cellular component (C). (*p < 0.05, FDR q value <0.05).

**Figure S10.** Heat maps represent statistically significant gene lists involved in inflammatory response, extracellular matrix, and negative regulation of cell population proliferation that are up- (red) or down-regulated (green) in tmx-treated muscle. (Fold change (FC) ≥ 1.5 or ≤ 0.666, *p < 0.05).

**Figure S11.** (A, B) Volcano plot for representing 877 statistically significant genes. (Fold change (FC) ≥ 1.5 or ≤ 0.666, *p < 0.05). Upregulated genes in tmx-treated muscles are labelled as red while downregulated genes are labelled as green, grey represents other genes, including Muscle regulatory factors (MRFs), Fibroblast growth factors (Fgfs), Fibroblast growth factor receptors (Fgfrs) and Hepatocyte growth factor (Hgf).

**Figure S12.** (A) Immunoblot analysis for Cdon, ERK2, pERK1/2, pFAK, FAK, and p21 in C2C12 cells which were grown on Matrigel- or poly-L-lysine-coated Petri dishes. (B) Fold-change from panel A. pERK or pFAK were normalized by levels of ERK2 or FAK, respectively. The value of the control muscle was set to 1. (n = 3, **p < 0.01, ***p < 0.001). (C, D) SA-β-gal and BrdU staining of C2C12 myoblasts (n = 3, ***p < 0.001).

**Figure S13.** (A) Lysates of 293 T cells transfected with control, Itgb1, and/or Cdon vectors as indicated were subjected to immunoprecipitation with Cdon antibodies and immunoblotting. (B) Control and Cdon-depleted C2C12 cells were subjected to biotinylation and pulldown with streptavidin bead followed by immunoblotting for Itgb1 and Cadherin.

**Table S1.** List of primer sequences

**Table S2.** List of antibodies used in this body

**Conflict of interest**

Ju-Hyeon Bae, Mingi Hong, Hyeon-Ju Jeong, Hye-Been Kim, Sang-Jin Lee, Dongryeol Ryu, Gyu-Un Bae, Sung Chun Cho, Young-Sam Lee, Robert S. Krauss, and Jong-Sun Kang declare that they have no conflict of interest.

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