**RESOURCE**

**Atlas of Circadian Metabolism Reveals System-wide Coordination and Communication between Clocks**

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**Summary**

Metabolic diseases are often characterized by circadian misalignment within and across different tissues. Yet how altered coordination and communication among tissue clocks is related to specific pathogenic mechanisms remains largely unknown. To gain novel perspectives on these relationships we applied an integrated systems biology approach. Performing circadian metabolomics profiling on several different tissues simultaneously, we present a comprehensive temporal and spatial atlas of *in vivo* circadian metabolism in the context of systemic energy balance, and under chronic nutrient stress (high fat diet, HFD). Comparative analysis reveals how the repertoires of tissue circadian metabolism are linked and gated to specific temporal windows under conditions of energy balance. In addition, we have identified and characterized a vast array of common and tissue-specific metabolite alterations elicited by HFD. Our study reveals highly specialized communication and coherence among tissue clocks, and their rewiring in the context of nutrient challenge. Overall, we illustrate how dynamic metabolic relationships can be reconstructed across time and space, and how integration of circadian metabolomics data from multiple tissues can improve our understanding of health and disease.

**Keywords**

Circadian, metabolism, metabolites, CircadiOmics, high fat diet, metabolomics

**Introduction**

The importance of circadian clocks in determining human health or disease is supported by a wealth of epidemiological evidence (Ball et al., 2016; Drake et al., 2004; Kawachi et al., 1995; Knutsson, 2003; Parkes, 2002; Scheer et al., 2009; Schernhammer et al., 2001; Sharifian et al., 2005; Sigurdardottir et al., 2012). Multiple mouse models of total and tissue-specific circadian clock disruption have likewise revealed a close association between circadian dysfunction and metabolic diseases (Lowrey and Takahashi, 2011).

Cells and tissues must compartmentalize metabolic pathways within precise temporal and spatial windows in order to orchestrate the complex set of biochemical reactions required for life (Tu and McKnight, 2006). The circadian clock plays a central role in this process by directly regulating the rhythmic transport and metabolism of various metabolites, as well as the release and tissue-specific sensitivity to various oscillating hormones. Metabolism likewise impinges upon the circadian system, and several recent studies have demonstrated that specific metabolic pathways (i.e. polyamines or the pentose phosphate pathway) are key regulators of circadian clocks (Rey et al., 2016; Zwighaft et al., 2015). Furthermore, oscillating metabolites synchronize central and peripheral tissue clocks, modulate transcriptional activity of core clock proteins, and regulate chromatin accessibility (Aviram et al., 2016; Nakahata et al., 2009; O'Neill et al., 2008; O'Neill et al., 2011; Peek et al., 2013; Ramsey et al., 2009). As such, metabolism and the circadian clock are inextricably linked (Asher and Sassone-Corsi, 2015). Extending these concepts to organism-wide energy balance, clocks across tissues are thought to be coordinated and in temporal alignment under conditions of energy balance, while disruption of tissue communication is thought to increase the risk of metabolic diseases (Roenneberg et al., 2012; Roenneberg and Merrow, 2016).

Since metabolites lie at this important interface between circadian clocks and metabolism and serve as a means of communication among tissues (Dyar and Eckel-Mahan, 2017), they are prime candidates for investigation. Metabolites are currently used as surrogate diagnostic and predictive biomarkers to define health or pathology, but their origins and precise associations with the diseases they describe are not satisfactorily explored (DeBerardinis and Thompson, 2012). While blood metabolite profiling is common, circadian metabolite crosstalk between tissues, and how blood metabolite signatures correlate with tissue-specific circadian function or dysfunction remains largely undefined. Chronic metabolic diseases, such as type II diabetes mellitus (T2DM), and cardiovascular diseases can take years to develop, and impact multiple tissues. The initial site of metabolic derangement may thus lie distal to the specific biomarker used, complicating any real understanding of disease cause and progression, and any subsequent development of effective therapeutic interventions. Multi-tissue metabolite comparisons may thus be extremely helpful in providing additional context and perspective that has previously been missing.

With the advent of high-throughput metabolomics, it is now possible to map metabolite dynamics across the diurnal cycle at the level of organelles (Aviram et al., 2016; Chen et al., 2016), cultured cells, and different tissues (Dallmann et al., 2012; Davies et al., 2014; Dyar et al., 2014; Eckel-Mahan et al., 2012; Fustin et al., 2012; Giskeodegard et al., 2015; Kim et al., 2014; Krishnaiah et al., 2017; Martinez-Lozano Sinues et al., 2014; Masri et al., 2014). A very limited number of studies have combined metabolomics data from more than one tissue under healthy or diseased states (Lu et al., 2017; Sugimoto et al., 2012) (see http://mmmdb.iab.keio.ac.jp/), and circadian metabolomics data from many important metabolic tissues, whether healthy or diseased, is currently lacking. Integration of circadian metabolomics data from multiple tissues is thus poised to push this type of analysis not only beyond simple diagnostics and biomarker detection, but towards a deeper understanding of how metabolic pathways are related within and across tissues in health and disease (Brown, 2016; Goodacre, 2007).

To begin constructing a systems-level view of circadian metabolism, we have performed and assembled global circadian metabolite profiling across 8 different tissues under conditions of energy balance (standard chow) or chronic nutrient stress (high fat diet). We show how metabolic pathways are gated to specific temporal and spatial windows in a tissue-specific manner. Mapping temporal metabolite correlations both within and across tissues, we have also reconstructed a comprehensive overview of how dynamic cellular events and biochemical processes are related among tissues over 24 hours. Importantly, our data reveal how metabolites and various metabolic networks are altered under nutrient stress, and we identify novel tissue-specific and inter-organ circadian metabolite dynamics particularly related to diet-induced obesity. Our data presents a uniquely rich starting point for hypothesis generation and validation. Indeed, circadian metabolite alterations revealed by our approach have major implications for muscle insulin sensitivity, hepatic steatosis, atherosclerosis, and energy expenditure. This comprehensive overview of circadian metabolism thus provides actionable knowledge for preventative medicine, and better predictions of how metabolite profiles can be controlled to affect a desired metabolic outcome (German et al., 2005; Gooley and Chua, 2014).

**Results**

**Temporal and Tissue-Specific Metabolite Profiling**

To characterize tissue-specific metabolism over 24 hours, and compare how multiple tissues respond to chronic nutrient stress, we performed circadian metabolomics profiling on 8 different murine tissues. These include two distinct brain regions, the suprachiasmatic nucleus (SCN) and the medial prefrontal cortex (mPFC), in addition to gastrocnemius skeletal muscle, interscapular brown adipose tissue (BAT), epididymal white adipose tissue (WAT), liver, serum, and cauda epididymal sperm (**Figure 1A**). Tissues were collected every four hours across the light/dark cycle from a single cohort of C57BL/6J mice (n=5 mice per *zeitgeber* time per diet) after 10 weeks of *ad libitum* access to standard chow or high fat diet (HFD, 60% kcal, see Experimental Procedures for details). Importantly, mice fed HFD were confirmed to have rhythmic energy intake indistinguishable from that of the vivarium chow-fed mice (Eckel-Mahan et al., 2013). Metabolites in each tissue sample were profiled by mass spectrometry (LC/MS and GC/MS, see Experimental Procedures).

Using this approach we detected hundreds of metabolites in each tissue, and obtained comprehensive coverage across a wide range of metabolite classes, including a variety of ‘lipids’ with diverse biological functions, amino acids, carbohydrates, nucleotides, peptides, xenobiotics, cofactors and vitamins, and ‘energy’ metabolites involved in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (**Figures 1B** and **1C)**. All data have been integrated into the existing CircadiOmics platform (http://circadiomics.igb.uci.edu/) allowing users to search for and view diurnal metabolite dynamics under chow and high fat diet in tissues of interest. The CircadiOmics platform integrates these circadian experimental data with background data mined from online and offline sources using statistical machine learning (Patel et al., 2012). Interactive networks have been generated and are freely available for the data pertaining to this circadian atlas.

Tissue-specific differences in the number of metabolites detected, their temporal oscillation, and the relative impact of HFD were readily apparent by direct comparison (**Figure 1B**). While relative class distribution of detected metabolites was similar across tissues (**Figure 1C**), relative metabolite abundance reflected tissue composition (**Figure 1D**). For example, WAT and BAT revealed a predominance of lipids, whereas brain and muscle revealed mostly amino acids and lipids. Liver and serum were similar, containing substantial amounts of carbohydrates, and comparable amounts of amino acids, lipids, and energy metabolites, while sperm contained mostly lipids and energy metabolites.

As shown in **Figure 1E**, the number of metabolites significantly impacted by HFD (diet effect *p*<0.05, linear regression model) varied among tissues independently of the number of metabolites detected (**Figure 1C**). For example, while ~540 total metabolites were detected in both mPFC and muscle, >3-fold more metabolites were altered by HFD in muscle than in mPFC (232 significantly impacted muscle metabolites versus 75 mPFC metabolites). Overall, WAT, liver, BAT, muscle and serum showed the greatest impact from HFD, with between 40%-60% of detected metabolites showing significant alterations (**Table 1**). Brain and sperm metabolites were more resistant to diet-induced metabolite alterations, with SCN, mPFC and sperm showing alterations in only 27%, 14%, and 8% of detected metabolites, respectively.

To obtain a more focused picture of common and tissue-specific metabolite classes impacted by HFD, we performed a metabolite subpathway enrichment analysis on significantly altered tissue metabolites. As shown in **Table S1**, specific lipid metabolites, especially long chain fatty acids, but also polyunsaturated fatty acids (PUFA), diacylglycerols, phospholipids, sphingolipids, glycerolipids, and lysolipids were among the most enriched metabolite classes altered by HFD. Metabolites involved in amino acid metabolism, particularly the urea cycle metabolites arginine and proline, but also lysine, the branched chain amino acids (BCAA) leucine, isoleucine and valine, and dipeptides were also highly enriched among metabolite groups altered by HFD.

We noted that differences in the relative abundance of significantly altered metabolites were characteristic of tissue-specific pathology (**Figure 1F**). For example, lipids comprised only 11% of metabolites detected in livers from chow-fed mice (**Figure 1D**), yet accounted for 52% of HFD-induced liver metabolite alterations, whereas liver carbohydrates, comprising 53% of liver metabolites under chow diet, made up only 8% of metabolites altered by HFD. This large accumulation of liver lipids relative to carbohydrates likely reflects HFD-induced hepatic steatosis. Skeletal muscles of HFD-fed mice also exhibited a relatively large accumulation of lipids known to negatively impact insulin sensitivity. On the other hand, serum carbohydrate levels were comparable to liver under chow conditions, yet made up 62% of serum metabolites altered by HFD, reflecting known increases in various blood sugar species after chronic HFD feeding. Overall, our data provide a comprehensive overview of tissue metabolite composition and fluctuations over 24 hours. In addition, metabolite alterations induced by HFD largely reflect tissue-specific susceptibility to chronic nutrient stress.

**Tissue-specific Impact of Nutritional Challenge on Circadian Metabolism**

To map circadian metabolism within and across tissues and characterize how high fat diet rewires circadian rhythms, we analyzed tissues for 24-hour oscillating metabolites (JTK\_CYCLE *p*<0.05) (Hughes et al., 2010). We determined that BAT, serum, liver, skeletal muscle, and mPFC are all highly circadian tissues (see **Figure 2**, *“% detected metabolites*” and “*# of circadian metabolites*”), with around 20%-50% of detected metabolites showing circadian oscillation regardless of diet. Major differences in tissue-specific circadian metabolism became apparent when comparing the relative percentages, numbers, amplitudes and class distributions of circadian metabolites among tissues, and between diets. For example, under chow diet, BAT displayed the highest circadian metabolite oscillation amplitude of all tissues (see **Figure 2**, “*amplitude*”). However, after HFD, amplitude was blunted in BAT, as well as in mPFC and serum. While lipids comprised the largest class of metabolites detected in each tissue (**Figure 1C**), the numbers of lipids showing circadian oscillation varied widely among tissues under chow and high fat diet (**Figure 2**). For example, there were far more circadian lipids detected in BAT, mPFC and serum under chow diet compared to SCN, muscle, WAT and liver. Furthermore, BAT, mPFC and serum all showed a massive loss of circadian lipid oscillation after HFD, while SCN surprisingly gained around 100 *de novo* circadian lipids. We also noted a marked temporal gating of circadian lipids to the rest (light) phase in mPFC, BAT, and serum under chow diet, but peaks were slightly different for each tissue. Circadian lipids peaked around ZT6 in mPFC, ZT6-ZT8 in serum, and ZT8-ZT10 in BAT, highlighting tissue-specific circadian lipid dynamics.

 Sperm, collected to generate caudal epidydimal fractions as shown in **Figures S1A** and **S1B**, displayed relatively few oscillating metabolites (**Figure 2**). However, glycolytic intermediates 2- and 3- phosphoglycerate oscillated only under chow conditions (**Figure S1C**). Furthermore, the levels of more than 40% of all detected lysolipids are significantly reduced under HFD conditions during the dark phase (**Figure S1D**) (*p*<0.05, ANOVA). Changes in lysolipid content can reflect alterations in membrane turnover or impaired lysosomal function, among other cellular metabolic pathways, suggesting that one or more of these processes may be impacted by HFD.

Compared to other tissues, skeletal muscle showed a more diverse and temporally compartmentalized distribution of circadian metabolite peaks under chow diet. Muscle circadian carbohydrates peaked around ZT2, whereas nucleotides peaked around ZT8, and amino acids mainly around ZT18, in the middle of the active and feeding (dark) phase. These peaks were drastically altered under HFD, as the main circadian peak of muscle amino acids shifted to around ZT8-ZT10, the end of the resting phase, with a secondary peak at ZT20. These alterations suggest that HFD causes a massive reorganization of circadian amino acid metabolism in skeletal muscle. Disease-associated metabolite enrichment analysis (Xia and Wishart, 2016) of all 42 circadian metabolites found to peak between ZT8-ZT10 in HFD revealed a significant association with “degradation of skeletal muscle” (*p*=0.006), highlighting a potential pathologic underpinning behind this reorganization of muscle circadian amino acids. Indeed, short-term high fat feeding in mice for 3 weeks was shown to accelerate skeletal muscle atrophy of the soleus upon denervation (Roseno et al., 2015), while 16-weeks of chronic high fat feeding in rats causes atrophy of the gastrocnemius muscle and upregulation of ubiquitin ligase *Murf1* (Sishi et al., 2011).

Circadian metabolite subpathway enrichment analysis further revealed a wide array of common and tissue-specific circadian metabolite classes (**Table S2**). For example, independent of diet, serum and muscle maintained robust circadian oscillation of BCAA metabolites (leucine, isoleucine, valine, and associated metabolites). However, under HFD, both serum and muscle gained additional circadian enrichment of metabolites involved in glycine, serine and threonine metabolism. Muscle also gained circadian oscillation of metabolites related to alanine and aspartate metabolism under HFD, while BAT gained oscillation of guanine-containing purine nucleotides.

To highlight specific metabolic pathways associated with circadian metabolites and the relative impact of nutrient stress, we performed KEGG pathway enrichment analyses on all circadian metabolites (**Figure S2**). Circadian metabolites were mostly linked to processes involved in metabolite transport, lipid biosynthesis, and especially nitrogen balance, including several pathways involved in protein synthesis, amino acid metabolism, and purine and pyrimidine metabolism. Interestingly, both serum and muscle showed similarly increased circadian enrichment for protein metabolism pathways under HFD, including protein digestion and absorption, and aminoacyl-tRNA biosynthesis. Overall, our comparative analyses revealed major differences in how circadian metabolism is organized among different tissues. While BAT, serum, liver, muscle, and mPFC are all highly rhythmic tissues under conditions of energy balance, common circadian alterations in protein metabolism were revealed in serum and muscle under HFD. These common metabolic changes elicited by HFD highlight coordinated and potentially pathogenic multi-tissue metabolic responses to chronic nutrient stress.

**Metabolite Correlations Reveal Tissue-specific Temporal Gating of Metabolic Pathways**

Coherent temporal gating of potentially incompatible metabolic pathways is thought to be essential for efficient maintenance of tissue homeostasis (Noguchi et al., 2013). To analyze how various dynamic metabolic processes are related within tissues over time under conditions of energy balance or metabolic imbalance, we examined the temporal correlation between metabolites within each tissue. We hypothesized that metabolites sharing a strong positive temporal correlation may have common origins or biological functions, and belong to the same metabolic pathway or a closely related metabolic network, whereas metabolites with a strong negative correlation may reflect temporal gating of incompatible pathways. Furthermore, temporal correlation of metabolites within a tissue can serve as a general index for synchronization of individual cells within a tissue, since dynamic metabolic pathways should be highly correlated among synchronized cells. As shown in **Figure 3A**, we detected extensive positive (red) and negative (blue) temporal correlations among metabolites in all tissues. Our analysis revealed that many tissues appear to lose metabolite correlation under HFD, with serum, BAT, WAT, and mPFC clearly showing less metabolite correlations under HFD. Such trends in SCN, liver and muscle were not clearly evident, whereas sperm appeared to gain temporal correlations under HFD.

 To identify the most robust temporal correlations among metabolites in each tissue we applied 100 random permutations to the replicate samples and estimated correlation coefficients and significance (Pearson correlation). Metabolites with a correlation *p*-value<0.05 in 99% of all tests were considered significant. Serum, liver, BAT and muscle all showed a high degree of temporal correlation among metabolites under chow diet, and all four tissues lost temporal correlation under HFD (**Figure 3B**). Loss of correlation was especially severe in serum and BAT, which lost 98% and 74%, respectively. On the other hand, liver and muscle lost only 34% and 39%, respectively, suggesting maintenance and/or reorganization of coordinated metabolic pathways under HFD.

We visualized connections between metabolite classes in each tissue by plotting significant positive and negative temporal correlations according to metabolite class (**Figure 3C**). Serum lipids showed the greatest degree of temporal correlation with other metabolites, especially amino acids. Importantly, these were all lost or severely reduced under HFD, with only serum amino acids and peptides showing correlation under HFD. The major positive correlations among BAT metabolites under chow, especially BAT nucleotides, were also mostly lost under HFD. While muscle and liver similarly lost temporal correlation among metabolite classes, they also maintained substantial metabolite correlation under HFD, particularly among amino acids and carbohydrates. The fact that serum displayed such a severe loss of intra-tissue metabolite correlation has substantial implications for the extent to which circadian misalignment among tissues is exacerbated under conditions of nutritional challenge.

Subpathway enrichment analysis of significantly correlated metabolites revealed a general maintenance of tissue carbohydrate correlations under HFD, with muscle glycolytic intermediates and liver sugar metabolites retaining temporal correlation (**Table S3**), in agreement with circadian metabolite enrichment analysis (**Table S2**). However, under HFD, serum lost temporal correlation among essential fatty acids, in addition to several medium chain fatty acids, and lysolipids, while muscle lost correlation among acylcarnitines and TCA cycle metabolites. Furthermore, serum and muscle both gained significant temporal correlations among BCAA and other amino acids, again suggesting a coordinated response linking serum and muscle amino acids under HFD.

Using these metabolite temporal relationships described by the data, we reconstructed entire metabolic pathways over 24-hours. For example, muscle glycolytic intermediates and acylcarnitines respectively reflect cytosolic or mitochondrial ATP production. Both metabolite classes showed dynamic oscillation over 24-hours (**Figure 3D**). Under chow diet, the peaks were confined to distinct temporal windows, reflecting a clear delineation of diurnal fuel availability. Glycolytic intermediates declined progressively across the light phase, whereas acylcarnitines increased towards the end. Acylcarnitines were lowest in the middle of the dark phase when glycolytic intermediates were increased. However, under HFD, oscillation of glycolytic intermediates was blunted during the dark phase, while acylcarnitines remained at peak levels. These coordinated metabolite alterations clearly imply impaired metabolic flexibility and insulin resistance (Goodpaster and Sparks, 2017), and give a perspective on when differences may be most pathophysiologically relevant.

**Inter-tissue Clock Communication: Metabolite Coherence or Dissonance**

Spatial compartmentalization of specific metabolic pathways within tissues and organs is another means to efficiently coordinate tissue-specific functions and maintain homeostasis (Tu and McKnight, 2006). To understand how metabolite oscillations are related among different tissues, we analyzed inter-tissue metabolite temporal correlations. Serum, liver, BAT and muscle metabolites all showed a relatively high degree of inter-tissue temporal correlation under chow diet, underscoring tight metabolic coupling between these tissues (**Figure 4A**). Importantly, serum emerged as the main source of metabolite temporal correlation, reflecting the role of circulating nutrients and hormones in communicating timing information to the periphery, and maintaining temporal coherence among different tissues under conditions of energy balance (Abbondante et al., 2016).

 HFD was associated with a severe loss of temporal coherence (**Figures 4A-C**). This was reflected by a loss of correlation between serum and muscle, liver, and BAT, in addition to a general loss of correlation among all tissues. Interestingly, the vast majority of inter-tissue metabolite correlations present under HFD were not shared under normal chow, highlighting various general and coordinated multi-tissue responses to HFD. These *de novo* tissue correlations were especially prevalent in muscle and liver, suggesting HFD-induced metabolic reprogramming in response to the increased lipid load (Eckel-Mahan et al., 2013).

To provide a global perspective on the relationships between tissue metabolites under chow diet, and to better visualize the massive loss of tissue metabolite correlations under HFD, we plotted all significant inter-tissue correlations according to metabolite class, tissue, and diet (**Figure 4C**). We noted serum metabolites shared a particularly high degree of temporal correlation with metabolites in other tissues, especially muscle, but also liver and BAT. Importantly, these were again lost or severely reduced under HFD. However, amino acids in serum, muscle, and liver retained a relatively high degree of correlation, again suggesting a coordinated response linking amino acids within these tissues under HFD. This link may be due in part to the known increase in gluconeogenesis under conditions of HFD-induced obesity, and the resulting requirement of the liver for converting amino acids into gluconeogenic precursors.

Comparative inspection of significant cross-tissue correlations between serum and liver, muscle, and BAT further emphasized the massive loss and reorganization of metabolite correlation induced by HFD (**Figure 5)**.Normally abundant liver-serum and muscle-serum metabolite correlations under chow were reduced around 60% under HFD **(Figure S3A**), while BAT-serum correlations under HFD were reduced 74%. Lipids and amino acids were the main metabolite classes correlated between serum and other tissues under chow diet, and together comprised around 60-70% of all significant inter-tissue metabolite correlations. Interestingly, BAT and serum nucleotides also showed a relatively high degree of temporal correlation. Importantly, most of these cross-tissue metabolite correlations were lost under HFD (**Figure S3B**), with inter-tissue lipids losing around 80% correlation in all tissues, and amino acids losing 35%-66%, depending on the tissue (**Figure S3C**).

Overall, our data demonstrate how diurnal metabolite oscillations are temporally linked within and across various tissues. The relatively large number of metabolite correlations under chow diet, especially among serum, muscle, liver, and BAT, reflects their shared metabolic roles and temporal gating of linked metabolic pathways. Cross-tissue metabolite correlation analysis also emphasized the importance of serum as a main communication conduit between tissues, especially among muscle, BAT and liver. As such, serum metabolites both direct and reflect tissue-specific metabolism. Accordingly, our data show how inter-tissue communication is largely disrupted under chronic nutrient stress, with substantial loss of metabolite temporal correlation among serum, muscle, liver, and BAT. Finally, we noted several novel and interesting cross-tissue metabolite correlations detected only under HFD, highlighting coordinated metabolic responses between tissues in response to this nutritional challenge (see below).

**Increased Muscle Protein Turnover under HFD Temporally Linked to Increased Liver Gluco- and Glyceroneogenesis**

While metabolite temporal correlations and circadian oscillations were generally disrupted under HFD, we noted several important tissue metabolites gained *de novo* circadian oscillation and temporal correlations under HFD, suggesting coordinated multi-tissue metabolic responses to chronic nutrient stress. Specifically, our data revealed a high association between circadian muscle and serum amino acids under chow and HFD, and several significant HFD-induced alterations characteristic of increased muscle protein degradation and liver gluco- and glyceroneogenesis, particularly during the light phase.

We first noted that diurnal rhythms of glutamine and alanine abundance in muscle and liver were gated to distinct temporal windows under chow diet, oscillating anti-phase to one another with increased glutamine abundance during the resting phase (light) and increased alanine abundance during the active phase (dark) (**Figure 6A**). This inverse circadian relationship between glutamine and alanine likely reflects physiological differences in precursor availability (A. J. Garber, 1984). However, circulating alanine remained relatively stable throughout the circadian cycle in chow-fed mice, yet became highly circadian and significantly increased during the light phase under HFD. This effect was even more pronounced in skeletal muscle, in which alanine levels remained at constitutively high levels throughout the light phase under HFD. There were no major HFD-induced changes in alanine levels in liver or WAT, whereas BAT showed slightly reduced levels. Circulating glutamine was also increased by HFD, but the greatest difference was seen during the dark phase, coinciding with modestly increased glutamine in HFD skeletal muscle at ZT16. Circadian glutamine oscillation remained normal in HFD liver, but was reduced in WAT and BAT.

Increased alanine production and release by skeletal muscle normally occurs in response to increased protein catabolism during periods of starvation (Felig, 1973). The rate of muscle protein degradation is often clinically inferred by measuring production and release of the surrogate metabolite 3-methylhistidine, a unique amino acid released by myofibrillar proteolysis and excreted in the urine rather than being reutilized for protein synthesis or oxidized for energy (Elia et al., 1981). We detected 3-methylhistidine only in serum and skeletal muscle, with both showing circadian oscillation under chow and HFD (**Figure 6B**). However, the relative abundance of 3-methylhistidine was significantly increased throughout the circadian cycle after HFD, particularly during the normal peak (ZT8) and trough (ZT16) observed in chow-fed mice, suggesting increased muscle protein degradation under HFD. We also noted increased creatinine in serum after HFD, and a similar trend for increased levels in muscles, indicating that muscle mass is maintained or even slightly increased despite increased protein breakdown. Together this suggests an increased rate of muscle protein turnover in mice under HFD.

The majority of alanine released from muscle is synthesized *de novo* by transamination of pyruvate, with nitrogen originating mostly from BCAA via glutamate (Odessey and Goldberg, 1979). Serum, muscle and liver BCAA showed circadian oscillation under chow and HFD, with increased levels during the dark phase, and reduced levels during the light phase (**Figure S4A**). Serum and muscle from HFD mice also showed increased BCAA levels at various time points across the circadian cycle compared to chow mice, whereas BCAA levels in liver, WAT, and BAT were all significantly reduced under HFD. While most amino acids are transaminated in liver, BCAA are transaminated mainly in skeletal muscle, resulting in formation of branched-chain keto acids (BCKA) α-ketoisocaproate, α-ketomethylvalerate, and α-ketoisovalerate. All three BCKAs were drastically reduced and lost circadian oscillation in muscle after HFD (**Figure S4B**), yet serum BCKA levels were significantly increased across the light/dark cycle, suggesting increased export from muscle and/or reduced uptake from other tissues.

A striking effect of HFD was the general increase in glucogenic amino acids in muscle and serum, while levels were consistently reduced in liver, WAT and BAT (**Figure S4A**), possibly reflecting increased rates of gluco- and glyceroneogenesis in liver (Song et al., 2001) and reduced amino acid uptake and metabolism in WAT and BAT (Serra et al., 1994). Moreover, glycerol 3-phosphate, a major precursor for liver glyceride-glycerol and VLDL synthesis (Nye et al., 2008), was increased around 50% throughout the day/night cycle under HFD (**Figure 6C)**, and oscillated in phase with muscle and serum alanine, indicating increased conversion of serum alanine (and other glucogenic amino acid substrates resulting from muscle degradation) to liver glyceride-glycerol (Nye et al., 2008).

Muscle protein degradation and liver gluco- and glyceroneogenesis are all transcriptionally-regulated processes under tight hormonal control, mainly by opposing actions of circulating glucocorticoids and insulin (Hatting et al., 2018; Schiaffino et al., 2013; Shimizu et al., 2015). Interestingly, we noted the temporally-linked rise and fall in muscle and serum 3-methylhistidine under chow diet largely reflected 24-hour oscillation of endogenous serum corticosterone (**Figure 6D**) and insulin (Jouffe et al., 2016). Interestingly, endogenous serum corticosterone levels were not increased, and continued to oscillate normally under HFD. This is intriguing, considering the well-established associations between chronic high fat feeding and insulin resistance of skeletal muscle and liver (Oakes et al., 1997) largely mirror metabolic complications associated with chronic glucocorticoid administration, including impaired insulin-dependent glucose uptake and metabolism by muscle, and reduced insulin-mediated suppression of hepatic glucose production leading to hyperglycemia (Dunford and Riddell, 2016). Our results thus indicate a major metabolic complication of chronic high fat feeding may be locally enhanced sensitivity to the effects of endogenous glucocorticoids. Accordingly, serum glucose remained constitutively elevated at all time points across the light/dark cycle under HFD, while the normal circadian oscillation of glucose in liver and muscle from chow-fed mice was blunted and likewise elevated, but only at ZT12 (**Fig. 6E**), corresponding to the peak of endogenous glucocorticoids. Altogether, our data suggests that HFD is associated with increased supply of gluconeogenic precursors from the muscle to the liver, and highlights the coordinated interplay between tissues controlling glucose homeostasis under normal and chronic nutrient stress.

The proposed pathogenic relationships between the muscle, serum, and liver metabolites increased under HFD are summarized in **Figure 6F**. Increased cycles of muscle protein turnover lead to increased production and release of alanine and other amino acids destined for uptake mostly by the liver. The importance of serum alanine in this process is supported by arteriovenous differences in rat and human showing preferential hepatic uptake of alanine above all other amino acids (Pozefsky et al., 1969), and although glutamine is the only amino acid released by muscle at higher rates than alanine, it is destined mostly for kidneys and the small intestine, which in turn release alanine (Snell and Duff, 1979). Excess amino acids not utilized for liver protein synthesis would be metabolized and converted to glucose and glyceride-glycerol, contributing to elevated glycemia, increased production of hepatic triacylglycerol (TAG) and secretion of VLDL. Hypertriglyceridemia and increased VLDL secretion are associated with an array of metabolic diseases linked to poor diet, lack of exercise, and obesity, including insulin resistance and cardiovascular disease (Eckel et al., 2005). Increased muscle FFA would activate pyruvate dehydrogenase kinase 4 (PDK4), leading to inhibition of pyruvate dehydrogenase, further promoting alanine production (Zhang et al., 2014). Our study thus reveals an inter-related network of metabolites and common metabolic pathways between muscle and liver that are coordinately modified under chronic nutrient stress. Overall, our data suggests that muscle protein turnover, and particularly muscle alanine production and release may play a previously underappreciated pathogenic role in a host of related metabolic diseases. The temporal and context-dependent associations between metabolites across tissues highlighted here provide a wealth of research leads to be explored in future analyses by, for example, pulse-chase experiments using labeled amino acids.

***De novo* Oscillation of Purine Nucleotides in BAT under HFD Linked to Impaired UCP1 Activity**

Our metabolomics analyses revealed BAT as the most circadian tissue. BAT displayed the highest percentage of circadian metabolites of any tissue (~50%; **Figure 2**), showed the highest amplitude of metabolite oscillations, and suffered the greatest loss of circadian metabolites under HFD, with lipids, nucleotides and amino acids the most affected. Under chow diet, we noted a distinct peak of circadian metabolites from ZT8-ZT10 that was greatly reduced under HFD. Inspection of these 67 circadian metabolites peaking between ZT8-ZT10 in BAT from chow-fed mice revealed the majority to be lipids (63%), nucleotides (16%), and amino acids (13%) (**Figure S5**).

To identify a potential source of oscillating lipids in BAT from chow-fed mice, we examined metabolites involved in lipid turnover and inter-organ lipid fluxes in relevant tissues. In chow-fed mice, glycerol and long-chain non-esterified fatty acids (NEFAs; both saturated and unsaturated) were increased in the serum during the rest phase, and reduced during the active phase (**Figure 7A**), in agreement with previous reports (Shostak et al., 2013). Diurnal oscillation of circulating NEFAs in chow-fed mice was largely mirrored by WAT, BAT, and muscle, although the precise timing and range of oscillation varied among tissues: WAT NEFAs remained higher throughout the resting phase, whereas BAT NEFAs showed a much higher and narrower peak around ZT8, near the end of the resting phase. Muscle NEFAs showed a biphasic distribution, with peaks towards the end of the dark phase and the middle of the light phase. Interestingly, liver NEFAs remained more stable over 24 hours, showing considerably lower oscillation than other tissues, and with a modest peak at the light/dark transition.

Tissue-specific temporal changes in glycerol and NEFA abundance reflect the continuous turnover and transport of lipids among tissues in a state of so-called “dynamic equilibrium” (Guggenheim, 1991), in which total body fat content remains constant in the midst of high rates of degradation, inter-conversion and re-esterification of lipid stores within and across tissues over 24 hours. Interestingly, high fat diet blunted oscillation of circulating NEFAs, increased levels of serum glycerol, and severely reduced NEFA levels and oscillation in WAT and BAT (**Figure 7A**), suggesting an impaired diurnal turnover of lipid stores in both fat depots. In contrast, liver glycerol and NEFAs were dramatically increased after HFD, similar to skeletal muscle, highlighting known pathological deposition of biologically active lipids observed in insulin resistance and obesity. The massive reduction in circadian BAT NEFAs is of particular interest, since they are known to directly stimulate thermogenic proton leak via activation of uncoupling protein 1 (UCP1) (Nicholls and Locke, 1984). Although the precise mechanism of NEFA-stimulated proton leak remains unresolved (Divakaruni and Brand, 2011), loss of thermogenic capacity and thus reduced BAT-derived energy expenditure is known to contribute to the development of obesity (Himms-Hagen, 1983). Altogether, our data suggest impaired circadian lipolysis of adipose tissue during the resting phase, dramatically reduced BAT activation at ZT8-ZT10, and increased biologically active NEFAs accumulating in the liver and muscle, especially at ZT12, during the light-dark transition.

In the absence of activating fatty acids, extra-mitochondrial purine nucleotides restore energy conservation in BAT mitochondria by direct binding and inhibition of UCP1 (Shabalina et al., 2004). However, recoupling efficiency varies among the purine nucleotides with guanine nucleotides showing higher recoupling efficiency than adenine nucleotides due to their higher affinity for UCP1 and higher concentration in the mitochondrial intermembrane space (Jastroch et al., 2010). Guanosine diphosphate (GDP) is clearly the most effective re-coupler at physiological levels, followed by guanosine monophosphate (GMP), guanosine triphosphate (GTP) and adenosine triphosphate (ATP), while adenosine diphosphate (ADP) is even less effective than ATP, and adenosine monophosphate (AMP) shows only a minor recoupling effect (Rafael, 1977). While we did not detect GDP, we noted that both 5’-GMP and its precursor guanosine, which showed no circadian oscillation in BAT from chow-fed mice, displayed *de novo* circadian oscillation in BAT after HFD, and a massive increase during the light phase, peaking at ZT8 (**Figures 7B&C**). Brown adipose tissue also contains high levels of the enzyme GMP reductase (GMPr), which catalyzes the reductive deamination of guanine nucleotides to inosine monophosphate (IMP), thus facilitating the activation of UCP1 by releasing endogenous inhibition by these nucleotides (Salvatore et al., 1998). IMP can be further converted to AMP, and dephosphorylated to form adenosine, another activator of brown adipocytes (Gnad et al., 2014). In agreement with increased 5’-GMP in BAT from mice on HFD, IMP, AMP and adenosine were all increased in BAT from HFD mice, although there was a gradual reduction in their abundance across the circadian cycle depending on their metabolic vicinity to GMP (**Figures 7B&C**).

Due to their important roles in metabolic regulation and signaling, intracellular concentrations of adenine and guanine nucleotides are normally kept under tight control by continuous inter-conversion and catabolism (Bender, 2011). Degradation can follow alternate routes (**Figure 7D**), but several steps rely on reduction of the cofactor nicotinamide adenine dinucleotide (NAD+) to NADH, including conversion of IMP to xanthosine monophosphate (XMP) via IMP dehydrogenase (IMPDH), conversion of hypoxanthine to xanthine, and conversion of xanthine to uric acid, both of which occur via xanthine dehydrogenase (XDH). The mammalian XDH enzyme is also inter-convertible with xanthine oxidase (XO), which transfers electrons from xanthine to oxygen instead of NAD+, and produces both superoxide anion and hydrogen peroxide rather than NADH (Nishino et al., 2008). Interestingly, similar to the liver (Eckel-Mahan et al., 2013), circadian NAD+ oscillation was completely lost in BAT after HFD, and NAD+ remained at constitutively low levels throughout the circadian cycle (**Figure 7E**), supporting a scenario of impaired BAT purine nucleotide catabolism after HFD.

While our results cannot distinguish between mitochondrial and nuclear pools, NAD+ provides essential circadian metabolic regulation in both compartments (Mori et al., 2005; Scher et al., 2007). Similarly, the downstream metabolite nicotinamide (NAM), produced from Sirt3 deacetylase activity, and precursor for NAD+ salvage via nicotinamide phosphoribosyltransferase (NAMPT) also lost oscillation after HFD (**Figure 7E**). Finally, circadian oscillation of downstream metabolites and end products of purine catabolism like hypoxanthine, xanthine and allantoin were all severely blunted in BAT after HFD (**Figure 7F**), further suggesting reduced purine catabolism**.**

Interestingly, *Sirt3* and *Nampt* are both circadian genes in BAT (**Figure S6B**; see also http://circadb.hogeneschlab.org/ and CircadiOmics), and they are both direct BMAL1 targets (Koike et al., 2012; Nakahata et al., 2009; Rey et al., 2011). It is thus tempting to speculate that HFD-induced attenuation of BAT circadian clock function may directly impair *Sirt3* and *Nampt* circadian expression. Indeed, we found that core circadian clock genes *Bmal1* and *Clock* showed significantly altered circadian gene expression profiles under HFD (**Figure S6A**). Likewise, we noted that *Nampt* and *Sirt3* both showed altered 24-hr expression profiles (**Figure S6B**); however, *Nampt* showed modestly reduced expression during the light phase, while *Sirt3* was significantly increased specifically during the dark phase. Additional relevant circadian genes in BAT (http://circadb.hogeneschlab.org/), such as *Gmpr* (coding for GMP reductase)*,* *Impdh1* (coding forthe predominant IMP dehydrogenase expressed in BAT)*,* and *Ucp1*, also showed drastic alterations in circadian gene expression under HFD feeding (**Figure S6B and C**). It is plausible that the precise connection to the BAT clock may result from a combination of circadian alterations under HFD and local clock misalignment.

Accumulation of purine nucleotides in the absence of activating fatty acids will result in UCP1 inhibition, reduction in BAT-derived energy expenditure, and promote obesity, but the physiological significance of ~7-8-fold increased GMP levels observed in BAT after HFD (**Figure 7B**) is unclear due to its 50-fold lower binding affinity for UCP1 (Klingenberg and Huang, 1999). We could not find reports quantifying endogenous levels of GMP and GDP in mouse brown adipose tissue, but 120-130uM GMP and 50uM GDP has been reported in mouse heart tissue by HPLC (McKee et al., 1999) and ~20-fold differences of GMP concentration in whole cell (~100uM) versus mitochondrial matrix (~4uM) of HeLa cells by LC/MS (Chen et al., 2016). To investigate the possible role of GMP and IMP *per se* in driving proton leak inhibition, we utilized isolated mitochondria from stable human embryonic kidney cells (HEK293) ectopically expressing mouse UCP1 (Hirschberg et al., 2011). Importantly, mouse UCP1 displays native behavior in this system (i.e. fatty acid and GDP sensitivity) without inducing artificial uncoupling. As shown in **Figure 7G** (left panel), addition of purines GDP, GMP or IMP to mitochondria from control HEK293 cells had no effect on respiration driving proton leak. However, in the presence of UCP1 (**Figure 7G**, middle panel), there was an upwards shift in the proton leak kinetics (relationship between respiration rate and membrane potential) indicating increased uncoupling (Divakaruni and Brand, 2011) which was prevented to a similar extent after addition of GDP or GMP. At the highest common membrane potential, addition of GMP or GDP to UCP1-containing mitochondria caused a similar decrease in proton conductance relative to the control (**Figure 7G**, right panel). Addition of IMP had no effect on respiration rate or membrane potential, suggesting the guanine moiety is particularly important for UCP1 inhibition by purine nucleotides. Altogether, our data suggests that the increased GMP observed in BAT after HFD is physiologically relevant and sufficient to significantly impair proton leak, especially within the context of drastically reduced fatty acid levels after HFD (**Figure 7A**). This would severely reduce the contribution of brown adipose to global energy expenditure, and promote expansion and accumulation of lipid stores.

**Discussion**

Homeostasis is maintained through internal stability within an organism, and its capacity to integrate physiological responses in a coordinated manner among organs and tissues (Roenneberg and Merrow, 2016). Circadian control appears central to homeostasis, as metabolic pathways interplay with the clock through different mechanisms in different organs (Eckel-Mahan and Sassone-Corsi, 2013). Despite recent advances, understanding how circadian metabolism is coordinated among various clocks has remained limited. Our study provides a comprehensive map of both temporal and spatial distribution of circadian metabolites, and reveals in exquisite detail the various intra- and inter-tissue temporal relationships under conditions of energy balance and energy imbalance. Importantly, we uncover that metabolites are linked within and across various tissues over time, and that these connections are modified by nutrient stress in the form of chronic high fat diet feeding. This global atlas thus captures the dynamic nature of tissue metabolism and inter-organ communication over 24 hours. Our comparative analysis of the number, amplitude, class, and peak distributions of circadian metabolites in each tissue classifies tissues in terms of their circadian metabolism, and defines precise temporal windows of common and tissue-specific metabolic pathways. This uniquely systems-level view of how tissue metabolism is organized over time has the potential to reveal novel biomarkers previously unappreciated by more targeted and focused approaches, and previously undetected metabolite dynamics and associations that could be strategically important for therapeutic exploitation.

A major finding of our study was the severe loss of temporal correlation within and among tissue metabolites under high fat diet. This demonstrates that metabolic or behavioral alterations associated with high fat diet feeding disrupt a normally dynamic and coherent temporal organization of multiple metabolic networks across tissues. Comparing inter-tissue metabolite correlations further emphasized the importance of serum metabolites in establishing and maintaining inter-tissue metabolic communications, and the pathogenic implications when this metabolic coupling between tissues is lost. While all metabolite classes showed a loss of intra- and inter-tissue temporal correlation, the impact was particularly severe for lipids and amino acids. Feeding/fasting cycles are determinants of rhythmic fluxes of circulating lipids and amino acids (Hatori et al., 2012). However, mice used in this study showed normal diurnal feeding patterns under chronic HFD as compared to standard diet (Abbondante et al., 2016; Eckel-Mahan et al., 2013). Thus, feeding time alone does not explain the substantial loss of temporal correlation among lipids and amino acids in serum, muscle, liver, and BAT. In *ad libitum* chow-fed mice, peak physiological levels of circulating lipids are found during the middle of the resting phase (Abbondante et al., 2016). However, under HFD, these oscillations are severely blunted, with residual oscillating lipids peaking during the end of the active phase. This indicates that consuming a disproportionately high amount of dietary lipids during the feeding phase may perturb normal physiological oscillations of serum lipids, presumably leading to alterations in metabolic signaling and energy sensing in other tissues.

Our data also underscored the significant temporal association between muscle and serum amino acids and peptides under chow and HFD, and significant HFD-induced metabolite changes suggesting increased muscle protein degradation during the resting phase. Accelerated turnover of muscle protein and amino acid release occurs largely by selective breakdown of myofibrillar proteins (actin and myosin), which make up around 60-70% of muscle protein (Mitch and Goldberg, 1996). In particular, muscle proteins serve as the main source of glucogenic amino acids during starvation, with glutamine and alanine together accounting for more than half of all α-amino acids released (Cahill, 2006; Felig, 1975). However, glutamine and alanine account for less than 15% of total amino acid residues in muscle protein (Ruderman and Goodman, 1973). Their preferential release is due to their selective *de novo* synthesis in muscle at the metabolic expense of other amino acids, especially BCAA (Goldstein and Newsholme, 1976).

Increased blood concentrations of specific amino acids and their metabolites have been known to be associated with obesity and T2DM since the late 1960s (Adibi, 1968; Felig et al., 1969), including BCKA (Adams, 2011). Renewed interest in elevated amino acids, particularly BCAAs, recognized their utility as predictive biomarkers for future type 2 diabetes risk, and for predicting successful therapeutic intervention several years before clinical diagnosis (Batch et al., 2013; Newgard, 2012; Newgard et al., 2009; Wang et al., 2011). However, their origin is debatable (Herman et al., 2010; Herrero et al., 1997; Zhao et al., 2016), as is whether they are cause or consequence of insulin resistance, and the utility of trying to control them in normalizing clinical profile. Since much of the available human data has been obtained from T2DM or obese patients after fasting, it is unlikely that alterations in serum amino acids are a direct result of nutrient metabolism (Lynch and Adams, 2014). Our data support a scenario in which increased circulating amino acids, including BCAA, mostly reflect increased skeletal muscle protein turnover. Specifically, in the context of HFD we observed a general increase of amino acids throughout the circadian cycle in both serum and skeletal muscle, but not in other tissues (**Figures 6A&S4**). Our data indicate that the increased circulating levels of alanine and glutamine seen after HFD most likely originate from muscle proteolysis and metabolism of BCAA, with increased release of BCKA.

In light of the temporal associations we uncovered between 24-hour serum corticosterone levels, and muscle and serum 3-methylhistidine, and considering the well-known associations between muscle and liver insulin resistance and diet-induced obesity, it is tempting to speculate that high fat diet enhances local tissue sensitivity to endogenous glucocorticoids. In support of this interpretation, streptazoticin-induced diabetic rats showed increased daily urinary excretion of 3-methylhistidine and creatinine compared to non-diabetic control rats (Tomas, 1982). Furthermore, while excretion of 3-methylhistidine and myofibrillar protein fractional degradation rates were increased in control and diabetic rats after corticosterone treatment, the effect was greater for the diabetic animals, demonstrating that insulin-deficient states are indeed associated with increased sensitivity to glucocorticoids. In humans, infusion of cortisol within the upper physiological range was also shown to increase proteolysis and *de novo* synthesis of alanine within 4 hours, without impacting plasma insulin, C-peptide, or glucagon (Simmons et al., 1984). While additional experimental validation is required to establish whether this is also the case for endogenous glucocorticoids under high fat diet conditions, the metabolic complications of exogenous glucocorticoid treatment already appear to be significantly amplified when combined with a high fat diet (Dunford and Riddell, 2016).

Our time-resolved multi-tissue metabolomics atlas also suggests further investigation of serum and muscle alanine as novel biomarkers for T2DM and cardiovascular disease risk is warranted, in light of its known metabolic relationships with existing predictive biomarkers (e.g. BCAA) and its potential links to other pathogenic risk factors (such as increased VLDL). Interestingly, elevated alanine aminotransferase (ALT) activity in the blood is already considered a predictive biomarker for T2DM (Vozarova et al., 2002; Zhou et al., 2013), metabolic syndrome (Marchesini et al., 2005; Schindhelm et al., 2007b), hepatic steatosis (Schindhelm et al., 2006), and coronary heart disease (Schindhelm et al., 2007a). Total ALT activity in the blood is a measurement of combined “leakage” of ALT1 and ALT2 isoforms from liver and muscle, respectively (Rafter et al., 2012). Therefore, a common “risk network” of related metabolites and enzymatic biomarkers surrounding alanine metabolism already exists for many linked metabolic diseases, suggesting a common pathogenic mechanism. In support of this interpretation, a study in which the authors profiled blood metabolites across the circadian cycle in patients with T2DM, as well as in obese but non-diabetic, or lean and non-diabetic individuals, recently established alanine as a novel T2DM biomarker in humans (Isherwood et al., 2017).

Another significant result uncovered by our study is the severe loss of BAT metabolite circadian oscillation in conjunction with *de novo* circadian oscillation of purine nucleotides, known inhibitors of UCP1 activity (**Figure 7B**). The primary physiological role of brown adipose tissue is thermogenesis, and the primary fuel for brown adipose thermogenesis is lipid, derived either from its own or WAT stores, and diet (Himms-Hagen, 1989). Our results underscore that ZT8-ZT10, the pre-awakening hours immediately before the active phase, is a particularly important circadian time for normal BAT physiology and activation state. This time window coincides with the peak of the brown adipose circadian glucose uptake rhythm, measured in mice by micro–positron emission tomography/computerized tomography (van der Veen et al., 2012), and peak expression of Rev-Erbα, a critical regulator of circadian BAT function (Gerhart-Hines et al., 2013). Likewise, human brown adipose has recently been shown to exhibit a similar “dawning” phenomenon, i.e. coupling of the glucose uptake rhythm to heat production in the hours before awakening (Lee et al., 2016), which also coincides with the peak of diurnal plasma catecholamine levels (Scheer et al., 2010). Interestingly, the brown adipose circadian activation rhythm appears to be an anticipatory rhythm driven endogenously by the brown adipocyte circadian clock, as it persists *ex vivo*, without external circadian neural, hormonal or metabolic time cues (Lee et al., 2016).

Loss of circadian NAD+ oscillation during the resting phase can explain reduced/impaired circadian purine nucleotide degradation, and may contribute to UCP1 inhibition during this critical time point. While the link between increased GMP abundance and reduced UCP1 activity was not previously known, elevated levels of GMP, IMP and AMP are observed in erythrocytes from diabetic patients (Dudzinska and Hlynczak, 2004). Furthermore, chronically elevated blood purine nucleotides and signaling are common pathophysiological consequences of many metabolic diseases (Park et al., 2015). One mechanism that can contribute to the loss of NAD+ and NAM oscillations in BAT after HFD is circadian disruption of enzymes involved in their formation (Eckel-Mahan et al., 2013). Indeed, we show that circadian expression of core clock genes (*Bmal1* and *Clock*) and BAT circadian genes regulating NAD+ formation (*Sirt3* and *Nampt)* are altered by HFD (**Figure S6A and B**). Likewise, circadian expression of genes regulating purine nucleotide metabolism (*Impdh1* and *Gmpr)* and thermogenesis (*Ucp1)* were severely impacted by HFD. Our results indicate that circadian regulation of BAT function is likely controlled at multiple points, and involves a close coordination between metabolism and transcriptional regulation. Additional experiments, including global gene expression profiling of BAT using tissue-specific gain- and loss-of-function models of various clock components, and genome-wide profiling of clock transcription factor binding in BAT are needed to fully elaborate these associations.

In summary, the circadian metabolomics atlas presented here thus constitutes a rich and robust starting point, both for hypothesis generation and validation. In addition, it fills a major gap in the development of predictive models, allowing for the temporal reconstruction of complex metabolic networks across multiple tissues and under different feeding paradigms. Building upon a wealth of existing knowledge regarding known metabolic alterations in specific tissues under feeding/fasting conditions, or under pathogenic conditions or diseased states, our data is uniquely situated to help identify novel biomarkers and biomarker networks, and to uncover previously unknown pathogenic mechanisms. Additional studies are needed to further elaborate subcellular metabolite localization and dynamics (Aviram et al., 2016; Dyar and Eckel-Mahan, 2017; Mauvoisin et al., 2017), and to monitor inter-organ metabolite flux suggested by this initial survey of the 24-hour metabolic landscape. It will also be important to further investigate how these oscillating metabolites are impacted under conditions of global or tissue-specific circadian misalignment.

Overall, our study sheds light on a highly coordinated, multi-tissue metabolic logic underlying physiological feeding/fasting rhythms, and the complex maintenance of whole body energy homeostasis. We have presented only a few interesting examples of the type of information encapsulated within this rich repository, and we show how the data can be used to reconstruct complex metabolic networks and to infer pathologic underpinnings. A wide range of intra- and inter-tissue metabolite correlations describing potentially novel predictive biomarkers and pathogenic networks remains to be fully explored and verified. Rather than a standalone atlas, we hope this resource will serve as a foundation for future studies integrating subcellular metabolomics data, global and tissue-specific clock gene mutants, additional diets, restricted feeding regimes, various exercise protocols, and higher temporal resolution.

**Author Contributions**

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**Figure Legends**

**Figure 1. Global metabolite profiling of mouse tissues over 24-hours under conditions of chow or high fat diet reveals common and tissue-specific metabolic signatures.**

(**A**) Experimental design. Suprachiasmatic nucleus (SCN), medial prefrontal cortex (mPFC), gastrocnemius skeletal muscle, interscapular brown adipose tissue (BAT), epididymal white adipose tissue (WAT), liver, serum, and cauda epididymal sperm collected every four hours across the light/dark cycle from a single cohort of C57BL/6J mice (n=5 mice per *Zeitgeber* Time, ZT, per diet) after 10 weeks of *ad libitum* access to standard chow or high fat diet (HFD, 60% kcal, see methods for details). 6 time points were profiled for SCN, mPFC, muscle and sperm; a 7th replicate time point, ZT24, was additionally profiled for serum, liver, BAT and WAT.

(**B**) Tissue-specific metabolite heatmaps. Rows reflect normalized (z-score) metabolite abundance across the light/dark cycle (white bar=ZT0, 4, 8 & 24; black bar=ZT12,16 & 20). Metabolite class is indicated at the right of each heatmap.

(**C**) Histogram showing counts and class of detected metabolites for each tissue in absolute numbers.

(**D**) Pie charts revealing tissue metabolite class composition according to relative metabolite masses (sum of standardized abundances).

(**E**) Histogram showing counts and class of metabolites significantly impacted by diet (diet effect *p*<0.05, linear regression model) in absolute numbers.

(**F**) Pie charts of HFD-altered metabolite class composition for each tissue. (relative metabolite masses affected by HFD).

**Table 1. Percent metabolites impacted by high fat diet** (diet effect *p*<0.05, linear regression model)

**Figure 2. Comparative analysis of circadian metabolites highlights heterogeneity of tissue circadian function.**

Metabolites oscillating with a 24-hr period (*p*<0.05, JTK\_CYCLE). Circadian metabolites in each tissue are plotted from left to right according to 1) Percent of detected metabolites (chow versus HFD), 2) absolute numbers (Venn diagrams indicate whether metabolite is circadian under chow, HFD or both), 3) % distribution of circadian metabolites (chow, HFD or both), 4) amplitude of circadian oscillation, 5) histograms showing absolute counts and class distribution of circadian metabolites, 6&7) radar plots showing class, absolute number, and phase distribution of circadian metabolites under chow or HFD according to peak time point.

**Figure 3. Tissue-specific metabolite correlations illustrate how temporal coherence and normal gating of metabolic pathways is maintained or altered by nutritional challenge.**

(**A**) Correlation heatmaps for all detected metabolites in each tissue. Correlation coefficient rho is shown as red (positive) or blue (negative) as indicated. The upper right triangles refer to chow diet, the lower left triangles to HFD. Metabolites were ordered according to metabolite classes as indicated in the color bar. (**B**) Histogram showing the number of significant metabolite correlations detected in each tissue under each diet. (**C**) Graphical visualization of significant metabolite correlations. Each ribbon indicates a significant metabolite correlation (positive=red, negative=blue) between or within each metabolite class. Ribbon thickness refers to number of significantly correlated metabolites. Metabolites were ordered according to metabolite class as indicated in colored bar around the circumference.(**D**) “Fuzzy plots” show the range (colored area) between minimum and maximum abundance for members of each metabolic pathway. Cubic-splines interpolation estimated continuous abundance.

**Figure 4. Massive loss of inter-tissue metabolite correlations under high fat diet.**

(**A**) Venn diagrams showing the number of correlated metabolites between each tissue under chow (blue), HFD (yellow) or both (overlap). Circle area refers to absolute number of significant metabolite correlations.

(**B**) Histogram shows the total number of significant inter-tissue metabolite correlations detected under each diet.

(**C**) Graphical summary of all inter-tissue metabolite temporal correlations. Each ribbon indicates a significant inter-tissue metabolite correlation (positive=red, negative=blue). Ribbon thickness refers to number of significantly correlated metabolites. Metabolites were ordered according to metabolite class for each tissue as indicated in the inner colored bar around the circumference.

**Figure 5. Cross-tissue metabolite correlations between serum and liver, muscle and BAT are severely altered by nutritional challenge.**

Networks of significantly correlated metabolites detected under chow or HFD. Each node refers to a single metabolite. Node shape indicates tissue or tissues showing correlation. Node color refers to metabolite class. Edges are drawn for each significant inter-tissue correlation, and edge colors refer to the sign of correlation coefficient (red=positive, blue=negative).

**Figure 6. Increased circadian oscillation of muscle and serum metabolites linked to increased liver precursors for gluco- and glyceroneogenesis.**

(**A-E**) Diurnal variations of selected metabolites in mouse tissues under chow or HFD (mean±SEM; n=5 per group per time point). (**F**) Simplified scheme showing interrelationships between HFD-induced metabolite alterations detected muscle, serum, and liver. Significantly increased metabolites are indicated by red text.

**Figure 7. Loss of circadian lipid oscillation in BAT linked to reduced purine catabolism, *de novo* oscillation of purine nucleotides, and impaired UCP1 activation.**

(**A**) Representative diurnal variations of substrates from the inter-organ triglyceride/fatty acid cycle in relevant tissues (mean±SEM; n=5 per group per time point). “Fuzzy plots” of all detected medium and long-chain nonesterified fatty acids (NEFAs), indicate the range (colored area) between minimum and maximum abundance for all medium and long-chained NEFAs. (**B**&**C**) Diurnal variations of BAT purine nucleotides and nucleosides (mean±SEM; n=5 per group per time point). GMP reductase (GMPr), converts GMP to IMP. (**D**) Scheme showing how metabolites of BAT purine catabolism are interrelated and altered under HFD. Metabolites significantly increased by HFD are indicated by red text, while significantly reduced metabolites are blue. Black metabolites are unchanged, and grey metabolites were not measured. Enzymes are shown as pink boxes, and are all coded by circadian genes in BAT. (**E**) Diurnal variations of NAD+ metabolites (mean±SEM; n=5 per group per time point). Nampt and Sirt3 are coded by circadian genes in BAT. (**F**) Diurnal variations of selected purine catabolism metabolites in BAT (mean±SEM; n=5 per group per time point). (**G**) Respiration driving proton leak measured in isolated mitochondria from control HEK293 cells (empty, left panel) or HEK293 cells ectopically expressing mouse UCP1 (middle panel) in the presence of selected purine nucleotides. Note that the upward shift in leak kinetics in the presence of UCP1 is prevented to a similar extent by GDP and GMP addition. Right panel shows data plotted at the highest common membrane potential.

**Experimental Procedures**

**Animals, diets and tissue collection**

Six week old C57BL6/J male mice were purchased from JAX / Jackson Labs (Stock Number: 000664). Experiments were performed in accordance with the institutional animal care and use committee guidelines at the University of California at Irvine. Mice were maintained on a 12hr light/12hr dark cycle (ZT0 corresponds to lights on and ZT12 to lights off in the animal facility), and fed *ad libitum* for 10 weeks with either standard chow diet (Prolab RMH 2500) or high fat diet (HFD) composed of 60% Kcal from fat (OpenSource DIETS, D12492). Body weight was measured weekly. Animals were separated into individual cages 1 week before sacrifice. Five male mice for each time point/diet were euthanized by cervical dislocation, and tissues immediately collected and stored at -80°C until further processing/analysis. Serum was prepared from an abdominal/thoracic blood sample and stored at -80°C. Sperm was collected after swimming out from the caudal portion of the epididymis in non-capacitating media (MEM without BSA) for 10min at 37°C. Sperm cells were then washed with PBS twice at low centrifugation speed and the sperm pellet was rapidly frozen in liquid nitrogen and stored at -80°C.

**Global metabolite profiling**

Non-targeted metabolite profiling, peak identification, and curation was performed by Metabolon (Durham, NC, USA) and by the Genome Analysis Center (GAC), Helmholtz Zentrum München (Neuherberg, Germany). Liver, serum, and sperm were processed and run by Metabolon on an HD3 system using described methods (Abbondante et al., 2016; Eckel-Mahan et al., 2012). Briefly, this analytical system combines a Linear Ion Trap MS/MS (LTQ XL, Thermo Scientific) coupled with UPLC (Acquity, Waters), and consisted of 2 reverse phase (RP)/UPLC-MS/MS methods: 1) with positive ion mode electrospray ionization (ESI) optimized for acidic species, and 2) with negative ion mode ESI optimized for basic species. An additional GC/MS platform for volatile compounds was used in parallel. WAT and BAT samples were processed and run by the GAC on the same analytical system, with the exception of the GC/MS platform, and with curation again performed by Metabolon. Skeletal muscle and brain tissues (SCN & mPFC) were processed and run by Metabolon on their HD4 platform, which runs with High Resolution Accurate Mass (HRAM) MS/MS (QExactive, Thermo Scientific) also coupled with UPLC (Acquity, Waters) as detailed in the the supplementary methods.

Overall, we processed and analyzed a total of 70 tissues each of liver, serum, BAT, and WAT (5 replicates x 2 groups x 7 time points, including additional time point ZT24), and 60 tissues each for SCN, mPFC, gastrocnemius skeletal muscle, and sperm (5 replicates x 2 groups x 6 time points). One biological replicate each from chow-fed SCN at ZT20 and from HFD-fed mPFC at ZT4 were lost during sample processing, leaving 4 remaining replicates each for these particular time points/diets. Two biological replicates from chow-fed mPFC at ZT4 were likewise lost, leaving 3 remaining replicates.

**Metabolomics data processing and analysis**

For further analysis, we used raw metabolomic intensity data (“origscale”). Data processing closely followed procedures reported in (Shin et al., 2014). Run day correction was performed for each metabolite by setting the run day medians equal to 1. We removed metabolites with more than 50% missing values and transformed data to log10. Data points outside 4 times the standard deviation for each metabolite were considered as outliers and removed. Missing data were imputed by k-nearest-neighbor. (Missing Values: 5% BAT, 25% BrainPFC, 5% BrainSCN, 5% Liver, 17% Muscle, 6% Serum, 17% Sperm, 8% WAT).

**Statistical analysis of metabolites**

Heatmaps were generated using the median of 5 replicates for each time point. Hierarchical clustering was performed with euclidean distance and Ward’s minimum variance linkage algorithm. Metabolites were categorized according to Metabolon superpathways: Amino Acids, Carbohydrates, Cofactors & Vitamins, Energy, Lipids, Nucleotides, Peptides and Xenobiotics. To identify metabolites that show significant change over time and/or diet we used a linear regression model of the formula $y \~ β\_{0}+ β\_{1}\*time+ β\_{2}\*diet+β\_{3}\*diet\*time+ε$. Significance of each fixed effect term in the linear model was estimated using ANOVA. Enriched KEGG pathways were identified using hypergeometric distribution test. Subpathway enrichments were calculated using Fisher’s exact test. To identify 24-hr cycling metabolites, we used the nonparametric test JTK\_CYCLE (Hughes et al., 2010) using an adjusted p<0.05 as described in (Abbondante et al., 2016; Eckel-Mahan et al., 2012). Subpathway enrichment analyses were performed applying Fisher’s exact test. For data entered into CircadiOmics, the rhythmicity test, BIO\_CYCLE is also used to define circadian oscillations, as described in (Agostinelli et al., 2016).

**Robust Correlations**To identify significant temporal correlations among metabolites in each tissue, we applied a robust permutation test, performing 100 random permutations of the replicate samples and estimated correlation coefficient and significance (Pearson correlation). Metabolites with a correlation pvalue < 0.05 in 95% (99% within tissue circos plots) of all permutation tests were considered as significant. Cross-tissue correlation networks were generated by connecting metabolites that show significant positive and negative correlations between tissues (Bonferroni corrected). Calculations were done using MATLAB R2016b, Statistics Toolbox.

***In vitro* functional characterization of proton leak kinetics**

HEK293 cells with stable expression of mouse UCP1 were generated as described (Jastroch et al., 2012). Mitochondria were isolated and proton leak kinetics measured according to (Jastroch, 2012). In brief, 0.35 mg/ml of mitochondrial protein were incubated in buffer containing 0.3% defatted bovine serum albumin with either 500 uM inositol-monophosphate (IMP), or guanosine-monophosphate (GMP), or guanosine-diphosphate (GDP) for seven minutes before energization (all from Sigma-Aldrich).

**BAT qPCR**

BAT mRNA was prepared from whole BAT tissue using Trizol (Invitrogen, 15596018). 1µg of RNA was used to synthesize cDNA using the Maxima H Minus cDNA Synthesis Master Mix (Thermo Scientific, M1662) and with an extension time of 60 min. at 50°C. qPCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, 1725270) using the following parameters:

1. 95°C- 10 min.
2. 95°C- 30 sec.
3. 60°C- 1 min.
4. 40 cycles of steps 2 and 3
5. 95°C- 15 sec.

Amplification was performed using a QuantStudio 3 Real-Time PCR System. Analysis was performed using the 2ΔΔ*CT* method and data were normalized relative to *18SrRNA* expression.

**qPCR Primers sequences**

|  |  |
| --- | --- |
| Primer | Sequence |
| *mBmal1\_F* | 5'-GCAGTGCCACTGACTACCAAGA-3' |
| *mBmal1\_R* | 5'-TCCTGGACATTGCATTGCAT-3' |
| *mClock\_F* | 5'-ACCACAGCAACAGCAACAAC-3' |
| *mClock\_R* | 5'-GGCTGCTGAACTGAAGGAAG-3' |
| *mPer2\_F* | 5'-CGCCTAGAATCCCTCCTGAGA-3' |
| *mPer2\_R* | 5'-CCACCGGCCTGTAGGATCT-3' |
| *mDbp\_F* | 5'-AATGACCTTTGAACCTGATCCCGCT-3' |
| *mDbp\_R* | 5'-GCTCCAGTACTTCTCATCCTTCTGT-3' |
| *mRevErba\_F* | 5'-AGGCTGCTCAGTTGGTTGTT-3' |
| *mRevErba\_R* | 5'-CTCCATCGTTCGCATCAATC-3' |
| *mNampt\_F* | 5'-GGTCATCTCCCGATTGAAGT-3' |
| *mNampt\_R* | 5'-TCAATCCAATTGGTAAGCCA-3' |
| *mSirt3\_F* | 5'-TGCTACTCATTCTTGGGACCTC-3' |
| *mSirt3\_R* | 5'-GGGCACTGATTTCTGTACTGC-3' |
| *mUcp1\_F* | 5'-CCGAAACTGTACAGCGGTCT-3' |
| *mUcp1\_R* | 5'-CCGAGAGAGGCAGGTGTTTC-3' |
| *mImpdh1\_F* | 5'-CCATGATGTACTCAGGAGAGC-3' |
| *mImpdh1\_R* | 5'-ACCCGTAGTGCAAATCTGTGG-3' |
| *mGmpr\_F* | 5'-GGCAGAAGCTGAAACTCT-3' |
| *mGmpr\_R* | 5'-TCCACGTCCCCTTTGTAA-3' |
| *18S\_rRNA\_F* | 5'-CGCCGCTAGAGGTGAAATTC-3' |
| *18S\_rRNA\_R* | 5'-CGAACCTCCGACTTTCGTTCT-3' |

**Data availability**

Metabolomics data are available from CircadiOmics (<http://circadiomics.igb.uci.edu>).