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Zhang & Manning reply

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In our study¹, we found that, as a balance to its acute effects on anabolic processes, prolonged activation of mTORC1 increases cellular proteasome content and degradation capacity through the NRF1/NFE2L1 transcription factor. In their communication, Zhao et al criticize our methods while failing to recognize the importance of distinguishing between mTORC1 and mTORC2 in studying an mTOR-regulated process.

One valid technical point they focus on involves continued label incorporation during early stages of the chase period in a standard pulse-chase assay. Sustained incorporation of ³⁵S-Met is evident in our published data¹, with net loss of protein only being measured following the first chase time point, which was taken rather late at 8 h. Most importantly, after this initial phase, cells with active mTORC1 were found to degrade protein at higher rates when compared directly to cells with inactive mTORC1, an effect that was blocked by inhibitors of the proteasome but not the lysosome¹. Consistent with the well-established induction of autophagy upon mTORC1 inhibition, the ability of long-term rapamycin treatment to slow protein turnover was even more pronounced in autophagy-deficient cells¹. Given that mTORC1 promotes protein synthesis and, thus, ³⁵S-Met reincorporation, if anything, we might have underestimated the positive effects of mTORC1 on protein degradation in these assays. This technical issue has no bearing on our interpretation of these experiments or their contribution to the central conclusions of our study.

To address whether ³⁵S-Met reincorporation influenced our conclusions, as proposed, we repeated these experiments using ³H-Phe in both a standard 30-min pulse-chase assay and the 20-h labeling experiment preferred by Zhao et al. In both cases, the data are similar to that presented in our paper, but without detectable label reincorporation (accompanying figure). In these experiments, we treated with rapamycin only during the chase period to assure that we are measuring turnover of the same pool of proteins. As published¹, TSC2-deficient cells with activated mTORC1 displayed enhanced rates of protein turnover relative to rapamycin-treated or TSC2-expressing cells, where mTORC1 is inactive. The differences are most evident at the later time points (>5 h), consistent with the NRF1-dependent transcriptional mechanism delineated in our study¹ and discussed in detail elsewhere². Thus, the discrepancy with Zhao et al's communicated findings has nothing to do with the assay, but rather is due to differences in the chosen culture conditions.

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A challenging but essential aspect of studying mTOR signaling is the need to clearly distinguish between mTORC1 and mTORC2, two functionally distinct mTOR complexes, in order to gain mechanistic insights into a cellular process³. TSC1/2 null cells, which exhibit growth factor-independent activation of mTORC1, have emerged as a powerful genetic tool, but must be cultured and compared to control cells in a manner that properly isolates mTORC1 from mTORC2. Zhao et al present experiments using TSC2 null MEFs, without reconstituted control cells, grown in full serum and treated with Torin1 or a dose of rapamycin (300 nM) at least 100-fold higher than the IC50 for mTORC1. Under these conditions, one cannot distinguish between effects on mTORC1 or mTORC2, as serum activates both complexes and these doses of inhibitors block both complexes. In contrast, the low serum growth conditions and treatments we use with these cells are specifically designed to separate effects of mTORC1 from mTORC2, where a clear mTORC1-dependent enhancement of long-term protein degradation is observed (ref. 1 and accompanying figure).

We stand by our conclusions that mTORC1 activation promotes the production of proteasomes leading to enhanced proteasome-mediated protein turnover through an increase in NRF1^{1,2}, a transcription factor now established in multiple independent studies to control cellular proteasome levels^{1–8}.

The authors provide this response on behalf of all of the authors of our published manuscript¹, and each author has reviewed and approved of its contents.

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Figure 1. Active mTORC1 signaling enhances protein degradation in both pulse-chase and long-term labeling assays with ^3H -Phe

a, *Tsc2*^{-/-} MEFs reconstituted with TSC2 or empty vector (Vec) were cultured for 16 h in medium containing 0.5% serum, with triplicate samples per condition and time point. Cells were pulse labeled for 30 min with ^3H -Phe, washed three times with chase medium containing 2 mM cold Phe, and chased in medium containing vehicle or rapamycin (20 nM). The amount of radioactivity in TCA-precipitable cellular protein was measured by scintillation, with the rate of protein degradation shown as the fraction of radiolabelled protein, relative to time 0, remaining over time. Immunoblot inset: mTORC1 signaling under these experimental conditions, as detected with phospho (p)-S6K1 (T389), at the 16 h time point. **b**, Cells were treated as in **a**, except radiolabeled for 20 h with ^3H -Phe and washed for 2 h with chase medium containing 2 mM cold Phe before measuring degradation of the remaining protein over time. Except for the serum and rapamycin concentrations, this assay is identical to that described in the Zhao et al communication. Data are composite of two independent experiments with three biological replicates each and are expressed as mean $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$.