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SUMMARY

Diabetes, obesity and cancer affect upwards of 15% of the world's population. Interestingly, all three diseases juxtapose dysregulated intracellular signaling with altered metabolic state. Exactly which genetic factors define stable metabolic setpoints in vivo remains poorly understood. Here, we show that Hedgehog signaling rewires cellular metabolism. We identify a cilium-dependent Smo-Ca⁺⁺-Ampk axis that triggers rapid Warburg-like metabolic reprogramming within minutes of activation and is required for proper metabolic selectivity and flexibility. We show that Smo-modulators can uncouple the Smo-Ampk axis from canonical signaling and identify cyclopamine as one of a new class of "selective partial agonists", capable of concomitant inhibition of canonical and activation of non-canonical Hedgehog signaling. Intriguingly, activation of the Smo-Ampk axis in vivo drives robust insulin-independent glucose uptake in muscle and brown adipose tissue. These data identify multiple non-canonical endpoints pivotal for rational design of Hedgehog modulators and provide a new therapeutic avenue for obesity and diabetes.

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HIGHLIGHTS

- Hedgehog signaling rapidly reprograms energy metabolism
- Smoothened activates a rapid primary cilium-dependent Smo-Ampk axis
- Cyclopamine and GDC-0449 uncouple canonical and non-canonical Smo-signaling
- Smoothened drives insulin-independent glucose uptake in muscle and BAT in vivo

A Smoothened-Ampk Axis Rewires Metabolism

Hedgehog partial agonism drives

Warburg-like metabolism in

muscle and brown fat

Raffaele Teperino,^{1,13} Sabine Amann,^{2,13} Martina Bayer,^{2,13} Sean L. McGee,³ Andrea Loipetzberger,⁴ Timothy Connor,³ Carsten Jaeger,⁵ Bernd Kammerer,⁵ Lilli Winter,⁶ Gerhard Wiche,⁶ Kevin Dalgaard¹, Madhan Selvaraj¹, Jeremy Reiter,⁷ Michael Gaster,⁸ Robert S. Lee-Young,⁹ Mark A. Febbraio,⁹ Claude Knauf,¹⁰ Patrice D. Cani,¹¹ Fritz Aberger,⁴ Josef M. Penninger,¹² J. Andrew Pospisilik,^{1,*} and Harald Esterbauer^{2,*}

1. Max Planck Institute of Immunobiology and Epigenetics, Stuebeweg 51, D-79108, Freiburg, Germany

2. Department of Laboratory Medicine, Medical University Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

3. Metabolic Remodeling Laboratory, Metabolic Research Unit, School of Medicine, Deakin University, 75 Pigdons Road, Geelong, Victoria, 3220, Australia

4. Department of Molecular Biology, University of Salzburg, Hellbrunnerstrasse 34, A-5020 Salzburg, Austria

5. Metabolomics Core, Center for Biological Systems Analysis (ZBSA), Habsburgerstrasse 49, D-79104 Freiburg, Germany

6. Department of Developmental Biology & Disease Mechanisms, Max F. Perutz Laboratories, Dr. Bohr-Gasse 9, A-1030 Vienna, Austria

7. Department of Biochemistry and Biophysics, Cardiovascular Research Institute, University of California, 600 16th Street, San Francisco, 94158, USA

8. Departments of Pathology and Endocrinology, Odense University Hospital, J.B. Winsløvsvej 25, DK-5000 Odense, Denmark

9. Cellular and Molecular Metabolism Laboratory, Baker IDI Heart and Diabetes Institute, 75 Commercial Road, Melbourne, 3004, Australia

10. INSERM U1048, Team 3, Institut de Medecine Moleculaire de Rangueil, Paul Sabatier University, Bat. L4, IFR150, CHU Rangueil, BP 84225, F-31432 Toulouse Cedex 4, France

11. Université catholique de Louvain, Louvain Drug Research Institute, Metabolism and Nutrition research group, Av. E. Mounier 73, B-1200 Brussels, Belgium

12. Institute of Molecular Biotechnology of the Austrian Academy of Science, Dr. Bohrgasse 3, A-1030 Vienna, Austria

13. These authors contributed equally to this work

*Correspondence:

pospisilik@ie-freiburg.de (J.A.P.), harald.esterbauer@meduniwien.ac.at (H.E.)

SUMMARY

Diabetes, obesity and cancer affect upwards of 15% of the world's population. Interestingly, all three diseases juxtapose dysregulated intracellular signaling with altered metabolic state. Exactly which genetic factors define stable metabolic setpoints in vivo remains poorly understood. Here, we show that Hedgehog signaling rewires cellular metabolism. We identify a cilium-dependent Smo-Ca⁺⁺-Ampk axis that triggers rapid Warburg-like metabolic reprogramming within minutes of activation and is required for proper metabolic selectivity and flexibility. We show that Smo-modulators can uncouple the Smo-Ampk axis from canonical signaling and identify cyclopamine as one of a new class of "selective partial agonists", capable of concomitant inhibition of canonical and activation of non-canonical Hedgehog signaling. Intriguingly, activation of the Smo-Ampk axis in vivo drives robust insulin-independent glucose uptake in muscle and brown adipose tissue. These data identify multiple non-canonical endpoints pivotal for rational design of Hedgehog modulators and provide a new therapeutic avenue for obesity and diabetes.

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64

INTRODUCTION

Hedgehog was initially described as a morphogen defining body patterning (Nusslein-Volhard and Wieschaus, 1980). In mammals, ligand binding to the inhibitory cell surface receptor Patched (Ptch) permits Smoothed (Smo) translocation to the tip of the cilium and liberates zinc-finger family transcription factors Gli1, Gli2 and Gli3 from proteasomal degradation and inhibitory tethering leading to activation of Gli-target genes (Hui, 2010; Ingham et al., 2011; Riobo and Manning, 2007; Rohatgi and Scott, 2007; Ruiz i Altaba et al., 2007). Three highly homologous mammalian ligands exist for *Drosophila* Hedgehog, namely Desert, Indian and Sonic Hedgehogs (Dhh, Ihh and Shh respectively). While the majority of Hedgehog-induced biological effects are thought to result from transcriptional regulation, several Gli-independent, non-canonical modules have also been reported (Riobo and Manning, 2007; Teglund and Toftgård, 2010). Shh, for instance, rapidly stimulates Src family kinases to spatially direct axonal outgrowth in vitro (Yam et al., 2009) and all three hedgehog ligands have been shown to promote cytoskeletal rearrangement via a Smo-Gi-RhoA axis in endothelial cells (Chinchilla et al., 2010). Furthermore, coupling of hedgehog to Rho small GTPases has been linked to the activation of fibroblast migration (Polizio et al., 2011). More recently, Shh has been shown to boost Ca^{++} and IP_3 transients in spinal neurons through a G-protein and Ca^{++} dependent pathway (Belgacem and Borodinsky, 2011).

Hedgehog activation impacts dozens of cellular processes including stem cell renewal, lineage decision, cell cycle-regulation, migration, and mitogenic signaling (Hui, 2010; Ingham et al., 2011; Rohatgi and Scott, 2007; Ruiz i Altaba et al., 2007; Teglund and Toftgård, 2010). Accordingly, activating mutations in Hedgehog pathway members have

been causally implicated in a number of disparate cancers including medulloblastoma, basal cell carcinoma, pancreatic cancers and cancers of the intestinal tract (Ng and Curran, 2011; Teglund and Toftgård, 2010). Also, Hedgehog signaling specifically blocks white but not brown adipogenesis (Pospisilik et al., 2010; Suh et al., 2006). This biological effect is sensitive to inflammatory state (Todoric et al., 2011) and represents one of the few known signaling nodes capable of differentially regulating brown vs. white adipocyte differentiation (Lowe et al., 2011). All three factors, inflammation, adipocyte turnover, and adipose sub-type distribution show substantial imbalance in obesity and represent targets for therapeutic intervention (Lumeng and Saltiel, 2011; Whittle et al., 2011). Inhibitors of Hedgehog signaling from at least 7 pharmaceutical companies are currently in clinical trials (Ng and Curran, 2011). Intriguingly, despite their advanced development, a universal mechanism underlying Hedgehog inhibitor efficacy is lacking. Specifically, potency of canonical Hedgehog pathway inhibition does not match potency of inhibition of cancer growth (Yauch et al., 2008). One proposed mechanism to explain these anomalies has been indirect cancer modulation through paracrine or mesenchyme-tumour interaction (Chen et al., 2011; Chinchilla et al., 2010; Theunissen and de Sauvage, 2009; Yauch et al., 2008).

In the current study we show that Hedgehog stimulates metabolic reprogramming towards a Warburg-like glycolytic state. The effect, mediated by a rapid non-canonical Smo-Ca⁺⁺-Ampk signaling arm effects robust glucose uptake in vivo in the mouse and in human myocytes in vivo, and surprisingly is also induced by several canonical hedgehog inhibitors. The results provide critical insights into the substantial Hedgehog inhibitor side effects in humans, into the complexity of hedgehog signaling itself, and they raise new therapeutic strategies for obesity and type-1 diabetes.

RESULTS

Hedgehog Reprograms Energy Metabolism

Hedgehog signaling regulates multiple cellular processes including two with finely tuned metabolic programs, namely cancer and adipose tissue development. We asked whether Hedgehog might determine metabolic state. We chose 3T3-L1 adipocytes as a model since they exhibit some of the best characterized metabolic regulatory circuits and they respond fully to Hedgehog (Pospisilik et al., 2010). Intriguingly, stimulation of mature 3T3-L1 adipocytes with SAG (*Smoothened Agonist*) (Chen et al., 2002b), a small molecule activator of Smo, led to acidification of culture media within 24 hours (Figure 1A). Quantitation revealed increased extracellular acidification (ECAR) and reduced oxygen consumption (OCR) rates upon SAG stimulation (Figure 1B), findings consistent with reduced glucose and an increased lactate levels in the culture medium (Figure 1C). Further, levels of the oxidized redox equivalents NAD^+ and NADP^+ (Figure 1D) were elevated, indicating substrate re-routing away from energy production. Importantly, reduced forms of the redox equivalents NADH and NADPH remained unchanged as did ATP levels, yielding an overall increase in redox ratios (Figure 1E and Figure S1A and S1B). This metabolic reset was reminiscent in many ways to the Warburg effect observed in cancer, and to the metabolic rearrangements that characterize stem cell renewal and immune cell activation (Hsu and Sabatini, 2008; Shi et al., 2011; Vander Heiden et al., 2009). Importantly, no evidence of proliferation was found in these terminally differentiated, growth arrested cells. Pathway stimulation using the endogenous ligand Sonic hedgehog (Shh) recapitulated all key aspects of the metabolic phenotype (Figure

1F-1H and S1C), and, whereas wild-type primary mouse embryonic fibroblasts (MEFs) exhibited significant SAG-stimulated metabolic shifts, *Smo*^{-/-} MEFs (Varjosalo et al., 2006) showed none (Figure 1I-1K), thus ruling out potential chemical artifacts. Importantly, these findings were recapitulated in the 3T3-L1 system upon *Smo* knockdown (Figure S1D-F). Thus, Hedgehog signaling reprograms adipocyte metabolism towards aerobic glycolysis.

Hedgehog Triggers Rapid Metabolic Rewiring via Ampk

Next, we performed a time-course of glucose removal from and lactate accumulation into 3T3-L1 culture supernatants. Surprisingly, marked glucose removal and lactate generation was observed within minutes of SAG addition (Figures 2A and 2B), a finding completely inconsistent with transcriptionally-driven canonical Hedgehog signaling (Figure S2A). To understand how *Smo* activation could lead to such rapid metabolic reprogramming we performed a phosphoproteomic survey of the acute Hedgehog signaling response. Two-dimensional gel electrophoresis was used to resolve phospho-enriched lysates from differentiated 3T3-L1 adipocytes treated for 10 minutes with or without SAG (Figure 2C). Of the 632 spots consistently detected in three independent experiments, 55 spots comprising 39 specific phospho-proteins showed reproducible differential regulation >1.5-fold (Table S1). This dataset is to our knowledge the first unbiased snapshot of rapid non-canonical Hedgehog signaling reported to date and identifies numerous downstream signaling activities not previously associated with Hedgehog. Intriguingly, in a KEGG pathway analysis, all significantly enriched genesets save one constituted compartments of primary energy metabolism including *glucose*, *ketone body*, *fatty acid*, *pyruvate*, and *ketogenic amino acid metabolism* as well as the *TCA cycle* (Figure 2D and Table S2). Numerous key regulators of glucose and energy

metabolism were identified including Acetyl-CoA acetyltransferase (Acat1), Glyceraldehyde-3-phosphate dehydrogenase (Gapdh), Transaldolase 1 (Taldo1), Hormone-sensitive lipase (Lipe), Phosphoglycerate mutase (Pgam1), Glycogen synthase (Gys), and Pyruvate dehydrogenase alpha 1 (Pdha1; Figure 2E) indicating that a complex metabolic signaling network exists that is rapidly accessible to Smoothed. Of interest, the only non-metabolic pathway enriched in our analysis was the *proteasome*, a well-known and integral component of canonical Hedgehog signaling (Hui, 2010; Teglund and Toftgård, 2010). Thus, Hedgehog rapidly signals to the machinery of primary energy metabolism.

To further characterize the Hedgehog-driven metabolic phenotype, we performed Western blot time-course analysis of Pdha1, AMP-activated protein kinase (Ampk) and Pyruvate kinase M1/M2 (Pkm2) phosphorylation events. All three proteins have key roles in deciding glycolytic substrate fate, anabolic-to-catabolic transitions and have been implicated in the regulation of the Warburg effect and cancer cell growth (Hitosugi et al., 2009; Jeon et al., 2012; Vander Heiden et al., 2009). Intriguingly, robust phosphorylation signals were observed from all three proteins, within two minutes of either SAG (Figure 2F) or Shh (Figure S2B), modifications consistent with enhanced glycolysis.

Could Ampk, Pdha1 and Pkm2 be responsible for the observed metabolic phenotype? shRNA-knockdown of Ampk in 3T3-L1 adipocytes (Figure S2C) completely abolished SAG-stimulated disruption of redox balance, extracellular glucose/lactate levels, and OCR/ECAR flux levels (Figures 2G, 2H and S2D). Similarly, pre-incubation of cells with compound C, an inhibitor of Ampk activity, abrogated detectable metabolic rewiring effects following SAG stimulation in both fully differentiated 3T3-L1 adipocytes (Figure

S2D and S2E-F) and MEFs (Fig. S2G-H). Importantly, OCR/ECAR measures confirmed that neither Ampk knockdown nor Ampk inhibition alone reduced metabolic rate. Knockdown of Pdha1 on the other hand (Figure S2I), a genetic mimic of its phosphorylation-mediated inhibition, showed no apparent effect on SAG-induced glucose uptake (Figure 2G), but substantially enhanced cellular lactate release (Figure 2H) and redox rebalancing (Figure 2I). Disruption of Pkm2 (Figure S2I), interestingly, blunted any sign of a SAG-inducible glucose/lactate response and increased basal lactate accumulation (Figures 2G and 2H and 2H inset). Collectively, these results suggest that Ampk and Pkm2 mediate SAG-induced glucose uptake while Pkm2 and Pdha1 focus substrate flux towards lactate. In keeping with a largely Ampk-driven process, SAG-induced glucose uptake was Glut4- but not Glut1-, or insulin receptor-dependent (Figure 2J-K and S2I). Thus, Smo activation drives Ampk/Pkm2/Pdha1-dependent metabolic rewiring.

To better define the kinetics of the non-canonical response we used AMPKAR, a Foerster resonance energy transfer (FRET) probe for AMPK activity (Tsou et al., 2011). Since transfection and FRET signal detection in mature adipocytes is poor, we used HEK293 cells, which have previously been used for high throughput Hedgehog screening (Roudaut et al., 2011). Intriguingly, addition of SAG to AMPKAR expressing HEK293 cells revealed rapid and sustained AMPK-FRET signals detectable within 20 seconds of SAG addition (Figure 2L). Thus, Hedgehog stimulates rapid activation of AMPK in human and mouse cells, and across disparate cell types.

A New Smo-Camkk2/Lkb1-Ampk Axis

While predominantly studied as an activator of Gli-dependent transcriptional regulation over hours or days, Smo is a Class-F G-protein coupled receptor (Ogden et al., 2008; Polizio et al., 2011), a class of proteins that effect rapid G-protein coupled second messenger signals. Ampk is principally stimulated by increased AMP/ATP ratio and by the upstream kinases Serine/threonine-protein kinase 11 (Lkb1) and Calcium/calmodulin-dependent protein kinase kinase 2 (Camkk2) (Canto and Auwerx, 2011; Hardie, 2011). Using GC/MS, no marked dysregulation in AMP, ADP, or ATP was observed (Figure S3A), indicating normal AMP to ATP balance. Knockdown of either Lkb1 or Camkk2 (Figure S3B-D), however, resulted in near-complete abrogation of SAG-stimulated metabolic sequelae (Figure 3A-C and S3E), suggesting that both proteins couple Smo to Ampk. Importantly, while resistant to Smo-dependent metabolic rewiring, both Lkb1 and Camkk2 knockdown adipocytes responded fully to AICAR (Figure S3F-H), indicating that Ampk signaling in these cells remained intact.

In line with a key role for Camkk2, SAG stimulation of adipocytes preloaded with the Ca^{++} -sensitive fluorophore Fluoforescein-AM revealed a Ca^{++} response more rapid and intense than a standard 1 μM ionomycin stimulation (Figure 3D). The same was true for the endogenous ligand Shh (Figure S3I). Administration of the extracellular calcium chelator EGTA under the same conditions completely abrogated SAG- and Shh-induced responses, indicating that Smo activation promotes opening of plasma membrane Ca^{++} channels rather than mobilizing intracellular stores (Figure 3D and Figure S3I). Importantly, the L-type calcium channel blocker Nifedipine markedly blunted both Ampk activity (Figure 3E and Supplementary Figure S3J-K) and the observed metabolic phenotype (Figure 3F-H). Moreover, Pertussis toxin (Ptx), a potent inhibitor of receptor-to- $\text{G}\alpha_i$ G-protein coupling, blunted Ampk activation and metabolic rerouting (Figure 3E-

3H). Thus, a rapid Ca^{++} - and $\text{G}\alpha_i$ -dependent non-canonical Smo-Ampk signaling axis rewires metabolism.

Non-canonical Rewiring of Metabolism is Primary Cilium Dependent

Canonical Hedgehog signaling requires Smo translocation to the primary cilium (Corbit et al., 2005; Rohatgi et al., 2009; Rohatgi et al., 2007; Wang et al., 2009). We used cilia-deficient *Ift88*^{-/-} and *Kif3a*^{-/-} MEFs and their respective control cell lines to probe the cilia dependence of our system. Ift88 (intraflagellar transport protein 88) and Kif3a (kinesin family member 3A) are required for proper ciliogenesis and their loss results in lack of canonical Hedgehog signaling competency and of a functional primary cilium. Strikingly, whereas control MEFs showed full non-canonical responses to SAG-induction, glucose uptake and lactate production were severely blunted in the mutant lines (Figure 4A and 4B). Importantly, both mutant cell lines exhibited a significant Ca^{++} -response to SAG (Figure 4C), but neither exhibited any sign of Ampk activation by in-cell Western blot (Figure 4D and S4A), indicating that the cilium is necessary to couple Smo-induction to Ampk activity. Of note, both lines mounted significant metabolic responses to AICAR, indicating uncompromised Ampk and downstream signaling (Figure S4B-D). Thus, the Smo-Ampk response is dependent on the primary cilium.

To corroborate these findings in the adipocyte system we tested the ability of several Smo variants to rescue the stable loss-of-function in our Smo-knockdown 3T3-L1 cells (Figure 4E and 4F). As expected, wild-type Smo-GFP rescued non-canonical signaling competency in the mature knockdown adipocytes. Transfection with a Cilia Localization Deficient Smo mutant however, was completely incapable of restoring responsiveness (Smo-CLD; Figure 4E, 4F and S4E). This variant harbors a mutation in 2 amino acids

264 necessary for Smo ciliary translocation (Corbit et al., 2005). Surprisingly, reconstitution
265 with the constitutively active SmoM2 mutant also failed to rescue metabolic
266 responsiveness to SAG (Figure 4E, 4F and S4E). Of note though, SmoM2 reconstituted
267 3T3-L1 cells did exhibit a moderate increase in glucose uptake and lactate accumulation
268 in the basal state. Knowing that canonical activation requires a second step of Smo-
269 translocation from the base to the tip of the cilium, and that SmoM2 accumulates towards
270 the tip (Han et al., 2008), these findings suggested that the base of the cilium could be
271 critical in linking Smo induction to Ampk activation. This interpretation is consistent with
272 the observations that *Ptch*^{-/-} MEFs (constitutively active canonical signaling) exhibit
273 metabolic rewiring in the basal state and show a lack of responsiveness to SAG that can
274 be reversed by long-term treatment with the Ampk blocker Compound C. Also, both the
275 basal hyper-activation and the lack-of-responsiveness phenotypes can be rescued by
276 long-term pre-incubation with SANT-1 (Figure 4G and 4H), which blocks Smo
277 translocation to the cilium base.

278 Boehlke et al. recently reported a cilium-based system in kidney epithelial cells coupling
279 mechanical sensation of fluid flow to cell-size regulation via basal-body restricted Lkb1
280 and Ampk (Boehlke et al., 2010). We used co-immunoprecipitation to test whether an
281 analogous system might be at play here. Importantly, Lkb1 and Ampk enriched in the
282 gamma-tubulin immunoprecipitation of mature 3T3-L1 cells (Fig. 4I, third column),
283 indicating presence of both proteins at the basal body. Similarly, we observed Lkb1 and
284 Ampk signals pulled down by acetylated alpha-tubulin (Fig. 4I, second column) indicating
285 their presence in the cilium. Critically, upon SAG activation we observed (i.) robust
286 enrichment of P-Ampk with gamma-tubulin pulldown, identifying a spatial node for non-
287 canonical signaling at the basal body (Fig. 4I, third column, top panel), (ii.) depletion of
288 Lkb1 from the acetylated alpha-tubulin compartment, and (iii.) apparent accumulation of

Lkb1 at gamma-tubulin. Thus, non-canonical Smo-signaling to Ampk appears compartmentalized at the basal-body.

Selective Partial Agonism of Metabolic Rewiring In Vitro and In Vivo

Numerous inhibitors of canonical Hedgehog signaling are in clinical trials for their anti-cancer potential (Ng and Curran, 2011). To tease apart the relative contributions of canonical Gli-dependent and non-canonical Ampk-dependent signaling we began to test well-characterized Hedgehog antagonists for their potential to block the identified rapid metabolic response. Surprisingly, when adding the classic Hedgehog antagonist cyclopamine (Chen et al., 2002a), initially as a negative control, we observed rapid and robust metabolic reprogramming analogous to that induced by SAG (Figure 5A-C). These effects were observed at 100 nM where cyclopamine binds efficiently to Smo but is incapable of displacing SAG from their shared binding site to block canonical signaling (Chen et al., 2002a; Chen et al., 2002b). Importantly, Cyclopamine is known to cause Smo translocation to the basal body of the cilium at these concentrations. Further, cyclopamine induced rapid and robust Ca^{++} - and AMPKAR responses sensitive to both extracellular EGTA and Ptx (Figure 5D and 5E), suggesting that SAG and cyclopamine induce a common non-canonical signal. Critically, cyclopamine-dependent glucose uptake, lactate production, and redox rebalancing were also all efficiently counteracted by addition of either Nifedipine, Ptx or the Ampk inhibitor compound C (Figure 5F-5H), and via knockdown of Smo in the 3T3-L1 system (Figure 5I and 5J). Thus, i. Smo-modulators can activate non-canonical signaling independently of the canonical arm, ii. canonical and non-canonical signaling arms can be uncoupled via the SAG/cyclopamine binding site of Smo, and iii. cyclopamine is a potent selective partial agonist of the non-canonical pathway.

314
315 Of chief importance, these findings provided us with a tool to probe the relevance of the
316 non-canonical axis in vivo. To test whether Smo selective partial agonism could boost
317 metabolism in vivo we performed oral glucose tolerance tests (OGTT) on wild-type
318 C57BL/6J mice in the presence or absence of acute cyclopamine pre-treatment.
319 Remarkably, mice receiving 10 mg/kg cyclopamine 15 minutes prior to testing displayed
320 a moderate but significant improvement in glucose tolerance (Figure 5K). Noteworthy,
321 neither basal nor glucose-stimulated insulin secretion appeared affected by the pre-
322 treatment (Figure S5A). Since insulin is known to counteract Ampk activity we reasoned
323 that an acute insulin response might be masking the full potential of the response. We
324 therefore examined the response in hyperglycemic-insulinemic clamps exploiting
325 continuous somatostatin infusion to suppress acute insulin responses. Impressively,
326 under these conditions cyclopamine infusion elicited robust glucose uptake (glucose
327 infusion rate) within 10-20 minutes of stimulation (Figure 5L). Thus, Smo selective partial
328 agonism induces metabolic rewiring and acute glucose uptake in vivo.

329 330 **Selective Partial Agonism Specifically Activates Muscle and Brown Fat**

331 In addition, the clamp studies were designed to trace tissue-specific glucose uptake via
332 introduction of a non-metabolizable 2-deoxyglucose radiolabel. Intriguingly, rather than
333 the anticipated white adipose tissue (WAT) glucose uptake, brown adipose tissue (BAT)
334 and muscles of multiple fiber types (extensor digitorum longus, EDL, glycolytic; and
335 soleus, Sol, oxidative) exhibited the dominant cyclopamine-induced glucose uptake
336 responses in vivo (Figure 6A). To explore the cell autonomy of this surprising finding we
337 stimulated C2C12 myotubes and differentiated primary brown adipocytes (pBAT) with
338 cyclopamine and measured glucose, lactate and NAD^+/NADH endpoints as before. In

keeping with the clamp results, both C2C12 and pBAT cells exhibited robust glucose uptake within 10 minutes of cyclopamine stimulation (Figure 6B). Brown adipocytes produced significant increases in lactate and NAD^+/NADH , whereas the myotubes, whose 10-fold lower lactate release rates suggest very high oxidative capacity, resisted both downstream metabolic sequelae (Figures 6C and 6D). These findings suggest that the exact metabolic end-state resulting from Smo stimulation depends on the machinery of the tissue type in question. Importantly, we observed prominent P-Ampk responses to cyclopamine in both cell models (Figure 6E). Equally important, all key endpoints including in vivo glycemic-insulinemic clamps, were recapitulated for SAG (Figure S6), corroborating the selectivity of cyclopamine-induced metabolic rewiring. Thus, selective partial agonism of the Smo-Ampk axis stimulates muscle and brown fat glucose uptake in mice.

These findings, observed under somatostatin infused conditions, raised the intriguing question whether the system might be effective even in the absence of insulin. To test this hypothesis, we treated mice with high-dose streptozotocin (STZ) to selectively neutralize insulin secretory capacity and generate a type-1 diabetes like state. Remarkably, despite a complete lack of circulating insulin, STZ-treated mice showed significant and robust glucose clearance within ~30 minutes of cyclopamine injection (Figure 6F). Further, in direct support of an endogenous glucose fate including brown adipose tissue, cyclopamine increased core body temperature $\sim 1.0^\circ\text{C}$ in the same mice over a 1 hour period (Figure 6G), suggesting even that selective partial agonism of the Smo-Ampk axis activates thermogenesis in vivo. Thus, Smo selective partial agonism induces insulin-independent and tissue-selective metabolic rewiring in vivo.

Smo Determines Metabolic Tone, Specificity and Flexibility

A number of studies indicate that Smo itself may have endogenous ligands, including two oxysterols 20(s)-OHC and 25-OHC (Corcoran and Scott, 2006; Nachtergaele et al., 2012). Given the potent gluco-regulatory effects of SAG and cyclopamine we revisited our in vitro system to ask if endogenous Smo-signaling might regulate metabolism. Indeed, Smo-knockdown reduced the anaerobic to aerobic ratio (lactate generation / glucose uptake) in both 3T3-L1 and C2C12 cells, manifest as either an increase in glucose uptake (3T3-L1), or a reduction in lactate generation (C2C12) (Figure 7A and 7B). Thus, Smo appears to modulate metabolic tone independent of exogenous ligand stimulation.

Since Smo-knockdown myocytes exhibited significant hyper-contractility to even minor perturbations we moved forward with Smo-knockdown 3T3-L1 cells. Intriguingly, despite a high metabolic rate, Smo-knockdown cells were incapable of mounting a substantial switch to glucose utilization when shifted from no-glucose to high-glucose medium, responding with a 5-fold smaller OCR burst than control cells (Figure 7C, 7D and S7A). Probing further, we used a panel of ~180 different carbon and nitrogen sources to fingerprint Smo-knockdown cells metabolically. The test, performed in fuel-depleted medium, measures substrate utilization for a spectrum of fuel sources, one substrate at a time. Intriguingly, Smo-knockdown 3T3-L1 adipocytes exhibited increased fuel utilization for the majority of substrates tested, including a remarkable 29 substrates completely unprocessed in control adipocytes (Figure 7E). Removal of Smo appeared to short-circuit cell-type restriction of fuel use, leaving cells able to metabolize a much wider substrate repertoire. Thus, Smo regulates metabolic flux, flexibility and substrate specificity.

Non-Canonical Signaling, Metabolism and Side Effects

389 This year, the FDA approved the first Hedgehog inhibitor, Vismodegib (GDC-0449), for
390 cancer therapy use in humans. Clinical data is currently available for five hedgehog
391 antagonists: GDC-0449, LDE-225, XL-139, IPI-926 and PF-0449913 (Li et al., 2012; Lin
392 and Matsui, 2012; Low and de Sauvage, 2010; Von Hoff et al., 2009). Unfortunately, the
393 first reports indicate side effects so severe that dropout rates have exceeded a
394 discouraging 50% (Sekulic et al., 2012; Tang et al., 2012). Intriguingly, the physiological
395 effects of our key non-canonical endpoints are highly consistent with the two major
396 reported side effects, weight loss (Ampk; catabolism) and muscle cramping (Ca^{++} -influx).
397 We probed our system to gain insight into whether non-canonical Smo-signaling might be
398 responsible for the side effect in humans. Intriguingly, initial testing revealed that all
399 compounds except jervine induced contraction in myocytes (Figure 7F). Because
400 contraction itself induces Ca^{++} and Ampk-dependent glucose uptake, this finding
401 precluded use of myocytes for quantitative determination of the non-canonical arm. We
402 therefore interrogated the canonical antagonists cyclopamine, jervine, SANT-1, GDC-
403 0449, LDE-225, XL-139 and agonists purmorphamine, SAG, the oxysterols 20(s)-OHC
404 and 25-OHC for their non-canonical and canonical potencies in mature 3T3-L1
405 adipocytes and Shh-Light2 cells (Figure 7F and 7G). Insulin, as a glucose uptake positive
406 control, and Tomatidine, as a steroid-like negative control, were also tested.
407 Interestingly, not all Smo-modulators induced non-canonical glucose uptake.
408 Cyclopamine and GDC-0449 did, making them selective partial agonists, as did SAG and
409 the two oxysterols, thus, dual agonists (Figure 7F bottom and S7B-D). Of these, all five
410 elicited significant Ca^{++} -responses and muscle contractility (Figure 7F top). Importantly
411 though, the reverse was not true. Not all Ca^{++} -inducing modulators elicited a glucose
412 uptake response (e.g. SANT-1, XL-139, LDE-225). So whereas Smo-ligand induced
413 glucose uptake appears to require Ca^{++} -induction, the processes can be

pharmacologically uncoupled. Of note, SANT-1, XL-139, and LDE-225 all block the initial Smo-translocation step to the cilium, additional support for the basal-body dependent mechanism presented above, and consistent with recently proposed multi-step activation models of Smo action. Thus, while Ca^{++} -influx is required for the glucose uptake response, Smo-dependent Ca^{++} induction alone does not induce metabolic rewiring.

Focusing on contractility, all Smo-modulators with the exception of jervine induced Ca^{++} -influx in the nanomolar range (Figure 7F). Interestingly, not all canonical agonists induced glucose uptake. Despite significant Ca^{++} and canonical activation signatures, purmorphamine showed no induction of non-canonical activity. Similarly, jervine showed no induction of any non-canonical response, Ca^{++} or glucose. It will be of great interest to see how Smo-“activation” by SAG, cyclopamine, jervine and Purmorphamine differ at the protein structure level. Together, these four compounds can independently uncouple canonical activation, canonical inhibition, Ca^{++} and Ampk signals presumably all via the same receptor.

Perhaps most relevant to muscle side effects in humans though, the finding that jervine exhibits canonical inhibition absent of both Ca^{++} and glucose uptake responses makes the prediction that development of small molecule inhibitors minimally impacting muscle contractility is possible. To gauge this hypothesis in vitro we have now tested SAG, cyclopamine, GDC-0449 and jervine in primary human myocytes. Importantly, in keeping with the 3T3-L1 data, SAG, cyclopamine and GDC-0449 all induced robust glucose uptake and P-AMPK responses (Figure 7H and S7E) in human muscle. They do so in the nanomolar range, and, in keeping with our Ca^{++} -data all three compounds elicit twitching of the human muscle fibers. Again and critically important, jervine showed no induction of glucose uptake, Ca^{++} or myocyte twitching. Thus, Smo-induced non-canonical glucose uptake and muscle contraction is conserved in primary human muscle cells.

Considering the implications for design of future Hedgehog therapeutics for cancer and other diseases, a rigorous analysis of the Smo-induced contractile responses appears to be of immediate and paramount importance.

DISCUSSION

Here, we show that a Smo-dependent non-canonical signal rewires metabolism in vitro and in vivo. This Smo-Camkk2-Ampk axis can be uncoupled from the canonical pathway at the level of Smo, and, cyclopamine and GDC-0449 are selective partial agonists of this rapid rewiring effect, where selective partial agonists are defined as compounds capable of differentially regulating two independent pathways via the same receptor. These demonstrations provide fundamental insights into Smo signaling, have substantial implications for ongoing Smo-modulator development for cancer, and open the door for Smo selective partial agonism as a new therapeutic strategy for obesity and diabetes.

We previously reported that mice with constitutively active canonical Hedgehog signaling in adipose tissue exhibit a unique healthy phenotype despite striking white adipose specific lipoatrophy (Pospisilik et al., 2010). Those findings identified Hedgehog signaling as one of the first hormonal axes capable of differentially regulating WAT and BAT adipogenesis. Interestingly, we once again observe BAT selectivity in the in vivo glucose uptake response to non-canonical agonism. What distinguishes the responsive tissues (BAT / red muscle) from the less responsive ones (WAT / white muscle)? Systematic analysis across over 25 mouse tissues showed no evidence for expression-based mechanism of either the canonical or non-canonical Hedgehog arms. Several of our own unpublished observations, however, support the concept that metabolic profile might determine responsiveness, including a strong TZD-dependence of the phenotype and

metabolic epistasis that appears to favor glycolytic metabolic profiles (R.T., J.A.P., H.E. *unpublished*). Certainly critical for the lack of WAT response, white adipocytes are aciliated (J. Reiter, *unpublished*) and rather than increasing glucose uptake as in most tissues, Ampk actually reduces glucose uptake in white adipose (Towler and Hardie, 2007).

Indeed, our data reveal a profound role for Smo-signaling in regulating metabolic flux, specificity and flexibility (Figure 7). These data are supported by our own transcriptional profiling of 3T3-L1 cells after 2 days of Shh stimulation that reveal highly significant enrichment scores for data sets associated with invasive cancer, hypoxia, and the transcriptional regulator Hif1 α , signatures that clustered around down-regulation of the KEGG/Biocarta pathways *oxidative phosphorylation*, *pyruvate metabolism*, and the *TCA cycle* among others (J.A.P. and H.E., *unpublished*). This finding indicates that canonical signaling reinforces the observed non-canonical metabolic routing by initiating a transcriptional aerobic glycolysis response and modulating expression of the entire machinery of primary metabolism.

Transcriptional reinforcement aside though, we see Smo selective partial agonism doubling whole body glucose uptake within ~30 minutes. Also, we observe an apparent parallel activation of thermogenesis with similar kinetics. The data therefore raise Smo selective partial agonism as a novel therapeutic option for obesity and types-1 and -2 diabetes, but more fundamentally, they demonstrate a dominant rapid physiological response of non-canonical hedgehog signaling in vivo. What additional non-canonical responses exist physiologically? Do endogenous ligands exist that selectively activate either canonical or non-canonical signaling? Certainly the two oxysterols 20(s)OHC and

25OHC are capable. To what extent do these factors underlie previous “canonical” effects? All these questions are open.

Many molecules have been shown to stimulate Smo-dependent signaling (Chen et al., 2002b; Corcoran and Scott, 2006; Nachtergaele et al., 2012; Sinha and Chen, 2005; Wang et al., 2010). While screening efforts typically rely on canonical readouts such as Gli-responsive luciferase reporters, few have interrogated non-canonical signaling in parallel. Systematic characterization of selective partial agonism should provide insights into the biochemical requirements necessary for canonical versus non-canonical stimulation and structural rationale for design of Smo selective partial agonists. Based on what we know, such Smo-modulators, much like the very pleiotropic SERM’s (*Selective Estrogen Receptor Modulators*) (Riggs and Hartmann, 2003), should have the potential to impact multiple Ampk-dependent pathways in and beyond metabolism, including cell size (kidney) and immune cell polarity (macrophages).

The uncoupling of canonical / non-canonical signals and activation of the Smo-Ampk axis by both SAG and cyclopamine, represents a paradigm shift. The finding adds a new level of complexity with which a cell can establish downstream signaling diversity and cell-type specificity. Cyclopamine has been shown to inhibit some non-canonical signals (Chinchilla et al., 2010; Polizio et al., 2011; Riobo et al., 2006). What distinguishes our findings from those? First, kinetics. Previous reports have used long-term measures to assess their effects. Perhaps more likely though is the second messenger system in question and sub-cellular localization, for instance at the cilium.

The last decade of Hedgehog research has seen substantial advances towards unraveling a “Gli-code” and linking ligand-binding to transcriptional output (Hui, 2010; Ruiz i Altaba et al., 2007). That said, a number of deep-seated paradigms remain enigmatic: the nature of Gli-target specification, Gli-target geneset expansion with signal intensity, and the inconsistencies in potency of pathway inhibition vs. potency of cancer growth inhibition. The demonstrations that Hedgehog rewires metabolism, and that canonical and non-canonical paths are independent, reveals functional plasticity that may – at least in part – explain these notions. For instance metabolic state alone can drive dramatic cell fate transitions and expression changes (e.g. immune cell polarity, activation state, cancer invasiveness, etc.) (Hitosugi et al., 2009; Hsu and Sabatini, 2008; Jeon et al., 2012; Shi et al., 2011). Indeed, it will be intriguing to see to what extent Hedgehog’s multiple pro-cancer effects depend on acute or chronic changes in metabolic state. We feel that to properly understand hedgehog phenotype, future analyses must include secondary messenger repertoire of the system in question and its respective non-canonical outputs.

Along similar lines, if canonical and non-canonical arms are independent then as long as Smo is expressed, the potential for a non-canonical response remains. GPCRs have the potential to signal when internalized (Calebiro et al., 2010), some even change their downstream effectors accordingly. Oxysterol binding to Smo has recently been shown to allosterically amplify hedgehog output (Corcoran and Scott, 2006; Nachtergaele et al., 2012). We now show they activate metabolic rewiring. These cell permeable lipid derivatives are quantitatively dynamic, found throughout the cell, and respond to the metabolic milieu. Should the notion of Hedgehog-responsiveness be fine-tuned? Are

537 some Hedgehog non-responsive cell-types still competent for non-canonical signals and
538 metabolic rewiring? These questions are now open.
539

EXPERIMENTAL PROCEDURES

Cell Culture

Primary brown preadipocytes, MEF's, C2C12's and mouse 3T3-L1 pre-adipocytes (ATCC) were grown and differentiated as described (Pospisilik et al., 2010; Todoric et al., 2011; Varjosalo et al., 2006; Wong et al., 2009). Before stimulations cells were starved over night in DMEM containing 1% BSA. All stimulations were performed in starvation medium. Human myoblast cultures and myocytes were established from four human study subjects according to (Gaster et al., 2002).

Cellular O₂/CO₂ exchange

A Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience) was used to analyze bioenergetic function of cells. Cellular DNA was measured to adjust for potential differences in cell densities.

Metabolites

NAD⁺, NADP⁺, NADH, NADPH, glucose and lactate were determined using commercially available kits (Bioassay Systems). AMP, ADP and ATP were determined as described (Liu et al., 2010).

shRNA

Lentiviruses expressing targeting shRNAs were used to transduce cells according to standard protocols. Puromycin was used to select stable clones.

Phospho-Protein Enrichment, 2D-PAGE and Mass Spectrometry

Phosphoproteins of total adipocyte protein were enriched by affinity column purification, isoelectric focused, and separated by SDS-PAGE. Detection, matching, background subtraction, normalization and quantitative comparison of scanned 2D spots used Progenesis SameSpots software (Nonlinear Dynamics). Spots were excised, tryptic digested, identified and quantified using an XCT-Ultra mass spectrometer.

AMPKAR-Foerster Resonance Energy Transfer Assay

Real-time AMPK activation was recorded using the AMPKAR-FRET construct as described (Tsou et al., 2011).

Ca⁺⁺ Signal Measurements

FluoForte AM (Enzo Lifescience) was used to measure dynamic changes of intracellular calcium levels according to the manufacturer's instructions.

Glucose Uptake

Basal glucose uptake of human myotubes was determined by capturing 2-[1-¹⁴C]-deoxy-glucose (Perkin Elmer) as previously described (Gaster et al., 2002).

Quantitative RT-PCR Analysis

Total RNA was extracted using TRIzol. cDNA was prepared with the iScript cDNA Synthesis Kit (Bio-Rad). qPCR was run using intron-spanning and junction primer pairs on an AbiPRISM 7500fast real-time cycler (Applied Biosystems) using the iQ SYBR Green Supermix (Bio-Rad).

Phenotype Microarrays

Phenotype microarray experiments were performed following protocols provided by Biolog Inc. (<http://www.biolog.com/>).

Animal Experiments

Eight to eleven week old, over-night fasted C57BL/6J male mice were used for in vivo studies. For cyclopamine testing mice were injected intraperitoneally (i.p.) with vehicle (45% HBS) or cyclopamine (10 mg/kg) either alone or in conjunction with a 1 g/kg OGTT. STZ induction was i.p. (200 mg/kg) followed by a 72 hour stabilization period. For clamps, femoral catheterized mice were infused continuously with 1 µg/kg/min somatostatin, and glycemia clamped to 12.5 mM by variable infusion of 10% glucose. Mice were maintained at steady-state for 90 minutes prior to cyclopamine administration.

Statistical Analysis

Data sets were compared for statistical significance using a two-tailed Students t-test, and data are expressed as mean ± standard error of the mean (SEM) unless otherwise specified. $p < 0.05$ was considered significant.

Other Methods

See Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental information includes Extended Experimental Procedures, 3 tables and 7 figures and can be found with this article online at doi:

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REFERENCES

- Belgacem, Y.H., and Borodinsky, L.N. (2011). Sonic hedgehog signaling is decoded by calcium spike activity in the developing spinal cord. *Proceedings of the National Academy of Sciences of the United States of America* *108*, 4482-4487.
- Boehlke, C., Kotsis, F., Patel, V., Braeg, S., Voelker, H., Bredt, S., Beyer, T., Janusch, H., Hamann, C., Gödel, M., *et al.* (2010). Primary cilia regulate mTORC1 activity and cell size through Lkb1. *Nature Cell Biology* *12*, 1115-1122.
- Calebiro, D., Nikolaev, V.O., Persani, L., and Lohse, M.J. (2010). Signaling by internalized G-protein-coupled receptors. *Trends in Pharmacological Sciences* *31*, 221-228.
- Canto, C., and Auwerx, J. (2011). Calorie Restriction: Is AMPK a Key Sensor and Effector? *Physiology* *26*, 214-224.
- Chen, J.K., Taipale, J., Cooper, M.K., and Beachy, P.A. (2002a). Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes and Development* *16*, 2743-2748.

639 Chen, J.K., Taipale, J., Young, K.E., Maiti, T., and Beachy, P.A. (2002b). Small molecule
640 modulation of Smoothened activity. *Proceedings of the National Academy of Sciences of*
641 *the United States of America* 99, 14071-14076.

642 Chen, W., Tang, T., Eastham-Anderson, J., Dunlap, D., Alicke, B., Nannini, M., Gould, S.,
643 Yauch, R., Modrusan, Z., Dupree, K.J., *et al.* (2011). Canonical hedgehog signaling
644 augments tumor angiogenesis by induction of VEGF-A in stromal perivascular cells.
645 *Proceedings of the National Academy of Sciences of the United States of America* 108,
646 9589-9594.

647 Chinchilla, P., Xiao, L., Kazanietz, M.G., and Riobo, N.A. (2010). Hedgehog proteins
648 activate pro-angiogenic responses in endothelial cells through non-canonical signaling
649 pathways. *Cell Cycle* 9, 570-579.

650 Corbit, K.C., Aanstad, P., Singla, V., Norman, A.R., Stainier, D.Y., and Reiter, J.F.
651 (2005). Vertebrate Smoothened functions at the primary cilium. *Nature* 437, 1018-1021.

652 Corcoran, R.B., and Scott, M.P. (2006). Oxysterols stimulate Sonic hedgehog signal
653 transduction and proliferation of medulloblastoma cells. *Proceedings of the National*
654 *Academy of Sciences of the United States of America* 103, 8408-8413.

655 Gaster, M., Petersen, I., Hojlund, K., Poulsen, P., and Beck-Nielsen, H. (2002). The
656 diabetic phenotype is conserved in myotubes established from diabetic subjects:
657 evidence for primary defects in glucose transport and glycogen synthase activity.
658 *Diabetes* 51, 921-927.

659 Han, Y.G., Spassky, N., Romaguera-Ros, M., Garcia-Verdugo, J.M., Aguilar, A.,
660 Schneider-Maunoury, S., and Alvarez-Buylla, A. (2008). Hedgehog signaling and primary

661 cilia are required for the formation of adult neural stem cells. *Nature Neuroscience* 11,
662 277-284.

663 Hardie, D.G. (2011). AMP-activated protein kinase--an energy sensor that regulates all
664 aspects of cell function. *Genes and Development* 25, 1895-1908.

665 Hitosugi, T., Kang, S., Vander Heiden, M.G., Chung, T.W., Elf, S., Lythgoe, K., Dong, S.,
666 Lonial, S., Wang, X., Chen, G.Z., *et al.* (2009). Tyrosine phosphorylation inhibits PKM2 to
667 promote the Warburg effect and tumor growth. *Science Signaling* 2, ra73.

668 Hsu, P.P., and Sabatini, D.M. (2008). Cancer Cell Metabolism: Warburg and Beyond.
669 *Cell* 134, 703-707.

670 Hui, C.-c. (2010). Gli Proteins in Development and Disease. *Annual Review of Cell and*
671 *Developmental Biology* 27, 513-537.

672 Ingham, P.W., Nakano, Y., and Seger, C. (2011). Mechanisms and functions of
673 Hedgehog signalling across the metazoa. *Nature Reviews Genetics* 12, 393-406.

674 Jeon, S.-M., Chandel, N.S., and Hay, N. (2012). AMPK regulates NADPH homeostasis to
675 promote tumour cell survival during energy stress. *Nature*.

676 Li, Y., Maitah, M.a.i.Y., Ahmad, A., Kong, D., Bao, B., and Sarkar, F.H. (2012). Targeting
677 the Hedgehog signaling pathway for cancer therapy. *Expert Opin Ther Targets* 16, 49-66.

678 Lin, T.L., and Matsui, W. (2012). Hedgehog pathway as a drug target: Smoothened
679 inhibitors in development. *OncoTargets and therapy* 5, 47-58.

680 Liu, Q., Gauthier, M.S., Sun, L., Ruderman, N., and Lodish, H. (2010). Activation of AMP-
681 activated protein kinase signaling pathway by adiponectin and insulin in mouse

682 adipocytes: requirement of acyl-CoA synthetases FATP1 and Acsl1 and association with
683 an elevation in AMP/ATP ratio. *Faseb J* 24, 4229-4239.

684 Low, J.A., and de Sauvage, F.J. (2010). Clinical experience with Hedgehog pathway
685 inhibitors. *Journal of Clinical Oncology* 28, 5321-5326.

686 Lowe, C.E., O'Rahilly, S., and Rochford, J.J. (2011). Adipogenesis at a glance. *J Cell Sci*
687 124, 2681-2686.

688 Lumeng, C.N., and Saltiel, A.R. (2011). Inflammatory links between obesity and
689 metabolic disease. *Journal of Clinical Investigation* 121, 2111-2117.

690 Nachtergaele, S., Mydock, L.K., Krishnan, K., Rammohan, J., Schlesinger, P.H., Covey,
691 D.F., and Rohatgi, R. (2012). Oxysterols are allosteric activators of the oncoprotein
692 Smoothened. *Nature Chemical Biology* 8, 211-220.

693 Ng, J.M.Y., and Curran, T. (2011). The Hedgehog's tale: developing strategies for
694 targeting cancer. *Nature Reviews Cancer* 11, 493-501.

695 Nusslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number
696 and polarity in *Drosophila*. *Nature* 287, 795-801.

697 Ogden, S.K., Fei, D.L., Schilling, N.S., Ahmed, Y.F., Hwa, J., and Robbins, D.J. (2008).
698 G protein Gai functions immediately downstream of Smoothened in Hedgehog signalling.
699 *Nature* 456, 967-970.

700 Polizio, A.H., Chinchilla, P., Chen, X., Kim, S., Manning, D.R., and Riobo, N.A. (2011).
701 Heterotrimeric Gi Proteins Link Hedgehog Signaling to Activation of Rho Small GTPases
702 to Promote Fibroblast Migration. *Journal of Biological Chemistry* 286, 19589-19596.

703 Pospisilik, J.A., Schramek, D., Schnidar, H., Cronin, S.J.F., Nehme, N.T., Zhang, X.,
704 Knauf, C., Cani, P.D., Aumayr, K., Todoric, J., *et al.* (2010). *Drosophila* Genome-wide
705 Obesity Screen Reveals Hedgehog as a Determinant of Brown versus White Adipose
706 Cell Fate. *Cell* 140, 148-160.

707 Riggs, B.L., and Hartmann, L.C. (2003). Selective estrogen-receptor modulators --
708 mechanisms of action and application to clinical practice. *New England Journal of*
709 *Medicine* 348, 618-629.

710 Riobo, N.A., and Manning, D.R. (2007). Pathways of signal transduction employed by
711 vertebrate Hedgehogs. *Biochemical Journal* 403, 369.

712 Riobo, N.A., Saucy, B., Dilizio, C., and Manning, D.R. (2006). Activation of heterotrimeric
713 G proteins by Smoothened. *Proceedings of the National Academy of Sciences of the*
714 *United States of America* 103, 12607-12612.

715 Rohatgi, R., Milenkovic, L., Corcoran, R.B., and Scott, M.P. (2009). Hedgehog signal
716 transduction by Smoothened: pharmacologic evidence for a 2-step activation process.
717 *Proceedings of the National Academy of Sciences of the United States of America* 106,
718 3196-3201.

719 Rohatgi, R., Milenkovic, L., and Scott, M.P. (2007). Patched1 regulates hedgehog
720 signaling at the primary cilium. *Science* 317, 372-376.

721 Rohatgi, R., and Scott, M.P. (2007). Patching the gaps in Hedgehog signalling. *Nature*
722 *Cell Biology* 9, 1005-1009.

723 Roudaut, H., Traiffort, E., Gorojankina, T., Vincent, L., Faure, H., Schoenfelder, A., Mann,
724 A., Manetti, F., Solinas, A., Taddei, M., *et al.* (2011). Identification and mechanism of

725 action of the acylguanidine MRT-83, a novel potent Smoothed antagonist. Molecular
726 Pharmacology 79, 453-460.

727 Ruiz i Altaba, A., Mas, C., and Stecca, B. (2007). The Gli code: an information nexus
728 regulating cell fate, stemness and cancer. Trends Cell Biol 17, 438-447.

729 Sekulic, A., Migden, M.R., Oro, A.E., Dirix, L., Lewis, K.D., Hainsworth, J.D., Solomon,
730 J.A., Yoo, S., Arron, S.T., Friedlander, P.A., *et al.* (2012). Efficacy and safety of
731 vismodegib in advanced basal-cell carcinoma. New England Journal of Medicine 366,
732 2171-2179.

733 Shi, L.Z., Wang, R., Huang, G., Vogel, P., Neale, G., Green, D.R., and Chi, H. (2011).
734 HIF1 -dependent glycolytic pathway orchestrates a metabolic checkpoint for the
735 differentiation of TH17 and Treg cells. Journal of Experimental Medicine 208, 1367-1376.

736 Sinha, S., and Chen, J.K. (2005). Purmorphamine activates the Hedgehog pathway by
737 targeting Smoothed. Nature Chemical Biology 2, 29-30.

738 Suh, J.M., Gao, X., McKay, J., McKay, R., Salo, Z., and Graff, J.M. (2006). Hedgehog
739 signaling plays a conserved role in inhibiting fat formation. Cell Metabolism 3, 25-34.

740 Tang, J.Y., Mackay-Wiggan, J.M., Aszterbaum, M., Yauch, R.L., Lindgren, J., Chang, K.,
741 Coppola, C., Chanana, A.M., Marji, J., Bickers, D.R., *et al.* (2012). Inhibiting the
742 hedgehog pathway in patients with the basal-cell nevus syndrome. New England Journal
743 of Medicine 366, 2180-2188.

744 Teglund, S., and Toftgård, R. (2010). Hedgehog beyond medulloblastoma and basal cell
745 carcinoma. Biochimica et Biophysica Acta 1805, 181-208.

746 Theunissen, J.W., and de Sauvage, F.J. (2009). Paracrine Hedgehog signaling in cancer.
747 Cancer Res 69, 6007-6010.

748 Todoric, J., Strobl, B., Jais, A., Boucheron, N., Bayer, M., Amann, S., Lindroos, J.,
749 Teperino, R., Prager, G., Bilban, M., *et al.* (2011). Cross-Talk Between Interferon-
750 gamma and Hedgehog Signaling Regulates Adipogenesis. Diabetes 60, 1668-1676.

751 Towler, M.C., and Hardie, D.G. (2007). AMP-Activated Protein Kinase in Metabolic
752 Control and Insulin Signaling. Circulation Research 100, 328-341.

753 Tsou, P., Zheng, B., Hsu, C.-H., Sasaki, A.T., and Cantley, L.C. (2011). A Fluorescent
754 Reporter of AMPK Activity and Cellular Energy Stress. Cell Metabolism 13, 476-486.

755 Vander Heiden, M.G., Cantley, L.C., and Thompson, C.B. (2009). Understanding the
756 Warburg effect: the metabolic requirements of cell proliferation. Science 324, 1029-1033.

757 Varjosalo, M., Li, S.-P., and Taipale, J. (2006). Divergence of Hedgehog Signal
758 Transduction Mechanism between Drosophila and Mammals. Developmental Cell 10,
759 177-186.

760 Von Hoff, D.D., LoRusso, P.M., Rudin, C.M., Reddy, J.C., Yauch, R.L., Tibes, R., Weiss,
761 G.J., Borad, M.J., Hann, C.L., Brahmer, J.R., *et al.* (2009). Inhibition of the hedgehog
762 pathway in advanced basal-cell carcinoma. New England Journal of Medicine 361, 1164-
763 1172.

764 Wang, J., Lu, J., Bond, M.C., Chen, M., Ren, X.R., Lyster, H.K., Barak, L.S., and Chen,
765 W. (2010). Identification of select glucocorticoids as Smoothed agonists: potential
766 utility for regenerative medicine. Proceedings of the National Academy of Sciences of the
767 United States of America 107, 9323-9328.

768 Wang, Y., Zhou, Z., Walsh, C.T., and McMahon, A.P. (2009). Selective translocation of
769 intracellular Smoothed to the primary cilium in response to Hedgehog pathway
770 modulation. *Proceedings of the National Academy of Sciences of the United States of*
771 *America* 106, 2623-2628.

772 Whittle, A.J., López, M., and Vidal-Puig, A. (2011). Using brown adipose tissue to treat
773 obesity - the central issue. *Trends in Molecular Medicine* 8, 405-411.

774 Wong, S.Y., Seol, A.D., So, P.L., Ermilov, A.N., Bichakjian, C.K., Epstein, E.H., Jr.,
775 Dlugosz, A.A., and Reiter, J.F. (2009). Primary cilia can both mediate and suppress
776 Hedgehog pathway-dependent tumorigenesis. *Nature Medicine* 15, 1055-1061.

777 Yam, P.T., Langlois, S.D., Morin, S., and Charron, F. (2009). Sonic Hedgehog Guides
778 Axons through a Noncanonical, Src-Family-Kinase-Dependent Signaling Pathway.
779 *Neuron* 62, 349-362.

780 Yauch, R.L., Gould, S.E., Scales, S.J., Tang, T., Tian, H., Ahn, C.P., Marshall, D., Fu, L.,
781 Januario, T., Kallop, D., *et al.* (2008). A paracrine requirement for hedgehog signalling in
782 cancer. *Nature* 455, 406-410.

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FIGURE LEGENDS

Figure 1. Hedgehog Reprograms Adipocyte Metabolism. (A – E) Differentiated 3T3-L1 adipocytes treated with SAG (200 nM) acidify growth medium (A); display reduced O₂-consumption (OCR) and increased extracellular acidification (ECAR) (B); utilize more glucose and produce more lactate (C); and show increased NAD⁺, NADP⁺ (D) and their ratios within 24 hours of treatment (E). (F – H) Similarly, treatment with Sonic Hedgehog (Shh) increased glucose consumption (F); lactate production (G) and NAD⁺/NADH ratio (H). (I – K) Unlike wild-type (Wt) MEFs, Smo^{-/-} MEFs are not responsive to SAG in terms of glucose consumption (I); lactate production (J); or change in NAD⁺/NADH ratio (K). Results are mean ± SEM where n = 4-6 independent experiments. *p < 0.05 compared to vehicle-treated samples. See also Figure S1.

Figure 2. Hedgehog Triggers Rapid Metabolic Rewiring via Ampk. (A and B) SAG-treated 3T3-L1 adipocytes increase glucose consumption (A) and lactate production (B) within minutes. (C) Phospho-2D-Gel highlighting spots changed within 10 min of SAG addition. (D) KEGG pathway analysis of differentially regulated phospho-proteins. (E) Two significantly phosphorylated spots corresponding to Pdha1. (F) Western blot time course of SAG-induced protein phosphorylation in 3T3-L1 adipocytes. (G – I) Ampk and Pkm2 knockdown abrogates SAG-induced glucose disposal (G); lactate production (H) and elevation in NAD⁺/NADH ratio (I). Glut4 knockdown blunts SAG-induced glucose uptake (J) and lactate production (K). (L) HEK293 cells an AMPK activity FRET reporter exhibit a rapid and robust signal upon SAG treatment. Results are mean ± SEM where n = 4-6 independent experiments. *p < 0.05 compared to vehicle-treated samples (A and B) or scrambled shRNA-transfected cells (G – K). See also Figure S2.

812

813 **Figure 3. A Novel Smo-Camkk2-Ampk Axis.** (A – C) Lkb1 and Camkk2 knockdown
814 abrogates SAG-induced glucose disposal (A); lactate production (B) and NAD⁺/NADH
815 elevation (C) in 3T3-L1 adipocytes. (D) SAG-treated adipocytes show a rapid and robust
816 calcium response, completely blunted by the extracellular calcium chelator EGTA (1 μ M).
817 (E) SAG-induced AMPK activity in AMPK-AR reporter expressing HEK293 cells is
818 inhibited by both Nifedipine and Ptx. (F – H) Nifedipine (20 μ M) or Ptx (100 ng/ml) treated
819 3T3-L1 adipocytes are refractory to SAG-induced glucose disposal (F); lactate production
820 (G) and NAD⁺/NADH elevation (H). Results are mean $\pm$ SEM where n = 4-6 independent
821 experiments. *p < 0.05 compared to scrambled shRNA-transfected cells (A – C) or
822 vehicle-treated samples (F – H). See also Figure S3.

823

824 **Figure 4. Non-canonical Rewiring of Metabolism is Primary Cilium Dependent.** (A-
825 D) MEFs derived from either *Ift88* or *Kif3a* knockout mice show no SAG-induced glucose
826 disposal (A), lactate production (B) or Ampk activation (D), despite an intact Ca⁺⁺
827 response (C). (E, F) Smo knockdown abrogates SAG-induced glucose disposal (E) and
828 lactate production (F) in 3T3-L1 adipocytes, effects rescued by the reintroduction of wild-
829 type Smo but not SmoM2 and Smo-CLD mutants. (G, H) MEFs derived from *Ptch* ^{-/-}
830 mice show increased basal glucose disposal (G) and lactate production (H) with no
831 further SAG response. Responsiveness is restored by chronic treatment with 100 nM
832 SANT-1. (I) Co-immunoprecipitation analysis of the Smo-Lkb1-Ampk axis. Results are
833 mean $\pm$ SEM where n = 4-6 independent experiments. *p < 0.05 compared to vehicle-
834 treated samples (A – H). §p < 0.05 compared to Smo-KD (E, F) or *Ptch*^{-/-} cells (G, H).
835 See also Figure S4.

836

Figure 5. Smo-Ampk Selective Partial Agonism Stimulates Glucose Uptake in vivo.

(A – C) Cyclopamine (100 nM) induces metabolic reprogramming in differentiated 3T3-L1 adipocytes increasing glucose consumption (A); lactate production (B) and NAD^+/NADH (C). (D) Cyclopamine elicits a rapid EGTA-sensitive Ca^{++} response. (E) AMPK-AR expressing HEK293 cells show rapid induction of AMPK activity upon cyclopamine treatment, an effect blunted by both Nifedipine and Ptx. (F – H) Cyclopamine-induced glucose consumption (F), lactate production (G) and elevation of NAD^+/NADH (H) are also abrogated by Nifedipine, Ptx and compound C. (I, J) Smo-knockdown 3T3-L1 adipocytes show no sign of cyclopamine-induced glucose disposal (I) and lactate production (J). (K) C57BL/6J mice display improved glucose tolerance ($n = 12$) when pretreated with cyclopamine (10 mg/kg, i.p.; $n=12$) relative to vehicle alone (HBS). (L) Increased glucose-infusion rate (GIR) during glycemic-insulinemic clamps ($n = 6-10$). Results represent mean $\pm$ SEM where $n = 4-6$ independent experiments in vitro. * $p < 0.05$ compared to vehicle-treated samples. See also Figure S5.

Figure 6. Insulin Independent Glucose Uptake in Muscle and Brown Fat. (A)

Glucose tracers revealed that cyclopamine enhances glucose uptake in BAT, EDL and Soleus muscles (EP, epididymal adipose; BAT, brown adipose tissue; VL, vas lateralis; EDL, extensor digitorum longus; Sol, soleus). (B – D) Whereas C2C12 myotubes exhibit only increased glucose disposal, primary brown adipocytes (pBAT) show increased glucose consumption (B); lactate production (C) and elevation of NAD^+/NADH (D) upon cyclopamine treatment. The chemical Ampk agonist AICAR (0.5 mM) only partially recapitulates the phenotypes. (E) Western Blot of cyclopamine-induced Ampk phosphorylation in pBAT and C2C12 myotubes. (F and G) Cyclopamine treatment (10 mg/kg) induces glucose clearance and elevation in body temperature ($n = 6-26$) in

862 insulin-deficient STZ-treated C57BL/6J mice. Results are mean $\pm$ SEM where n = 4-6
863 independent experiments. *p < 0.05 compared to vehicle-treated samples. See also
864 Figure S6.

865

866 **Figure 7. Smo Controls Metabolic Flexibility and Skeletal Muscle Contraction.** (A, B)
867 Smo-knockdown increases basal glucose consumption in adipocytes (A) and decreases
868 lactate production in C2C12 cells (B). Both effects are rescued by the re-introduction of
869 wild-type Smo (A, B). (C, E) Smo knockdown 3T3-L1 adipocytes exhibit increased
870 metabolic rate (C) and complete lack of glycolytic flexibility upon glucose exposure (D).
871 (E) Smo knockdown adipocytes show increased metabolism of a broad range of carbon
872 substrates. (F) Assessment of a panel of Smo-modulators revealed tight correlation
873 between induction of a Ca^{++} -response and contraction, and largely uncoupled canonical
874 (red circles = IC50; green open circles = EC50 Gli-induction in Shh-Light2 cells) and non-
875 canonical (green solid circles = EC50 glucose uptake) responses. (G) Dose-response
876 curves for all compounds inducing glucose uptake. (H) SAG, cyclopamine and GDC-
877 0449 induce glucose disposal in primary human myocytes. Results are mean $\pm$ SEM
878 where n = 4-6 independent experiments in vitro. *p < 0.05 compared to vehicle-treated
879 samples. See also Figure S7.

Figure 1

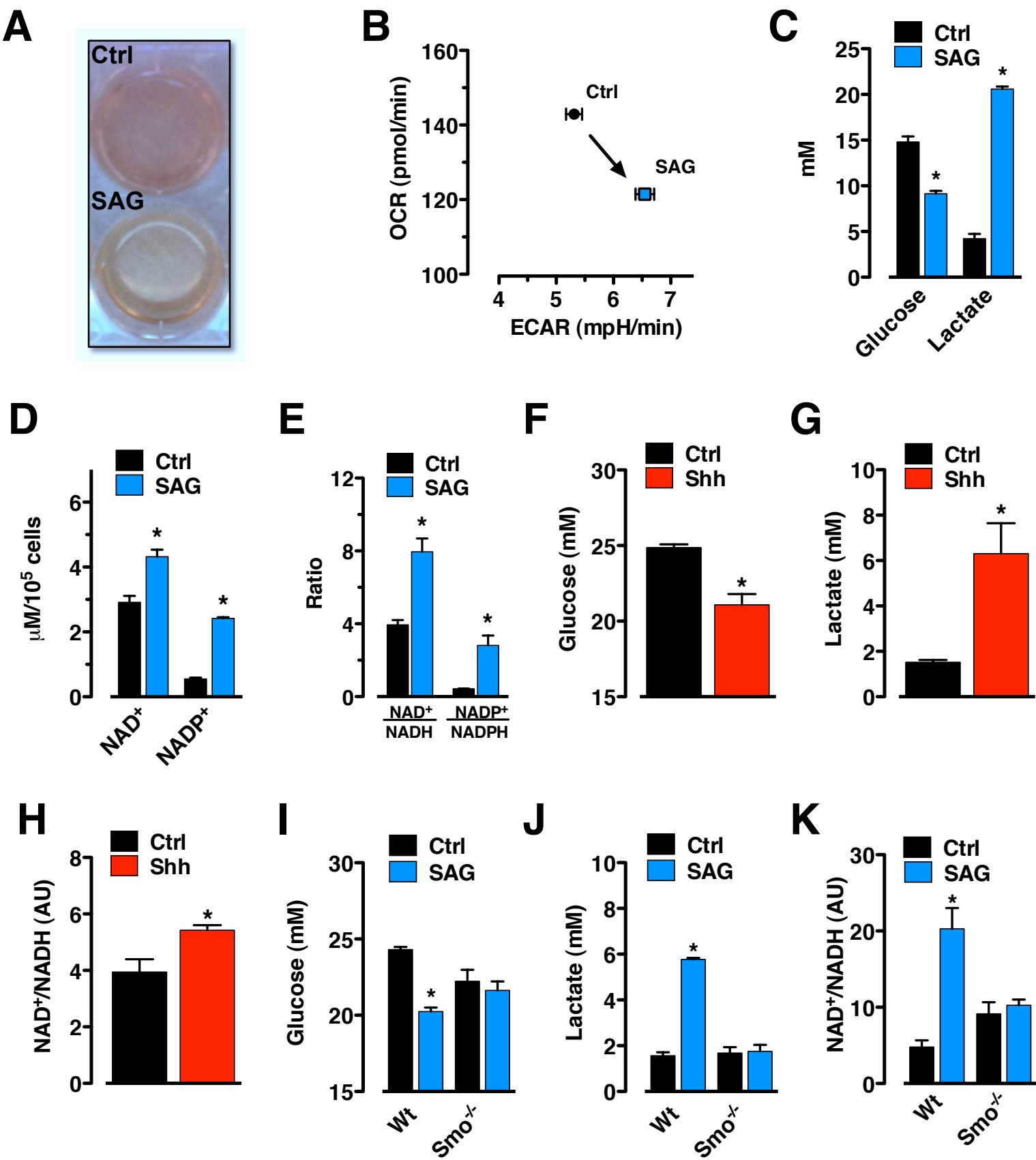


Figure
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Figure 2

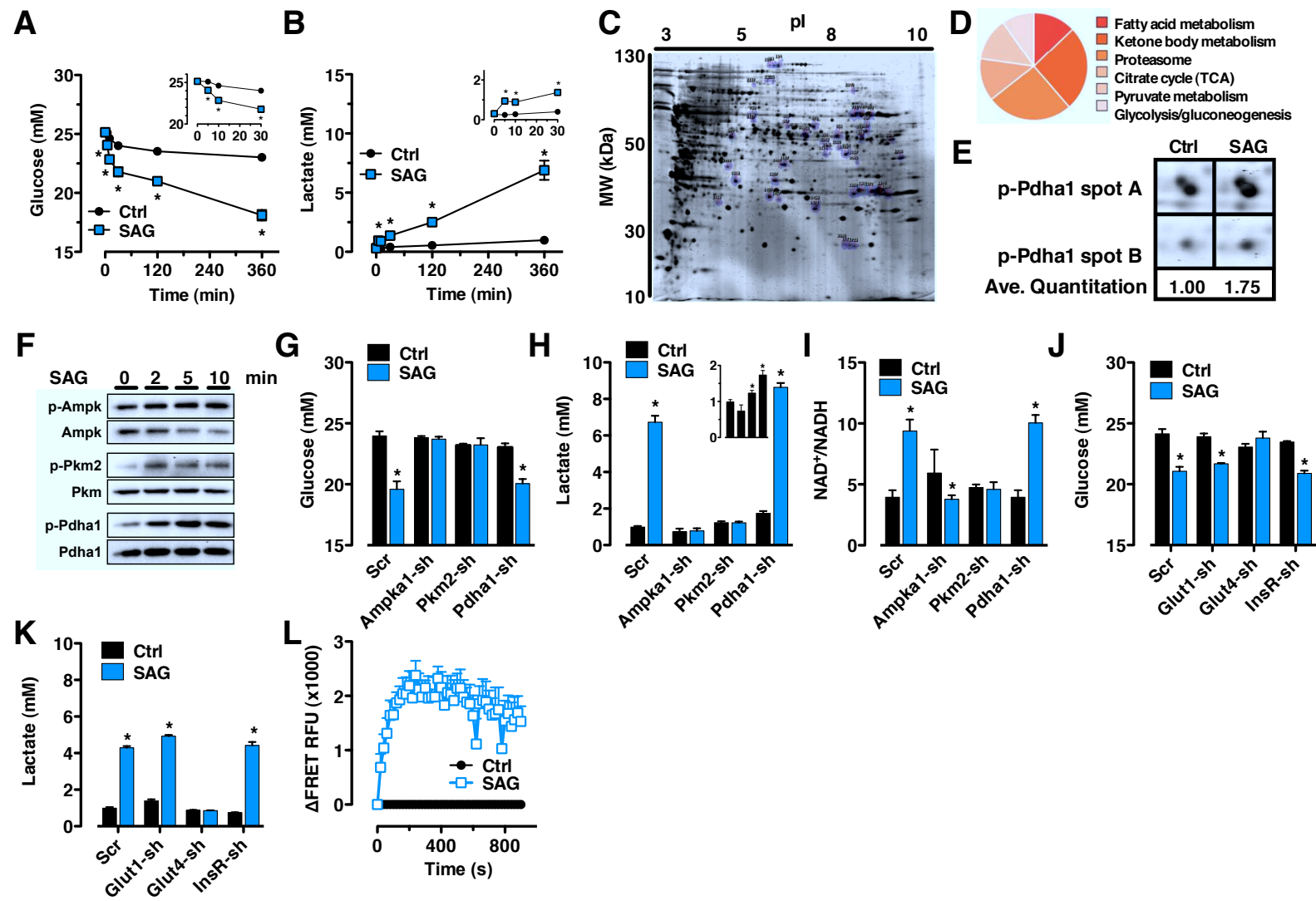


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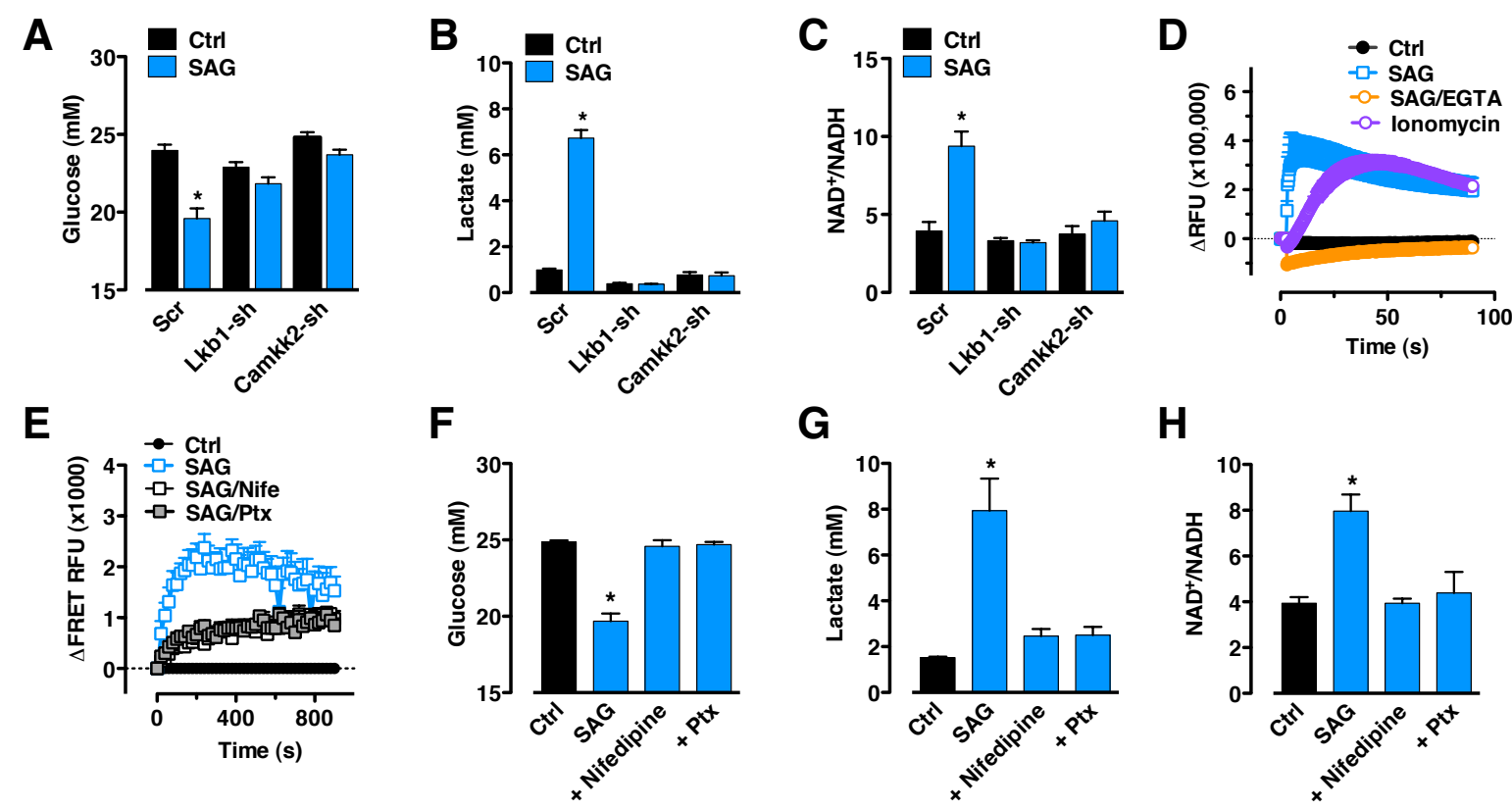


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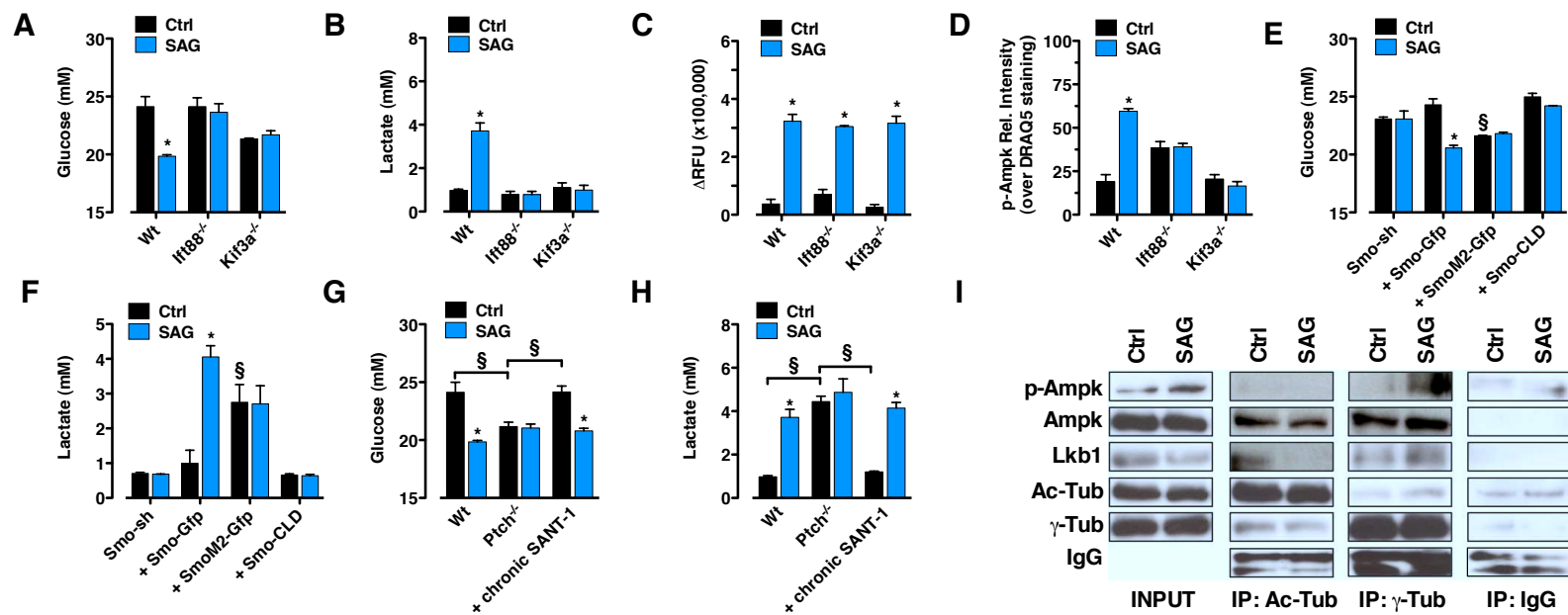


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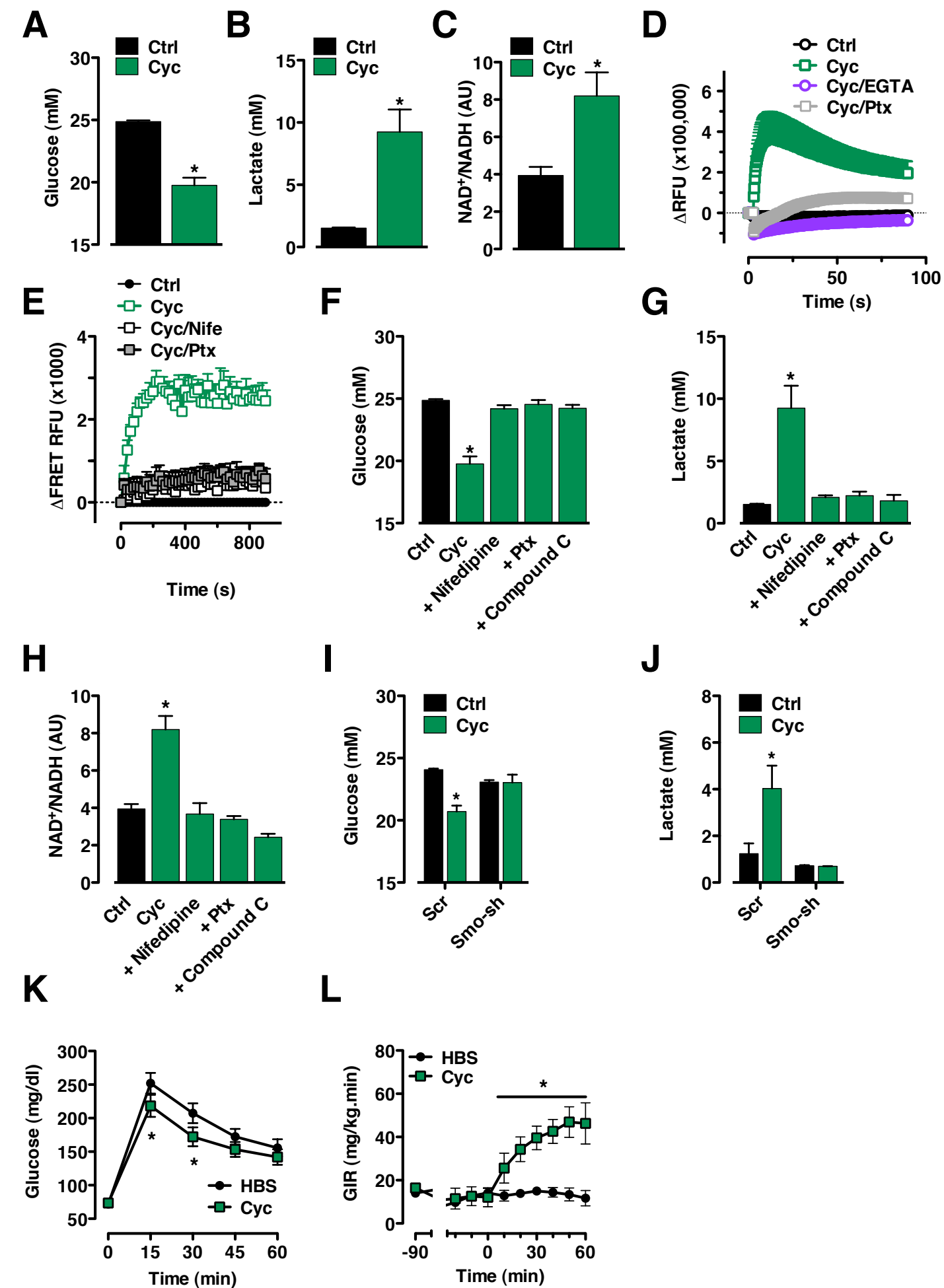


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Figure 6

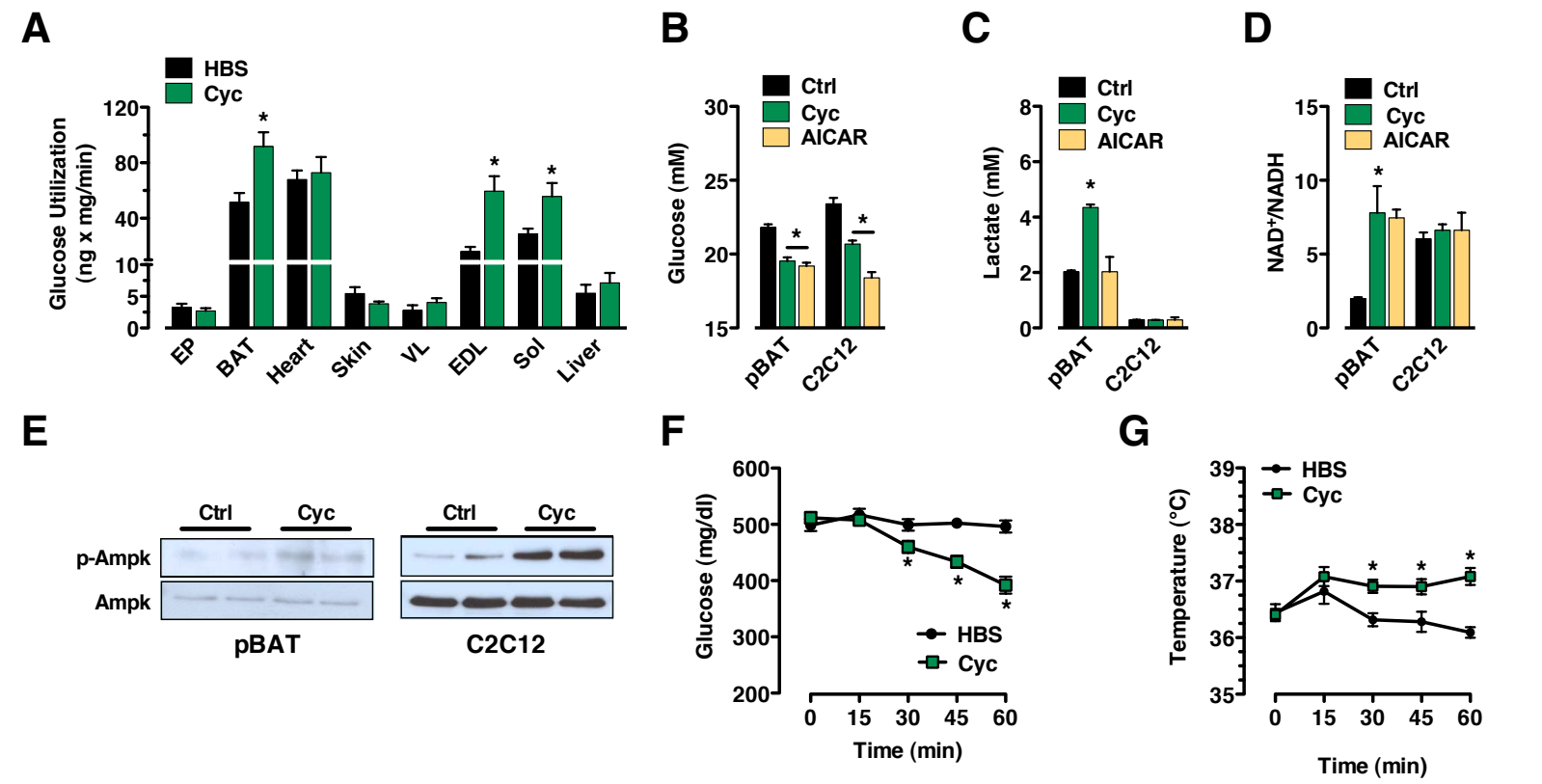
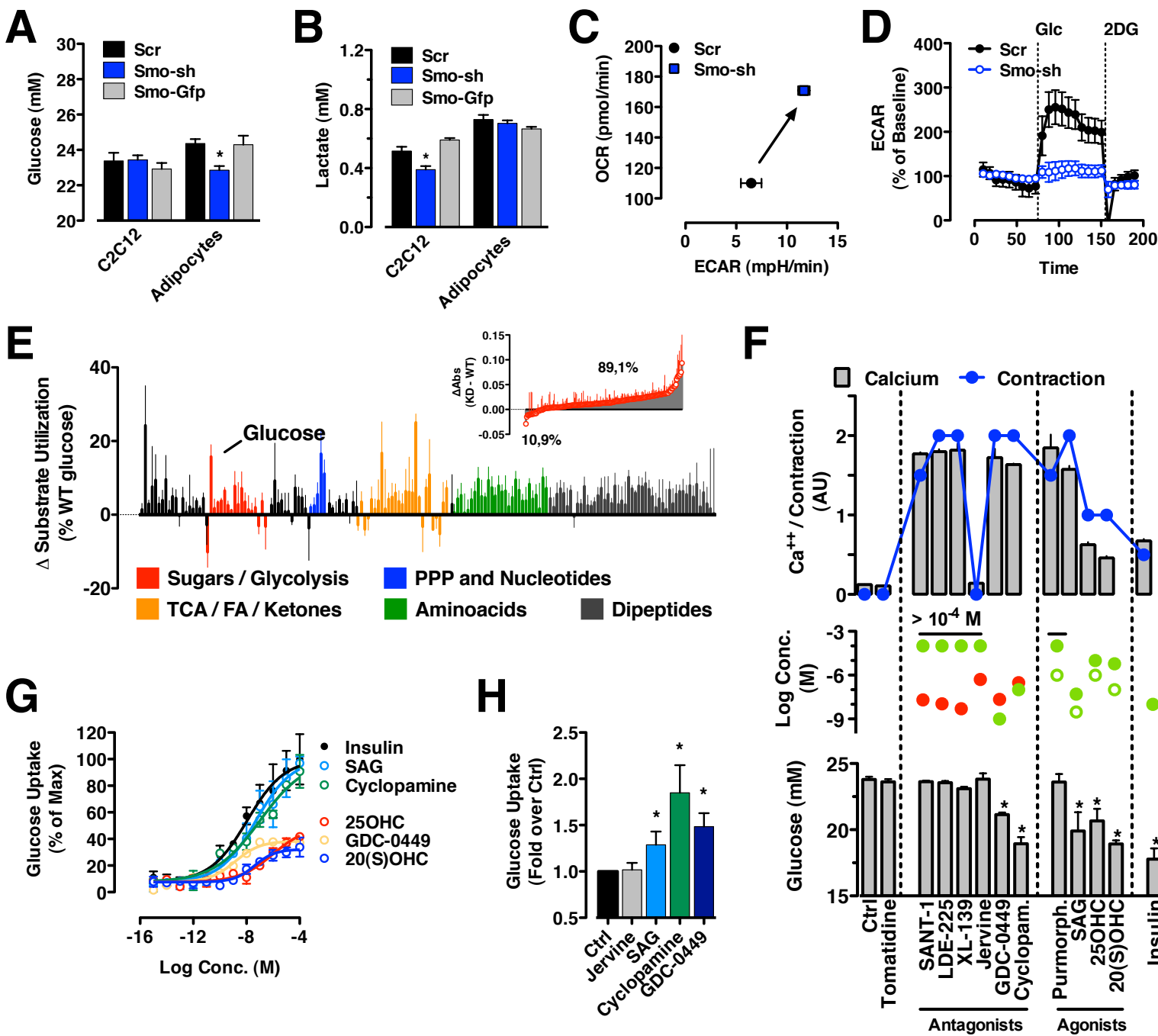


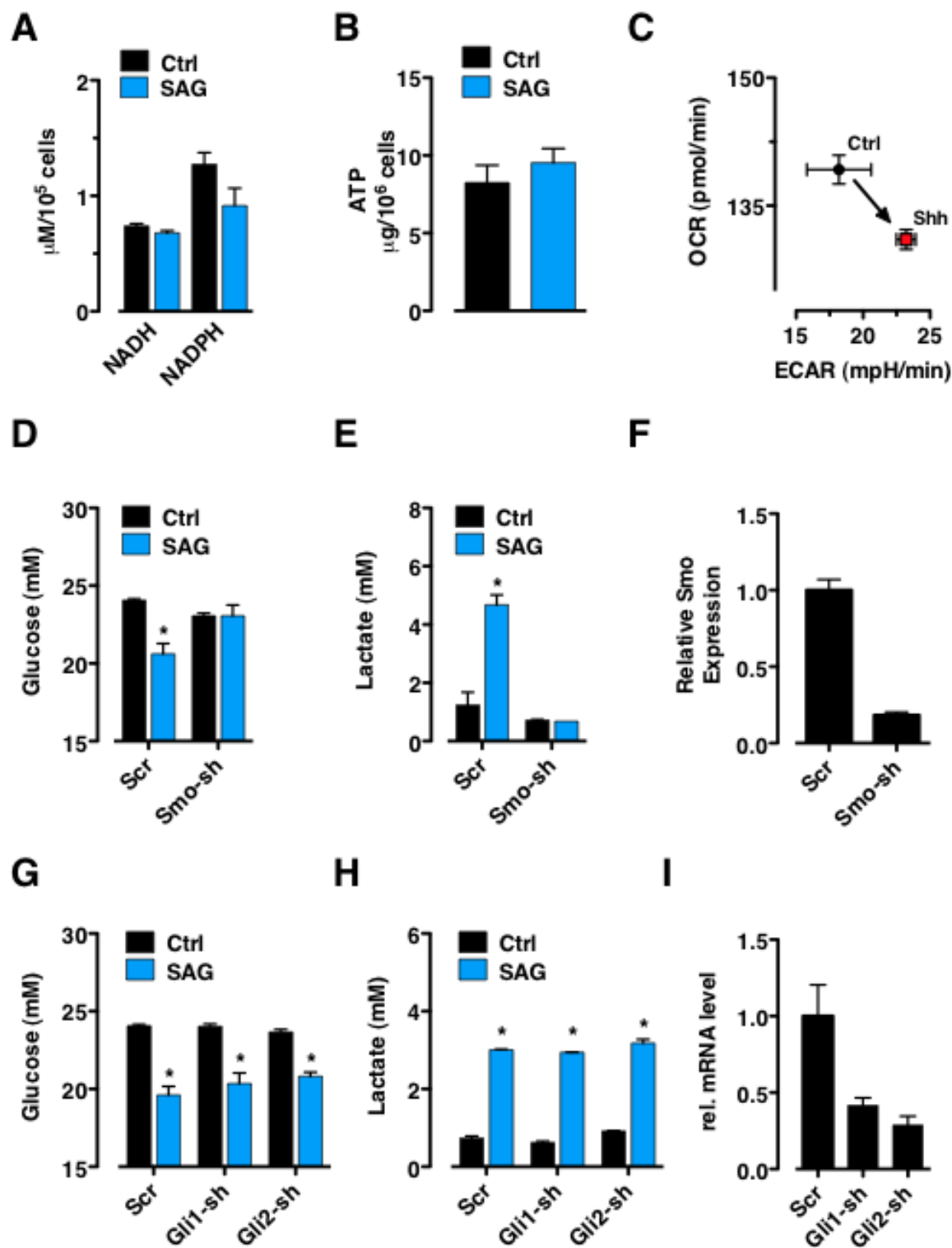
Figure 7



1 Supplemental Information

2

Supplementary Figure S1



3

Figure S1. Hedgehog Reprograms Adipocyte Metabolism, Related to Figure 1

SAG-treated 3T3-L1 adipocytes show no differences in NADH and NADPH (A), as well ATP levels (B). (C) Shh-treated 3T3-L1 adipocytes show reduced O₂ Consumption Rate (OCR) and increased Extra Cellular Acidification Rate (ECAR). (D-F) SAG-induced glucose consumption (D) and lactate production (E) are blunted upon Smo knockdown in 3T3-L1 adipocytes. (G-I) SAG-induced glucose consumption (G) and lactate production (H) are unaffected by Gli1 and Gli2 knockdown in 3T3-L1 adipocytes (I). Results represent mean $\pm$ SEM (n = 3).

Supplementary Figure S2

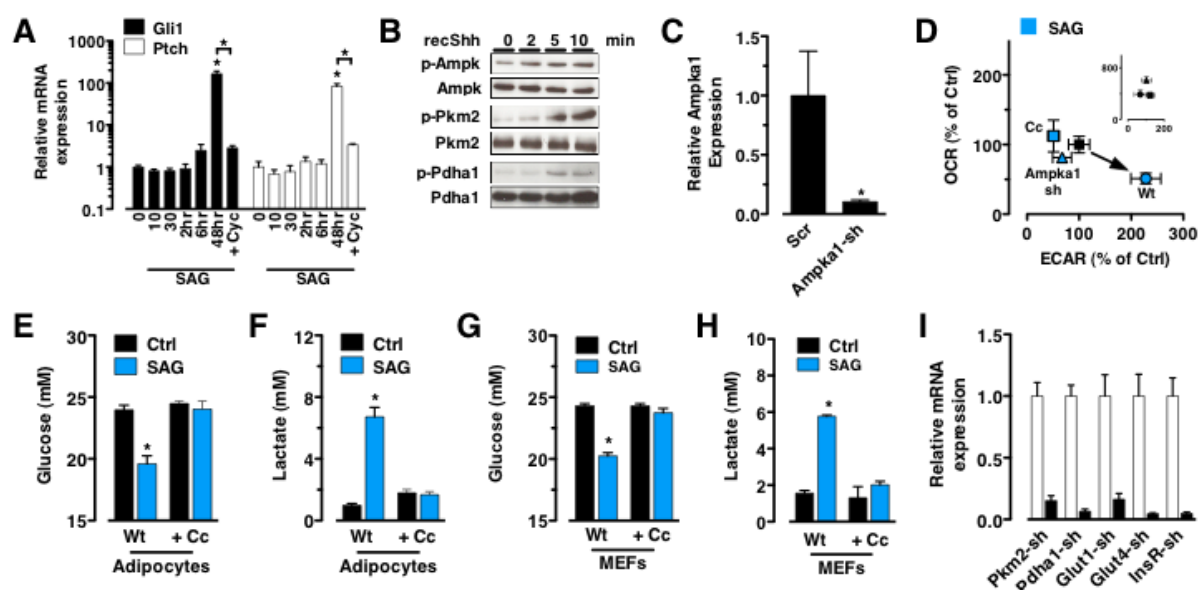


Figure S2. Hedgehog Triggers Rapid Metabolic Rewiring via Ampk, Relates to Figure 2

(A) Real-Time PCR time course of SAG-induced Gli1 and Ptch expression in 3T3-L1 adipocytes. (B) Western-blot time-course of Shh-induced protein phosphorylation in 3T3-

L1 adipocytes. (C) Real-time PCR evaluation of Ampk expression in knock-down 3T3-L1 adipocytes. (D) Ampka1 knockdown in 3T3-L1 adipocytes blunts SAG-induced (blue shapes) reduction in OCR and increase in ECAR. Basal OCR/ECAR rates are shown in the insert. (E-H) Chemical inhibition of Ampk activity with Compound C (Cc 10 μ M) blunts SAG-induced glucose consumption (E; G) and lactate production (F; H) in 3T3-L1 adipocytes (E-F) and wild-type MEFs (G-H). (I) Real-time PCR evaluation of Pkm2, Pdha1, Glut1, Glut4 and Insulin Receptor (InsR) expression in knock-down 3T3-L1 adipocytes. Results represent mean $\pm$ SEM (n = 3).

Supplementary Figure S3

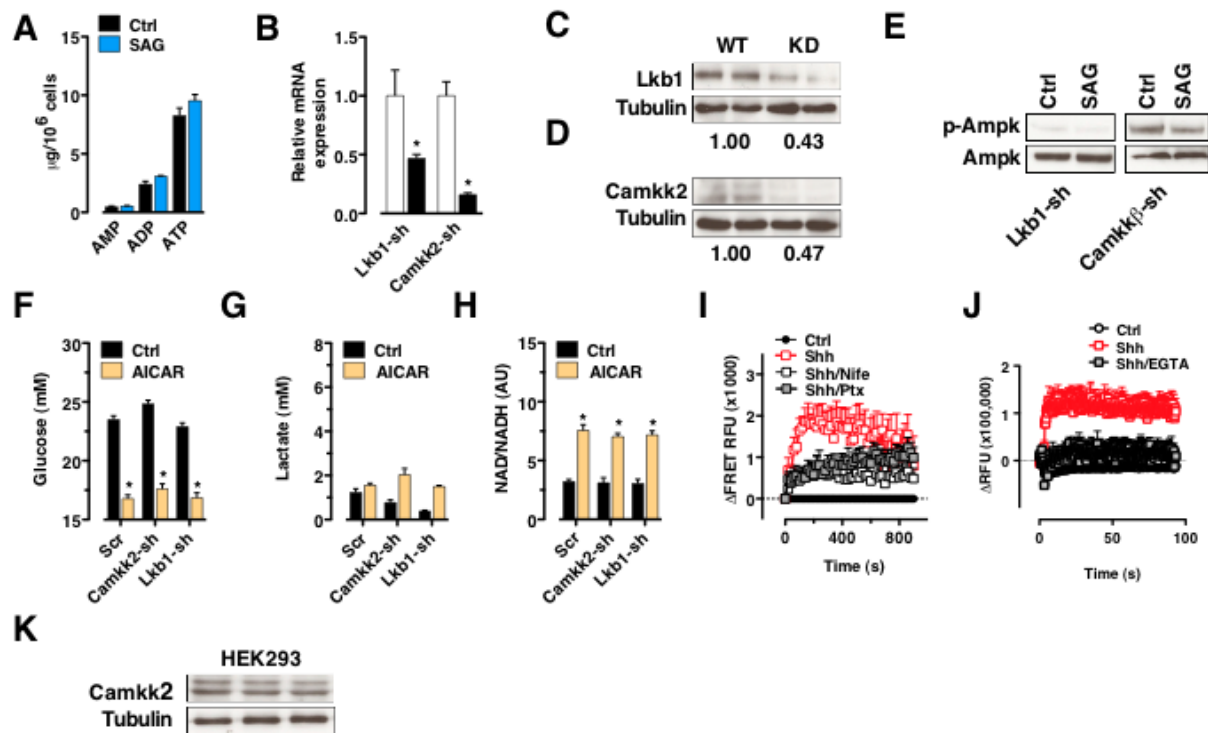


Figure S3. A Novel Smo-Lkb1/Camkk2-Ampk Axis, Relates to Figure 3

(A) SAG-treated 3T3-L1 adipocytes show no differences in AMP, ADP and ATP levels.

(B) Real-Time PCR evaluation of *Stk11/Lkb1* and *Camkk2* expression in knockdown 3T3-L1 adipocytes. (C) *Lkb1* protein expression in wild-type and knockdown 3T3-L1 adipocytes. (D) *Camkk2* protein expression in wild-type and knockdown 3T3-L1 adipocytes. (E) Western blot analysis of SAG-induced *Ampk* phosphorylation in *Lkb1* and *Camkk2* knockdown 3T3-L1 adipocytes. (F-H) Effect of AICAR treatment on glucose consumption (F), lactate production (G) and NAD^+/NADH ratio (H) in *Camkk2* and *Lkb1* knockdown 3T3-L1 adipocytes. (I) 3T3-L1 adipocytes loaded with the calcium dye FluoForte-4AM exhibit a rapid and robust calcium response upon *Shh* stimulation, completely blunted by the extracellular calcium chelator EGTA ($1\ \mu\text{M}$). (J) *Shh*-induced AMPK activity in HEK293 cells transfected with the AMPK-AR reporter construct is inhibited by both Nifedipine and Ptx treatment. (K) *Camkk2* protein expression in Human Embryonic Kidney (HEK293) cells. Results represent mean $\pm$ SEM ($n = 3$).

Supplementary Figure S4

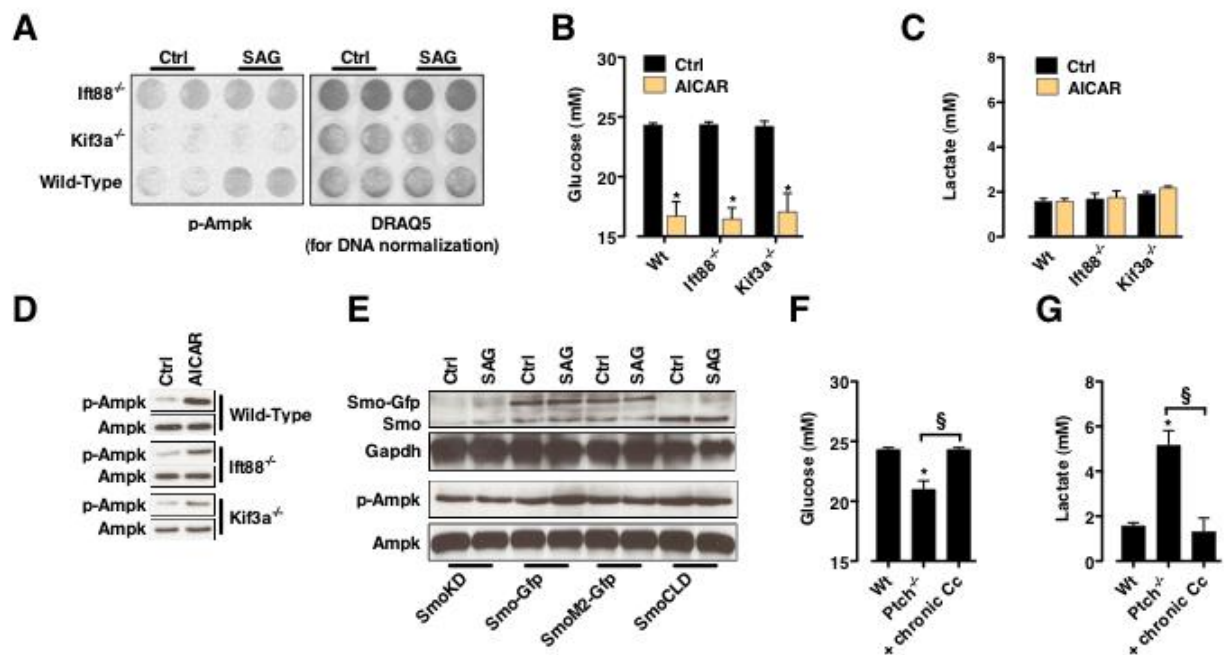
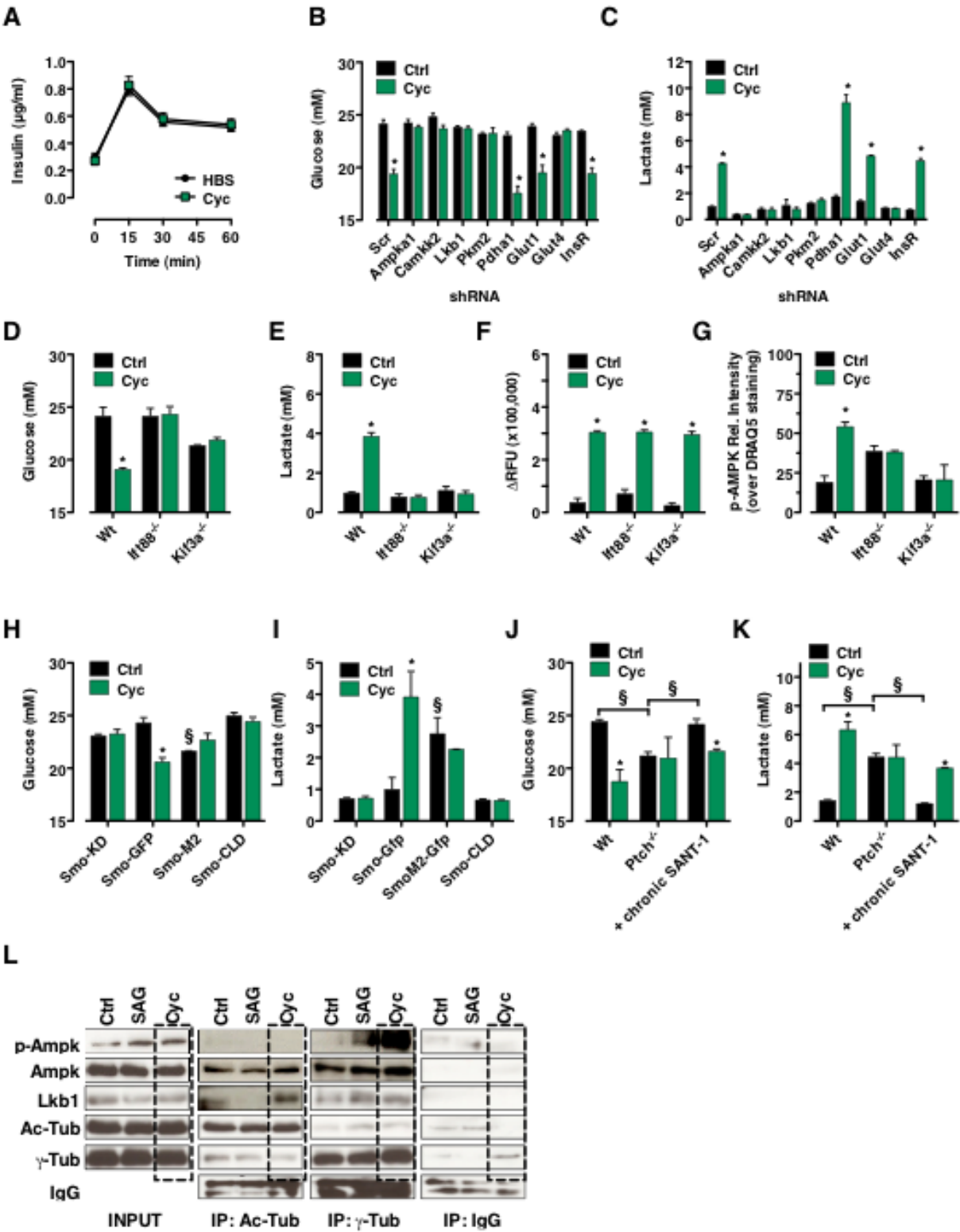


Figure S4. Non-canonical Rewiring of Metabolism is Primary Cilium Dependent.

Relates to Figure 4. (A) InCell Western Blot analysis of SAG-induced Ampk phosphorylation in wild-type, *Ift88*^{-/-} and *Kif3a*^{-/-} MEFs. (B-D) Effect of AICAR treatment on glucose consumption (B), lactate production (C) and Ampk phosphorylation (D) in wild-type, *Ift88*^{-/-} and *Kif3a*^{-/-} MEFs. (E) Western blot evaluation of Smo protein expression and SAG-induced Ampk phosphorylation in Smo knockdown 3T3-L1 adipocytes rescued with wild-type Smo-Gfp, SmoM2-Gfp and Smo-CLD. (F-G) Effect of long-term Compound C treatment (10 μ M) on glucose consumption (F) and lactate production (G) in *Ptch*^{-/-} MEFs. Results represent mean $\pm$ SEM (n = 3).

Supplementary Figure S5



58

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Figure S5. Uncoupling of Canonical and Non-Canonical Signaling by Cyclopamine Reveals Non-Canonical Signaling Capable of Effecting Glucose Uptake *in vitro* and *in vivo*, relates to figure 5.

(A) Treatment of C57BL/6J mice with Cyclopamine (10 mg/kg) does not affect insulin secretion (n = 12). (B-C) Cyclopamine-induced glucose consumption (B) and lactate production (C) in wild-type and *Ampka1*, *Camkk2*, *Lkb1*, *Pkm2*, *Pdha1*, *Glut1*, *Glut4* and *InsR* knockdown 3T3-L1 adipocytes. (D-G) Cyclopamine-induced glucose consumption (D), lactate production (E) Ca^{++} influx (F) and Ampk phosphorylation (G) in wild-type, *Ift88^{-/-}* and *Kif3a^{-/-}* MEFs. (H-I) *Smo* knockdown 3T3-L1 adipocytes are resistant to Cyclopamine-induced glucose consumption (H) and lactate production (I). Both are rescued by the reintroduction of wild-type *Smo*, but not with either *SmoM2* or *Smo-CLD* mutants (H-I). (J-K) Effect of chronic SANT-1 (100nM) treatment on Cyclopamine-induced glucose consumption (J) and lactate production (K) in *Ptch^{-/-}* MEFs. (L) Co-immunoprecipitation analysis of the *Smo*-*Lkb1*-*Ampk* axis in Cyclopamine treated (third column) 3T3-L1 adipocytes. Results are mean $\pm$ SEM (n = 3).

Supplementary Figure S6

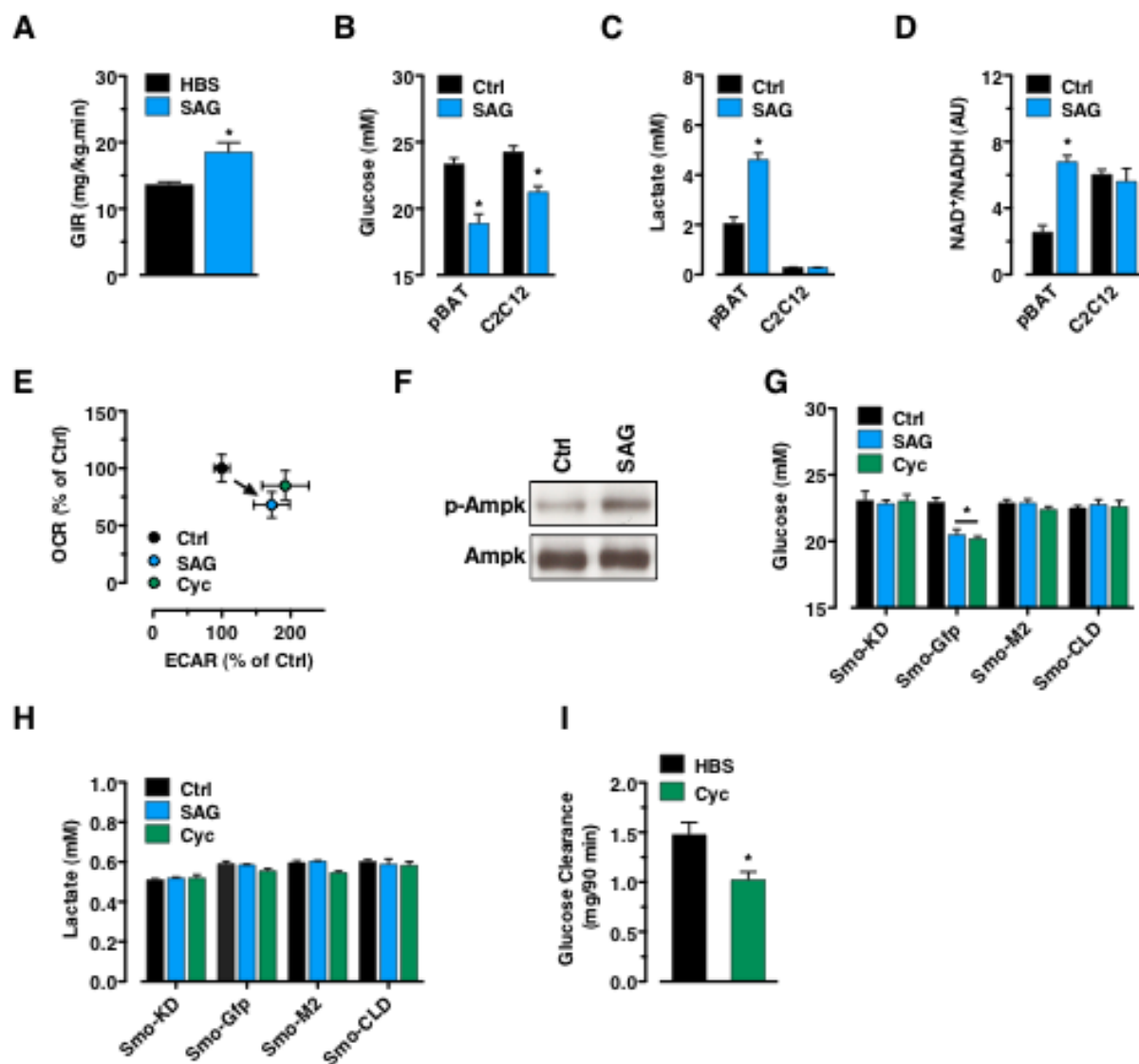
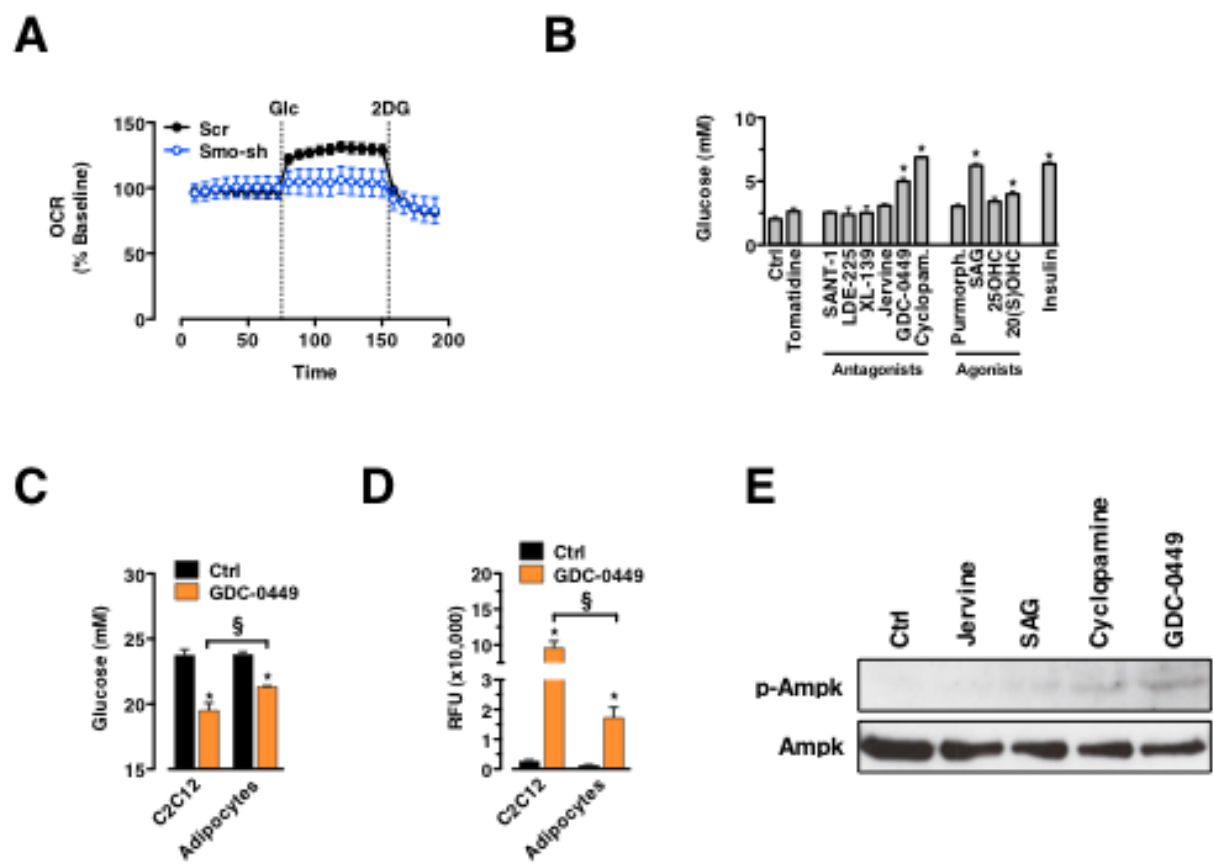


Figure S6. Specific insulin-independent glucose uptake in muscle and brown fat.

Relates to Figure 6. (A) SAG-treated mice also exhibit increased glucose-infusion rate (GIR) during glycaemic-insulinemic clamps (n = 6-10). (B-D) SAG treatment (200nM) induces glucose uptake (B), lactate production (C) and elevated NAD⁺/NADH ratio (D) in differentiated primary brown adipocytes and C2C12 myotubes. (E) SAG and Cyclopamine induce reduced Oxygen Consumption Rate (OCR) and increased Extracellular Acidification Rate (ECAR) in C2C12 myotubes. (F) Western blot evaluation

85 of SAG-induced Ampk phosphorylation in C2C12 myotubes. (G-H) Smo knockdown
 86 C2C12 myotubes show no signs of SAG-and Cyclopamine-induced glucose disposal (G)
 87 and lactate production (H). Both are rescued by the reintroduction of the wild-type Smo
 88 (G, H) but not with either SmoM2 and Smo-CLD mutants (E, F). (I) Cyclopamine
 89 treatment (10mg/kg) reduces urinary glucose clearance in insulin-deficient STZ-treated
 90 C57BL/6J mice (n = 6). Results are mean $\pm$ SEM (n = 3).

Supplementary Figure S7



92 **Figure S7. Smo Controls Metabolic Flexibility and Skeletal Muscle Contraction.**
 93 **Relates to Figure 7.** (A) Smo knockdown 3T3-L1 adipocytes exhibit only minimal
 94 increase in Oxygen Consumption Rate (OCR) in response to a Glycolytic Stress Test. (B)
 95 Lactate production induced by a panel of SAG modulators in 3T3-L1 adipocytes (C-D)

97 GDC-0049-induced glucose consumption (C) and Ca^{++} flux (D) in C2C12 myotubes and
98 3T3-L1 adipocytes. (E) Western Blot analysis of Ampk phosphorylation in human primary
99 myocytes in response to different Smo modulators. Results are mean $\pm$ SEM (n = 3).

100

101

EXTENDED EXPERIMENTAL PROCEDURES

Reagents

Unless otherwise stated all chemicals and reagents were obtained from SIGMA.

Cell Culture

Murine 3T3-L1 preadipocytes (ATCC) were maintained in DMEM containing 10% heat inactivated calf serum (CS) at 37°C and 5% CO₂. Two days post-confluence, adipocyte differentiation was induced by treating cells with 0.25 mM 3-Isobutyl-1-methylxanthine (IBMX), 1 µM dexamethasone, 1.74 µM insulin and 5 µM Troglitazone in DMEM containing 10% fetal bovine serum (FBS). After 48 hr, the medium was replaced with DMEM supplemented with 10% FBS, insulin and Troglitazone. After an additional 48 hr, cells were switched to DMEM with 10% FBS for the rest of the differentiation process and the medium was changed every other day. Seven days post-induction, differentiated 3T3-L1 adipocytes were starved overnight in DMEM containing 1% bovine serum albumin (BSA) before starting different treatments as indicated in the text. For treatments the following compounds and concentrations were used: 200 nM SAG (Alexis Biochemicals), 0.25 µg/ml recombinant Shh (R&D Systems), 100 nM cyclopamine, 10 µM compound C, 20 µM nifedipine and 100 ng/ml pertussis toxin (Ptx; Calbiochem).

Primary mouse embryonic fibroblasts (MEFs) were grown in DMEM with 10% FBS at 37°C and 5% CO₂. MEFs lacking functional Ptch (Ptch^{-/-}) were derived from Ptch^{-/-} mouse embryos as described previously (Todoric et al., 2011). Kif3^{-/-} and Ift88^{-/-} MEFs were derived from the respective mouse embryos (Wong et al., 2009). Smo^{-/-} MEFs were kindly provided by Jussi Taipale (Varjosalo et al., 2006). For experiments, MEFs were

grown to confluence and, 2 days post-confluence, were starved in DMEM with 1% BSA for 16 hr. The next day, treatments were performed as indicated in the text.

C2C12 myoblast cells (<10 passages after purchase from ATCC) were grown in DMEM containing 10% FBS. For differentiation, cells were allowed to reach 80% confluency and induced to differentiate in DMEM containing 2% horse serum. C2C12 cells were considered as myotubes after 96 hr of differentiation, as previously reported (Cantó et al., 2009).

Primary brown pre-adipocytes were obtained by collagenase digestion from mouse interscapular brown adipose tissue depots as previously described (Pospisilik et al., 2010). In brief, minced brown adipose tissue depots from 6-8 week old mice was digested at 37°C for 45-60 min in a shaking water bath with a cocktail consisting of collagenase II (1 mg/ml, Worthington), Dispase 1 (1.2 U/ml, Worthington) in DMEM containing 3% fatty acid free BSA and DNase I (100 Units/ml). After digestion, the slurry was passed through a 100 µm cell strainer (Becton Dickinson). Filtered cells were pelleted by centrifugation at 250 g for 5 min, washed with DMEM/F12 containing 10% FBS, and red blood cells were lysed using RBC lysis buffer. After a final washing step cells were passed through a 30 µm cell strainer (Partec) to remove clumps and most of the endothelial cell aggregates. Isolated cells were plated directly into the respective experimental dishes and differentiated as described (Pospisilik et al., 2010).

Human myoblast cultures and myocytes were established from four human study subjects as previously described (Gaster et al., 2001a; Gaster et al., 2001b). Four healthy lean subjects participated in the study. Muscle biopsies were obtained from the *vastus lateralis* muscle by needle biopsy (Bergstrom, 1975) under local anesthesia. The subjects had normal glucose tolerance and no family history of diabetes. All subjects gave written, informed consent, and the local ethics committee of Funen and Vejle

County approved the study. Muscle tissue was minced, washed and dissociated for 60 min. by three treatments with 0.05 % trypsin-EDTA. The cells obtained were seeded for up-scaling on ECM-gel coated dishes after 30 minutes of preplating. Growth medium contained DMEM supplemented with 2% FCS, 2% Ultrosor G (Pall Biosepra), 50 U/ml penicillin, 50 µg/ml streptomycin and 1.25 µg/ml amphotericin B. Cells were subcultured twice before final seeding. At 75% confluence, the growth medium was replaced by basal medium (DMEM supplemented with 2% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin, 1.25 µg/ml amphotericin B, and 25 pM insulin) in order to induce differentiation. Human myotubes were allowed to differentiate for 4 days. The cells were cultured in humidified 5% CO₂ atmosphere at 37°C, and medium was changed every 2-3 days.

shRNA

Transduction ready lentiviruses expressing shRNAs targeting Ampka1 (RMM3981-9591411), Lkb1/Stk11 (RMM3981-9591552), Camkk2 (RMM3981-9593143), Glut1 (RMM3981-97067625), Glut4 (RHS3979-9610846), Insulin receptor (RMM3981-9590983), Pkm2 (RHS3979-9593029) and Pdha1 (RHS3979-9595502) were obtained from OpenBiosystems. A lentivirus harboring a scrambled shRNA was used as control (OpenBiosystems, RHS002). For lentiviral transduction, preadipocytes were seeded overnight in 12-well plates (Corning) in DMEM containing 10% calf serum (CS). The following morning 5 MOI of lentivirus were complexed with 5 mg/ml Polybrene (Santa Cruz Biotechnology), added to the cells and left 24 hr before being removed and replaced with DMEM containing 10% CS. Puromycin was used to select stable clones. For knock-down of Gli1, Gli2 and Smo in 3T3-L1 and C2C12 cells the following lentiviral shRNA constructs were used: non-targeting control shRNA (shc002), shGli1 (TRCN0000054886), shGli2 (TRCN0000238361), shSmo (TRCN0000026245). Lentivirus

production and transductions were done as described previously (Kasper et al., 2007). For validation of knockdown efficiency, confluent cells were starved over night in 0.5% CS and then stimulated with 100nM SAG for 48 hr (Alexis Biochemicals) to induce detectable levels of Gli1 mRNA.

The functionality of the shRNA knockdown was analyzed by SybrGreen qPCR using the following primers: Gli1 (forward 5'-CACCGTGGGAGTAAACAGGCCTTCC-3'; reverse 5'-CCAGAGCGTTACACACCTGCCCTTC-3'), Gli2 (forward 5'-CTAAATGATGCCAACCAGAACA-3'; reverse 5'-CCTCAGCCTCAGTCTTGACC-3'), Smo (forward 5'-CTCTTCCTGGTTAGCACCTTAAAAC-3'; reverse 5'-GTGTGTGAGAGTGTATGTGTGTGTG-3'), Rplp0 (forward 5'-TGCACTCTCGCTTTCTGGAGGGTG-3'; reverse 5'-AATGCAGATGGATCAGCCAGGAAGG-3') was analyzed as reference control gene.

Transfection

HEK293 cells (ATCC) were grown in DMEM containing 10% FBS. 2×10^6 cells were seeded in 10 cm dishes and transfected with 5 μ g of the AMPK-AR construct (Tsou et al., 2011) using the GeneJuice Reagent (Merck) according to manufacturer's instructions. 48 hr after transfection, cells were resuspended in PBS and used for Foerster resonance energy transfer (FRET) assays.

Oxygen Consumption and Extracellular Acidification Rate Measurements

Analysis of oxygen consumption (OCR) and extracellular acidification (ECAR) rates was performed using a Seahorse XF24 Flux Analyzer (Seahorse Bioscience). Briefly, differentiated 3T3-L1 adipocytes were seeded into XF 24-well cell culture microplates. On the day of testing, adherent cells were pretreated for 6 hr with either vehicle or SAG (200

nM). A final volume of 600 μ l of assay medium was added to each well prior to the experimental protocol. Cells were then transferred to a CO₂ free incubator, maintained at 37°C for 1 hr before the start of the assay. Following instrument calibration, pretreated cells were transferred to the XF24 Flux Analyzer to record oxygen consumption as well as extracellular acidification rates. The measurement protocol consisted of 4 min mixture, 2 min wait and 2 min OCR/ECAR measurement times. At the end of the assay, media was aspirated and cellular DNA was measured using CyQuant (Invitrogen) to adjust for potential differences in cell densities.

Metabolite Measurements

NAD⁺, NADP⁺, NADH, and NADPH were determined using the Enzychrom NAD⁺/NADH and the Enzychrom NADP⁺/NADPH assay kit, respectively (BioAssay Systems) according to manufacturer's instructions. To measure glucose consumption and lactate production, the Glucose RTU and Lactate RTU kits (Biomerieux) were used according to manufacturer's instruction and normalized to cell number. AMP, ADP and ATP were determined as previously described (Liu et al., 2010). In brief, differentiated 3T3-L1 adipocytes, were starved overnight in DMEM containing 1% BSA and treated as indicated. At the end of the experiment, cells were washed once with ice cold PBS and proteins were precipitated with 1% trichloroacetic acid (TCA). Repeated ether washes were used to remove TCA from the aqueous extract phase. Extracts were dried and stored at -80°C until further processing. To determine nucleotide phosphates by reversed-phase HPLC cell extracts were lyophilized for 3 days at -20 °C. Dried samples were dissolved in mobile phase A (60 mM K₂HPO₄ and 40 mM KH₂PO₄, adjusted to pH 7 with 0.1 M KOH). 20 μ l were injected into an Agilent 1200 HPLC system (Agilent) and nucleotides were separated by gradient elution from an Agilent Eclipse XDB-C18 4.6 x

150 mm column at a flow rate of 1 ml/min. The gradient was 99% phase A from 0-5 minutes, 99% phase A to 40% A from 5-20 minutes and 99% phase A from 20-32.5 minutes. 100% acetonitrile served as mobile phase B. Eluting nucleotides were monitored on a UV detector at 254 nm. ATP, ADP and AMP standards were used to generate calibration curves for peak quantifications.

Glucose Uptake

Basal glucose uptake was determined by capturing 2-[1-¹⁴C]-deoxy-glucose (Perkin Elmer) as previously described (Gaster et al., 2002). The day before determination of basal glucose uptake FBS was exchanged for bovine serum albumin. Myotube cultures were exposed for 10 minutes to final concentrations of: a) 0.6 and 6.0 μ M Jervine, b) 10 and 100 nM GDC-0449, c) 100 and 200 nM SAG, d) 100 and 200 nM Cyclopamine and e) vehicle as treatment control. For the determination of nonspecific deoxyglucose uptake, sister cultures were incubated with phloretin. Glucose transport activity was calculated as fold change above control values.

Phospho-Protein Enrichment and 2D-PAGE

Phosphoprotein enrichment was performed by affinity column purification of total adipocyte protein using a PhosphoProtein Purification Kit (Qiagen) according to the manufacture's instructions. Briefly, $\sim 10^7$ cells were washed twice with ice cold HEPES buffer on ice, resuspended in 5 ml lysis buffer containing protease and phosphatase inhibitors and Benzonase nuclease, provided by the manufacturer. The cell suspension was incubated on ice for 30-60 min with vortexing every 10 min. Subsequently, cell lysates were centrifuged at 10,000 g and 4°C for 30 min. PhosphoProtein purification columns were equilibrated with lysis buffer and 2.5 mg protein loaded on the column. For

2D-PAGE, immobilized pH gradient (IPG) strips (pH 3-10, 17 cm; GE Healthcare) were passively rehydrated with 50-100 µg of proteins eluted from the PhosphoProtein purification columns. Isoelectric focusing (IEF) was performed on an Ettan IPGphor II system (GE Healthcare). Focused IPG strips were equilibrated in equilibration buffer (6 M Urea, 50 mM Tris pH 8.8, 2% SDS, 20% glycerol) with 100 mM 1,4-dithio-D,L-threitol (DTT) and 2.5% iodacetamide (IAA) for 10-15 min. Equilibrated IPG strips were placed on top of 12% acrylamide/1,4-Bis (acryloyl) piperazine (AA/PDA) gels and overlaid with 0.5% low-melting agarose. Proteins were separated by SDS-PAGE using an Ettan DALTtwelve (GE Healthcare) at 5 W per gel for 30 min followed by 15 W per gel until the dye front completely ran off. Gels were stained with Ruthenium II tris bathophenanthroline-disulfonate (RuBPS), and scanned with a FluorImager 595 (GE Healthcare) at a resolution of 100 µm. Detection, matching, background subtraction, normalization and quantitative comparison of 2D spots were accomplished using the Progenesis SameSpots software (Nonlinear Dynamics).

Silver Staining and In-Gel Digest

After washing, 2D-gels were sensitized in 0.02% Na₂S₂O₃, stained with ice-cold 0.1% AgNO₃ and developed with 3% Na₂CO₃/0.05% formaldehyde. Spots of interest were excised and stored in 1% acetic acid until further processing or immediately destained (150 mM potassium hexacyanoferrate, 500 mM sodium thiosulfate) followed by washing in 50% methanol/10% acetic acid over night. pH was neutralized with 50 mM NH₄HCO₃, and proteins were reduced with 10 mM DTT in 50 mM NH₄HCO₃ at 56°C for 30 min and alkylated with 50 mM iodacetamide in 50 mM NH₄HCO₃ in the dark for 20 min. Subsequently, gel-pieces were washed with acetonitrile and dried in a vacuum centrifuge. Dry gelspots were digested with 0.1 mg/ml trypsin (Roche) in 50 mM NH₄HCO₃. Digested

peptides were eluted by adding 5% formic acid in 50% acetonitrile. The supernatant was transferred into silicon-coated tubes and the elution procedure was repeated two times. Between each elution step the gel-pieces were ultrasonicated at 160 W for 10 min. Finally, the peptide solution was concentrated in a vacuum centrifuge to a total volume of approximately 20 μ l.

LC/MSMS Analysis

Tryptic digests were loaded on a Zorbax 300SB-C8 column (5 μ m, 0.3 mm, 5 mm) using the HPLC-Chip technology (Agilent) and separated by nanoflow LC (1100 Series LC system, Agilent) with a Zorbax 300SB-C18 column (5 μ m, 75 mm, 150 mm) at a flow rate of 250 nl/min using a gradient from 0.2% formic acid and 3% acetonitrile to 0.2% formic acid and 45% acetonitrile over 12 min. Peptide identification was accomplished by MSMS fragmentation analysis with an iontrap mass spectrometer (XCT-Ultra, Agilent) equipped with an orthogonal nanospray ion source. The MSMS data, including peak list generation and search engine, were interpreted by the Spectrum Mill MS Proteomics Workbench software (Version A.03.03, Agilent) allowing for two missed cleavages and searched against the SwissProt Database for murine proteins (Version 14.3 containing 20.328 entries) allowing for precursor mass deviation of 1.5 Da, a product mass tolerance of 0.7 Da and a minimum matched peak intensity (% Scored Peak Intensity) of 70%. Carbamidomethylation of cysteines was set as fixed modification. To assess the reliability of the peptide scores, searches against the corresponding reverse database were performed. 6.0% positive hits were found with peptides scoring >9.0, while 0.2% positive hits were found with peptides scoring >13.0. Consequently, the threshold for protein identification was set scoring 14.0 or higher, resulting in a false discovery rate of 0.1%.

Data were further processed and quantified with the Scaffold software tool (Version 3.0, Proteome Software). Protein identifications were accepted if they contained at least two identified peptides. Proteins that contained similar peptides and could not be differentiated based on MSMS analysis alone were manually verified.

Immunoblotting

Cells were washed twice with ice cold PBS on ice and lysed in RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors (Roche). Cell lysates were cleared by centrifugation at 4°C at 16,000 g for 30 min. Protein concentration in the supernatant was determined using the BCA Protein Assay Kit (Pierce). 20 µg of proteins were resolved by SDS-PAGE and transferred to PVDF membranes (GE Healthcare). Membranes were blocked with 5% BSA in Tris-buffered saline containing 0.2% Tween-20 (TBS-T), and incubated with primary antibodies at 4°C over night. The following antibodies were used (all from Cell Signaling unless indicated otherwise): anti-phospho-AMPK (pThr172) (1:5,000; Cat.-Nr. 2535), anti-total AMPK (1:5,000; Cat.-Nr. 2532), anti-phospho-PKM2 (pTyr105) (1:5,000; Cat.-Nr. 3827), anti-total PKM2 (1:10,000; Cat.-Nr. 3198), anti-phospho-PDHA1 (1:5,000; ab77844, Abcam), anti-total PDHA1 (1:5,000; Cat.-Nr. 3205), and HRP-linked anti-rabbit IgG (1:5,000; Cat.-Nr. 7074). Antigen-specific binding of antibodies was detected with SuperSignal West Femto and Pico Kits (Pierce) using a ChemiDoc XRS Imager (Bio-Rad). Image analysis was performed using Image Lab Software Version 3.0.1 (Bio-Rad).

AMPKAR-Foerster Resonance Energy Transfer Assay

Cells transfected with the AMPK-AR construct (Tsou et al., 2011) were resuspended in PBS at a concentration of 10^5 cells/ml. A Synergy 4 microplate reader (BioTek) was used for measuring Foerster resonance energy transfer (FRET) signals. In brief, compounds were pipetted into a 96-well plate at the concentrations indicated in the text. Cell suspensions were pre-loaded in the reader to be injected at 10^4 cells/well. FRET signals were detected using 420/50 nm and 528/20 nm as excitation and emission settings. Baseline values for each well were acquired before injection and subtracted afterwards.

Ca²⁺ Signal Measurements

Cells were cultured in black wall/clear bottom 96-well plates (Corning). At day seven post-induction, cells were starved overnight in DMEM containing 1% BSA and loaded with the calcium dye FluoForte AM (Enzo Lifescience) according to the instructions provided by the manufacturer. Intracellular calcium levels were measured using a Synergy 4 microplate reader (BioTek). Briefly, compounds were injected at the reported concentration and calcium flux was measured by detecting fluorescence at 488/530 nm (excitation/emission). Baselines for each well were acquired before injection and subtracted afterwards. Ionomycin and EGTA were injected in concentrations of 1 μ M.

Quantitative RT-PCR Analysis

Total RNA was extracted from cells using TRIzol according to the manufacturer's instructions. Isolated total RNA was reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative RT-PCR was performed on an AbiPRISM 7500fast real-time cycler (Applied Biosystems) using the iQ SYBR Green Supermix (Bio-Rad). Post-amplification melting curve analysis was performed to check for unspecific products and primer-only controls were included to ensure the absence of primer dimers.

349 Threshold cycles (C_t -values) of all replicate analyses were normalized to hypoxanthine-
350 guanine phosphoribosyltransferase (*Hprt*). To compare the effect of various treatments
351 with untreated controls, $2^{-\Delta\Delta C_t}$ values were calculated to obtain fold expression levels.
352 Mouse specific primers for *Ampka1* (forward 5'-ACGGCCGAGAAGCAGAAGCAC-3';
353 reverse 5'-TCGTGCTTGCCCACCTTCAC-3'), *Camkk2* (forward 5'-
354 CCGTTCGAAGGTAGCCGCCG-3'; reverse 5'-CGCTGCCTTGCTTCCTTGGGC-3'),
355 *Stk11/Lkb1* (forward 5'-TGA CTGCGGCCACCACTCT-3'; reverse 5'-
356 CGGAACCAGCTGTGCTGCCTA-3'), *Ptch1* (forward 5'-
357 TGACGGGGTCCTCGCTTACAA-3'; reverse 5'-CTACCAGACGCTGTTTAGTCA-3'),
358 *Smo* (forward 5'-GGCCTGACTTTCTGCGTTGCACACC-3'; reverse 5'-
359 GGGTTGTCTGTTTCGCACCAAGG-3'), *Gli1* (forward 5'-AGTGCCATGCCGCAGCAG-3';
360 reverse 5'-CATCTCCACGCCGCTGTC-3'), *Glut1* (forward 5'-
361 AGAACCAATGGCGGCGGTCC-3'; reverse 5'-ATGCGTCTCCCGCCAGACCT-3'), *Glut4*
362 (forward 5'-TCCCACCAGACCCGCCCTTT-3'; reverse 5'-
363 AGTCACTCGCTGCCGAGGGG-3'), *InsRb* (forward 5'-GGGCTGCGTTGTTTACGGGC-
364 3'; reverse 5'-GCTCTGGGTGGGTCGCGTTG-3'), *Pkm2* (forward 5'-
365 AGGCAGCCATGTTCCACCGT-3'; reverse 5'-TGATAGGAGCCCGAGGGCGG-3'),
366 *Pdha1* (forward 5'-AGAAGCCGGCAAGCCGAGTG-3'; reverse 5'-
367 GCTCCATCCGGCGCACAGTC-3') and *Hprt* (forward 5'-
368 CGCAGTCCCAGCGTCGTGAT-3'; reverse 5'-CGAGCAAGTCTTTCAGTCCTGTCC-3')
369 have been used.

370

371 **Phenotype Microarrays**

372 Phenotype microarray experiments were performed following protocols provided by
373 Biolog Inc. (<http://www.biolog.com/>). Briefly, scrambled and *Smo*-shRNA infected 3T3-L1

adipocytes were grown, differentiated as described above and inoculated in the BIOLOG plates PM-M1 and PM-M2 for at least 16 hours at 37°C containing 5% CO₂ prior to the assay. The provided proprietary redox reagent was added to quantitate substrate use at 590 and 750 nm in a Sinergy 4 Microplate reader (BIOTEK).

Mouse Studies

C57BL/6J mice were maintained on a standard rodent chow diet and a 12 hr light: 12 hr dark cycle. Eight week old, over-night fasted C57BL/6J male mice were injected intraperitoneally (i.p.) with vehicle (45% hydroxypropyl- β -cyclodextrin in HBS) or cyclopamine (10 mg/kg). 15 min after injection, an oral glucose tolerance test (OGTT) was performed by administering 2 g/kg glucose by oral gavage. Tail-vein blood was collected to measure glucose and insulin levels. Insulin was measured using an ultrasensitive insulin ELISA kit (Mercodia), according to the manufacturer's recommendations. For cyclopamine tolerance tests eight week old C57BL/6J mice were i.p. injected with streptozotocin (STZ, 200 mg/kg body weight) to destroy pancreatic β -cells. 72 hr later STZ-treated mice were separated into two groups and injected i.p. with either vehicle or cyclopamine (10 mg/kg). Blood glucose and body temperature were recorded at the beginning of the test and every 15 min during the experiment. All animal experiments were carried out according to an ethical animal license protocol in accordance to the animal protection law and approved by the Regierungspräsidium Freiburg (Tierversuchsvorhaben Registrier-Nr.: G-10/94).

Glycemic-Insulinemic Clamp Studies

Indwelling catheters were placed into the left femoral vein and externalized in the interscapular region of 11 week old C57BL/6J mice (Knauf et al., 2005; Pospisilik et al.,

2010). The animals were allowed to recover for 5 days and fasted for 16 hr before the experiment. Somatostatin (SST14 Bachem, Switzerland) was infused at a rate of 1 $\mu\text{g/kg/min}$ and hyperglycemia (12.5 mM) was maintained by variable infusion of 10% glucose. Steady state was maintained for 90 min before cyclopamine injection (2/3 bolus 1/3 infusion; 10 mg/kg), or HBS (control). Whole blood was sampled from the tail every 10 min during the last hour using a glucometer (AccuCheck Aviva; Roche Diagnostics).

Statistical Analysis

All data unless otherwise indicated are shown as the mean $\pm$ standard error of the mean (SEM). Data sets were compared for statistical significance using two-tailed Student's *t*-tests for unpaired samples. All figures and statistical

SUPPLEMENTAL REFERENCES

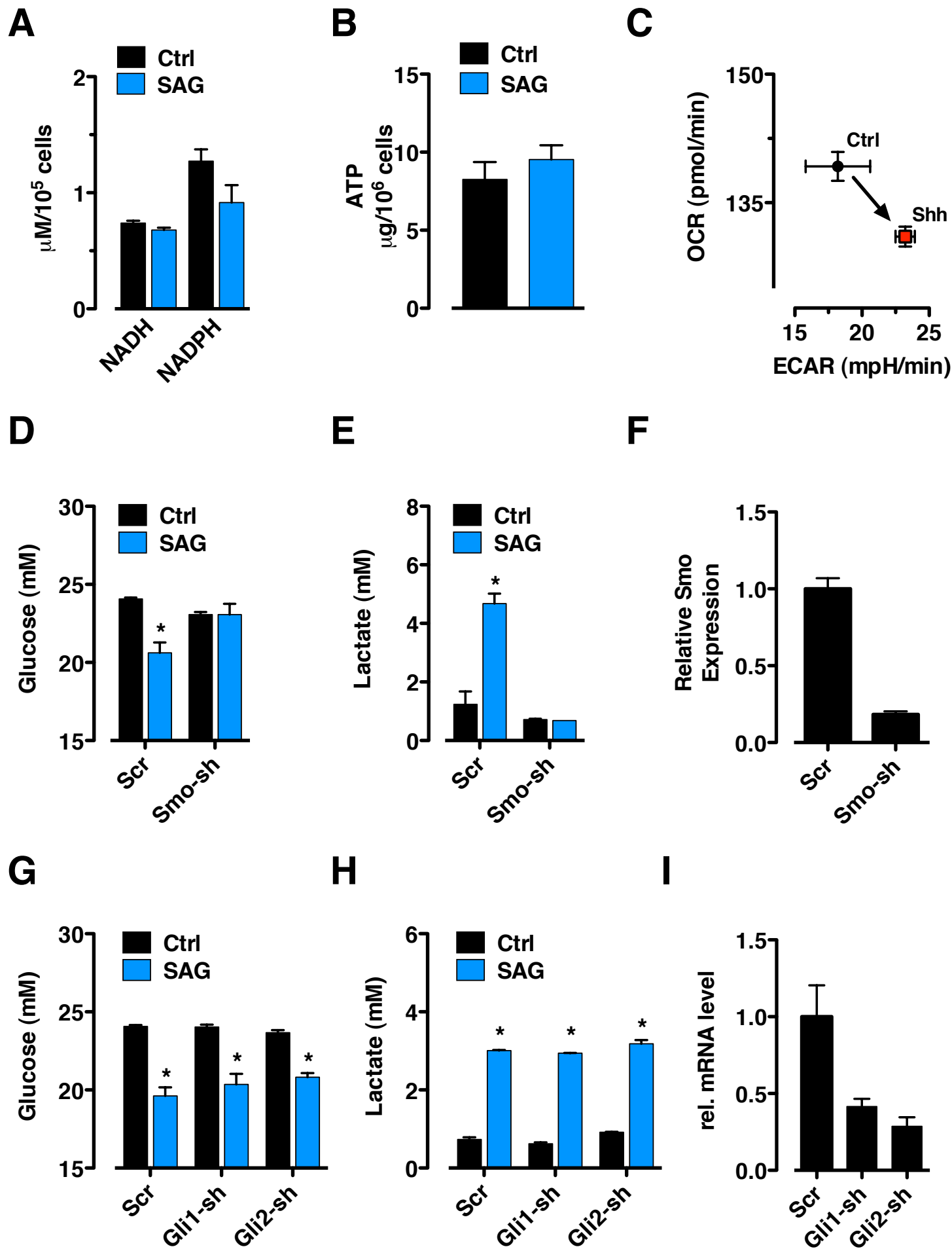
Bergstrom, J. (1975). Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest* 35, 609-616.

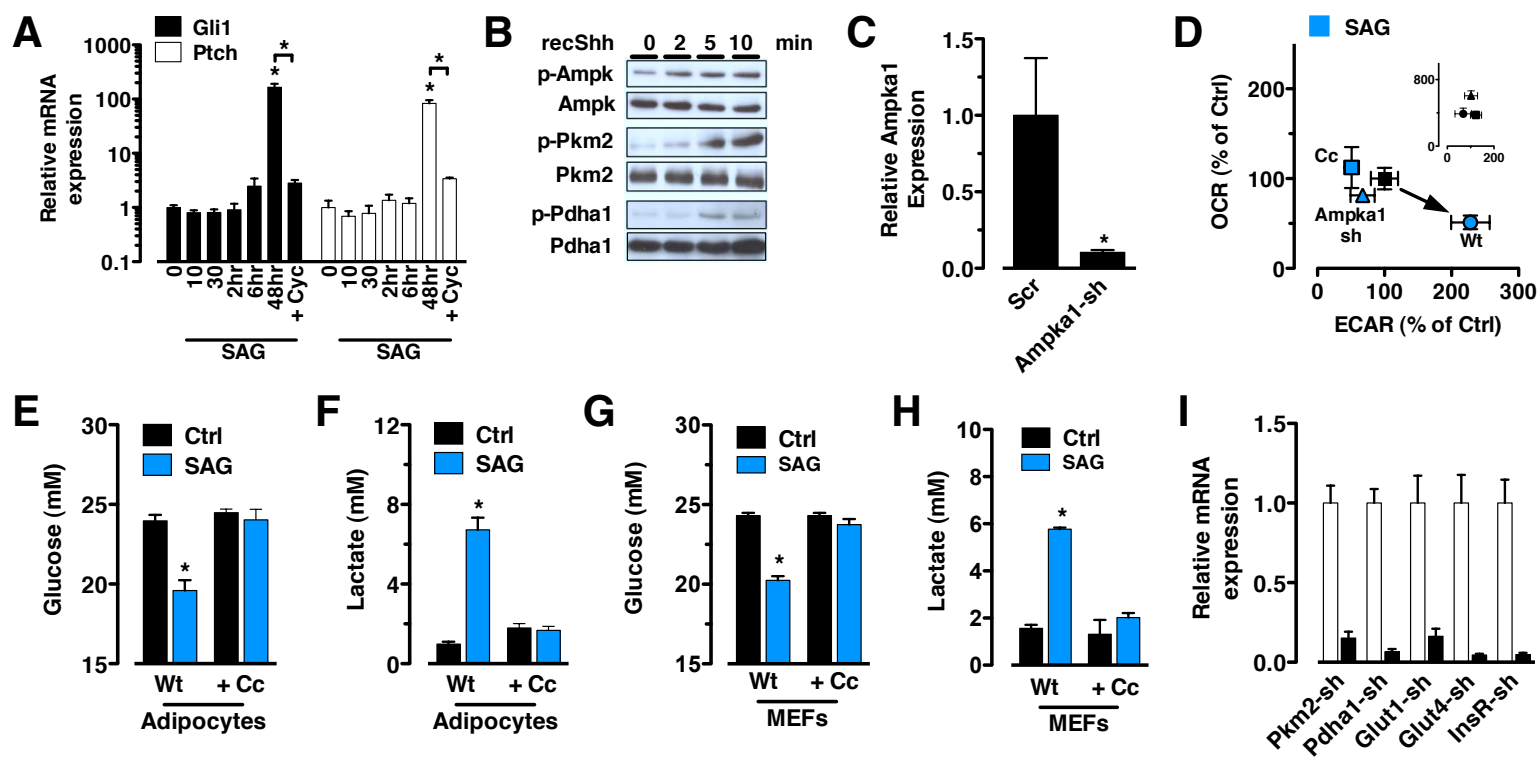
Cantó, C., Gerhart-Hines, Z., Feige, J.N., Lagouge, M., Noriega, L., Milne, J.C., Elliott, P.J., Puigserver, P., and Auwerx, J. (2009). AMPK regulates energy expenditure by modulating NAD⁺ metabolism and SIRT1 activity. *Nature* 458, 1056-1060.

Gaster, M., Kristensen, S.R., Beck-Nielsen, H., and Schroder, H.D. (2001a). A cellular model system of differentiated human myotubes. *APMIS* 109, 735-744.

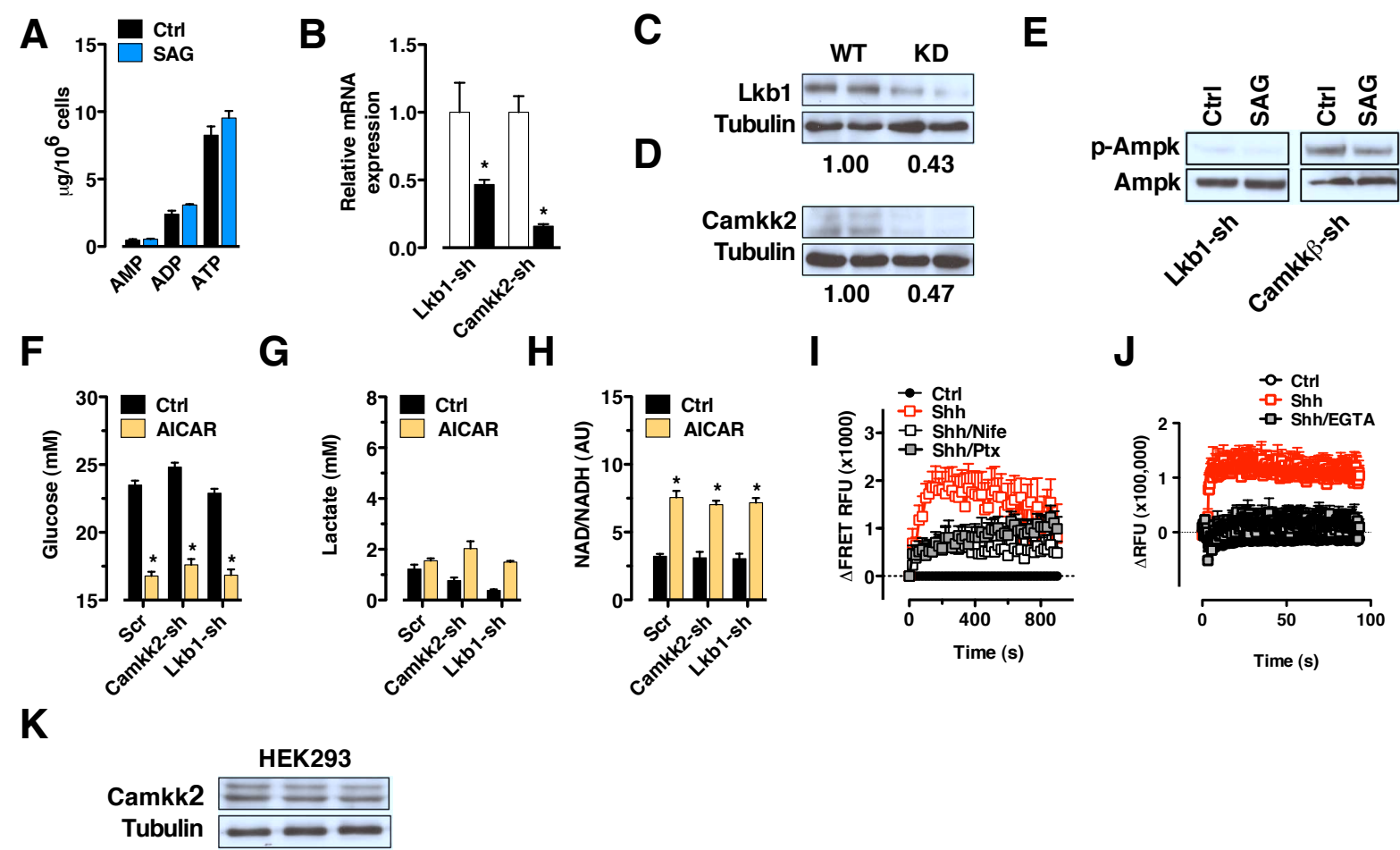
Gaster, M., Schroder, H.D., Handberg, A., and Beck-Nielsen, H. (2001b). The basal kinetic parameters of glycogen synthase in human myotube cultures are not affected by chronic high insulin exposure. *Biochim Biophys Acta* 1537, 211-221.

Supplemental Figure S1

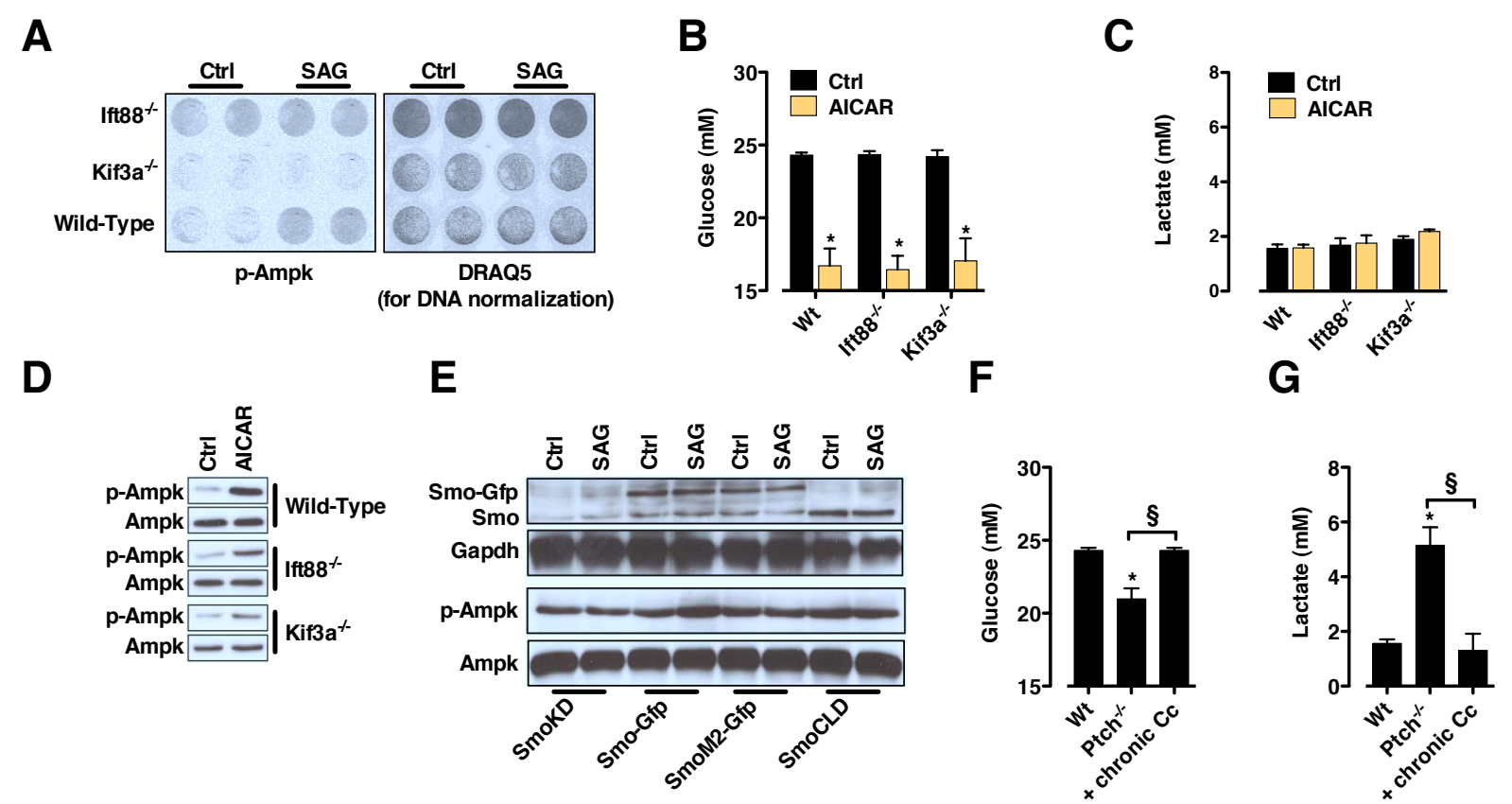




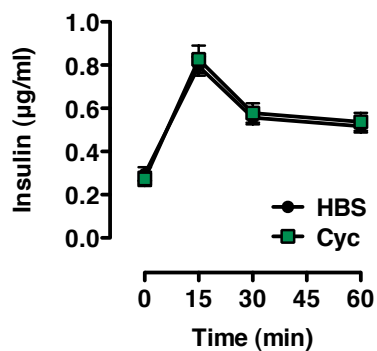
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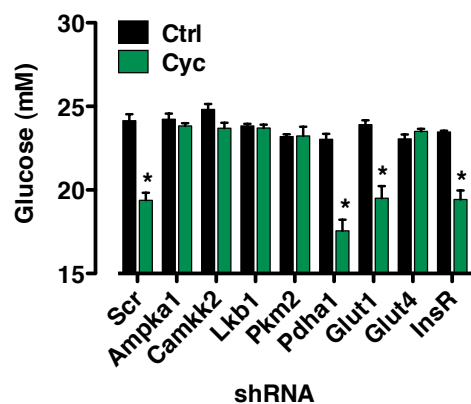
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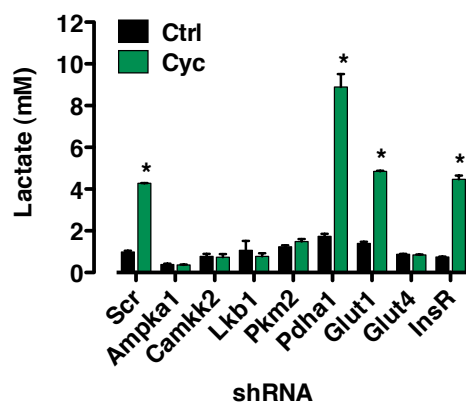
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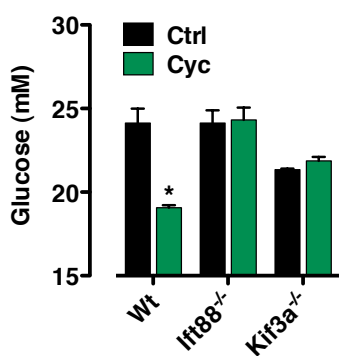
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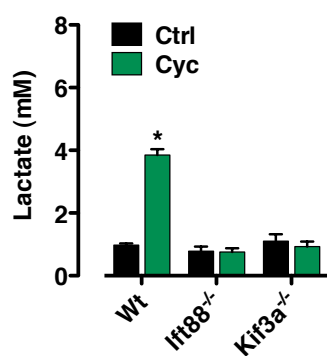
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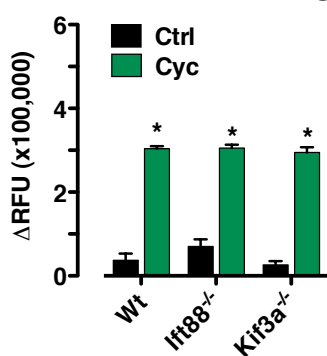
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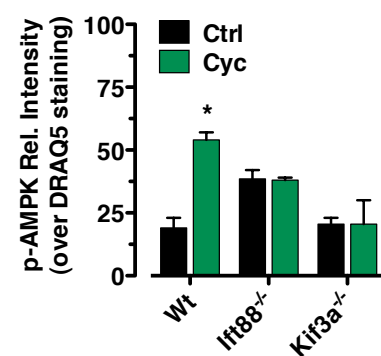
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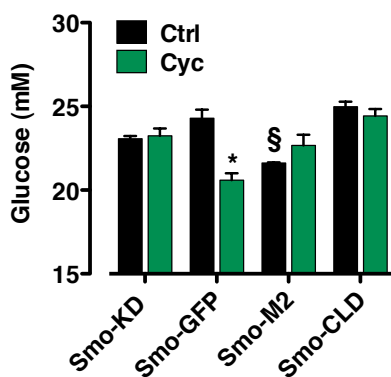
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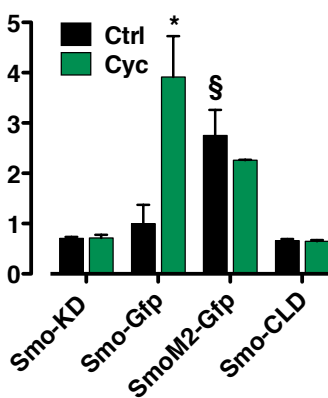
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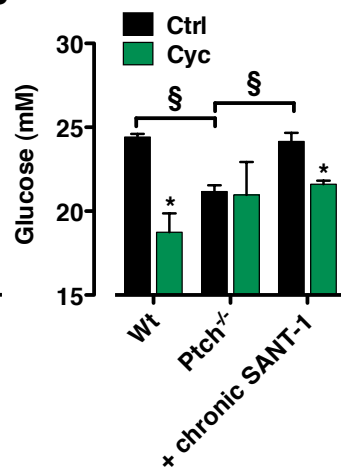
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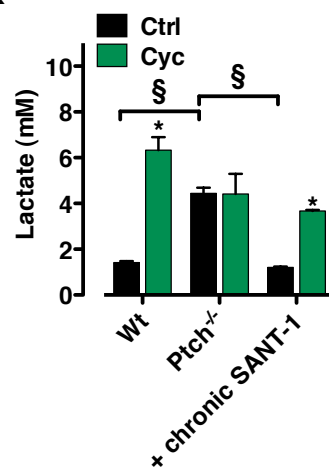
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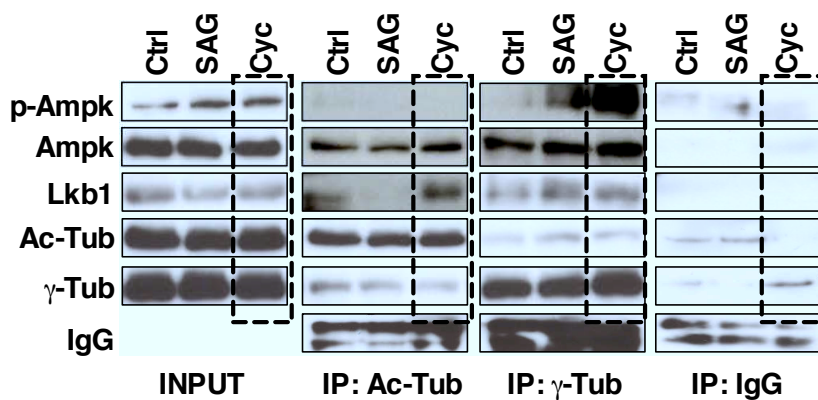
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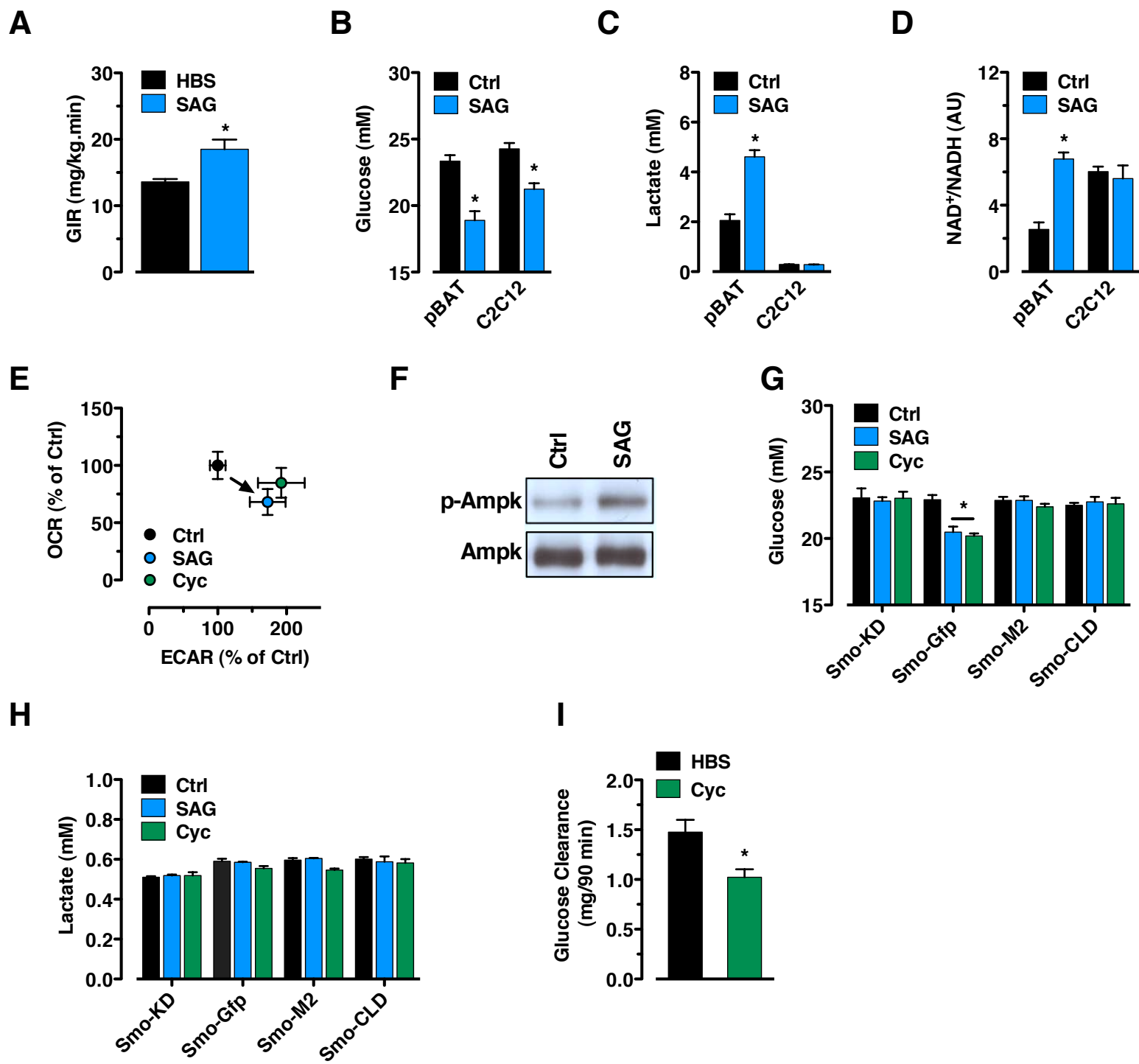
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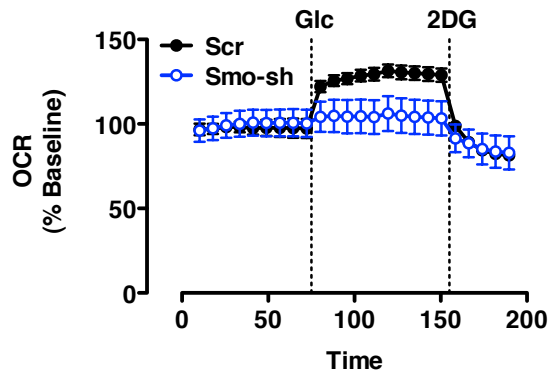


Supplementary Figure S6

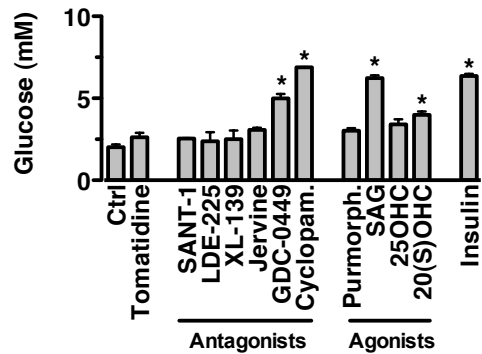


Supplementary Figure S7

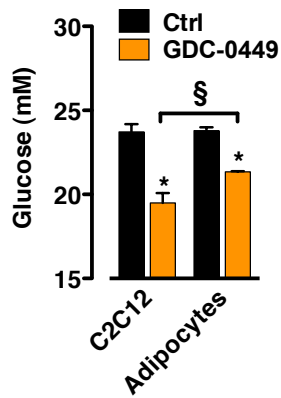
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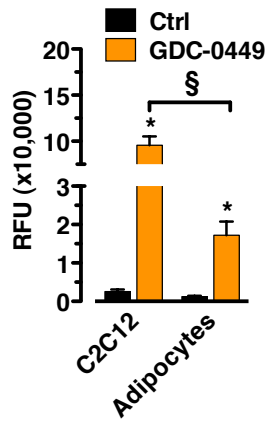
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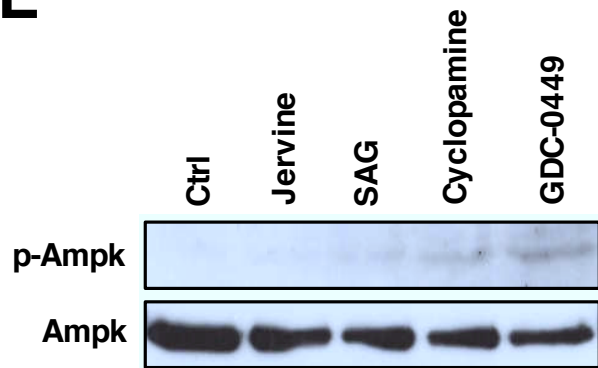
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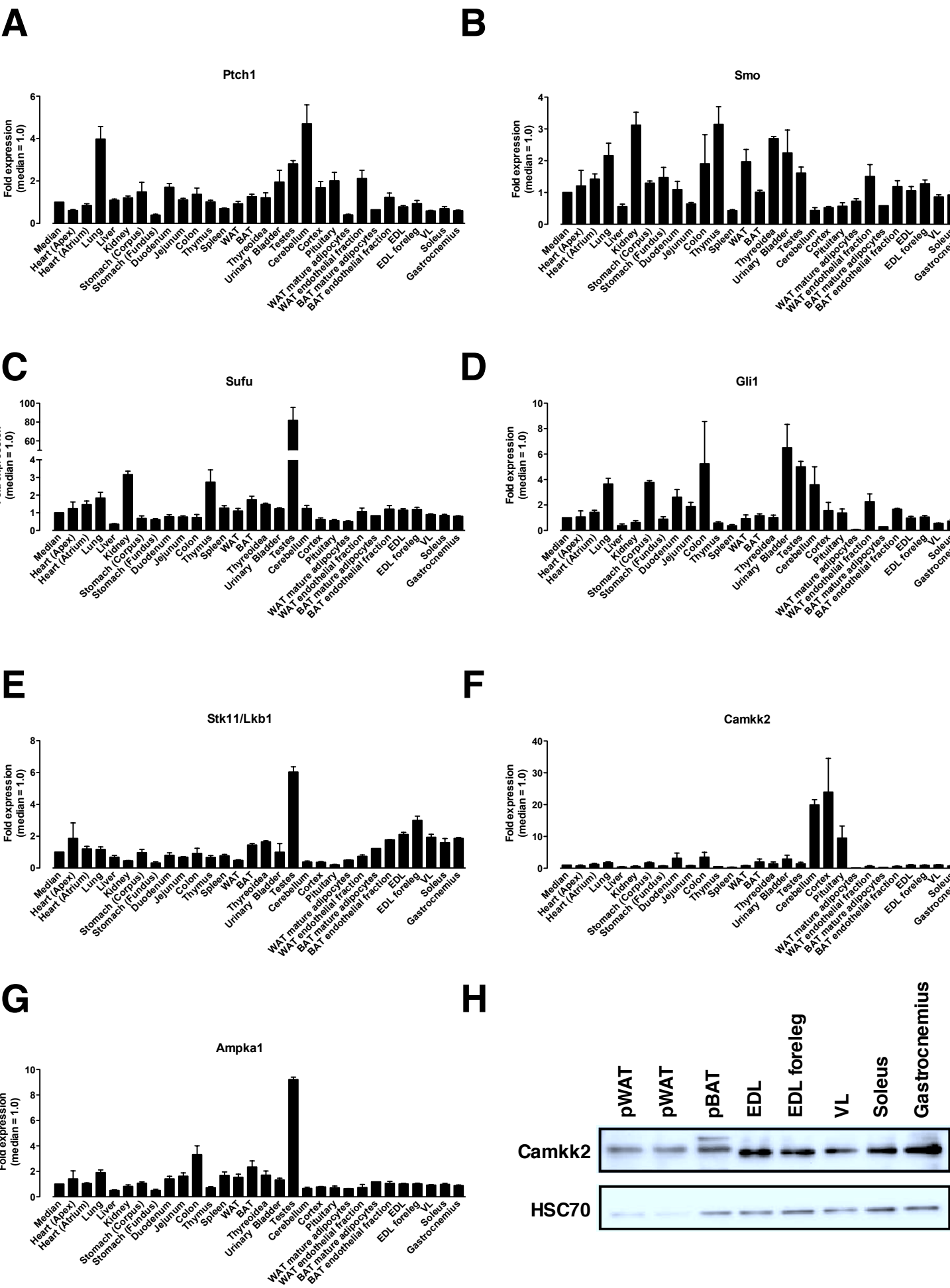


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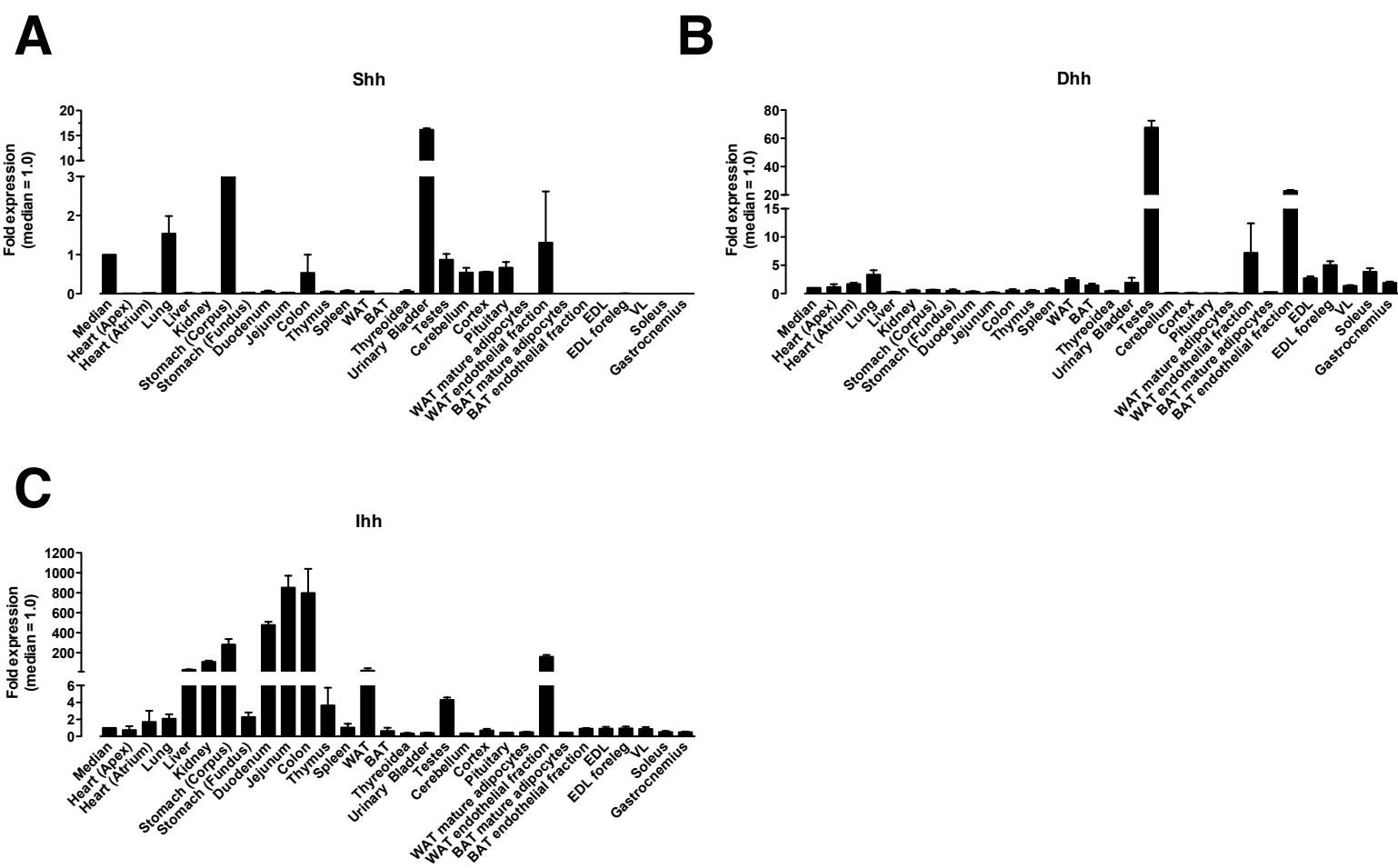


E





Supplementary Figure R2 - For Reviewers only



Diabetes, obesity and cancer affect $\sim 1/6^{\text{th}}$ of the world's population and share one key etiological feature, namely, altered metabolic state. Here, we identify a new Hedgehog-induced regulatory circuit that rewires cellular metabolism within minutes, prompting glucose uptake in in vitro, and in muscle and brown fat in vivo. Surprisingly, several hedgehog "antagonists" activate the pathway, dramatically altering our understanding of hedgehog signaling and forcing a re-examination of a decade of published work. Implications of the work are profound, particularly for the development of cancer, diabetes, and obesity therapeutics.