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Nocturnin: A Circadian Target of Pparg- Induced Adipogenesis

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Abstract

Nuclear receptors (NRs) control cell fate and regulate tissue function. Some of the NRs are expressed in a circadian and tissue specific manner. Clock genes are part of the circadian network and fine tune gene expression in adipose and skeletal tissues. Pparg, a master transcription factor that determines adipogenesis exhibits a circadian expression pattern in white adipose tissue and liver. In this paper we found that message and protein for a peripheral clock gene, nocturnin, is markedly up-regulated with Pparg activation in adipocytes and bone marrow stromal cells. Nocturnin is also expressed in relatively high amounts in other tissues which may have physiologic relevance for bone, including the brain and hypothalamus. Importantly, we found polymorphic strain differences in bone marrow nocturnin expression that relate to phenotypic determinants of skeletal acquisition. Defining the function of nocturnin in peripheral tissues should provide new insights into lineage allocation and the intimate relationship between nuclear receptors and physiologic timekeeping.

Introduction

Obesity has grown to epidemic proportions in the United States and around the world. The consequences of energy excess are manifested in multiple tissues although the cardiovascular system is particularly vulnerable, in part because of impaired lipid disposal and inflammatory cytokine release from adipocytes. The origins of obesity are multifactorial and include genetic, environmental and hormonal determinants, although the final common pathway of this disease can be traced to adipogenic function. Adipocytes originate from a multipotent stem cell that can differentiate into adipocytes, chondrocytes, osteoblasts or myocytes. These cells exist in the bone marrow as well as most adipose depots, and their recruitment occurs during environmental stresses or hormonal manipulation [1]. Osteoporosis is also a disease that is growing in prevalence. Its origin is multifactorial and like the adipocyte, the osteoblast is often dysfunctional [2]. In particular, age-related osteoporosis is characterized by reduced osteoblast- mediated bone formation accompanied by increased Pparg expression and adipocytic infiltration of the bone marrow [3]. These features have raised the intriguing possibility that under certain circumstances marrow stromal cells can preferentially enter the adipogenic rather than the osteogenic lineage.

Timekeeping in mammals modulates many homeostatic processes including adipogenesis [4]. Its main pathway is through the master ‘clock’ genes, Bmal1 and CLOCK, which are regulated by Cry and Per genes in an antiphase pattern. Clock genes are expressed in multiple tissues, including bone marrow stromal cells and provide ‘real-time’ regulation in response to cyclic changes in the physical environment [5]. The master oscillator in the suprachiasmatic nucleus integrates light and hormonal signals and uses the sympathetic nervous system to entrain peripheral clock gene expression. However, a growing body of evidence supports a hypothalamic- independent peripheral role for these genes in adipose and skeletal tissue, serving as immediate sensors for external cues such as feeding and exercise [6]. Not surprisingly, more than 25% of genes in the calvarial bone transcriptome

exhibit circadian rhythms, a proportion similar to that shown for adipose tissue [7]. Furthermore, circadian dysfunction is implicated in the pathogenesis of obesity, Cushings' disease, and the metabolic syndrome.

Members of the nuclear receptor (NR) superfamily can influence the expression patterns of critical genes in peripheral tissues through circadian pathways [8]. For example, Pparg2 in white but not brown adipose tissue, and in liver, has a circadian pattern of expression which is accentuated by a high fat diet [9]. Expression of Rev-erb α and β, orphan nuclear receptors that are transcriptional suppressors, are also circadian in nature, up-regulated by Bmal1 and CLOCK and suppressed by Per and Cry [10]. Moreover, Rev-erb α and β are markedly enhanced during 3T3-L1 growth in dexamethasone/IBMX/insulin supplemented conditioned media, or with rosiglitazone [9,10]. These observations support the hypothesis that specific NRs can integrate physiologic events from external environmental cues. Overall, the control of gene expression in adipose tissue involves a series of signals from transcription factors which are modulated by the influence of circadian factors. In this paper, we found that a circadian clock gene, *nocturnin,* is up-regulated not only by CLOCK and Bmal1 but also by Pparg2. Furthermore, in this study we propose that *nocturnin* is an important downstream effector of adipogenic differentiation.

Material and Methods

Mice

All strains utilized for the studies reported herein were obtained from research colonies at The Jackson Laboratory, Bar Harbor, Maine. All mice were produced by pair matings, with progeny weaned at 22-25 days of age and housed in groups of 2-5 of the same sex in polycarbonate cages (324 cm^2) with sterilized White Pine shavings. Colony environmental conditions included 14:10 hour light:dark cycles, with free access to acidified water (pH 2.5 with HCl to retard bacterial growth) that contains 0.4 mg/ml of vitamin K (menadione Na bisulfite), and irradiated NIH31 diet containing 6% fat, 19% protein, Ca:P of 1.15:0.85, plus vitamin and Mineral fortification (Purina Mills International, Brentwood, MO). All procedures involving mice were reviewed and approved by the Institutional Animal Care and Use Committee of The Jackson Laboratory.

B6.C3H.6, i.e..6T mice were generated as previously described by introgressing a region of mouse chromosome 6 from C3H/HeJ onto C57BL/6J mice for ten generations [11, 12]. The 6T congenic has low bone mass, increased marrow adiposity, reduced cortical thickness and impaired osteoblast differentiation in vitro [13]. Only female 6T mice at 16 weeks of age were used for gene expression studies of bone marrow and liver and the expression patterns were compared to B6 and C3H female mice of the same age. Mice were genotyped by preparing genomic DNA from digestion of 1 mm tail tips in 0.5 ml of 50 mM NaOH for 10 min at 95° C, then pH was adjusted to 8.0 with 1M Tris-HCl. Genotyping of individual mouse DNAs was accomplished by polymerized chain reaction (PCR) using oligonucleotide primer pairs (Mit markers, www-genome.wi.mit.edu/cgi-bin/mouse/index) from several sources (Research Genetics, Birmingham, AL; Invitrogen, Carlsbad, CA; IDT, Coralville, IA; Qiagen, Valencia, CA). These primer pairs amplify simple CA repeated sequences of anonymous genomic DNA that are of different sizes and, via gel electrophoresis, can discriminate between B6 and C3H genomes.

Skeletal Phenotyping

6T mice were phenotyped by micro CT (SCANCO-40) for trabecular bone volume fraction and cortical thickness as described previously[11-13]. Briefly, femurs were scanned using a Micro CT40 Microcomputed Tomographic instrument (Scanco Medical AG, Bassersdorf,

Switzerland) to evaluate trabecular bone volume fraction and microarchitecture in the secondary spongiosa of the distal femur. Daily quality control of the instrument's operation was checked with a manufacturer-supplied phantom. The femurs were scanned at low-resolution energy level of 55KeV, and intensity of 145 μA. Approximately 100 slices were measured just proximal to the distal growth plate, with an isotropic pixel size of 12 μm and slice thickness of 12 μm. Trabecular bone volume fraction (%BV/TV) and microarchitecture properties were evaluated in the secondary spongiosa, starting ~ 0.6 mm proximal to the growth plate, and extending proximally 1.5 mm. Marrow fat was measured by counting adipocyte 'ghosts' by H+E staining on plain histological samples.

Gene expression—Primers for the following genes were used for real time quantitative PCR using MacVector sequence analysis package (Version 7.1.1, Accelrys Inc):

NOC: Forward: acc agc cag aca tac tgt gc; Reverse: ctt ggg gaa aaa cgt gcc t Size: 101 bp

Pparg2: Forward: aaa ctc tgg gag att ctc ctg ttg Reverse: gaa gtg ctc ata ggc agt gca Size: 70bp Cycle sequencing of PCR-DNA templates was performed using Applied Biosystems' BigDye Terminator v3.1 cycle sequencing kit. Sequencing reactions were then purified using Agencourt's CleanSEQ magnetic bead purification system. Purified reactions were run on Applied Biosystems 3730×l DNA Analyzer using POP 7 polymer. Raw data files are analyzed using Applied Biosystems DNA Sequencing Analysis Software, Version 5.2.

Micro-array

Studies of gene expression in liver, neonatal calvariae, and bone marrow of 6T, B6 and C3H as well as U-33 cells transfected with Pparg and exposed to rosiglitazone were performed using the following protocol as described previously [14]. Liver, marrow flush, or calvarial osteoblast mRNA was collected from three 8wk old B6, three C3H, and three 6T female mice. Mice were fasted for 5 hours before tissue collection. Tissue samples were stored in RNAlater (Ambion, Austin, TX) following dissection and later homogenized in TRIzol (Invitrogen, Carlsbad, CA). Total RNA was isolated by standard TRIzol methods according to the manufacturer's protocols, and quality was assessed using a 2100 Bioanalyzer instrument and RNA 6000 Nano LabChip assay (Agilent Technologies, Palo Alto, CA). Following reverse transcription with an oligo(dT)-T7 primer (Affymetrix, Santa Clara, CA), double-stranded cDNA was synthesized with the Superscript double-stranded cDNA synthesis custom kit (Invitrogen). In an in vitro transcription (IVT) reaction with T7 RNA polymerase, the cDNA was linearly amplified and labeled with biotinylated nucleotides (Enzo Diagnostics, Farmingdale, NY). Fifteen micrograms of biotin-labeled and fragmented cRNA was then hybridized onto MOE430v2.0* GeneChip™ arrays (Affymetrix) for 16 hours at 45°C. Post-hybridization staining and washing were performed according to manufacturer's protocols using the Fluidics Station 450 instrument (Affymetrix). Finally, the arrays were scanned with a GeneChip™ Scanner 3000 laser confocal slide scanner. The images were quantified using GeneChip(TM) Operating Software(GCOS) v1.2. Probe level data were imported into the R software environment and expression values were summarized using the Robust MultiChip Average (RMA) function in the R/affy package as previously described.[15] Using the R/maanova package, an analysis of variance (ANOVA) model was applied to the data, and F1, F2, F3 and Fs test statistics were constructed along with their permutation p-values. False discovery rate was then assessed using the R/q value package to estimate q-values from calculated test statistics [15]. The data in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible: GEO Series accession number GSE5959.

Data Bases and Sequencing Manual sequencing was performed by standard methods at The Jackson Laboratory for the nocturnin gene as previously described. In silico analyses were performed using several data bases:

Gene expression studies used the following data base: http://biogps.gnf.org/#goto=welcome;

SNPs in the nocturnin gene

http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn=snps/door

Western Blot—Immunoprecipitation of nocturnin was performed by western blot using a polyclonal antibody donated by Dr. C Green.

Statistics

For gene array studies ANOVA with F statistics were applied. For strain differences in gene expression between C3H and B6, similar applications were used. For comparisons of western blot density between differentiated and undifferentiated adipocytes and for mRNA expression of nocturnin, students' t test was applied with a p value <0.05 considered significant.

Results

Micro array of 6T liver, bone and marrow reveal common downstream pathways for Pparg2

Phenotypic examination of the female congenic 6T mouse which carries a 30cM region of C3H genome on a 99% B6 background, demonstrated a lean animal with enhanced insulin sensitivity but low bone mass, increased marrow and hepatic adiposity and impaired osteoblast differentiation [12, 13]. Previous genetic studies in the 6T mouse established there was a 'gain of function' polymorphism in the Pparg gene on mouse Chr 6 [16]. This contributed to a unique metabolic phenotype which included insulin sensitivity but significant bone loss following high fat feeding[16]. To address the question of which downstream genes were activated in this metabolic phenotype, we performed micro-array gene expression studies in livers from B6 and 6T mice [12,13]. Not surprisingly, genes related to lipid storage and Pparg activation were up regulated (e.g. *Srebp, Spot 14, Scd and Fasn*) significantly ($p<0.0001$) in 6T, while genes associated with cell proliferation (*c-jun, Igf1, Igf2,Egfr)* were markedly suppressed ($p<0.0001$). When we examined gene array profiles from bone marrow of 6T and B6, as well as calvarial OBs, we once again found that the most highly expressed genes were associated with adipogenesis; these included *Fasn, Srebp, Pparg,* and *Spot 14.* On the other hand, the same cell proliferation genes in liver were suppressed in bone. Markers of osteoblast differentiation including Runx 2 and Msx1 were also reduced in calvarial osteoblasts from 6T compared to B6 ($p<0.05$). Most intriguingly, in all three tissues, mRNA for *Ccrn4l (nocturnin)*, a circadian clock gene, was consistently increased (2-3.5 fold, $p<0.0001$) in 6T compared to B6 littermate control tissue. Thus 6T mice exhibit up-regulation of most Pparg target genes, as well as *nocturnin* in liver, bone and fat.

Pparg activation in marrow stromal cells enhances *Nocturnin* expression

To test whether activation of Pparg in mesenchymal stromal cells affected the expression of *nocturnin*, we used U-33 cells transfected with Pparg2(U-33+g2) and exposed them to rosiglitazone as described recently [14]. Timed gene expression studies at 2, 24, 72 hrs by micro-array confirmed that lipid synthesis genes were up-regulated early and significantly while pro-osteoblastic genes such as Runx 2, Osterix and Dlx5 were markedly suppressed at 24 and 72 hours. Remarkably, *nocturnin* was enhanced 28 fold by gene array at 24 hours and

was one of the top genes affected by rosiglitazone after 72 hours in the presence of Pparg2. To confirm this finding, we performed real time PCR on the U-33+g2 cells exposed to rosiglitazone for 24 hours and found an 8 fold up-regulation of *nocturnin* compared to no treatment of the U-33+g2 cells or rosiglitazone exposure of U-33 cells without Pparg ($p<0.0001$). Furthermore, *nocturnin* mRNA was persistently increased at 72 hours to nearly 3 fold ($p<0.01$). Finally, using *in silico* data bases for the nocturnin promoter region, we were able to find a putative PPRE site. Hence, *nocturnin* is markedly upregulated by Pparg2 activation and may be an important downstream gene in the adipogenic pathway.

Nocturnin is up regulated in differentiated adipocytes

Next, we asked whether fully differentiated adipocytes expressed *nocturnin.* In 3T3-L1 cells exposed to insulin, IBMX, and dexamethasone for 4-6 days, *nocturnin* mRNA was increased more than 5 fold vs control 3T3-L1 cells not differentiated ($p<0.01$). To confirm that enhanced gene expression resulted in increased protein levels of nocturnin, we performed western blot using a polyclonal nocturnin antibody kindly provided by Dr. Carla Green, in sets of 3T3-L1 cells exposed to insulin, IBMX and dexamethasone for either 5 or 6 days. At day 5, nocturnin protein was nearly 3 fold greater in the differentiated cells than controls (data not shown). At day 6 when adipocyte differentiation was complete (Figure 1) there was two fold increase in nocturnin protein (i.e. lanes 4-6 in Figure 1) compared to undifferentiated controls (lanes 1-3). Both time points for protein changes in nocturnin corresponded to expression studies noted above. Thus we concluded that *nocturnin* is highly expressed in differentiating adipocytes.

Inbred strains exhibit genotypic and phenotypic differences in *nocturnin* expression

Previously we demonstrated that C3H/HeJ have much higher trabecular and cortical bone mass than B6 mice at the time of peak bone acquisition [17, 18]. Yet we reported this strain also had a ‘gain of function’ polymorphism in Pparg resulting in enhanced insulin sensitivity in response to high fat feeding [16]. To determine how nocturnin affected the skeletal and metabolic phenotype of B6 and C3H mice we interrogated several data bases. As shown in Figure 2, there were genotypic differences between B6 and C3H in both introns and exons of the *nocturnin* gene. Most importantly, we identified and confirmed by sequence analysis a non-synonymous SNP in exon 2 of the *nocturnin* gene in C3H (Figure 2). Next, we performed gene expression profiling for adipogenic pathways from 16 week femurs (whole bone and marrow) of C3H and B6 mice. There were significant increases ($p<0.01$) in genes located downstream of Pparg including *Fasn, Lipoprotein lipase, CD 36, Spot14, Ucp1 and Scd* in C3H vs B6 ($p<0.01$), but surprisingly two clock genes, *nocturnin* and *per1* were both markedly suppressed in C3H compared to B6 (−7.0 and −3.0 fold respectively, $p<0.01$) (see Figure 3). Thus we found significant genotypic and phenotypic differences between two inbred strains of mice in *nocturnin* expression. The marked reduction in *nocturnin* expression in the C3H mouse, despite its ‘gain of function’ in Pparg. Whether these genotypic differences are responsible for the strain specific phenotypic response to high fat feeding in these two strains remains to be determined.

Discussion

Nocturnin is a mammalian circadian deadenylase first identified in Xenopus retinae and subsequently called *Ccrn4l* [19]. It is a member of the *CCR* family of yeast transcription factors, but its structure differs from CCR members due to the absence of an N-terminal portion that contains a transactivating domain [20]. In mammals, *nocturnin* is highly expressed in liver, nervous tissue, pituitary, hypothalamus, fat, and bone marrow (http://biogps.gnf.org/#goto=welcome). In liver and fat it exhibits a circadian rhythm and its expression is regulated by CLOCK and Bmal1 through binding to the E box of the *nocturnin* promoter [21].

In this paper, we determined that *nocturnin* is not only regulated by circadian genes, but is also immediately downstream of Pparg and is highly expressed in the differentiated adipocyte. Hence we believe that nocturnin is a transcriptional target of Pparg. In addition, we found significant genotypic differences in the expression of *nocturnin* across inbred strains that may be important not only for adipocyte function and insulin sensitivity, but also skeletal acquisition.

It is still not clear how *nocturnin* functions in adipose tissue or in bone marrow mesenchymal stromal cells. Presumably it acts by deadenylating long 3′UTR mRNAs in collaboration with one or more RNA binding proteins, although its targets have not yet been defined. Interestingly, *Noc* −/− mice are similar to wild type in respect to body composition parameters albeit the loss of circadian Pparg expression in liver and protection against obesity and hepatic steatosis with high fat feeding [22]. Yet even though the Noc−/− animals are lean, there is some impairment in insulin sensitivity with a high fat diet [22]. These findings suggest *nocturnin* may play an indirect role in mediating Pparg's actions on glucose transport. Furthermore, *Noc* −/− mice have reduced basal body temperatures implying there may could be a defect in brown adipogenesis, a process controlled through activation of Pparg and the recruitment of specific so-activators [22]. Hence, a physiologic role for *nocturnin* in adipocyte differentiation is very likely. Indeed, it is conceivable, based on our data in C3H mice, that *nocturnin* may be down regulated in response to persistent up-regulation of Pparg activity as a means of fine tuning Pparg's transcriptional effects. Whether the 3′UTR of Pparg mRNA, which is quite long, itself is a target for nocturnin's enzymatic activity remains to be determined, but identification of such transcripts should provide valuable information about its relative importance in the adipogenic differentiation pathway [16].

More intriguing is the interface between adipocyte differentiation and the circadian clock system. As noted, Rev-erb is a transcriptional repressor regulated by the CLOCK/Bmal1 master oscillating genes during adipogenic differentiation[9]. *Nocturnin* is another output gene, also regulated by CLOCK/Bma1I as well as Pparg2 [19]. If these two systems worked in synchrony or in antephase, another mechanism of circadian control over adipogenesis would be plausible. Indeed, it has already been established that Pparg is expressed in a rhythmic fashion in white fat and liver [8, 22]. Further studies are needed to determine the precise pattern of nocturnin expression relative to other clock genes during both day and night. A proposed interaction between the NRs and the circadian system in adipocytes is illustrated in Figure 4.

Notwithstanding a discrete mechanism of action for *nocturnin*, several lines of evidence support an important role for clock genes in disordered fat deposition as well as impaired skeletal acquisition [23]. For example, dexamethasone synchronizes the oscillating expression of several clock genes in murine calvariae including Rev-erb α and β as well as DBP [24]. Excess glucocorticoids also cause centripetal obesity as well as low bone mass. Changes in the frequency or magnitude of circadian gene expression in both hard and soft tissues in response to glucocorticoids may underlie these long term treatment sequelae. Moreover, gene deletion of Cry and Per genes results not only in lean mice but also in a high bone mass phenotype[25]. In addition, disordered timekeeping, for example, in night shift workers or patients using second generation anti-psychotic drugs, is associated with profound changes in adipose deposition and obesity. These individuals may also have skeletal abnormalities related to altered circadian rhythms with or without changes in serum prolatin (Maloney, personal observation).

A notable finding from this study is the strain differences in *nocturnin* expression. B6 is one of the most frequently used mouse strains in laboratory studies. However, these mice have

low bone mass and a strong tendency to develop insulin resistance and obesity with age or on a high fat diet. In contrast, C3H/HeJ has high bone mass, and is less prone to diet induced obesity, possibly because of polymorphic differences in several adipogenic genes including *nocturnin*. Our finding that *nocturnin* mRNA is markedly reduced in bone and marrow of C3H compared to B6 mice and is associated with a non synonymous SNP in exon 2 is surprising considering the presence of enhanced marrow fat and increased Pparg expression in bone. These data imply that nocturnin may play an important role in regulating Pparg both in a positive (adipocyte differentiation) and negative (limiting further Pparg activity) direction. More importantly, polymorphisms in the *nocturnin* gene may have relevance for human conditions such as obesity and osteoporosis. Indeed, recent unpublished work from our collaborator, Dr. Juan Pablo Rodriguez, revealed marked up regulation of *nocturnin* in marrow stromal cells from postmenopausal women with osteoporosis compared to those with degenerative arthritis (Rodriguez, unpublished communication).

In sum, we established that the circadian de-adenylase, *nocturnin* is highly expressed in adipose tissue, is regulated by activation of Pparg, and may play an important role in lineage allocation. Studies to determine the mechanism of action of *nocturnin* in bone and fat is ongoing and should provide significant insight into the interface of nuclear receptor action with the peripheral circadian clock system.

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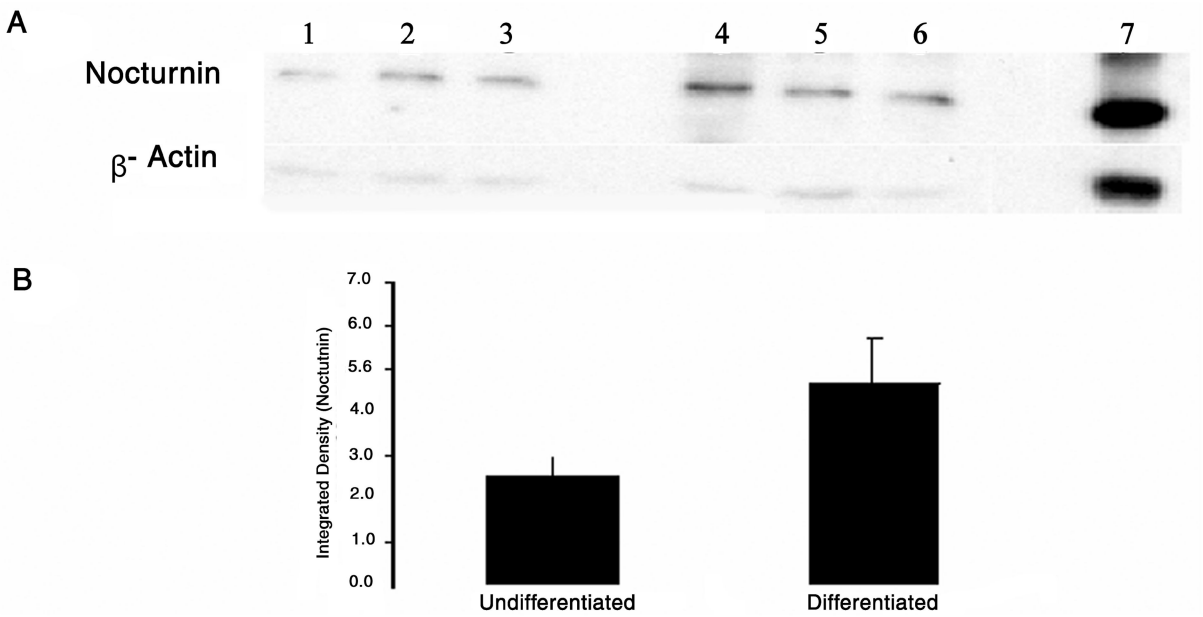

Figure 1.
Nocturnin protein is increased in adipocytes that are fully differentiated vs undifferentiated 3T3-L1 cells.

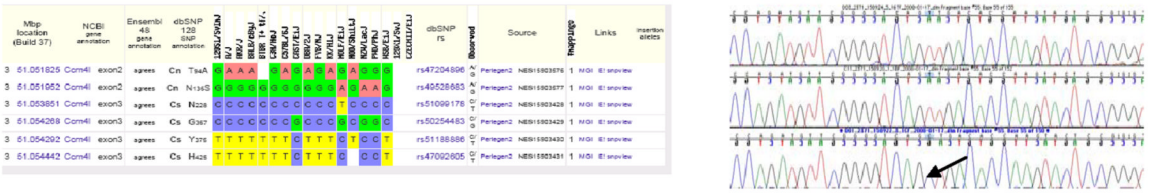

Figure 2.
Perlegen database representing a Cn (non-synonymous) SNP in exon 2 with a G (C3H) to A (B6) substitution. This was confirmed by manual sequencing of exon 2 in *Noc*. The black arrow in C3H (lowest panel) shows the G-A substitution. The two top lanes represent B6 and 6T respectively.

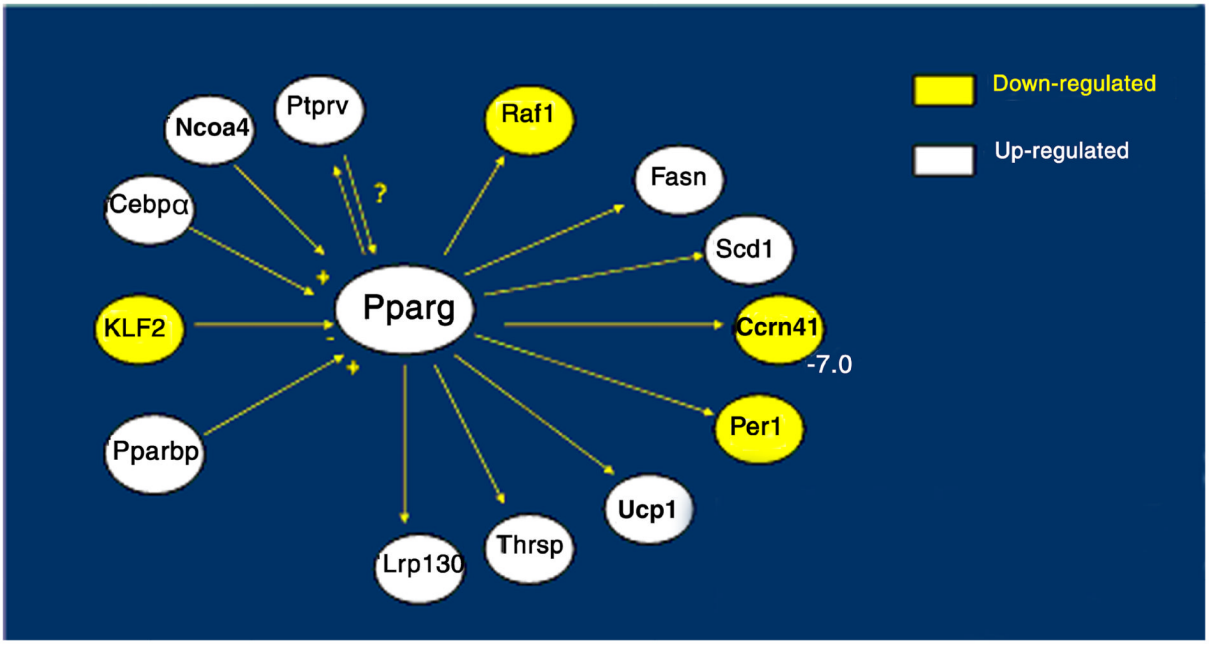

Figure 3.
Femur (Bone+ Marrow) Array of genes in the Pparg pathway of C3H mice compared to B6 mice.

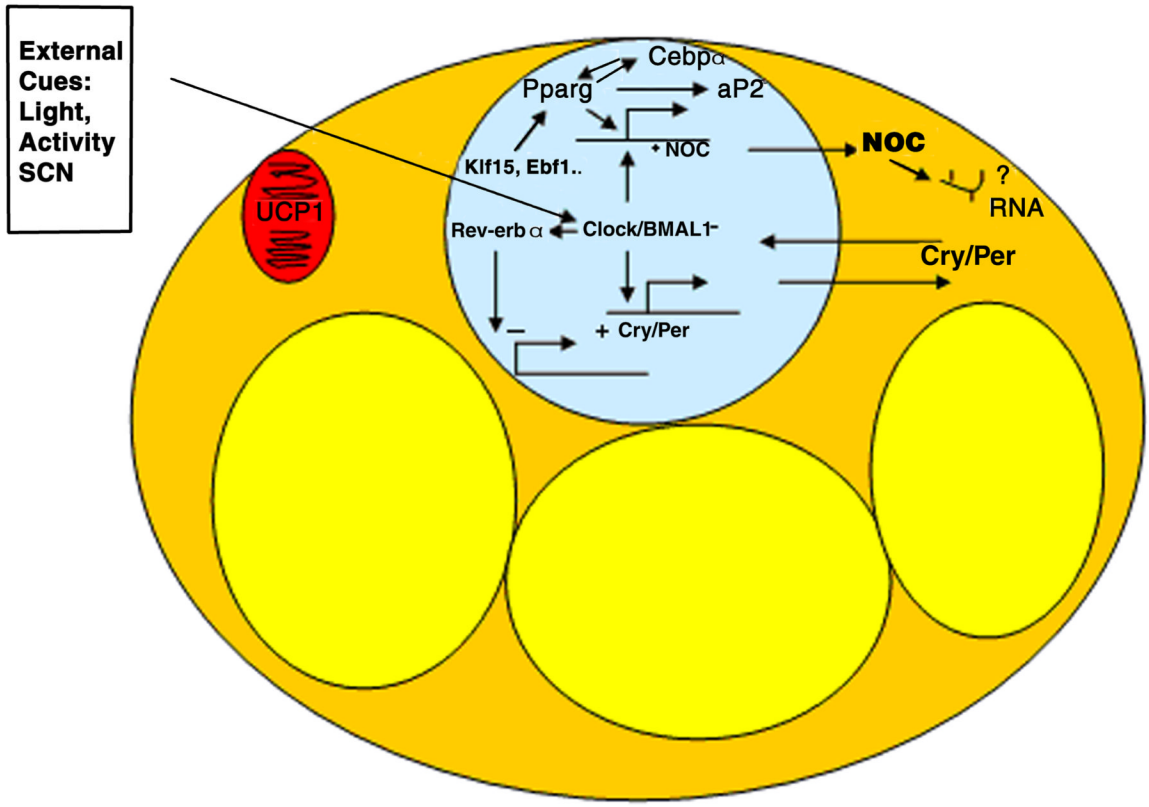

Figure 4.
The central role of Pparg and Clock Genes in Adipogenic differentiation.