A CRISPR-based genome-wide screen for adipogenesis reveals new insights into mitotic expansion and lipogenesis.

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Summary

In response to excess nutrients, white adipose tissue expands by both generating new adipocytes and by upregulating lipogenesis in existing adipocytes. Here, we performed a genome-wide functional genomics screen to identify regulators of adipogenesis in the mouse 3T3-L1 cell model. The pooled screening strategy utilized FACS to isolate populations based on lipid content by gating for fluorescence intensity of the lipophilic, green fluorescent BODIPY 493/503 dye. Additionally, this approach categorized if genes functioned during mitotic expansion or lipogenesis. Cellular mechanisms regulating the rates of protein translation and protein stability were found to be critical for adipogenesis and lipogenesis. These mechanisms were further supported by proteomic analyses, which demonstrated that many changes in protein abundance during 3T3-L1 adipogenesis were not driven by transcription. Within these themes, we illustrate that hypusination is critical for translating adipogenic inducers of mitotic expansion and that the neddylation/ubiquitin pathway modulates insulin sensitivity to regulate lipogenesis.

Keywords

Adipogenesis, obesity, mitotic expansion, lipogenesis, CRISPR, ubiquitin, lipid, hypusine, Nedd8, translation

Introduction

Obesity, characterized by an excessive expansion of white adipose tissue, is a world-wide epidemic and its comorbidities, including diabetes, cancer, cardiovascular and hepatic disease, pose a significant and costly challenge to public health (Bluher, 2019). White adipose tissue expands in response to overnutrition by both increasing the size of existing adipocytes and by

generating new adipocytes via *de novo* adipogenesis of preadipocytes (Haczeyni, et al., 2018). Notably, white adipose tissue consisting of smaller adipocytes, even if greater in number, is metabolically healthier (Ghaben and Scherer, 2019). Thus, there is substantial interest in understanding the molecular mechanisms that selectively regulate mitotic expansion versus lipogenesis.

The process of adipogenesis can be functionally subdivided into distinct processes: 1) activation of preadipocytes, 2) expansion of preadipocyte numbers, 3) differentiation into an adipocyte, and 4) lipogenesis (Tang, et al., 2003b; Rosen, et al., 2000). Much of our current understanding of adipogenesis has been gained from the study of a murine cell line model of adipogenesis, 3T3-L1 cells (Green and Meuth, 1974). These cells are immortalized and differentiate robustly and uniformly upon addition of a differentiation cocktail, greatly enabling biochemical and genetic characterization of adipogenesis. Specifically, in response to the differentiation factors insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX), confluency-arrested 3T3-L1 cells are activated to re-enter the cell cycle, undergo 2-3 rounds of specialized mitosis (termed mitotic differentiative expansion, MDE) and then activate a well-characterized transcriptional cascade before accumulating lipids in response to insulin (Zebisch, et al., 2012). A multitude of transcription factors and transcriptional coregulators of adipogenesis have been identified which in turn activate the master adipogenic transcription factors C/EBPα and PPARγ (MacDougald and Lane, 1995). Non-transcriptional mechanisms regulating adipogenesis have received less attention.

In vivo, quiescent preadipocytes are activated in response to physiological cues such as dietary fatty acids (FAs) and insulin to re-enter the cell cycle to self-renew and to differentiate into adipocytes (Hilgendorf, et al., 2019; Jeffery, et al., 2015). In contrast to this asymmetric cell division, 3T3-L1 MDE is symmetric with both daughter cells uniformly differentiating into adipocytes, an advantage for both biochemical and genetic analyses. Of note, while some other cell line models of adipogenesis appear to not undergo complete mitoses, these cells still need to progress through S-phase (Cho and Jefcoate, 2004). Thus, the replication of DNA, both *in vivo* and *in vitro* is a critical though poorly understood aspect of adipogenesis.

Lipogenesis is a well-regulated metabolic process converting excess energy into triglycerides, the predominant constituent of lipid droplets in white adipocytes. This process is required for both the formation of lipid droplets in newly differentiating preadipocytes as well as for storing additional energy in existing adipocytes, increasing their size. In response to insulin, the differentiating preadipocyte/expanding adipocyte takes up excess energy (e.g. glucose, FAs) and activates enzymatic pathways leading to the formation of acetyl-CoA and malonyl-CoA, the building blocks of FAs (Song, et al., 2018). Esterification of FAs to glycerol leads to the formation of triglycerides to form or grow lipid droplets. Since lipid droplets are highly dynamic, composed of a multitude of macromolecules, and are critical to protect cells from lipotoxicity, their biology has become the focus of intense investigation (Olzmann and Carvalho, 2019; Walther and Farese, 2012).

We sought to systematically identify genes involved in adipogenesis by conducting a genome-wide screen using CRISPR/Cas9 to disrupt gene function. To date, efforts to identify adipogenic genes faced technical limitations for large scale screens as they relied on arrayed libraries (Sohle, et al., 2012; Guo, et al., 2008). Alternatively, pooled screening formats require a mechanism to isolate cell populations with a desired phenotype. Consequently, most studies to date have used pooled screening formats to screen for genes that directly affect cell growth or

cell death, or have used engineered reporter cell lines to establish a selection platform to indirectly screen for genes of interest (van Beekum, et al., 2012).

Here, we have developed a lipid-based sorting strategy to directly isolate differentiated adipocytes versus undifferentiated preadipocytes. We used 3T3-L1 preadipocytes expressing Cas9 constitutively and a single guide RNA (sgRNA) lentiviral library targeting the mouse genome. By differentiating the sgRNA-infected pool of 3T3-L1 cells and sorting based on lipid content, we identified novel adipogenic regulators and characterized their relative importance to MDE versus lipogenesis. Intriguingly, we further show that cellular mechanisms regulating translation and protein stability are critically important for adipogenesis. We illustrate the strength of this screen by describing how hypusination, a regulator of translation initiation, and neddylation control of ubiquitin-dependent proteolysis regulate MDE and lipogenesis, respectively.

Results

A lipid-based sorting strategy enables pooled screening for regulators of adipogenesis.

Pooled CRISPR/Cas9 screening strategies are based on the ability to isolate cell populations which enrich or deplete for a desired phenotype, followed by deep sequencing to quantify changes in relative sgRNA abundance between populations. Since the ability to differentiate does not affect relative cell abundance in a population, we instead developed a sorting-based strategy based on fluorescent intensity of the lipophilic, green fluorescent dye BODIPY 493/503 (Fig. 1A). Briefly, 3T3-L1 preadipocytes expressing Cas9 protein were infected at low multiplicity of infection with a previously developed mouse sgRNA library (Breslow, et al., 2018; Morgens, et al., 2017). This library covers the entire mouse genome with 10 sgRNAs per gene as well as more than 10,000 non-targeting/safe sgRNAs. To avoid a bottleneck effect, the 3T3-L1 pool was maintained at ~1000x coverage per sgRNA at all times (~200 million cells in total). The 3T3-L1 pool was passaged for more than 2 weeks prior to initiating the experiment to select against sgRNAs targeting genes essential for viability, allowing them to drop out of the pool. The surviving pool of 3T3-L1 cells (~97% of initial sgRNA library) was grown to confluence and differentiated upon addition of differentiation cocktail. After 4 days of differentiation, the 3T3-L1 pool was stained with BODIPY followed by large scale cell sorting for green fluorescence by flow cytometry. The Day 4 time point was chosen, since adipocytes at this time point contain small lipid droplets, but are not too large to hinder sorting.

First, we confirmed the robustness and uniformity of adipogenesis as well as our ability to isolate differentiated adipocytes versus undifferentiated preadipocytes by FACS in parental 3T3-L1 cells. The staining protocol was able to separate the two populations based on green fluorescent intensity with only ~5% of cells falling into the incorrect category (negative and positive, Fig. 1B). As a proof of principle, we next infected 3T3-L1 Cas9 cells with a sgRNA targeting the master adipogenic transcription factor PPARγ. Consistent with PPARγ being required for adipogenesis, the bulk of these cells fell into the negative category even after 4 days of differentiation (Fig. 1B, yellow). Thus, this screening strategy is capable of isolating cell populations which enrich or deplete for the presence of intracellular lipid in cells and therefore the ability to undergo differentiation.

Two sequential rounds of screening were performed in duplicates (Fig. S1A). First, we used the genome-wide sgRNA library and gated for the top 50% BODIPY positivity. This more lenient cutoff was chosen to enable sorting of a sufficiently large cell population to maintain a ~1000x

coverage per sgRNA even in the sorted cell population. Genes affecting adipogenesis were identified by quantifying and comparing sgRNA abundance in the Day 4 BODIPY$^{top\ 50\%}$ cell population versus the bulk Day 0 cell pool. Effect size was calculated using the Mann-Whitney U test for the 10 sgRNAs per gene target compared to the non-targeting/safe sgRNA population, and the significance cut-off was set at an estimated FDR of 5% (Storey and Tibshirani, 2003). This first screen identified ~1500 gene targets as candidate regulators of adipogenesis (Table S1). We selected 10 of these gene targets for manual confirmation. Selection was based on gene targets scoring as highly significant but having little to no previously known function in regulating adipogenesis. We confirmed that CRISPR-mediated loss of 9 out of the 10 gene targets inhibited adipogenesis, including two gene targets whose loss had comparable effects to loss of C/EBPβ and PPARγ (Fig. S1B). As an additional confirmation, two high scoring yet unknown genes, E130309D02RIK and GM14939, were knocked out in 3T3-L1 cells, which subsequently failed to properly differentiate (Fig. S1C). This argues that the list of gene targets obtained from screen 1 is strongly enriched for regulators of adipogenesis.

A second round of screening was conducted under more stringent sorting conditions to identify strong and high-confidence regulators of adipogenesis. Briefly, we developed a new custom library targeting the top 1500 hits of the first screen as well as additional genes of interest related to signal transduction pathways (Fig. S1A). We repeated the screening strategy in duplicates, this time sorting for both the top and bottom quartile of BODIPY signal intensity. Additionally, bulk cell populations on day 0 and day 4 (unsorted) were collected. Together, these four cell populations allowed for the identification of three different but related properties that can contribute to overall BODIPY signal (Fig. 1C, Table S2). Genes affecting differentiation (henceforth referred to as DIFF) were identified by comparing sgRNA abundance in the Day 4 BODIPY^{high} cell population versus the bulk Day 0 cell pool, reflecting depletion or enrichment of sgRNAs in the most clearly differentiated population. Genes affecting mitotic differentiative expansion (MDE) were identified by comparing sgRNA abundance in the bulk Day 4 versus the bulk Day 0 cell populations. Genes affecting lipid accumulation (henceforth referred to as LIPID) were identified by comparing sgRNA abundance in the Day 4 BODIPY^{high} versus BODIPY^{low} cell populations, allowing us to distinguish direct effects on lipolysis and lipogenesis irrespective of MDE. Taken together, the second screen not only identified putative regulators of adipogenesis but categorized their relative importance to MDE versus LIPID. Notably, the first screen successfully culled the number of adipogenic regulators found in the whole genome, because hits from the first screen were significantly enriched among the hits identified for DIFF, MDE, and LIPID (Fig. S1D).

Since adipogenesis is a function of both the ability to undergo MDE and to accumulate lipid, we next sought to establish how the three phenotypic scores relate to each other. Interestingly, the size of the DIFF effect strongly correlated with the simple sum of the MDE and LIPID effects (Fig. S1E, F). This argues that the ability to categorize adipogenic genes into regulators of MDE versus LIPID can provide significant functional discrimination for assigning the molecular mechanism of action for both known and novel hits identified in this screen. To validate this insight, we investigated adipogenic genes known to regulate MDE and/or LIPID. The insulin receptor, the BMP1A receptor, and C/EBPβ are known to be required for adipogenesis and all three are known inducers of MDE (Bowers and Lane, 2007; Tang, et al., 2003b; Gregoire, et al., 1998). Consistently, all three genes showed significant, negative DIFF and MDE effects (Fig. 1D purple in heatmap and cartoon). In addition, the ligands insulin and BMP4 promote lipid accumulation (Modica, et al., 2016; Kersten, 2001) and their associated INSR and BMPR1A

receptors accordingly also showed a significant negative LIPID effect (Fig. 1D purple). Similarly, our screen validated that the cell cycle regulator RB1 is required for adipogenesis, but this was a consequence of dual, opposing roles for RB1, with: (1) a significant positive MDE effect, consistent with it being required to exit MDE; and (2) a significant negative LIPID effect, consistent with RB1 promoting expression of lipogenic genes (Fig. 1D blue) (Moreno-Navarrete, et al., 2013). Finally, loss of adipose triglyceride lipase or the mTOR pathway inhibitor TSC2 resulted in increased adipogenesis, which was entirely driven by a positive LIPID effect, consistent with increased lipid accumulation (Fig. 1D red) (Schreiber, et al., 2019; Duvel, et al., 2010). Thus, our screen not only identified candidate adipogenic regulators, but the MDE and LIPID scores provide testable hypotheses to understand how both known and novel adipogenic genes function during adipogenesis.

Assessment of screen performance.

Having established the relationship between our three phenotypic scores, we next sought to assess our ability to detect known regulators of adipogenesis as well as MDE and LIPID. Focusing on specific gene targets first, inspection of our screen data showed that the known adipogenic regulators INSR, PIK3CA, C/EBPβ, PPARγ, KLF5, RB1, and ATGL scored as strong and significant regulators of adipogenesis (Fig. 2A, bottom). Further analysis showed that INSR, PIK3CA, C/EBPβ, SREBF2, and PPARγ score as significant inducers of MDE, while RB1 scores as a significant inhibitor of MDE (Fig. 2A top). Similarly, INSR, IRS1, PIK3CA, AKT2, RB1, and PPARγ score as significant inducers of LIPID, while ATGL scores as a significant inhibitor of LIPID (Fig. 2A middle). Thus, our screen detects well-established regulators of adipogenesis.

Next, we assessed the ability of our screen to recover hits in pathways known to regulate MDE and LIPID. Overlaying our MDE scores onto the KEGG pathway for the canonical cell cycle showed that many cell cycle genes were significant hits (Fig. 2B). This enrichment was observed even though many cell cycle genes were also required for viability and therefore dropped out of the screen. Similarly, overlaying our LIPID scores onto the KEGG pathways for the TCA cycle, FOXO signaling, and PI3K-AKT signaling, known to be important for lipogenesis and insulin signaling, showed that a considerable fraction of the components tested in these pathways were significant hits (Fig. 2C, Fig. S2A-B). Our screen thus identified components in diverse cellular processes, in accordance with the multiple pathways organizing adipogenesis.

Finally, we performed a Gene Ontology Enrichment Analysis for biological processes on significant hits in DIFF. Strikingly, all GO terms obtained fell into three general categories: signaling, regulation of transcription, and regulation of post-transcriptional processes (Fig. 2D). Adipogenesis is known to be regulated by a cascade of adipogenic transcription factors and co-regulators. Similarly, signal transduction pathways are known to regulate the initiation of adipogenesis and lipogenesis, including phosphorylation of key transcriptional regulators in response to initiation of adipogenesis (Rabiee, et al., 2017; Humphrey, et al., 2013). However, the prevalence of hits relating to the general regulation of translation and protein stability was striking, suggesting that these core machineries are integral to understanding adipogenesis.

Many changes in protein abundance during adipogenesis are not driven by transcriptional mechanisms.

Given the strong representation of hits in processes reflecting the central dogma for molecular biology, we next sought to assess the representation of our hits in the core machineries of

transcription, post-transcriptional modifications, translation, and protein degradation (Fig. 3). Notably, this analysis not only underscored the importance of these gene families and complexes to MDE, LIPID, and DIFF, but also highlighted the ability of this screen to identify which members of a gene family or molecular machinery are functionally most relevant. For example, many components of the translation initiation complex were significant hits, but specific isoforms of some components were unique. The scaffolding protein EIF4G consists of three family members. EIF4G3 is expressed at low levels and did not score in the screen. EIF4G1 is the major isoform and had dual, opposing roles in adipogenesis, being required for MDE yet inhibiting lipid accumulation. In contrast, the third isoform EIF4G2/DAP5 scored as the most highly significant inhibitor of adipogenesis and inhibited both MDE and lipid accumulation (Fig. S3). EIF4G2 is not well-understood, but generally thought to be responsible for approximately 20% of protein translation, notably including cap-independent translation (de la Parra, et al., 2018). Thus, EIF4G1 and EIF4G2 have distinct functions during MDE, suggesting that the composition of the initiation complex may regulate translation of specific proteins with relevance to MDE. Similarly, analysis of cullin-ring complexes not only revealed that protein stability is critical in the regulation of adipogenesis, but identified that specific cullins, adaptor proteins, and F-box and other substrate recognition receptors are likely functionally important. This suggests that the degradation of specific target proteins is critical for adipogenesis. Further, the specific targets of these degradation mechanisms are likely to have been identified as controllers of adipogenesis in our screen.

There have been numerous gene expression profiles conducted to assess changes in gene expression during 3T3-L1 adipogenesis to identify candidate adipogenic regulators (Ahmed and Kim, 2019). However, our data argued that processes regulating protein abundance directly, namely differential translation of mRNA and degradation of proteins, are also critical for adipogenesis. This suggests that this mRNA-centric view of adipogenesis may have obscured important biology. We therefore sought to correlate changes in mRNA and protein concentrations during 3T3-L1 adipogenesis. Specifically, duplicate samples at 0, 24, 48, and 96 hours post-differentiation were collected and analyzed by shotgun proteomics to quantify changes in protein abundance during 3T3-L1 adipogenesis. (Fig. 4A, Table S3). Newly obtained and previously published gene expression data for the same time points were combined to obtain a consensus of mRNA expression during 3T3-L1 adipogenesis (Fig. S4A). Scatter plot analysis showed that there is very poor correlation between mRNA and protein abundance at any of the time points assessed. In fact, this analysis shows that only approximately 25% of protein abundance can be explained by mRNA concentration (Fig. 4B). Numerous studies have shown that the relationship between protein and mRNA is complicated by the variable contribution of other processes, including regulatable translation rates, protein stability, and protein secretion (Liu, et al., 2016). However, the correlation observed in this study is even poor compared to the more typically observed squared Pearson correlation coefficient of ~0.40 (Vogel and Marcotte, 2012). To determine how changes in mRNA expression related to changes in protein abundance over the time course of adipogenesis, we next performed hierarchical clustering of the proteomics time course data (Fig. 4C, Table S4). This analysis yielded 10 clusters with differential changes in protein expression during adipogenesis. Next, we assessed mRNA expression changes for the proteins found in each cluster by performing hierarchical clustering (Fig. 4D, Fig. S4B, Table S4). A number of specific patterns linking gene expression to protein abundance emerged from this analysis. Some proteins accumulate concurrently with mRNA expression (e.g. Fig. 4D cluster 2.A). Other proteins accumulate

appreciably later than mRNA, suggesting that that either translational control or regulation of protein stability could regulate accumulation of proteins important for differentiation (e.g. Fig. 4D cluster 7.A). Finally, many of the transcript changes bore little to no resemblance to the changes in protein concentration (e.g. Fig. 4D cluster 3.B). For example, both TSC2 and NCOA6 protein abundance transiently increases during adipogenesis, while mRNA expression remains unchanged or transiently decreases during adipogenesis (Fig. 4D cluster 3.B, Table S4). This suggests that proteins are selectively translated (or selectively stabilized) upon addition of differentiation cocktail and then specifically degraded upon completion of MDE. Consistent with its known function as an mTOR pathway inhibitor, TSC2 inhibited LIPID in our screen (Table S2). The expression pattern suggests that TSC2 protein is transiently upregulated during early adipogenesis to prevent premature accumulation of lipids. Similarly, NCOA6 is a known co-activator of LXRs and critical for liver lipid metabolism (Kim, et al., 2003). Moreover, it was identified in a GWAS study for body fat distribution (Winkler, et al., 2015; Heid, et al., 2010). In our screen, NCOA6 is required for LIPID (Table S2). The expression pattern suggests that NCOA6 protein is upregulated during early MDE to initiate transcriptional expression of genes important for lipogenesis. Finally, NUBP2 protein transiently decreases at the 48h time point, when mRNA levels are maximal (Fig. 4D cluster 4.B, Table S4). We found that NUBP2 is a novel regulator of adipogenesis, required for MDE (Fig. S1B, Table S2). Since NUBP2 is required for the formation of iron-sulfur clusters and hence proliferation (Christodoulou, et al., 2006), the expression pattern during adipogenesis suggests that NUBP2 may be degraded at the end of MDE to enable cell cycle exit. Additional proteins that are strongly controlled at the level of protein abundance and important for adipogenesis were found to be highly linked to GWAS studies supporting roles in obesity (such as body mass index), including MED11, TAOK1, FNT, and WDR82 (Table S2, Table S4). Other proteins were linked to metabolic disorders. Thus, we conclude that focusing on changes in protein abundance, rather than mRNA abundance, may identify interesting adipogenic regulators. An examination of conserved processes regulating protein abundance directly may uncover key mechanisms regulating adipogenesis. Here, we focus on the importance of hypusination, a highly conserved pathway regulating translation of proteins containing consecutive proline residues, and neddylation, a ubiquitin-like posttranslational modification known to activate Cullin-Ring E3 ubiquitin ligases.

Hypusination is required to translate proline-rich adipogenic regulators important for MDE.

Examination of the screen showed that proteins important for the translation of polyproline sequences are important for adipogenesis (Fig. 3). The eukaryotic initiation factor 5A is important for the efficient translation of mRNAs that contain four or more consecutive proline codons (Gutierrez, et al., 2013). There are two isoforms, the ubiquitously expressed isoform EIF5A and the testes and brain-specific isoform EIF5A2 (Jenkins, et al., 2001). Hypusine is a unique post-translational modification of EIF5A, and it is essential for EIF5A function. In a highly conserved pathway, the enzymes deoxyhypusine synthase (DHPS) and deoxyhypusine hydroxylase (DOHH) use the polyamine spermidine as a substrate for hypusination of a conserved lysine residue in EIF5A (Wolff, et al., 1995; Abbruzzese, et al., 1986; Park, et al., 1981). In our initial validation of the first screen, we confirmed that loss of DHPS inhibited adipogenesis (Fig. S1B). The second screen identified DOHH and EIF5A, but not EIF5A2, as required for MDE (Fig. 5A, Fig. S5A). Of note, loss of DHPS also resulted in a very negative MDE score, but this did not reach the significance cut-off, likely because more than half of the targeting sgRNAs were not recovered in the any of the experimental time points, consistent with

loss of DHPS affecting cell viability (Lee, et al., 2002). To further validate the importance of hypusination to MDE, we therefore showed that acute pharmacological inhibition of this pathway reduced 3T3-L1 adipogenesis. Specifically, we treated 3T3-L1 cells with an inhibitor of ornithine decarboxylase (ODC1), the rate-limiting enzyme in spermidine synthesis (Meyskens and Gerner, 1999). The inhibitor was added two days prior to addition of differentiation cocktail to sufficiently deplete the spermidine pool prior to MDE and the inhibitor was maintained with the cocktail. Inhibition of hypusination resulted in a dose-dependent inhibition of MDE and adipogenesis (Fig. 5B, Fig. S5B). Of note, spermidine has previously been shown to be required for 3T3-L1 adipogenesis (Vuohelainen, et al., 2010). Our data argues that loss of spermidine inhibits adipogenesis at least in part by inhibiting hypusination.

We hypothesized that hypusination is required for MDE because hypusinated EIF5A is required for the translation of a subset of mRNAs that contain consecutive prolines and that one or more of these gene products is in turn required for MDE (Fig. 5C). We therefore sought to generate a list of candidate adipogenic proteins containing multiple consecutive prolines (Fig. 5D). Specifically, we sought genes that were identified in the screen as regulating MDE (Fig. 5D red circle), containing four or more consecutive prolines (yellow circle), and which are translated during MDE (blue circle). This analysis identified 27 candidates (Fig. 5E). Notably, this list contains both well established and candidate adipogenic genes. C/EBPβ is a known early adipogenic transcription factor required for MDE (Tang, et al., 2003a). Consistent with our model, a previous study has shown that loss of spermidine prevents the translation, but not the transcription of C/EBPβ in early adipogenesis (Hyvonen, et al., 2013). Other candidate proteins are known to be required for adipogenesis, including RBM14 (Firmin, et al., 2017) and BRD2 (Denis, et al., 2010; Wang, et al., 2009), but their importance to MDE is less-well appreciated. Thus, we propose that the hypusination pathways regulates adipogenesis by enabling the translation of regulators of MDE with multiple consecutive prolines, including C/EBPβ.

Neddylation is required to prevent precipitous lipid accumulation in preadipocytes and hepatocytes.

Examination of our screen showed that the ubiquitin-like protein NEDD8 was a highly significant hit (Fig. 6A, top row). We were particularly intrigued by the dual role of NEDD8 in adipogenesis. While NEDD8 was required for adipogenesis, this was driven by a requirement for MDE. The screen argued that NEDD8 also independently inhibits lipid accumulation. NEDD8 is a ubiquitin-like post-transcriptional modification and its best validated substrate is the cullin subunit of the Cullin-Ring E3 family of ubiquitin ligases (CRLs) (Petroski and Deshaies, 2005). Neddylation is required to activate CRLs, which in turn promotes ubiquitin-mediated degradation of their substrates. CRLs are multi-subunit complexes containing a cullin, a RING-box protein, an adapter, and a specific substrate recognition receptor. There are eight cullins, two RING-box proteins, 4 adapter proteins, and a multitude of substrate recognition receptors (including F-box proteins), making this the largest superfamily of E3 ubiquitin ligases. Consequently, CRLs are responsible for ubiquitination of ~20% of proteins targeted for ubiquitin-mediated degradation and play a critical role in many cellular processes (Zhao and Sun, 2013). The best-established function for CRLs is the regulation of the cell cycle and consistent with this, we observed a significant negative MDE score in the screen (Fig. 6A, top row). There are eight cullin genes, six of which were tested in the second screen. Strikingly, examination of their scores allowed for the identification of which cullins and other CRL components functioned in MDE versus LIPID. Neddylation of CUL3 was required for MDE (Fig. 6A, second row). In contrast, the screen

identified that neddylation of CUL1 or CUL5, as well as their association with the CUL5 adapter proteins ELOB and ELOC, was required for inhibiting lipid accumulation (Fig. 6A). We decided to further explore how neddylation of this CRL regulates lipid accumulation.

First, we sought to confirm that inhibition of neddylation resulted in increased lipid accumulation. Specifically, 3T3-L1 preadipocytes were treated with a specific inhibitor of NAE, the E1 in the neddylation pathway (iNAE, MLN4924). No differentiation cocktail was added. Lipid content was assessed using the lipophilic dyes BODIPY or LipidTOX. Within 2 days, inhibition of neddylation led to the formation of many small lipid droplets in 3T3-L1 preadipocytes (Fig. S6A, top panels). The timeframe of this lipid accumulation as well as the disorganized appearance of the lipid droplets argued against this being a consequence of canonical adipogenesis. Accordingly, iNAE promoted lipid accumulation even in the presence of an inhibitor of PPARγ (Fig. S6B). Co-staining with BODIPY and PLIN, a protein upregulated during adipogenesis to coat lipid droplets, confirmed that the intracellular lipid accumulated in response to neddylation inhibition was not organized into the typical lipid droplet known to form in differentiating preadipocytes (Fig. S6A, bottom panels). To further study this intracellular lipid, 3T3-L1 preadipocytes were grown in the presence of a fluorescently labeled fatty acid (FA) with and without iNAE (Fig. 6B). In the absence of neddylation, 3T3-L1 preadipocytes accumulate lipids precociously.

Since neddylation of CRLs is required for ubiquitin-mediated degradation of substrates, including cell cycle proteins, we next sought to determine if lipid accumulation was a consequence of cell cycle arrest or the stabilization of other CRL substrates. 3T3-L1 preadipocytes were treated with inhibitors of neddylation, the proteasome (MG132), or S-phase (hydroxyurea, HU). As expected, inhibition of neddylation or S-phase inhibited proliferation in a dose-dependent manner (Fig. S6C). Only inhibition of neddylation or the proteasome, and not cell cycle inhibition, resulted in lipid accumulation in a dose-dependent manner, as assessed by green-fluorescent BODIPY staining over time (Fig. 6C). This lipid accumulation was observed within hours of inhibition. Since inhibition of the proteasome phenocopied the effect of loss of neddylation, we hypothesized that a neddylated (and hence active) CRL constitutively promotes proteasomal degradation of a substrate which normally promotes lipid accumulation. A candidate for this substrate would be stabilized in the absence of neddylation. To identify proteins stabilized in the absence of neddylation, 3T3-L1 preadipocytes treated with iNAE or vehicle were analyzed by shotgun-proteomics (Table S5). PANTHER pathway analysis of differential protein abundance revealed a significant enrichment for cell cycle regulators, consistent with the known role of CRLs in regulating the cell cycle (Fig. S6D, orange and teal). Notably, proteins involved in insulin signaling were also among the top hits, including the insulin signaling adapter IRS1 (Fig. S6D, purple and teal). IRS1 has previously been identified as a substrate for CRLs in other cell contexts (Scheufele, et al., 2014; Hu, et al., 2012; Xu, et al., 2012). Immunoblot staining confirmed that inhibition of neddylation resulted in activation of insulin signaling within hours of treatment, as assessed by staining for phosphorylated IRS1 and AKT (Fig. 6D). Thus, we propose that in 3T3-L1 preadipocytes the neddylated CRL consisting of CUL5, ELOB/ELOC and SOCS1 and SOCS3 targets components of the insulin signaling pathway, including IRS1, for degradation. Consistent with this, our screen confirmed that IRS1 is required for lipid accumulation (Fig. 6A, last row). In the absence of neddylation, components of the insulin signaling pathway, including IRS1, would thus accumulate, resulting in uncontrolled insulin signaling and lipogenesis (Fig. 6E).

To test this model, 3T3-L1 preadipocytes treated with an inhibitor of neddylation were supplemented with additional insulin. This potentiated lipid accumulation in the presence of iNAE (Fig. 6F). Similarly, addition of an inhibitor of PI3K signaling, required for AKT activation, prevented iNAE-induced lipid accumulation in a dose-dependent manner (Fig. 6G). Next, we tested if inhibition of neddylation resulted in increased *de novo* lipogenesis. First, we assessed the ability of 3T3-L1 preadipocytes treated with the inhibitor of neddylation to take up glucose, the macromolecule commonly catabolized to generate the building blocks for FA synthesis. Addition of iNAE resulted in a dramatic and highly significant increase in the uptake of the fluorescent glucose analog 2-NBTG (Fig. S6E). Moreover, inhibition of neddylation only resulted in lipid accumulation if 3T3-L1 cells were cultured in the presence of glucose (Fig. S6F). Thus, iNAE-induced lipid accumulation is the consequence of insulin-signaling induced glucose catabolism to synthesize intracellular lipids *de novo*.

Finally, we wanted to assess if this role of neddylation is conserved in other cell contexts sensitive to insulin-induced lipogenesis. Notably, preadipocytes isolated from gonadal white adipose tissue, but not from inguinal white adipose tissue, showed iNAE-induced lipogenesis in culture both in the presence and absence of differentiation cocktail (Fig. S6G and data not shown). Since gonadal preadipocytes show little adipogenesis in culture even in the presence of differentiation cocktail while inguinal preadipocytes differentiate spontaneously even in the absence of differentiation cocktail, this further supports the hypothesis that neddylation is required to specifically prevent precocious activation of insulin signaling and lipogenesis. Finally, we investigated the importance of neddylation in hepatocytes. Non-alcoholic fatty liver disease is strongly correlated with obesity and in part driven by increased hepatic lipogenesis (Divella, et al., 2019). Inhibition of neddylation in AML12 hepatocytes resulted in a significant increase in *de novo* lipogenesis, further potentiated by insulin in a dose-dependent manner (Fig. 6H). Thus, neddylation is required to prevent precocious, insulin-induced lipogenesis in multiple cellular contexts of relevance to human health.

Discussion

Obesity-related diseases are among the leading causes of morbidity and preventable death. Identifying regulators of adipogenesis may advance several potential therapeutic avenues. Here, we present a genome-wide screen for regulators of adipogenesis, both validating well-characterized regulators of adipogenesis and identifying novel candidates. We also quantify the relative importance of adipogenic hits by MDE and lipogenesis. To illustrate the power of the screen, we show that hypusination is required for translating inducers of MDE and that neddylation is required to prevent premature and precipitous lipogenesis in both preadipocytes and hepatocytes. This follow-up analysis highlights the importance of post-transcriptional mechanisms to the regulation of adipogenesis and challenges the common use of mRNA-centric approaches in the field.

Resources provided in this study.

The functional genomics screen enumerates many candidate adipogenic regulators. To further aid future investigators, we list as an additional parameter which hits were identified in a GWAS study for body fat distribution (Table S2) (Winkler, et al., 2015; Heid, et al., 2010). Moreover, this study also provides a compilation of public gene expression data during 3T3-L1 adipogenesis. Finally, we performed a proteomic analysis to quantify changes in protein abundance during

3T3-L1 adipogenesis. We used publicly available mRNA expression data and performed a proteogenomic comparison of 3T3-L1 adipogenesis (Table S4).

A main conclusion uncovered in our analysis is that mechanisms regulating translation of subsets of mRNA and protein stability play a critical role in adipogenesis. Notably, the screen provides testable hypothesis for how these post-transcriptional mechanisms function during MDE and lipogenesis, as illustrated for hypusination and neddylation. For example, our data showed that a translation initiation complex containing EIF4G1 is required for MDE, while translation initiation utilizing EIF4G2/DAP5 inhibits MDE and adipogenesis. Previous studies have shown that EIF4G2 drives translation of a select group of mRNAs important for diverse cellular processes, including the transition of mESCs from pluripotency to differentiation (Sugiyama, et al., 2017; Yoffe, et al., 2016). Additional teratogenesis studies with mESCs lacking EIF4G2 have demonstrated a requirement for all tissues of mesodermal origin, further implicating this gene in the earliest of embryonic differentiation (Yamanaka, et al., 2000). These studies suggest that EIF4G1 and EIF4G2 selectively translate proteins that are either required for or inhibiting to MDE, respectively; and that these roles are vital for proper mesoderm differentiation. Further investigation may discover the identity of these adipogenesis-associated mRNAs.

Finally, while much of the screen validation in this study focused on post-transcriptional control of adipogenesis, we note that this screen also identified a number of putative adipogenic transcription factors, including Zmynd8, which has known GWAS association for body fat distribution (Table S2). Other significant hits were associated with the cytoskeleton and mechanisms of cellular trafficking. Moreover, the screen also identified candidate adipogenic genes that are annotated but to-date uncharacterized, including GM14393 and E130309D02Rik (Fig. S1C). Follow-up investigation will establish how these genes regulate steps in adipogenesis and lipogenesis and may suggest potential therapeutic targets for obesity and metabolic health.

Limitations of the screen.

To avoid the confounding effects of viability genes in this screen, the infected pool of 3T3-L1 cells was passaged for two weeks prior to initiating the experiment (defined as time of plating, 4 days prior to Day 0). This time frame was selected to (1) allow for sufficient turnover of target genes to disrupt gene function and (2) select against any sgRNA targeting viability genes, since a decrease in sgRNA abundance over time due to loss of viability could be interpreted as a requirement for adipogenesis and MDE. While the two-week time frame should be sufficient to select against sgRNA with strong lethality, we cannot exclude the presence of some sgRNA in the pool that resulted in a slight decrease in cell growth over time. However, since the average recovery of sgRNA elements per gene for the duplicates was 19.34 out of 20 sgRNA total, we do not believe that this is a major concern. Likewise, some genes required for viability may have additional, but separable roles in adipogenesis, which we would fail to report here.

The screen is based on the ability to enrich for cells with high or low amounts of intracellular lipid by sorting for green fluorescent intensity upon addition of the lipophilic dye BODIPY. However, if a cell is infected with a sgRNA that substantially affects total cell number, then this cell may be enriched in the BODIPY^{high} or BODIPY^{low} cell populations independent of adipogenesis. We observed this effect for components of the Hippo signaling pathway. Absence of Hippo signaling results in an inability to both confluency-arrest and to exit MDE. Since the 3T3-L1 adipogenesis protocol specifies that cells remain confluency-arrested for two days prior

to addition of differentiation cocktail and cells normally exit MDE after 2 days of differentiation, loss of Hippo signaling effectively resulted in four additional days of proliferation compared to cells infected with non-targeting sgRNAs. This yielded a positive DIFF score due to increased representation of sgRNAs in the Day 4 cell population (Table S1), despite the fact that Hippo signaling is required for adipogenesis and consequently leads to decreased lipid content (data not shown) (Ardestani, et al., 2018). We cannot exclude the possibility that other pathways regulating confluency are similarly over/underrepresented, confounding the analysis. Data related to these pathways should therefore be viewed with caution. We note however that the screen was sufficiently robust to accurately screen cell cycle regulators, including the RB1/E2F signaling pathway.

Homopolymeric proline proteins driving MDE

Our screen showed that the highly conserved hypusination pathway, required for translating proteins with sequences of consecutive prolines, is important for MDE. We generated a list of candidate drivers of MDE that contain a sufficiently long sequence of prolines to require hypusinated EIF5A for efficient translation. Of note, the murine C/EBPβ sequence contains five consecutive prolines, and spermidine depletion is known to inhibit translation of C/EBPβ while not affecting mRNA expression (Hyvonen, et al., 2013). Translation of C/EBPγ, which functions similarly to C/EBPβ but does not contain this stretch of prolines, is not affected by spermidine depletion (Hyvonen, et al., 2013). Murine C/EBPα contains a stretch of eight prolines, and therefore also likely requires the hypusination pathway for efficient translation, albeit later in the adipogenic program. Importantly, human C/EBPβ and C/EBPα also contain homopolymeric proline sequences with nine and seven consecutive prolines, respectively. Thus, the hypusination pathway is likely also critical for adipogenesis of human preadipocytes. Further investigation is required to confirm the importance of hypusination to the translation, but not transcription of the other candidates listed. Moreover, it is unclear if the failure to undergo MDE in the absence of hypusination is a consequence of loss of any single protein or instead the consequence of the sum of protein losses. Finally, the list of candidates contains both known and novel adipogenic regulators. However, the screen argues that even the known adipogenic regulators, such as BRD2 and KDM1A, are critical for adipogenesis because they are specifically required for MDE. Follow-up investigation will establish how these proteins function to promote MDE.

CRLs regulate lipogenesis

The screen identified that neddylation is strongly required for differentiation due to its requirement in MDE. In addition, NEDD8 had a significant positive LIPID score, arguing that neddylation inhibits lipid accumulation. Here, we show that neddylation of a CRL is required to prevent premature activation of insulin signaling and thus lipogenesis. We propose that this CRL is composed of CUL5 and ELOB/ELOC. Based on our proteomics analysis and previously published data (Hu, et al., 2012), we further propose that IRS1 is a critical substrate of this CRL. We note that our proteomics analysis identified several other components of insulin signaling which are also stabilized as a consequence of neddylation inhibition, including PIK3R1, SRFBP1, PHIP, and IGFBP3. Moreover, PANTHER pathway analysis of differential protein abundance shows enrichment of pathways other than cell cycle and insulin. Further investigation is required to establish any of these additional proteins as CLR substrates.

Inhibition of neddylation promotes lipid accumulation in both preadipocytes and hepatocytes. Obesity is associated with an increased risk for hepatic steatosis and non-alcoholic fatty liver disease. Our data suggests that loss of NEDD8 and CRL function can drive this phenotype. Further investigation is needed to establish if dysregulation of this pathway contributes to human disease.

Systems level analysis to define the adipogenesis and lipogenesis programs in new contexts

The list of strong genetic requirements for adipogenesis and lipogenesis we discovered here facilitates many specific functional tests in preadipocytes and other cell types. The genome-wide comprehensive screen defines a core set of adipogenic subprograms for both known and new pathways causing specific metabolic changes throughout adipogenesis. This systems-level analysis is enabled by a global map of RNA and protein abundance through differentiation, coupled to knowledge of which gene products are critical. Specific subsets of these subprograms may be employed in other tissues that create fat deposits including bone marrow, muscle, liver, or specific fat depots. These subprograms include the translational and protein stability programs outlined here. Single cell profiles and shotgun proteomics in specific tissues may help pinpoint which of these adipogenic subprograms operate in specific subpopulations of preadipocytes and fat. New efforts can generalize from the preadipocyte requirements defined here to define how the adipogenesis program is molded to adapt fat tissue for specific needs in different tissues.

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Author Contributions

Conceptualization, KIH, CTJ, MCB, and PKJ; Methodology, KIH, CTJ, KH; Software, KH, AR and JD; Investigation, KIH, CTJ, AR, RC, YZ, and ZJ; Resources, MCB, KJS, and PKJ; Writing – Original Draft, KIH and CTJ; Writing – Review & Editing, KIH, CTJ, AR, and PKJ; Supervision, KIH and PKJ; Funding Acquisition, KIH, KH, MCB and PKJ.

Declaration of Interests

The authors declare no competing interests.

Figure titles and legends

Figure 1: Development of an adipogenesis screen using a lipid-based sorting strategy

a. Overview of screening strategy. 3T3-L1 cells receiving a non-targeting control sgRNA are shaded in white, cells receiving positive or negative regulators of adipogenesis are shaded in color. Lipophilic green-fluorescence BODIPY dye is denoted in green.
b. Flow cytometry histograms of undifferentiated and differentiated 3T3-L1 cells treated with BODIPY. Cells lacking PPARG fail to differentiate and remain BODIPY-negative. Top and bottom quartile were sorted to obtain a Day 4 low and Day 4 high population.
c. Phenotypic scores were calculated by comparing sgRNA abundance relative to non-targeting control sgRNAs between two distinct cell populations. The differentiation score (DIFF) is calculated by comparing Day 0 to Day 4 high, the MDE score is calculated by comparing Day 0 to bulk (unsorted) Day 4, and the lipid accumulation score (LIPID) is calculated by comparing Day 4 low to Day 4 high.
d. Heatmap displays phenotypic scores obtained for known adipogenic regulators regulating MDE and/or LIPID. Schematic representation of results for each category. Cells receiving a non-targeting control sgRNA are shaded in white, cells lacking positive regulators of MDE are shaded in yellow, cells lacking a negative regulator of MDE which is also a positive regulator of LIPID are shaded in blue, and cells lacking a negative regulator of LIPID are shaded in red.

Figure 2: Overview of adipogenesis screen results

a. Volcano plot of q-value versus effect size for MDE, LIPID, and DIFF. Known adipogenic regulators are labeled.
b. KEGG pathway of cell cycle with gene targets that scored significantly in MDE analysis denoted in red.
c. KEGG pathway of TCA cycle with gene targets that scored significantly in LIPID analysis denoted in red.
d. GO term analysis of the DIFF analysis identifies terms associated with signaling, regulation of transcription, and post-transcriptional regulation of protein translation and stability.

Figure 3: Complexes Important to Adipogenesis.

Gene families and complexes important for core processes in the central dogma for molecular biology are strongly represented among hits. Light red color indicates the genes that had a significant score ($q<0.05$) for MDE, LIPID, or DIFF. Border denotes a significance score in the top 100 in MDE, LIPID, or DIFF and * denotes a positive pZ in MDE. This analysis highlights which subunit or gene family member is critical for adipogenesis.

Figure 4: Protein translation and stability drive substantial dynamic changes in expression during adipogenesis

a. Schematic for analysis of RNA and protein abundance during 3T3-L1 adipogenesis. RNA abundance was obtained from published data, protein abundance was determined using shot-gun proteomics.

b. Scatter plot showing $\log_2$ of RNA versus protein abundance at 0, 24, 48, and 96 hours of 3T3-L1 adipogenesis. Only 25% of protein abundance can be explained by mRNA abundance.
c. Heatmap showing hierarchical clustering of protein abundance during 3T3-L1 adipogenesis. Changes in protein concentration fall into 10 clusters.
d. Profile plot showing fold change in protein expression over time (after z-scoring for each time point) for four clusters. For all proteins in each cluster, corresponding RNA concentrations were analyzed, and hierarchical clusters yielded three profile plots. Number of proteins/genes in each cluster is indicated below each cluster. Color of profile denotes distance from center, where red is zero and green is maximal change.

Figure 5: Hypusination Pathway is critical for mitotic clonal expansion and adipogenesis

a. Heatmap displays phenotypic scores of components of the hypusination pathway. Loss of DHPS, DOHH, and EIF5A inhibits MDE.
b. 3T3-L1 preadipocytes treated with DFMO, an inhibitor of the hypusination pathway, fail to undergo adipogenesis in a dose-dependent manner as determined by a green fluorescence mask generated by BODIPY staining. Mean of two experiments +/- SD. ***$p < 0.001$.
c. Model describing importance of hypusination to MDE. Hypusination is required for translation of homopolymeric proline tracts. At least one protein containing a stretch of prolines is important for MDE.
d. Venn Diagram to identify candidate regulators of MDE whose translation is dependent on the hypusination pathway. Candidates are regulators of MDE, contain a stretch of four or more prolines and are expressed during MDE. This analysis identifies 27 candidate regulators
e. Heatmap displays phenotypic scores of candidate regulators and the longest stretch of prolines in sequence. This list contains a number of proteins known to be important for adipogenesis and specifically MDE.

Figure 6: Neddylation Pathway inhibits premature accumulation of lipids

a. Heatmap displays phenotypic scores of NEDD8 and Cullin-RING Ubiquitin ligase complexes. Neddylation of some CRLs is required for adipogenesis, likely driven by the requirement for MDE. Loss of neddylation also promotes lipid accumulation via a distinct CRL.
b. 3T3-L1 preadipocytes treated for 32 hours with an inhibitor of neddylation accumulate the green fluorescent BODIPY-C_{12} FA.
c. 3T3-L1 preadipocytes treated with an inhibitor of neddylation or with an inhibitor of proteasome degradation, but not with an inhibitor of proliferation, accumulate lipid in a dose-dependent manner as measured by BODIPY fluorescent intensity. Data points represent average of 3 independent experiments ±SE.
d. Inhibition of neddylation in 3T3-L1 preadipocytes promotes activation of the insulin signaling pathway as determined by immunoblotting for phosphorylated IRS1 and AKT. Immunoblot shows three independent experiments per time point
e. Model describing how neddylation inhibits *de novo* lipogenesis. In the absence of neddylation, components of the insulin signaling pathway are stabilized, leading to activation of the pathway and lipid accumulation

f. Insulin potentiates accumulation of lipids induced by inhibition of neddylation in 3T3-L1 preadipocytes as measured by BODIPY fluorescent intensity. Data points represent average of 3 independent experiments ±SE.
g. Inhibition of PI3K attenuates the accumulation of lipids promoted by inhibition of neddylation in a dose-dependent manner as measured by BODIPY fluorescent intensity. Data points represent average of 3 independent experiments ±SE.
h. Inhibition of neddylation promotes lipogenesis in AML12 hepatocytes as determined by incorporation of [3H]-acetate. Insulin potentiates this effect. Bar graphs are average of 3 independent experiments ±SD. * $p<0.05$; *** $p<0.001$;

Supplemental Information titles and legends

Figure S1: related to Figure 1. Genome-wide sgRNA screen of 3T3-L1 adipogenesis screen using a lipid-based sorting strategy.

a. Workflow of primary and secondary screen. 3T3-L1 cells expressing CAS9 were transduced with a sgRNA library targeting all genes in the genome targeted with 10 distinct sgRNAs. The pool of knockout cells was differentiated in duplicate, treated with the BODIPY fluorescent dye, and the top 50% of differentiated cells were sorted as determined by green fluorescent intensity. Effect of each sgRNA was determined by comparing abundance relative to non-targeting control sgRNA in the Day 0 undifferentiated cell population versus Day 4 BODIPY-positive cell population. Significance was set at an FDR of 5%. Custom libraries of significant hits, any additional known adipogenic regulators based on literature search, and gene targets involved in signaling were generated. 3T3-L1 cells expressing CAS9 were transduced with the sgRNA sublibrary (10 sgRNAs pre gene). The pool of knockout cells was differentiated in duplicate, treated with the BODIPY fluorescent dye, and the top and bottom quartile of differentiated cells were sorted as determined by green fluorescent intensity. Effect of each sgRNA on differentiation, MDE, and lipid accumulation was determined by comparing relative sgRNA abundance between cell populations.
b. 3T3-L1 preadipocytes lacking one of 10 genes identified in screen 1 were generated and differentiated. C/EBPB and PPARG were used as positive controls. Experiments were conducted in triplicates and bar graphs with error bars represent average of two independent experiments, each conducted in triplicates and normalized to its GFP positive and negative control. Error bar is ±SD. * $p<0.05$; ** $p<0.01$
c. 3T3-L1 preadipocytes lacking one of two uncharacterized genes, GM14393 and E130309D02Rik, that scored as significant in screen 1 were generated and differentiated. Experiments were conducted in triplicates and bar graphs with error bars represent average normalized to safe sgRNA control. Error bar is ±SD. *** $p<0.001$
d. The primary screen identified 1494 significant hits (FDR of 5%). A total of 4603 genes were tested in the secondary screen. This screen identified 448 hits important for differentiation, 1014 hits important for MDE, and 570 hits important for lipid accumulation (FDR of 5%). Hits identified in each category in the secondary screen were significantly enriched for hits identified in the primary screen as determined by a chi-squared test. This argues that the primary screen was successful in narrowing down the list of real adipogenic regulators.

e. Scatter plot showing effect size in the differentiation screen versus the sum of the effect sizes obtained in the MDE and lipid accumulation screens. Only gene targets that scored as significant in at least one of the screens are plotted. More than half of the differentiation scores can be explained by the simple addition of the two components of adipogenesis, MDE and lipid accumulation.
f. Scatter plot showing effect size in MDE screen versus lipid accumulation screen. Color gradient describes differentiation effect size. Only gene targets that scored as significant in at least one of the screens and with at least 12 sgRNA elements (out of 20 total in duplicate runs) recovered are plotted. Gene targets listed in Figure 1D are annotated.

Figure S2: related to Figure 2, Adipogenesis screen results

a. KEGG pathway of PI3K-AKT signaling with gene targets that scored significantly in lipid accumulation screen denoted in red.
b. KEGG pathway of FOXO signaling with gene targets that scored significantly in lipid accumulation screen denoted in red.

Figure S3: related to Figure 3, Heatmap of screen scores for genes in Figure 3

Heatmap displays phenotypic scores for complexes important for adipogenesis. Significance, qValue < 0.05, is denoted by filled circles.

Figure S4: related to Figure 4, post-transcriptional regulatory mechanisms are critical to describe changes in expression during adipogenesis

a. PCA analysis of publicly available RNAseq data at 0, 24, 48, and 96 hour post-differentiation post-batch correction. RNA expression clusters by time point and independent of experimental source.
b. Profile plot showing fold change in protein expression over time (after z-scoring for each time point) for six clusters as defined in Fig. 4C. RNA concentrations corresponding to the proteins in each cluster were analyzed and clustered hierarchically to yield three profile plots. Number of proteins/genes in each cluster is indicated below each cluster. Color of profile denotes distance from center, where red is zero and green is maximal change.

Figure S5: related to Figure 5, Inhibition or knockout of genes required for EIF5A hypusination lead to a loss of MDE in 3T3-L1 preadipocytes

a. 3T3-L1 preadipocytes without DOHH or EIF5A have reduced Nuclei Count at 4 days post cocktail, consistent with a failure to undergo MDE in the absence of hypusination. Bar graphs represent normalized nuclei count ±SE *** $p<0.001$.
b. 3T3-L1 preadipocytes were treated with 1mM aminoguanidine and the labeled concentrations of DMFO (iODC1) for two days prior to and after cocktail addition (4 days total). Lipid Confluence (black, left axis) and Nuclei Count (blue, right axis) were imaged and assessed 4 days after cocktail addition. Inhibition of ODC1 leads to a rapid loss of lipogenesis, and a nearly complete abrogation of MDE. Bar and scatter dot represent Lipid Confluence and Nuclei Count, respectively, ±SE.

Figure S6: related to Figure 6, inhibition of neddylation promotes accumulation of lipids

a. 3T3-L1 preadipocytes treated for 36 hours with an inhibitor of neddylation accumulate lipid as determined by staining with the lipophilic fluorescent dyes BODIPY and LipidTox. This intracellular lipid appears fragmented and disorganized as determined by lack of PLIN staining after 48 hours of neddylation inhibition. As a positive control 3T3-L1 cells undergoing normal adipogenesis were stained with PLIN at 72 hours post differentiation media (DM) addition. Scale bar is 10µm.
b. 3T3-L1 preadipocytes treated with an inhibitor of neddylation accumulate intracellular lipid even in the presence of a PPARγ inhibitor. Data points represent average of 3 independent experiments ±SE.
c. 3T3-L1 preadipocytes treated with an inhibitor of neddylation or with an inhibitor of proliferation show decreased doubling time in a dose-dependent manner as measured by the confluency mask of the Essen Incucyte live-imaging system.
d. Volcano plot of q-value versus $\log_2$ fold change of protein abundance in 3T3-L1 preadipocytes treated with vehicle versus inhibitor of neddylation for 14 hours as determined by shot-gun proteomics (left). Samples were run in triplicates. Panther pathway analysis of all protein with a $\log_2$ fold change greater than 1 (and identified in at least 3 out of 6 samples) shows an enrichment for proteins associated with cell cycle regulation and insulin signaling (right). Orange denotes cell cycle regulators, purple denotes components of insulin signaling, and teal describes hits associated with both the cell cycle and insulin signaling.
e. Inhibition of neddylation in 3T3-L1 preadipocytes promotes accumulation of the fluorescent glucose analog 2-NBTG. Bar graphs are average of 3 independent experiments ±SD. * $p<0.05$; *** $p<0.001$;
f. Inhibition of neddylation in 3T3-L1 preadipocytes only promotes lipid accumulation in the presence of glucose, consistent with *de novo* insulin-induced lipogenesis. Data points represent average of 3 independent experiments ±SE.
g. Inhibition of neddylation in primary gonadal preadipocytes promotes lipid accumulation in a dose-dependent manner as measured by BODIPY fluorescent intensity (top). Inhibition of neddylation in primary inguinal preadipocytes does not promote lipid accumulation (bottom). Data points represent average of 6 independent experiments for samples treated with iNAE and 3 independent experiments for control (0µM iNAE) ±SE.

Supplemental Table 1: Raw data of first screen

Supplemental Table 2: Raw data of second screen

Supplemental Table 3: Protein expression during 3T3-L1 adipogenesis

Supplemental Table 4: Hierarchical clustering of changes in protein and RNA expression during 3T3-L1 adipogenesis

Supplemental Table 5: Neddylation inhibitor proteomics

Supplemental Table 6: Sublibraries

Supplemental Table 7: Primers used

Star Methods

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Peter Jackson (pjackson@stanford.edu).

Materials availability

All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and code availability

The published article includes all datasets generated during this study. Code used to analyze datasets are available at https://github.com/biohank/CRISPR_screen_analysis

EXPERIMENTAL MODEL AND SUBJECT DETAILS

In vivo animal studies

C57Bl/6J mice were purchased from Jackson Laboratory (000664). All mice were maintained at the Stanford animal care facility and all experiments were approved by the Administrative Panel on Laboratory Care at Stanford University. Animals were housed in groups of 5 adults per cage. Male mice between 6-8 weeks of age were used for isolation of murine primary preadipocytes.

Cell line models

3T3-L1 cells were cultured in DMEM medium containing 10% Bovine Calf Serum, 1% Pen/Strep, and 1% GlutaMAX and switched to DMEM containing 10% FBS, 1% Pen/Strep, and 1% GlutaMAX during adipogenesis.

Murine primary preadipocytes were isolated from inguinal or epididymal white adipose tissue from C57Bl/6J male mice using mouse protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Stanford University. Primary preadipocytes were maintained and differentiated in DMEM medium containing 10% FBS, 1% Pen/Strep, and 1% GlutaMAX.

AML12 mouse hepatocytes were purchased from ATCC (#CRL-2254) and cultured with DMEM/F12 medium (Gibco) supplemented with 10% FBS, 10 µg/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone, and 15 mM HEPES in a humidified atmosphere containing 5% CO_2 at 37°C. AML12 hepatocytes were passaged once reached confluency.

METHOD DETAILS

Plasmids

pMCB306 and pMCB302 (lentiviral vector, loxP-mU6-sgRNAs-puro resistance-EGFP-loxP and loxP-mU6-sgRNAs-puro resistance-mCherry-loxP) and p293 Cas9-BFP were gifts from Prof.

Michael Bassik (Stanford University). pCMV-VSV-G and pCMV-dR8.2 dvpr were gifts from Bob Weinberg (Addgene plasmid #8454; http://n2t.net/addgene:8454 ; RRID:Addgene_8454 and #8455; ; http://n2t.net/addgene:8455 ; RRID:Addgene_8455) (Stewart, et al., 2003). pHCMV-EcoEnv was a gift from Miguel Sena-Esteves (Addgene plasmid # 15802; http://n2t.net/addgene:15802; RRID:Addgene_15802) (Sena-Esteves, et al., 2004). Lentiviral vectors containing sgRNA were generated by ligating annealed sgRNA oligonucleotides into pMCB306 vector digested with BstXI and BlpI restriction enzymes.

Cell Line generation

Lentiviral vectors carrying the gene of interest were co-transfected with pCMV-VSV-G or pHCMV-EcoEnv and pCMV-dR8.2 dvpr into 293T cells using Fugene6 (Promega). Media was replaced after 24h and virus was harvested 48 and 72h post-transfection. Virus was filtered with a 0.45µm OVDF filter (Millipore) or freeze-thawed and subjected to centrifugation at 3000rpm for 10min. Cell lines were infected with virus in 10µg/ml polybrene (Millipore). Media was replaced after 24h and infected cells were isolated either via antibiotics selection or FACS sorting after 48-72h post-infection.

3T3-L1 cells expressing Cas9-BFP were generated by infection of virus harvested from 293T cells transfected with p293 Cas9-BFP, pCMV-VSV-G and pCMV-dR8.2 dvpr. 3T3-L1 Cas9-BFP cells were sorted for BFP positivity. To generate Crispr/Cas9 knockout cells, 3T3-L1 Cas9-BFP cells were infected with lentivirus containing the sgRNA of interest. Primers listed in Table S7.

3T3-L1 sgRNA pools for screening were generated as follows: sgRNA sublibraries were cloned from oligonucleotide pools (Agilent) as previously described (Morgens, et al., 2017). Genome-wide sgRNA library was synthesized in 20 sublibraries with 10 different sgRNAs per gene and we generated 2 additional sublibraries for second screen (Table S6). Oligonucleotides in each sublibrary were amplified with a sublibrary specific library adaptor protein, digested with BstXI and BlpI restriction enzymes, and ligated into pMCB320 vector. 10µg of each sublibrary, 7µg of pCMV-dR8.2 dvpr and 3µ of pHCMV-EcoEnv was co-transfected using Fugene6 and OptiMem onto one 15cm plate of 293T cells. Media was replaced after 24h and 25ml of viral supernatant was collected each at 48 hours and 72 hours post-transfection and frozen. For each sublibrary, 1.5e6 3T3-L1 Cas9-BFP cells were plated into 1050 cm^2, smooth PETG roller bottles (Fisher, 12-575-120). 96 hours post-plating, 50ml of viral supernatant of each sublibrary was thawed, spun for 10min at 300rpm and added to each roller bottle in a total of 200ml media volume containing 10µg/ml polybrene. After 24 hours, cells were trypsinized and passaged into a 1800 cm^2, smooth roller bottles (Fisher, 12-565-529). After 24 hours, cells were selected in 2 µg/ml puromycin. Cells were grown for 2 weeks, maintained at any time at 1000x the number of sgRNA elements present in the sublibrary (~1000 genes per sublibrary, resulting in ~10,000 sgRNA elements per sublibrary, requiring maintenance at ~10 million cells per sublibrary), and then frozen as separate sublibrary pools in multiple aliquots of 10^7 cells. 1 aliquot of each sublibrary was subsequently combined for each experimental replicate and time-point.

In vitro Adipogenesis

3T3-L1 cells were grown to confluency in DMEM containing 10% Bovine Calf Serum, followed by another 2 days at confluency in DMEM containing 10% Bovine Calf Serum. Adipogenesis was then induced using DMEM containing 10% FBS and differentiation cocktail consisting of 1µg/ml insulin, 1µM Dex, and 0.5mM IBMX. After 2 days of differentiation cocktail, media was changed to DMEM containing 10% FBS and 1µg/ml insulin. Maintenance media was changed

every 2-3 days for a total differentiation time of 4-8 days. Where indicated, cells were treated with inhibitors to enzymes in the hypusination pathway for a total of four days, starting 2 days prior to addition of differentiation cocktail and ending on Day 2 of differentiation. Cells treated with hypusination inhibitors GC7 and DFMO were supplemented with 1mM Aminoguanidine.

Mouse primary preadipocytes were grown to confluency in DMEM containing 10% FBS. Adipogenesis was induced using DMEM containing 10% FBS and differentiation cocktail consisting of insulin, dexamethasone, IBMX and/or 10μM NAE inhibitor MLN4924 (Selleckchem, S7109, Pevonedistat). After 3 days of differentiation cocktail, media was changed to DMEM containing 10% FBS, 1μg/ml insulin and 10μM NAE inhibitor MLN4924. Maintenance media was changed every 2-3 days.

Functional genomics screen

The screen was conducted in duplicate. For each condition (Day 0 bulk, Day 4 bulk, Day 4 for sorting), 1 aliquot of 10^7 cells for of each 3T3-L1 Cas9 sublibrary pool was combined in 30ml of fresh media and centrifuged for 5min at 1300g. Screen was performed in duplicates/condition. Pellets were resuspended and filtered using 100μm cell filter. Cell pools were then plated in multiple 1800 cm^2, smooth roller bottles such that each roller bottle contained equivalent numbers of cells ($5x10^7$ cells) from only 1 condition and replicate. Cells were grown to confluency, followed by media change. Cells were kept at confluency for 48 hours.

Day 0 bulk samples were collected by trypsinization and frozen in media supplemented with 10% DMSO. For remaining samples, adipogenesis was induced using DMEM containing 10% FBS and differentiation cocktail consisting of 1μg/ml insulin, 1μM Dex, and 0.5mM IBMX. After 48 hours, media was changed to DMEM containing 10% FBS and 1μg/ml insulin. After 48 hours, Day 4 bulk samples were collected by trypsinization and frozen in media supplemented with 10% DMSO. To the remaining samples, BODIPY 493/503 (ThermoFisher, D-3922) was added to media for a final concentration of 5μM for 1 hour at 37°C (incubator). Cells were collected by trypsinization, combined into each duplicate and filtered using 100μm cell filter. Cells were washed with PBS and pellets were resuspended in PBS containing 1% serum.

Samples were gated for mCherry+ and FITC as indicated in the figure legend (top 50% for first screen, top and bottom 20% for second screen) on a FACSAria II (BD) instrument. Sorted populations were pelleted and frozen in media supplemented with 10% DMSO. DNA was isolated with a QiaAMP DNA Blood Maxi Kit (Qiagen) concurrently for all samples. Genomic DNA was then amplified with Herculase II polymerase (Agilent) as previously described (Deans, et al., 2016). Briefly, outer primers were used to amplify the sgRNA cassette and inner primers were used to amplify the PCR product and to introduce Illumina sequencing barcodes and adapters (Table S7). PCR products were gel-extracted, quantified using dsDNA HS kit (Thermo Fisher), and pooled for deep sequencing using NextSeq 500 sequencer with high-output v2 kits (Ilumina). Sequencing was performed using a custom primer against the sgRNA protospacer.

Phenotypes from CRISPR screens were analyzed as previously described (Han, et al., 2020). Briefly, log2 fold enrichments (log2e) of sgRNAs were calculated between undifferentiated and differentiated 3T3-L1 samples and subsequently normalized to the median log2e of negative control sgRNAs (Morgens, et al., 2017) in libraries. Log2e of all sgRNAs were then divided by the standard deviation of negative control sgRNAs to yield phenotype Z scores (pZ) of sgRNAs. A median pZ score of ten sgRNAs for a given gene was measured and used as the phenotype

of the gene. The probability that the distribution of all ten sgRNAs targeting a given gene was significantly different from that of negative control sgRNAs was measured using Mann-Whitney U (MWU) test to calculate statistical significance of gene phenotypes. Lastly, to correct for multiple hypothesis testing, we calculated the adjusted p values (q values) based either on the genome-wide distribution of p values (Storey and Tibshirani, 2003) or on the Benjamini–Hochberg procedure implemented in statsmodels, a python statistical module (https://www.statsmodels.org/stable/index.html). pZ scores of sgRNAs from two experimental replicates were pooled together to calculate combined phenotypes of genes. Scripts used to analyze the data were published previously (Han, et al., 2020) and are available at https://github.com/biohank/CRISPR_screen_analysis.

To overlap screen hits with GWAS, GWAS summary statistics were obtained from the publicly available GIANT consortium's download site: http://portals.broadinstitute.org/collaboration/giant/images/f/f0/All_ancestries_SNP_gwas_mc_merge_nogc.tbl.uniq.gz.

Flow cytometry analysis

Cells were treated with 5µM BODIPY 493/503 for 1 hour at 37°C and collected by trypsinization. Cells were washed twice with PBS and pellets were resuspended in PBS containing 1% serum. Samples were analyzed for FITC on a FACSCalibur (BD) and data was analyzed by FlowJo (TreeStar).

KEGG pathway and Gene Ontology enrichment analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was conducted to identify the biological role of the hits from our screen (PMID: 30321428, PMID: 10592173, PMID: 27899662). All the genes recovered in the second screen were used as background for the enrichment analysis. KEGG pathway analysis revealed that hits from MDE screen were enriched for cell cycle activity, the hits from LIPID screen were mainly involved in TCA cycle and oxidative phosphorylation, and the hits from DIFF screen were enriched for insulin signaling, PI3K-AKT, mTOR, and FOXO pathways. Genes visualized in white were not tested in the second screen, genes visualized in grey were tested but did not score as significant hits in MDE, LIPID, or DIFF, and genes visualized in light red were tested and scored as significant (adjusted $P<0.05$) in one or more of the analyses (MDE, LIPID, DIFF).

Gene Ontology enrichment for biological processes was performed on all significant DIFF hits ($q<0.05$) with a background of all genes analyzed in the second screen. Panther Pathway Analysis was performed on hits from the neddylation inhibitor proteomics dataset. 3T3-L1 cells in triplicates were treated with 10µM NAE inhibitor MLN4924 for 14 hours. Hits were defined as having a peptide count in at least 3 out of the 6 samples and at least a 2 fold increase in expression in the presence of the inhibitor.

Combining public RNA expression data sets

Publicly available RNAseq data sets (Ahmed and Kim, 2019) were analyzed. Data sets used are available: http://bioconductor.org/packages/release/data/experiment/vignettes/curatedAdipoRNA/inst/doc/curatedadiporna_vignette.html

Briefly, low mean 'per base sequence quality' samples (< 34) were filtered out, and samples for 4 time points (0, 24, 28 and 96 hrs) were picked for further analysis. Low expression genes that did not meet the criteria of (count > 10 in at least 2 samples) were filtered out. The resulting dataset was analyzed by DESeq2 using time and study name as variables. Genes with log2-fold change > 1 and adjusted p-value < 0,05 were considered significant.

Proteomics analysis

3T3-L1 cells were cultured and differentiated as described above. Where noted, samples were treated with 10µM NAE inhibitor MLN4924 for 14 hours. At described time points, cells were washed twice with PBS and then collected using a cell scraper in PBS. Samples were centrifuged and pellets were flash-frozen in liquid nitrogen. Pellets were thawed and resuspended in 500µl freshly made Cell Lysis Buffer (50mM HEPES pH 8.5, 75mM NaCl, 8M Urea, and protease and phosphatase inhibitors (ThermoFischer, A32961). Cells were lysed using QIAshredder columns (Qiagen, 79656) at 5000rpm for 1 min at 4°C and clarified by centrifugation at 15,000rpm for 10min at 4°C. Protein concentration of supernatant was quantified using a BCA protein assay kit (ThermoFischer, 23225). 20µg of each lysate were reduce using 5mM TCEP (ThermoFischer, PG82080) for 25min at room temperature and alkylated using 10mM CAA (freshly made, Sigma, C0267) for 30min at room temperature in the dark, followed by quenching using 15mM DTT for 15min at room temperature in the dark. Samples were precipitated by adding lysis buffer to final volume of 100µl, adding 100µl methanol and vortexing, 50ul chloroform and vortexing, and 100µl of water (for UHPLC; Sigma, 900682), followed by centrifugation at 14,000*g* for 3 min at 4°C. Methanol was removed followed by speed-vac to dryness. Samples were resuspended in 50µl of 200mM HEPES pH 8.5 for a final protein concentration of 0.4µg/µl. Samples were digested overnight by adding 0.2µg LysC and Trypsin (Promega, V5073; 10x stock was supplemented with 1% ProteaseMax (Promega, V2072) and Ammonium bicarbonate) and shaking at 250rpm at 37°C. Samples were acidified in TFA (ThermoFischer, L06374-AC) to reach pH 2 to stop digestion. Samples were cleaned up using C18 stagetips. Briefly, 5 layers of C18 (Empore, 320907D) were punched out with 0.9mm needle and packed into a 200µl pipette tip, resin was activated with 50µl methanol, followed by centrifugation at 2000g for 2min at 4°C. Stagetip was washed three times with 150 µl 5% acetonitrile (ACN; Sigma, 34998) 0.5% TFA, centrifuge 2000g for 2min at 4ºC. Samples were bound by adding to C18 column and centrifuging at 500g for 5min at 4ºC. Samples were washed twice with 150 µl 5% ACN 0.5% TFA and centrifugation at 2000g for 2min at 4ºC. Samples were eluted with 25 µl 50% ACN 0.1% Formic Acid (FA; ThermoFischer A117-50) and centrifugation at 2000g for 2min at 4ºC. Elution was repeated two more times followed by speed-vac. Peptides were resuspended in 20ul Solution A (2% CAN, 0.1% FA in water for UHPLC) and quantified using Quantitative Colorimetric Peptide Assay (ThermoFischer, 23275).

Samples were diluted to 100ng/µl and 200ng of sample was analyzed using the timsTOF Pro (Bruker Daltonics), an ion-mobility spectrometry quadrupole time of flight mass spectrometer. Specifically, a nanoElute (Bruker Daltonics) high pressure nanoflow system was connected to the timsTOF Pro. Peptides were delivered to the sample trap (5 cm x 300µm i.d., ThermoFisher Scientific, 160454) in line with a reversed phase analytical columns (25 cm x 75 µm i.d., IonOpticks, AUR2-25075C17A-CSI) with pulled emitter tips. Liquid chromatography was performed at 45 °C and peptides were separated on the analytical column using a 120 min gradient (solvent A: 2% ACN, 0.1% FA; solvent B: 0.1% FA, in ACN) at a flow rate of 400 nl/min. A linear gradient from 2-15 % B was applied for 60 min, followed by 15-23 % B for the next 30

min and followed by a step to 35% B for 10 min and a step to 80% B for 10 min followed by 10 min of washing at 80% B. The timsTOF Pro was operated in PASEF mode with the following settings: Mass Range 100 to 1700m/z, 1/K0 Start 0.6 V·s/cm2, End 1.6 V·s/cm2, Ramp time 100ms, Lock Duty Cycle to 100%, Capillary Voltage 1600V, Dry Gas 3 l/min, Dry Temp 200°C, PASEF settings: 10 MS/MS, charge range 0-5, active exclusion for 0.4 min, Scheduling Target intensity 20000, Intensity threshold 2500, CID collision energy 59eV.

Raw data files from Bruker timsTOF Pro were analyzed using Byonic software (Protein Metrics, Inc) to identify peptides and proteins with the following settings: precursor mass tolerance 20 ppm, fragment mass tolerance: 40 ppm using QTOF/HCD fragmentation type. Fully specific tryptic digest with maximum 2 missed cleavages were specified. The following modifications were searched for the differentiation time course: carbamidomethyl (fixed), Met oxidation, Ser/Thr/Tyr phosphorylation and Asn/Gln deamidation (common), N-term acetylation and Gln/Glu to pyro-Glu conversion (rare) with total common max = 2 and total rare max = 1. The following modifications were searched for the neddylation inhibition experiment: carbamidomethyl (fixed), Met oxidation, Ser/Thr/Tyr phosphorylation and Asn/Gln deamidation (common), N-term acetylation and GlyGly (for ubiquitin modification, neddylation, ...) and ValGly (Ufm1 modification) modifications on Lys (rare) with total common max = 2 and total rare max = 1. We used a mouse refseq proteome library (June 2018) supplemented with common contaminants. To estimate false discovery rates, we applied a target-decoy library strategy using reverse sequences with FDR set at 1%. For differential expression analysis we used the msmsTests package.

Heatmap and Clustering

The $\log_2$ values of protein ratios were normalized by z-normalization among different time points. The z-normalized proteins were imported in Perseus (PMID: 27348712) and hierarchical clustering analysis was performed for proteins (rows). K-means clustering of all quantified proteins across the timespan resulted in 10 clusters. For clustering, we adopted an average linkage method, and also the Euclidean distance to compute pairwise distances. The profile plots of ten clusters with distinct behavior were generated in Perseus with the y-axis representing the z-score and the x-axis representing the time point. The colors represent the distance from the center with red being closer to the center of the trend and green being more far from the center.

Each of the ten protein clusters was categorized in 3 sub-clusters based on the regulation pattern of their corresponding RNAs.

Sample preparation and immunoblot

Cells were lysed in 1x LDS buffer containing DTT and incubated at 95C for 5min. Proteins were separated using NuPage Novex 4-12% Bis-Tris protein gels (Thermo Fisher Scientific, WG1402BOX) in NuPage MOPS SDS running buffer (50 mM MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7), followed by transfer onto nitrocellulose membranes (Life Technologies, LC2001) in Towbin Buffer (25 mM Tris, 192 mM glycine, pH 8.3) containing 10% methanol. Membranes were blocked in LI-COR Odyssey Blocking Buffer (LI-COR, NC9232238) for 30 min at room temperature, followed by incubation with primary antibody in blocking buffer for at least 1h at room temperature. The membrane was washed 3 times for 10min in TBST buffer (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.5) at room temperature, incubated with secondary IRDye antibodies (LI-COR) in blocking buffer for 30 min at room temperature, and

then washed 3 times for 10min in TBST buffer. Membranes were scanned on an Odyssey CLx Imaging System (LI-COR), with protein detection at 680 and 800 nm. Antibodies were used as follows: pIRS1 (Cell signaling 2381, 3203, 5610, 1:1000), pAKT (Cell signaling, 4051, 1:1000), Tubulin (Sigma, 9026, 1:5000).

Immunofluorescence and Lipid staining

Cells were grown on acid washed 12mm round coverslips and fixed with 4% paraformaldehyde (433689M, AlfaAesar) in PBS at room temperature for 10min. Samples were blocked with 5% normal donkey serum (017-000-121, Jackson ImmunoResearch) in IF buffer (3% BSA and 0.1% NP-40 in PBS) at room temperature for 30min. Samples were incubated with primary antibody in IF buffer at room temperature for 1h, followed by 5 washes with IF buffer. Samples were incubated with fluorescent-labeled secondary antibody at room temperature for 30min, followed by a 5 min incubation with 4',6-diamidino-2-phenylindole (DAPI) in PBS at room temperature for 5min and 5 washes with IF buffer. Coverslips were mounted with Fluoromount-G (0100-01, SouthernBiotech) onto glass slides followed by image acquisition. Antibodies were used as follows: Anti-Perilipin-1 antibody (ab61682, 1:100).

To confirm fatty acid composition upon treatment with NAE inhibitor MLN4924, 3T3-L1 cells were plated onto coverslips and grown to confluency. Cells were treated with DMSO control or NAE inhibitor MLN4924 as well as methanol control or 10µM BODIPY C12-fatty acid (Invitrogen, D3822) for 32 hours. Samples were fixed in 4% paraformaldehyde in PBS at room temperature for 10min and stained with DAPI in PBS at room temperature for 5min. Coverslips were mounted with Fluoromount-G onto glass slides followed by image acquisition.

To stain lipids using BODIPY or LipidTOX, cells on coverslips were fixed in 4% paraformaldehyde in PBS at room temperature for 10min and stained with LipidTOX Deep Red Neutral Lipid Stain (ThermoFisher, H34477, 1:200), BODIPY 493/503 (ThermoFisher, D-3922, 20uM), and 1µg/ml DAPI in PBS at room temperature for 30min. Coverslips were mounted with Fluoromount-G onto glass slides followed by image acquisition.

Epi-fluorescence and confocal imaging

Images were acquired on an Everest deconvolution workstation (Intelligent Imaging Innovations) equipped with a Zeiss AxioImager Z1 microscope and a CoolSnapHQ cooled CCD camera (Roper Scientific) and a 40x NA1.3 Plan-Apochromat objective lens (420762-9800, Zeiss) was used.

Confocal images were acquired on a Marianas spinning disk confocal (SDC) microscopy (Intelligent Imaging Innovations).

Isolation of primary preadipocytes

Inguinal or epididymal white adipose tissue was removed and minced. Minced tissue was incubated in Collagenase Buffer (3,000 U/ml type II collagenase powder (Sigma, C6885), 100 U/ml DNase (Worthington, LS006344), 1 mg/ml poloxamer 188 (P-188) (Sigma P5556), 1 mg/ml BSA, 20 mM HEPES buffer, and 1 mM $CaCl_2$ in Medium 199 with Earle's salts (Sigma, M4530)) for 10min at 37C, followed by 20min shaking (250rpm) at 37C. Digested samples were strained through a 100µm filter and diluted 1:1 in cell suspension buffer (2% FBS, 1 mg/ml P-188, and 1% pen/strep in PBS), followed by centrifugation (1300rpm, 5min).

For isolation of SVF cells depleted for RBCs and WBCs, the cell pellet was resuspended in cell suspension buffer and layered onto Histopaq-1077 (Sigma, 10771), followed by 20min RT centrifugation at 2000rpm. The cell layer was removed and centrifuged (5min, 1300rpm), and cell pellet was resuspended in cell suspension buffer containing anti-CD45 microbeads (Miltenyi Biotec, 130-052-301) and anti-TER119 microbeads (Miltenyi Biotec, 130-049-901) for 30min at 4C, followed by MACS depletion using an LD column. Flow-through was collected and centrifuged (1300rpm, 5min). Cell pellet containing primary preadipocytes were resuspended in DMEM containing 10% FBS, 1% Pen/Strep, and 1% GlutaMAX.

Oil Red O staining and quantification

Cells are fixed in 4% PFA/PBS for 10min at room temperature, followed by 3 rinses in PBS. Samples were incubated in 60% isopropanol for 5min at room temperature and then allowed to dry completely. Samples are then incubated in freshly diluted 60% Oil Red O staining solution in water (stock is 0.5% Oil Red O (Sigma, 00625) in isopropanol) for 20min at room temperature, followed by 3 rinses in water. Samples were allowed to dry completely and imaged. To quantify, Oil Red O was extracted by incubating dried samples stained on the same day in 100% isopropanol for 5min at room temperature and absorbance was measured at 510nm.

Live Cell Imaging Analysis for lipid accumulation and proliferation

Live imaging for kinetics quantification of adipogenesis and for proliferation assay was performed using the IncuCyte Live Cell Analysis Imaging System (Essen Bioscience) with images acquired every 2h using the 10x objective. 3T3-L1 cells were grown in the presence of indicated concentrations of NAE inhibitor MLN4924, proteasome inhibitor MG132, and DNA replication inhibitor hydroxyurea (HU). Proliferation was assessed using a confluency mask generated by the IncuCyte Zoom Analysis Software using phase images. For the kinetic quantification of adipogenesis, 3T3-L1 cells were differentiated as described above or supplemented with inhibitors as described above and supplemented with 2µM BODIPY 493/503. Green fluorescence images were acquired every 2h using default IncuCyte setting. Total green fluorescence intensity was determined from a green fluorescent mask generated by the IncuCyte Zoom Analysis Software.

Glucose uptake assay

3T3-L1 cells were plated in a 96 well optical bottom plate and grown to confluency. Cells were treated with DMSO control or 10µM NAE inhibitor MLN4924 for indicated time periods in the presence of 100µM 2-NBDG (Cayman, 11046) or ethanol control. Cells were imaged using the IncuCyte Live Cell Analysis Imaging System (Essen Bioscience) after 2 PBS washes using default IncuCyte settings to quantify total green fluorescence intensity from a green fluorescent mask generated by the IncuCyte Zoom Analysis Software.

Lipogenesis assay

AML12 hepatocytes were washed with warm PBS twice and starved in serum-free DMEM/F12 overnight. Indicated concentrations of insulin with or without NAE inhibitor MLN4924 was added at the same time. After 24 hours incubation, a mixture of 10 µM cold acetate and 2 µCi 3H-Acetate (#NET003H005MC, PerkinElmer) was added to each well and cells were incubated for another 4 hr. Cells were washed with PBS twice and lysed using 0.1 N hydrogen chloride. Lipids were extracted by 2:1 chloroform-methanol (v/v). After 10 min centrifugation at 3000 x g, lower

phase was transferred to scintillation vial and 3H activity was measured by liquid scintillation counting.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical parameters including the statistical test used, exact value of n, what n represents, and the distribution and deviation are reported in the figures and corresponding figure legends. Most data are represented as the mean ± standard deviation and the p-value was determined using two-tailed Student's t tests.

Unless otherwise stated, statistical analyses were performed in Microsoft Excel and GraphPad Prism.

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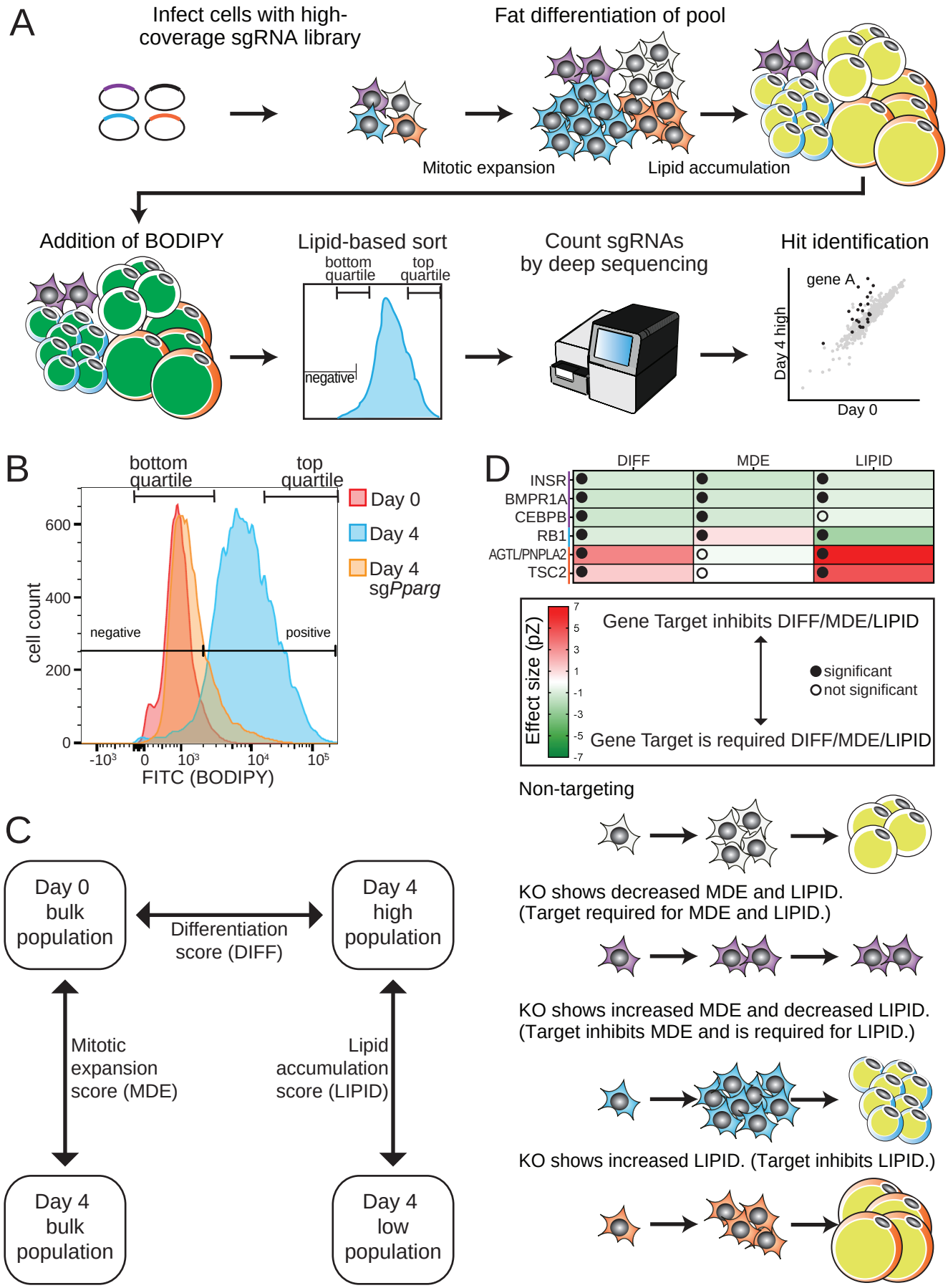

A
Infect cells with high-coverage sgRNA library
Fat differentiation of pool
Mitotic expansion
Lipid accumulation
Addition of BODIPY
Lipid-based sort
bottom quartile
top quartile
negative
Count sgRNAs by deep sequencing
Hit identification
gene A
Day 4 high
Day 0
B
bottom quartile
top quartile
Day 0
Day 4
Day 4 sgPparg
cell count
negative
positive
FITC (BODIPY)
C
Day 0 bulk population
Differentiation score (DIFF)
Day 4 high population
Mitotic expansion score (MDE)
Lipid accumulation score (LIPID)
Day 4 bulk population
Day 4 low population
D
DIFF
MDE
LIPID
INSR
BMPR1A
CEBPB
RB1
AGTL/PNPLA2
TSC2
Effect size (pZ)
Gene Target inhibits DIFF/MDE/LIPID
significant
not significant
Gene Target is required DIFF/MDE/LIPID
Non-targeting
KO shows decreased MDE and LIPID.
(Target required for MDE and LIPID.)
KO shows increased MDE and decreased LIPID.
(Target inhibits MDE and is required for LIPID.)
KO shows increased LIPID. (Target inhibits LIPID.)

Figure 1

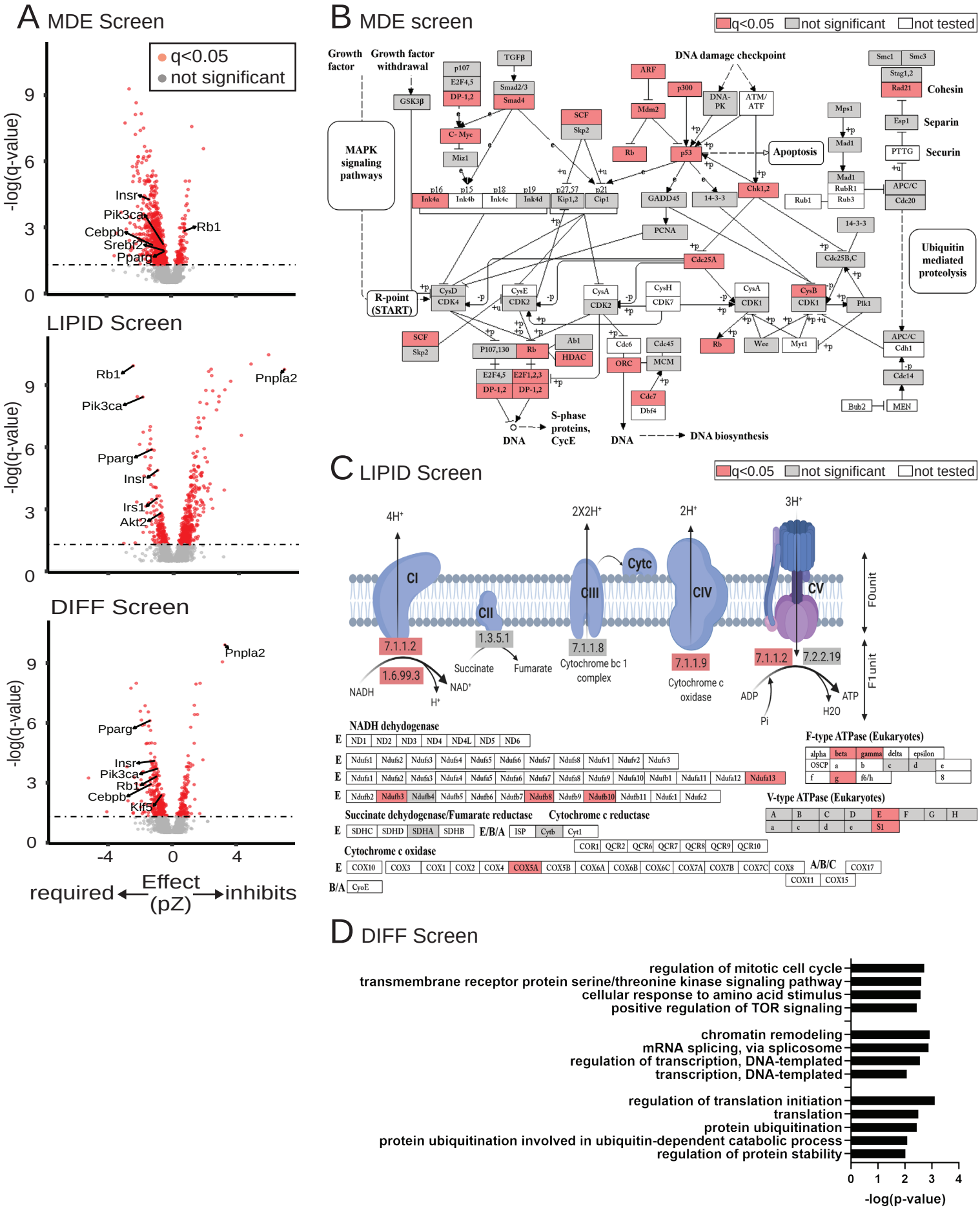

A MDE Screen
q<0.05
not significant
-log(q-value)
Insr
Pik3ca
Cebpb
Srebf2
Pparg
Rb1
LIPID Screen
Rb1
Pik3ca
Pnpla2
Pparg
Insr
Irs1
Akt2
DIFF Screen
Pnpla2
Pparg
Insr
Pik3ca
Rb1
Cebpb
Klf5
-4
0
4
required
Effect (pZ)
inhibits
B MDE screen
q<0.05
not significant
not tested
Growth factor
Growth factor withdrawal
MAPK signaling pathways
DNA damage checkpoint
Cohesin
Separin
Securin
Apoptosis
Ubiquitin mediated proteolysis
R-point (START)
S-phase proteins, CycE
DNA
DNA biosynthesis
C LIPID Screen
q<0.05
not significant
not tested
4H+
2X2H+
2H+
3H+
CI
CII
CIII
CIV
CV
Cytc
FOunit
F1unit
7.1.1.2
1.6.99.3
NADH
NAD+
H+
1.3.5.1
Succinate
Fumarate
7.1.1.8
Cytochrome bc 1 complex
7.1.1.9
Cytochrome c oxidase
7.1.1.2
7.2.2.19
ADP
Pi
ATP
H2O
NADH dehydogenase
F-type ATPase (Eukaryotes)
V-type ATPase (Eukaryotes)
Succinate dehydogenase/Fumarate reductase
Cytochrome c reductase
Cytochrome c oxidase
D DIFF Screen
regulation of mitotic cell cycle
transmembrane receptor protein serine/threonine kinase signaling pathway
cellular response to amino acid stimulus
positive regulation of TOR signaling
chromatin remodeling
mRNA splicing, via splicosome
regulation of transcription, DNA-templated
transcription, DNA-templated
regulation of translation initiation
translation
protein ubiquitination
protein ubiquitination involved in ubiquitin-dependent catabolic process
regulation of protein stability
0
1
2
3
4
-log(p-value)

Figure 2

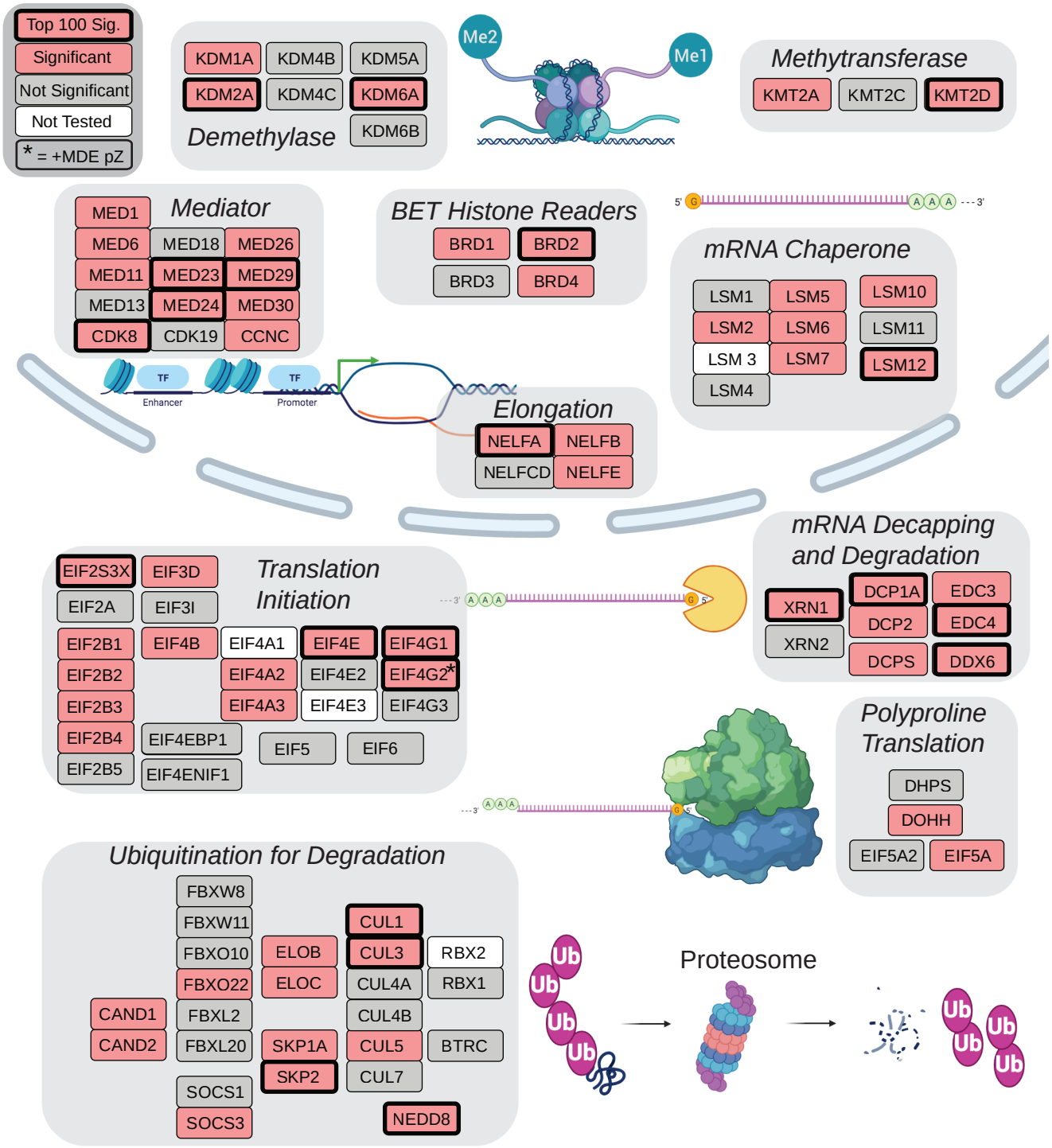

Top 100 Sig.
Significant
Not Significant
Not Tested
* = +MDE pZ
KDM1A
KDM4B
KDM5A
KDM2A
KDM4C
KDM6A
KDM6B
Demethylase
Me2
Me1
Methytransferase
KMT2A
KMT2C
KMT2D
Mediator
MED1
MED6
MED18
MED26
MED11
MED23
MED29
MED13
MED24
MED30
CDK8
CDK19
CCNC
BET Histone Readers
BRD1
BRD2
BRD3
BRD4
mRNA Chaperone
LSM1
LSM5
LSM10
LSM2
LSM6
LSM11
LSM 3
LSM7
LSM12
LSM4
TF
Enhancer
TF
Promoter
Elongation
NELFA
NELFB
NELFCD
NELFE
mRNA Decapping and Degradation
XRN1
DCP1A
EDC3
DCP2
EDC4
XRN2
DCPS
DDX6
Translation Initiation
EIF2S3X
EIF3D
EIF2A
EIF3I
EIF2B1
EIF4B
EIF4A1
EIF4E
EIF4G1
EIF2B2
EIF4A2
EIF4E2
EIF4G2*
EIF2B3
EIF4A3
EIF4E3
EIF4G3
EIF2B4
EIF4EBP1
EIF5
EIF6
EIF2B5
EIF4ENIF1
Polyproline Translation
DHPS
DOHH
EIF5A2
EIF5A
Ubiquitination for Degradation
FBXW8
FBXW11
FBXO10
FBXO22
FBXL2
FBXL20
CAND1
CAND2
SOCS1
SOCS3
ELOB
ELOC
SKP1A
SKP2
CUL1
CUL3
CUL4A
CUL4B
CUL5
CUL7
RBX2
RBX1
BTRC
NEDD8
Ub
Proteosome

Figure 3

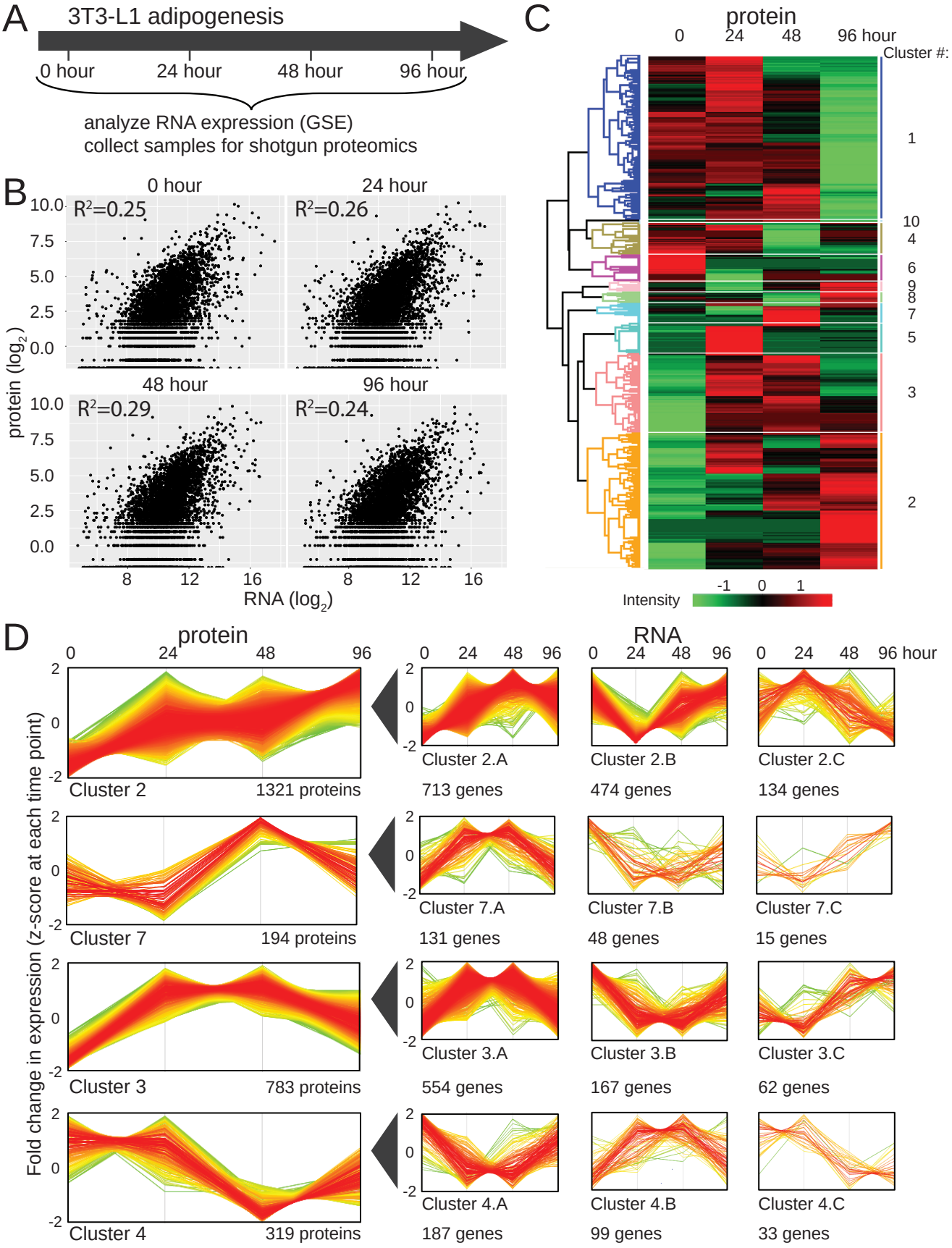
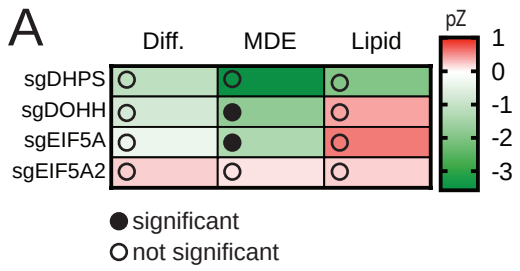
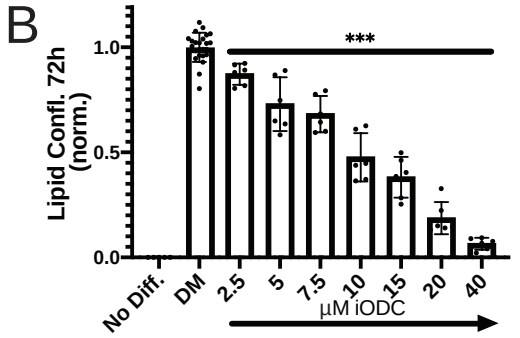
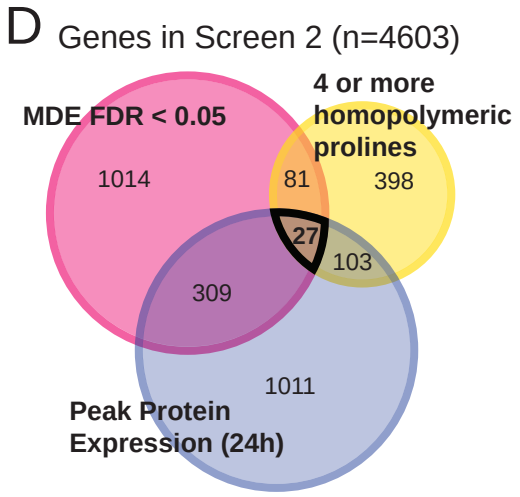
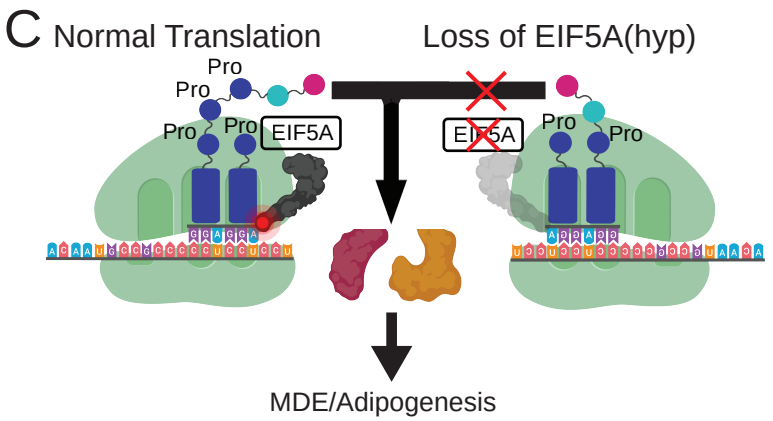

A
3T3-L1 adipogenesis
0 hour
24 hour
48 hour
96 hour
analyze RNA expression (GSE)
collect samples for shotgun proteomics
B
0 hour
24 hour
48 hour
96 hour
R²=0.25
R²=0.26
R²=0.29
R²=0.24
protein (log2)
RNA (log2)
C
protein
0
24
48
96 hour
Cluster #:
1
10
4
6
9
8
7
5
3
2
Intensity
-1
0
1
D
protein
RNA
Fold change in expression (z-score at each time point)
Cluster 2
1321 proteins
Cluster 2.A
713 genes
Cluster 2.B
474 genes
Cluster 2.C
134 genes
Cluster 7
194 proteins
Cluster 7.A
131 genes
Cluster 7.B
48 genes
Cluster 7.C
15 genes
Cluster 3
783 proteins
Cluster 3.A
554 genes
Cluster 3.B
167 genes
Cluster 3.C
62 genes
Cluster 4
319 proteins
Cluster 4.A
187 genes
Cluster 4.B
99 genes
Cluster 4.C
33 genes

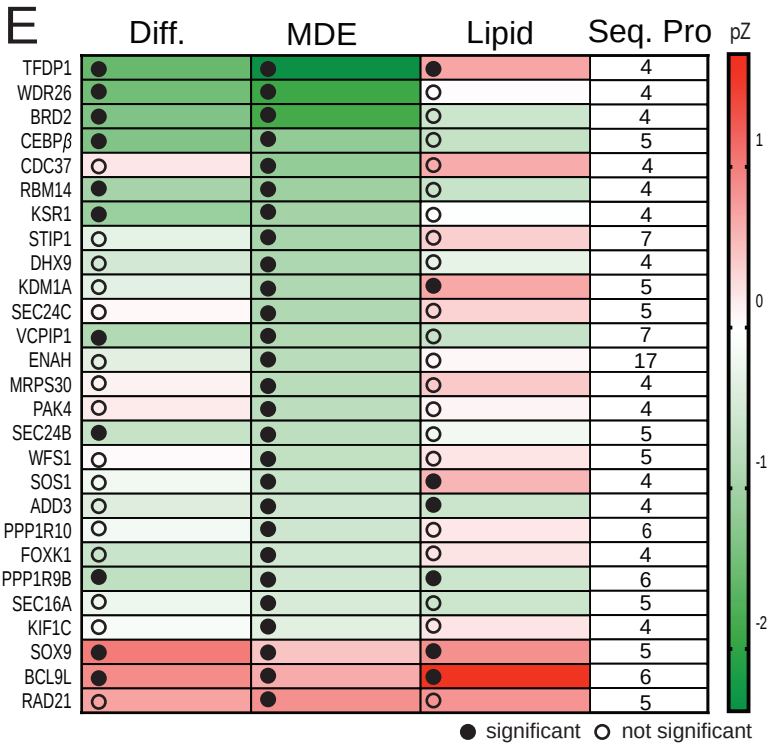
Figure 4


A
Diff.
MDE
Lipid
pZ
1
0
-1
-2
-3
sgDHPS
sgDOHH
sgEIF5A
sgEIF5A2
significant
not significant


B
Lipid Confl. 72h (norm.)
1.0
0.5
0.0
***
No Diff.
DM
2.5
5
7.5
10
15
20
40
µM iODC


D
Genes in Screen 2 (n=4603)
MDE FDR < 0.05
4 or more homopolymeric prolines
Peak Protein Expression (24h)
1014
81
398
27
103
309
1011


C
Normal Translation
Loss of EIF5A(hyp)
Pro
EIF5A
MDE/Adipogenesis


E
Diff.
MDE
Lipid
Seq. Pro
pZ
TFDP1 4
WDR26 4
BRD2 4
CEBPβ 5
CDC37 4
RBM14 4
KSR1 4
STIP1 7
DHX9 4
KDM1A 5
SEC24C 5
VCPIP1 7
ENAH 17
MRPS30 4
PAK4 4
SEC24B 5
WFS1 5
SOS1 4
ADD3 4
PPP1R10 6
FOXK1 4
PPP1R9B 6
SEC16A 5
KIF1C 4
SOX9 5
BCL9L 6
RAD21 5
1
0
-1
-2
significant
not significant

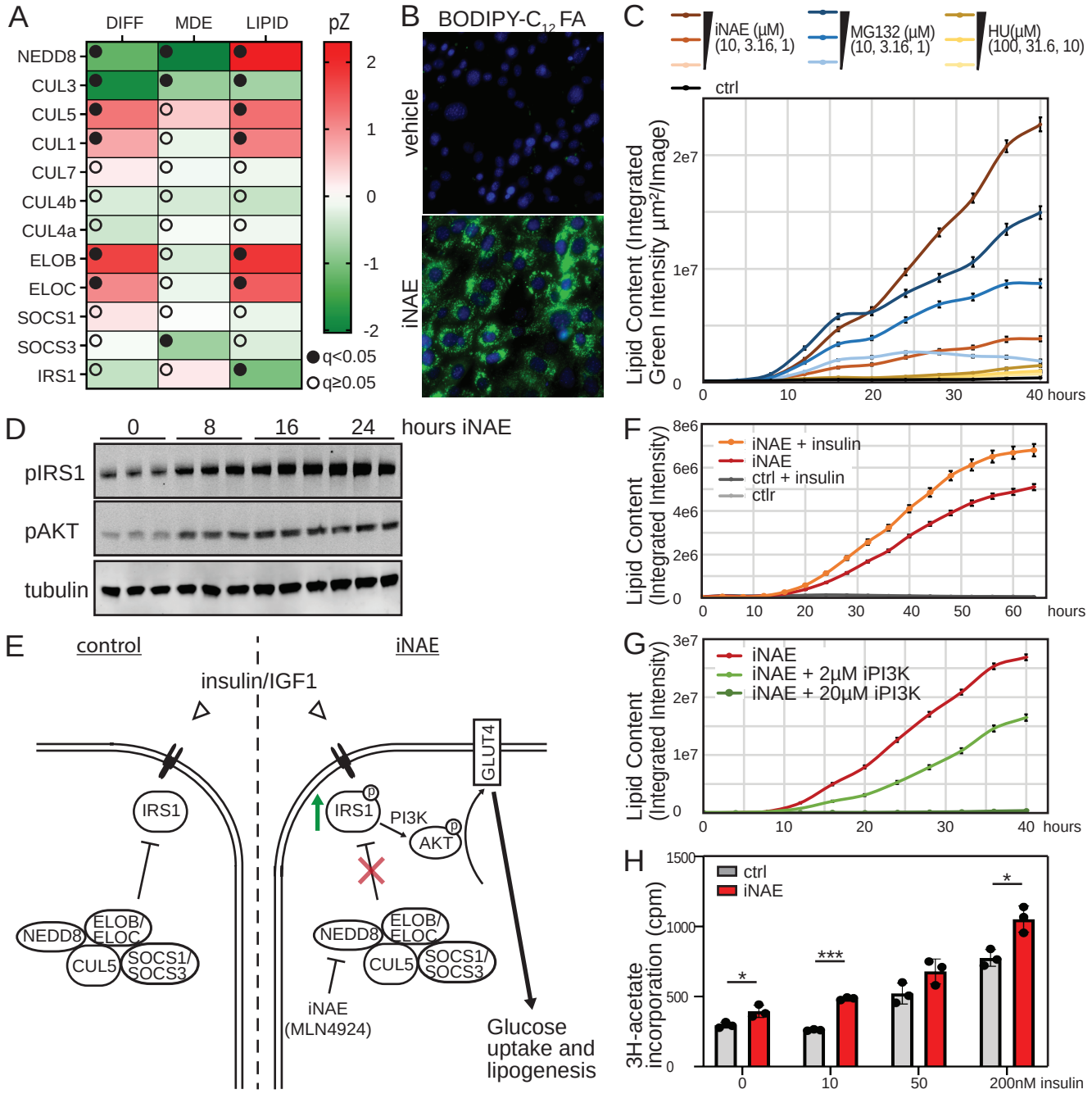
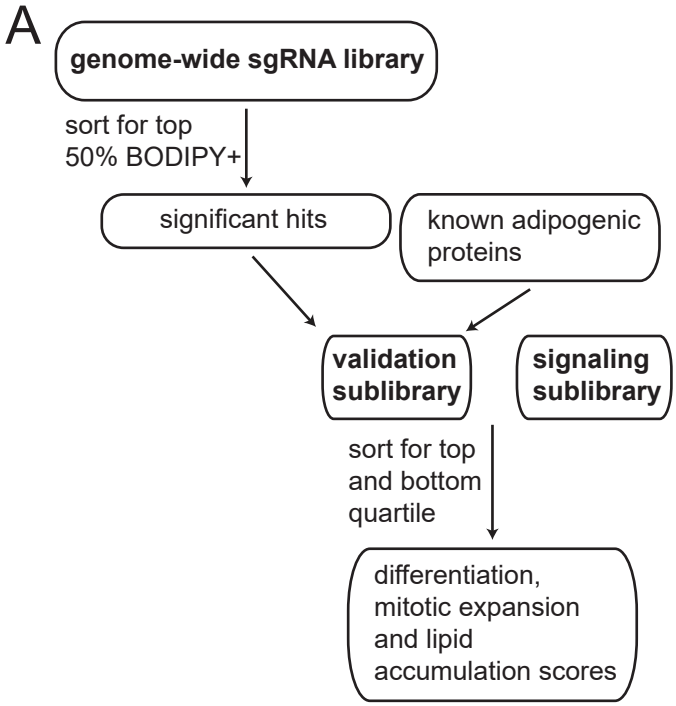
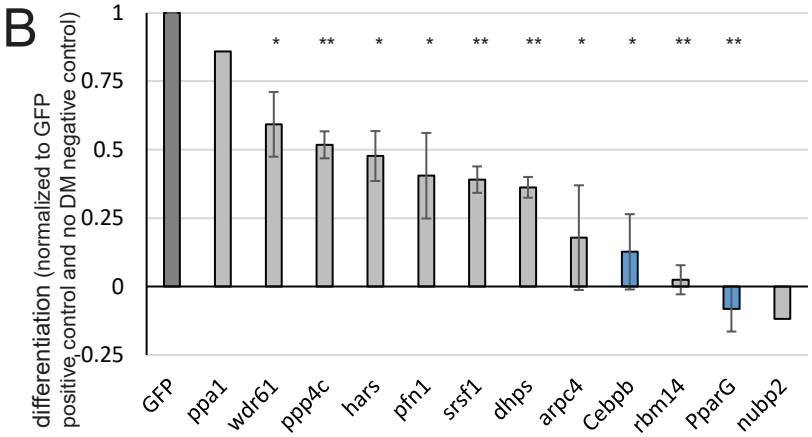
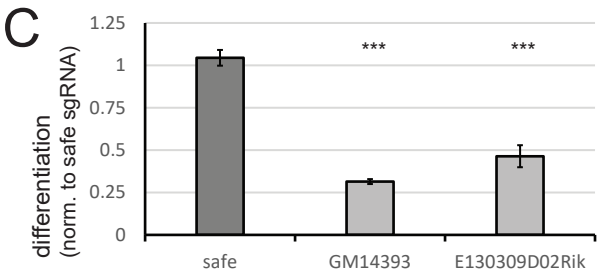

A
DIFF
MDE
LIPID
pZ
NEDD8
CUL3
CUL5
CUL1
CUL7
CUL4b
CUL4a
ELOB
ELOC
SOCS1
SOCS3
IRS1
2
1
0
-1
-2
q<0.05
q≥0.05
B
BODIPY-C12 FA
vehicle
iNAE
C
iNAE (μM) (10, 3.16, 1)
MG132 (μM) (10, 3.16, 1)
HU(μM) (100, 31.6, 10)
ctrl
Lipid Content (Integrated Green Intensity μm²/Image)
2e7
1e7
0
0
10
20
30
40 hours
D
0
8
16
24
hours iNAE
pIRS1
pAKT
tubulin
F
iNAE + insulin
iNAE
ctrl + insulin
ctlr
Lipid Content (Integrated Intensity)
8e6
6e6
4e6
2e6
0
0
10
20
30
40
50
60
hours
E
control
iNAE
insulin/IGF1
IRS1
NEDD8
ELOB/ ELOC
CUL5
SOCS1/ SOCS3
GLUT4
IRS1
p
PI3K
AKT
p
NEDD8
ELOB/ ELOC
CUL5
SOCS1/ SOCS3
iNAE (MLN4924)
Glucose uptake and lipogenesis
G
iNAE
iNAE + 2μM iPI3K
iNAE + 20μM iPI3K
Lipid Content (Integrated Intensity)
3e7
2e7
1e7
0
0
10
20
30
40 hours
H
ctrl
iNAE
3H-acetate incorporation (cpm)
1500
1000
500
0
*
***
*
0
10
50
200nM insulin

Figure 6

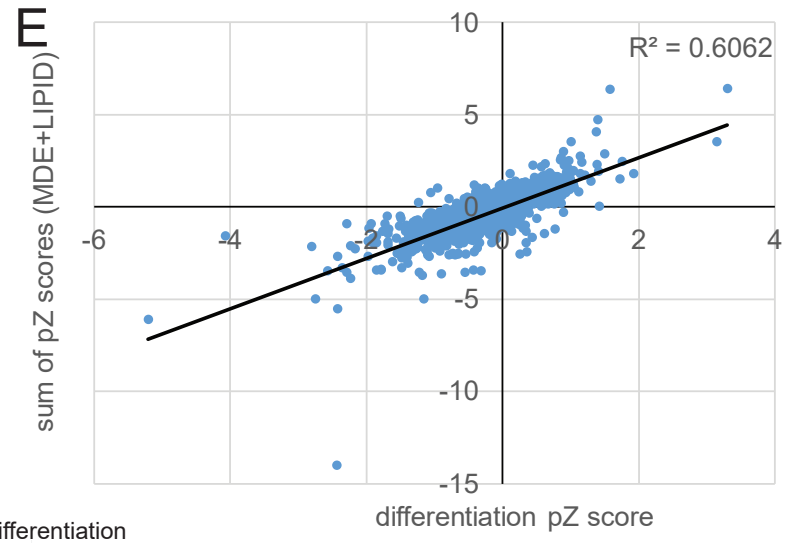






D

	significant hits (q=0.05	overlap with primary hits	enrichment p-value (chi$^{2)}$
primary screen	1494	n/a	n/a
Differentiation score	448	210	<0.0001
MDE score	1014	407	<0.0001
LIPID score	570	238	<0.0001
Total genes analyzed in second screen	*4603*		



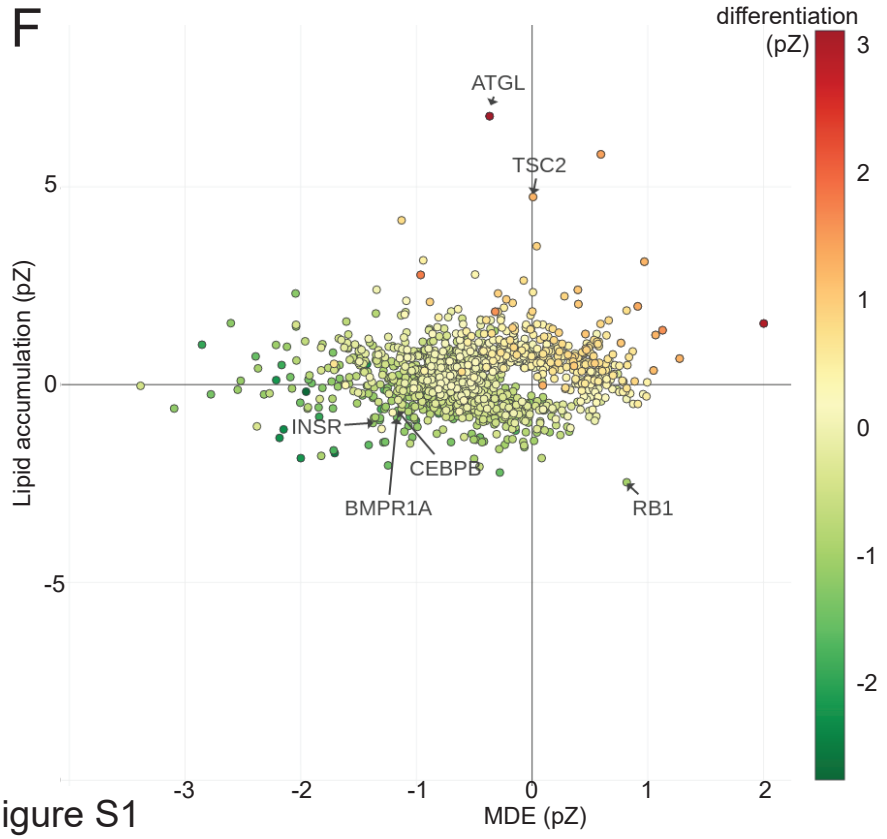


Figure S1

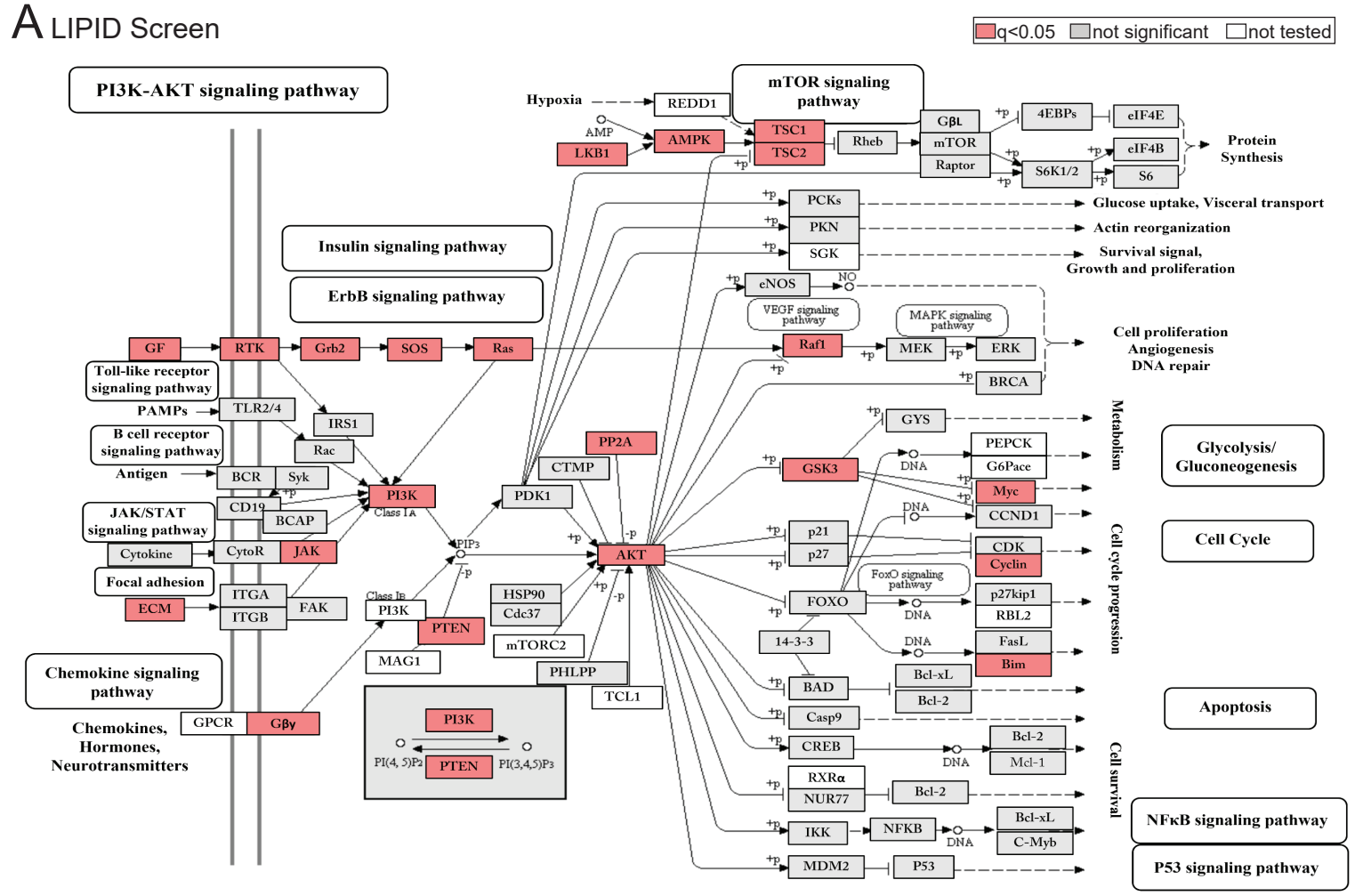
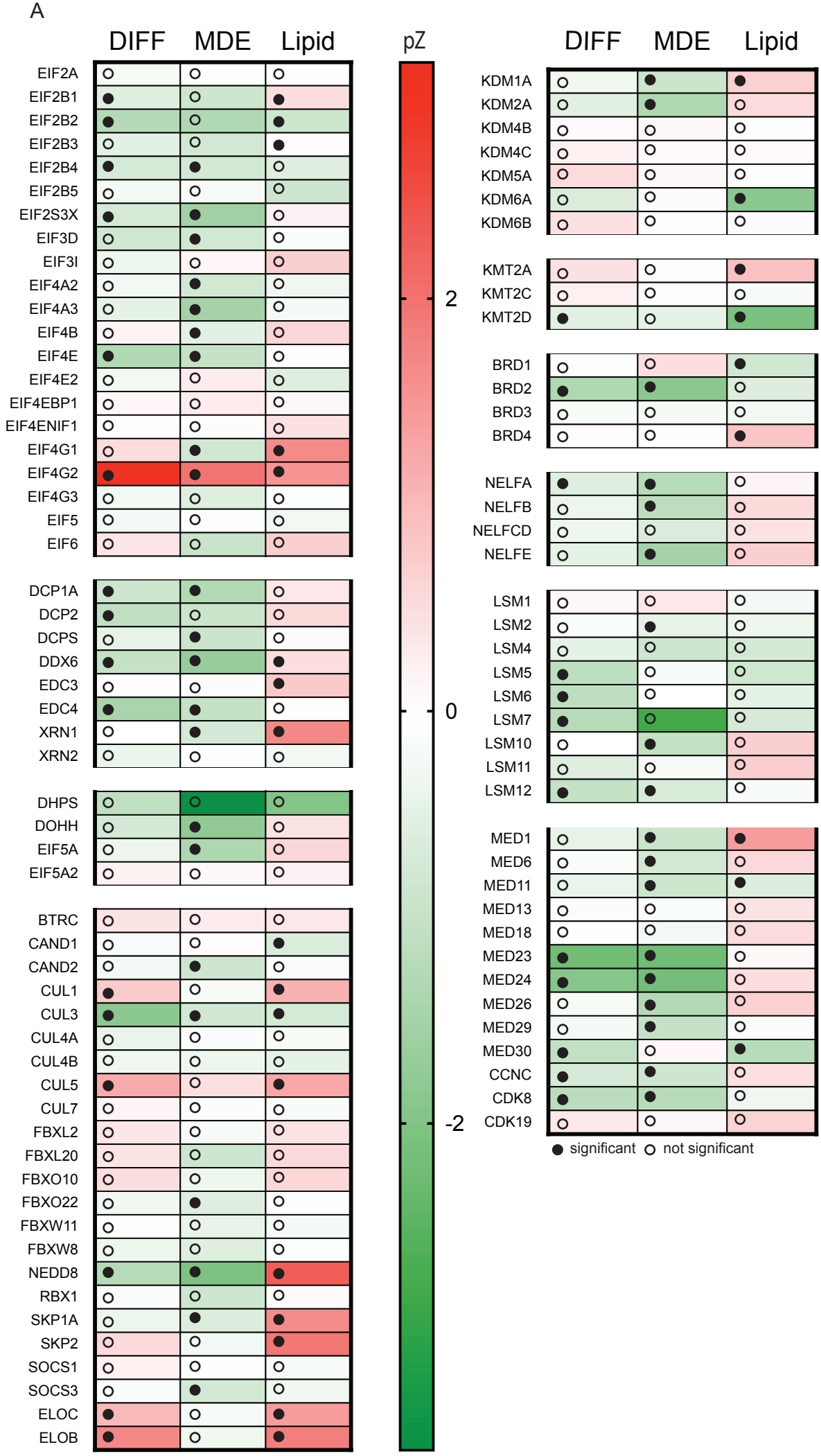
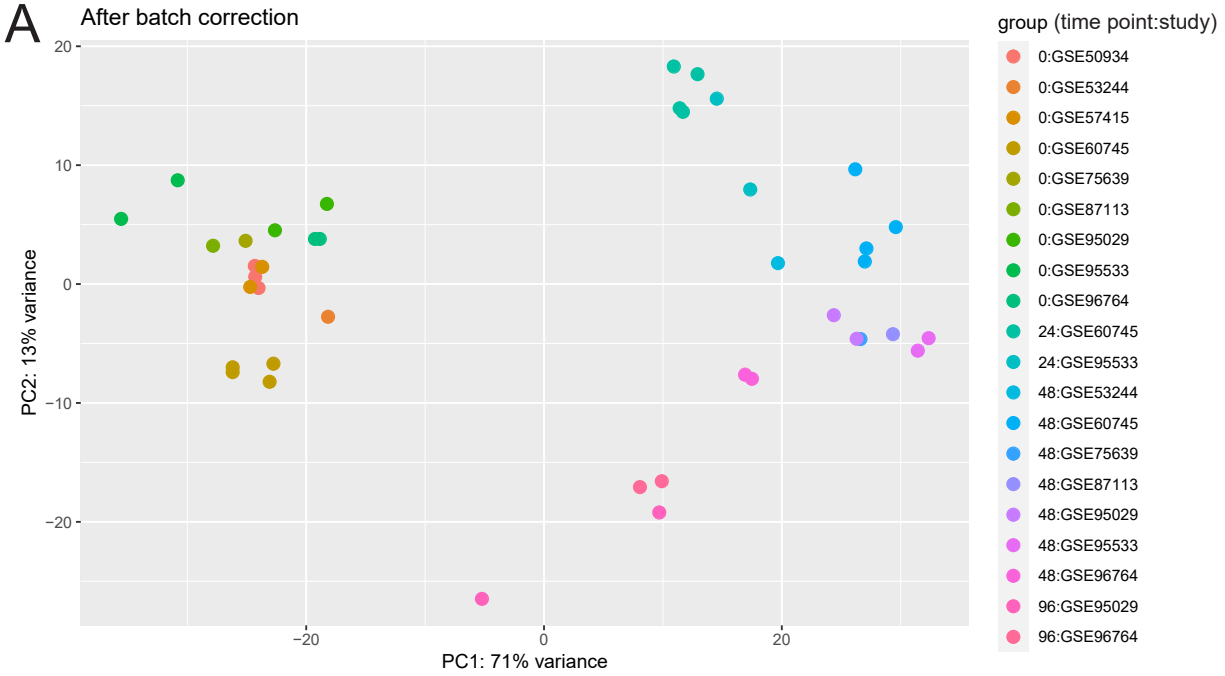

A LIPID Screen
q<0.05
not significant
not tested
PI3K-AKT signaling pathway
mTOR signaling pathway
Hypoxia
REDD1
AMP
LKB1
AMPK
TSC1
TSC2
Rheb
GβL
mTOR
Raptor
4EBPs
eIF4E
eIF4B
S6K1/2
S6
Protein Synthesis
PCKs
PKN
SGK
Glucose uptake, Visceral transport
Actin reorganization
Survival signal, Growth and proliferation
Insulin signaling pathway
ErbB signaling pathway
eNOS
NO
VEGF signaling pathway
MAPK signaling pathway
GF
RTK
Grb2
SOS
Ras
Raf1
MEK
ERK
BRCA
Cell proliferation
Angiogenesis
DNA repair
Toll-like receptor signaling pathway
PAMPs
TLR2/4
IRS1
B cell receptor signaling pathway
Antigen
BCR
Syk
Rac
PI3K
Class IA
CD19
BCAP
JAK/STAT signaling pathway
Cytokine
CytoR
JAK
Focal adhesion
ECM
ITGA
ITGB
FAK
PIP3
PDK1
PP2A
CTMP
AKT
Class IB
PI3K
PTEN
HSP90
Cdc37
mTORC2
MAG1
PHLPP
TCL1
Chemokine signaling pathway
Chemokines, Hormones, Neurotransmitters
GPCR
Gβγ
PI3K
PI(4, 5)P2
PTEN
PI(3,4,5)P3
GYS
Metabolism
GSK3
DNA
PEPCK
G6Pace
Glycolysis/ Gluconeogenesis
Myc
CCND1
p21
p27
CDK
Cyclin
Cell Cycle
Cell cycle progression
FoxO signaling pathway
FOXO
p27kip1
RBL2
14-3-3
FasL
Bim
BAD
Bcl-xL
Bcl-2
Apoptosis
Casp9
CREB
Bcl-2
Mcl-1
Cell survival
RXRα
NUR77
Bcl-2
IKK
NFKB
Bcl-xL
C-Myb
NFκB signaling pathway
MDM2
P53
P53 signaling pathway
B LIPID Screen
q<0.05
not significant
not tested
FOXO signaling pathway
TGF-β signaling
Smad4
TGF-β
TGFβR
Smad3
Smad4
Smad3
Low nutrition (glucose, O2)
ADP
AMP
Energy metabolism
LKB1
Glycolysis/ Gluconeogenesis
Ubiquitin mediated proteolysis
AMPK
Ubiquitination
Citrate cycle/ TCA cycle
JNK
MST1
ROS
Glucose
GLUT4
Oxidative phosphorylation
NLK
SKP2
MDM2
SET9
Acetylated
FOXO
IGF1
IGF1R
Insulin signaling pathway
PTEN
IKKα/β
CBP
SIRT1
INS
INSR
IRS
PI3K
PDK1/2
AKT
FOXO
DNA
PIP3
SGK
FOXO
Deacetylated
Homer
PIKE-L
PI3K-AKT signaling
PRMT1
USP7
Glutamate
mGluR
CK1
IL-6
IL-10
STAT3
CDK2
DNA damage
Jak/STAT signaling pathway
p38
EGF
EGFR
Grb2
SOS
Ras
Raf
MEK
ERK
MAPK signaling
Neuro developmental signal
FOXG1
CyclinB
CyclinD
CyclinG2
p15
p19
p21
p27
Cell cycle
p130
PIK
Gadd45
FasL
Bim
TRAIL
Bcl-6
Apoptosis
BNIP3
Atg12
ATG8
Autophagy
Catalase
Mn-SOD
Gadd45
ATM
Oxidative stress, resistance and DNA repair
PEPCK
G6PC
Metabolism
Glycolysis/ Gluconeogenesis
Bcl-6
IL-7R
KLF2
EDG1/6
RAG-12
Immuno-regulation
Atrogin-1
Muscle autophagy

Figure S2

A


DIFF
MDE
Lipid
pZ
EIF2A
EIF2B1
EIF2B2
EIF2B3
EIF2B4
EIF2B5
EIF2S3X
EIF3D
EIF3I
EIF4A2
EIF4A3
EIF4B
EIF4E
EIF4E2
EIF4EBP1
EIF4ENIF1
EIF4G1
EIF4G2
EIF4G3
EIF5
EIF6
DCP1A
DCP2
DCPS
DDX6
EDC3
EDC4
XRN1
XRN2
DHPS
DOHH
EIF5A
EIF5A2
BTRC
CAND1
CAND2
CUL1
CUL3
CUL4A
CUL4B
CUL5
CUL7
FBXL2
FBXL20
FBXO10
FBXO22
FBXW11
FBXW8
NEDD8
RBX1
SKP1A
SKP2
SOCS1
SOCS3
ELOC
ELOB
2
0
-2
KDM1A
KDM2A
KDM4B
KDM4C
KDM5A
KDM6A
KDM6B
KMT2A
KMT2C
KMT2D
BRD1
BRD2
BRD3
BRD4
NELFA
NELFB
NELFCD
NELFE
LSM1
LSM2
LSM4
LSM5
LSM6
LSM7
LSM10
LSM11
LSM12
MED1
MED6
MED11
MED13
MED18
MED23
MED24
MED26
MED29
MED30
CCNC
CDK8
CDK19
significant
not significant


A
After batch correction
PC2: 13% variance
PC1: 71% variance
20
10
0
−10
−20
−20
0
20
group (time point:study)
0:GSE50934
0:GSE53244
0:GSE57415
0:GSE60745
0:GSE75639
0:GSE87113
0:GSE95029
0:GSE95533
0:GSE96764
24:GSE60745
24:GSE95533
48:GSE53244
48:GSE60745
48:GSE75639
48:GSE87113
48:GSE95029
48:GSE95533
48:GSE96764
96:GSE95029
96:GSE96764

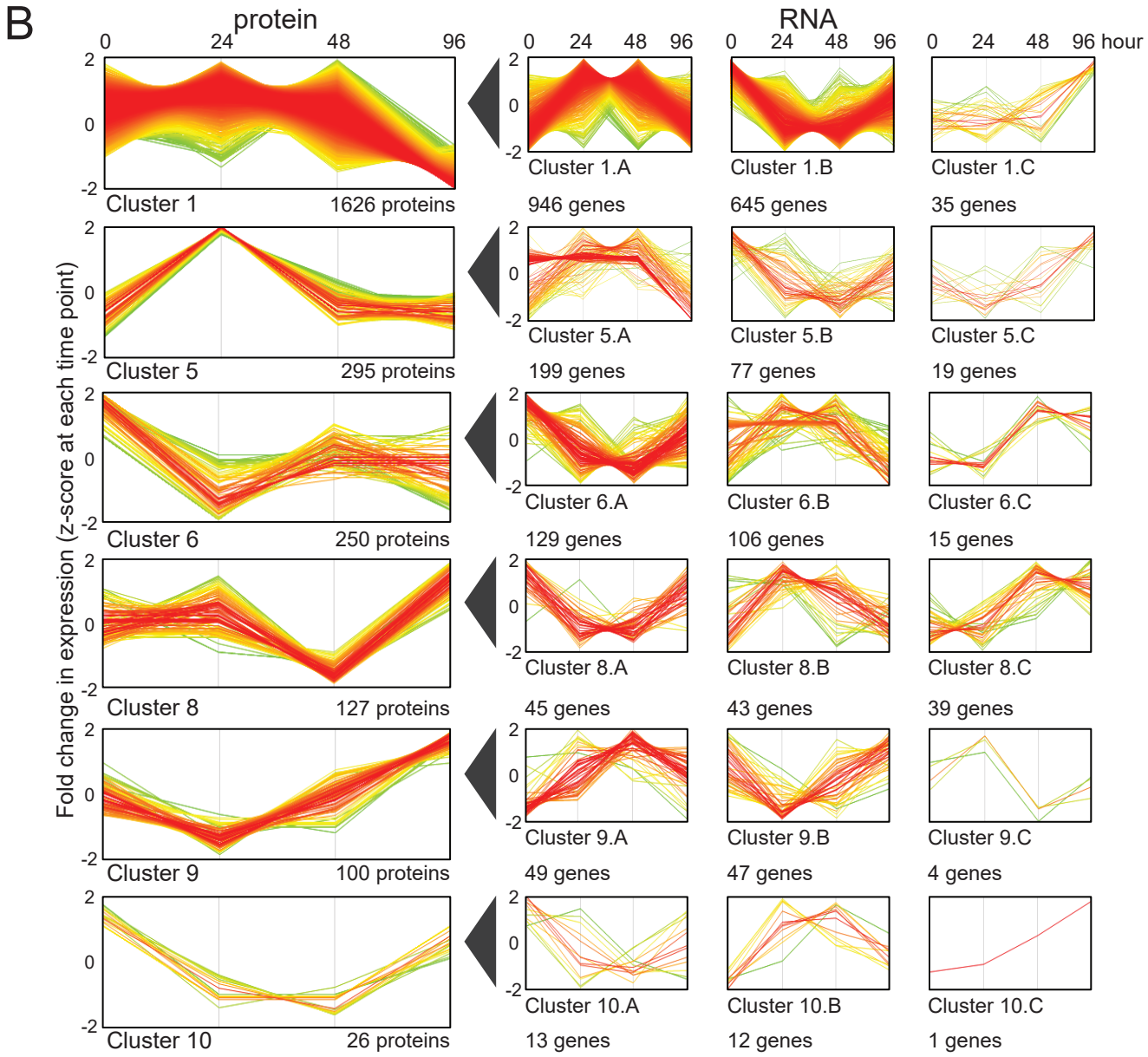
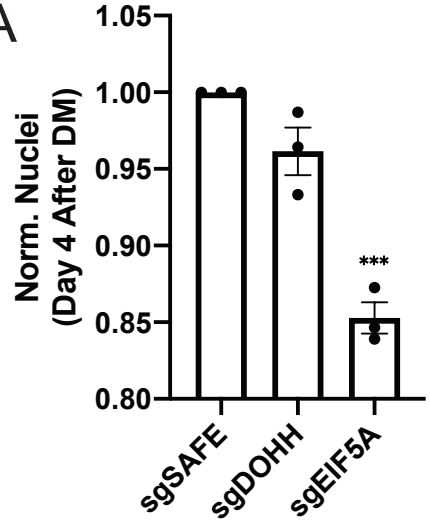
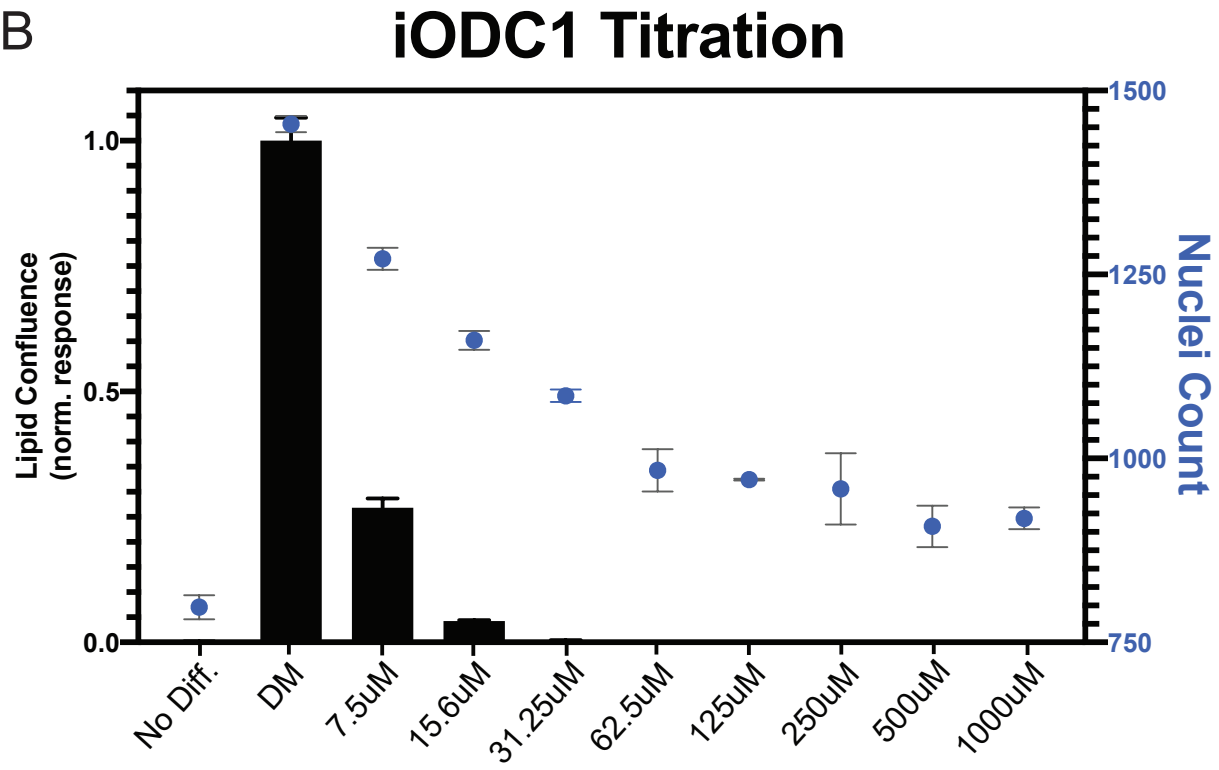

B
protein
RNA
0 24 48 96
0 24 48 96 hour
Fold change in expression (z-score at each time point)
Cluster 1
1626 proteins
Cluster 1.A
946 genes
Cluster 1.B
645 genes
Cluster 1.C
35 genes
Cluster 5
295 proteins
Cluster 5.A
199 genes
Cluster 5.B
77 genes
Cluster 5.C
19 genes
Cluster 6
250 proteins
Cluster 6.A
129 genes
Cluster 6.B
106 genes
Cluster 6.C
15 genes
Cluster 8
127 proteins
Cluster 8.A
45 genes
Cluster 8.B
43 genes
Cluster 8.C
39 genes
Cluster 9
100 proteins
Cluster 9.A
49 genes
Cluster 9.B
47 genes
Cluster 9.C
4 genes
Cluster 10
26 proteins
Cluster 10.A
13 genes
Cluster 10.B
12 genes
Cluster 10.C
1 genes

Figure S4

A


1.05
1.00
0.95
0.90
0.85
0.80
Norm. Nuclei
(Day 4 After DM)
***
sgSAFE
sgDOHH
sgEIF5A

B


iODC1 Titration
1.0
0.5
0.0
Lipid Confluence
(norm. response)
1500
1250
1000
750
Nuclei Count
No Diff.
DM
7.5uM
15.6uM
31.25uM
62.5uM
125uM
250uM
500uM
1000uM

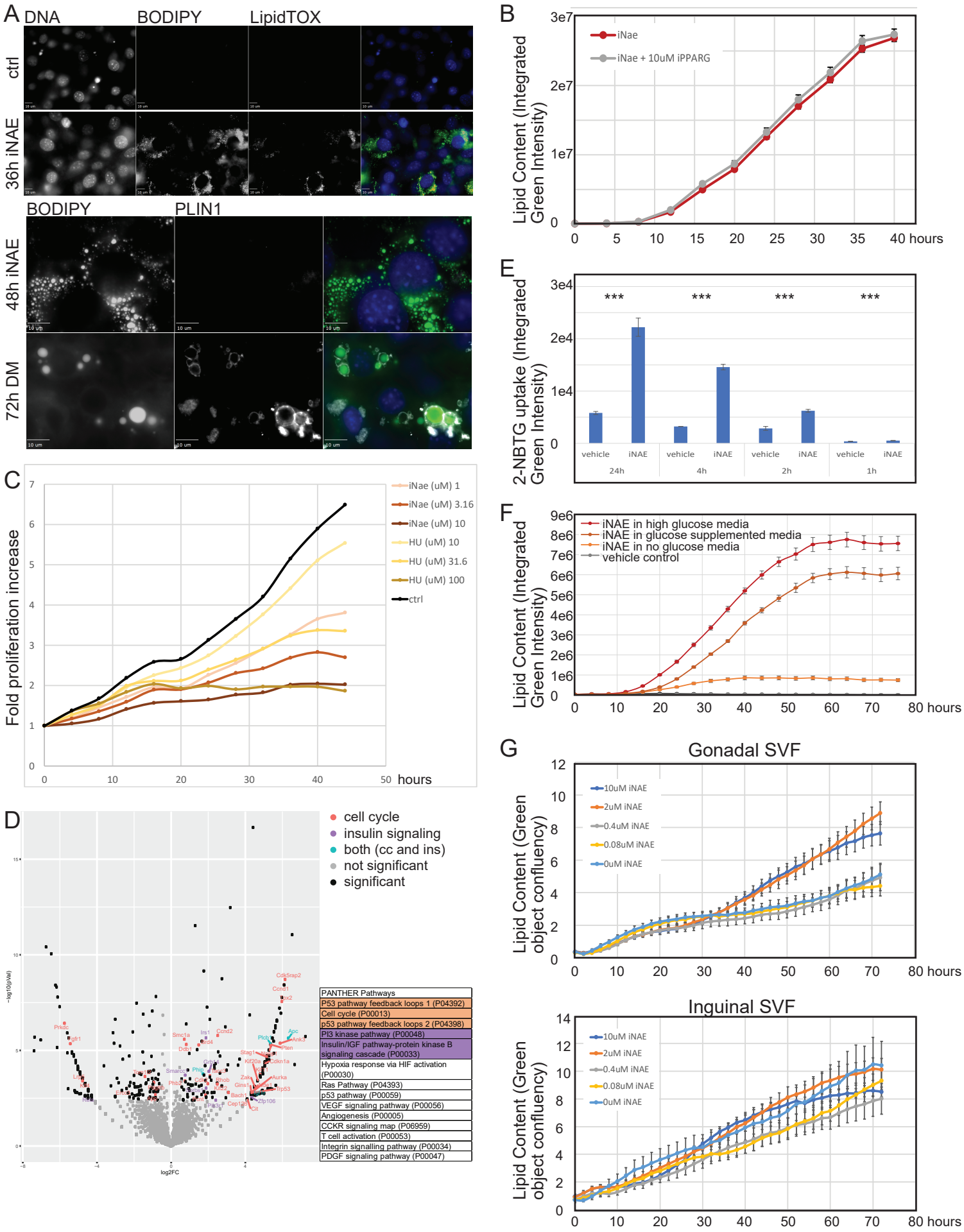

A
DNA
BODIPY
LipidTOX
ctrl
36h iNAE
BODIPY
PLIN1
48h iNAE
72h DM
B
Lipid Content (Integrated Green Intensity)
iNae
iNae + 10uM iPPARG
hours
C
Fold proliferation increase
iNae (uM) 1
iNae (uM) 3.16
iNae (uM) 10
HU (uM) 10
HU (uM) 31.6
HU (uM) 100
ctrl
hours
D
cell cycle
insulin signaling
both (cc and ins)
not significant
significant
PANTHER Pathways
P53 pathway feedback loops 1 (P04392)
Cell cycle (P00013)
p53 pathway feedback loops 2 (P04398)
PI3 kinase pathway (P00048)
Insulin/IGF pathway-protein kinase B signaling cascade (P00033)
Hypoxia response via HIF activation (P00030)
Ras Pathway (P04393)
p53 pathway (P00059)
VEGF signaling pathway (P00056)
Angiogenesis (P00005)
CCKR signaling map (P06959)
T cell activation (P00053)
Integrin signalling pathway (P00034)
PDGF signaling pathway (P00047)
log2FC
E
2-NBTG uptake (Integrated Green Intensity)
vehicle
iNAE
24h
4h
2h
1h
F
Lipid Content (Integrated Green Intensity)
iNAE in high glucose media
iNAE in glucose supplemented media
iNAE in no glucose media
vehicle control
hours
G
Gonadal SVF
Inguinal SVF
Lipid Content (Green object confluency)
10uM iNAE
2uM iNAE
0.4uM iNAE
0.08uM iNAE
0uM iNAE
hours

Figure S6