Impact of *Retinoic acid induced-1* (*Rai1*) on Regulators of Metabolism and Adipogenesis

The mammalian system undergoes ~24 hour cycles known as circadian rhythms that temporally orchestrate metabolism, behavior, and physiology through the manipulation of a complex transcriptional circuitry. In mammals, a central "master" clock located in the SCN of the hypothalamus generates signals that control peripheral clocks in other tissues of the body to regulate body temperature, hormone product, sleeping and feeding patterns, and other biological activities. Given the complexity of this regulator **(Fig 1)**, it is not surprising that differential expression of clock components may have widespread effects on downstream genes, notably those that regulate energy metabolism, an idea supported by the observation that *Clock* gene mutant mice develop obesity and metabolic syndrome (Turek *et al.* 2005). A recent study in the Elsea lab demonstrated that alteration of transcription factor *Retinoic acid induced-1* (*RAI1*) impacts the expression of *CLOCK*, leading to a wide number of abnormalities (Williams *et al.* 2012). Haploinsufficiency of *RAI1* is the primary cause of Smith-Magenis syndrome (OMIM 182290), a complex congenital disease characterized by neurobehavioral complications, craniofacial abnormalities, obesity, and an inverted circadian rhythm (Elsea & Williams 2011). A large portion of SMS patients exhibit aberrant fat distribution leading to truncal obesity. Mouse models of SMS also display obesity along with high levels of leptin and corticosterone **(Fig 2).** Moreover, *Rai1*-Tg (over expressing *Rai1*) mice are underweight, indicating the importance of *Rai1* dosage (Burns *et al.* 2010). Interestingly, SMS patients do not have an increased risk for metabolic syndromes, including insulin resistance

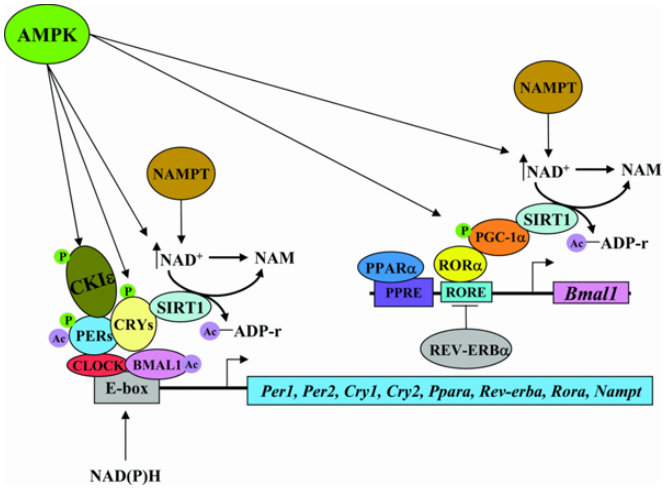

Fig 1. The transcriptional circuitry that integrators circadian oscillator and metabolism (Froy, 2011)

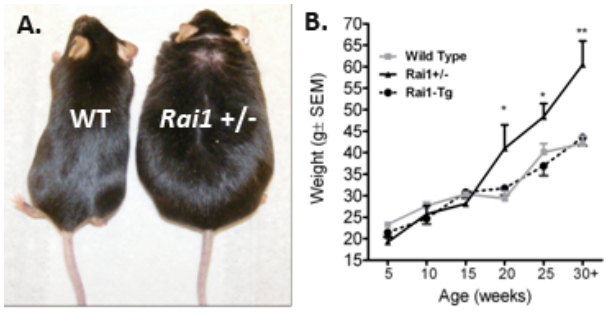

Fig 2. (A) Wild type (normal littermates) and *Rai1*$^{+/-}$ (heterozygous) mice of same age are shown. Older *Rai1*$^{+/-}$ animals may grow to >70 g. **(B)** Shown are mouse weights at 5-30 weeks of age. Older *Rai1*$^{+/-}$ animals may grow to >70 g (Burns *et al.* 2010).

and diabetes, suggesting a more complicated mechanism of pathogenesis. Despite preliminary data regarding *Rai1* dosage on levels of several circadian and metabolic genes, the etiology of the metabolic complications observed in persons with SMS remains largely unexplored. *Rai1* regulates the core components of the circadian clock, but how does *Rai1*dosage impact its downstream transcriptional circuitry involved in metabolic regulation? In the present work, we propose to investigate the expressional dynamics of key metabolic genes in *Rai1* haploinsufficient and over-expressing mouse metabolic tissues, using wet-lab and computational approaches. We will also conduct a histological analysis of adipose and liver tissue to investigate lipid accumulation **(Fig 3)**. The data generated from the following experimental plan will establish a foundation for designing future studies regarding potential treatments targeting affected pathways to systemically alter circadian regulators and facilitate healthy metabolism in persons with SMS.

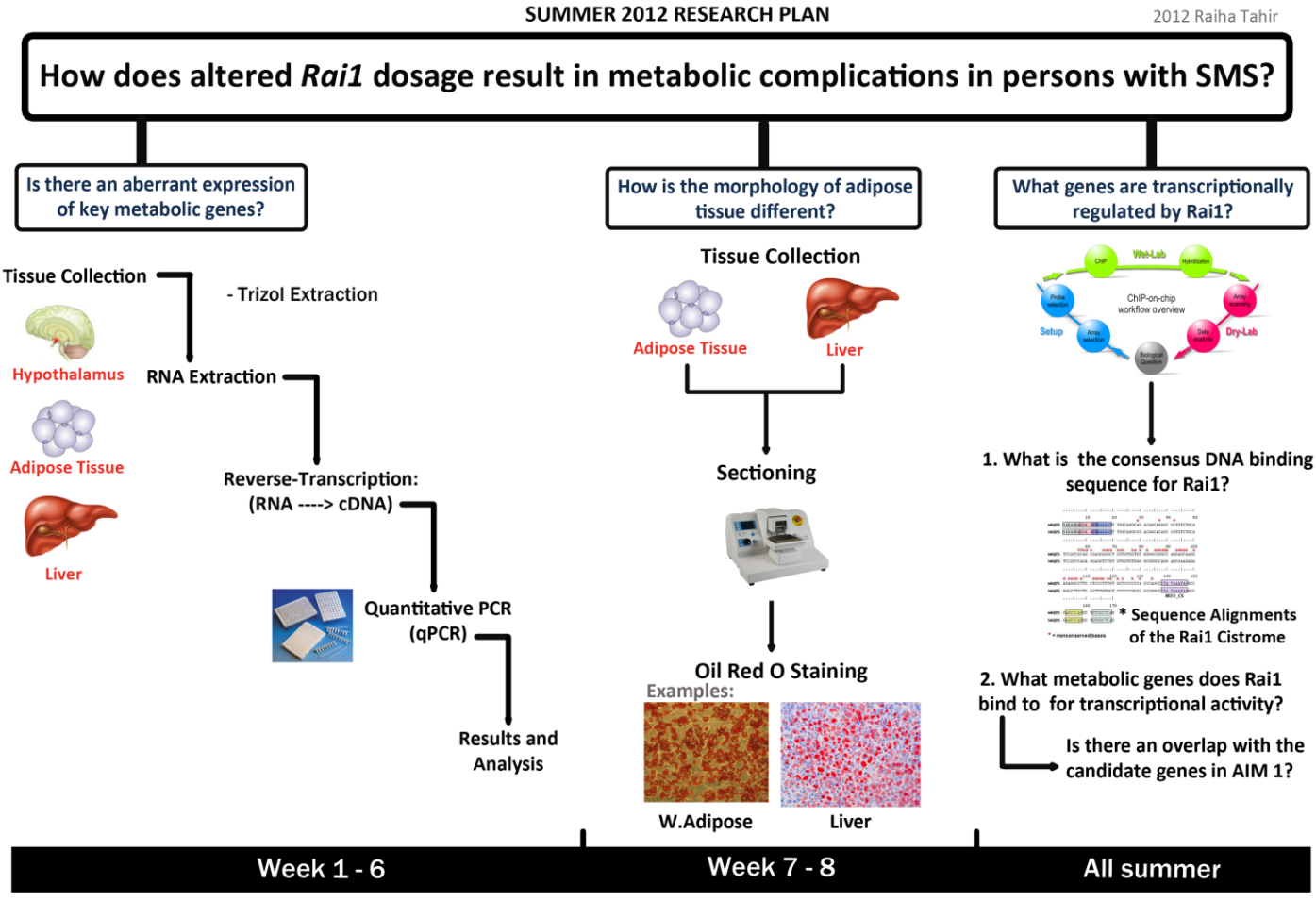

Fig 3: A summary of the work plan to investigate our research question regarding *Rai1* and its impact on metabolic processes. The plan is designed to reach three main aims that will be reached through the shown work flow.

Aim 1: Determining the expression levels of key metabolic genes (6 weeks)

The transcription network at the intersection between the central circadian regulator and the peripheral metabolic tissues is complex, and to begin understanding the role of *Rai1* dosage in metabolic regulation, we must first determine its impact on the key elements of this network. Therefore, we propose to assess the expression levels of several key metabolic regulator as well as adipocyte-specific genes in *Rai1*+/- and *Rai1*-Tg mice tissue **(Table 1)**. White adipose tissue (WAT), muscle, and hypothalamic tissues, key metabolic sites in humans and mice, will be utilized for this assessment. Tissues will be collected from 3-5 mice for each genotype

Metabolic Regulator Genes	
Genes	**Function**
PPAR-γ (Pparg)	Differentiation of adipocytes
Rev-erbβ (Nr1d2)	Links circadian rhythm and metabolism
PGC1-α (Ppargc1a)	Mitochondrial biogenesis

Adiposity Genes	
Genes	**Function**
Srebf1	Lipid homeostasis
Pepck	Gluconeogenesis
Fabp4	Fatty acid uptake/transport

Table 1: Key metabolic regulator and adiposity genes for this investigation. Expression levels of these genes will be assessed in *Rai1* dosage altered tissues from mice.

in accordance with standard protocol and frozen at -80°C. In order to implement this project, I will obtain training and approval to work on Dr. Elsea's IACUC protocol (AM10101) prior to summer. The expression levels of these genes will be determined using quantitative real-time PCR. Total cellular RNA will be extracted from all three tissues using RNeasy kit (Qiagen protocol), and then reverse transcribed to obtain cDNA. Left and right primers for each gene in Table 1 will be designed using Taqman assays. qPCR results will be analyzed using the software accompanying the qPCR machine. This study will result in graphical data that will quantify the expression each chosen gene relative to expression in WT mouse tissues. Given the regulatory role of *Rai1* on the *Clock* gene, our current prediction is that many of these downstream metabolic and adiposity genes will also show aberrant expression levels. These data will allow us to do more complicated analysis and focus future studies on manipulating these genes in an effort to alter the dynamics of the affected pathways.

Aim 2: Assessing the characteristics of *Rai1* altered adipose tissue (2-3 weeks)

In addition to investigating the molecular changes that occur due to altered *Rai1* dosage, we will conduct a phenotypic analysis of the adipose tissue from *Rai1* heterozygous and transgenic mice. Currently published work shows that adipocytes in *Rai1* haploinsufficient tissue show consistent size and seem to be "full" relative to WT fat cells (Burns *et al*. 2010). Interestingly, an increased number of evenly shaped fat cells are

seen in a given section of tissue rather than adipocytes enlargement, which is normally observed due to excess adiposity. This perhaps indicates increased lipid accumulation, and can be easily investigated using an Oil Red O (ORO) staining protocol that shows lipid localization in tissues. Frozen or fixed adipose tissue will be sent to VCU Pathology lab for sectioning and will be subsequently stained using a standard protocol. Fat will be seen as red droplets in the tissue, with a blue nuclear counter stain. This visualization will help us evaluate the lipid accumulation in *Rai1* deficient or over-expressing adipose tissue, and based on results, various other staining might also be conducted to further characterize the cell count and morphology in these tissues.

Aim 3: Identifying a consensus DNA binding site sequence for *Rai1* (All summer)

Alongside investigations using wet-lab techniques, it will be essential to approach our research question from an *in silico* perspective through analysis of data available regarding *Rai1* transcriptional activity at the genome-wide scale. What promoters does Rai1 protein bind to? Which of these genes are involved in metabolism? The answers can be explored through computational analysis of Chromatin Immunoprecipitation with microarray (ChIP-Chip) data sets that are currently available to the Elsea lab. Briefly, this data contains information regarding Rai1 protein interaction with functional elements in the mouse genome, and has DNA sequence of sites that *Rai1* protein binds to in an *in vivo* environment. Determination of a consensus binding site sequence for *Rai1* using these sequence fragments will allow us to search promoters of metabolic genes in other genomes and identify the direct targets of *Rai1* transcriptional activity. This can be done by generating sequence alignments using ClustalW2, and visualizing them using a Jalview platform. It will be of interest to see whether some of the candidate genes from this analysis overlap with the differentially expressed genes we identify through q-PCR. In addition, we will generate a list of all metabolic genes that are reported in the ChIP-chip data sets and classify them based on their role in various metabolic processes. Comparison with a previous microarray data set, which lists genes that are differentially expressed when *Rai1* is down regulated in mouse hypothalamus, will be crucial during this analysis, and we expect to find *Rai1* associated genes that are common to both data sets.

Work Plan and Future Studies

Assuming 20 – 40 hours in the lab, a tentative work plan for each week is shown in **Table 2**. A mid-term project report regarding the data obtained from this ten week research investigation will be submitted August 1, 2012 and the research project results will be presented at the VCU Undergraduate Symposium/Poster Day in Spring 2013. This study, however, will not conclude with these three aims. Based on the preliminary data that will be obtained this summer, we will be able to determine additional lines of study. Integration of expression data will help determine if certain metabolic pathways are affected. If so, we can design studies that alter mouse feeding schedule, etc, to see whether we can manipulate the circadian-metabolic oscillator and therefore the expression of downstream genes that innervate the affected metabolic pathways.

	Aim*	Methodology
Week 1:	1	Design primers for target genes: Taqman Assays
Week 2:		Collect tissues from transgenic mice
Week 3:		Collect tissues from wild type mice
Week 4:		RNA Extraction from tissues: preparation of cDNA
Week 5:		qPCR and expression data analysis
Week 6:		Result replication and Analysis **(Starting preparation of mid-term report)**
Week 7:	2	Adipose tissue sectioning at the VCU Pathology Dept.
Week 8:		Oil Red O staining protocol
Week 9:		Analysis and imaging of stained tissues
Week 10:		Submit mid-term report on August 1st.
*Aim 3 (ChIP-Chip data analysis) is not included as it will be conducted all summer.		

Table 2: A detailed 10-week work plan for implementation of the proposed work.

References:

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2. Burns, Brooke, et al. "Rai1 haploinsufficiency causes reduced Bdnf expression resulting in hyperphagia, obesity and altered fat distribution in mice and humans with no evidence of metabolic syndrome." *Human Molecular Genetics* 19.20 (2010):4026-4042.
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4. Nadler, S T, et al. "The expression of adipogenic genes is decreased in obesity and diabetes mellitus." *Proceedings of the National Academy of Sciences* 97.21 (2000):11371-11376.
5. Turek, Fred W, et al. "Obesity and metabolic syndrome in circadian Clock mutant mice." *Science* 308.5724 (2005):1043-1045.
6. Williams SR, Zies, D, Mullegama SV, Grotewiel MS, Elsea SH 2012. Smith-Magenis syndrome results in disruption of CLOCK gene transcription and reveals an integral role for RAI1 in the maintenance of the circadian rhythmicity. (Manuscript Submitted).