Role of nuclear lamins in modulating circadian gene expression in cancer cells

A thesis submitted towards partial fulfilment of
BS MS Dual Degree programme

By

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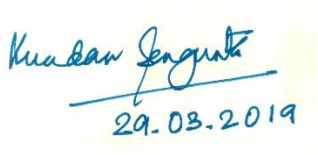
Certificate

This is to certify that this dissertation entitled “Role of nuclear lamins in modulating circadian gene expression in cancer cells” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER) Pune, represents original research carried out by Anuvind K G at CHROMOSOME BIOLOGY LAB (CBL), IISER Pune and Molecular and Epigenetic Regulation of Biological Clocks Lab, IGFL, ENS Lyon under the supervision of Dr. Kundan Sengupta, Assistant Professor, IISER Pune and Dr. Kiran Padmanabhan, CR1, IGFL, ENS Lyon during the academic year 2018-2019.



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Date: 27 March 2019

Declaration

I hereby declare that all the matter embodied in the report entitled “Role of nuclear lamins in modulating circadian gene expression in cancer cells” are the results of the investigations carried out by me at the Department of Biology, Indian Institute of Science Education and Research (IISER) Pune and the Department of Biology, Institut de Génomique Fonctionelle de Lyon, ENS Lyon, under the supervision of Dr. Kundan Sengupta, Assistant Professor, IISER Pune and Dr. Kiran Padmanabhan, CR1, IGFL, ENS Lyon and the same has not been submitted anywhere else for any other degree.

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Abstract

Circadian clocks drive the rhythmic expression of ~5-15% of expressed genes in mammals in a cell-type and tissue-specific manner. Nuclear envelope, a global regulator of the genome, interact with the circadian core-clock transcription factor BMAL1 through Man1. LBR and lamin B1 were also found to have similar regulatory roles as Man1 in regulating rhythmic gene expression. We hypothesize that B-type lamins and especially lamin B2 might modulate and have regulatory roles in clock function. Further studies also show that the expression profiles of the circadian genes differ in human colorectal cells from that of mouse embryonic fibroblasts (MEFs). Therefore clock disruption in MEFs may not be translated to clock disruption in human colorectal cancer cells. We also find that while AKT2 and KRAS show fluctuations in their expression levels across time, SNAI1 and RUNX2 levels are relatively constant. The nuclear envelope is likely to associate and regulate core circadian clock genes and maintain the periodicity of clock-controlled genes in cells.

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INTRODUCTION

1.1 Circadian Rhythm

Most organisms on Earth have a self-sustaining rhythmic clock that governs their metabolism, physiology and behaviour (Aschoff, 1981). These rhythms could span a few hours, a day, or months. The ultradian rhythms are a recurrent period repeated throughout a day. Heart rate, blood circulation and eye blinking are a few examples. These periods are shorter than a day (Wollnik, 1989). Infradian rhythms, on the other hand, are those rhythms that have durations longer than a day. Examples of these include menstrual cycle, breeding rhythms and tidal rhythms. Circadian rhythms (circa means approximately and diem means a day) are those rhythms which have a duration of about a day, i.e. 24 hours. These rhythms include the sleep-wake cycle, the body temperature cycle and hormone-secretion cycles (Schibler, 2005).

Circadian Rhythms are self-sustaining and are entrainable by external environmental cues like light, nutrition and temperature cycle. These are known as Zeitgebers (time givers) and synchronise the biological clock to that of the earth's 24-hour period of the day (Reppert and Weaver, 2002).

The Master Regulator of the circadian clock is the Suprachiasmatic Nucleus (SCN) which is at the base of the Hypothalamus. The SCN controls the circadian rhythms of molecular, endocrine, physiological and behavioural functions in the body. The SCN is synchronised to the environmental light-dark cycle through the retina. The light information reaches the SCN directly through the retinohypothalamic tract (RHT). Various studies establish that the RHT is necessary and sufficient for efficient photic entrainment of the circadian rhythm. Originating from a distinct set of retinal ganglion cells, the RHT terminates majorly in the SCN (Reppert and Weaver, 2002). The retinal ganglion cells are notably distinct from the rods and cones which give rise to the visual pathways. Normal circadian rhythms are observed in retinally degenerate strains of mice which lose nearly all rods and cones by early adulthood. Genetically engineered mice which do not have rods and cones are also observed to be light-entrainable, thanks to a third opsin - melanopsin (Hattar et al., 2003).

1.2 Mechanism of Circadian Rhythm

In mammals, the Circadian rhythm is established by a set of genes referred to as the core-clock genes. The circadian clock mechanism can be described by the Transcription Translation Feedback Loop (TTFL). The circadian transcription factors Circadian Locomotor Output Cycles Kaput (CLOCK) and Aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL) or BMAL1 form a heterodimer and bind to the E-box response elements in the promoter regions of target genes which drive the positive transcription arm of the TTFL. Two families of these target genes that encode the circadian proteins Period (PER1, PER2 and PER3) and Cryptochrome (CRY1 and CRY2) are rhythmically transcribed and translated in the cytoplasm. PERs and CRYs interact with each other and translocate back into the nucleus to inhibit the activity of the BMAL1-CLOCK complex. Eventual degradation of the PER-CRY complex leads to reactivation of BMAL1: CLOCK and a new cycle (Fig 1.1). Other accessory proteins like D-site albumin Binding Protein (DBP), and the nuclear orphan receptors retinoic acid (RORs) and REV-ERBs modulate the activity of the loop through activation and repression of BMAL1 transcription. Additional population dependent regulation of PER and Cry adds an extra layer to efficient time-keeping of the clock. Casein Kinase-1 δ/ε (CK1δ/ε) phosphorylates PER proteins and facilitates the entry of the Per-Cry complex into the nucleus. Furthermore, Protein Phosphatase 1 (PP1) dephosphorylates Per proteins resulting in their cytoplasmic localisation. Hence, the balance between CK1δ/ε and PP1 is an important post-translational regulator of the circadian rhythm.

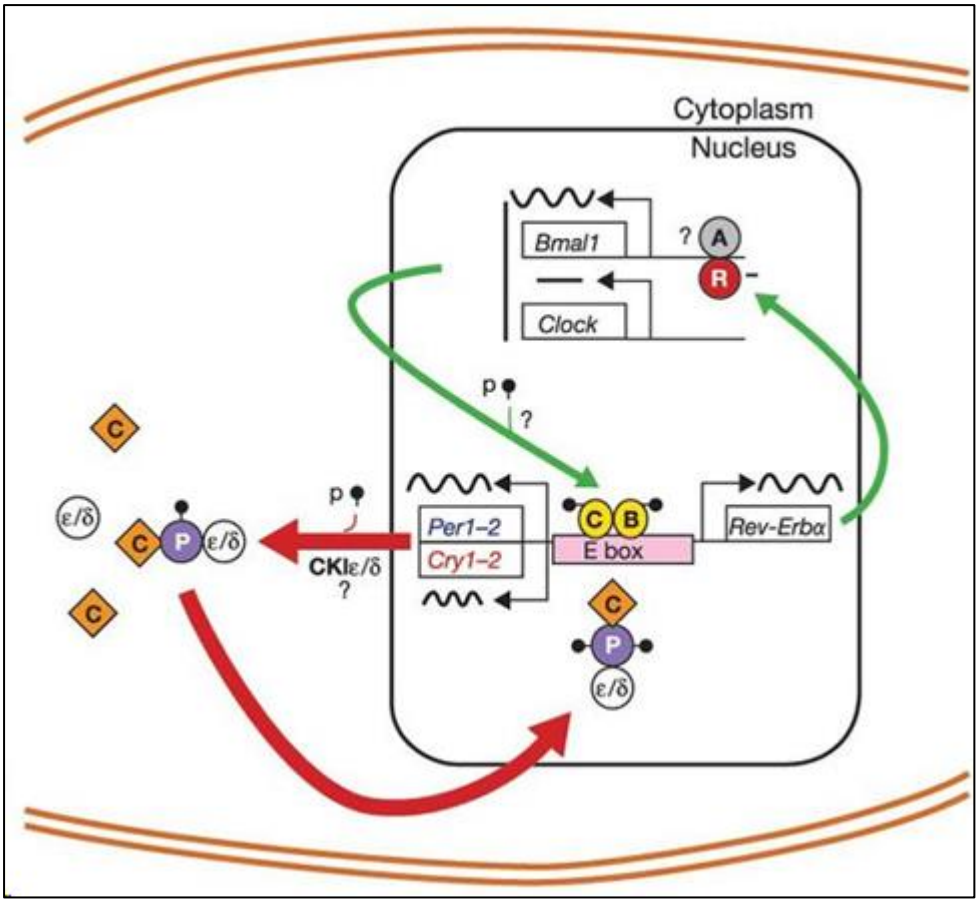

Fig 1.1| Mammalian molecular Circadian Rhythm. The mammalian clock mechanism consists of positive (green) and negative (red) interactive feedback loops. CLOCK (C, oval) and BMAL1 (B, oval) form heterodimeric complex to activate Per, Cry and Rev-Erbα genes through E-box enhancers. PER (P, blue circle) levels increase and they complex with CRY (C, diamond) and Casein Kinases CK1δ/ε (δ/ε, circle) and get phosphorylated. CRY-PER-CK1δ/ε complex in the nucleus, associate with the CLOCK-BMAL1 complex to shut down their transcription forming the first negative feedback loop. REV-ERBα (R, circle) levels increase and act through the Rev-Erb/ROR response elements in the Bmal1 promoter to repress Bmal1 transcription. CRY-mediated inhibition of CLOCK-BMAL1 mediated transcription de-represses (activates) Bmal1 transcription. An activator (A, circle) might interact with mPER2 or act alone to positively regulate the clock. There might be other kinases (?) that are involved in the phosphorylation of the clock proteins. (Reppert and Weaver, 2002).

Circadian Studies in Cell Culture

Through ablation and transplant experiments, SCN was demonstrated to harbour the master circadian pacemaker. The circadian oscillations in SCN appear to occur by cell-autonomous mechanisms. Isolated SCN neurons kept in culture were observed to display circadian firing frequencies for almost six days. The firing frequencies although different in wild type hamsters and homo- or heterozygous tau mutant hamsters, appear to correspond to the observed wheel-running activity of the donor animals. Other neural tissues like hamster retina and primary chick pineal gland cells

kept in tissue culture secrete melatonin in a circadian manner. However, the mechanisms involved in circadian output pathways are poorly understood as they involve neural and humoral signals. Later it was found that peripheral tissue cultures also showed circadian oscillation for PER2 gene (Isojima et al., 2003). Serum shock induces circadian rhythm in Rat-1 fibroblast cells and also H35 hepatoma cells (Balsalobre et al., 1998). It has been observed that when the growth medium was changed to a high serum-containing medium, circadian rhythmicity was induced for the clock genes. Similar effects have been observed for glucocorticoids. Dexamethasone – a glucocorticoid agonist is used for circadian rhythm synchronisation in cultured cells apart from temperature entrainment and light entrainment (Balsalobre et al., 2000).

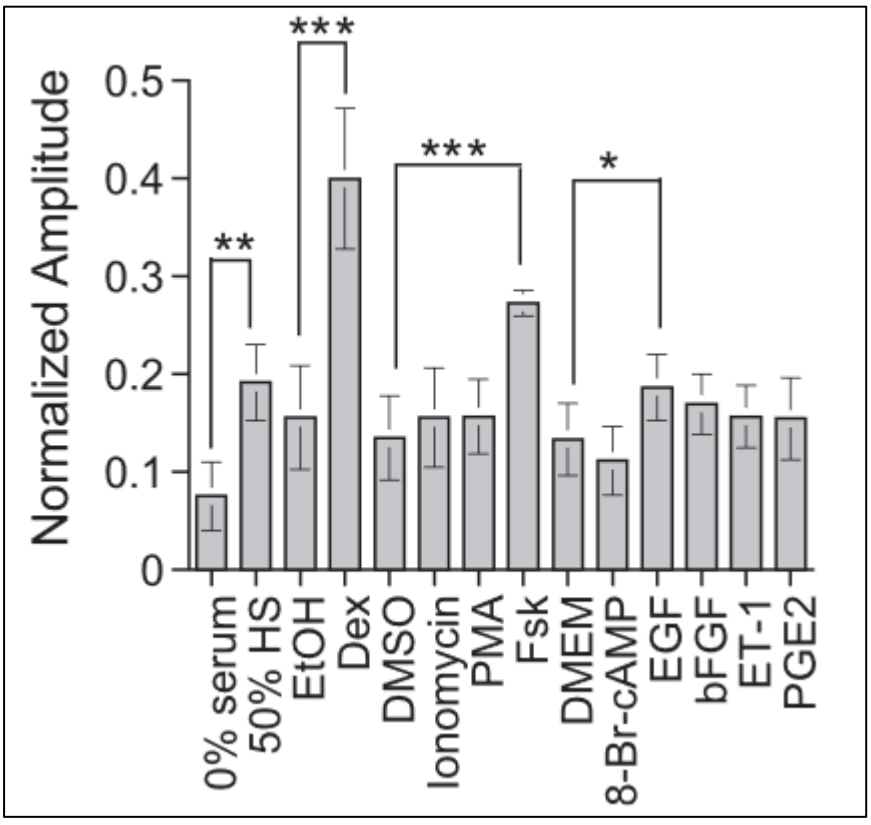

Fig 1.2| Histogram showing the relative amplitudes of oscillation on treatment with various molecules. It is noteworthy to observe that Dex has the highest amplitude oscillations (Izumo et al., 2006).

Fig 1.2 shows relative circadian amplitudes induced by different molecules in Rat-1 fibroblast cells. Dexamethasone induces the highest amplitude of oscillations (Izumo et al., 2006). For the same reason, dexamethasone synchronisation is being used in our studies in HCT116 cells.

1.3 Circadian Rhythm and Cell Cycle

The cell cycle and the circadian cycle are two distinct oscillators whose functions are tightly timed and regulated. Accumulating evidence suggests that the two pathways have molecular links between them and hence a bidirectional control on themselves. The cell cycle regulates cell cycle progression through the cyclic expression of key cell cycle molecules (cyclins) in a sequential unidirectional manner. There are four phases in the cell cycle: G1 phase, S phase (synthesis phase that includes DNA replication), G2 phase and M phase (mitotic phase). There also exists a G0 phase in which the cells are in a state of quiescence and are temporarily non-dividing. Each phase transition point is called a cell cycle checkpoint where the cell cycle can be regulated.

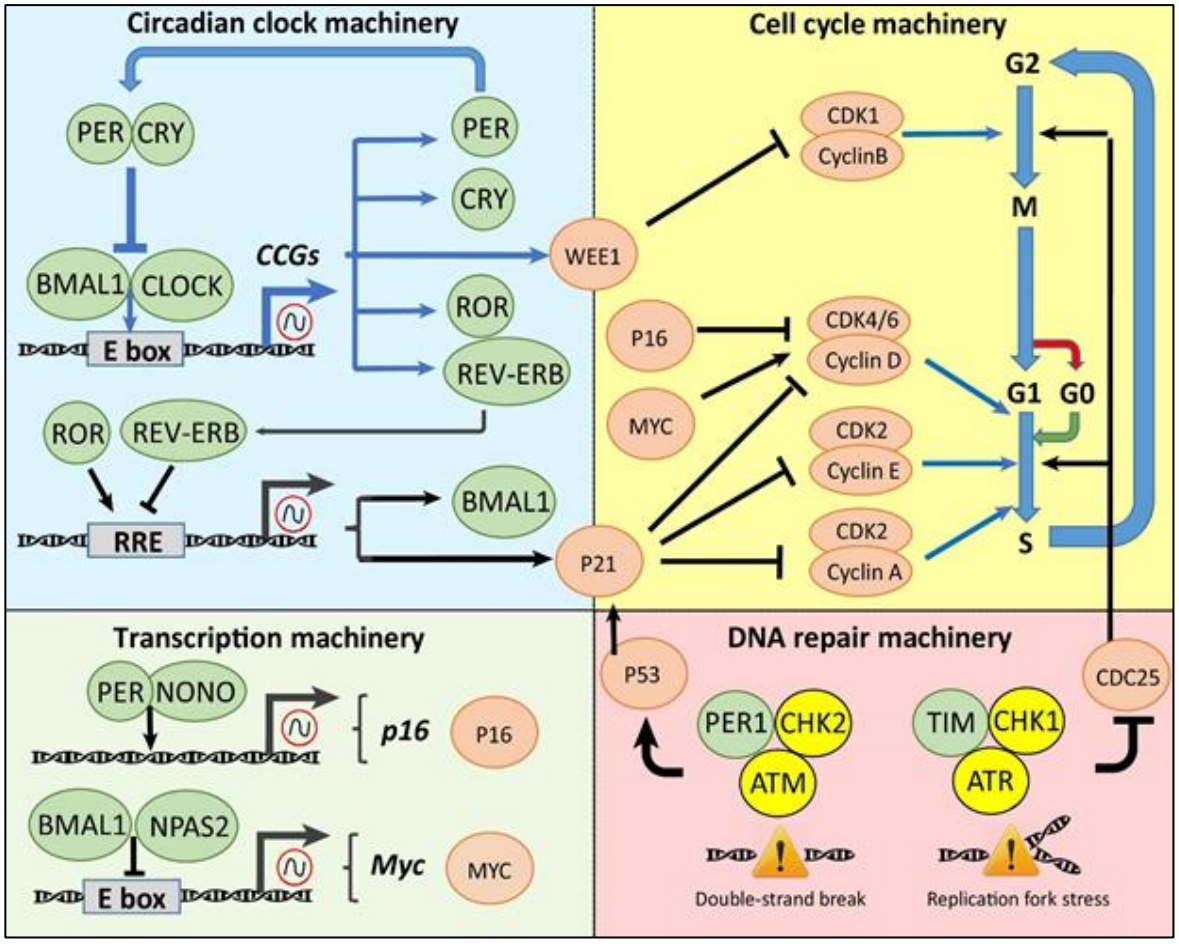

Fig 1.3| Crosstalk between the circadian rhythm and cell cycle.The circadian clock is involved in controlling the expression of cell cycle genes viz. WEE1, P21, P16 and MYC (blue and green box), which regulate the cell cycle at the G1-S, S-phase and G2-M checkpoints (yellow box). The circadian clock also involves in DNA repair events which in turn impact the cell cycle progression (red box). (Gaucher et al., 2018)

At the molecular level, the cell cycle progresses through the sequential activation of cyclin-dependent kinases (CDKs) that form complexes with cyclins. The molecular circuits in the pathway are critical to maintaining oscillations in the levels and activity of CDKs and cyclins and thereby crucial for cell cycle progression. The circadian clock controls specific parts from this circuit for temporal regulation of their expression.

Studies have shown that the circadian rhythm can affect the cell cycles at different levels. As shown in Fig 1.3, G1-to-S transition of the cell cycle appears to be under the circadian control through the CLOCK-BMAL1 mediated cyclic transcription of the cell cycle inhibitor gene p21WAF1. Furthermore, studies also suggest that the Ink4a/Arf tumour suppressor also alters the circadian period thereby reinforcing the interplay between the circadian clock and the cell cycle network. It is also noteworthy that WEE1 that controls the G2/M phase of the cell cycle is also under circadian control through CLOCK-BMAL1 (Gaucher et al., 2018).

Additionally, it was also found that the coupling between circadian rhythm and cell cycle in synchronised cells are in the ratio 3:2 (i.e. three cell cycles for two clock cycles). In healthy cells, the cell cycle is actively coupled with circadian rhythm while specific circadian rhythm disruption leads to premature epidermal ageing of cells (Feillet et al., 2015).

1.5 Circadian Rhythm and Cancer

In 2007, the International Agency for Research on Cancer (IARC) categorised “shift work that involves circadian disruption [as] probably carcinogenic to humans." Moreover, the circadian clock regulates the expression of genes such as thymidylate synthase, p21 and Wee-1, which control DNA synthesis, cell division cycle and proliferation; coordinating these physiological processes in a circadian manner. In mice, it was observed that disrupted circadian rhythm correlates with tumour development and progression (Relógio et al., 2014). PER1 and PER2 gene expression levels decreased in familial breast tumours when compared with normal breast tissues.

Clock disruption can promote tumorigenesis through multiple mechanisms. Downregulation of PER2 leads to an increase in β catenin levels and its target Cyclin D which in turn leads to cell proliferation in colon cancer cell lines and intestinal and

colonic polyp formation. Suppression of β catenin inhibits cellular proliferation in intestinal adenomas (Savvidis and Koutsilieris, 2012).
Downregulation of PER1 also leads to a higher risk of cancers. In summary, circadian rhythms and the cell cycle are tightly regulated, and hence disruption of circadian rhythm leads to a higher risk of cancer. Also, since a vast majority of genes involved in drug metabolism follow a circadian pattern in expression, treatment regimens also need to take circadian rhythmicity of metabolic pathways into consideration.

1.6 The Nuclear Envelope and Circadian Rhythm

The cytoplasm is separated from the nucleus by the Nuclear Envelope (NE). The double membrane structure is composed of an Outer Nuclear Membrane (ONM) and Inner Nuclear Membrane (INM). The ONM and INM are continuous with the ER, yet have distinct protein compositions. Entry and exit to the nucleus is through the Nuclear Pore Complex (NPC). Beneath the INM is a meshwork of intermediate filamentous proteins called lamins. The nuclear lamina is composed of two types of lamins - A and B (Shimi et al., 2015).The structural composition of each type of lamin is different from the other, and so is the function (Fig 1.4). Until recently, nuclear lamins were known to maintain the structural integrity of the nucleus and provide mechanical support to the nucleus. However, in recent years, with advancements in microscopy and imaging, nuclear lamins have been found to have a significant role in global gene regulation. The nuclear lamina is represses gene expression, and hence genes may be localised to the nuclear periphery for repression (de Leeuw et al., 2018).
Circadian rhythm is regulated by the nuclear envelope. The INM protein Man1 interacts with the promoter of BMAL1 directly and positively regulate its expression. It was observed that Man1 knockdown leads to a reduction in the circadian period while overexpression resulted in an extended circadian period suggesting a positive regulation of the clock. LBR and Lamin B1 regulate the circadian clock. LBR and Lamin B1 knockdown enhances the circadian period while overexpression resulted in a shorter period. Although their mechanism of action is proposed to be via Man1 (Lin et al., 2014), it is also possible that they directly interact with chromatin and regulate the rhythm.

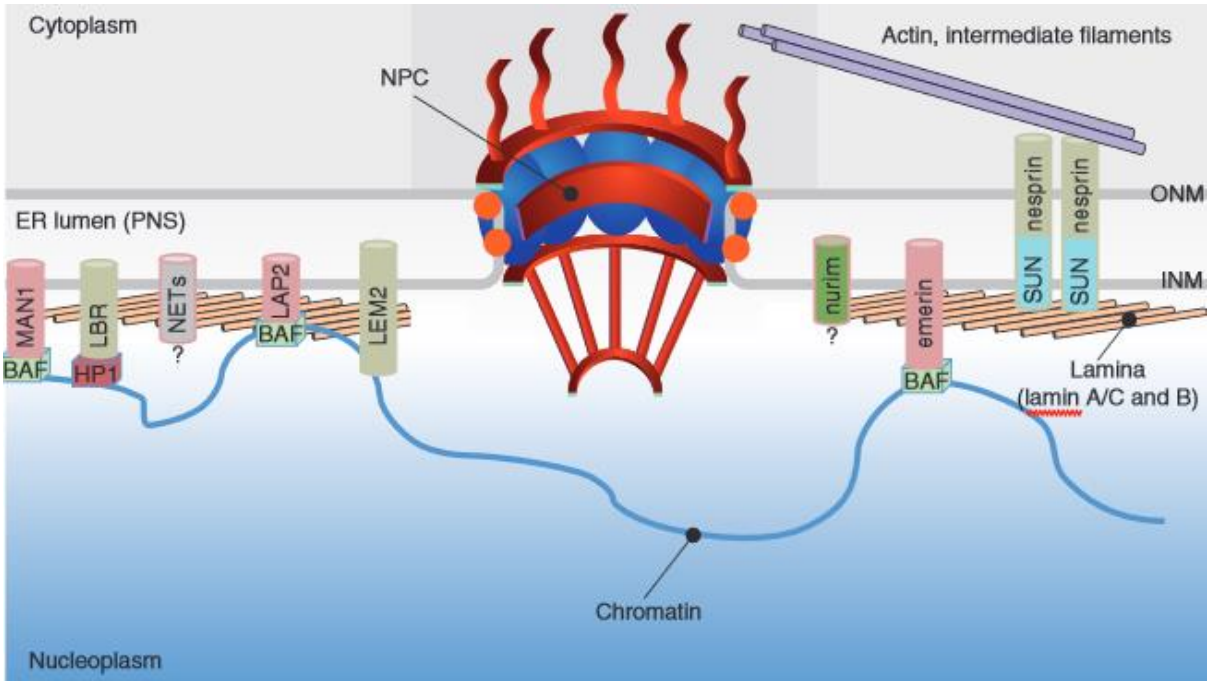

Fig 1.4| The Nuclear Envelope and its components (Hetzer, 2010).

1.7 The Nuclear Envelope in Cancer

As mentioned previously, nuclear lamins have various functions in the cell. As it is unlikely that nuclear lamins and nuclear envelope transmembrane proteins (NETs) would be attributed with such a wide variety of disparate functions, it is suggested that nuclear envelope acts as a scaffold to which many different functional complexes assemble. Tumour cells have been known to have variations in their nuclear size and shape since as early as the 1830s. Loss of lamin-A has been used as a prognostic indication for various tumours (Prokocimer et al., 2006).
Colon carcinomas and adenomas and gastric cancers have reduced lamin B1 levels as well as lamin A/C levels. The NE has regulatory roles in genome regulation, genome stability, cytoskeletal stability, cell cycle regulation and cell migration and hence can contribute to cancers in many different ways. The presence of tissue-specific NETs also REVeals that there could be tumour specific NE contributions. This, without a doubt, makes the NE a solid and potent therapeutic target for cancer (de Las Heras et al., 2013).

This project aims to study the effect of nuclear lamins on circadian rhythm. Since Lamin B Receptor (LBR) and Lamin B1 are known to have regulatory roles on the

circadian clock, we intend to find if the other nuclear Lamins (Lamin A/C and Lamin B2) have any roles in circadian rhythm regulation. Furthermore, we also try to find if there exists a correlation between circadian rhythm and the nuclear localisation of some cancer-associated genes as well as the genes coding for lamins thereby establishing positional significance for cancer-associated genes in following circadian rhythms.

Materials and Methods

2.1 Cell Culture

SW480 (human colon adenocarcinoma), HCT116 (human colon adenocarcinoma), BLI (mouse embryonic fibroblast) and MLI (BMAL1 knockout mouse embryonic fibroblast) cells were cultured in DMEM (Invitrogen, 11995) in 10% heat-inactivated Fetal Bovine Serum (FBS, Invitrogen, 6140-079 Carlsbad, USA) and supplemented with Penicillin (100 units) and Streptomycin (100 µg/ml, Invitrogen, 15070-63). Cells were grown at 37ºC and 5% CO_2. HCT116 cells transfected with shLBR construct were grown in DMEM medium in the presence of 10% heat-inactivated FBS and supplemented with Penicillin (100 units) and Streptomycin (100 µg/ml) and additionally with Puromycin (100 µg/ml, Sigma)

2.2 Dexamethasone Synchronization

Confluent cells (~$2x10^6$ cells) were treated with 100 nM Dexamethasone. Medium Changed to DMEM in 10% heat-inactivated FBS and supplemented with Penicillin (100 units) and Streptomycin (100 µg/ml) after (2 hours).

2.3 Preparation of metaphase spreads

HCT116 cells were grown to a confluency of ~60%. 10 µl/ml of Invitrogen Colcemid Solution (Roche, 10 295 892 001) was added to arrest the cells at metaphase. The cells were incubated at 37°C in 5% CO_2 for 80 minutes. The medium was removed and cells harvested by trypsinization. Cells were centrifuged at 1000 rpm, 10°C for 5 minutes; the supernatant was discarded and the pellet was re-suspended in 4 ml of 0.075 M Potassium Chloride (pre-warmed to 37°C). This suspension was incubated in a water-bath at 37°C for 30 minutes. After the incubation, few drops of the fixative (freshly prepared 3:1 solution of Methanol: Acetic Acid) was added to terminate the reaction. This suspension was centrifuged at 1200 rpm and 4°C for 10 minutes and washed thrice in fixative. ~10 µl of this suspension was dropped onto glass slides from a height. The metaphase spreads were allowed to dry. The metaphases were stained with 0.05 µg/ml DAPI solution for 2 mins and mounted in Invitrogen SlowFade Gold Antifade Reagent.

2.4 RNA Extraction and qRT PCR

Primers used for RT PCR reaction:

Gene	Primer Sequences
BMAL1	(F) 5'- GCCCATTGAACATCACGAGTAC - 3' (R) 5'- CCTGAGCCTGGCCTGATAGTAG - 3'
PER1	(F) 5'- TCTGTAAGGATGTGCATCTGGT - 3' (R) 5'- CAGGCAGTTGATCTGCTGGT - 3'
PER2	(F) 5'- AGTTGGCCTGCAAGAACCAG - 3' (R) 5'- ACTCGCATTTCCTCTTCAGGG - 3'
Cry2	(F) 5'- CGTGTTCCCAAGGCTGTTCA - 3' (R) 5'- CTCCGTCACTACTTCCACACC - 3'
REV-ERBA	(F) 5'- TGGACTCCAACAACAACACAG - 3' (R) 5'- GTGGGAAGTAGGTGGGACAG - 3'
DBP	(F) 5'- GTTGATGACCTTTGAACCCGA - 3' (R) 5'- CCTCCGGCACCTGGATTTTT - 3'
LBR	(F) 5'- TGGGTGATCTCATCATGG – 3' (R) 5'- ATCGAACCTGACCGTACACTTCTCGGTGGACAAGC – 3'
Lamin A	(F) 5'- CCGCAAGACCCTTGACTCA – 3' (R) 5'- TGGTATTGCGCGCTTTCAG – 3'
Lamin B1	(F) 5'- CGACCAGCTGCTCCTCAACT – 3' (R) 5'- CTTGATCTGGGCGCCATTA – 3'
Lamin B2	(F) 5'- AGTTCACGCCCAAGTACATC – 3' (R) 5'- CTTCACAGTCCTCATGGCC – 3'
Man1	(F) 5'- TTGGCCCTGAGGAAGAATTG – 3' (R) 5'- ACCATCACTACACCTAAGCATAA – 3'
AKT2	(F) 5'- GACTATCTCAAACTCCTTGGC – 3' (R) 5'- GCATACTTCAGCGCAGTG – 3'
BCL2	(F) 5'- GTTTGTACGAGTTCAGTGGAG- 3' (R) 5'- GCTTCTAGGTGGAGTTGGAA- 3'
KRAS	(F) 5'- GTGAGGGAGATCCGACAATAC – 3' (R) 5'- GCATCATCAACACCCAGATTAC – 3'

RUNX2	(F) 5'- GCAAGGTTCAACGATCTGAG – 3' (R) 5'- GCGGTCAGAGAACAAACTAG – 3'
SNAI1	(F) 5'- CCCCAATCGGAAGCCTAACT – 3' (R) 5'- GCTGGAAGGTAAACTCTGGAT – 3'
HPRT	(F) 5'- AGAGCTATTGTAATGACCAGT- 3' (R) 5'- CTTCCACAATCAAGACATTCTT- 3'

Quantitative Real-Time PCR carried out on BMAL1, PER1, PER2, CRY2, KRAS, AKT2, RUNX2, LMNB1, LMNB2, LBR, and MAN1. Primer Sequences are given below.

2.4.1 RNA Extraction

a. Sample Homogenization: Media was removed from the 35mm dishes and was washed using 1x PBS. To the washed dishes 500µl TRIZOL reagent (Invitrogen, 15596-026) was added to homogenize the cells. The plates were incubated in RT for 5 minutes followed by collection of the samples in 1.5ml Eppendorf tubes for phase separation.

b. Phase separation: To the sample, 100µl Chloroform (PER 500 µl TRIZOL) was added. The mixture was inverted ten times without vortexing and was incubated in RT for 10 minutes. The sample was centrifuged for 15 minutes at 4^{o}C and 12000g. The aqueous phase was collected, and equal volumes of Isopropanol was added to each Eppendorf tube. The mixture was incubated in RT for 10 minutes followed by centrifugation for 15 minutes at 4^{o}C and 12000g.

The pellet was dislodged using 75% ethanol in nuclease-free water (NFW). The sample was centrifuged for 15 minutes at 4^{o}C and 12000g and was kept for drying at RT. 10-15µl of NFW was added, and samples were incubated at 65^{o}C for 5 minutes followed by 37^{o}C for 10 minutes. The collected RNA can be stored at -80^{o}C.

2.4.2 cDNA Preparation

cDNA was prepared using Verso Reverse Transcriptase System. The scheme is given below:

a. RNA mix (5µl reaction)

Component	Stock Concentration	Final Concentration	Volume Added
Purified RNA		1 µg	x µl
Oligo dT	50 µg	2.5 µg	1 µl
NFW			5 – (1+x) µl
Total			5 µl

Samples are incubated at 70^{o}C for 5 minutes followed by quick chill in ice for 2 minutes.

b. Reverse Transcriptase mix (15 µl reaction)

Components	Stock Concentration	Final concentration	Volume added
5x cDNA Synthesis Buffer	5x	1x	4 µl
dNTP mix	10mM	0.5mM	2 µl
RT Enhancer	20 U	1 U	1 µl
Verso Enzyme Mix	20 U	1 U	1 µl
Nuclease Free Water			7 µl
RNA Mix from (a.)			5 µl
Total			20 µl

PCR Reaction Scheme:

1. 25^{o}C for 15 minutes (Primer Annealing)
2. 42^{o}C for 60 minutes (Extension)
3. 70^{o}C for 5 minutes (Enzyme denaturation)
4. 4^{o}C

2.4.3 Quantitative RT PCR

a. Kapa Sybr Mix (4 µl reaction)

Components	Stock Concentration	Final Concentration	Volume Added
KAPA Sybr Green KAPA Biosystems (KM4100)	2x	1x	2.5 µl
Forward+Reverse Primer cocktail	10 µM	400nM	0.2 µl
Nuclease Free Water			1.3 µl
cDNA (1:2 diluted)			1 µl
Total			5 µl

RT PCR (Biorad CFX96) reaction scheme:

a. 95^{o}C for 5 minutes (Initial Denaturation)
b. 95^{o}C for 20 seconds (Denaturation)
c. 60^{o}C for 1 minute (Annealing, extension, and read fluorescence)

Transcript levels were normalized to HPRT and further normalized with respect to the transcript levels of CT0. Relative levels were obtained using $2^{-\Delta\Delta CT}$ method

2.5 BMAL1-Luciferase Assay

Lentiviral transfection of luciferase gene into HCT116 cells under the control of BMAL1 promoter. Cells passaged under Hygromycin selection for two weeks. 100nM luciferin added into complete DMEM and fed into the lumicycle for luminescence measurement every 10 minutes for five days (However, this experiment did not work).

2.6.1 BAC DNA Isolation

Nick Translation

Component	Volume (µl)
5x Labelling Mix	4
DNA Template	x
Nick Translation Mix	4
NFW	20-(8+x)
Total	20

5x Labelling Mix:

Component	Volume (µl)
2.5mM dATP	5
2.5mM dCTP	5
2.5mM dGTP	5
2.5mM dTTP	3.4
1mM fluorescently labelled dUTP	4
NFW	27.6
Total	50

The Nick Translation Mix is from ROCHE (already contains DNA Polymerase 1 and DNase 1 mixture)

The components are to be added in the order NFW, DNA Template, 5x Labelling Mix followed by Nick Translation Mix. Mix the components well, spin down and keep the mixture at 15^{o}C for 3 hours (Make sure that the reaction is in the dark). After 3 hours, add 1µl of 0.5M EDTA to each tube to quench the reaction. Keep the reaction at 65^{o}C for 10 minutes. Take out 3µl from the 150µl into a 0.2ml Eppendorf tube to run on a gel. To the remaining 102µl, add one-tenth the volume of sodium acetate (3M,

pH 5.2). Then add 3x the volume of 100% ethanol (molecular biology grade) for DNA precipitation. Keep at -80^{o}C overnight.

Statistical Analysis

Two sampled t-test has been carried out for all the paired graphs that compare control and experimental profiles. Two sample t-test is used to check whether the average difference between two groups of data is significant or due to random chance. Graphpad Prism 8.0 software has been used for all the plotting as well as statistical analysis.

RESULTS

In the first part of the project, we aim to study the effects of nuclear lamins in the regulation of circadian rhythm. The study has been done in human colorectal adenocarcinoma cell line - HCT116. The second part of the project aims to compare HCT116 with that of MEFs. Additionally we also aimed to study the effect of clock disruption in MEFs on cancer-associated genes namely AKT2, KRAS, RUNX2 and SNAI1.

HCT116 has previously been demonstrated to have an autonomous clock. (Relógio et al., 2014) We first synchronize HCT116 cells that has reached confluence using dexamethasone. Cells are collected in TRIZOL every four hours after the synchronization. Quantitative real-time PCR is carried out with the mRNAs extracted to compare the transcript profiles of various genes.

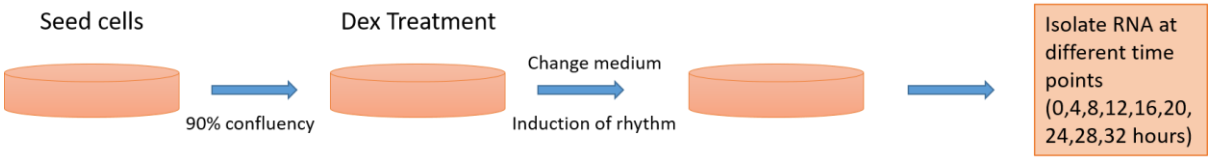

Fig 3.1| Design of experiment for wild type circadian expression profiling of genes.

3.1 Clock gene expression in the human colorectal cancer cell line - HCT116

We first checked the expression profiles of core-clock genes in dexamethasone synchronized HCT116 cells using quantitative real-time PCR to determine if the cell line exhibits circadian rhythms in gene expression. We first synchronized the cells using dexamethasone and collected RNA at intervals of 4 hours for 48 hours. We then quantified the expression levels of BMAL1, PER1, PER2 and DBP and normalized to the housekeeping gene HPRT. We found that BMAL1 oscillates with a peak at synchronized time (ST; 0 hours for ST is 24 hours after the dexamethasone synchronization followed by change of medium) 16 hours. DBP on the other hand has a peak expression at ~24 hours and is expected to continue the oscillation from ~28 hours. PER1 also peak at ~16 hours contrary to the expected peak at ~8 hours. PER2 however does not show a rhythmic expression pattern.

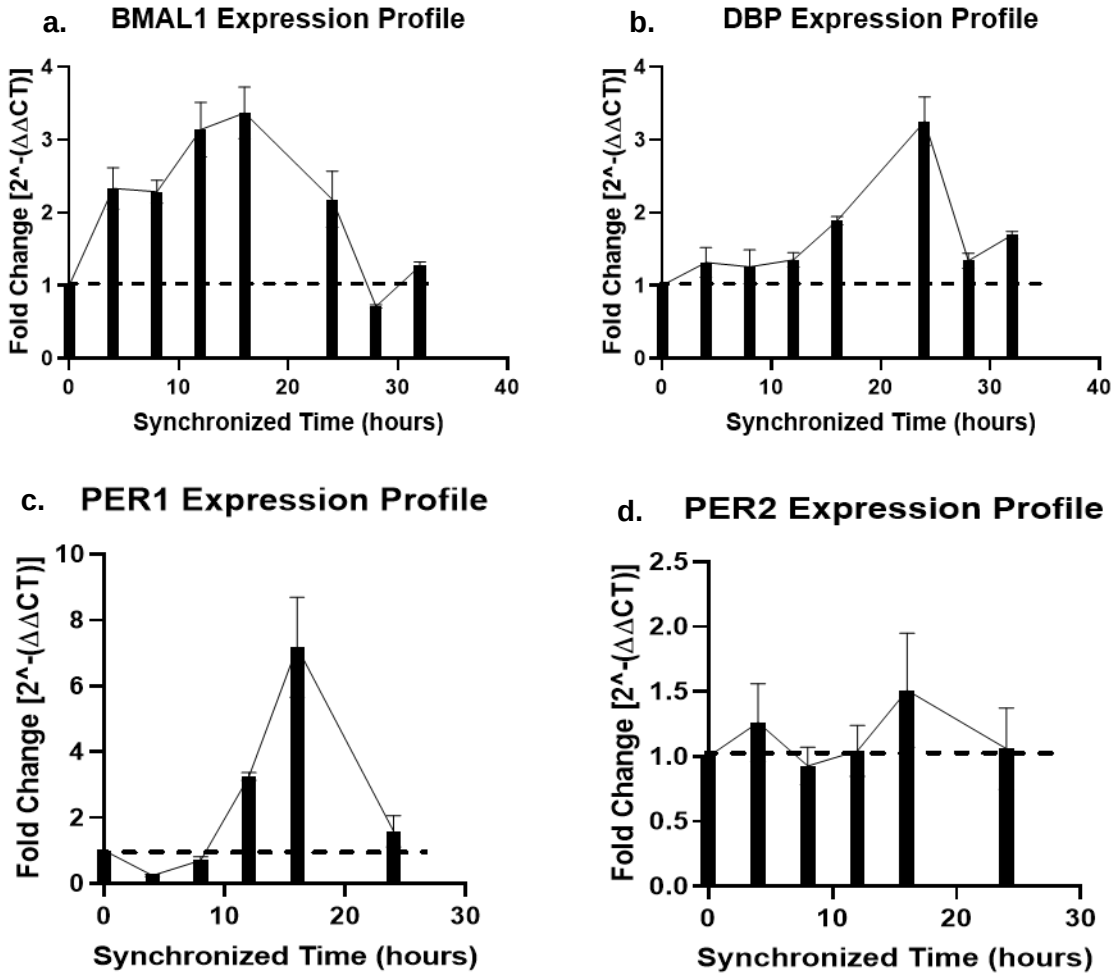

Fig 3.2| Expression Profile of Core-Clock Genes upon Dexamethasone Synchronization in HCT116 cells. The activator BMAL1 (a.) levels peaks at ST16 hours. DBP (b) steadily increases till ST24 and fall as expected. However, the repressor protein PER1 (c), contrary to the published literature, peaks at ST16 hours. PER2 (d) levels seem nearly flat. Data from 3 biological replicates (N=3; n=6). Error bars represent SE.

3.2 Nuclear Lamins have a Circadian Expression Profile

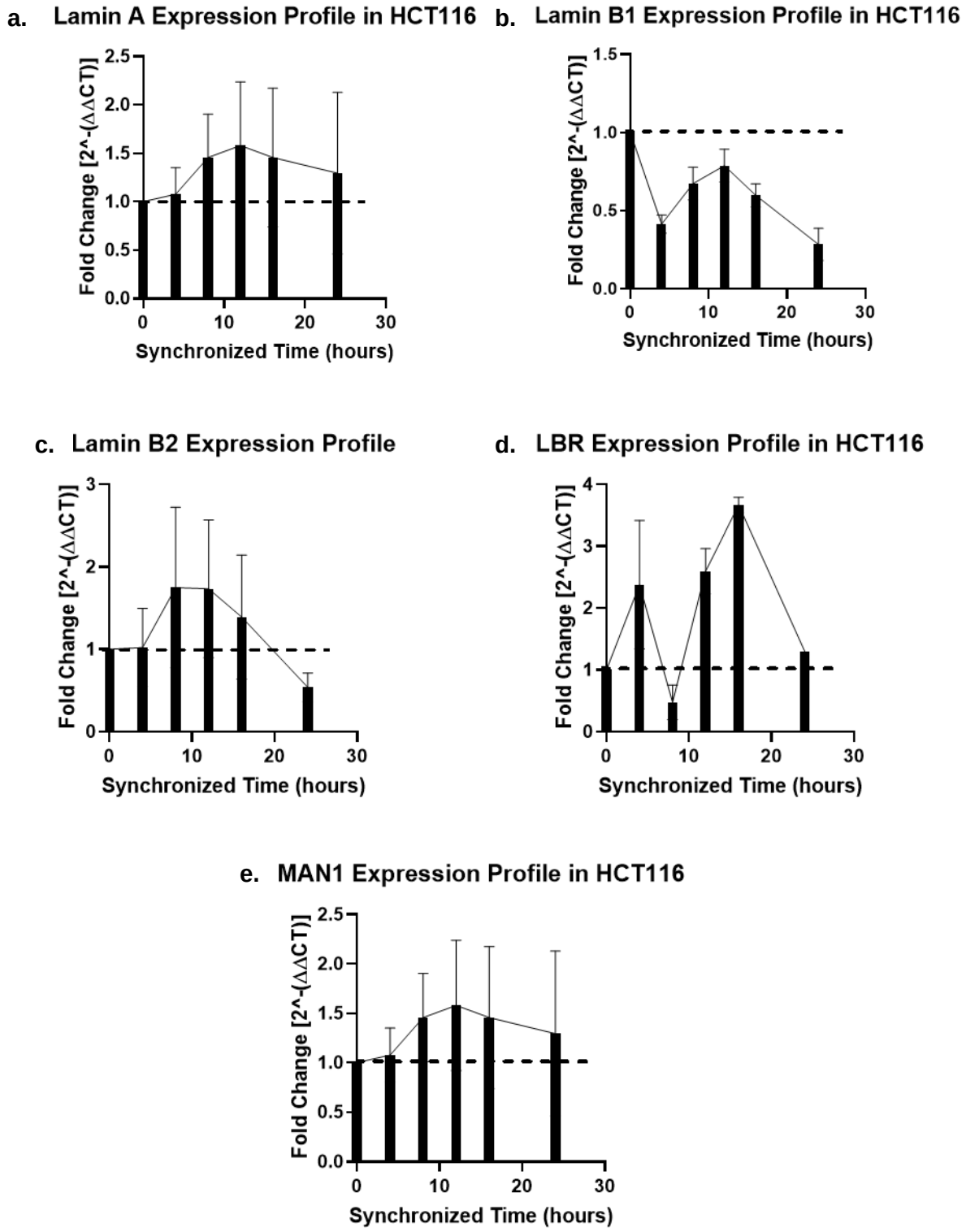

Fig 3.3| Expression Profile of Nuclear Envelope genes upon Dexamethasone synchronization in HCT116 cells. From top left (a) lamin A, (b) lamin B1, (c) lamin B2, (d) LBR and (e) MAN1. Data obtained from 3 biological replicates (N=3; n=6). Error bars represent SE.

While lamin B2 levels peak at ~8 hours (Fig 3.3c), lamin B1 levels reduce at ~4 hours and reaches a maximum level at ~12 hours and gradually goes down again (Fig 3.3 b). Man1 levels (Fig 3.3e), although do not show a high fold change in expression level, peak at ~16 hours and follow a pattern similar to that of BMAL1. LBR also peaks at ~16 hours (Fig 3.3d). Although LBR also has a high expression level at ~4 hours, essentially having two peaks. Lamin A however seem to show a consistent expression profile with a slight variation in its transcript levels across time (Fig 3.3a).

3.3 Role of LBR in rhythmic transcription in HCT116

LBR is already shown to regulate the circadian rhythm. (Lin et al., 2014) To determine if LBR regulates circadian gene expression in HCT116 cells, we generated shRNA mediated doxycycline inducible stable shLBR knockdown HCT116 cells. Doxycycline addition leads to significant reduction in 48 hours. We synchronize the doxycycline treated HCT116 cells after 24 hours so as to collect RNA at 48 hours after the Dox treatment. RT PCR was performed to check the effect of LBR on circadian rhythm.

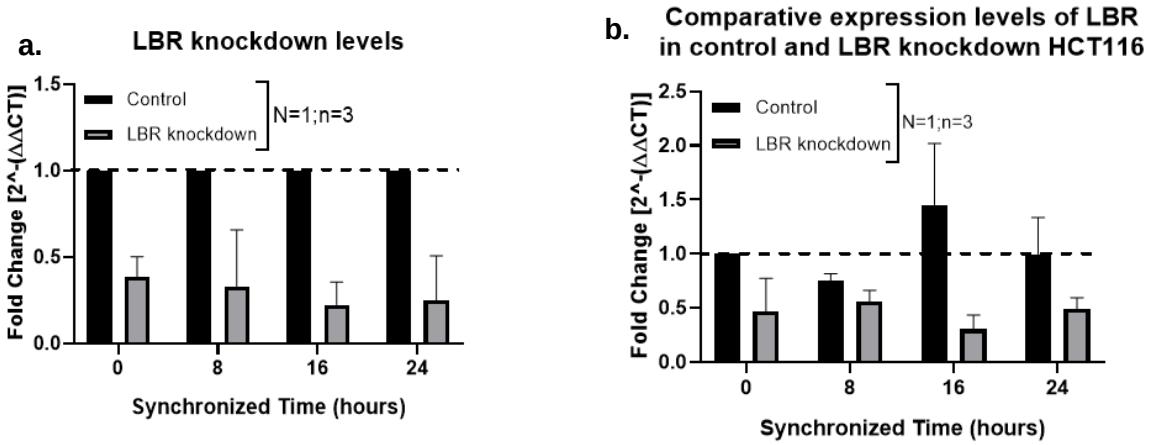

Fig 3.4| (a) LBR levels after Doxycycline induction of LBR knockdown. (b) LBR expression levels across time normalized to control. Data obtained from 1 biological replicate. Error bars represent SD.

There has been nearly 50% knockdown of LBR for ~0 and ~8 hours while about 60% knockdown for ~16 and ~24 hours. Hence, the shRNA mediated knockdown has worked effectively (Fig 3.4a). It is noteworthy that the LBR knockdown affected the CR of itself. Figure 3.4b suggests that the circadian period for LBR has been reduced in the LBR knockdown cells.

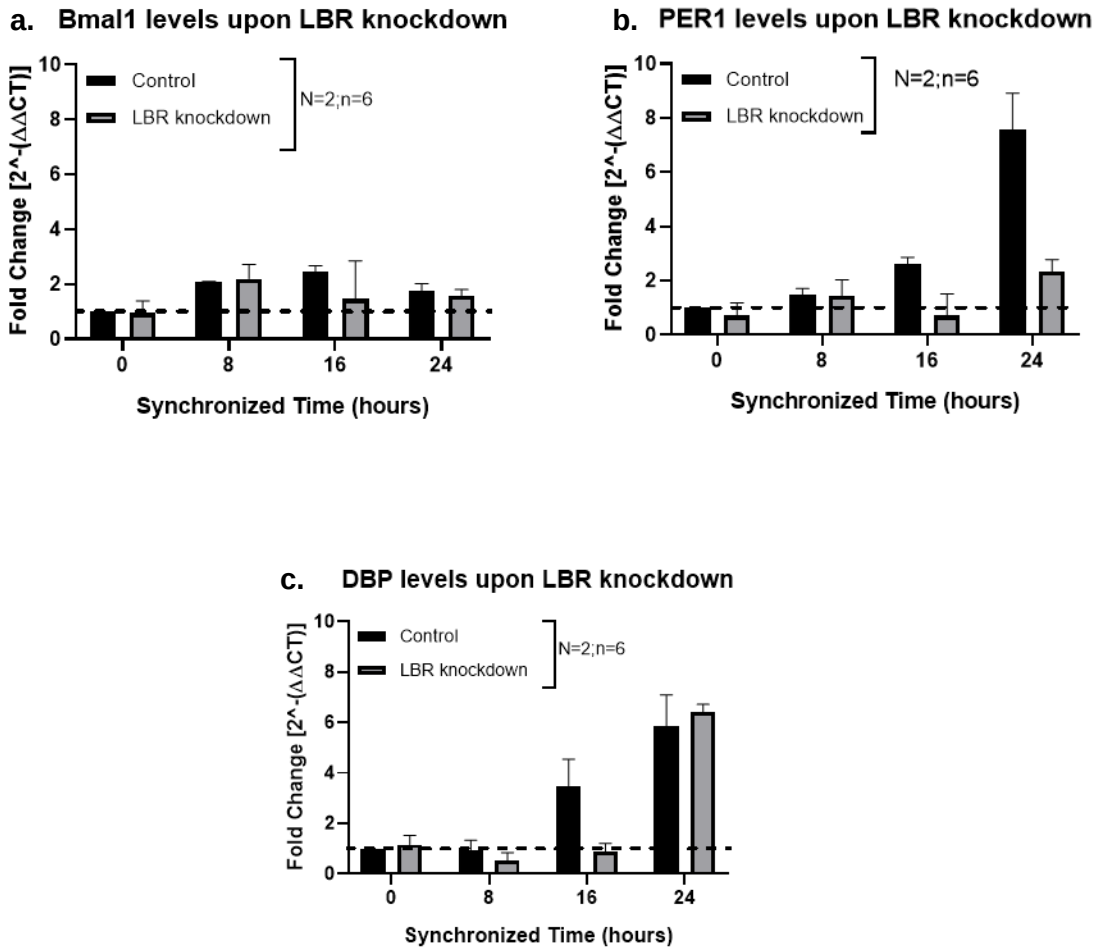

Fig 3.5| Expression Profiles of core-clock genes upon LBR knockdown. BMAL1 (a) levels are almost the same for control and LBR knockdown HCT116 cells. PER1 (b) levels do not seem to peak even at ST24 in LBR knockdown cells. DBP (c) levels seem to peak slower than the control. PER1 and DBP levels suggest a longer period for the clock. Data obtained from 2 biological replicates. Error bars denote SD. (two sample t test, p= ns)

BMAL1 expression levels suggest that the clock is shortened with its peak at ~16 hours shifted to ~8 hours (Fig 3.5a). Although the error bars seem to make the ST16 fold change comparable for control and the LBR knockdown cells. However, from DBP expression levels, it appears that the LBR knockdown cells take slightly longer time to reach its peak expression levels than the control cells (Fig 3.5b). PER1 levels show a considerable reduction upon LBR knockdown (Fig 3.5c).

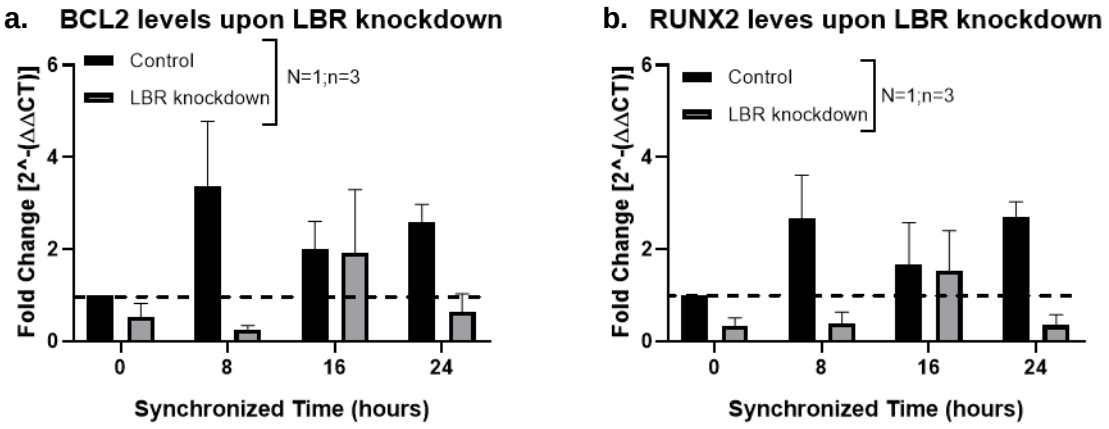

Fig 3.6| Expression Profiles of cancer-associated genes BCL2 and RUNX2 after LBR knockdown and circadian rhythm induction using dexamethasone synchronization. While BCL2 and RUNX2 seem to show a considerable change their expressions, RUNX2 is shown to have a significant change in its expression profile (two sample t-test, (a.) p=ns; (b.) p< 0.05).

3.4 Impact of Lamin A knockdown on Expression levels of circadian genes

siRNA mediated Lamin A knockdown was carried out followed by dexamethasone synchronization. RT PCR was performed on candidate core-clock genes to check the effect of Lamin A on circadian rhythm.

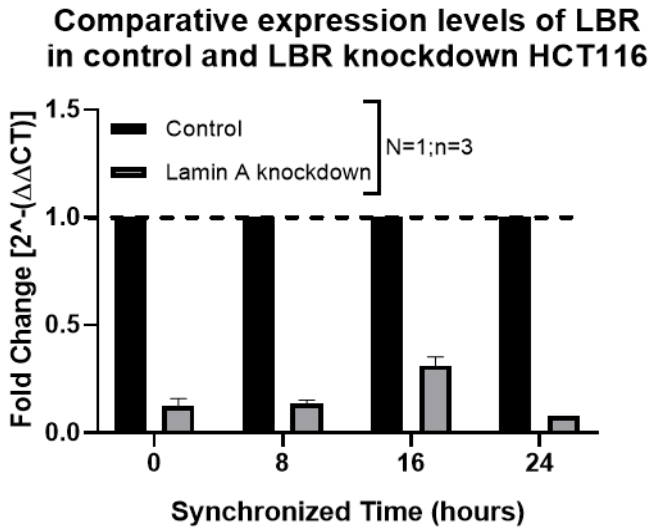

Fig 3.7| Lamin A levels after siRNA transfection. siRNA mediated Lamin A knockdown was carried out followed by Dexamethasone synchronization of HCT116 cells. A knockdown of ~80% has been achieved. Data obtained from 1 biological replicate. Error bars represent SD.

There has been nearly 80% knockdown of Lamin A after the siRNA mediated knockdown (Fig 3.7). Hence, the knockdown has worked efficiently.

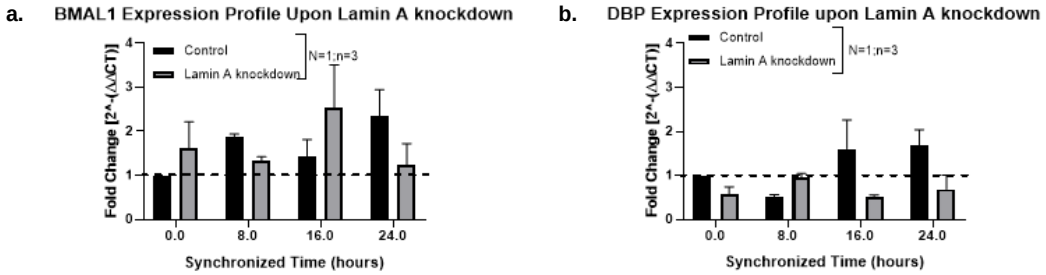

Fig 3.8|Expression Profile of BMAL1 and DBP after Lamin A knockdown. BMAL1 (a) levels are similar in both control and lamin A knockdown cells. DBP (b) expression is altered with the maximum expression at ST 8 hours which is comparable to the ST 0 hours levels of control cells. All time points normalized to ST 0 hours. Error bars represent SD (two tailed t-test, p= ns)

Expression levels of BMAL1 and DBP has been checked to check the effect of Lamin A on circadian rhythm. It appears that BMAL1 and DBP levels in Lamin A knockdown cells remain nearly the same as that of control cells. Although there is a difference in ~16 hours expression in BMAL1 which could be attributed to experimental error. In summary, Lamin A knockdown do not seem to have an effect on circadian rhythm (Fig 3.8).

3.5 Lamin B2 depletion leads to decrease in levels of circadian genes

siRNA mediated knockdown on Lamin B2 was carried out followed by dexamethasone synchronization. RT PCR was performed on candidate core-clock genes to check the effect of Lamin B2 on circadian rhythm in HCT116 cells.

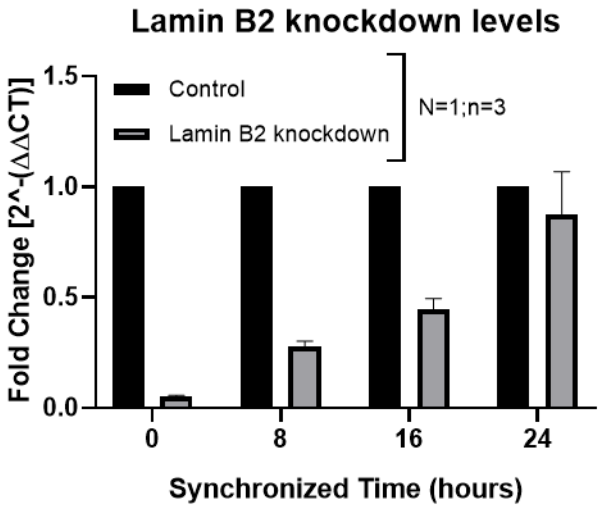

Fig 3.9| Transcript levels of Lamin B2 after siRNA mediated Lamin B2 knockdown. siRNA mediated knockdown of lamin B2 was carried out followed by dexamethasone synchronization. Although a knockdown of ~80% is achieved at ST 0 hours, the lamin B2 levels seem to be rescued as time progresses. Data obtained from 1 biological replicate (N=1; n=3). Error bars denote SD.

Although there is a nearly 90% knockdown of Lamin B2 at 0 hours, Lamin B2 levels increase and show a knockdown of ~60% at 8 hours, 50% at 16 hours and ~20% at 24 hours (Fig 3.9).

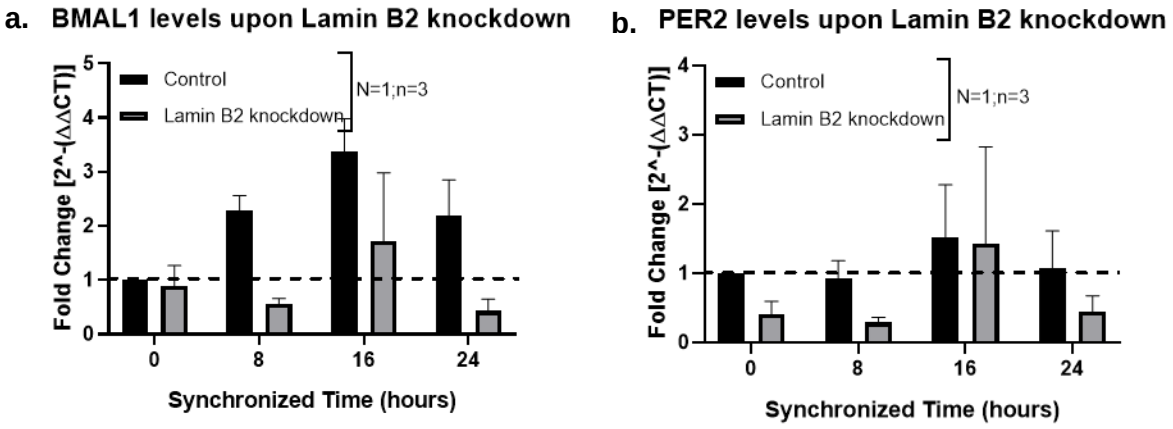

Fig 3.10|Expression Profiles of BMAL1 and PER2 after Lamin B2 knockdown. For both BMAL1 and PER2, the knockdown levels are considerable lower than the control (except ST16 hours for PER2) suggesting an overall reduction in the amplitude of the rhythm. Data obtained from 1 biological replicate (N=1; n=3). Error bars represent SD (two sample t-test, p=ns)

Overall expression profile of BMAL1 and PER2 remain the same. However, the relative transcript levels/ fold change for BMAL1 is considerably reduced in Lamin B2 knockdown cells compared to the control cells (Fig 3.10a). PER2 transcript levels are reduced to ~50% of that of control but the trend of the CR remains the same (Fig 3.10b). Further validation of the results are ongoing.

3.6 Role of Spatial Position of a gene in its circadian rhythm expression

Chromatin architecture has been found to be altered in circadian rhythm (Aguilar-Arnal and Sassone-Corsi, 2015). To follow up this with respect to cancer, we checked the expression profiles of cancer-associated genes-KRAS, AKT2, RUNX2, SNAI1 to check if these genes also follow a circadian rhythmic expression. We further planned to check if their expression correlates with their position in the nucleus. Also, if their expression and position are correlated, if the nuclear envelope has any roles in the same.

Quantitative RT PCR was performed for cancer associated genes in HCT116 cells to screen for their rhythmicity if any. Clearly, candidate genes AKT2, KRAS, SNAI1

show fluctuations in their expression levels across time, transcript levels of other genes RUNX2 do not change across time (Fig 3.11).

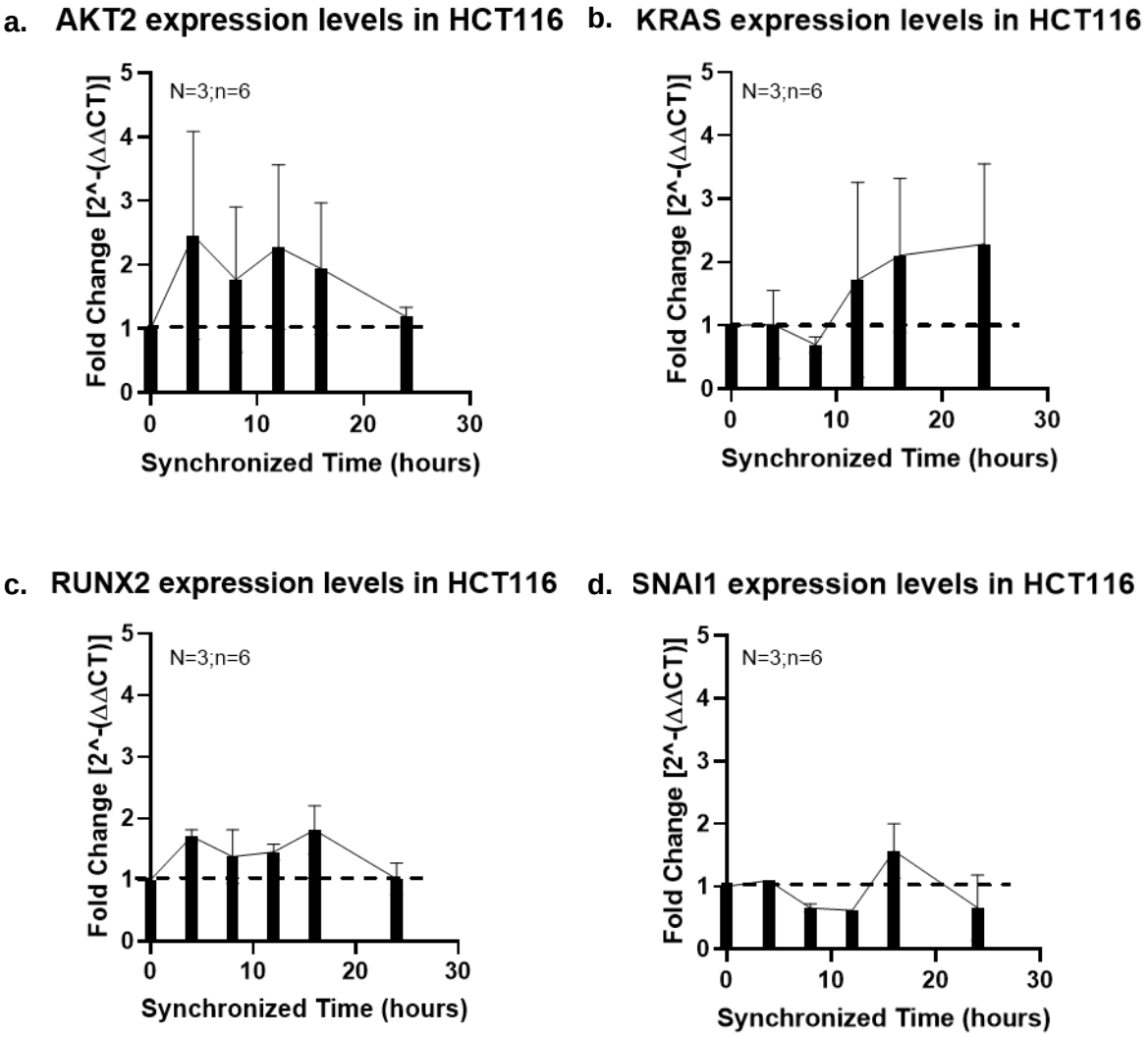

Fig 3.11|Expression Profiles of the Cancer-Associated Genes AKT2, KRAS, RUNX2 and SNAI1. AKT2 (a) levels peak at ST 4, 12 hours. KRAS (b) levels increase later at ~ST 12 hours. RUNX2 (c) levels are nearly the same throughout the time interval with slight increase at ST 4 and 16 hours. SNAI1 (d) levels also are nearly the same throughout the measurement window with slightly lower levels at ST 8 and 12 hours and a peak at ST 16 hours. Data obtained from 3 biological replicates (N=3;n=6). Error bars represent SEM.

Expression profiles of AKT2, KRAS, RUNX2 and SNAI1 have been plotted. It can be seen that while AKT2 and KRAS show considerable fold change in their expression with AKT2 peaking at ~4 hours (Fig 3.11a) and KRAS peaking at ~16 hours (Fig 3.11b), RUNX2 follow a biphasic expression profile with very slight variation in its expression across time (Fig 3.11c). RUNX2 has peaks at ~4 and ~16 hours. SNAI1

levels reduce at ~8 hours, reaches a peak at ~16 hours and then reaches back to its normal levels at ~24 hours (Fig 3.11d).

Expression Profiles of the cancer associated genes was checked in mouse embryonic fibroblast cells. It is clearly seen that the expression profiles of the genes differ in HCT116 and MEFs.

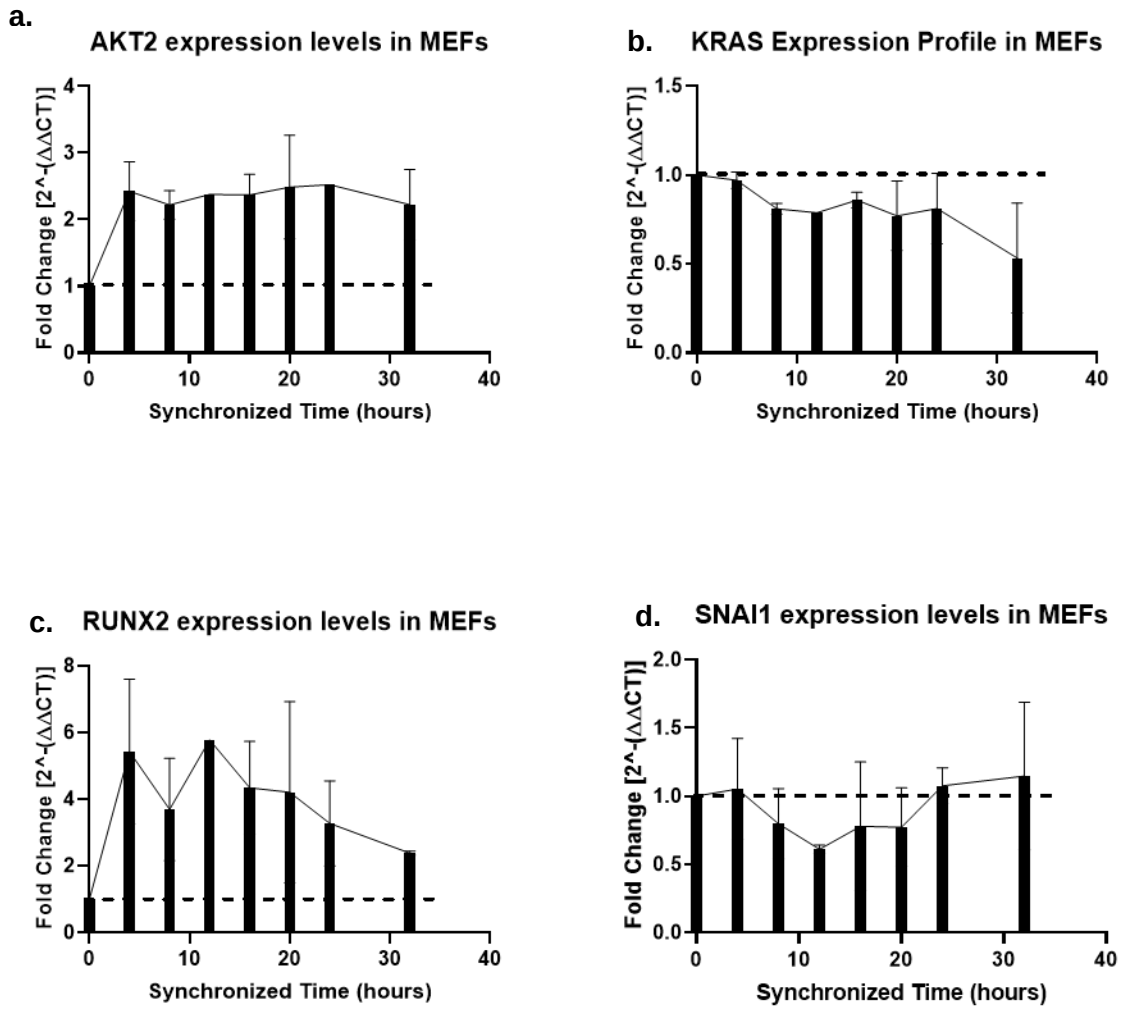

Fig 3.12| Expression Profiles of the Cancer-Associated Genes in MEF cells. AKT2 (a) levels increase at ST 4 and seem to plateau further. KRAS (b) levels go down gradually and RUNX2 (c) levels seems to show a rhythmic profile with a peak at ~ST12. SNAI1 (d) levels are nearly the same across time with a slightly lower level at ~12 hours. Data collected from 3 biological replicates (N=3; n=6). Error bars represent SEM.

RUNX2 in MEFs is highly expressed and show fluctuations in their levels across time with the highest expression at ~12 hours. AKT2 levels rise at ~4 hours and remain constant throughout the day. DBP levels go down till ~12 hours and then

increase. SNAI1 levels are nearly constant across time while KRAS levels show fluctuations in their levels.

Expression Profiles of nuclear Envelope Proteins in mouse embryonic fibroblasts were obtained by performing RT PCR for different times of the day.

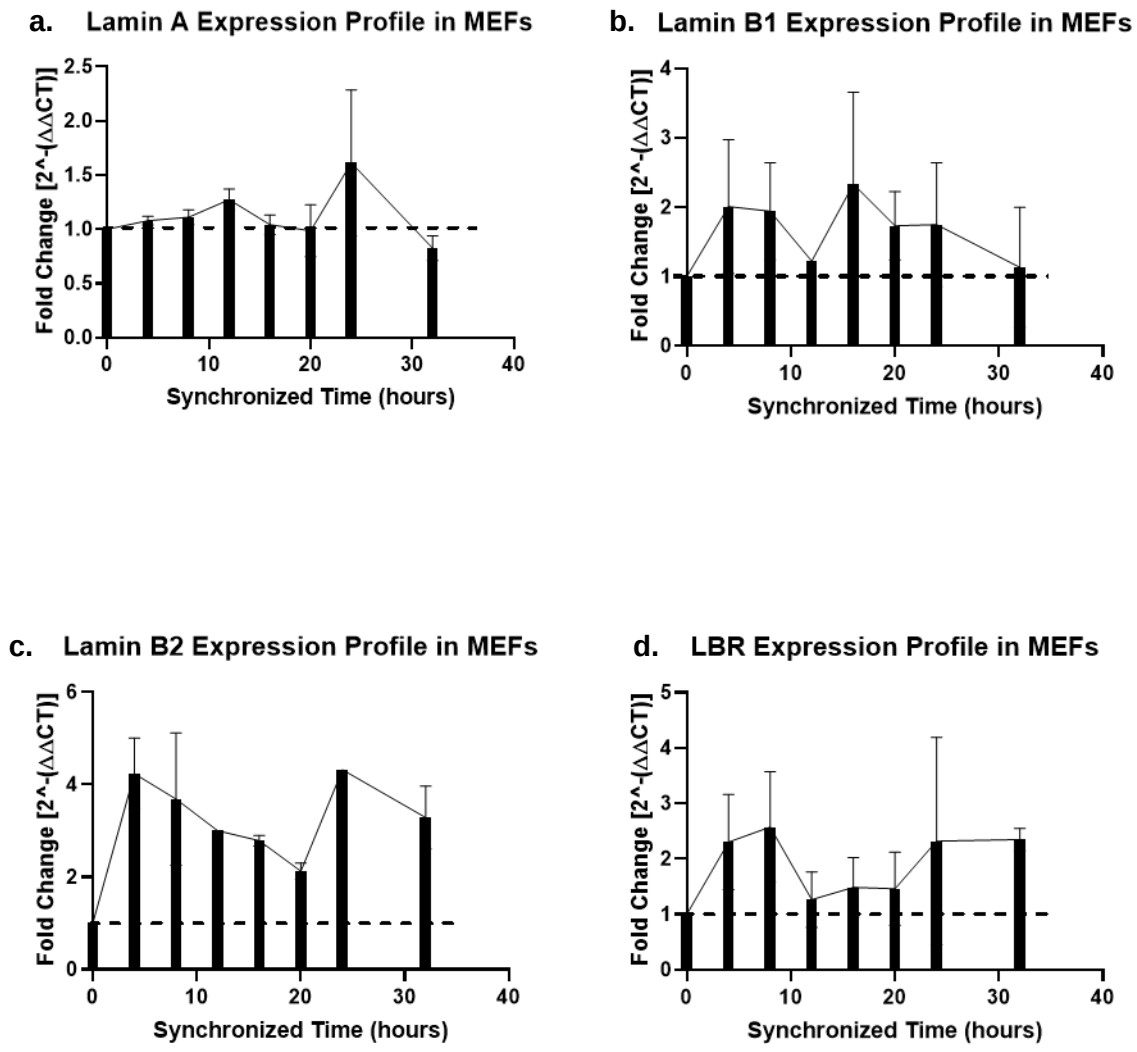

Fig 3.13|Expression Profiles of Nuclear Envelope Molecules- Lamin A (a), B1 (b), B2 (c) and LBR (d) in MEFs. This suggests that the expression profiles of nuclear envelope genes in MEFs and HCT116 are different. Data obtained from 3 biological replicates (N=3; n=6). Error bars represent

Lamin B2 shows the maximum extent of fold change across time with peak expression at ~4 hours. However, it nearly reaches its basal expression level at ~20 hours and rises again. Lamin A levels remain nearly the same while Lamin B1 shows a biphasic distribution with Lamin B1 peaks at ~8 and ~16 hours. LBR peaks at ~8 hours.

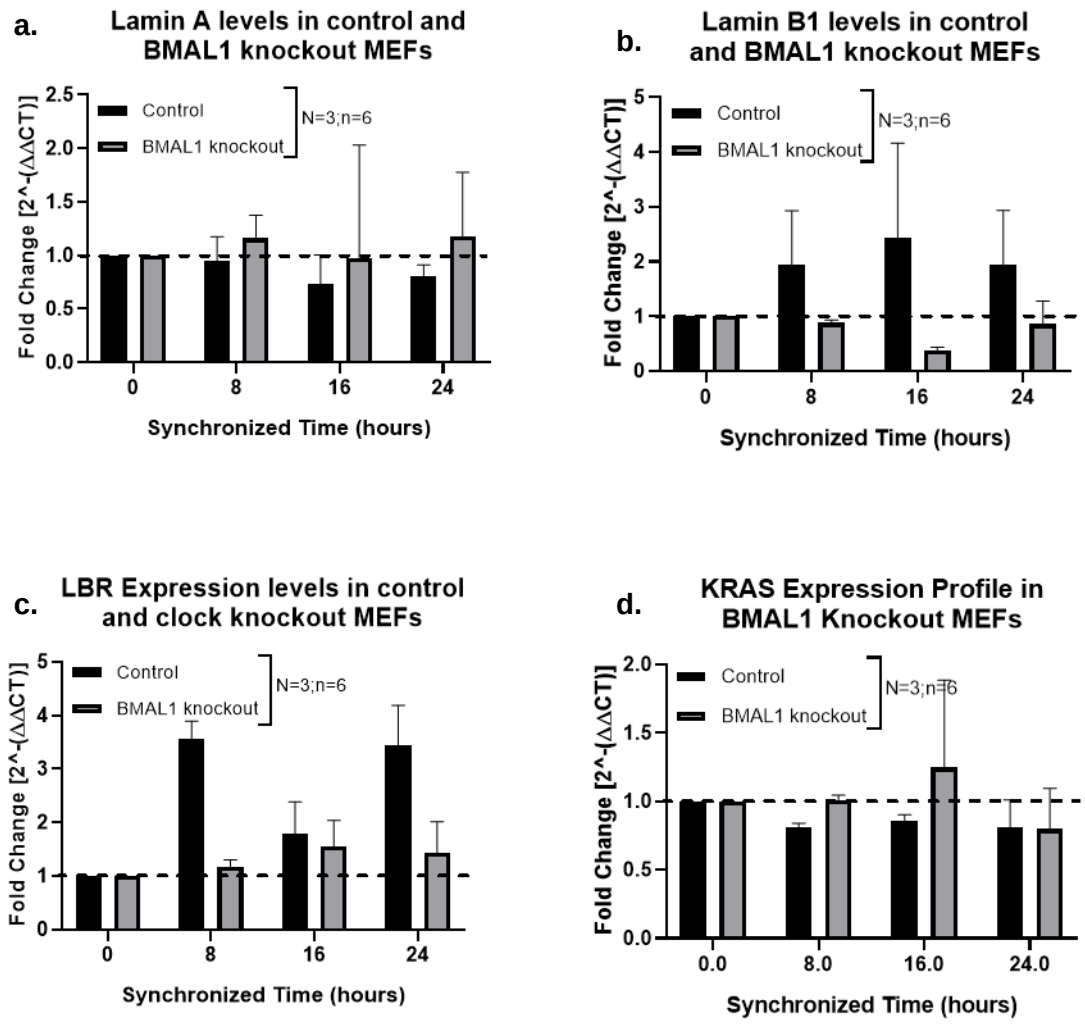

Fig 3.14| Comparative expression levels of control and BMAL1 knockout MEFs. The clock disruption do not seem to have a significant effect on the above genes. Data collected from 3 biological replicates (N=3; n=6). Error bars represent SEM. (two tailed t-test, p= (a.) ns, (b.) ns, (c.) <0.05, (d.) <0.05)

Expression profiles of BMAL1 knockout MEFs did not reveal much significant results. Although, clearly the levels of the genes (KRAS, Lamin A, Lamin B1, LBR) remain relatively constant across time in the clock knockout MEFs as expected.

DISCUSSION

The circadian clock is known to regulate the expression of ~5-15% of genes in mammals (Panda et al., 2002). The “clock” is necessary for proper functioning of body metabolism, physiology and behaviour. Recent studies have demonstrated that the nuclear envelope regulates the expression of various genes through epigenetic modifications (Doi et al., 2006; Ripperger and Schibler, 2006).

We aimed to check if the circadian clock isregulated by Lamin A/C and/or Lamin B2. Lamin A/C is a known interactor of Lamin B1 and Lamin B2 is a known interactor of LBR. LBR and Lamin B1, as mentioned previously, are already known to regulate the circadian clock.

Dexamethasone is a glucocorticoid agonist which induces circadian rhythmicity in cells. It increases the Per1 mRNA levels in rat-1 fibroblasts, kick-starting the molecular circadian rhythm (Balsalobre et al., 2000). Expression levels are measured through real-time quantitative PCR.

We selected HCT116 cell line as it was previously successfully entrained using dexamethasone (Relógio et al., 2014) (Fig 4.1, 4.2).

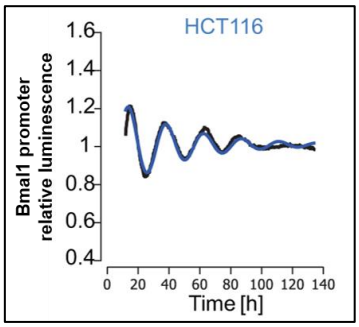

Fig 4.1| HCT116 is confirmed to have a cell autonomous clock (Relógio et al., 2014).

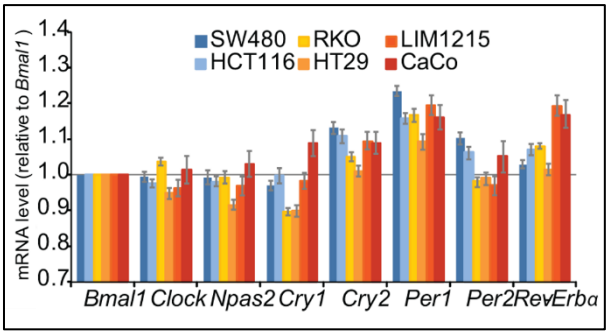

Fig 4.2| mRNA levels of different core clock genes in different colorectal cancer cell lines (Relógio et al., 2014).

HCT116 cell lines are derived from human colorectal cancer cells and has been used for various cell culture related circadian studies. We first validated if the cells we sensitive to dexamethasone treatment by comparing transcript levels of synchronised and unsynchronised cells by RT PCR.

Since the dexamethasone treatment synchronizes cellular clocks, we further characterised the transcript profiles of core clock genes (CCGs) in HCT116 using RT PCR (Fig 3.2).

Aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL), also known as BMAL1 is a core clock gene that encodes a transcription factor which has a basic helix-loop-helix (bHLH) and two PAS domains. In humans, BMAL1 is located on the Chr. 11, and the BMAL1 protein generates the molecular circadian rhythms in mammals. The transcript levels rise gradually from 0 hours, reach a peak at ~16 hours (16 hours after synchronisation) and gradually decreases to continue the cycle (Fig 3.1a).

D-box binding protein (DBP), a member of the PAR leucine zipper transcription factor family, is a primary clock-controlled gene and shows a robust circadian expression in SCN and other peripheral cells. DBP binds to an upstream promoter on the insulin gene. We observe that DBP increases its transcript levels similar to BMAL1 till CT16 and starts to decrease after CT16 to continue the period (Fig 3.1b).

Period 1 and 2 (Per1 and Per2) along with Cryptochrome (Cry 1 and 2) are one of the essential core clock genes that drive the negative feedback loop of the circadian cycle. In HCT116 cells, contrary to the common findings, Per1 transcript levels also peak at CT16 and rapidly reduces the transcript levels after CT16 while Per2 does not seem to be circadian in its expression pattern (Fig 3.1c and d).

Rev-Erbα drives the second negative loop by directly interacting with the BMAL1 promoter and thereby reducing its own transcription. Rev-Erbα transcript levels seem to be increasing till 24 hours and then sudden fall at further time points.

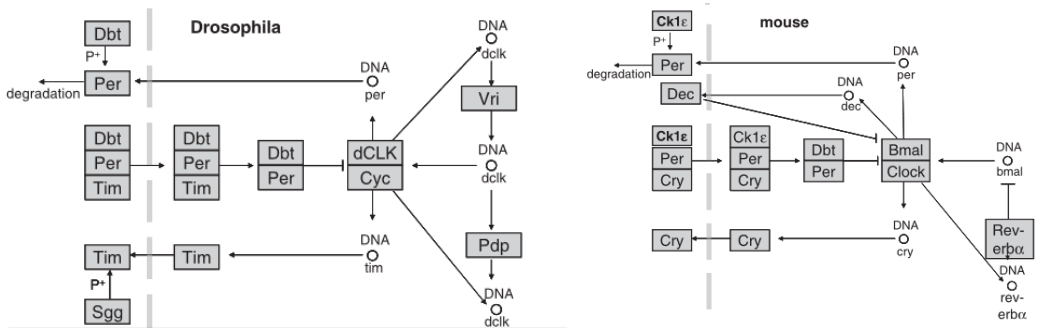

Fig 4.3| Circadian rhythm pathway in D. melanogaster and Mouse (Fuhr et al., 2015).

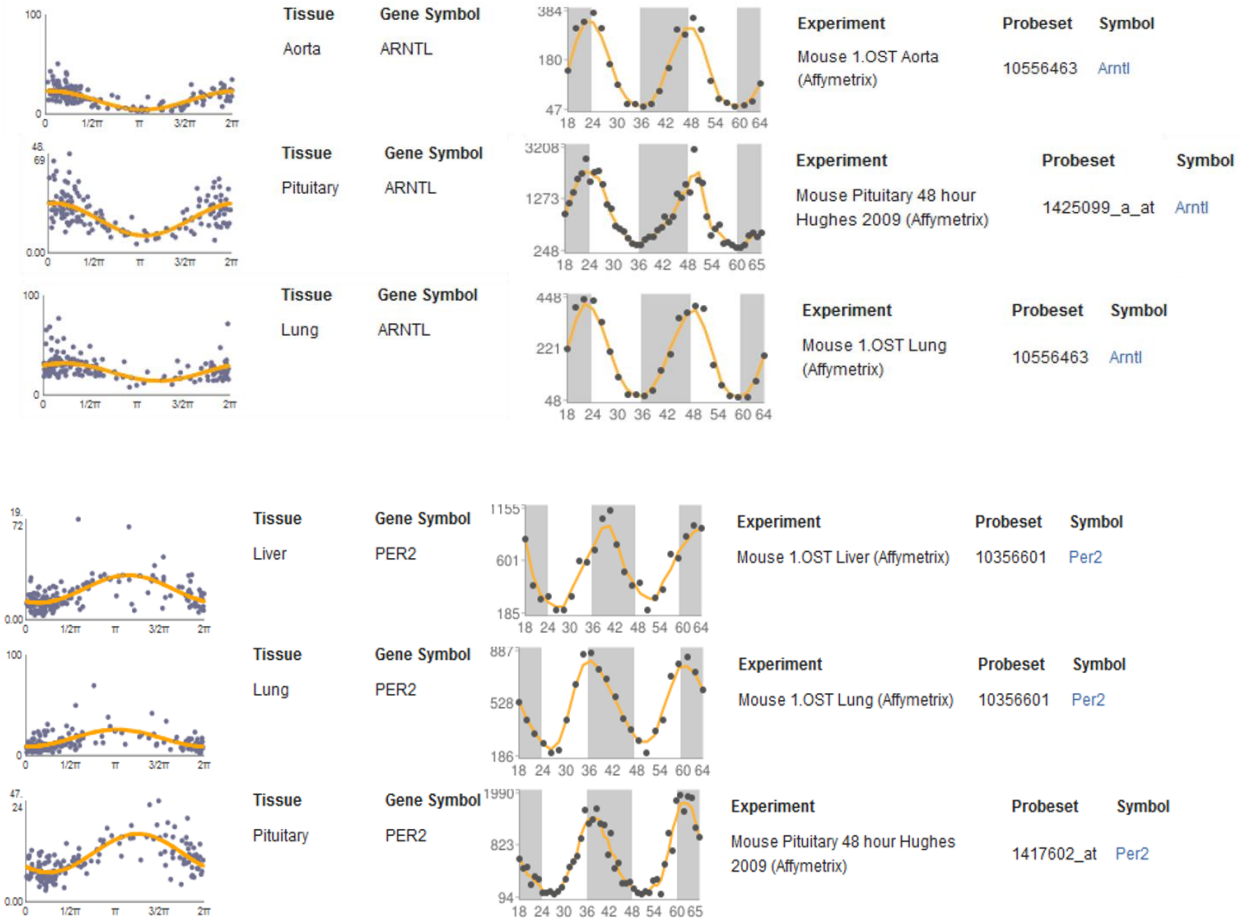

Fig 4.4| Expression profiles of BMAL1 and Per2 in different tissues from human and mouse. For human data (in left), the x-axis is CYCLOPS-estimated sample phase in radians. The y-axis is the TPM from RNA seq. Each point on the graph represent each individual. For mouse (in right), x-axis is the time in hours and y-axis is the same as in humans (GTEx Consortium, Nat. Genet. 2013).

As mentioned in previously, cell-cycle and circadian rhythm are mutually regulated by each other. To minimize the effects of cell cycle, we performed experiments on confluent dishes. Confluent dishes would reduce the number of cell cycles and hence would reduce its effect on the CR. Moreover, the circadian rhythm in cell lines are desynchronized with time (Relógio et al., 2014). This makes it difficult to conduct long term measurement of the circadian rhythm. To address this, we tried to generate stable cell line with BMAL1-luciferase reporter gene. Even though the cells survived lentiviral transfection and 2 weeks of Hygromycin selection, the cells showed no significant luminescence when checked in the lumicycle (the instrument which measures luminescence). Hence we were unable to monitor the circadian clock for more than 32 hours.

The nuclear envelope is known to regulate circadian rhythm through Man1 which directly binds to the upstream promoter site of BMAL1. The study also showed similar effects as Man1 with LBR and Lamin B1. However, their mechanism of action is unclear (Lin et al., 2014). We further moved forward to ask if the remaining lamins (Lamin A/C and Lamin B2) regulate circadian rhythm or not. We observe that Lamin A/C show a relatively flat expression level across time suggesting a constant expression pattern throughout the day (Fig 3.2a). This suggests that Lamin A/C is not a clock controlled output gene. However, lamin B2 shows a rhythmic expression pattern.

LBR was knocked down using shLBR construct. Although the DBP levels did not show considerable change in neither LBR knockdown nor lamin B1 knockdown cells, it could mean that the core-clock genes are the only genes affected while the clock controlled genes would take some more time of entrainment to follow the same pattern.

To find the effect of the nuclear envelope on circadian rhythm, we performed RT PCR of nuclear envelope molecules namely Lamin A/C, B1 and B2, LBR and Man1 in dexamethasone synchronised HCT116 cells. The cells were synchronised and collected over 32 hours at an interval of 4 hours. The expression profiles showed that Lamin B1 and B2, LBR and Man1 follow a circadian pattern of expression. Furthermore, we also conclude that the Lamin B2 regulate the circadian rhythm similar to that of LBR although the fold change is lower in Lamin B2 than LBR.

In the second part, since cell cycle is tightly regulated by the circadian clock and also by the nuclear envelope, we aimed at focusing on the disease that is most closely related to cell cycle- cancer. Circadian rhythm disruption has been reported in various cancers. LBR disruption as well as lamin disruption are prognostic tools in cancers. Hence, we studied the expression profiles of some cancer associated genes in HCT116 to check the effect of their circadian expression on LBR, lamin A and lamin B2 knockdown conditions. The Cancer-associated genes selected for the study are given below.

KRAS: Kirsten RAS oncogene homologue is a well-known proto-oncogene from the mammalian RAS gene family. The encoded protein is a member of the small GTPase superfamily. KRAS is involved in cell signalling pathways that control cell growth, cell maturation and apoptosis. A single amino acid substitution results in various malignancies including lung adenocarcinoma, mucinous adenoma, ductal

carcinoma of the pancreas and colon carcinoma.

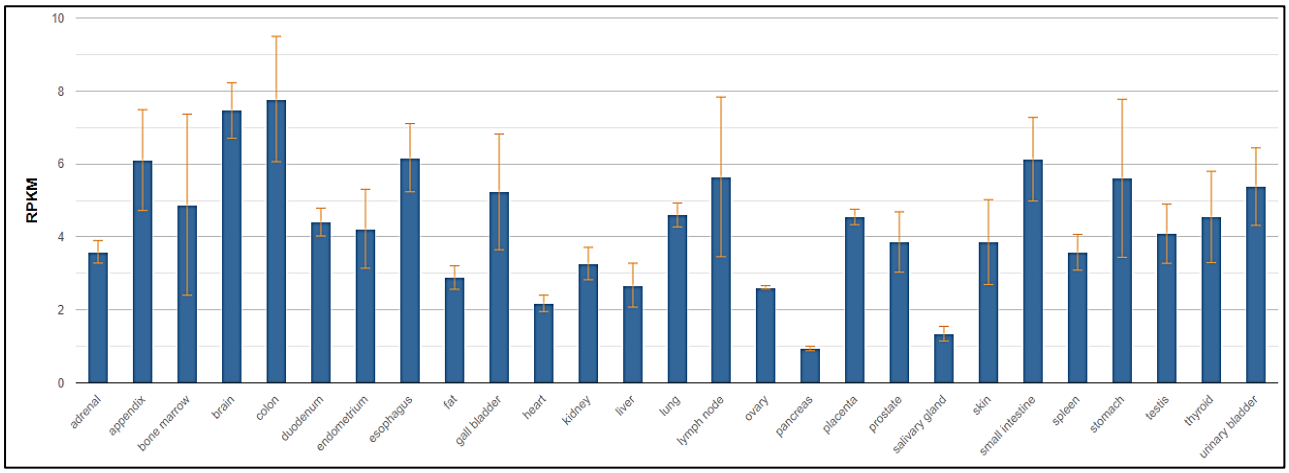

Fig 4.5| Transcript levels of KRAS in different normal tissues. RNA-seq was performed of tissues from 95 human individuals representing 27 different tissues to determine tissue-specificity of all protein-coding genes.
(https://www.ncbi.nlm.nih.gov/gene/3845)

In normal colon tissue, the transcript levels of KRAS is near to 7.8 RPKM. However, a mutation in the KRAS gene results in the substantially increased transcript levels in the same tissues as shown below. Since KRAS is one of the highly mutated oncogenes in colon cancers and is highly expressed in colon cancer cells, KRAS was chosen for the study.

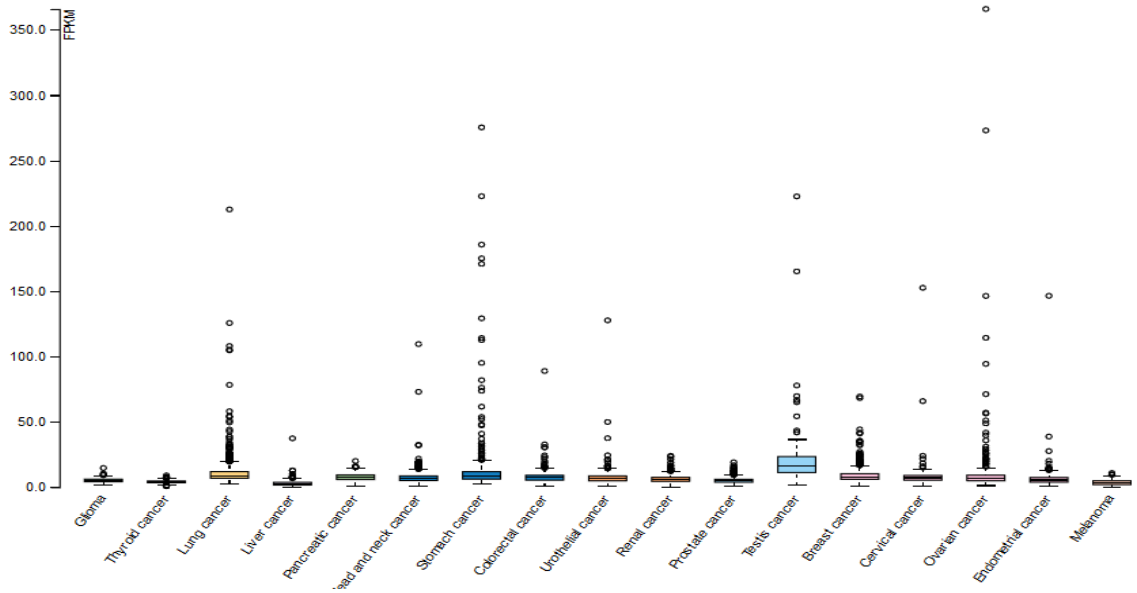

Fig 4.6| Transcript levels of KRAS in different cancer tissues. TCGA dataset that gives pan-cancer expression profiles of KRAS gene.

Mechanism of action of KRAS in colon cancers is given below. The pathway obtained through KEGG analysis suggests that a mutation in KRAS would affect cell proliferation through the MAPK signalling pathway. Also, note that Erb-β is circadian in its expression and hence KRAS is also expected and does follow a circadian expression profile.

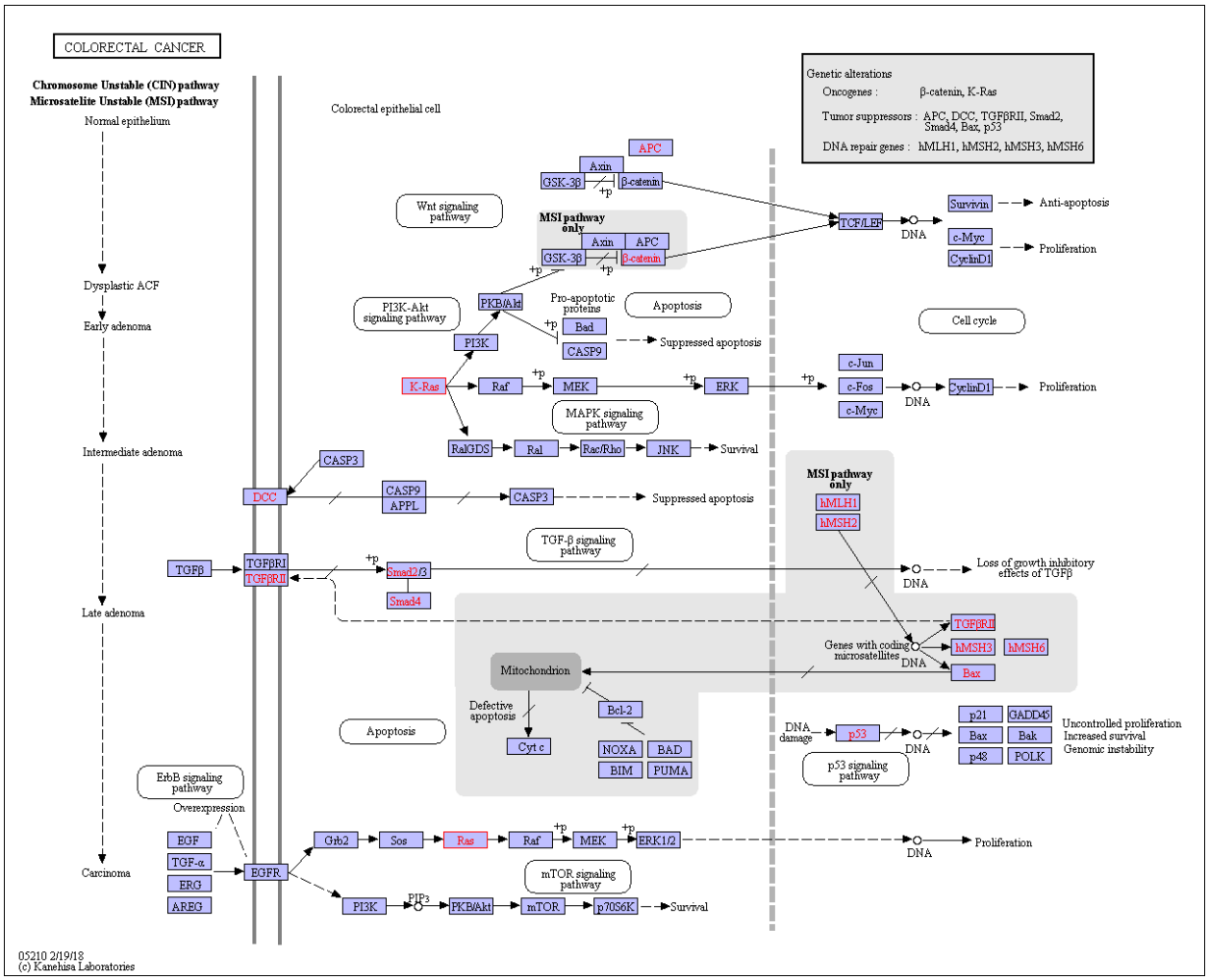

Fig 4.7| Kegg pathway analysis of KRAS gene in colorectal cancers.

RUNX2: Runt-related transcription factor 2 is a member of the RUNX family of transcription factors. The nuclear protein has a DNA-binding domain and is essential for osteoblastic differentiation and skeletal morphogenesis. According to TCGA, RUNX2 is not prognostic in colon cancers. The transcript levels are similar in colon cancer as well as the normal colon. However recent studies suggest that RUNX2 is a potent prognostic factor in human colon carcinoma patients through the promotion of cell proliferation, migration and invasive properties of carcinoma cells. RUNX2 is

partly upregulated by estrogen signals through ERβ of carcinoma cells. (Sase et al., 2012)

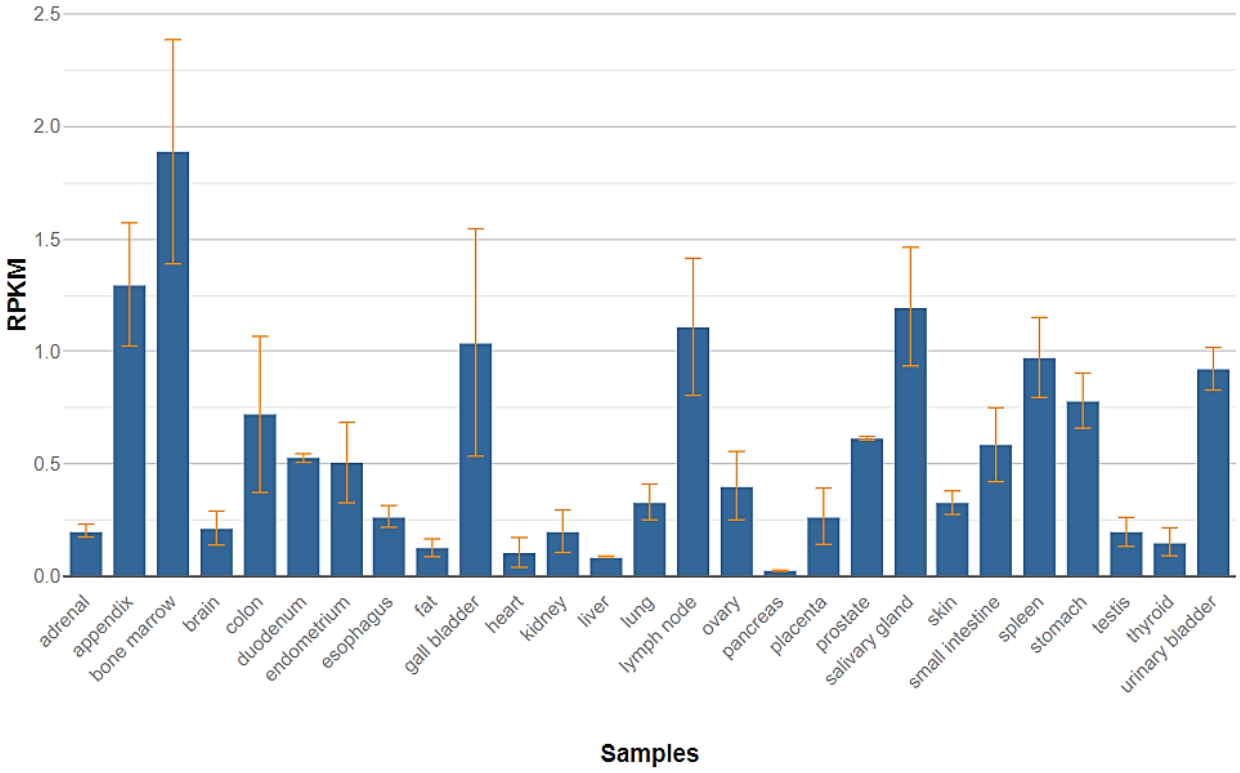

Fig 4.8| Transcript levels of RUNX2 in different normal tissues. (https://www.ncbi.nlm.nih.gov/gene/860)

The transcript levels of RUNX2 in different cancers are given below. There is a slight increase in the expression level of RUNX2 in colon cancers.

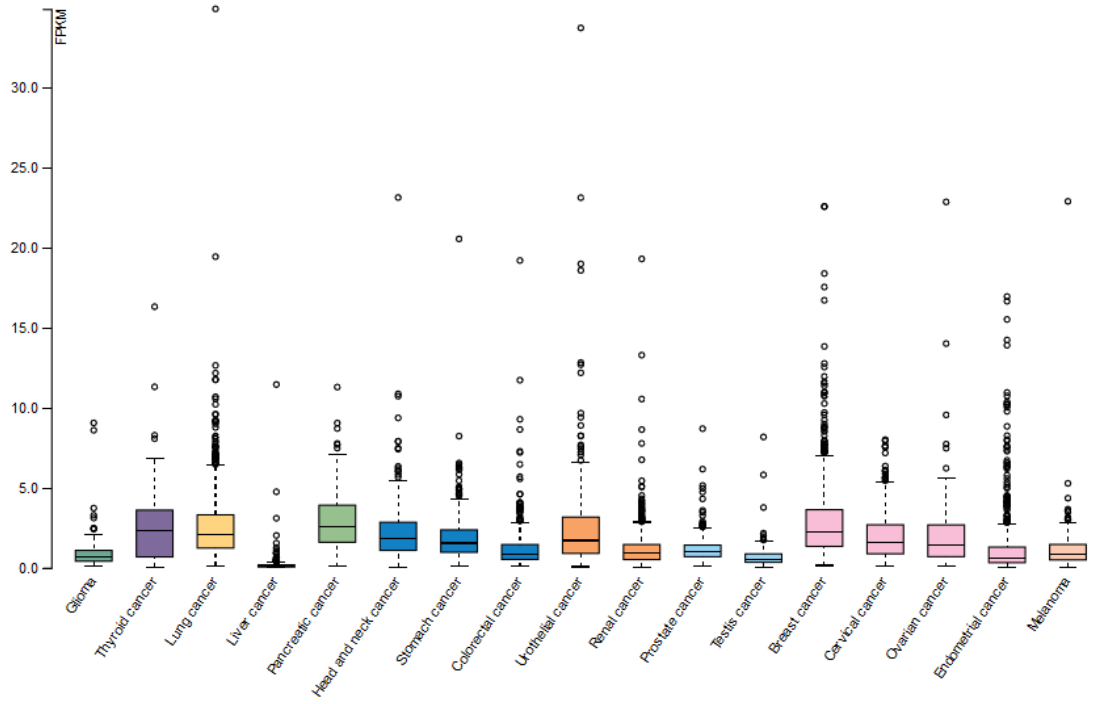

Fig 4.9| Transcript levels of RUNX2 in different cancer tissues. TCGA dataset that gives pan-cancer expression profiles of RUNX2 gene.

SNAI1: Snail family transcriptional repressor 1 is an embryonic zinc finger transcriptional repressor which downregulates the expression of ectodermal genes within the mesoderm. In the colon, normal expression is near to 0.8 RPKM. SNAI1 is prognostic in colon cancers, and high expression levels of the same are unfavourable in colorectal cancers.

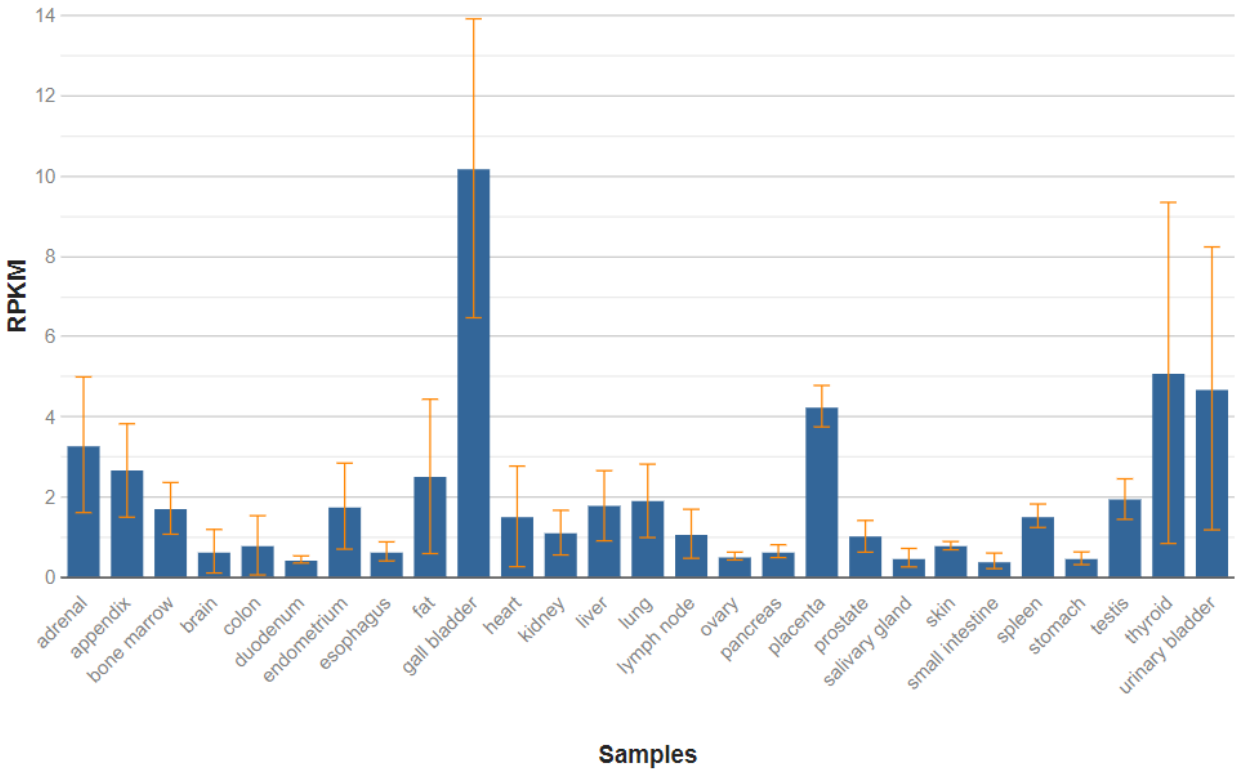

Fig 4.10| Transcript levels of SNAI1 in different normal tissues. (https://www.ncbi.nlm.nih.gov/gene/6615)

Transcript levels of SNAI1 in various cancers from TCGA are given below.

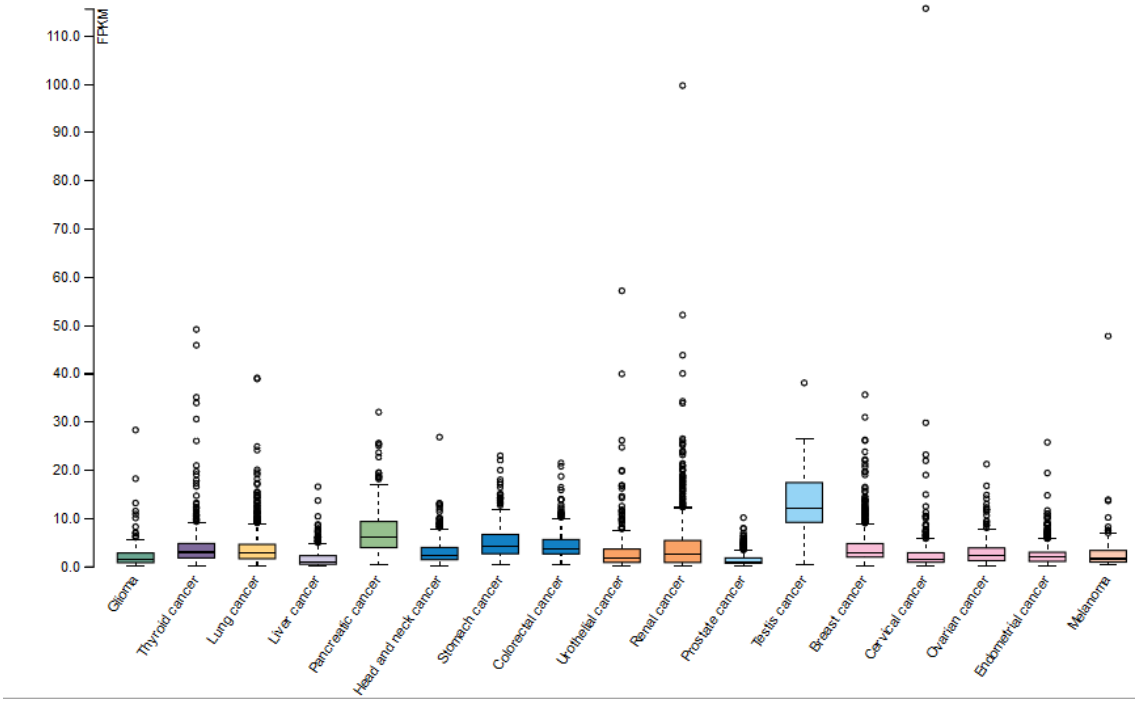

Fig 4.11| Relative expression levels of SNAI1 in various cancers in humans. Data obtained from TCGA datasets.

Median Expression levels of SNAI1 in colon cancers is about 3.6 FPKM which is relatively much higher than basal expression levels.

TNFα induces epithelial-mesenchymal transition (EMT) thereby promotes colorectal cancer. TNFα treatment increases the SNAI1 levels which result in EMT in colorectal cancer cells. SNAI1 stability is increased by activation of the AKT pathway and subsequent repression of GSK-3β activity, thereby decreasing the association of SNAI1 with GSK-3β on TNFα treatment. (Wang et al., 2013)

AKT2: AKT serine/threonine kinase 2 is a putative oncogene encoding a protein that belongs to a subfamily of serine/threonine kinases that contains SH2-like (Src homology 2- like) domains. AKT2 primarily found to be overexpressed in ovarian cancers also contributes to the malignant phenotype of a subset of ductal pancreatic cancers. The transcript levels of AKT2 in normal tissues is given below. In normal colon, AKT2 has an RPKM value of 7.8 according to NCBI.

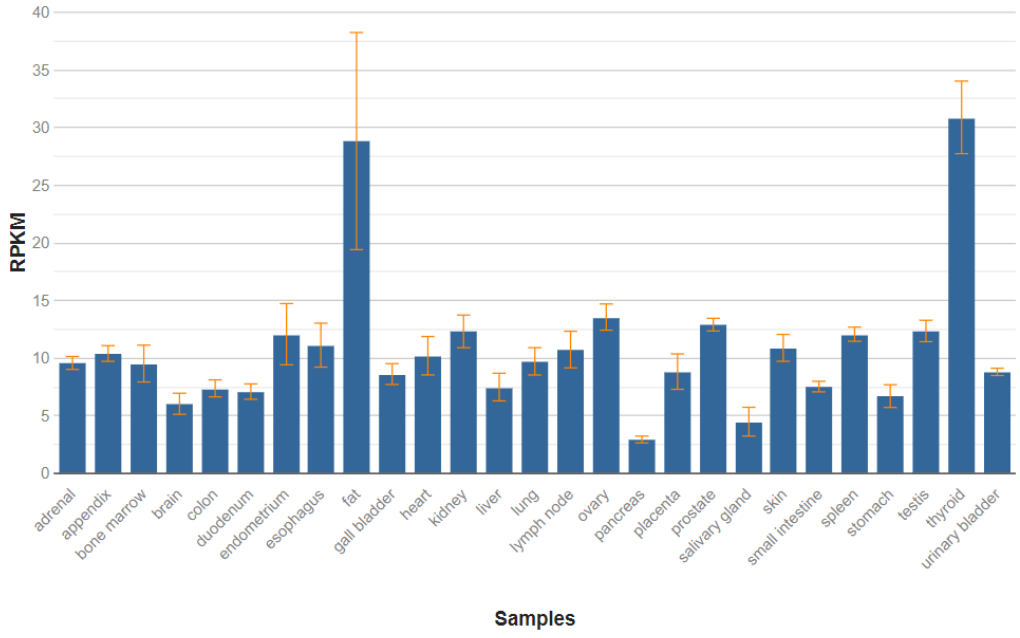

Fig 4.12| Transcript levels of AKT2 in various normal human tissues. (https://www.ncbi.nlm.nih.gov/gene/208)

In cancers, however, AKT2 is overexpressed. Given below are AKT2 transcript levels in various cancers.

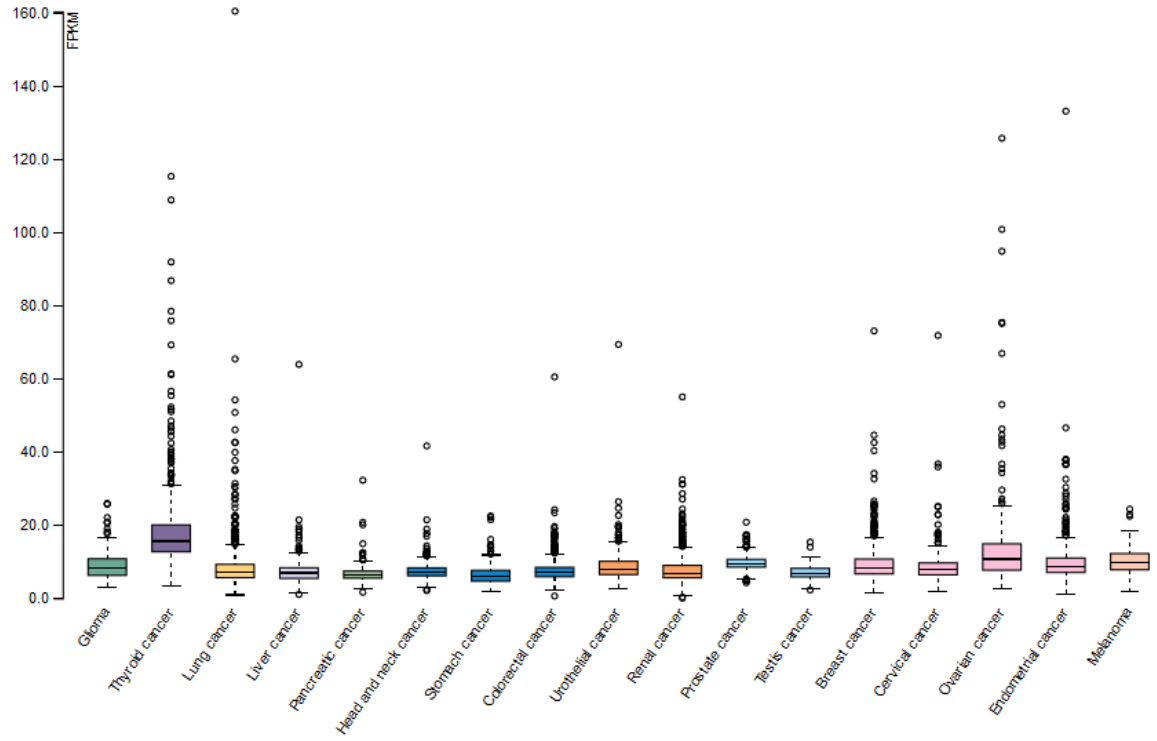

Fig 4.13| Relative expression levels of AKT2 in various cancers in humans. Data obtained from TCGA datasets.

Although TCGA claims that AKT2 is not prognostic in colon cancers, studies also suggest that AKT2 is critical in metastasis of colorectal cancer cells. AKT2 overexpression correlates with last stage colorectal cancers. AKT2 serves as a major target of PI3K for colorectal cancer metastasis establishment. PI3K pathway deregulation is a common occurrence in different tumours. AKT2 overexpression influences metastatic phenotype by promoting extravasation at the secondary metastatic sites, and along with PTEN deficiency that preferentially favours persistence and growth of metastases result in colorectal cancers. Hence AKT2 is currently considered a potential drug target in cancer therapy. (Rychahou et al., 2008)

To confirm the effect of the circadian rhythm of cancer-associated genes, we checked the expression profile of the same genes in BMAL1 knockout mouse embryonic fibroblast (MEF) cells. The transcript levels of cancer-associated genes, however, do not seem to show much variation across different time points in wild type MEF cells. Circadian gene expression confirmed that the synchronisation and hence the circadian rhythm has been induced in the cells.

The difference in expression pattern in HCT116 and MEF cells could be due to the lack of expression of these genes in the mouse embryonic fibroblast itself. However, the same genes in HCT116 cells show rhythmicity. Hence, we concluded that the expression of circadian rhythm is different in different cell lines. Extrapolating the same, we could also claim that circadian rhythms are different in different species.

CONCLUSION AND FUTURE DIRECTIONS

The project started with an objective of studying the effects nuclear envelope on circadian rhythm and vice versa in a cancer model. LBR, Lamin A/C and Lamin B2 knockdown do not seem to show statistically significant difference in gene expression. However, the published data already show that LBR affects the expression profiles of circadian genes in U2OS cells.

Further, we compared the expression profiles of genes in HCT116 and MEFs. We conclude that different genes follow different expression profiles with some following similar profiles while other different. Hence, we cannot entirely compare the two model systems definitely.

We also checked the effect of circadian clock disruption on the expression levels of candidate genes- Lamin A, B1,LBR and KRAS and find different results for different genes. This suggests that not all genes are clock controlled and hence circadian disruption need not affect all the genes alike.

As lamin knockdown study do not show an effect on the circadian rhythm (although preliminary), overexpression of lamin A and lamin B2 on cells and study their effect on the core clock gene expression.

Perform 3D FISH of cancer-associated genes that show a circadian rhythm, viz. KRAS, AKT2 at highest expressing and lowest expressing time points to compare and contrast between their position on the nucleus and hence their functional correlation to location in the nucleus.

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Supplementary

HPRT Values across time