mTORC1 controls the adaptive transition of quiescent stem cells from G₀ to Gatility

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A unique property of many adult stem cells is their ability to exist in a non-cycling, quiescent state1. Although quiescence serves an essential role in preserving stem cell function until the stem cell is needed in tissue homeostasis or repair, defects in quiescence can lead to an impairment in tissue function2. The extent to which stem cells can regulate quiescence is unknown. Here we show that the stem cell quiescent state is composed of two distinct functional phases, G₀ and an ‘alert’ phase we term GAlert. Stem cells actively and reversibly transition between these phases in response to injury-induced systemic signals. Using genetic mouse models specific to muscle stem cells (or satellite cells), we show that mTORC1 activity is necessary and sufficient for the transition of satellite cells from G₀ into GAlert and that signalling through the HGF receptor cMet is also necessary. We also identify G₀-to-GAlert transitions in several populations of quiescent stem cells. Quiescent stem cells that transition into GAlert possess enhanced tissue regenerative function. We propose that the transition of quiescent stem cells into GAlert functions as an ‘alerting’ mechanism, an adaptive response that positions stem cells to respond rapidly under conditions of injury and stress, priming them for cell cycle entry.

Adult stem cells have been presumed to exist in one of two states: (1) the quiescent state in which the cell is not actively cycling and (2) the activated state where the cell has committed to or is in the cell cycle3,4. In contrast to the cell cycle, which can be subdivided into distinct phases, quiescence is not as well characterized. Emerging data suggest that stem cells can regulate quiescent functional properties5,6. Studying the regulation of the transition of satellite cells (SCs) from the quiescent to the activated state, we made a curious observation—SCs in a muscle contralateral to the muscle in which we induced an injury (contralateral satellite cells, CSCs) responded to that distant injury and had cycling properties that were different from those in a non-injured animal (quiescent satellite cells, QSCs) and from the injured tissue (activated satellite cells, ASCs) (Fig. 1a). Using the Pax5CreER driver and Rosa26EYFP lineage tracer to specifically label SCs7,8 (Extended Data Fig. 1a), we found that these CSCs showed markedly increased, but overall still low, propensity to cycle when compared to QSCs, as measured by BrdU (5-bromodeoxyuridine) incorporation in vivo (Fig. 1b). Upon isolation and culturing ex vivo, CSCs displayed accelerated cell cycle entry as measured by EdU incorporation and time required to complete the first cell division compared to QSCs (Extended Data Fig. 1b). Subsequent cell divisions of progeny of CSCs and QSCs occurred at similar rates to those of ASCs. This functional response was not limited to SCs in muscle groups directly contralateral to the injury or to the agent of muscle injury (Extended Data Fig. 1c–e).

One of the most obvious changes in ASCs is a dramatic increase in cell size relative to QSCs (Fig. 2a). We found that ASCs displayed a very slight, but significant, increase in cell size relative to QSCs (Fig. 2a, b and Extended Data Fig. 2a, b). Similarly, we also observed that ASCs had stronger EYFP intensity from the Rosa26EYFP reporter, elevated levels of pyronin Y staining, and increased incorporation of the ribonucleotide EU compared to QSCs (Extended Data Fig. 2c–e), which suggests increased transcriptional activity. Principle component analysis (PCA) of the transcriptional profiles of QSCs, CSCs and ASCs showed that CSCs fall between QSCs and ASCs along the first component axis (PC1) (Fig. 2c). Transcriptionally, CSCs were highly correlated with both QSCs and ASCs, more strongly than QSCs and ASCs were correlated (Fig. 2c), which also

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Collectively these data describe a set of properties that distinguishes CSCs from QSCs and ASCs: kinetics of cell cycle entry, propensity to cycle, cell size, transcriptional activity and mitochondrial metabolism. Importantly, CSCs, like QSCs, are still quiescent in that, as a population, almost all CSCs are not actively cycling. Because the injury-induced phenotype of CSCs is intermediate between QSCs and ASCs, we refer to CSCs as ‘alert’ SCs and the set of properties that distinguishes these cells as the ‘alert’ phenotype. The characteristics of this alert phenotype described above have a common thread in that they have all been previously linked, in other systems, to the mTORC1 signalling pathway (reviewed in ref. 9). For example, we observed induction of phospho-S6 (pS6), a surrogate of mTORC1 activity, in alert SCs (Fig. 2g, h and Extended Data Fig. 3e–g). Furthermore, we found that by sorting SCs for properties of the alert state (Extended Data Fig. 3b), we enriched for a population of pS6+ SCs that also possessed the other attributes of the alert state (elevated propensity to cycle and reduced time to first division) (Extended Data Fig. 3i–n). Together these data show that there is a strong correlation between activation of mTORC1 signalling and the alert phenotype in SCs.

To test if any aspects of the alert response were directly regulated by mTORC1 signalling, we used the Pax7CreER driver to specifically ablate TSC1, an inhibitor of mTORC1 signalling, in SCs. As a genetic model of mTORC1 activation10, TSC1 knockout (KO) QSCs displayed induction of mTORC1 activity (Extended Data Fig. 4a, b). TSC1 KO QSCs also displayed all aspects of the alert phenotype in an otherwise non-injury context: increased propensity to cycle, accelerated cell cycle entry, increased MitoTracker Deep Red staining and increased cell size (Fig. 3a–c and Extended Data Fig. 4c). To test whether the alert response requires mTORC1, we used a conditional allele of Raptor (Rptor)11, an essential component of the mTORC1 signalling complex, with the Pax7CreER driver to specifically ablate Raptor expression and suppress mTORC1 signalling in SCs (Extended Data Figs 4b and 5a–c). Overall, we found that Raptor KO SCs contralateral to a muscle injury were completely unresponsive to the injury and did not manifest any of the injury-induced characteristics of QSCs.

To gain further insight into what distinguishes CSCs from QSCs, we analysed the molecular pathways enriched in genes induced in the CSC transcriptome relative to the QSC expression profile. We found that two annotation groups were significantly enriched in genes upregulated in CSCs relative to QSCs: cell cycle and mitochondrial metabolism (Extended Data Fig. 3a). To further investigate mitochondrial metabolism in CSCs, we performed MitoTracker Deep Red staining and measured mtDNA content and found that, relative to QSCs, CSCs displayed evidence of elevated mitochondrial activity (Fig. 2d, e). Consistent with these findings, and keeping with the increase in cell size, we also found that CSCs have increased levels of cellular ATP (Fig. 2f and Extended Data Fig. 3b–d).

Figure 2 | Satellite cells that are distant from an injury become ‘alert’. a, Representative images of QSCs, CSCs and ASCs immediately after isolation. b, CSCs are larger than QSCs and ASCs (along PC1) as shown by PCA and Pearson’s r values (r = 3). c, CSCs have a transcriptional profile that is intermediate between QSCs and ASCs (along PC1) as shown by PCA and Pearson’s r that is intermediate between QSCs and ASCs (along PC1) as shown by PCA and Pearson’s r values (r = 3). d, Increased mitochondrial activity in CSCs compared to QSCs (representative FACS plot, n = 4). Unst, unstained. e, CSCs have increased mtDNA content relative to QSCs (n = 3), measured by qRT–PCR and normalized to genomic DNA (gDNA). f, CSCs have more intracellular ATP than QSCs (n = 4). g, Immunofluorescence immunohistochemistry (IF-IHC) staining of tibialis anterior (TA) muscle showing representative pS6+ and pS6− SCs. h, Quantification of IF-IHC staining for pS6 in SCs (n ≥ 3; significance is versus non-injured).

Figure 3 | Activation of mTORC1 is necessary and sufficient for the alert phenotype. a–c, TSC1 KO QSCs display characteristics of alert SCs: increased propensity to cycle in vitro (a, n ≥ 6); reduced time to first division (b, n = 3); increased mitochondrial activity (c, representative FACS plot n = 3). d–f, Raptor KO suppresses induction of the alert state. Raptor KO SCs show no differences in: propensity to cycle in vitro (d, n ≥ 6); time to first division (e, n = 3); and mitochondrial activity (f, representative FACS plot, n = 3). g–i, cMet KO CSCs show no injury-induced regulation of: propensity to cycle in vivo (g, n ≥ 4); time to first division (h, n ≥ 3); and mitochondrial activity (i, representative FACS plot, n = 3).
changes that alert wild-type CSCs display (Fig. 3d–f and Extended Data Fig 5d, e). These data combined show that mTORC1 signalling in SCs is necessary and sufficient for the alert response.

Next, we focused on the signals upstream of mTORC1 which initiate the alert response and which are regulated by injury. Latent hepatocyte growth factor (HGF) is found in the extracellular matrix of many tissues; upon injury it is processed into an active form by serum proteases. Active HGF can regulate mTORC1 via PI3K-Akt signalling. Furthermore HGF is known to influence SC behaviour. To test if HGF signalling has a role in the alert response, we used conditional ablation of the HGF receptor, cMet, to suppress HGF signalling in SCs. Ablation of cMet in SCs completely blocked the activation of mTORC1 signalling, as measured by pS6 staining, in cultured SCs and in vivo in SCs following injury (Extended Data Figs 4b and 5f, g). Consistent with our hypothesis that mTORC1 activation is required for the alert response in SCs, cMet KO CSCs did not exhibit any functional response to injury (Fig. 3g–i and Extended Data Fig. 5h). Collectively, these data suggest that signalling downstream of cMet is critical for the induction of the alert response in SCs.

To gain further understanding of the molecular pathways underlying the functional transition into the alert state, we analysed the transcriptional profiles from the SC-specific genetic models described above. We found that induction of genes involved in mitochondrial metabolism strongly correlated with the ability to transition into the alert state.

Wild-type CSCs and TSC1 KO QSCs show induction and Rptor KO and cMet KO CSCs do not (Extended Data Figs 3a and 7a–e). These data suggest that regulation of mitochondrial metabolism is a crucial aspect of stem cell quiescence.

The function of SCs in response to injury is to proliferate, differentiate and form new muscle tissue. As such, we tested whether the functional changes of CSCs affected their differentiation and muscle regenerative abilities. Following isolation and ex vivo culturing, CSCs displayed enhanced kinetics of differentiation as measured by expression of myogenin (MyoG) and cell fusion (Fig. 4a, b and Extended Data Fig. 8a). To translate these observations in vivo, we assessed the ability of CSCs to participate in muscle regeneration. Three days before injury of the left tibialis anterior (TA) muscle, we performed an ‘alerting’ injury to the right limb to transition SCs in the left TA into the alert state (Fig. 4c).

We found that animals that received an ‘alerting’ injury displayed strikingly enhanced muscle regeneration at all time points following injury when compared to the normal muscle regenerative process (Fig. 4d, e). These data show that the functional properties of alert SCs translate into enhanced muscle regenerative ability in response to injury.

The markedly enhanced muscle regenerative function of CSCs prompted us to investigate other conditions which may induce the alert state in SCs. We found that SCs adopted functional aspects of the alert response to bone injuries and to minor skin wounds (Extended Data Fig. 8b, c), injuries for which the role of SCs is not apparent. These data suggest that SCs can adopt the alert state in response to multiple types of injuries and may be a general response of SCs to injury. Therefore, we tested if other populations of quiescent stem cells could similarly adopt properties of the alert state. We found that fibro-adipogenic progenitors (FAPs), a resident mesenchymal stem cell population in skeletal muscle, responded in a similar way as SCs. CFAPs (FAPs in muscles of a limb contralateral to the site of muscle injury) displayed an induction of mTORC1 signalling, accelerated cell cycle entry, increased propensity to cycle and increased cell size when compared to quiescent FAPs from non-injured animals (QFAPs) (Fig. 4f–h and Extended Data Fig. 9a–c). Additionally, we found that long-term haematopoietic stem cells (LT-HSCs) displayed...
activation of mTORC1 signalling in response to muscle injury (Fig. 4i and Extended Data Fig. 9d). To test if mTORC1 activation in LT-HSCs caused increased functional potential, as it does in SCSs, we then administered interferon-gamma (IFN-$\gamma$), to the animals to stimulate LT-HSC activation\textsuperscript{32}. Similar to the effect of an ‘alerting’ injury on muscle regeneration, LT-HSCs primed by muscle injury were more sensitive to IFN-$\gamma$ and had a more robust response (Fig. 4i). Notably, and similar to what we demonstrated in SCSs, the induction of mTORC1 in HSCs increases their mitochondrial activity\textsuperscript{23,24}, which is consistent with a transition into the alert state. Collectively, these data indicate that activation of mTORC1 signalling in quiescent stem cells alters their properties, endowing them with enhanced functional potential, an alerting mechanism that prepares the cell for potential activation.

As it relates to stem cell biology, the data we present here demonstrate that stem cells undergo dynamic transitions between functional phases in the quiescent state. We propose a model in which $G_{\text{Alert}}$ and $G_0$ are phases within quiescence and form a quiescence cycle (Fig. 4k).

Although it has been suggested that not all quiescent cells are functionally equivalent\textsuperscript{25,26}, the in vivo relevance and the molecular mechanisms regulating functionally distinct states had not previously been elucidated. We propose that mTORC1 activity is a distinguishing aspect of at least two distinct phases within quiescence. Here we demonstrate how these phases of stem cell quiescence in vivo are regulated in the context of physiological conditions by mTORC1 (and, for SCSs, by cMet). Most importantly, our data indicate that the ability to transition between $G_0$ and $G_{\text{Alert}}$ is critical to the positioning of stem cell populations to be able to respond rapidly in tissue homeostasis and repair while maintaining a pool of deeply quiescent, reserve stem cells. This represents a newly identified form of cellular memory, an adaptive response akin to that in neuronal or immune cells, in which prior experience influences future responses.

METHODS SUMMARY

Unless stated otherwise, in the figure legend, all graphical data are presented as mean ± s.e.m., except for histograms, and significance was calculated using two-tailed unpaired Student’s t-tests: *$P<0.05$, **$P<0.01$. When sample size (n values) are reported as a range, exact sample size values can be found in the Methods section. Time to first division experiments are presented as a representative histogram plotting data from individual cells and, on the right, as a bar graph depicting the quantitative analysis of the mean time to first division in replicate experiments.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

**Received 15 January; accepted 13 March 2014.**

**Published online 25 May 2014.**

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Supplementary Information is available in the online version of the paper.

Acknowledgements We would like to thank members of the Rando laboratory for discussions critical to the preparation of this manuscript, especially T. Cheung and S. Biressi. We thank L. Rott for providing assistance with FACS. This work was supported by The Glenn Foundation for Medical Research, by a grant from the Department of Veterans Affairs to T.A.R., and grants from the National Institutes of Health to J.T.R. (K99 AG041764, K.Y.K. (K08 HL098889), M.A.G. (R01 DK092883) and to T.A.R. (R01 AG036695, R01 AG23806 and R01 AR062185). Authors Contributions J.T.R. conceived and designed most of the experiments reported. T.A.R. provided guidance throughout J.T.R., C.B. and N.M. performed experiments and collected data, J.T.R., J.O.B. and L.L. analysed the microarray data. J.T.R. and K.K.M. conceived and performed bioluminescence experiments, J.T.R. and M.J.C. designed primary regeneration experiments. J.T.R. and G.W.C. performed and analysed transplant experiments. K.Y.K., C.-R.T. and M.A.G. conceived, performed and analysed data from the experiments in HSCs, J.T.R. and T.A.R. analysed data and wrote the manuscript. Author Information Array data is deposited in GEO (accession numbers GSE55490 and GSE47177), as previously published (ref. 27) and as Supplementary Data Set 1. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to T.A.R. (rando@stanford.edu).
Satellite cell transplantation. Donor EYFP<sup>+</sup> SCs were FACS purified from Pax<sup>CreER<sup><sub>1</sub></sub></sup>–<sub>Rosa26<sup>EYFP<sup><sub>1</sub></sub></sup></sub> mice, 2.5 DPI (CSCs) or from non-injured animals (QSCs). Donor SCs were counted, washed with PBS and resuspended in PBS with 0.1% BSA at a concentration of 5 × 10<sup>5</sup> cells in 20 μL. The TA muscle of the host, 12-week-old male NSG mice, was prepared by BaCl<sub>2</sub> injury two days before transplantation. The transplantation was performed by anesthetizing the host mice, exposing the TA muscle by opening the skin and fascia, and slowly injecting 20 μl of the donor cell solution into the TA using a 50 μl Hamilton syringe. Each host mouse received transplantation of QSCs into the right TA and CSCs into the left TA. After injection, the skin over the TA was closed by sutures, the mice were administered analgesic and antibiotics and were allowed to recover. Two weeks after transplantation, SC engraftment was measured by FACS of EYFP<sup>+</sup> SCs from the TA muscle of donor mice.

Mitotracker staining. Prior to FACS analysis, 200 nM Mitotracker Deep Red (Invitrogen) was added to muscle digests and incubated for 1 h at 37 °C, with gentle shaking. Digests were washed once and Mitotracker staining was visualized on a BD FACSAria II or III in the APC channel.

Cell culture. SCs and FAPs were cultured on ECM (Sigma) coated poly-d-lysine 8-well chamber slides (BD). 15,000 cells were plated per well and cultured overnight in Hams F10 medium (Cellgro) with 10% fetal bovine serum (Gibco), the next day the medium was switched to Hams F10 with 10% horse serum (Gibco), and cells were cultured in this medium until fixation by PFA. In EdU incorporation experiments, 0.05 mM EdU (Invitrogen) was added to the culture medium and replenished every 12 h until cells were fixed. SCs were fixed 40 h after plating and FAPs were fixed 48 h after plating. For in vivo BrdU pulse labelling experiments, cells were isolated, plated and fixed 2 h after isolation.

Time-lapse microscopy. To perform time-lapse microscopy analysis, 10,000 SCs were plated onto ECM coated 8-well chamber slides and allowed to adhere for several hours to slides. After the cells adhered the medium was changed to Hams F10 with 10% HS and the slides were transferred to an environmentally controlled Zeiss Axiohot 200M equipped AxioCam. Time-lapse data acquisition and visualization was made using AxioVision software and images were captured every 15 min. The time required to complete the first division after plating was recorded only for cells that stayed within the acquisition field. Representative data from time-lapse experiments are displayed as histograms, bar graphs of time to first division display quantification of replicate experiments (mean ± s.e.m.).

ATP measurement. SC ATP levels were measured using the ATP Bioluminescence Assay Kit CLS II (Roche) according to the manufacturer’s instructions. Briefly, 20,000 SCs were counted with a haemocytometer immediately after isolation, pelleted, and boiled in 100 mM Tris, 4 mM EDTA, pH 7.4 for 2 min. After boiling, the debris was pelleted and supernatant was used for analysis.

Immunostaining. Immunofluorescence immunohistochemistry (IF-IHC) was performed on muscle tissue that was mounted with tragacanth gum and snap frozen in isopentane cooled in liquid nitrogen immediately after dissection. The 8-μm sections were fixed in 4% PFA for 5 min and blocked in donkey serum before staining. Pax7 and eMHC staining was performed with the M.O.M. kit (Vector) according to the manufacturer’s instructions. pS6 staining was performed following Pax7 staining by incubating the sections in a solution of PBS, 0.3% Triton X-100, 100 μM donkey serum and rabbit anti-pS6 antibodies at a dilution of 1:100, overnight. Secondary detection of pS6 was performed with donkey anti-rabbit Alexa 647 antibodies (Invitrogen) at a dilution of 1:500.

Immunocytochemistry (ICC) staining was performed on PFA fixed cells that had been cultured on chamber slides. EdU incorporation was visualized by Click-it (Invitrogen) according to the manufacturer’s instructions. For BrdU analysis, cells were fixed with 70% ethanol and treated with 2N HCl for 20 min before staining with BrdU antibodies. Image capture, analysis and quantification were performed using Velocity software.

All displayed immunostaining images are representative of at least 3 independent experiments.

Western blotting. Western blot analysis was performed on whole cell extracts of 1 × 10<sup>4</sup> SCs that were counted, washed and lysed in sample buffer immediately after FACS purification. Lysates were subject to SDS–PAGE, transferred to PVDF membrane, and probed with indicated antibodies. Between antibody probing, the PVDF membrane was stripped using Restore Western Blotting Stripping Solution (Pierce).

Bioluminescence. Photo-emission was measured using 10,000 (CD<sup>3<sup>1</sub></sup>, CD<sup>4<sup>−</sup></sub>, Sca<sup>−1</sup>, VMAC<sup>−</sup>) SCs purified from FACS of non-injured or 2.5 DPI Pax<sup>CreER<sup><sub>1</sub></sub></sup>–<sub>Rosa26<sup>EYFP<sup><sub>1</sub></sub></sup></sub> mice. SCs were allowed to adhere to ECM-coated 6-well plates for 1 h before addition of luminal and imaging with an IVIS imager.

mtDNA quantification. Total DNA was isolated from 10,000 SCs immediately after FACS isolation using QIAamp DNA mini kit (Qagen) according to the manufacturer’s instructions. mtDNA was quantified by qRT–PCR using primers amplifying the Cytochrome B region on mtDNA (forward primer: 5’-CATTATTAT

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TATCGGGGCGCTA-3', reverse primer: 5'-TGTGGTTGGTTGTGATCTG-3' relative to the β-globin region on gDNA (forward primer: 5'-GAAGCGATTTCTAGGAGCAG-3', reverse primer: 5'-GAAGCGATTTCTAGGAGTAGA-3').

Cell size measurements

FACS. Throughout the dissection, digestion, and processing of muscle tissue (described above), preparations were maintained at 37°C. Immediately upon completion of the isolation protocol, mononuclear rEF1™ cells were analysed in the forward scatter channel (FSC) by FACS to assess cell size. Data are presented as representative histograms.

Microscopy. Immediately after isolation, QSCs, 2.5 DPI CSCRs and 2.5 DPI ASCs were plated on 8-well chamber slides and allowed to adhere for 30 min at 37°C, after which the media was aspirated, chambers removed and a coverslip applied. Bright field images of the slides were acquired using an Axioskop 2 with a ×40 objective lens. The analysis of cell diameter was performed using AxioVision software (Zeiss).

Antibodies. Antibodies used in this study were: anti-Pax7 (ab27277) and anti-eMHC (F1.652) from DSHB; phospho-S253/365/370 (ab81584 and ab8577) and phospho-S2448/2451 mTOR (ab92439) from Cell Signaling; rabbit anti-Mac1 (ab218915) and anti-CD4 (ab24325), anti-CD8 (ab24331), anti-β2m (ab6271), anti-α-tubulin (ab6271), anti-actin (ab6271) from Sigma. For HSCs: lineage antibodies: anti-B220 (ab28394) and anti-CD45 (ab28395) from e Biosciences; anti-β2m (ab28394) from AbCam; anti-PDGFRα (ab10162) from R&D Systems; anti-Myc (566358) and anti-MyoD (554130) from BD; anti-BrdU (ab100030C) from Serotec; actin (ab3854) from Sigma. For HSCs: lineage antibodies: anti-B220 (515-0452-82), anti-CD4 (515-0042-82), anti-CD8 (515-0081-82), anti-Mac1 (515-0112-82), anti-Gr1 (515-5931-82), anti-Ter119 (515-5921-82); anti-Ly6A (Sca1) (52-5981-81), anti-CD117 (C174) (747-1171-82), anti-CD150 (12-1502-82), anti-CD48 (17-0481-82), anti-EPOR (17-2012-80) from eBioscience.

Transcriptional profiling and pre-processing. For each sample, total RNA was isolated from tissues using TRIzol (Invitrogen) extraction followed by RNeasy Plus Micro Kit (Qiagen) from approximately 400,000 SCs pooled from ≥4 mice. Hybridization to GeneChip Mouse Gene 1.0 ST Affymetrix arrays was performed by the Stanford Protein and Nucleic Acid Facility. Raw data files are available at the NCBI GEO database (accession numbers GSE55490 and GSE47177).

Intensities were pre-processed using the Expression Console (Affymetrix) for all analyses were performed on mean-centred log2-transformed expression levels.

General methods. Unless stated otherwise, sample size (n values) are reported as biological replicates of mice and/or SC isolations from separate mice performed on different days. In figure legends where sample size is reported as a range, the exact sample size values are reported below:

Fig. 1b: QSCs (n = 13), CSCs (1.5 DPI, n = 3; 2.5 DPI, n = 7; 3.5 DPI, n = 5), ASCs (1.5 DPI, n = 3; 2.5 DPI, n = 5; 3.5 DPI, n = 5).
Fig. 1c: CSCs (n = 11), ASCs (1.5 DPI, n = 2; 2.5 DPI, n = 2; 3.5 DPI, n = 2), ASCs (1.5 DPI, n = 2; 2.5 DPI, n = 2; 3.5 DPI, n = 2).
Fig. 2e: QSCs (n = 11), WT CSCs (n = 7), Raptor KO QSCs (n = 7), Raptor KO CSCs (n = 6).
Fig. 2f: wild-type QSCs (n = 11), WT KO QSCs (n = 7), cMet KO QSCs (n = 4), cMet KO CSCs (n = 5).
Fig. 4e: Normal regeneration (3.5 DPI, n = 6; 6 DPI, n = 5; 11 DPI, n = 3; 15 DPI, n = 3; 24 DPI, n = 4), Alert regeneration (3.5 DPI, n = 5; 6 DPI, n = 5; 11 DPI, n = 3; 15 DPI, n = 3; 24 DPI, n = 4).
Fig. 4h: QFAPs (n = 6), CFAPs (each point, n = 2), AFAPs (each point, n = 2).
Fig. 4j: non-injured (n = 4), injured (n = 5).
Fig. 4k: non-injured + PBS (n = 4), non-injured plus IFN-γ (n = 4), injured plus PBS (n = 3), injured plus IFN-γ (n = 4).

Extended Data Fig. 1c: non-injured (n = 2), injured (R-TA, n = 3; L-TA, n = 3; R-Quad, n = 2, triceps, n = 2).
Extended Data Fig. 3c: (n = 6), Extended Data Fig. 3k: (n = 5), Extended Data Fig. 3m: (n = 6).
Extended Data Fig. 5g: wild-type QSCs (n = 6), wild-type CSCs (n = 4), cMet KO QSCs (n = 3), cMet KO CSCs (n = 6).
Extended Data Fig. 6a: AQP1 (n = 5), 14 DPI (n = 3), 28 DPI (n = 3).
Extended Data Fig. 6b: AQP1 (n = 13), CSCs (1.5 DPI, n = 3; 2.5 DPI, n = 3; 3.5 DPI, n = 5; 7 DPI, n = 2; 14 DPI, n = 3; 21 DPI, n = 2; 35 DPI, n = 2), ASCs (1.5 DPI, n = 3; 2.5 DPI, n = 5; 3.5 DPI, n = 5; 7 DPI, n = 2; 14 DPI, n = 3; 21 DPI, n = 2; 35 DPI, n = 2).
Extended Data Fig. 6c: QSCs (n = 11), CSCs (2.5 DPI, n = 2; 7 DPI, n = 2; 14 DPI, n = 2; 21 DPI, n = 2; 28 DPI, n = 2; 35 DPI, n = 6), ASCs (2.5 DPI, n = 2; 7 DPI, n = 2; 14 DPI, n = 2; 21 DPI, n = 2; 28 DPI, n = 2; 35 DPI, n = 4).
Extended Data Fig. 9c: QFAPs (n = 6), CFAPs (each point, n = 2), AFAPs (each point, n = 2).
Extended Data Fig. 9d: non-injured (n = 4), injured (n = 5).

In most cases, the data presented were compiled over the course of 2 years, as mice with the appropriate genotype became available. Therefore the magnitude of the effect and variability in the measurements were primary factors in determining sample size and replication of data. Although samples were not explicitly randomized or blinded, mouse identification numbers were used as sample identifiers and thus the genotypes and experimental conditions of each mouse/sample were not readily known or available to the experimenters during sample processing and data collection. The only criteria used to exclude samples involved the health of the animals, such as visible wounds from fighting. In these cases, the animals were handled in accordance with approved IAUCUC guidelines.

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Extended Data Figure 1 | SCs distant from the site of an injury display a functional response to the injury. a, Representative FACS plot from isolation of EYFP^+ SCs from 10-week-old Pax7CreER<sup>+</sup>; Rosa26<sup>EYFP+</sup> mice 3 weeks following TMX treatment. Mononuclear cells from muscle digests were gated in FITC and Pac-Blue (autofluorescence) channels to separate EYFP^+ SCs. EYFP^+ SCs were usually 2–4% of all events from muscle digests. b, Progeny of CSCs and QSCs take comparable times to complete the second cell division. Analysis of the time required to complete the second division (QSCs 10.2 ± 2 h, n = 148 cells; CSCs 10.9 ± 2 h, n = 155), following the first cell cycle (Fig. 1d), shows that accelerated cell cycle kinetics of CSCs is limited to the first division. c, SCs throughout the body increase in propensity to cycle in response to injury. In injured animals, SCs isolated from indicated muscle groups show higher frequency of BrdU incorporation when compared to SCs from the same muscle groups from non-injured mice (n ≥ 2 animals). d, Muscle crush injuries increase the in vivo cycling propensity of CSCs. Twelve hours after BrdU pulse labelling, SCs isolated from TA and Gast muscles contralateral to muscle crush injury show elevated BrdU labelling frequency versus SCs from those muscles from non-injured mice (mean ± s.e.m.; non-injured, n = 5 animals; muscle crush, n = 3; **P < 0.01). e, SCs contralateral to a muscle crush injury have increased cell cycle entry kinetics. 2.5 DPI SCs contralateral to a muscle crush injury incorporate EdU more rapidly than QSCs when cultured ex vivo for 40 h (mean ± s.e.m., n = 3 animals, *P < 0.05).
Extended Data Figure 2 | CSCs are distinct from QSCs but retain stem cell characteristics. 

**a**, CSCs are slightly larger than QSCs and much smaller than ASCs. Immediately after isolation, analysis of cell diameters of QSCs, 2.5 DPI CSCs and 2.5 DPI ASCs, measured by phase contrast microscopy, shows that CSCs have a distribution that is shifted to the right compared to QSCs (histographic representation of data displayed in Fig. 2a, b). 

**b**, CSCs are larger than QSCs as measured by the FSC parameter by FACS (representative FACs plot, similar results observed in 4 independent experiments). 

**c**, CSCs have elevated intensity of an EYFP reporter. FACS analysis of EYFP intensity in the FITC channel shows that 2.5 DPI CSCs display a slight shift in EYFP distribution relative to QSCs, suggesting increased expression of this reporter from the Rosa26 locus (representative FACs plot, similar results observed in 4 independent experiments). 

**d**, CSCs show elevated levels of pyronin Y staining, suggesting an increased RNA content relative to QSCs, but substantially less than ASCs (representative FACs plot, similar results observed in 4 independent experiments). 

**e**, CSCs increase global transcriptional activity compared with QSCs. FACs analysis of EU incorporation, following pulse labelling by i.p. injection, shows that 2.5 DPI CSCs display a greater than 95% of SCs recovered were found to be Pax7+ as measured by ICC (data not shown).

**f**–**i**, Immunocytochemical (ICC) staining of QSCs, 2.5 DPI CSCs and 2.5 DPI ASCs immediately after isolation shows that CSCs are highly similar to QSCs in expression of the QSC marker Pax7 (**f**), as well as markers of SC activation, MyoD (**g**) and Ki67 (**h**), and myogenic differentiation, MyoG (**i**). (mean ± s.e.m.; n = 4 animals; *P < 0.05, **P < 0.01). 

**j**, CSCs have comparable ability to engraft as QSCs. EYFP+ QSCs and 2.5 DPI CSCs were isolated from donor mice (Pax7CreER/++; Rosa26EYFP/++). A total of 5 x 10^4 EYFP+ QSCs were transplanted into the left TAs and 5 x 10^4 EYFP+ CSCs were transplanted into the right TAs of host NSG mice. Two weeks after transplantation, EYFP+ SCs were isolated from TA muscles of host mice and SC engraftment efficiency was measured as the number of EYFP+ SCs that were recovered as a percentage of the number of donor SCs that were transplanted (n = 4, red line indicates mean). For both donor cell populations, greater than 95% of SCs recovered were found to be Pax7+ as measured by ICC (data not shown).

**k**, CSCs that incorporate BrdU self-renew. Following injury to one TA muscle, mice were administered BrdU continuously for 4 days followed by 21 days of chase (as shown in the diagram). IF-IHC analysis of the TA contralateral to the injury revealed BrdU+ Pax7+ cells in the satellite cell position beneath the basal lamina. An example of such a cell is illustrated here (top row of images). On the right is quantification of BrdU+ SCs after 21 days of chase by ICC after FACS isolation, showing that CSCs have self-renewal capacity similar to QSCs (mean ± s.e.m., n = 3 animals, *P < 0.05). Below is an example of a BrdU+ myonucleus in the contralateral fibre following proliferation.
Extended Data Figure 3 | CSCs have elevated mitochondrial and mTORC1 activity. **a**, Induction of genes involved in the cell cycle and mitochondrial metabolism in CSCs. Pathway analysis of genes that were induced in CSCs versus QSCs showed enrichment of genes involved in the cell cycle and mitochondrial metabolism. Redundant KEGG pathways that contain overlapping genes were assembled into annotation groups (details of array and enrichment analysis are found in the Methods section).

**b**, CSCs have slightly increased cell volume compared to QSCs. Cell volume was calculated from cell size measurements (Fig. 2b) (mean ± s.e.m., n = 4 animals, *P < 0.05, **P < 0.01 compared to QSCs).

**c**, CSCs have a slightly greater intracellular ATP concentration than QSCs (mean ± s.e.m., n = 4 animals, *P < 0.05 compared to QSCs).

**d**, Increase in photo emission from CSCs expressing luciferase reporter (LuSEAP). Immediately after isolation and plating, bioluminescence imaging of \(1 \times 10^4\) Pax7CreER; Rosa26 LuSeAP SCs shows that 2.5 DPI CSCs have greater luminescence than QSCs, ASCs have substantially elevated luminescence. Activated fibro-adipogenic progenitors (AFAPs) were isolated from the same injured muscle as ASCs and plated as a negative control for LuSEAP expression. Light emission from luciferase is dependent on the amounts of luciferase enzyme, ATP and luciferin. Increased ATP and increased expression from the Rosa26 locus in CSCs (Fig. 2) and Extended Data Fig. 2c) could both contribute to increased luminescence. Data presented are from a representative experiment with similar results observed in two independent experiments. **e**, Low magnification image of IF-IHC staining of TA muscle. Boxed areas are of the representative pS6 on and pS6 off SCs that are shown in Fig. 2g.

**f**, CSCs have increased levels of pS6 as shown by western blot analysis of whole-cell extracts from \(1 \times 10^5\) cells of each population collected immediately after isolation.

**g**, CSCs show a bimodal distribution of pS6 staining at 1 DPI, with peaks corresponding to the signal in pS6 off QSCs and pS6 on ASCs when analysed by FACS (representative FACS plot, similar results observed in 3 independent experiments). **h**, Sorting SCs for properties of the alert state (that is, high levels of MitoTracker Deep Red (MTDR) staining and YFP expression) enriches for SCs that display the other properties of alert SCs: elevated mTORC1 activity, reduced time to first division and increased propensity to cycle. Representative gating of MTDRHi;EYFPHi SCs (Hi) and MTDRLo;EYFP Lo SCs (Lo).

**i–m**, Sorting of Hi SCs reveals a sub-population of QSCs that displays characteristics of the alert state. Hi SC cells have increased mTORC1 activity (**i**), an increased propensity to cycle in vivo (**j**), and an accelerated time to first division (**k**). Both Hi and Lo SCs stain positive for the SC marker, Pax7 (**l**). 12 h after an in vivo EdU pulse, most SCs that incorporate nucleotide (quantified in **m**) stain positive for pS6 (**m**). Panels **i–m** are displayed as mean ± s.e.m., n = 3 animals, *P < 0.05, **P < 0.01.
Extended Data Figure 4 | TSC1 KO SCs show induction of pS6 and increased cell size. a, TSC1 KO increases SC pS6 levels. IF-IHC staining shows no pS6 staining of SCs in wild-type TA muscle and strong staining of SCs in TSC1 KO TA (representative images of low-magnification muscle section, numbered boxed regions are shown in high magnification below). b, Levels of pS6 in SC-specific KO models. TSC1 KO SCs show induction of pS6 when compared to wild-type QSCs, whereas Rptor KO QSCs and CSCs show no detectable pS6. cMet KO QSCs show comparable levels of pS6 as wild-type QSCs. However, unlike wild-type CSCs, cMet KO CSCs show no induction of pS6. Displayed is western blotting analysis of whole cell extracts from $1 \times 10^5$ cells per each population/genetic model collected immediately after isolation. The first three lanes (WT: QSCs, CSCs and ASCs) are the same as Extended Data Fig. 3f and are redisplayed for the purpose of comparison. c, TSC1 KO SCs are larger than wild-type SCs (representative FACS plot, similar results observed in 4 independent experiments).
Extended Data Figure 5 | Rptor and cMet KO SCs contralateral to injury display no ‘alerting’ response. a, Depletion of Rptor protein in Rptor KO SCs. ICC staining of EYFP+ SCs cultured for 40 h after isolation shows that Rptor protein is undetectable in Rptor KO SCs but clearly detectable in wild-type SCs. b, Absence of pS6 in Rptor KO SCs. ICC staining shows that after 40 h in culture, EYFP+ wild-type SCs stain strongly pS6+ whereas EYFP+ Rptor KO SCs do not exhibit any detectable pS6 signal. c, PCR verification of Rptor exon 6 excision in Rptor KO SCs. Using primers flanking the floxed exon 6 of the Rptor genomic locus, PCR analysis of genomic DNA from SCs isolated from a Rptor conditional KO animal (Rptor<sup>fl/fl</sup>;Pax7<sup>CreER<sub>1</sub></sup>;Rosa26<sup>EYFP<sub>1</sub></sup>) shows efficient recombination of the floxed allele, whereas analysis of genomic DNA from SCs from a wild-type animal (Rptor<sup>1/1</sup>;Pax7<sup>CreER<sub>1</sub></sup>;Rosa26<sup>EYFP<sub>1</sub></sup>) and FAPs from a Rptor conditional KO animal does not show recombination. d, FACS analysis reveals that Rptor KO SCs are slightly smaller and display a slight leftward shift in FSC distribution relative to wild-type SCs. e, Rptor KO SCs do not enlarge in response to contralateral injury. 2.5 DPI, Rptor KO CSCs show a nearly identical FSC distribution to that of Rptor KO QSCs and do not increase in size in response to contralateral injury as do wild-type CSCs (d). a–e, Representative data, similar results observed in at least 3 independent experiments. f, cMet is required for phosphorylation of S6 by HGF. In culture, wild-type SCs show a robust increase in the frequency of pS6+ SCs in response to a 1 h stimulation with HGF whereas cMet KO SCs show no change in pS6 staining frequency (mean ± s.e.m., n = 4, **P < 0.01). g, cMet KO prevents induction of pS6 in SCs contralateral to injury as measured by IF-IHC (mean ± s.e.m.; n = 3 animals, ≥ 50 Pax7<sup>+</sup> SCs analysed from each animal; *P < 0.05). h, cMet KO CSCs do not change in size. FACS analysis shows that cMet KO and wild-type QSCs have similar FSC distributions and that this distribution is not altered in cMet KO SCs contralateral to an injury (a representative FACS plot is shown; similar results were observed in 3 independent experiments).
Extended Data Figure 6 | The functional properties of alert CSCs revert back to the QSC state 28 DPI. 

**a**, Frequency of pS6 $^+$ CSCs returns to non-injured levels 28 DPI. Quantification of the percentage of pS6 $^+$ SCs by IF-IHC shows that immediately following injury, most CSCs (orange bars) become pS6 $^+$. The frequency of pS6 $^+$ CSCs decreases to levels observed in non-injured animals (black bar) by 28 DPI (mean ± s.e.m., n = 3 animals, 50 Pax7 $^+$ SCs analysed from each animal, **P < 0.01 versus non-injured).

**b**, The propensity of CSCs to cycle returns to the level of QSCs several weeks after injury. At various times after injury, mice were given an i.p. injection of BrdU. SCs were isolated 12 h later from the injured muscles (ASCs) or from the contralateral muscles (CSCs). The frequency of BrdU incorporation returned to QSC levels (dashed line) by approximately 21 days after injury for both ASCs and CSCs (mean ± s.e.m., n = 3 animals).

**c**, Cell cycle entry kinetics of CSCs returns to the level of QSCs several weeks after injury for both ASCs and CSCs (mean ± s.e.m., n = 2 animals).

**d**, CSCs isolated 28 DPI have a transcriptional profile very similar to QSCs as shown by PCA and Pearson’s $r$ value. Transcripome analysis was performed as in Fig. 2c, with the addition of data from CSCs 28 DPI.
The ability to adopt the alert state strongly correlates with expression of genes involved in mitochondrial metabolism.

a. Pathway analysis (as performed in Extended Data Fig. 3a) of the genes induced in TSC1 KO QSCs compared to wild-type QSCs shows that genes involved in mitochondrial metabolism are significantly enriched. Pathway analyses of the genes induced in Rptor KO CSCs compared to Rptor KO QSCs (b) and cMet KO CSCs compared to cMet KO QSCs (c) show that genes involved in mitochondrial metabolism are not enriched. d. Expression of genes involved in oxidative phosphorylation (KEGG ID mmu00190) is coupled with the alert state. Heat map of the expression of genes in the oxidative phosphorylation pathway shows that models of the alert state (CSCs and TSC1 KO QSCs) have elevated expression of these genes and that models of non-alert SCs (QSCs, Rptor KO SCs and cMet KO SCs) have low expression of these genes. Hierarchical clustering (Euclidean distance, complete linkage) shows that models of the alert state (CSCs and TSC1 KO SCs) cluster together and that models of non-alert SCs (QSCs, Rptor KO SCs and cMet KO SCs) form another cluster. e. Centroid-based clustering using oxidative phosphorylation genes (KEGG ID mmu00190) shows that grouping SCs into three clusters reveals an ‘alert’ cluster (wild-type CSCs and TSC1 KO QSCs), a ‘non-alert’ cluster (QSCs, CSCs 28 DPI, Rptor KO QSCs and CSCs and cMet KO QSCs and CSCs), and an ‘activated’ cluster (ASCs). Ellipses of dispersion show standard deviation (radius) and mean (centre) for each cluster using the first two components from PCA. Combined, these data show that induction of genes involved in mitochondrial metabolism strongly and consistently correlates with ability to adopt the alert state: wild-type CSCs and TSC1 KO QSCs are alert, and Rptor KO and cMet KO CSCs are not alert.
Extended Data Figure 8 | SCs enter the alert state in response to many types of injuries. a, Cultures of CSCs differentiate more quickly than do cultures of QSCs (representative ICC staining of MyoG, data quantified in Fig. 4a, b). b, SCs enter the alert state in response to injuries to non-muscle tissue. SCs contralateral to a tibial fracture (bone inj) and SCs in an animal that received a skin wound on the abdomen (skin inj) increase in propensity to cycle in vivo (mean ± s.e.m.; non-injured, n = 5 animals; bone injured, n = 2; skin injured, n = 6; **P < 0.01 versus non-injured). c, SCs increase cycle cell entry kinetics in response to non-muscle injuries. SCs contralateral to a tibial fracture injury and SCs from mice that received a skin injury have increased frequency of EdU incorporation when cultured for 40 h ex vivo compared to SCs from non-injured animals (mean ± s.e.m.; n = 3 animals; *P < 0.05 versus non-injured).
Extended Data Figure 9 | FAPs and LT-HSCs adopt an alert state in response to muscle injury. a, Increased frequency of pS6\(^+\) FAPs contralateral to muscle injury. Representative IF-IHC staining of TA muscle from a non-injured animal (top) or contralateral to injury (bottom) shows that the frequency of pS6\(^+\) (PDGFR\(\alpha\);CD31\(^-\)) FAPs is increased in contralateral muscle (data are quantified in Fig. 4g). Labelled boxes indicate regions for which higher magnification is displayed. b, CFAPs increase in size. FACs analysis shows that 2.5 DPI CFAPs increase in FSC distribution compared to QFAPs; AFAPs show a greater increase in size (a representative FACs plot is shown, similar results were observed in 3 independent experiments). c, CFAPs increase in propensity to cycle. Twelve hours following an i.p. injection of BrdU, CFAPs isolated at indicated DPIs have an elevated frequency of BrdU incorporation compared to QFAPs (0 DPI). d, Muscle injury increases the frequency of phospho-mTOR\(^+\) (pmTOR) LT-HSCs. FACs analysis of pmTOR in Lineage\(^-\), Sca-1\(^-\), cKit\(^-\), CD150\(^+\) HSCs isolated from bone marrow 1 DPI showed that LT-HSCs induce mTORC1 signalling in response to muscle injury (mean ± s.e.m.; n ≥ 4; *P < 0.05).