mTORC1 controls the adaptive transition of quiescent stem cells from G_0 to G_{Alert}

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Abstract

A unique property of many adult stem cells is their ability to exist in a non-cycling, quiescent state[1]. 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 function[2], 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: G_0 and an "alert" phase we term G_{Alert}, and that stem cells actively and reversibly transition between these phases in response to injury-induced, systemic signals. Using genetic models specific to muscle stem cells (or satellite cells (SCs)), we show that mTORC1 activity is necessary and sufficient for the transition of SCs from G_0 into G_{Alert} and that signaling through the HGF receptor, cMet is also necessary. We also identify G_0-to-G_{Alert} transitions in several populations of quiescent stem cells. Quiescent stem cells that transition into G_{Alert} possess enhanced tissue regenerative function. We propose that the transition of quiescent stem cells into G_{Alert} functions as an 'alerting' mechanism, an adaptive response that positions stem cells to respond rapidly under conditions of injury and stress without requiring cell cycle entry or a cell fate commitment.

Adult stem cells have historically 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 cycle[3–4]. In contrast to the cell cycle, which can be sub-divided into distinct phases, quiescence is less well characterized. Emerging data suggest that stem cells can regulate quiescent functional properties [5–6]. While studying the regulation of the transition of 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 responded to that distant injury and had cycling properties that were different from those in a noninjured animal (QSCs) and from the injured tissue (ASCs) (Fig. 1a). Using the Pax7[CreER] driver and Rosa26[EYFP] lineage tracer to specifically label SCs[7–8] (Extended Data Fig. E1a), we found that these contralateral SCs (CSCs) showed markedly increased, but overall still low, propensity to cycle when compared to QSCs, as measured by BrdU 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 (Figs. 1c, d). Subsequent cell divisions of progeny of CSCs and QSCs occurred at similar rates to those of ASCs (Fig. E1b). The response of CSCs was not limited to muscle groups directly contralateral to the injury or to the agent of muscle injury (Figs. E1c–e).

One of the most obvious changes in ASCs is a dramatic increase in cell size relative to QSCs (Fig. 2a). We found that CSCs displayed a very slight, but significant, increase in cell size relative to QSCs (Figs. 2a–b, E2a–b). Similarly, we also observed that CSCs had stronger EYFP intensity from the Rosa26[EYFP] reporter, elevated levels of Pyronin Y staining, and incorporation of the ribonucleotide EU compared to QSCs (Figs. E2c–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 suggests that CSCs are intermediate between QSCs and ASCs. However, detailed ICC analysis immediately after isolation showed that CSCs are phenotypically more similar to QSCs (Figs. E2f–i). To test if CSCs represent a population of stem cells or a population of committed progenitor cells, we performed transplantation and pulse-chase experiments and found no difference in the engraftment efficiency and capacity for self-renewal between CSCs and QSCs (Fig. E2j, k). Together, these data suggest that CSCs are similar to, but distinct from, QSCs and possess the stem cell characteristics of QSCs.

To gain further insight into what distinguishes CSCs from QSCs, we analyzed 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 (Fig. E3a). To further investigate mitochondrial metabolism in CSCs, we performed MitoTracker Deep Red (MTDR) staining and measured mtDNA content and found that, relative to QSCS, CSCs indeed displayed evidence of elevated mitochondrial activity (Figs. 2d, e). Along these lines, and keeping with the increase in cell size, we also found that CSCs have increased levels of cellular ATP (Figs. 2f, E3b–d).

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, the vast majority of 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 signaling pathway (reviewed in[9]). Indeed, we observed induction of phospho-S6 (pS6), a surrogate of mTORC1 activity, in alert SCs (Figs. 2g–h, E3e–g). Taking this a step further, we found that by sorting SCs for properties of the alert state (Fig. E3h) 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) (Figs. E3i–m). Together these data show that there is a strong correlation between activation of mTORC1 signaling and the alert phenotype in SCs.

To test if any aspects of the alert response were directly regulated by mTORC1 signaling, we used the Pax7[CreER] driver to specifically ablate TSC1, an inhibitor of mTORC1 signaling, in SCs. As a genetic model of mTORC1 activation[10], TSC1 KO QSCs displayed induction of mTORC1 activity (Figs. E4a–b). Importantly, TSC1 KO QSCs also displayed all aspects of the alert phenotype in an otherwise noninjured context: increased propensity to cycle, accelerated cell cycle entry, increased MTDR staining, and increased cell size (Figs. 3a–c, E4c). To test whether the alert response requires mTORC1, we used a conditional allele of Raptor (Rptr)[11], an essential component of the mTORC1 signaling complex, with the Pax7[CreER] driver to specifically ablate Raptor protein and suppress mTORC1 signaling in SCs (Figs. E4b, E5a–c). Overall, we found that Rptr KO SCs contralateral to a muscle injury were completely unresponsive and did not manifest any characteristics of an alert SC (Figs. 3d–f, E5d–e). These data combined show that mTORC1 signaling 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 activated by serum proteases[12–13]. Active HGF can regulate mTORC1 via PI3K-Akt signaling[14]. Furthermore HGF is known to influence SC behavior[15–16]. To test if HGF signaling has a role in the alert response, we used a conditional allele of the HGF receptor, cMet, to suppress HGF signaling in SCs[17]. Ablation of cMet in SCs completely blocked the activation of mTORC1 signaling, as measured by pS6 staining, in cultured SCs and *in vivo* in CSCs following injury (Figs. E4b, E5f–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 (Figs. 3g–i, E5h). Collectively, these data suggest that signaling downstream of cMet is critical for the induction of the alert response in SCs.

Following tissue repair after injury, activity of the HGF activation cascade gradually subsides[12]. We found the frequency of pS6+ CSCs following a distant injury declined to a level similar to that of noninjured animals 28 days after injury (Fig. E6a). Interestingly, we

found that, also at 28 DPI, the propensity to cycle and cell cycle entry kinetics of CSCs also returned to those of QSCs (Figs. E6b, c). Furthermore, the transcriptional profile of CSCs 28 DPI had returned to that of QSCs (Fig. E6d). These data suggest that the alert state is reversible and that the functional and transcriptional changes in alert CSCs that occur downstream of mTORC1 revert to the properties of QSCs when mTORC1 activity subsides.

To gain further understanding of the molecular pathways underlying the functional transition into the alert state, we analyzed 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 Rptr KO and cMet KO CSCs do not (Figs. E3a, E7a–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[18–19]. As such, we tested whether the functional changes of CSCs affected their differentiation and muscle regenerative abilities. Following isolation and culturing *ex vivo*, CSCs displayed enhanced kinetics of differentiation as measured by expression of MyoG and cell fusion (Figs. 4a, b, E8a). To translate these observations *in vivo*, we assessed the ability of CSCs to participate in muscle regeneration. Three days prior to injury of the left 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 dramatically enhanced muscle regeneration at all time points following injury when compared to the normal muscle regenerative process (Figs. 4d, e). These data show that the functional properties of alert SCs translates into enhanced muscle regenerative ability in response to injury.

The dramatically 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 (Figs. E8b, 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[20–21], responded much like SCs. CFAPs (FAPs in muscles of a limb contralateral to the site of muscle injury) displayed an induction of mTORC1 signaling, accelerated cell cycle entry, increased propensity to cycle, and increased cell size when compared to quiescent FAPs from noninjured animals (QFAPs) (Figs. 4f–h, E9a–c). Additionally, we found that long term hematopoietic stem cells (LT-HSCs) displayed activation of mTORC1 signaling in response to muscle injury (Figs. 4i, E9d). To test if mTORC1 activation in LT-HSCs caused increased functional potential, as it does in SCs, we then administered interferon-gamma (IFNγ), to the animals to stimulate LT-HSC activation[22]. Interestingly and similar to the effect of an 'alerting' injury on muscle regeneration, LT-HSCs primed by muscle injury were more sensitive to IFNγ and yielded a more robust response (Fig. 4j). Notably, and similar to what we demonstrated in SCs, the induction of mTORC1 in HSCs increases their mitochondrial

activity[23–24], which is consistent with a transition into the alert state. Collectively, these data suggest that activation of mTORC1 signaling in quiescent stem cells alters their properties, endowing them with enhanced functional potential, an alerting mechanism, should the stem cell be required in tissue repair.

As it relates to stem cell biology, the data we present here suggest that stem cells undergo dynamic transitions between functional phases in the quiescent state. We propose a model in which G_{Alert} and G_0 form a quiescence cycle (Fig. 4k). While it is clear that not all quiescent cells are functionally equivalent[25–26], the *in vivo* relevance and the molecular mechanisms regulating functionally distinct states have not 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 SCs, by cMet). Most importantly, our data suggest that the ability to transition between G_0 and G_{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 novel form a 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 ± SEM, except histograms, and significance was calculated using two-tailed unpaired Student *t*-tests: * denoting $P < 0.05$, ** denoting $P < 0.01$. Where sample size (n values) are reported as a range, exact sample size values can be found in Supplemental Methods. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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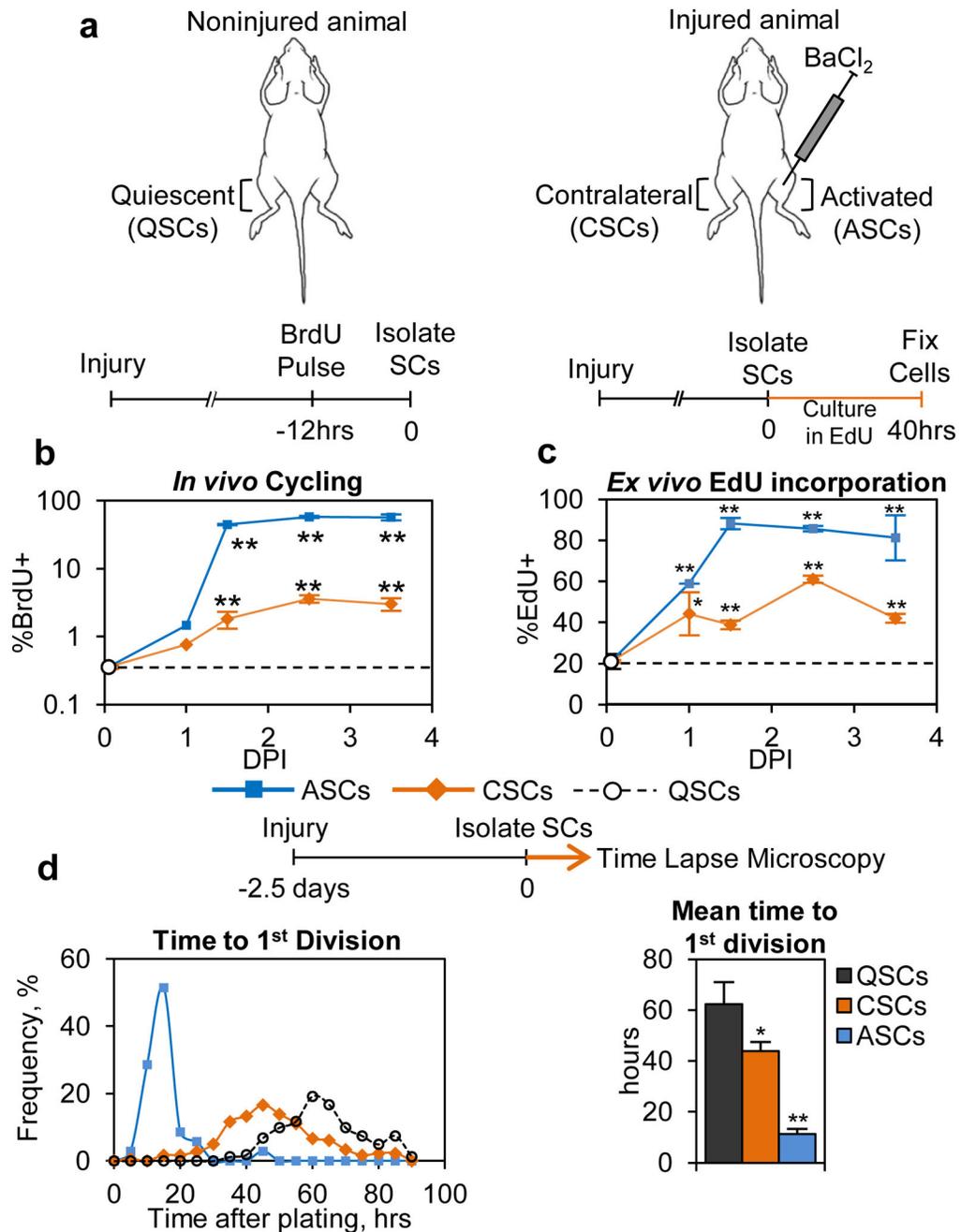
Figure 1. Satellite cells distant from the site of injury have different cell cycle kinetics than quiescent and activated satellite cells

(**a**) Schematic representation of the location of QSCs, CSCs, and ASCs in relation to muscle injury. (**b**) CSCs have greater propensity to cycle *in vivo* than do QSCs (n ≥ 3; significance is versus QSCs). (**c**) Higher percentages of CSCs incorporate EdU after 40 hrs than QSCs. Data from a representative experiment is presented (n ≥ 2; significance is versus QSCs). (**d**) CSCs require less time to compete the first division (n=3). Details on data presentation and sample size can be found in the Methods Summary and Supplemental Methods Sections.
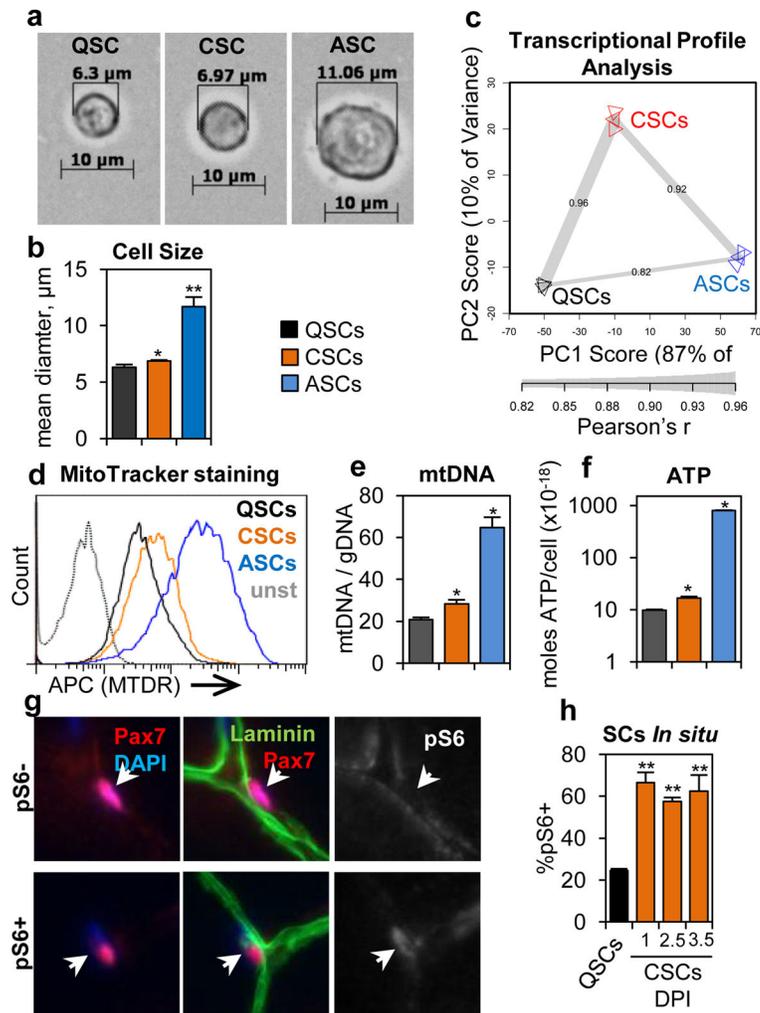
Figure 2. Satellite cells that are distant from an injury have become 'alert.'
(**a**) Representative images of QSCs, CSCs, ASCs immediately after isolation. (**b**) CSCs are larger than QSCs (n=3). (**c**) CSCs have a transcriptional profile that is intermediate between QSCs and ASCs (along PC1) as shown by PCA and Pearson's r values (n=3). (**d**) Increased mitochondrial activity in CSCs compared to QSCs. (representative FACS plot, n=4). (**e**) CSCs have increased mtDNA content relative to QSCs (n ≥ 3). (**f**) CSCs have more intracellular ATP then QSCs (n=4). (**g**) IF-IHC staining of TA muscle showing representative pS6− and pS6+ SCs. (**h**) Quantification of IF-IHC staining for pS6 in SCs (n ≥ 3; significance is versus noninjured).
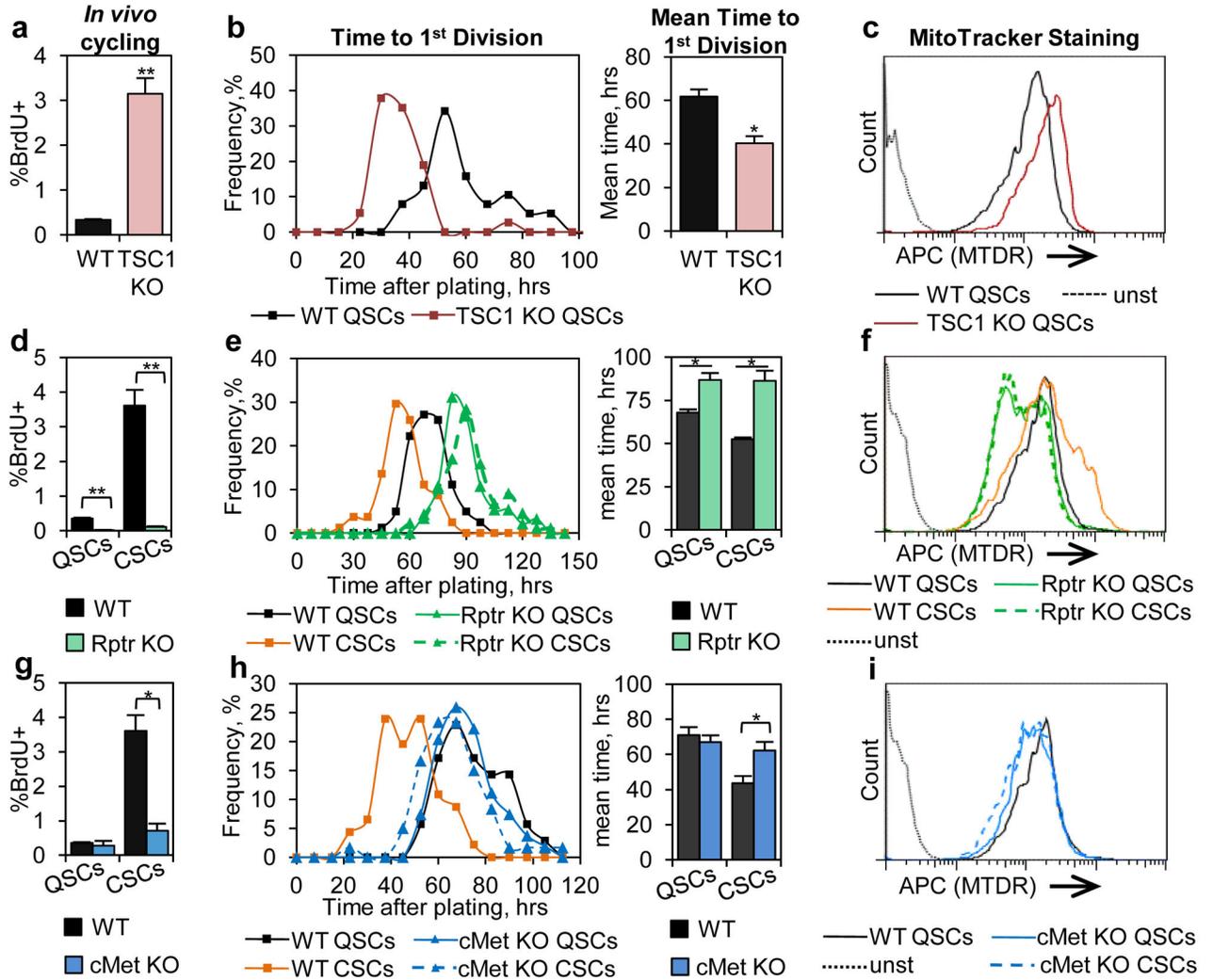
Figure 3. Activation of mTORC1 is necessary and sufficient for the alert phenotype
TSC1 KO QSCs display characteristics of alert SCs: (**a**) increased propensity to cycle *in vivo* (n 6); (**b**) reduced time to first division (n=3); and (**c**) increased mitochondrial activity (representative FACS plotn=3). Rptr KO suppresses induction of the alert state. Rptr KO CSCs show no differences in: (**d**) propensity to cycle *in vivo* (n 6); (**e**) time to first division (n=3); and (**f**) mitochondrial activity (representative FACS plot, n=3). cMet KO CSCs show no injury-induce regulation of: (**g**) propensity to cycle *in vivo* (n 4); (**h**) time to first division (n 3); and (**i**) mitochondrial activity (representative FACS plot, n=3).
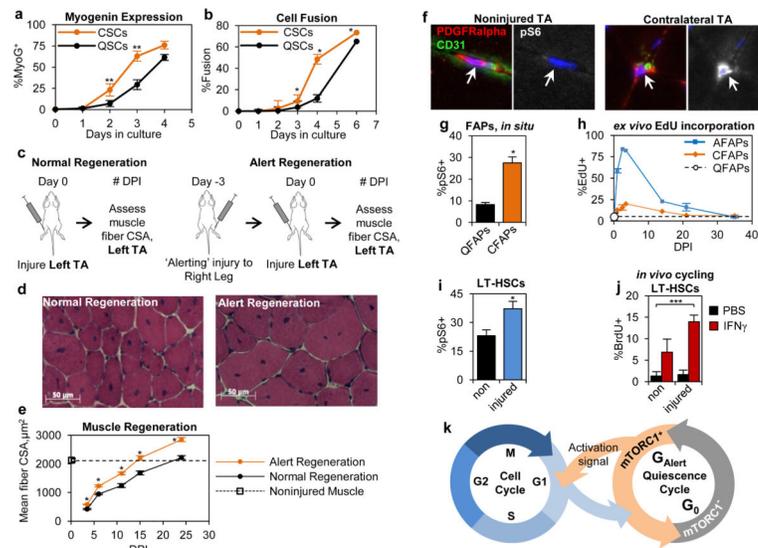
Figure 4. Stem cells in the alert state have enhanced functional properties
(**a–b**) CSCs have enhanced kinetics of myogenic differentiation *ex vivo*. They rapidly (a) express become MyoG+ and (b) fuse (n=3; significance is versus QSCs at same time point). (**c**) Schematic depiction of 'alert' regeneration experimental design. (**d–e**) A prior 'alerting' injury enhances the progress of muscle regeneration: (d) representative histological section and (e) quantification of nascent, centrally nucleated muscle fiber CSA (n 3). (**f–h**) FAPs adopt characteristics of the alert state: (**f**) higher frequency of pS6+ FAPs in muscles contralateral to injury (representative IF-IHC staining); (**g**) quantification of pS6 staining (n=4); and (**h**) accelerated kinetics of cell cycle entry (n 2). (**i–j**) LT-HSCs display characteristics of the alert state in response to muscle injury: (**i**) increased frequency of pS6 staining (n 4); and (**j**) enhanced activation response to IFNγ (n 3; *** $p<0.001$). (**k**) Model depicting quiescence cycle of G_0 and G_{Alert} phases.