Global phosphoproteomics reveals the skeletal muscle contraction signalling repertoire and novel regulation of sarcoplasmic reticulum calcium sensing machinery *via* AMPK

N.J. Hoffman,[1] B.L. Parker,[1] R. Chaudhuri,[1] T. Naim,[2] J.G. Burchfield,[1] J. Stöckli,[1] G.S. Lynch[2] and D.E. James,[1] [1]The Charles Perkins Centre, Education and Research Hub - D17, School of Molecular Bioscience, The University of Sydney, NSW 2006, Australia and [2]Department of Physiology, North Wing, Medical Building, University of Melbourne, VIC 3010, Australia.

Exercise plays an essential physiological role in regulating metabolic homeostasis. However, only a few signalling pathways are known that trigger its beneficial effects in skeletal muscle. We recently used global phosphoproteomics to map acute exercise signalling in human skeletal muscle, revealing over 1,000 exercise-regulated phosphorylation sites and a network of kinases and AMPK substrates (Hoffman *et al*., 2015). To further our understanding of the contraction-regulated mechanical signals underlying this complex exercise signalling network, we performed a global, unbiased mass spectrometry (MS)-based phosphoproteomic analysis of high-intensity contraction in rat skeletal muscle. *Tibialis anterior* muscles were obtained from five Wistar rats anaesthetized by intraperitoneal administration of pentobarbital and subjected to either sham surgery or a sciatic nerve stimulated high-intensity *in situ* contraction protocol (100 Hz; 1 s on, 3 s off; 5 min). The signalling response between each sham and contracted muscle was highly reproducible (average Pearson's correlation coefficient r=0.95). Of the 6,664 phosphorylation sites we quantified, 932 sites were significantly regulated by contraction *versus* sham surgery (adjusted P<0.05; >1.5 fold change). Strikingly, the upstream kinase(s) for only 23 of these 932 sites are known. Pathway analysis revealed that well characterized contraction signalling pathways were significantly regulated including the insulin, mitogen-activated protein kinase (MAPK), mammalian target of rapamycin (mTOR) and calcium signalling pathways. Given the central role of calcium in muscle contraction, we mined our data for calcium sensing proteins containing phosphorylation sites never previously known to be regulated by contraction. This revealed contraction-stimulated phosphorylation of several sites on stromal interaction molecule 1 (STIM1), a calcium sensing protein on the sarcoplasmic reticulum that participates in store-operated calcium entry (SOCE). We have observed two of these STIM1 phosphorylation sites, Ser257 and Ser521, to be regulated by exercise and AMP-activated protein kinase (AMPK; Hoffman *et al*., 2015), a kinase that is also activated by contraction. Considering the importance of the domain surrounding Ser257 in regulating STIM1 oligomerisation and activation to stimulate SOCE *via* interaction with ORAI1 channels, we generated a STIM1 Ser257 phospho-specific antibody to validate these MS findings. *In vitro* validation experiments using siRNA knockdown of STIM1 and AMPK in rat L6 skeletal muscle cells confirmed that STIM1 is a bona fide AMPK substrate. To uncover the biological significance of STIM1 Ser257 phosphorylation by AMPK, we have generated a STIM1 phospho-dead (S257A) mutant and are exploring the functional role of Ser257 using microscopy and calcium flux measurements. Taken together, the "contractome" signalling network contains a wealth of novel phosphoproteins for mechanistic dissection that will help reveal previously unappreciated biological mechanisms underlying this powerful mechanical stimulus. These data serve as an invaluable resource for future physiological studies of skeletal muscle contraction in health and disease.

Hoffman NJ, Parker BL, Chaudhuri R, Fisher-Wellman KH, Kleinert M, Humphrey SJ, Yang P, Holliday M, Trefely S, Fazakerley DJ, Stöckli J, Burchfield JG, Jensen TE, Jothi R, Kiens B, Wojtaszewski JF, Richter EA, James DE. (2015) Global phosphoproteomic analysis of human skeletal muscle reveals a network of exercise-regulated kinases and AMPK substrates. *Cell Metab* doi: 10.1016/j.cmet.2015.09.001