Circadian regulation of Hepatitis B virus.

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Abstract

Whilst the role that circadian rhythm plays in human disease is emerging rapidly, its role in infection is only beginning to dawn. HBV affects over 350 million people globally with no cure and limited treatment options. Currently available interventions will not curb this epidemic. Every living organism on earth has evolved a circadian rhythm, whereby key systemic processes including metabolism, growth and immunity undergo seismic changes on a daily basis to maximise energy conservation. In order to achieve organism wide control, there are a myriad of individual cellular functions like autophagy that receive cues from a central pacemaker to optimise their functioning. The HBV replication cycle is known to be dependent on a competent autophagic pathway. It is unknown if HBV is being modulated by circadian rhythm and whether such overarching regulation would include autophagy as an intermediate process. The work presented here shows that the HepaRG cell line is permissive to HBV infection and is capable of sustaining an endogenous circadian rhythm. The NTCP receptor -key for HBV entry- is shown to be regulated by REV-ERBα. Separately, autophagosome maturation is shown to be delayed by HBV replication through the use of a dual fluorophore LC3 reporter. Both autophagy and HBV are shown for the first time to be pharmacologically modulated by an agonist of REV-ERB called SR9009, in addition to known autophagy regulators, thereby suggesting an intersecting point of governing pathways. I present evidence that HBV transcription, translation of HBsAg and HBeAg as well as mature virion release are susceptible to modulation by SR9009. Finally, I describe how an *in vivo* murine HBV model shows decreased HBV pgRNA and HBeAg following treatment with SR9009.

Whilst the broader potential role of chrono-medicine in human pathogenesis is emerging rapidly, its place in infection is in its infancy. It is anticipated that these results could be a starting point for understanding and utilising the role of circadian rhythm in HBV replication for treatment development.

This thesis is dedicated to Stewart Attridge and the many hours we spent travelling between Birmingham and Oxford to our home.

Acknowledgements

I write this as the world is facing the pandemic caused by SARS-Cov-2. Never before has the word ‘virus’ become part of our daily lexicon. Despite the threat to humanity, I am more heartned by how our community has come together in a united response. It has shown the very best in us as people. From global to local, virologist or not, people have shared their expertise and resources in a manner many would have not though possible. I feel privileged to call many of these people my peers and indeed friends.

It is almost impossible to fully acknowledge the roles played by my supervisors Jane McKeating, Xiaodong Zhuang and Philippa Matthews, thank you.

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“*Hey! I'ma keep running 'Cause a winner don't quit on themselves*”.

“I dream it, I work hard, I grind 'til I own it”.

Finally to Stu, thanks for sticking by me. I couldn’t have done this without you. On the many difficult Friday evenings and Sunday nights. On the long commutes when we were apart - thank you. Thank you for being my chef, counsellor, greatest supporter and soulmate.

Statement of originality

I declare that the work presented within this thesis entitled “Circadian regulation of Hepatitis B replication” is my own. Contributions made by collaborators have been clearly acknowledged. I have not submitted this thesis, in whole, or in part for any other degree or qualification at the University of Oxford or elsewhere.

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List of Abbreviations

ACTH	Adrenocorticotropic hormone
AL	Autolysosome
ALT	Alanine transferase
AMPK	Adenosine monophosphate activated protein kinase
AP	Autophagosome
ART	Antiretroviral therapy
B2M	Beta 2 microglobulin
BCA	Bicinchoninic acid
bHLH-PAS	Basic helix loop helix-Per arnt sim
BMAL1	Brain and muscle arnt Like 1
cccDNA	Covalently closed circular DNA
CCG	Circadian controlled gene
CK	Caesin kinase
CLOCK	Circadian locomotor output cycles kaput
CREB	cAMP response element binding protein
CRH	Corticotropin releasing hormone
CRSD	Circadian rhythm sleep disorders
CRY	Cryptochrome
CS	Corticosteroid
DDBP1	Damaged DNA binding protein 1
DBD	D-site binding protein
DKRK1A	Dual-specificity tyrosine-phosphorylated regulated kinase 1A
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DR	Direct repeat
dsDNA	Double stranded DNA
dslDNA	Double stranded linear DNA
E-box	Enhancer box

E4BP4	E4 promoter binding protein 4
EASL	European association for the study of the liver
EGFP	Enhanced green fluorescent protein
EPI	Expanded programme on immunisation
ETV	Entecavir
FACN	Fatty acid synthase
FBS	Fetal bovine serum
FBXL3	F-Box protein 3
FCS	Fetal calf serum
FoxO3	Forkhead transcription factor O3
GRE	Glucocortocoid response element
GSK	Glycogen synthase kinase
HBc	Hepatitis B core
HBcrAg	Hepatitis B core related antigen
HBeAg	Hepatitis B e antigen
HBIg	HBV immunoglobulin
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
ipRGC	Intrinsically photosensitive retinal ganglion cell
LC3	Light chain 3
miRNA	Micro RNA
MOI	Multiplicity of infection
mRFP	Monomeric red fluorescent protein
mTOR	Mammalian target of rapamycin
NA	Nucleoside analogue
NR1D	Nuclear receptor subfamily 1 group D
NTCP	Sodium taurocholate co transporting peptide
OBI	Occult HBV infection

ORF	Open reading frame
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
PegIFN	Pegylated interferon
PER	Period
PFA	Paraformaldehyde
pgRNA	Pre genomic RNA
PVDF	Polyvinylidene fluoride
RAR	Retinoic acid receptor
rcDNA	Relaxed circular DNA
RIPA	Radioimmunoprecipitation assay
RLU	Relative light unit
ROR	Related orphan receptor
RORE	ROR response elements
RT	Reverse transcriptase
SCD	Stearoyl CoA desaturase
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulfate
SREBPs	Sterol regulatory element-binding proteins
TF	Transcription factor
TTFL	Transcription-translation feedback loops
vL	Viral load
ZT	Zeitgeber
βTrCP	β-transducin repeat-containing protein

Chapter 1: Introduction

1.1 Overview of Hepatitis B

Viruses are wholly dependent on cellular machinery for their replication. The flaviviruses Hepatitis C (HCV) and Zika have been shown to be subject to influence by circadian rhythm (Zhuang *et al.*, 2017). It is unknown if Hepatitis B virus (HBV) is modulated by circadian rhythm. This is a novel direction of research in the contemporary field of chrono-infection, where understanding of the mechanisms involved are in their infancy. The HBV epidemic is showing little signs of slowing down and new therapeutic options are needed.

Numerous sub-cellular processes are increasingly recognised to be co-regulated by components of the clock framework. I hypothesize that a proportion of any circadian mediated influence on HBV replication, could occur through the co-regulation of a crucial intermediate process like autophagy (**Figure 1.1**).

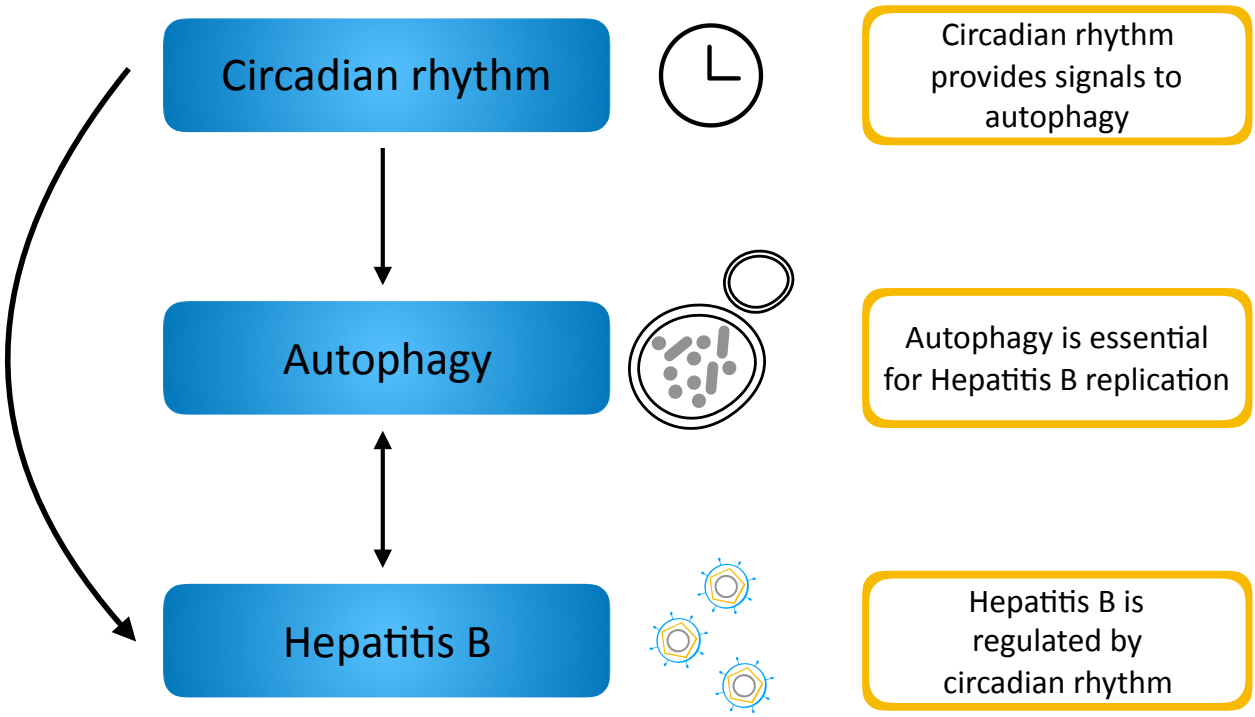


Figure 1.1: Proposed interplay between circadian rhythm, autophagy and HBV. Circadian rhythm provides cues to orchestrate autophagy. A competent autophagic pathway is known to be crucial to HBV replication. It is unknown if circadian modulation of HBV takes place and whether this would involve autophagy.

1.1.1 Epidemiology

Hepatitis B virus (HBV) causes an infection that poses an ever-increasing threat to public health. There is no cure, but an effective vaccine and suppressive antiviral treatments exist. A review of published literature between 1965 and 2013 estimates that 2 billion people have been infected with the virus, with almost 257 million people showing signs of ongoing or chronic infection (Schweitzer *et al.*, 2015). This is defined as the presence of hepatitis B surface antigen (HBsAg) in serum for longer than six months. Whilst average global prevalence of HBsAg is circa 3.61%; the burden of infection is unequally shared. Countries are broadly classified into three categories of infection: low ≤2% (Western Europe and North America), intermediate 2-8% (most of Asia and Eastern Europe, further sub-illustrated as low/high intermediate) and high ≥8% (much of sub-Saharan Africa and parts of Asia) (**Figure 1.2**). There are some particularly vulnerable patient groups at risk of HBV infection. The risk of developing chronic infection is inversely related to age. If infected perinatally the chance of developing chronic infection exceed 90%, this decreases to between 20-60% for those aged between six months and six years, and declines to under 5% if infection occurs as an adult (Hyams, 1995). Whilst access to vaccination and maternal treatment is decreasing the incidence in some highly endemic areas, these interventions remain far from universal. HBV attributed liver cirrhosis and hepatocellular carcinoma (HCC) cause approximately 887,000 deaths annually (World Health Organization, 2017). Disease awareness is poor, as of 2016 only 27 million people (10.5%) living with HBV were aware of their infection while only 4.5 million (16.7%) were receiving treatment (World Health Organization, 2017). A prevalence model suggests that 63 million new cases of chronic infection and 17 million HBV-related deaths will occur between 2015 and 2030 because of ongoing transmission and poor access to treatment (Nayagam *et al.*, 2016). A forecasting model suggests that even with combined neonatal HBV vaccination and treatment to prevent vertical transmission; it will still take centuries to eliminate HBV (McNaughton, Lourenço, *et al.*, 2019). Traditionally, rates of infection in

western countries were low with most infection due to sexual transmission or contact with infected blood through drug abuse. However, migration and population movement by refugees is increasing the prevalence of HBsAg carriage in these regions. A meta-analysis of 110 studies representing 209,822 immigrants and refugees estimated a seroprevalence of 7.2% infection and 40% immunity to HBV (Rossi *et al.*, 2012).

Ambitious international sustainable development goals set by the UN prompted the WHO to draft the Global Viral Hepatitis Strategy; if reached they will reduce the number of deaths by 65% and increase treatment rates from 1% to 80% (WHO, 2016). This is an epidemic that is showing little evidence of slowing down that will require new therapeutics and a combined global effort to curb.

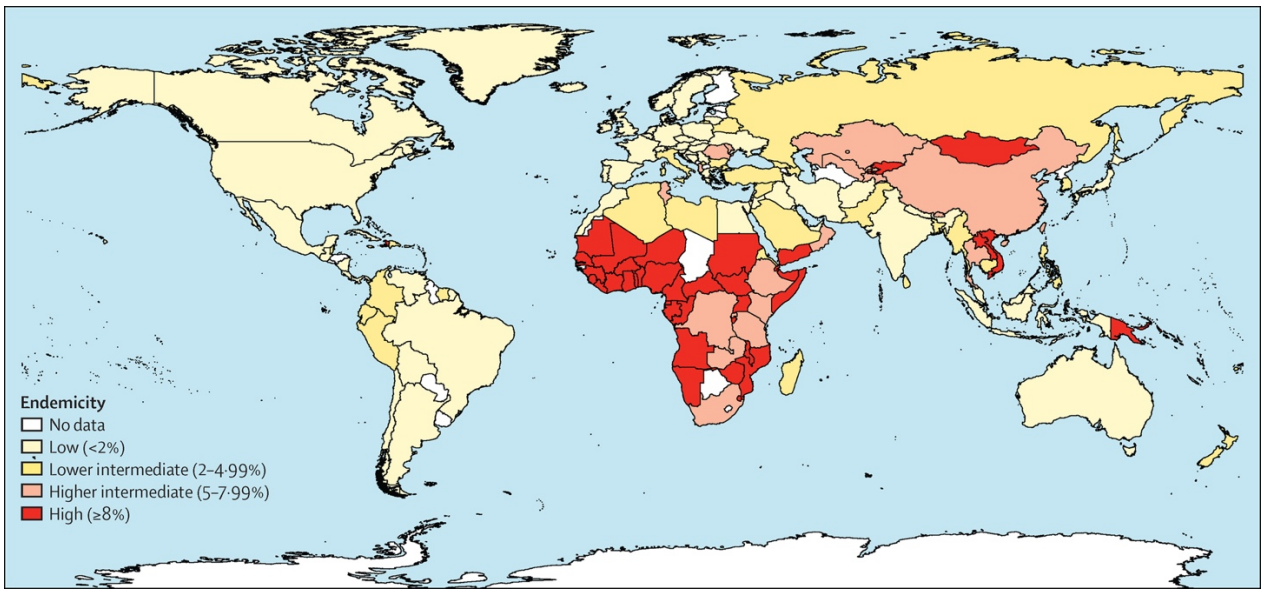


Figure 1.2: Global HBsAg endemicity by country. Global average HBsAg prevalence is 3.6% with a wide inter-country variation observed. Countries are grouped into four categories based on HBsAg prevalence. Taken from (Schweitzer *et al.*, 2015), permission obtained.

1.1.2 Clinical manifestations.

There is a wide spectrum of clinical manifestations possible in both acute and chronic HBV infection. Acute infection - defined as the presence of HBsAg in serum for less than six months - can range from subclinical to anicteric or icteric hepatitis to occasionally fulminant hepatitis. Manifestations of chronic infection can range from asymptomatic to inflammatory liver disease, signs of liver fibrosis or cirrhosis and HCC. Extra-hepatic manifestations of HBV infection have been described and include vasculitis, peripheral neuropathy and glomerulonephritis (Liang, 2009). HBV carries a 10-25% lifetime risk for developing HCC and in direct contrast to HCV, malignancy can develop in the absence of cirrhosis (Crissien and Frenette, 2014). Worldwide, approximately 54% of cases of HCC can be attributed to HBV infection alone, whilst patients with HBV and cirrhosis have a 2% annual risk of developing malignancy (European Association For The Study Of The Liver, 2018). Several studies have identified individual HBV parameters - genotype C (Yu *et al.*, 2005), Hepatitis B e Antigen (HBeAg) status (Yang *et al.*, 2002) and viral load (vL) (Chen *et al.*, 2006)- as independent risk factors for the development of HCC. Of these, the only readily modifiable risk factor amenable to currently available therapeutics is HBV viral load. There is a significant dose dependent relationship between serum HBV DNA and the incidence of HCC. Strikingly, there is a cumulative incidence for HCC of 14.9% for patients with a HBV DNA level of 1 million copies/mL or greater, compared with 1.3% for patients with an HBV DNA of 300 copies/mL per 100,000 person years (Chen *et al.*, 2006). Accordingly, current therapeutics focus on decreasing viral load which consequently decreases the risk of developing cirrhosis and HCC.

1.1.3 Natural history

Clinical HBV infection is a dynamic process reflecting the interaction between host immune response and the virus. Current guidelines stratify patients for therapy according to age, sex and markers of inflammatory or fibrotic liver disease ((alanine transferase (ALT), HBV DNA vL and a fibroscan or biopsy score). The initial clinical assessment and proposed nomenclature by the European Association for the Study of the Liver (EASL) for each disease stage is shown in (**Figure 1.3**). HBV infection has five classical disease stages. These are represented by dynamic levels of HBV DNA, the liver enzyme ALT, and HBsAg over time (**Figure 1.4**). In addition to the four illustrated, a fifth stage characterised by loss of HBsAg -with or without the presence of antibodies to HBsAg- termed occult HBV infection (OBI) is possible. OBI represents the most favourable stage for patients as although replication competent covalently closed circular DNA (cccDNA) persists in hepatocytes, serum DNA levels tend to be undetectable or very low. Therefore, despite the possibility of reactivation remains, it represents the most quiescent phase of HBV infection. The biology and detailed clinical impact of OBI is comprehensively reviewed by (Raimondo *et al.*, 2019). Notable exceptions exist to these staging criteria, the existence of which treating healthcare staff must be aware of e.g. HBsAg escape and pre-core HBeAg mutants. Additionally, the disease stages do not always occur in a linear fashion nor will all stages be experienced by every patient. They are used as a guide to anticipate and thus prevent periods of liver damage.

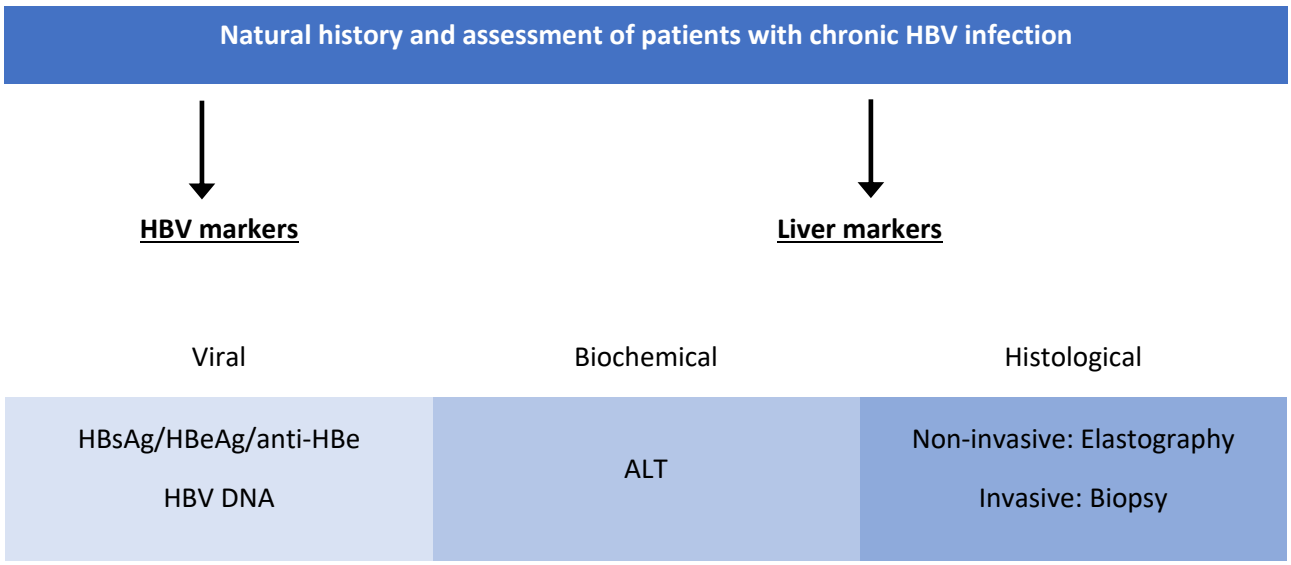
Significant gaps persist regarding the highly dynamic nature of HBV infection. In particular, the accuracy between the currently utilised clinical biomarkers and true disease activity, notably: transcriptional activity. The capability to quantitively measure cccDNA in a clinical setting is an unmet need and its absence is difficult to overstate. It should be noted that it is the transcriptional activity surrounding cccDNA rather than its total quantity that correlates with

disease progression. cccDNA is a dichotomy that represents both the most significant barrier and likely viral target towards achieving a sterilising cure. Levels of cccDNA are known to vary over time and do so roughly in line with the stages outlined by EASL, (European Association For The Study Of The Liver, 2017a). The true effectiveness of current and any purported future therapeutics will be gauged by the extent of their modulation of the cccDNA pool (Yang and Kao, 2014; Tu *et al.*, 2017).

Existing clinical biomarkers do not directly reflect the level of transcriptional activity of cccDNA. The requirement to perform a liver biopsy with a large sampling error to make even a rudimentary estimate is an ample deterrent. Consequently, there is a reliance on serum biomarkers. The quantity of serum HBsAg is known to fall on NA therapy but its use as a biomarker in EAg negative disease is problematic, likely due to the transcription of HBsAg from integrated DNA rather than cccDNA, as evidenced in primate models (Zoulim *et al.*, 2015; Wooddell *et al.*, 2017). Its use as a quantitative marker of cccDNA is highly debated (Chan *et al.*, 2011; Lebossé *et al.*, 2017). pgRNA is not directly targeted by NA therapy, is rarely measured clinically and so remains almost exclusively in the domain of academic research rather than diagnostic laboratories. HBeAg status is crudely used to make a preliminary distinction between active and inactive disease with numerous caveats. Peripheral DNA quantification is a key clinical parameter but even that does not give an accurate picture of the burden of hepatic cccDNA and crucially once treatment with NA is established it can no longer be used as such (suppressed peripheral HBV DNA due to NA therapy is not reflective of cccDNA transcriptional activity), thereby removing the most readily available biomarker.

A possible interim solution is HB core related antigen (HBcrAg). This represents a promising novel serum biomarker that has been shown to correlate accurately with intrahepatic cccDNA transcriptional activity (Kimura *et al.*, 2005; Seto *et al.*, 2014; Testoni *et al.*, 2019). This enables the efficacy of future therapies to be measured against this critical benchmark and would allow some of the gaps in our knowledge about the dynamics of HBV infection at different

stages to be elucidated. This is one of the reasons that measurements of cccDNA -where possible- were attempted in this thesis, in addition to the measurement of standard clinical HBV parameters. It is hoped it would allow retrospective comparison of new developments in HBV biomarker research and antiviral agents.



	HBeAg positive		HBeAg negative	
	Infection	Hepatitis	Infection	Hepatitis
HBsAg	High	High/intermediate	Low	Intermediate
HBeAg	Positive	Positive	Negative	Negative
DNA	>10^7 IU/mL	10^4-10^7 IU/mL	<2,000 IU/mL	>2,000IU/mL
ALT	Normal	Raised	Normal	Elevated
Liver disease	None/minimal	Moderate/severe	None	Moderate/severe
Old terminology	Immunotolerant	Immune reactive HBeAg positive	Inactive carrier	HBeAg negative chronic hepatitis

Figure 1.3: Initial clinical assessment of patients with and disease stages of HBV infection. Illustration showing the clinical markers used to assess patients with chronic HBV infection. Tabulated underneath are the HBV disease stages and associated expected clinical parameters. Adapted from (European Association For The Study Of The Liver, 2017b).

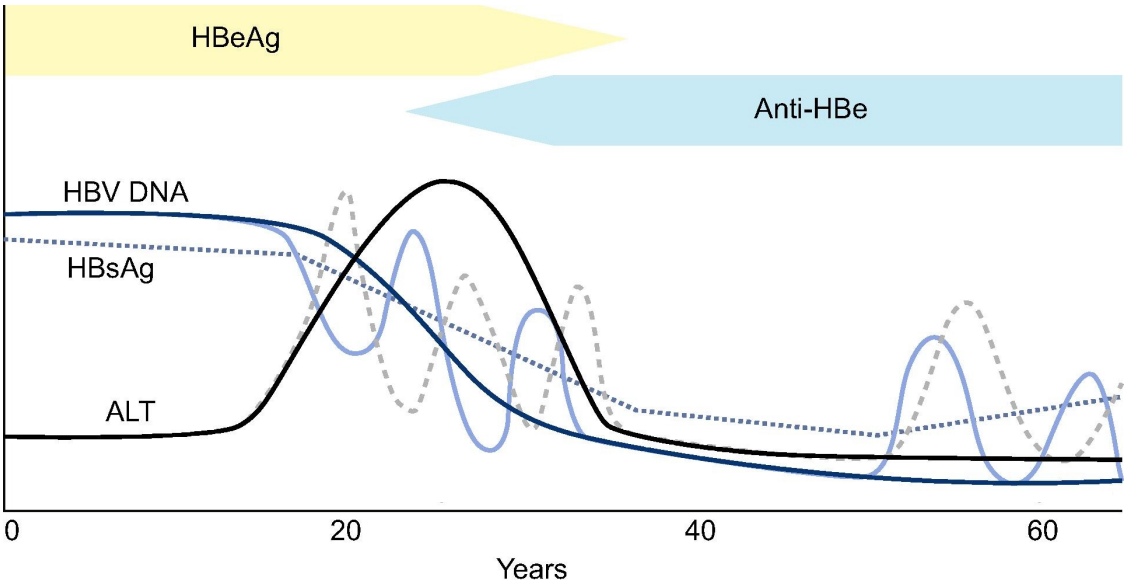


Figure 1.4: The evolution of viral and host parameters over time in response to HBV infection. Adapted from (Lok *et al.*, 2017).

1.1.4 HBV cure.

It is noteworthy that the concept of a 'cure' in HBV is difficult to define in practice. A sterilising cure would be defined as complete elimination of HBV, removing the need for further treatment with no risk of viral relapse or liver disease progression. This is generally regarded as uncommon due to two specific features of the HBV life cycle, namely the formation of a stable, long-lived, episomal minichromosome in the form of cccDNA in the nucleus of infected hepatocytes, and integration of viral DNA into the host genome (**Figure 1.5**) and discussed in greater detail in (**Appendix 1**). Current therapies do not have a direct influence on the cccDNA pool and leave viral integration entirely untouched. It is estimated that - using available studies on viral kinetics - it would take 14.6 years on continuous nucleosi(t)ide analogue (NA) therapy to diminish the cccDNA pool (Yang and Kao, 2014). Although integrated HBV DNA serves as a replicative dead-end for the virus, its oncogenic potential persists, and precise clinical implications remain unknown (reviewed comprehensively by (Tu *et al.*, 2017)). In an attempt to standardize endpoints for upcoming clinical trials involving a rapidly expanding cohort of new HBV therapeutic agents and expedite the pathway between discovery and regulatory approval, new consensus definitions needed to be agreed upon. This occurred at an international summit where a definition of 'functional cure' was accepted (Lok *et al.*, 2017). This is defined as a sustained, undetectable HBsAg and serum DNA with or without development of anti-HBs occurring after completing a finite course of treatment, with resolution of residual liver injury and a decrease in risk of HCC over time. It is noteworthy that spontaneous rates of HBsAg loss are 0.5-1% per year. Whilst the pursuit of a functional and ideally a sterilising cure is underway, there are two main classes of therapeutic agents currently available.

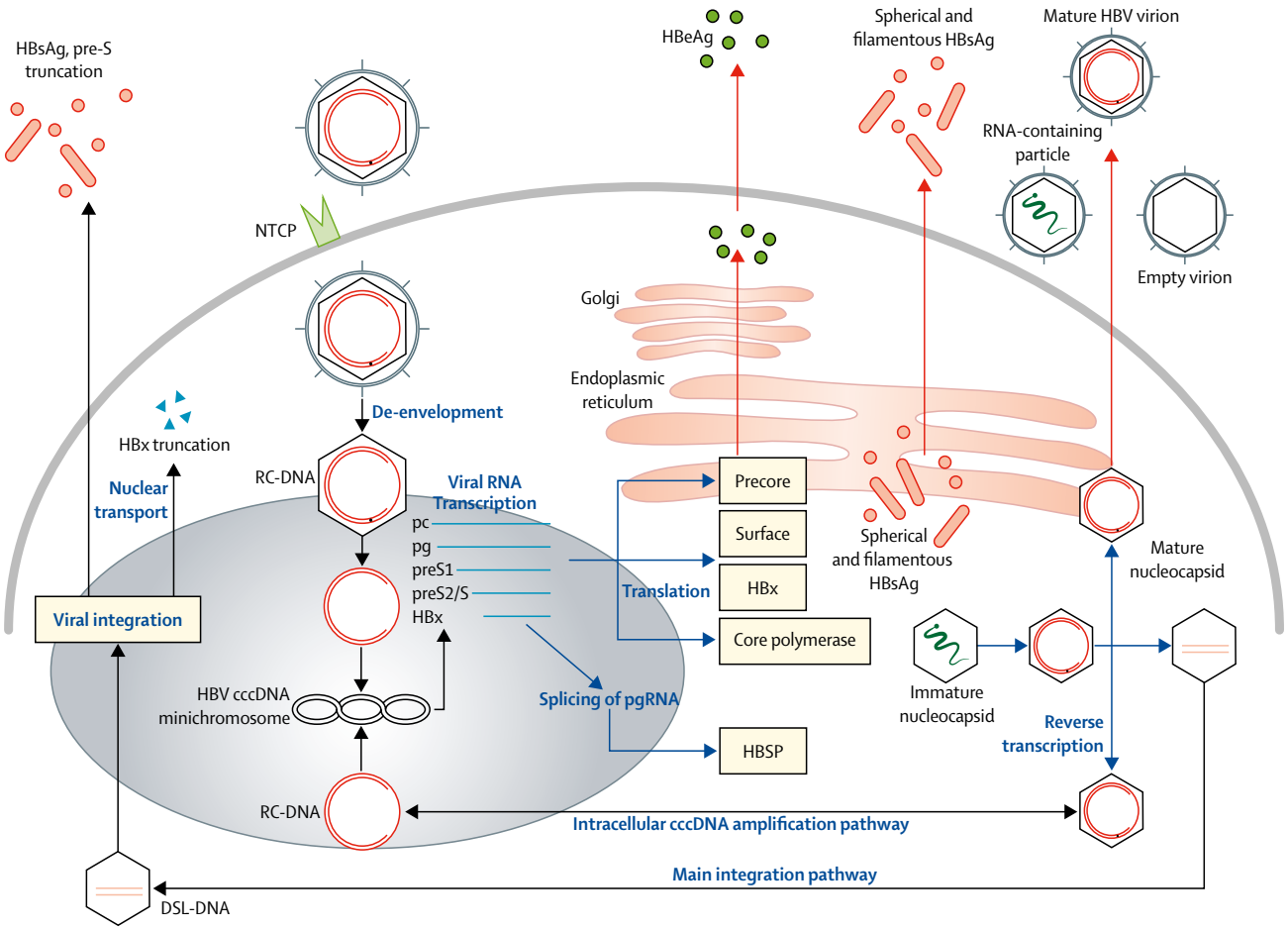


Figure 1.5: HBV replication cycle. Viral entry occurs via binding of the preS1 domain of the large HBsAg envelope protein to the NTCP receptor. After uncoating the nucleocapsid is transported to the cell nucleus. The incoming partially double-stranded genomic viral DNA is converted from its relaxed circular formation into cccDNA by host DNA repair machinery. This acts as the HBV transcriptional template and long-lived reservoir in the hepatocyte nucleus. The 3·2 kb HBV genome is organized into four overlapping but frame-shifted open-reading frames. Viral promoter and enhancer elements promote transcription of five major mRNAs, which are translated into seven viral proteins. The pgRNA is encapsidated with polymerase and reverse transcribed into rcDNA. Following viral DNA particle synthesis, mature nucleocapsids are either packaged with envelope proteins and exported as infectious virions or recycled back into the nucleus to amplify the cccDNA pool. Adapted from (Revill *et al.*, 2019), permission obtained.

1.1.5 HBV management.

There are numerous comprehensive clinical guidelines on the management of patients with HBV infection. UK based practitioners most commonly use NICE or EASL guidelines (NICE, 2013; Terrault *et al.*, 2016; European Association For The Study Of The Liver, 2017a). The overarching goal of care for patients with HBV is to improve survival by stopping disease progression to cirrhosis whilst decreasing likelihood of HCC development. Additional goals include; preventing transmission (vertical or horizontal), reducing the risk of reactivation, and preventing or treating extra-hepatic manifestations. These goals can be achieved by modifying various clinical parameters; long term serum HBV DNA suppression, HBeAg seroconversion, normalisation of ALT, HBsAg loss and improved histological markers. The main therapeutics available to achieve these goals are pegylated interferon α (PegIFNα) or NAs. The precise mechanism of action of PegIFN therapy is not known. It is thought to have a dual role both as a directly acting antiviral and also augmenting the body's own immune response to HBV infection by modulation of IFN-stimulated genes (Tan *et al.*, 2018). NAs like tenofovir or entecavir competitively bind to HBV viral polymerase (reverse transcriptase) and cause premature termination of DNA transcription with resultant chain termination (Delaney IV *et al.*, 2006). Each therapy has its own merits and disadvantages.

(i) NA therapy is administered daily orally, newer agents have a high genetic barrier to resistance, are potent and well-tolerated (Lok *et al.*, 2016). It is used to achieve HBV DNA suppression and for many patient groups (decompensated liver disease and pregnancy) represents the only suitable therapy.

(ii) Treatment with PegIFNα is composed of a weekly subcutaneous injection for a finite period of usually 48 weeks. The goal is to achieve enhanced long-term immunological control whether by seroconversion to anti-HBe or loss of HBsAg. PegIFNα is contraindicated in vulnerable patient groups (pregnancy and decompensated liver disease) and has numerous tolerability and safety concerns that preclude its wider use. Additionally, there is significant variation in the clinical response observed. These issues can be somewhat mitigated by optimal patient selection using predictive response criteria (HBeAg status, HBsAg levels, viral genotype) prior to commencing therapy. These are coupled with early cessation of therapy if specific benchmarks of progress are not met. Previously uncommon, quantitative HBsAg measurements are increasingly incorporated into aspects of HBV disease management as reviewed by (Cornberg *et al.*, 2017).

1.1.6 Immunization

Passive immunization with specific HBV immunoglobulin (HBIg) is obtained from the pooled plasma of human donors and is recommended - in conjunction with active immunization - when rapid (albeit temporary) protection from HBV is required, such as in the immediate perinatal period when a baby may be exposed to high maternal viral loads. UK guidelines for HBIg and the HBV vaccine are contained in Chapter 18: Hepatitis B, of the "Green Book" (Department of Health, 2017).

Active immunization with a recombinant vaccine consisting of HBsAg is available. Protection is based upon production of antibodies to HBsAg: anti-HBs. It is a safe and highly efficacious vaccine that in the majority of cases results in sufficient levels of anti-HBs to give protection in

>90% of healthy recipients (Van Damme and Van Herck, 2007). Vaccine response rates typically wane in patients over the age of 40 and those who are immunocompromised or on dialysis. It is normally given in three doses in a dosing schedule dependent on the clinical scenario. The HBV vaccine has proved an effective tool in preventing onward transmission. In conjunction with neonatal HBIg administration, vertical transmission can be reduced by almost 92% compared to no intervention (Lee *et al.*, 2006). Since 1987 the WHO has recommended universal infant or adolescent immunisation as part of the Expanded Programme on Immunisation (EPI). HBV mass vaccination plays an integral component of the global strategy towards eradication (Chen, 2009). In those highly endemic countries where mass vaccination programmes were implemented, a significant drop in HBsAg carriage - along with long lasting immunity in response to the vaccine - has been observed, which has resulted in a significant decrease in HBV related morbidity. Taiwan implemented its programme in 1984 and has seen a drop from 15% to 1.2% in HBsAg carriage and 20.6% to 2.9% in anti-HBc (Shepard *et al.*, 2006; Ni *et al.*, 2007). However, HBV vaccine-resistant escape mutants have emerged; this was first described in a child born to a HBeAg-positive mother who went on to develop acute HBV infection despite appropriate vaccination and passive immunization with HBIg (Zanetti *et al.*, 1988). This strain was found to contain a substitution mutation of a glycine to an arginine at position 145 (SG145A) in the HBsAg protein (Carman *et al.*, 1990). There is an increasing recognition of escape mutants that contain additional mutations in the envelope and polymerase regions; these infections bypass normal serological diagnosis, evade vaccine protection, contribute towards OBI and represent an emerging public health threat (Coleman, 2006; Sheldon and Soriano, 2008).

1.1.7 Viral genome and replication

HBV is a small enveloped partially double stranded DNA (dsDNA) virus of 3.2kb belonging to the *Hepadnaviridae* family. It is a hepatropic virus replicating almost exclusively in mature hepatocytes. The Sodium Taurocholate Co-transporting Polypeptide (NTCP) cell receptor was first described in 2012, and is crucial for both HBV and Hepatitis D virus entry into hepatocytes (Yan *et al.*, 2012; Ni *et al.*, 2014). The HBV replication cycle is outlined in (**Figure 1.5**) and discussed in detail in (**Appendix 1**). Although a DNA virus, HBV replicates through RNA intermediates utilising a viral reverse transcriptase (RT) enzyme. This aligns HBV replication to that of retroviruses, although notably integration is not a pre-requisite to successful replication. Mature virions are comprised of an icosahedral capsid which contain HBV relaxed circular DNA (rcDNA) and a viral polymerase. The nucleocapsid is wrapped in a surface envelope composed of HBsAg and lipids likely derived from host cell (Patient *et al.*, 2007).

Prior to molecular methods HBV was classified into serotypes based upon HBsAg response, but now is categorised into eight genotypes (A-H) based on a >8% difference in the genome, along with an additional putative two genotypes (I and J) and numerous subtypes. These genotypes, and subtypes, are associated with different disease outcomes and responses to IFN treatment (Palumbo, 2007; Schaefer, 2007). Genotypes make little difference to the efficacy of NA therapy due to a common viral RT. Unfortunately not all patients have access to treatment or are diagnosed late if at all; in this group genotype B and C are associated with worse outcomes (Kao *et al.*, 2000).

HBV overcomes the constraints of a small genome by using unique transcription and translation strategies to maximise its coding capacity. This compact viral genome contains four

overlapping open reading frames (ORFs) that correspond to four genes (Surface, Core, Polymerase and X) that encode seven proteins: HBeAg, HBcAg, HBV polymerase (Pol), three variants of HBsAg (denoted by their size as large (L) or pres-S1 , medium (M) or pre-S2, and small (S) glycoproteins) and HBx (X protein) (Seeger and Mason, 2015; Tong and Revill, 2016). The spatial organisation of the ORFs in the viral genome are illustrated in (**Figure 1.6**). Transcription is modulated by four promoters (Core, X, S1 and S2) and two enhancers (EnhI and EnhII) to produce at least five major mRNA transcripts namely; a 3.5kb precore mRNA, a 3.5kb core mRNA, a 2.4 kb preS1, a 2.1 preS2/S mRNA and a 0.7kb HBx mRNA. The RNA transcripts with corresponding HBV ORFs and resultant proteins are illustrated in (**Figure 1.7**). Both enhancers which are required for promoter activity have binding motifs for an increasingly recognised number of ubiquitous and liver specific transcription factors (TFs) that are thought to contribute towards viral hepatotropism. EnhI regulates all four HBV promoters and EnhII regulates Core (Guo, Bell and Ou, 1991).

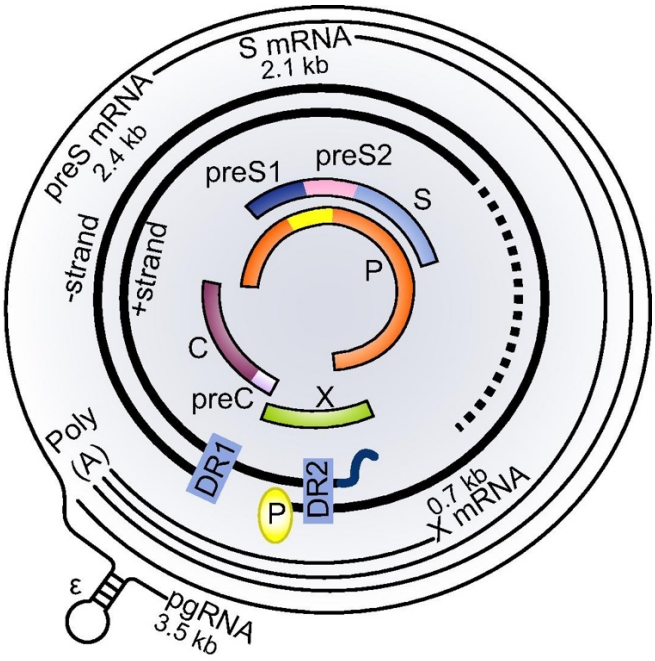


Figure 1.6: Organization of the HBV viral genome, viral RNA transcripts and ORFs. Adapted from (Tong and Revill, 2016)

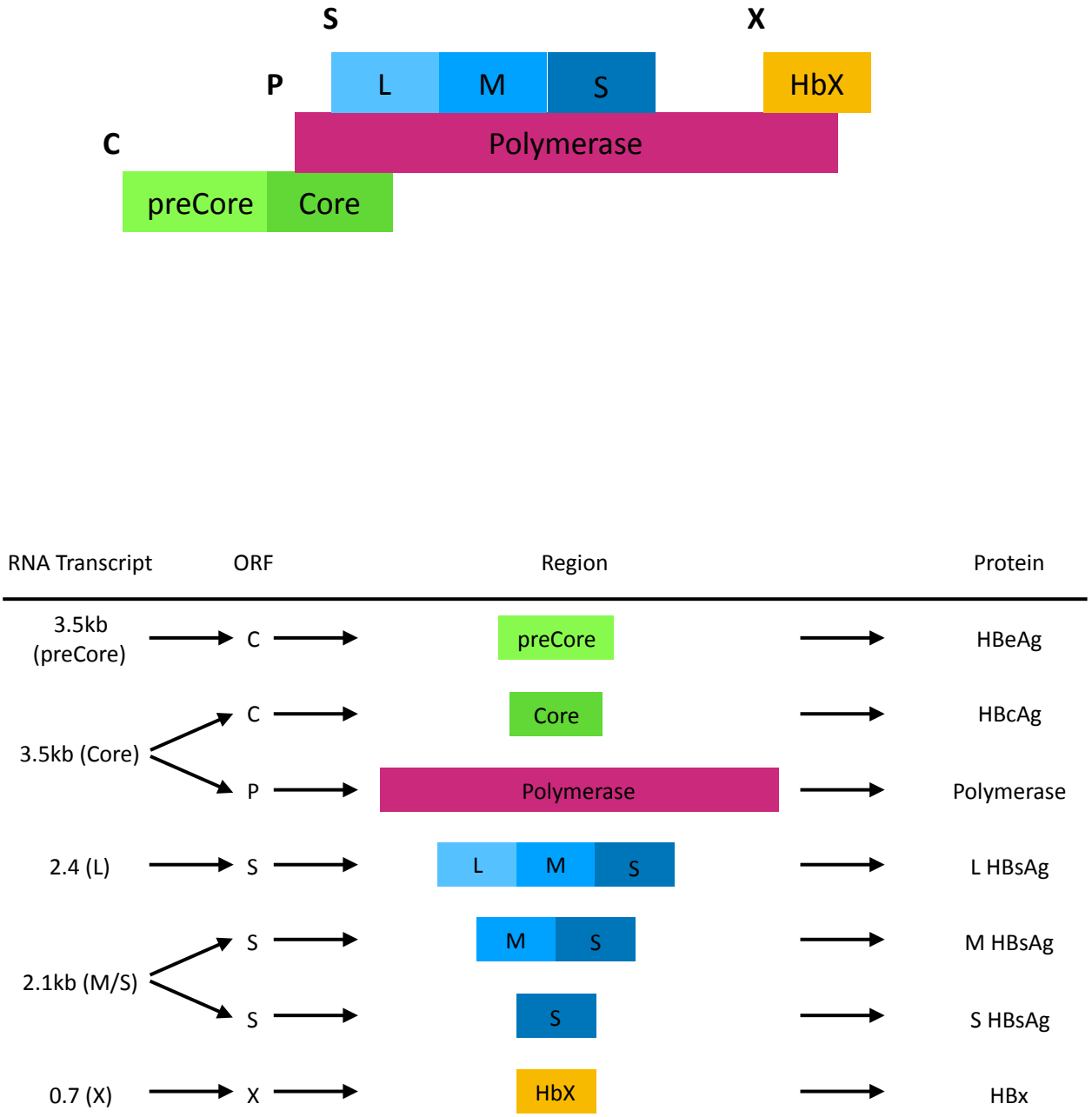


Figure 1.7: HBV ORFs with resultant mRNA transcripts and protein products. A linearized illustration of the four ORFs with corresponding protein product is illustrated above a tabulated summary. The Core ORF generates two transcripts that contain two in frame translational start codons that generate HBeAg and HBcAg. Polymerase is produced from the Core transcript but less frequently as its initiator codon is several hundred base pairs away at the 5' end of its ORF. The S ORF generates two transcripts with three start codons to produce three variant sizes of HBsAg. The X ORF produces a single transcript responsible for HBx protein production. Adapted from (Hadziyannis and Laras, 2018).

1.2 Overview of Circadian Rhythm

The cellular landscape undergoes daily seismic changes as part of its circadian rhythm. Viral replication is entirely dependent on these cellular processes. It is unknown if circadian rhythm could modulate HBV replication. Circadian rhythm is increasingly recognised as a component of human pathology.

1.2.1 Introduction to mammalian circadian rhythms

The word circadian is a derivative from the Latin words *circa* meaning "around" and *diēm* meaning "day". The earliest recorded account of a circadian process was in the 4th century BC, when a naval admiral of Alexander the Great called Androsthenes described the diurnal leaf movements of a tamarind tree (Bretzl, 1903). A diurnal rhythm refers to any physiological, behavioural or other biological process that repeats itself approximately every 24 hours, correlating with the natural period of the Earth's rotation. A period is the length of time a rhythm takes to repeat itself. Accordingly, a circadian rhythm is any diurnal process that is endogenously generated and self-sustaining within the organism. Circadian rhythms are experienced from organisms as diverse as cyanobacteria to complex eukaryotes (Bhadra *et al.*, 2017). It is noteworthy that although circadian rhythms are endogenous, they can respond to external cues e.g. light, feeding or sleeping which are called zeitgebers (ZT), derived from the German words *zeit* "time" and *geber* "giver". These ZT serve to adjust the phase - defined as the timing of the rhythm relative to a fixed point – of the organism's endogenous circadian system to match a changing environment. This is akin to humans adjusting to having travelled across multiple time zones, whereby the circadian rhythm synchronises to the new time zone in response to newly timed ZT. The phase of their circadian clock will advance or regress to

align with these newly timed stimuli to avoid asynchrony. This process of adjusting phase to ZT is called entrainment. Descriptive terms of a circadian waveform are illustrated in (**Figure 1.8**)

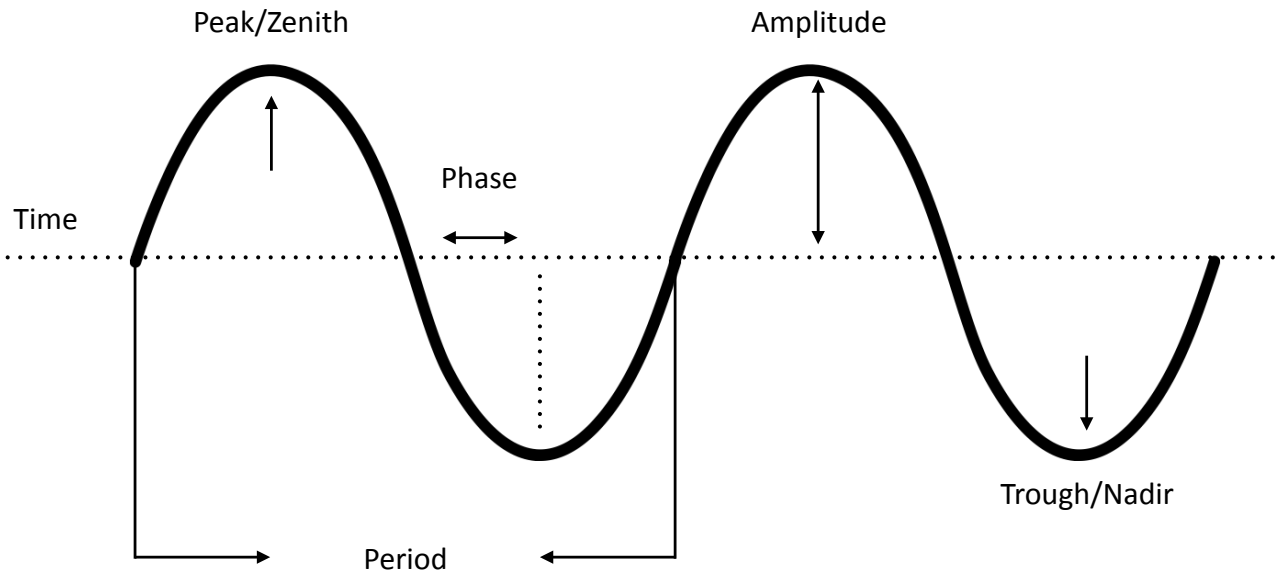


Figure 1.8: Structure of a circadian waveform. Modified from (Poggiogalle, Jamshed and Peterson, 2018).

Circadian rhythms have evolved to meet the requirement of living organisms to optimise energy conservation. This is achieved by temporal segregation of opposing metabolic processes (catabolism and anabolism) and by anticipation of activity-feeding-fasting cycles to maximise efficiency thus promoting survival (Gerhart-Hines and Lazar, 2015; Panda, 2016; Poggiogalle, Jamshed and Peterson, 2018). To achieve such broad metabolic control there are networks of intermediary targets that lie beneath the overall arc of circadian modulation.

(i) Corticosteroid: Exposure to dexamethasone is capable of inducing rhythmic gene expression in cultured fibroblasts and can change the phase of gene expression in multiple rat organs (liver, kidney and heart) (Balsalobre *et al.*, 2000). These observations demonstrate synchronisation with subsequent entrainment to a ZT *in vivo* and *in vitro*.

(ii) AMPK: the cellular energy sensor adenosine monophosphate activated protein kinase (AMPK) is capable of transmitting energy dependent signals - by phosphorylation - to the key mammalian clock regulator proteins cryptochrome (CRY) and PER thus rendering them inactive (Jordan and Lamia, 2013). This illustrates a crucial link between known metabolic cellular energy homeostatic pathways and modulators of the circadian clock. The observation that AMPKs subunit composition, cellular location and substrate phosphorylation showed time-dependent variation further enforces this relationship.

(iii) Cholesterol synthesis: Mice treated with an agonist of the circadian clock repressor REV-ERBα show reduced levels of plasma cholesterol in addition to reduced expression in an array of genes responsible for sterol biosynthesis (Sitaula *et al.*, 2017). This adds to an expanding body of evidence that REV-ERB plays a part in the

circadian modulation of genes on a broader systematic level, as well as its localised role in the negative transcription feedback loop of BMAL1.

(iv) Glucose homeostasis: During fasting periods, mammals maintain glucose homeostasis by hormonal stimulation of hepatic gluconeogenesis, activating the cAMP mediated phosphorylation of cAMP response element binding protein (CREB) (Nordlie, Foster and Lange, 1999). Rhythmically expressed CRY decreases hepatic gluconeogenesis by decreasing glucagon mediated increases in CREB phosphorylation. Consequently, overexpression of CRY1 decreases serum glucose and improves insulin sensitivity in an insulin resistant murine model (Zhang *et al.*, 2010). This highlights how perturbations of the mammalian clock may promote certain metabolic pathologies.

The composite effect is that the whole spectrum of metabolic processes - gluconeogenesis, insulin secretion, cholesterol synthesis, fat oxidation and energy expenditure - follow and contribute to regulation of a daily circadian clock (Li, Li and Wang, 2012; Gerhart-Hines and Lazar, 2015). Whilst metabolism appears to be the homeostatic process most finely tuned to circadian regulation, it is certainly not the only one. Autophagy, cellular regeneration, inflammation and immunity are all increasingly understood to be regulated by clock components (Ma, Panda and Jiandie D Lin, 2011; Scheiermann, Kunisaki and Frenette, 2013; Poggiogalle, Jamshed and Peterson, 2018). The circadian modulation of the autophagic pathway will be discussed separately.

Whilst the pharmacological development of REV-ERB agonists to permit a deeper analysis of circadian architecture is undoubtedly advantageous, it must not be forgotten that these first-generation agents will likely modulate numerous off- target effects (Thomes *et al.*, 2016;

Sitaula *et al.*, 2017; Sulli *et al.*, 2018). Modulation of REV-ERB by SR9009 will regulate gross elements of cellular functioning like metabolism and growth, in addition to any purportedly nuanced or more targeted components like autophagy. SR9009 itself is unlikely to ever be licenced for treatment in humans, although its use as a drug of abuse in athletes is acknowledged (Davies *et al.*, 2019; Thevis, Kuuranne and Geyer, 2020). It is impossible to fully control for wider off-target effects in a laboratory setting, so caution is required whilst interpretating results gained using these agents. Targeted organ specific modulation is a highly desirable goal.

1.2.2 The role of the suprachiasmatic nucleus

In humans the suprachiasmatic nucleus (SCN) is located in the anterior part of the hypothalamus superior to the optic chiasm (**Figure 1.9**). It is the master metronome and is primarily regulated by a light ZT. This was illustrated in experiments in which there was cessation of circadian activity in rats with complete SCN lesions, despite persistence of environmental light (Eastman, Mistlberger and Rechtschaffen, 1984). The SCN also controls the specific characteristics of the circadian clock that is to be followed in the mammal: when SCN transplants were introduced into arrhythmic SCN lesioned animals, it restored not only a rhythm but one with a period specific to the donor (Ralph *et al.*, 1990). The SCN receives signals from intrinsically photosensitive retinal ganglion cells (ipRGCs) via the retinohypothalamic tract. These cells represent a third photoreceptor in the mammalian retina and their discovery (Berson, Dunn and Takao, 2002) represented a solution to the observation that blind humans (Czeisler *et al.*, 1995; Lockley *et al.*, 1997) and murine models lacking rods

and cones (Freedman *et al.*, 1999) could maintain a fully entrained circadian rhythm. The SCN relays phase information to the organs in the rest of the body. The role that glucocorticoids and other ZT play in particular modulation of the hepatic clock are discussed in (**Appendix 2).**

Whilst variation to the extent any particular organ functions under circadian modulation is recognised, those that do possess their own independent clock (Mohawk, Green and Takahashi, 2012). Previously, it was thought that these peripheral clocks simply followed the SCN metronome, but this opinion was revised following demonstrations of independent clock activity in the liver (Mauvoisin *et al.*, 2014), cardiovascular system (Davidson *et al.*, 2005), skeletal muscle (Harfmann, Schroder and Esser, 2015) and adipose tissue (Gómez-Santos *et al.*, 2009). The SCN serves as the master clock, allowing a common point of reference for coordination of the peripheral clocks to maintain their phase. To achieve this, the SCN disseminates its primary cue via neural and hormonal signals (Buijs, Escobar and Swaab, 2013). The peripheral clocks are capable of receiving multiple ZT, both internally from the central clock and externally in the form of feeding, physical activity and sleep (Mohawk, Green and Takahashi, 2012). This allows for organ specific adjustments in metabolic and physiologic processes to be made collectively, that are concordant with the organisms' singular metronome. Therefore, a constant exchange of timing information is required to maintain each clock is in phase with respect to each other. Consequently, as different ZT could set different phases between the central clock in the SCN and those in the periphery, the two systems can become misaligned e.g. shift workers, jet-lag. This can result in a misaligned circadian rhythm and is increasingly linked with human pathology.

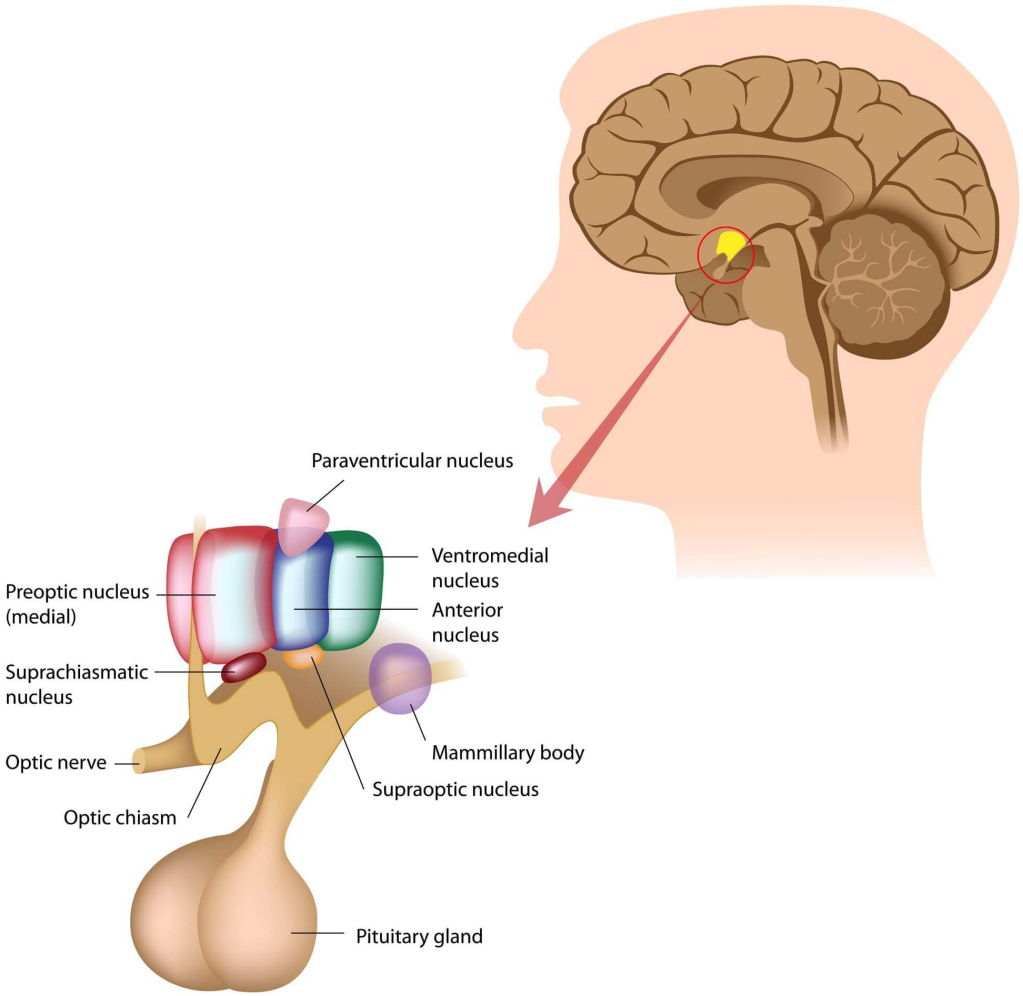


Figure 1.9: Location of the suprachiasmatic nucleus. Corticotropin releasing hormone (CRH) -released by the hypothalamus- stimulates the secretion of adrenocorticotropic hormone (ACTH) -released by the anterior pituitary- into the vasculature. ACTH then acts on the adrenal cortex to release amongst other hormones: cortisol. Serum cortisol levels show a robust circadian pattern. Adapted from Alila Medical media, *Shutterstock images*. Viewed 27th September 2019 < https://www.shutterstock.com/image-vector/nuclei-hypothalamus-112081064>.

1.2.3 The molecular basis of circadian rhythms

The mammalian molecular clock is based upon multiple transcription-translation feedback loops (TTFL) that are comprised of at least ten genes (**Figure 1.10**).

(i) Primary loop: The transcription factors (TF) BMAL1 (Brain and Muscle Arnt Like 1) and CLOCK (Circadian Locomotor Output Cycles Kaput) comprise the activating loop (Buhr and Takahashi, 2013). Both these proteins are part of the basic helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) domain family, characterised by two α-helices connected by a loop. Generally, TFs in this domain are dimeric, whereby each loop contains amino acid residues capable of binding to DNA. The PAS domain is a widespread component of signal transduction protein pathways, where they act as a molecular sensor and point of interaction via binding. The domain is named after the proteins in which the domains were first characterised (Moglich, Ayers and Moffat, 2009). BMAL1 and CLOCK form a heterodimer that can bind to a specific DNA element called an Enhancer box (E-box). The E-box has a palindromic sequence of CANNTG (where N can be any nucleotide) but has a canonical sequence of CACGTG (Kyriacou and Rosato, 2000). The BMAL1:CLOCK heterodimer activates transcription of circadian controlled genes (CCG) by binding to E-boxes in their promoters (Gekakis *et al.*, 1998; Yoo *et al.*, 2005). Targets of this activation include the Period (PER) and CRY proteins. PER and CRY are translated in the cytoplasm, dimerize and trafficked to the nucleus where they impose negative feedback on the transcription of BMAL1 and CLOCK. This cycle takes approximately 24 hours (Sangoram *et al.*, 1998; Griffin, Staknis and Weitz, 1999). Degradation of PER and CRY are a prerequisite to the initiation of transcription of BMAL1 /CLOCK and re-commencement of the central circadian TTFL loop. This degradation also contributes to the phase of the cycle. Both proteins are targeted for degradation in

the proteasome in different pathways. PER is phosphorylated by casein kinase (CK) ε and δ which marks it for ubiquitination by a β-transducin repeat-containing protein (βTrCP) with subsequent degradation by the 26S proteasome (Camacho *et al.*, 2001; Eide *et al.*, 2005; Shirogane *et al.*, 2005). CRY1 and CRY2 are targeted for ubiquitination by individual phosphorylation event and kinase pathways. CRY1 is phosphorylated by the nutrient sensor AMPK1 (Lamia *et al.*, 2009). CRY2 is phosphorylated at serine in positions 557 and then 553, by a sequential dual-specificity tyrosine-phosphorylated regulated kinase 1A (DYRK1A) and glycogen synthase kinase (GSK-3β) cascade (Harada *et al.*, 2005; Kurabayashi *et al.*, 2010). Subsequently, CRY is polyubiquitinated (i.e. both CRY1 and CRY2) by F-Box protein 3 FBXL3 (Busino *et al.*, 2007) and undergoes degradation in the proteasome. The paralogs of the PER (1-3) and CRY (1-2) proteins appear to offer a degree of compensation should a gene or family expression be reduced or even lost. Work by (Baggs *et al.*, 2009) documents the effects on period of both individual and collective circadian TF knockout by siRNA in U2OS cells.

(ii) Secondary loop: The BMAL1:CLOCK heterodimer initiates transcription of a second loop involving the orphan-nuclear receptor genes REV-ERB with its isoforms α and β -also known as nuclear receptor subfamily 1 group D member (NR1D)-, and retinoic acid receptor (RAR)-related orphan receptor (ROR) with isoforms α, β and γ. Whilst the REV-ERBs act to repress, the RORs enhance transcription of BMAL1 by competitively binding to the ROR response elements (ROREs) in the BMAL1 promoter (Solt, Kojetin and Burris, 2011; Everett and Lazar, 2014). Once considered merely an accessory feedback loop, it has since been demonstrated that the genome-wide cis acting targets (cistromes) in the murine liver of both REV-ERB isoforms share extensive overlap with the master circadian activator BMAL1 (Cho *et al.*, 2012). This suggests a more global repressor role for REV-ERB in

the circadian modulated proteome than mere negative transcription of BMAL1. Furthermore, double isoform REV-ERB knockout mice is lethal *in utero* and results in severely disrupted circadian clock and lipid homeostatic gene networks if induced in adult animals.

(iii) Third loop: A tertiary loop is becoming increasingly recognised (Yoshitane *et al.*, 2019), adding a layer of precision and robustness, as none of the genes involved are required for core clock function. Transcription of PER1 can be modulated by competitive binding between Albumin D-site-Binding Protein (DBP) which acts to increase and Adenovirus E4 promoter Binding Protein 4 (E4BP4) which acts to repress transcription. DBP and E4BP4 compete to bind a specific DNA element with the canonical sequence TTAYGTAA called a D-box (Yamaguchi *et al.*, 2000; Ueda *et al.*, 2005). It has been suggested that together these three known binding motifs provide a necessary delay to cycle at 24 hours by varying their times of peak activity: E-box in the morning, D-box in the day and RORE in the evening (Ukai-Tadenuma *et al.*, 2011).

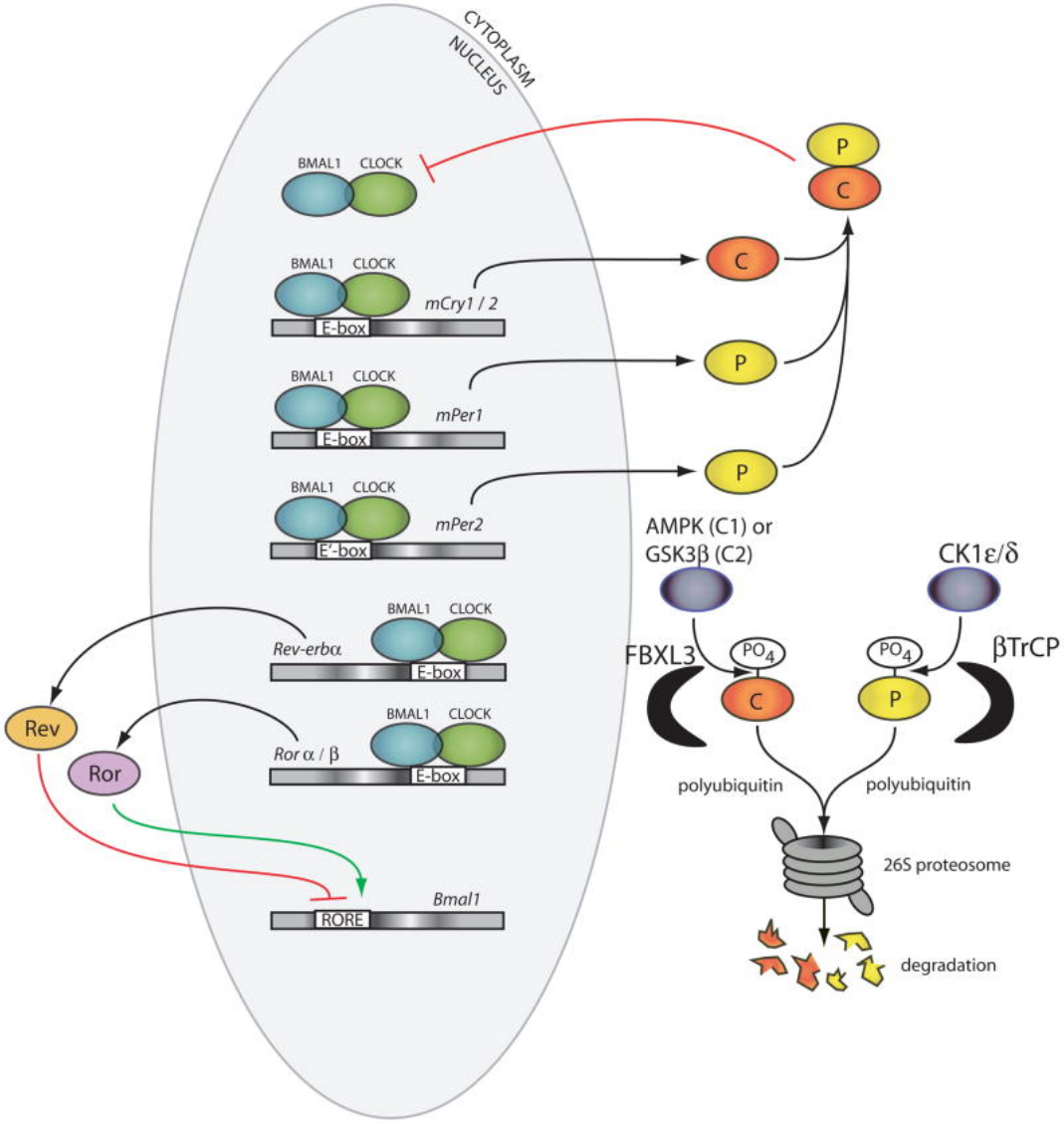


Figure 1.10: Molecular components of circadian rhythm. Adapted from (Buhr and Takahashi, 2013).

Expression profiling of clock components in humans has been attempted by a few groups (Bjarnason *et al.*, 2001; Boivin *et al.*, 2003; Archer *et al.*, 2008; Kusanagi *et al.*, 2008). PER3 has consistently been found to show the most robust oscillation. In 24 normal human volunteers who underwent actigraphy (non-invasive measurement of rest/activity cycle), the average zenith timings were 06:05 for PER3, 08:06 for PER2 and 15:06 for BMAL1 (Archer *et al.*, 2008). An individual's PER3 expression correlated highly with average time of peak serum concentration of melatonin (04:20) and cortisol (10:49). However, substantial inter-personal differences in clock parameters exist due to environmental variations which makes standardisation difficult.

The modulation of CCG expression is discussed in **Appendix 3**. It is a multi-layered process whereby technological limitations coupled with emerging knowledge of post transcriptional and translational modifications, pose significant barriers in defining how genes can be circadian regulated.

As our knowledge of the systems subject to this regulation expands, so will our understanding of how perturbations in the circadian clock contribute to disease pathology with the resultant development of a new speciality: chronobiology (Panda, 2019). This exciting new field prompts further questions about how key processes within the human body are under circadian regulation. By studying the resulting human pathology caused by disorders of circadian rhythm, a greater understanding of how integral the mammalian clock is obtained. The Circadian Rhythm Sleep Disorders (CRSD) are discussed in **Appendix 4**. Coupled with this is an evolving appreciation of the clock mediated immune response, with immediate implications for vaccine effectiveness as discussed in **Appendix 5.**

1.2.4 Viral hepatitis and circadian rhythm

HCV is a positive sense single stranded RNA flavivirus that infects the liver and replicates in the cytoplasm. Several reports suggest a role for components of the circadian clock to regulate HCV replication (Vinciguerra *et al.*, 2013):

(i) Stable expression of HCV core protein reduced both transcript and protein expression of PER2 and CRY2 in hepatoma cell cultures. The same effect was observed upon stable expression of the entire genome from a replicon. Conversely, overexpression of PER2 decreases HCV RNA levels (Benegiamo *et al.*, 2013).

(ii) BMAL1 knockout, overexpression of REV-ERBα or treatment with its agonist decreased both HCV entry and genome replication in hepatoma cell lines (Zhuang *et al.*, 2019). Transcripts and protein expression of CD81 and claudin-1 (cell receptors crucial for HCV entry) were modulated by knock-out of BMAL1 or treatment with a REV-ERB agonist (Zhuang *et al.*, 2019).

(iii) It was observed that liver transplant surgeries that took place in the morning had a rebound HCV viremia sooner than those that took place in the afternoon (Zhuang *et al.*, 2018).

There is a considerable gap in our knowledge as to whether HBV is modulated by circadian rhythm. To date, the following observations have been made:

(i) Stable expression of HBx results in a decrease in both BMAL1 transcripts and protein in hepatoma cells (Yang *et al.*, 2011).

(ii) HBV-associated HCC tissue shows lower levels of PER1-3 and CRY2 transcripts compared with peri-tumour tissue (Yang *et al.*, 2014). The authors also showed that expression of the HBx gene resulted in decreased PER3 and CRY1-2.

(iii) A disrupted clock is a feature of HCC and possibly acts as a risk factor for development of malignancy (Filipski, Li and Lévi, 2006; Filipski *et al.*, 2009; Lahti, Merikanto and Partonen, 2012; Savvidis and Koutsilieris, 2012; Li *et al.*, 2017).

This suggests that HBV infection or a component thereof -possibly HBx- is capable of disrupting the endogenous clock of the cell. It is hoped that research in this area would help progress the field and further understanding of the degree to which HBV replication is under circadian modulation.

1.3 Overview of Autophagy

It is unsurprising, given the temporal separation of eating and fasting imposed by circadian rhythm, that the survival mechanism responsible for maintenance of cellular nutrient homeostasis could also follow rhythmic expression. The concept that autophagy is under circadian modulation has generally been accepted (Sachdeva and Thompson, 2008).

As a competent autophagic pathway is a core requisite to HBV replication, any circadian modulation of HBV could occur through the co-regulation of autophagy.

1.3.1 The role of cellular autophagy

The word autophagy is derived from Greek meaning self-devouring. Autophagy is primarily a protective mechanism of cellular homeostasis. It occurs at a constant basal rate to remove damaged proteins or cellular organelles that occur as by-products of daily metabolic processes in eukaryotic cells. It is a dynamic process that can be upregulated in a matter of minutes to generate nutrients or energy in response to starvation, hypoxia or bioenergetic demands (Ravikumar *et al.*, 2010). The autophagic pathway is outlined in (**Figure 1.11**). It revolves around the central process of an autophagosome (AP) fusing with a lysosome to form an autolysosome (AL).

Autophagy plays a part in a variety of catabolic processes which is reflected in the many specialised forms that have been described:

- ***Macroautophagy*** -henceforth referred to as autophagy- is responsible for the clearance of protein aggregates which can accumulate due to oxidative stress or

mutations (Lamark and Johansen, 2012).

- ***Mitophagy*** is responsible for turnover of defective mitochondria following damage or oxidative stress (Wang and Klionsky, 2011).
- ***Lipophagy*** - Lipolysis of cellular triglycerides to meet cellular energy requirements (Singh *et al.*, 2009).
- ***Xenophagy*** attempts to help the cell respond to infection by attempting to eliminate invading bacteria or viruses. Xenophagy also plays an as yet ill determined role in the immune response by way of antigen presentation. Although most obligate intracellular pathogens have evolved ways to subvert this defence, a few, including HBV, have incorporated the autophagic process into their replication cycle (Levine, Mizushima and Herbert W Virgin, 2011).

Autophagy plays a key -albeit controversial- role in apoptosis. It is generally accepted that significant cross-talk occurs between apoptosis and autophagy but there is ongoing debate as to the net effect. Although primarily considered a survival mechanism, autophagy can represent an alternate cell death pathway called programmed cell death type II. This differentiates it from classic apoptosis or programmed cell death type I. Both share common upstream signals, but the outward results can be linked or mutually exclusive depending on the surrounding environment (Clarke, 1990; Maiuri *et al.*, 2007).

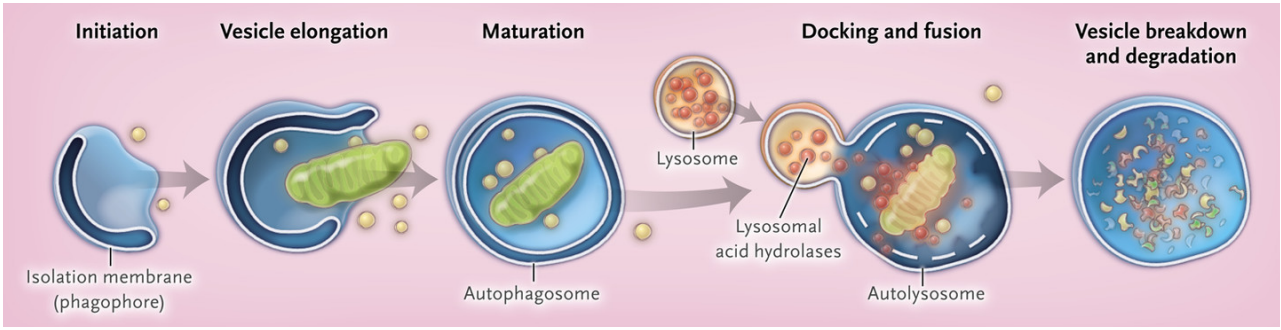


Figure 1.11: Autophagy sequence. The autophagic pathway involves the initial formation of a double membrane open structure called a phagophore. This forms around the targets for degradation, whereupon the open ends will fuse to form the now closed autophagosome. An autolysosome is formed when the AP fuses with a lysosome and the contents are subsequently degraded and released. Modified from (Choi, Ryter and Levine, 2013).

1.3.2 The molecular basis of autophagy

A variety of upstream cellular environmental cues can signal, via opposing regulatory pathways, the mobilisation of the autophagic process **Figure 1.12**. The molecular signalling systems involved in autophagy are complex and numerous (Wang and Klionsky, 2003; Levine and Klionsky, 2004; Meijer and Codogno, 2004; Ravikumar *et al.*, 2010)**.**

The most commonly undertaken autophagic assays centre around a pivotal step in the molecular control of AP membrane formation. AP membrane elongation is achieved via two ubiquitin like conjugation systems: the microtubule- associated protein light chain 3 (LC3)–ATG8 and the ATG5–ATG12 (Ravikumar *et al.*, 2010). The LC3-ATG8 conjugation results in the conversion of a cytosolic form of LC3 (LC3-I) to its focal AP membrane associated phosphatidylethanolamine conjugated form (LC3-II). This conversion marks the point of AP formation and a visible initiation of autophagy. LC3 can be pictured by microscopic immunofluorescence with differentiation between diffuse cytosolic LC3-I and focal puncta AP membrane LC3-II possible. Western blotting to differentiate LC3-I and LC3-II is also possible (Klionsky *et al.*, 2016). An important protein that interacts with LC3 by marking cargo for degradation is sequestosome 1 (p62) (Komatsu *et al.*, 2007). By acting as an indirect marker for complete autophagy degradation, quantitative measurements of p62 can be used to measure autophagic flux. This is an important point as measures of AP formation by LC3 alone are subject to numerous caveats and are wholly unable to comment on autophagic flux (Klionsky *et al.*, 2016). The molecular basic of autophagy is discussed is greater detail in **Appendix 6**.

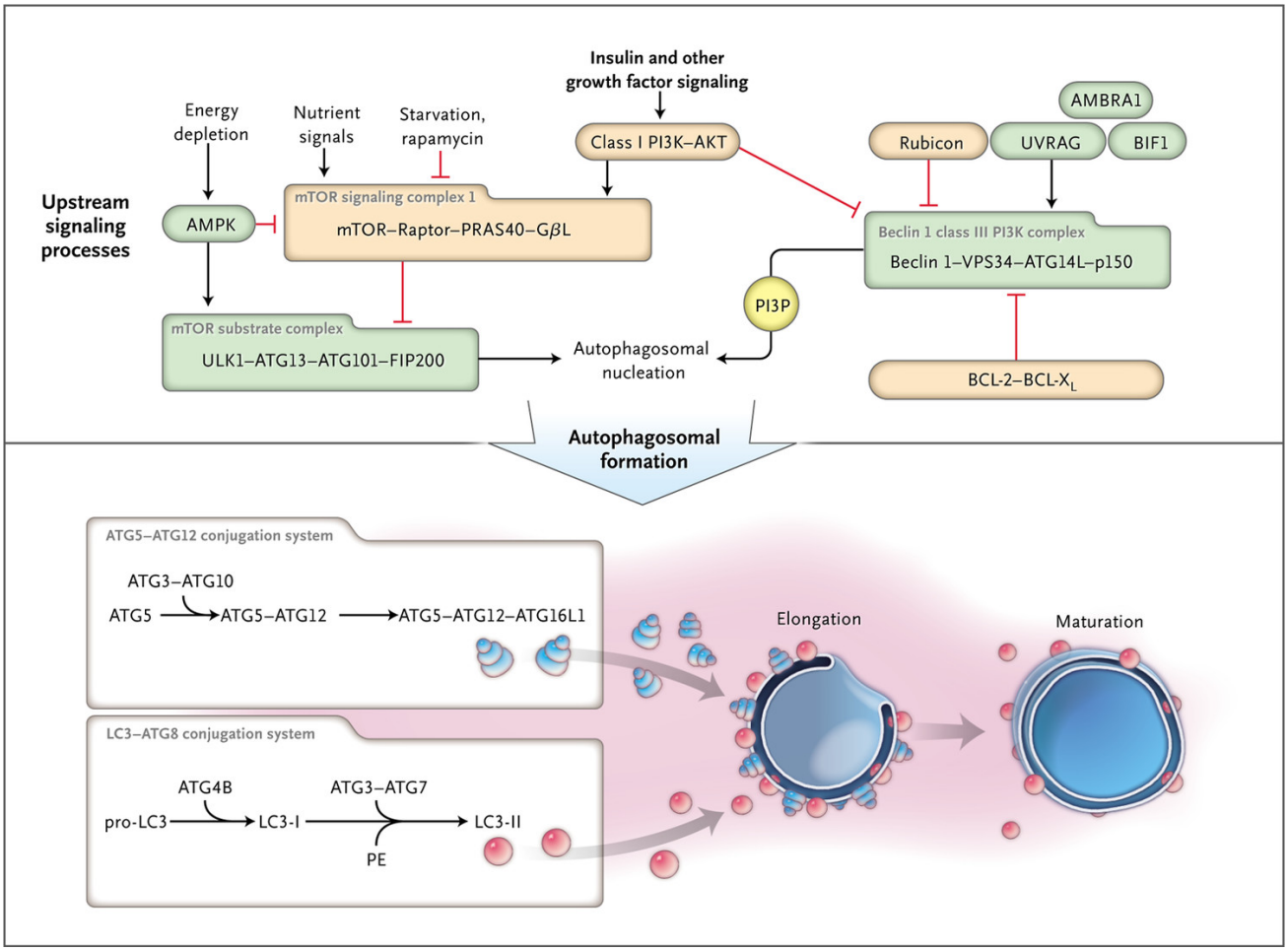


Figure 1.12: Molecular framework of autophagy. Modified from (Choi, Ryter and Levine, 2013).

1.3.3 Circadian regulation of autophagy

There is a general consensus that autophagy is under circadian modulation by virtue of its capabilities to receive nutrient and energy signals from the mammalian target of rapamycin (mTOR) and AMPK pathways (Sachdeva and Thompson, 2008). Early studies by electron microscopy showed the numbers of AP in rat renal tubule cells or retinal rod cells showed marked diurnal variation (Pfeifer, Scheller and Germany, 1975; Reme and Sulser, 1977), but it was not until more recently that several TFs were identified that regulate components of the autophagic pathway (**Figure 1.13**).

Forkhead transcription factor O3 (FoxO3) induces expression of several autophagy genes in the tissue of both the myocardium and skeletal muscle (Mammucari *et al.*, 2007; Zhao *et al.*, 2007). This was shown to contribute to muscle atrophy induced by starvation or neural denervation. Additionally, sterol regulatory element-binding proteins (SREBPs) which are important for cellular sterol homeostasis and transcription factor "EB" (TFEB) which is crucial for lysosome dynamics have both been shown to have autophagic modulating capabilities (Seo *et al.*, 2011; Settembre *et al.*, 2011). Whilst undoubtedly linked with metabolism, these TFs have not yet been demonstrated to modulate autophagy in a circadian fashion.

Work by (Ma, Panda and Jiandie D. Lin, 2011) identified the primary molecular link. The authors demonstrated the rhythmic protein expression of the crucial autophagic proteins LC3-I, LC3-II and p62 in multiple murine organ tissues. Previous observations using electron microscopy to illustrate rhythmic numbers of AP in muscle tissue was verified. Subsequent PCR analysis demonstrated numerous autophagic genes crucial for AP formation, mitophagy and lysosomal degradation would display a robust circadian oscillation. By examining a cohort of known hepatic TFs that can regulate either the starvation response or mammalian clock,

CCAAT/enhancer-binding protein β (C/EBP β) was identified as the molecular link through which circadian regulation of autophagy is mediated (Ma, Panda and Jiandie D. Lin, 2011). C/EBP β shows rhythmic expression that is capable of stimulating transcription of multiple autophagy genes, which results in enhanced protein degradation. In mice, a shift in clock phase -induced by an altered feeding schedule- also resulted in a shifted expression of C/EBP β, thereby highlighting its regulation from both nutritional and circadian signals. To further verify these findings, two murine knockdown models were used:

(i) In liver specific BMAL1 knockout mice, the rhythmic and peak expression of C/EBP β was lost compared to control animals. Interestingly, p62 and LC3 levels were only modestly affected. This suggests a degree of systematic redundancy, as basal autophagic flux was impaired but not eliminated.

(ii) In a second model, shRNA-mediated knockdown of C/EBP β resulted in a diminished basal and starvation-induced expression of multiple autophagy genes. Accordingly, rhythmic expression of these genes was interrupted. In contrast with the previous model, the levels of p62 were constitutively elevated suggesting that autophagic flux is almost abolished, highlighting the importance of C/EPB β as a regulator of autophagy.

In summary C/EBP β was identified a primary link between nutritional and circadian signals to modulate the rhythmic activation of autophagy. Other linking TF like FoxO3, SREBP2 and TFEB contribute to this crosstalk. As our understanding of the circadian modulation of autophagy increases, so too does the potential for therapeutic interventions to counteract the adverse metabolic effects of a perturbed circadian rhythm (Nohara, Yoo and Chen, 2015; Panda, 2019).

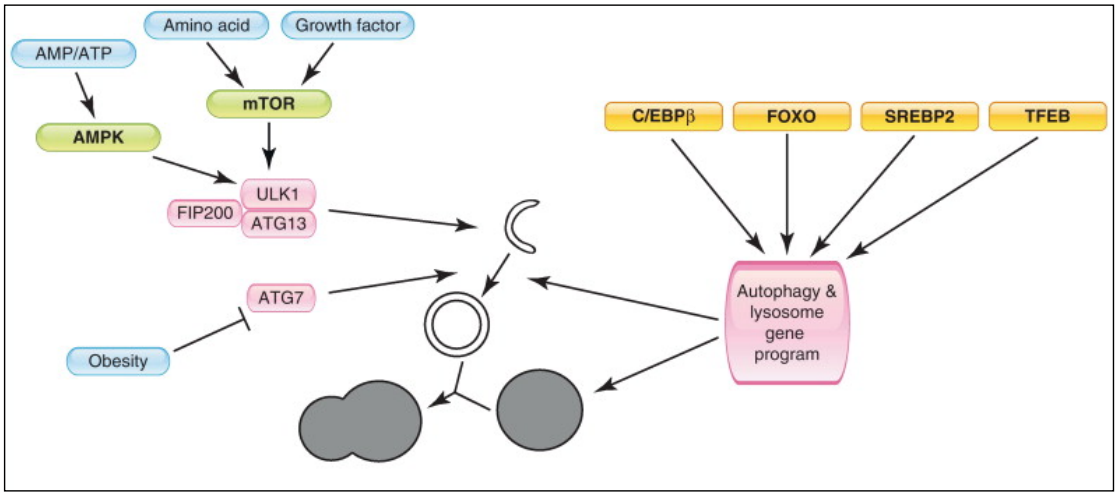


Figure 1.13: Metabolic and transcriptional regulation of autophagy. Modified from (Ma *et al.*, 2012).

1.3.4 HBV and autophagy

The role that autophagy plays in human disease is explored in (**Appendix 7**). In a similar fashion to (**Appendix 4**) where the pathology due to perturbations in circadian rhythm was discussed; much can be learned about the molecular architecture by studying disease phenotypes.

Xenophagy plays a specific role in shaping the innate and adaptive immune response and attempts to directly control invading microbes by acting as a cell autonomous line of defence. Infection by intracellular pathogens constitutes a significant cellular stress: a potent inducer of autophagy. Of course, pathogens have evolved counter measures to avoid elimination and, in some cases, to subvert the autophagic response for their own benefit (Deretic and Levine, 2009; Levine, Mizushima and Herbert W. Virgin, 2011; Lennemann and Coyne, 2015). The role that xenophagy plays in HBV and other viral infections is emerging, although numerous gaps and some inconsistencies remain (Pratt and Sugden, 2012).

(i) Pivotal work demonstrated HBV infection stimulates the early autophagic response in cell cultures, murine models and clinical samples (Sir *et al.*, 2010). The increase in autophagy did not result in enhanced autophagic flux and was dependent on HBx protein increasing the enzymatic activities of PI3K, which is essential to autophagy initiation. Mouse models deficient for autophagy when infected with HBV showed a 90% decrease in HBV DNA, a 50% decrease in HBeAg and a 60% decrease in HBsAg in serum (Tian *et al.*, 2011). HBV RNA transcription and packaging of core RNA was minimally affected but DNA was almost undetectable.

(ii) Examining the liver tissue revealed that the sub-cellular location of HBcAg was primarily cytoplasmic in the autophagy-deficient mice compared to a nuclear localisation in control animals. Together, this suggests a step immediately after packaging of core RNA, but prior to RT, as the component in the DNA replication cycle most reliant on a competent autophagic pathway (Tian *et al.*, 2011).

(iii) Another group reported that HBV stimulated the early autophagic response without increasing degradation and suggested a role for HBsAg stimulating ER stress (Li *et al.*, 2011). The authors suggested viral envelopment was the step most affected by autophagy inhibition.

(iv) A cell line stably integrated with HBV and treated with the autophagy inducer rapamycin showed increased HBV DNA and HBsAg production (Huang *et al.*, 2014). This could be reversed by treatment with the autophagy inhibitor 3-methyladenine.

(v) More recent work focussed on negating the oncogenic role of HBx by microRNA (miRNA). Preliminary work illustrated that the HBx mediated autophagy induction via beclin-1 could be reversed by miRNA-30a with resultant induction of apoptosis (Kumar *et al.*, 2015). This represents a possible autophagic target that could prevent HBx mediated malignancy.

It is difficult to immediately reconcile all these findings. The understanding of the full role played by autophagy in HBV infection and HBV derived HCC is in its infancy. It is noteworthy that these studies used different model systems, cell lines, and the majority of the HBV components were performed with viral integrants rather than *de novo* infection culture

systems. This epitomises the difficulties of HBV culture *in vitro* (Tang *et al.*, 2012). Crucially, the studies agree that perturbation of autophagy is an integral component of the HBV replication cycle and pharmacological modulation modifies key viral parameters.

In turn, it is likely that a proportion of any proposed circadian modulation of HBV could easily be affected through the intermediate process of circadian controlled autophagy.

1.4 Project aims.

Hypothesis 1: HepaRG cells support an entrainable circadian rhythm.

Objectives:

- Optimise a synchronisation technique that would not confound HBV replication parameters.
- Develop a real-time bioluminescence assay capable of both screening and measuring a circadian rhythm.
- Determine if HBV has an effect on rhythm generation.

Hypothesis 2: Circadian rhythm can modulate HBV through autophagy

Objectives:

- Develop an automated method of measuring autophagosome maturation using the dual fluorophore LC3 reporter and Cell profiler software.
- Define the effect HBV has on autophagosome maturation.
- Determine whether hepatic autophagy can be modulated by REV-ERB activation
- Determine if modulators of autophagy affect HBV replication.

Hypothesis 3: HBV replication is modulated at various points by REV-ERBα

Objectives:

- Characterise the components of the HBV replication cycle sensitive to circadian modulation *in vitro*.
- Investigate if REV-ERB activation by SR9009 shows antiviral activity in a murine HBV infection model.

Chapter 2: Materials and Methods

2.1 Tissue Culture

All the cell lines used in this study are described in (**Table 2.1**). Two types of media were used which were then supplemented with selection antibiotics as specified: Dulbecco's Modified Eagle Medium (DMEM) (Gibco) media with L-Glutamax, supplemented with 10% Fetal Bovine Serum (FBS), 1% Non- Essential Amino Acids (NEAA) with 50 units/ml of Penicillin/Streptomycin (P/S) (Gibco USA) and William's E Medium (Gibco) supplemented with 10% Fetal Clone II serums (FCS), 50 units/ml P/S, 6ml of Glutamax x100 (Gibco), 250uL Insulin human solution (Sigma) with 5 µG/mL hydrocortisone (Sigma). Cell lines were maintained at 37°C, 5% CO_2 in a humidified incubator (New Brunswick Galaxy 1705). Where directed, flasks and plates were collagen coated with a solution of 20% collagen (Sigma- calf skin) diluted in Phosphate Buffered Saline (PBS) (Gibco Life Technologies) for 1 hour and washed once with PBS before use. To passage, the old media was aspirated before being washed with PBS which was then discarded. Cells were removed from the surface by incubating with 0.05% Trypsin EDTA 1x (Gibco) (approximately 30% of incubating volume) for 6 minutes. The side of the flask was gently tapped to aid dissociation, and fresh media added to make up the original volume. 10% of this was then placed into a new flask with fresh media. Cells were passaged approximately every 3-4 days or when they reached 80-90% confluence.

Name	Tissue of Origin	Media	Source
HepG2 NTCP K7	Human hepatoma subclone with overexpressed NTCP	DMEM	Dr Ulrike Protzer, Technische University München, Munich, Germany.
Huh-7	Human hepatoma	DMEM	Dr Charles Rice, Rockefeller University, NY, USA.
HepG2.2.15	HepG2 derived cell line stably infected with HBV	DMEM	Dr Ulrike Protzer, Technische University München, Munich, Germany.
HepAD38	HepG2 derived cell line stably infected with HBV, under a CMV promoter	DMEM	Dr Ulrike Protzer, Technische University München, Munich, Germany.
HepaRG	Human hepatic progenitor	Williams	Dr Fabien Zoulim, Lyon University, France.
293t	Human embryonic kidney	DMEM	Prof McKeating, University of Birmingham, UK
U2OS	Human osteosarcoma	DMEM	Commercial ATCC
HepG2 NTCP K7 (Autophagy reporter)	As parent line with transduced LV containing mRFP/eGFP LC3 stable fluorescent reporter	DMEM + Puromycin 0.2uG/mL	As parent, LV construct from Dr Sovan Sarkar, University of Birmingham, UK
HepAD38 (Autophagy reporter)	As parent line with transduced LV containing mRFP/eGFP LC3 stable fluorescent reporter	DMEM + Puromycin 0.2uG/mL	As parent, LV construct from Dr Sovan Sarkar, University of Birmingham, UK
U2OS Bmal1 Luc	As parent line with transduced LV containing Bmal1 luciferase	DMEM + Puromycin 1uG/mL	As parent, LV construct commercial Addgene
HepaRG Bmal1 Luc	As parent line with transduced LV containing Bmal1 luciferase	Williams + Puromycin 2uG/mL	As parent, LV construct commercial Addgene
HepaRG NTCP Luc	As parent line with transduced LV containing NTCP luciferase	Williams + Puromycin 2uG/mL	As parent, LV construct from Dr Koichi Watashi, Institute of Infectious Diseases, Tokyo, Japan.

Table 2.1: Cell lines used in this study.

2.2 Transfection

All plasmids used are listed in (**Table 2.2**). Target cells are seeded in their usual maintenance media. Two hours prior to transfection this is switched to antibiotic free media. Cells were transfected using a Fugene (Promega) kit following the manufactures instructions. Transfection mix was made to a 10% volume of target well media with Optimem (Gibco). In experiments where multiple plasmids were transfected (co-transfection), the total amount of DNA was kept constant using pcDNA3.1 where necessary. Unless otherwise specified, co-transfections took place in 6-well plates where the total Well size and total DNA amounts are specified in the corresponding figure legends.

2.3 Antibodies

All antibodies used are listed in (**Table 2.3**). Dilutions are as listed unless otherwise specified.

Vector	Insert	Plasmid Source
pcBmal1 FLAG HA	Bmal1 with a 4kDa Haemagglutinin tag. Antibiotic resistance: Ampicillin	Prof McKeating, University of Oxford, UK
pcBmal1 MYC HIS	Bmal1 with a 12kDa x5MYC and X6HIS tag. Antibiotic resistance: Ampicillin	Prof McKeating, University of Oxford, UK
pcRev-erbα HALO	Rev-erbα with a 34kDa Halo tag. Antibiotic resistance: Ampicillin	Prof McKeating, University of Oxford, UK
pcmRFP/eGFP LC3	Dual fluorescent LC3 reporter. Antibiotic resistance: Ampicillin	Dr Sovan Sarkar, University of Birmingham, UK
pVSVG	Lentivirus envelope plasmid. Antibiotic resistance: Ampicillin	Dr Sovan Sarkar, University of Birmingham, UK
pGAG/POL	Lentivirus packaging plasmid. Antibiotic resistance: Ampicillin	Dr Sovan Sarkar, University of Birmingham, UK
pREV	Lentivirus packaging plasmid. Antibiotic resistance: Ampicillin	Dr Sovan Sarkar, University of Birmingham, UK
pTAT	Lentivirus packaging plasmid. Antibiotic resistance: Ampicillin	Dr Sovan Sarkar, University of Birmingham, UK
pBmal1 luc	Lentivirus transfer vector. Antibiotic resistance: Ampicillin	Commercial: Addgene
pNTCP luc	Lentivirus transfer vector. Antibiotic resistance: Kanamycin	Dr Koichi Watashi, Institute of Infectious Diseases, Tokyo, Japan.

Table 2.2: Plasmids used in this study.

Antibody	Source	Working concentration
Bmal1	Abcam	1:1000 (WB)
Rev-erbα	Thermofisher	1:1000 (WB)
GAPDH	Cell Signalling	1:1000 (WB)
Myc	Sigma	1:2500 (WB)
Flag	Sigma	1:2000 (WB)
LC3	Novusbio	1:4000 (WB)
P62	BD transduction	1:2000 (WB)
Actin	Sigma	1:2500 (WB)
Secondary anti rabbit	GE Healthcare	1:5000 (WB)
Secondary anti mouse	Dako	1:2000 (WB)

Table 2.3: Antibodies used in this study.

2.3 Lentivirus production

A 6 well plate was coated with poly-L-lysine (Thermofisher) for 5 minutes and rinsed with PBS. 293T cells were seeded at 4x10^5 cells/well and incubated overnight at 37°C, aiming for 50% confluence. Cells were transfected with the transfer vector plasmid of interest along with envelope and packaging plasmids following manufacture's protocols (Addgene). 2.5µG DNA was transfected per well in the ratio 2µG Vector:0.1µG TAT:0.1µG REV:0.1µG GAG-POL:O.2µG VSVG per well. A Fugene (Promega) transfection kit was used as per manufactures instructions. The transfection mix was incubated at 37°C for 24 hours. The next day the media was changed to standard DMEM. The supernatant containing lentivirus was collected 24 hours later by collecting the media and centrifuging at 1500rpm for 10 minutes. It was then filtered through a 0.45 µM filter, polyene at 8 µG/mL was then added and stored at 4°C for later use.

2.4 Transduction and selection

Intended cells for transduction were seeded at 2.5x10^5 cells/well in a 6 well plate in maintenance media with no antibiotics. Lentivirus containing media was added and cells were incubated for 24 hours. The following day this was removed and replaced with media containing the appropriate antibiotic for selection.

2.5 Western blotting and detection

Cell lysates were produced from cell cultures by aspirating culture media, washing once with PBS and adding 45µL of a specified lysis buffer for every 40,000 cells seeded. This ratio was kept constant throughout the study. The lysis buffers used were: Urea lysis buffer (H_2O, 8M urea, 8% glycerol, 20% sodium dodecyl sulphate, 1M Tris-HCL, pH7.5); radioimmunoprecipitation assay (RIPA) buffer (20mM Tris pH 7.5, 2mM EDTA, 150mM NaCl, 1% NP40) and NP40 (150 mM sodium chloride, 1.0% Triton X-100, 50 mM Tris, pH 8). The lysis buffer used is specified in the corresponding figure legend. This mixture was agitated around the well until viscous. The lysate was then mixed with x4 Sodium Dodecyl Sulfate (SDS) reducing buffer (H2O, 30% glycerol, 6% SDS, 0.02% bromophenol blue, 10% 2-β-mercaptoethanol and 0.2M Tris-HCL, pH6.8) at a ratio of 1:3, then heated at 95°C for 5 minutes and vortexed for 10 seconds. Lysates were briefly centrifuged prior to loading onto a gel. This gel was composed of a sample stacking component on top of a resolving gel component which was prepared using manufacturer's instructions (Protogel National Diagnostics UK) and the percentage is specified in the figure legend. Approximately 10-20uG of protein was loaded onto the gel and ran with 6µL of reference ladder (New England Biolabs Colour prestained Broad range) at 100V for 80 minutes. BioRad Powerpac were used for

running and transfer phases. Polyvinylidene fluoride (PVDF) membranes (Amersham UK) were activated in methanol for 30 seconds before assembly in their cassettes in transfer buffer and then ran at 350mAmps for 90 minutes. Running and transfer buffers made as per manufacturer's instructions (National Diagnostics). Following transfer, the membrane was moved to (Freedom Rocker Blotbot by Next Advance) at 4°C where it followed a standard immunoblotting sequence overnight. The membrane was first incubated in 5% milk PBST (PBS, 0.1% Tween-20 and 5% Marvel Dry Milk) for 2 hours. Then it was incubated with the primary antibody diluted in 1% milk PBST for 10 hours before a wash cycle with PBST (3x5 minutes and 2x10 minutes washes) took place. The membrane is then incubated with the secondary antibodies diluted in 1% milk PBST for 2 hours before another wash cycle. The membrane was developed using Super-signal West Dura extended substrate (Thermoscientific) in the imager (Bio-Rad).

2.6 Luciferase assay

45µL of sample lysed in x1 passive lysis buffer (Promega) was mixed with 45µL of working solution from the kit (Promega) and added to a 96 well white plate and read on a plate-reader (Thermo Electro Corp, Multiscan Ascent). All assays were firefly based. The reported relative light units (RLU) were subsequently plotted as RLU/µG of protein by performing a bicinchoninic acid (BCA) protein assay.

2.7 Bicinchoninic Acid (BCA) protein assay

Cells were lysed in x1 passive lysis buffer (Promega) and underwent a single freeze-thaw cycle (-80 for 30 minutes). 5µL of lysate was mixed with 200µL of working reagent from a BCA protein assay kit (Thermofisher Scientific) in a 96 well plate. 5µL of known protein concentration samples were made from this kit and used to generate a standard curve as per manufacturer's instructions. Plates were incubated at 37°C for 30 minutes before reading on a Multiscan Plate reader (Thermo Electron Corporation USA) with the absorbance value at 600nm. Protein concentration was interpolated from the standard curve.

2.8 Real time luciferase assay

The clariostar machine (BMG Labtech) was used for real time firefly luciferase measurement. Cells were seeded at 3x10^4 cells/well in a 96 well plate the day before in phenol free maintenance media (Gibco). Only the inner wells of the plate were used for analysis, the outer wells were filled with PBS to reduce evaporation. Commercial BMG luciferase substrate was added at 1:1000 to the media immediately before placing in machine. Plates were incubated at standard temperature and CO2 settings. Measurements were taken at 30 minute intervals.

2.9 Imaging of puncta in the autophagy reporter cell lines

Cells were seeded at 40,000/well/24 well plate on coverslips that had been washed in 70% ethanol, then thrice with PBS and subsequently collagen-coated. To fix, media was removed and

4% Paraformaldehyde (PFA) added for 15 minutes before washing off thrice with PBS before mounting with 5uL DAPI (Invitrogen Prolong Gold anti-fade) on coverslips (Thermoscientific 13mm) on clear glass slides (VWR premium slides) with the edges painted with clear nail polish (L'Oréal). Individual and composite images were taken at x100 on a Leica DM6000B fluorescence microscope. AP have dual red and green puncta while AL have red only due to loss of the acid labile green fluorescence.

2.10 Quantification of puncta using CellProfiler software.

Puncta were initially counted manually and later using the software CellProfiler (Jones *et al.*, 2008; Kamentsky *et al.*, 2011; McQuin *et al.*, 2018). The pipeline setting was calibrated for use on images obtained as outlined previously.

The pipeline had the following settings: Select image type; greyscale. Set intensity range; image metadata. Thresholding method; manual. Manual threshold; 0.005. Use advance settings; yes. Typical diameter; minimum 8 maximum 30. Discard objects outside the diameter range; yes. Discard objects touching the image border; yes. Threshold strategy; global. Thresholding method; minimum cross entropy. Threshold smoothing scale; 1.3488. Threshold correction factor; 1. Lower and upper bounds on threshold; 0 and 1. Method to distinguish clumped objects; intensity. Method to draw dividing lines between clumped objects; intensity. Automatically calculate size of smoothing filter for declumping; yes. Automatically calculate minimum allowed distance between local maxima; yes. Speed up by using lower resolution image to find local maxima; no. Fill holes in identified objects; after both thresholding and declumping. Handling of objects if excessive number of objects identified; continue.

2.11 HBsAg and HBeAg ELISAs

Media was collected, and instructions followed as per kit (Autobio Diagnostics). Briefly, sample supernatant was added to enzyme conjugate in the supplied 96 well plate and incubated for 60 minutes. The wells were washed, and mixed substrate solution was added before measurement of the resultant chemiluminescence on Polarstar Omega (BMG).

2.12 Quantitative PCR

To prepare DNA/RNA from target cells the media was removed, and samples were washed with PBS before being lysed using RLT+ lysis buffer according to manufacturer's instructions (Qiagen, Germany). Genome purification was then performed using the Allprep DNA and RNA purification kit (Qiagen, Germany).

2.12.1 HBV pgRNA quantification

HBV pgRNA copies were quantified with the One-step Takyon qPCR kit (Eurogentec) using a TaqMan primer set: forward 5'-GAGTGTGGATTCGCACTCC -3' and reverse 5'-GAGGCGAGGGAGTTCTTCTT-3' specific for pgRNA (Applied Biosystems, USA). PCR reaction volume was 10µl and performed on 40-100ng of DNase treated RNA. To DNase treat samples,clarified supernatants were incubated with 10 units of DNase at 37ºC for 30 minutes before incubation with PERT lysis buffer (0.1M TrisHCl pH7.4, 50mM KCl, 0.25% Triton X-100 (v/v), 40% Glycerol (v/v) at 65ºC for 10 minutes. Quantitative reverse-transcription PCR (qRT-PCR) was carried out in a microAmp 96 well optimal reaction plate (Applied Biosystems, USA) using a Light Cycler 96 (Roche) and data analysed with Light Cycler® 96 SW1.1 software. The

PCR cycling conditions were: 60 minutes at 50°C, 2 minutes at 95°C followed by 50 cycles of 95°C for 5 seconds and 60°C for 60 seconds. HBV pgRNA data represents copies as determined by a standard curve produced using a 10-fold dilution series of HBV1.3 Genotype D plasmid. pgRNA copies were normalized against the cellular housekeeper gene Beta-2 Microglobulin (B2M) (Thermofisher). Genes of interest represent relative mRNA expression as determined by the 2-ΔΔCt method for relative quantification; samples are normalized against this B2M endogenous control.

2.12.2 HBV particles

To quantify secreted HBV particles, supernatant was first DNase treated, as outlined previously (Thermofisher). HBV genomes were quantified by qPCR using an HBV specific primer set: forward 5'-TTTCACCTCTGCCTAATCATCTCT-3' and reverse: 5'-CTTTATAAGGGTCGATGTCCATGC-3'. Reaction master-mix consisted of 2X Sensifast SYBR no rox kit (Bioline) 400nM of each primer and 2ul of lysed supernatant in a 20µl reaction. Quantitative PCR (qPCR) was carried out in a microAmp 96 well optimal reaction plate (Applied Biosystems, USA) using Light Cycler 96 (Roche) and data analysed with LightCycler® 96 SW1.1 software. The PCR cycling conditions are as follows: 60 minutes at 50°C, 2 minutes at 95°C followed by 50 cycles of 95°C for 5 seconds and 60°C for 60 seconds. HBV copy numbers were interpolated from a 10-fold serial dilution of a HBV1.3 Genotype D plasmid.

2.12.3 BMAL1, REV-ERB & NTCP

RNA was amplified using the TaqMan primers (Applied Biosystems, USA) where the primer sequences were not disclosed. Reaction master-mix was designed using components of the Takyon qPCR master mix (Eurogentec, UK) according to manufacturer's instructions. qRT-PCR

was carried out in a microAmp 96 well optimal reaction plate (Applied Biosystems, USA) using a LightCycler® 96 Instrument system (Roche, Switzerland). The PCR cycling conditions are as follows: 30 minutes at 50 ^{O}C, 5 minutes at 95 ^{O}C followed by 50 cycles of 95 ^{O}C for 15 seconds and 60 ^{O}C for 60 seconds. Genes of interest represent relative mRNA expression as determined by the 2-ΔΔCt method for relative quantification; samples are normalized against this B2M endogenous control.

2.13 HBV infection

2.13.1 *De novo*

Target cells were seeded on collagen coated plates and treated with 2.5% DMSO for 3 days prior to inoculation. The cells were infected with HBV at a multiplicity of infection (MOI) of 200 genome equivalents per cell in the presence of 4% polyethylene glycol for 24 h. Viral inoculum was removed at 24 h post infection. The cells were washed three times with PBS and maintained in DMEM 10% FBS which was supplemented with 2.5% DMSO.

2.13.2 Adenovirus HBV model

This used an adenoviral-based vector that delivers a 1.3 overlength HBV genome containing a GFP reporter designated AdHBV described by (He *et al.*, 1998; Sprinzl *et al.*, 2001). Target cells were seeded at 80% confluence one day prior to inoculation. The MOI was determined in (**Figure 3.7**). The inoculum was applied for two hours before being removed and replaced with standard culture media. The nucleoside reverse transcriptase inhibitor entecavir was used as a transcription control.

2.13.3 HBV gaussian luciferase

This used a recombinant HBV virus whose genome contains a transgene for a gaussia luciferase that was cloned into the polymerase region -termed rHBVGLuc. This virus was a gift from Dr Jochen Wettengel of Ulrike Protzer group at the Technische Universität München, Munich. Target cells were seeded at 80% confluence one day prior to inoculation. MOI was determined in (**Figure 3.8B**). The inoculum was applied for six hours before being removed and replaced with standard media culture. The entry inhibitor mycrludex was used as a control to inhibit NTCP-dependent infection events.

2.14 Statistical analysis

All data are expressed as mean ± s.e.m. where * $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$ **** $P \leq 0.0001$. All statistical tests were performed using the GraphPad Prism 7 built-in analysis tool. The statistical test performed is detailed in individual figure legends.

Chapter 3: Identification of a hepatic cell line which supports circadian entrainment and HBV infection.

3.1 Introduction

Fundamental aspects of human physiology and behaviour are under circadian control (Poggiogalle, Jamshed and Peterson, 2018). Evolution in response to the eating/fasting and wake/sleep cycles have resulted in the temporal segregation of opposing metabolic processes to maximise their efficiency (Solt *et al.*, 2012; Gerhart-Hines and Lazar, 2015). The liver is at the forefront of this metabolic processing and, unsurprisingly, it is increasingly recognised as an organ highly influenced by circadian rhythm. Many key components of metabolism are under circadian regulation: glucose tolerance, gluconeogenesis, lipogenesis, cholesterol synthesis, glucocorticoid secretion and AMPK energy-sensing (Balsalobre *et al.*, 2000; Zhang *et al.*, 2010; Feng *et al.*, 2011; Jordan and Lamia, 2013). Murine RNA-Seq and microarray analysis suggest that between 10-18% of hepatic genes show a circadian pattern of expression (Panda *et al.*, 2002; Storch *et al.*, 2002; Zhang *et al.*, 2014). Measuring transcripts alone however likely underestimates the true extent of the circadian proteome largely due to the limited concordance between mRNA transcriptional rhythms and protein expression. This has been attributed to post-transcriptional modification of RNA (Ueda *et al.*, 2002; Menet *et al.*, 2012; Tahara and Shibata, 2016).

Bile salts are secreted in order to facilitate the digestion of fat in our food. Sodium taurocholate cotransporting peptide (NTCP) is a hepatic receptor that is responsible for the recycling of conjugated bile salts as part of the enterohepatic circulation (Watashi *et al.*, 2014). Additionally, NTCP is a crucial receptor for HBV entry that contributes towards its tropism (Yan *et al.*, 2012). Murine studies suggest that disruption of circadian rhythm by restricted feeding or double knockout of *PER1/2* decreased NTCP expression, increased serum bile acid levels and induced a transient liver injury mimicking hepatic cholestasis thereby suggesting a role for clock components in regulating NTCP expression and function (Ma *et al.*, 2009). It is unknown whether

human NTCP expression and whether NTCP mediated HBV entry could, in turn, be under circadian regulation.

One of the best recognised *in vivo* circadian parameters in humans is serum cortisol (Van Cauter, Leproult and Kupfer, 1996). Clinically, exogenous corticosteroid (CS) administration is known to exacerbate HBV morbidity by reactivation of otherwise quiescent disease or by inducing HBV replication which results in a disease flare. This observation is supported by a number of case-reports. A large cohort study of over 5000 patients (Wong *et al.*, 2019a) found that short exposure (<7days) to high dose dexamethasone (>40mg OD) was associated with a significant risk of a flare occurring. As exposure to CS is one of the best-established approaches to synchronize the circadian clock *in vitro*, preliminary work needs to investigate any potential effects of CS on HBV replication. This phenomenon is further discussed in (**Chapter 3 section 3.3**)

To investigate how circadian rhythm affects the HBV replication cycle, it is essential to establish a circadian rhythm in an HBV permissive cell line. Not all cell lines are entrainable to a circadian rhythm. The osteosarcoma U2OS cell line has a particularly robust circadian rhythm that can be used as a comparative benchmark (Hughes *et al.*, 2009). There exist numerous well-established protocols to perform *in vitro* cell synchronization which involve incubation at a lower ambient temperature, exposure to a high dose of CS or 50% FBS -also described as a 'serum shock'- (Balsalobre, Damiola and Schibler, 1998; Balsalobre *et al.*, 2000). Screening for a dynamic circadian rhythm requires repeated serial measurements with short intervals over a prolonged period. This labour intensive process has yielded the development of automated real-time luminescence recording methods (Ramanathan *et al.*, 2012) to record circadian rhythms.

Whether by transient transfection or stable transduction of clock promoter driven luciferase reporters e.g. *BMAL1, PERs or CRYs*, circadian rhythms can be monitored in real-time.

In vitro models that support HBV infection have several limitations: a reliance on hepatoma derived cancer cell lines, a lack of primate models, the dependence on a single genotype D virus, the requisite overexpression of NTCP protein, the use of dimethylsulfoxide (DMSO) to differentiate cells and the use of polyethylene glycol to facilitate infection (Thomas and Liang, 2016; Revill *et al.*, 2019). Research laboratories are biased towards use of genotype D virus, which is obviously not the only genotype encountered in a clinical setting. Different genotypes have differing outcomes (**Chapter 1.1.7**) (Kao *et al.*, 2000; Kramvis, Kew and François, 2005; Schaefer, 2005, 2007; European Association For The Study Of The Liver, 2017b). The almost ubiquitous use of DMSO and overexpression of NTCP to facilitate infection, whilst welcome developments to facilitate *in vitro* work undoubtedly carry off target side effects affecting cellular metabolism, signalling and growth (Choi *et al.*, 2009; Nikolaou *et al.*, 2016). Whilst the development of the HepaRG cell line marked a significant improvement (Gripon and Rumin, 2002), it did not remove the need to inoculate high titre virus on differentiated cells over many hours to establish an infection sufficient to study. This time taken to establish a robust *de novo* infection is particularly counterproductive to studying the relative short-lived 24h circadian rhythm. Hence an exploration into alternate HBV infection models was made. Observations derived from *in vitro* hepatoma cell lines and even the most contemporary murine models are far removed from the clinical bedside, therefore caution must be taken before their generalisation whilst taking care not to diminish their significance; this is a fine balance to achieve (Castell *et al.*, 2006; Donato, Jover and Gómez-Lechón, 2013; Ruoß *et al.*, 2020).

REV-ERBα is a nuclear receptor that acts as a potent transcriptional repressor with haem as its ligand (Mangelsdorf *et al.*, 1995; Raghuram *et al.*, 2007; Yin *et al.*, 2007). Nuclear receptors bind to specific DNA sequences of genes to regulate their transcription in a ligand-dependent

manner. REV-ERB binds to a RORE sequence to exert these repressive transcriptional effects. SR9009 is a synthetic REV-ERB agonist that mimics the action of physiological haem in activating the REV-ERBs providing a useful tool complementing *in vitro* and *in vivo* work (Solt *et al*., 2012).

3.2 Results

3.2.1 Establishing synchronisation protocols and circadian rhythm monitoring using U2OS cells.

To demonstrate the technical capability of detecting a circadian rhythm, a U2OS line stably expressing a BMAL1 promoter driven firefly luciferase reporter (U2OS-Bmal1-luc) was generated by lentivirus transduction and subsequent antibiotic selection.

Three synchronization techniques and maintenance media compositions were evaluated for entrainment of a circadian rhythm in U2OS-Bmal1-luc cells (**Figure 3.1**). The three synchronization techniques were:

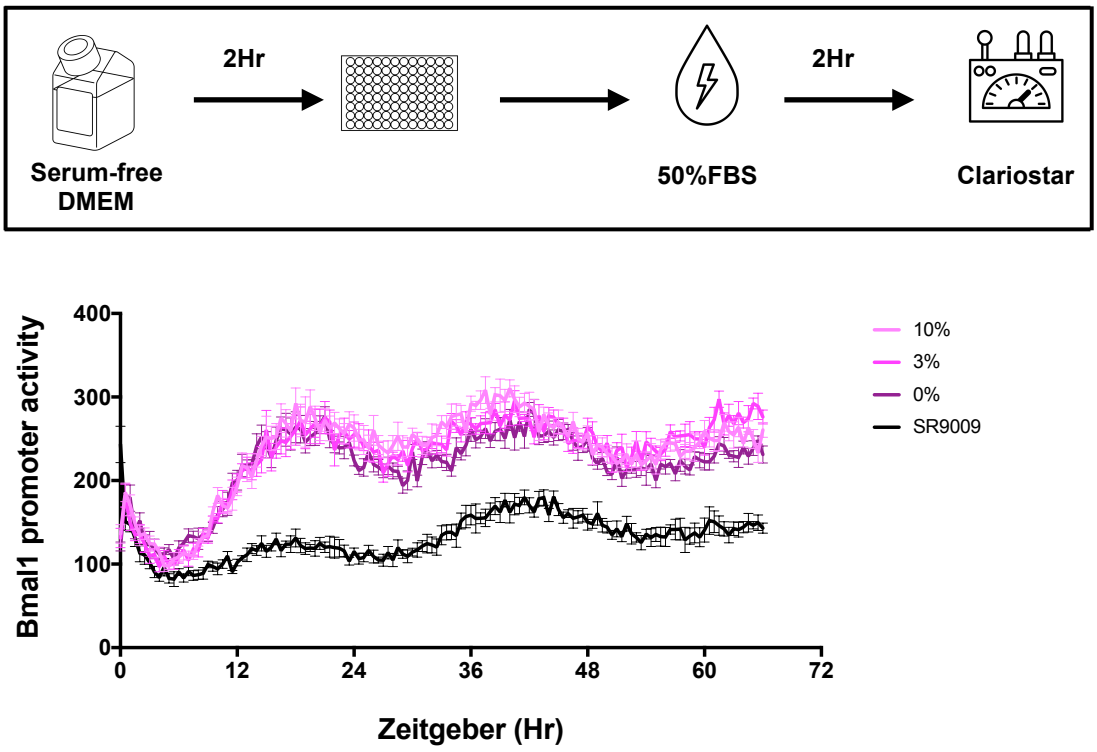
- A two-hour serum starvation period followed by a two hour incubation with 50% FBS.
- A two-hour incubation with 50% FBS.
- A two-hour incubation with 100 nM dexamethasone.

After synchronisation, maintenance media with varying FBS concentrations (0%, 3% or 10%) were used to investigate if cell growth would interfere with the rhythm generated. The REV-ERB agonist SR9009 that can inhibit BMAL1 promoter activity was included as a control to establish the basal levels of the promoter activity. While all three techniques were effective in producing a pattern consistent with a circadian rhythm that lasts over 66 hours, those exposed to the dexamethasone treatment showed the highest amplitude (**Figure 3.1A-C**). Henceforth, dexamethasone treatment was selected as the primary method to synchronise cells. The

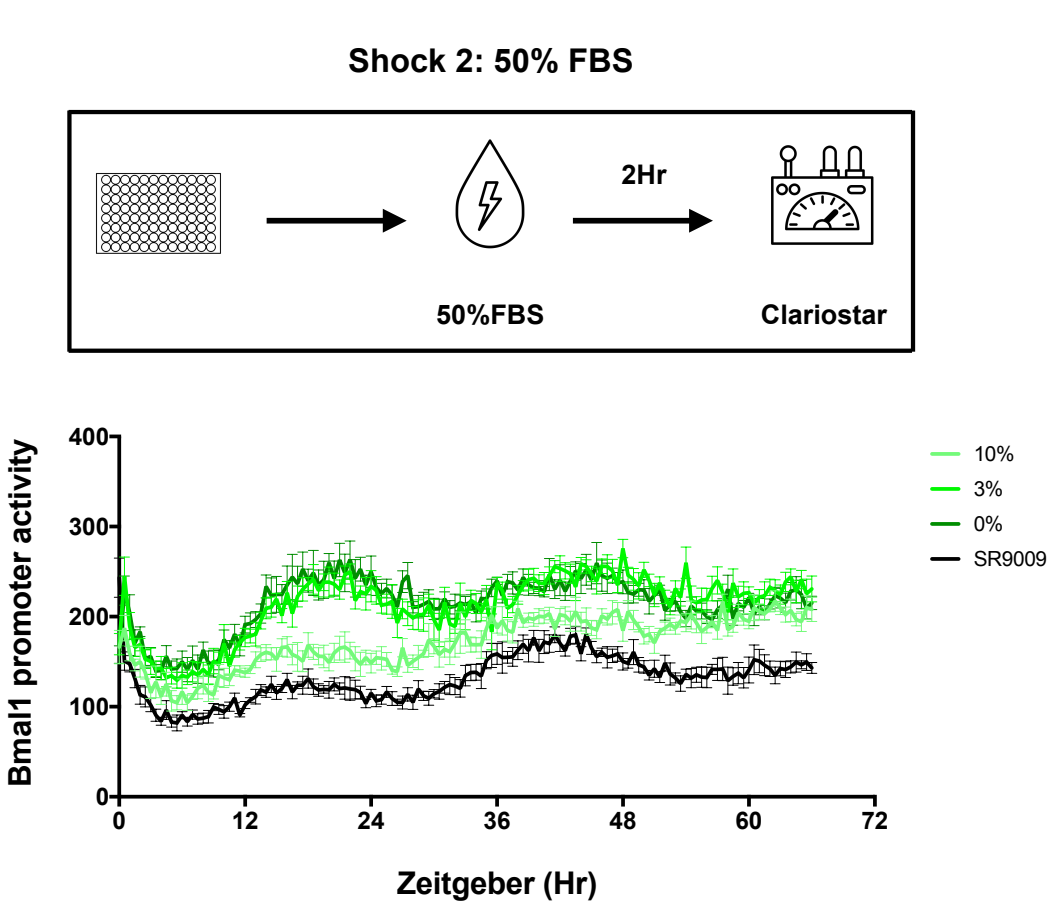
concentration of FBS in the maintenance media had a negligible effect on the amplitude and period. There was a significant dampening of the BMAL1 promoter amplitude with SR9009 treatment regardless of the synchronization technique, validating the rhythms generated by these different approaches.

These results demonstrate our technical ability to induce and monitor the circadian rhythm in the U2OS cells.

A


Shock 1: Serum starve then 50% FBS
2Hr
2Hr
Serum-free DMEM
50%FBS
Clariostar
Bmal1 promoter activity
400
300
200
100
0
0
12
24
36
48
60
72
Zeitgeber (Hr)
10%
3%
0%
SR9009

B


Shock 2: 50% FBS
2Hr
50%FBS
Clariostar
Bmal1 promoter activity
400
300
200
100
0
0
12
24
36
48
60
72
Zeitgeber (Hr)
10%
3%
0%
SR9009

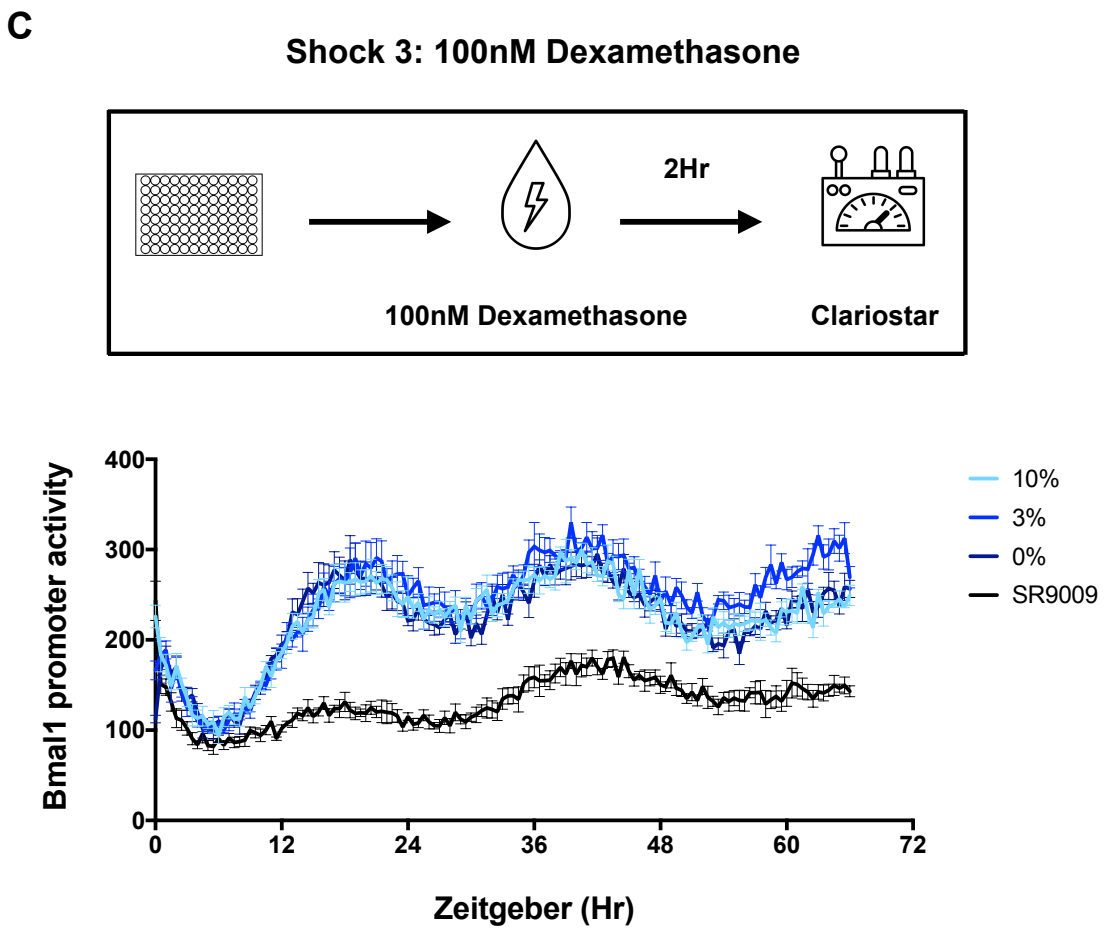


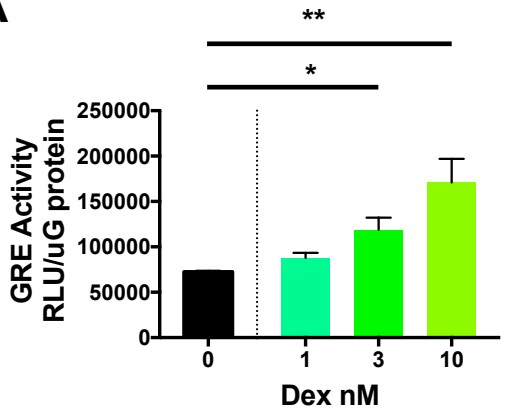
Figure 3.1: U2OS cells demonstrate a robust circadian rhythm in response to three synchronization techniques. U2OS cells stably expressing a BMAL1 promoter reporter expressing luciferase are shocked into synchrony using three different techniques. Exposure to; (A) 50% FBS preceded by a two-hour exposure to serum-free media, (B) 50% FBS alone or (C) 100nM dexamethasone for 2 hours according to the protocols shown. Cells were cultured in increasing percentage FBS maintenance media where luminescence was measured every 30 minutes for a total of 66hrs. As a control, cells were treated with the REV-ERB agonist SR9009 (20uM) that is known to repress BMAL1 promoter activity. A circadian rhythm -that is blunted by exposure to SR9009- is observed across all three shock techniques (A-C). The composition of maintenance media does not affect the rhythm observed. Data is expressed as relative light units and represent the mean ± SEM, n=6 per timepoint.

3.2.2 Assessment of HBV replication parameters response to dexamethasone.

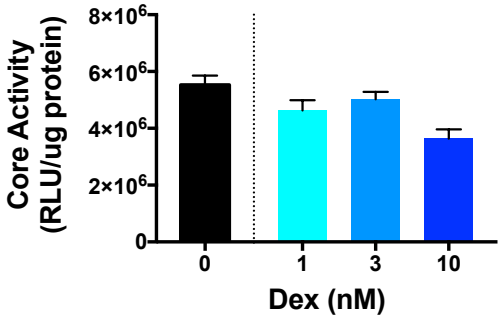
To explore whether the use of dexamethasone in the synchronization protocol would modulate HBV infection, key HBV parameters including viral promoter activity, pgRNA and HBsAg/HBeAg production were investigated for dexamethasone responsiveness.

To remove inherent CS contained in the FBS in standard maintenance media, all cells were initially incubated in serum-free media for 24 hours before treating with CS. A HepG2 line stably overexpressing the NTCP protein (HepG2-NTCP) was used. Luciferase reporter constructs driven by a glucocorticoid-responsive element (GRE) or one of the four HBV promoters: Core, X, S1 or S2 were transfected into HepG2-NTCP cells. The total amount of DNA transfected was kept constant between conditions using an irrelevant plasmid pcDNA3.1. Transfected cells were treated with a range of CS concentrations for 24-48 hours and cell lysates assessed for luciferase activity and protein quantification. The GRE reporter was used as a positive control where it expectedly showed increased activity in response to an increasing dose of dexamethasone (**Figure 3.2A**). The four HBV promoters -Core, X, S1 and S2- remained insensitive to the dexamethasone treatment both at 24h (**Figure 3.2B-E**) and 48h (data not shown).

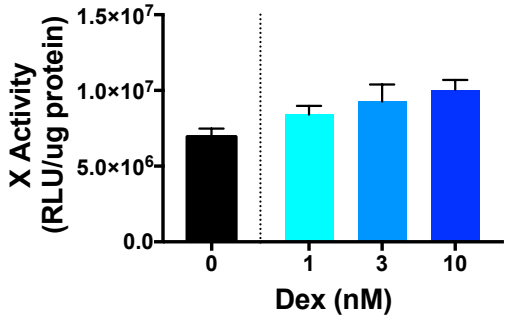
A


**
*
GRE Activity
RLU/uG protein
250000
200000
150000
100000
50000
0
0
1
3
10
Dex nM

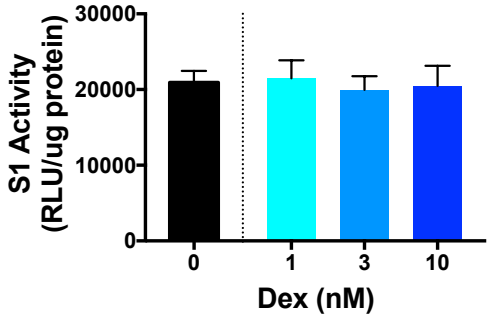
B


Core Activity
(RLU/ug protein)
8×10^6
6×10^6
4×10^6
2×10^6
0
0
1
3
10
Dex (nM)

C


X Activity
(RLU/ug protein)
1.5×10^7
1.0×10^7
5.0×10^6
0.0
0
1
3
10
Dex (nM)

D


S1 Activity
(RLU/ug protein)
30000
20000
10000
0
0
1
3
10
Dex (nM)

E

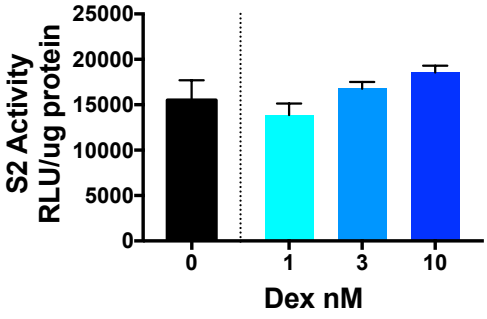

S2 Activity
RLU/ug protein
25000
20000
15000
10000
5000
0
0
1
3
10
Dex nM

Figure 3.2: Dexamethasone does not enhance HBV promoter activity. Either a glucocorticoid response element (GRE) or one of four HBV promoter reporters expressing luciferase were transiently transfected using PEI into HepG2 NTCP cells. They were serum starved for 24 hours before being exposed to dexamethasone. The GRE reporter was used as a positive control. Twenty-four hours later they were lysed, and luciferase measurements were taken. Increasing activity is seen with increasing dose of steroid in the GRE (A) but not in any of the HBV promoters (B-E). Data shown as mean ± SD, n=6. P= 0.019 (0 vs 3nM dexamethasone) and 0.002 (0 vs 10nM dexamethasone) using Kruskal-Wallis (A).

To assess whether dexamethasone affects viral replication, HepG2.2.15 cell line containing both HBV cccDNA and integrated HBV DNA was used. HepG2.2.15 cells were treated with dexamethasone for 48 hours and HBV pgRNA quantified by qRT-PCR. Whilst a downward trend in HBV pgRNA is observed, only in those treated with 100nM dexamethasone did it reach significance (**Figure 3.3A**). HBeAg and HBsAg in the supernatant were quantified at 48h or 72h post dexamethasone treatment. There was no significant change with 20nM or 100nM dexamethasone treatment compared with mock (**Figure 3.3B-C**).

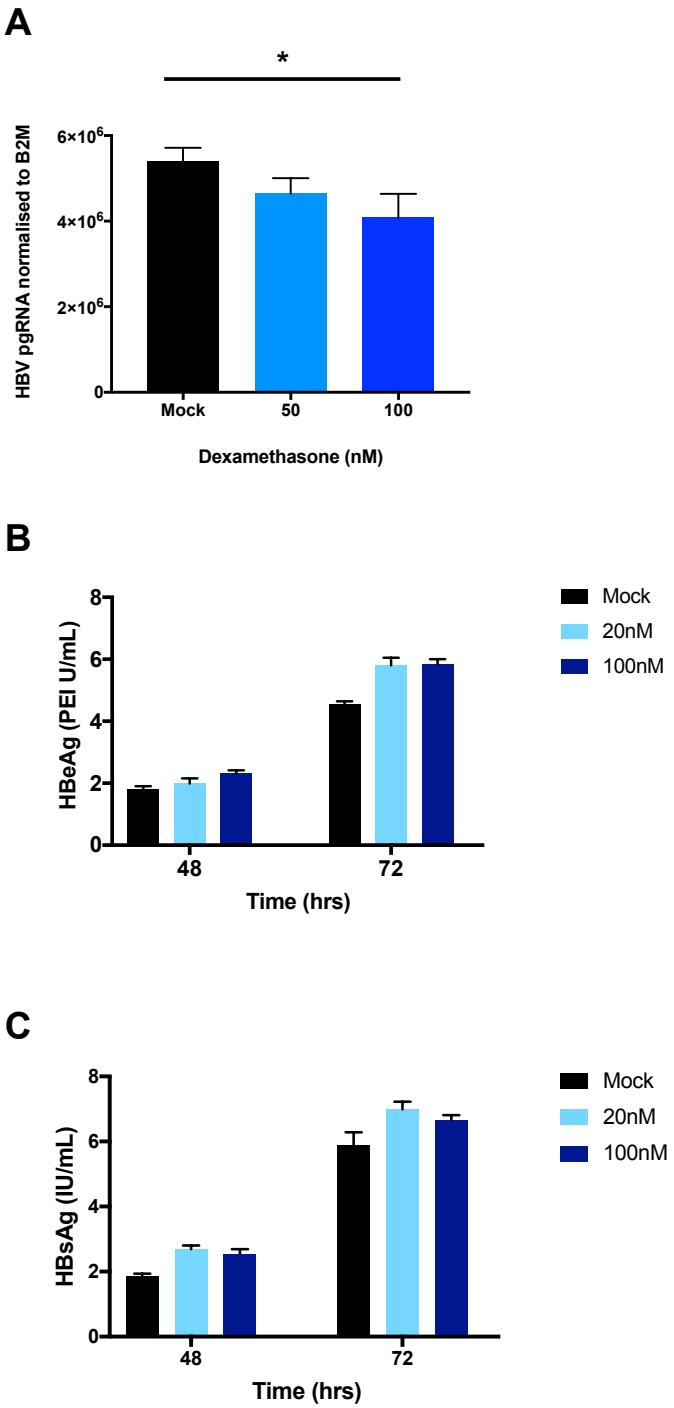


Figure 3.3: Dexamethasone modestly reduces pgRNA but does not affect HBeAg or HBsAg production in HepG2.2.15 cells. HepG2.2.15 cells were treated with dexamethasone for 48 hours and HBV pgRNA measured by qRT-PCR. Treatment with 100nM dexamethasone decreases pgRNA (A). Data shown as mean ± SD, n=4. P=0.01 using Kruskal-Wallis. The supernatant was collected at 48 and 72 hours before undergoing ELISA for HBeAg and HBsAg. No significant modulation is observed in the ELISAS (B-C). Data shown as mean ± SD, n=4.

These results suggest limited modulation in HBV promoter activity, transcription or antigen production in response to CS treatment. It is therefore unlikely that the use of both comparatively smaller concentration and durations of dexamethasone would preclude their use to synchronise a cell line when studying the HBV replication cycle.

3.2.3 HepG2 cells show a limited circadian rhythm.

HepG2 cells are commonly used in HBV research due to their permissivity to support viral replication following NTCP overexpression. HepG2-NTCP cells were transduced with a BMAL1 luciferase promoter using the same lentiviral system for producing the U2OS-BMAL1-luc line. In contract to the U2OS-BMAL1-luc cells, the HepG2-NTCP-BMAL1-luc cells do not demonstrate a circadian pattern of the promoter activity using serum shock or dexamethasone protocols (**Figure 3.4**). This demonstrates that the HepG2 cell line is not suitable for circadian experiments.

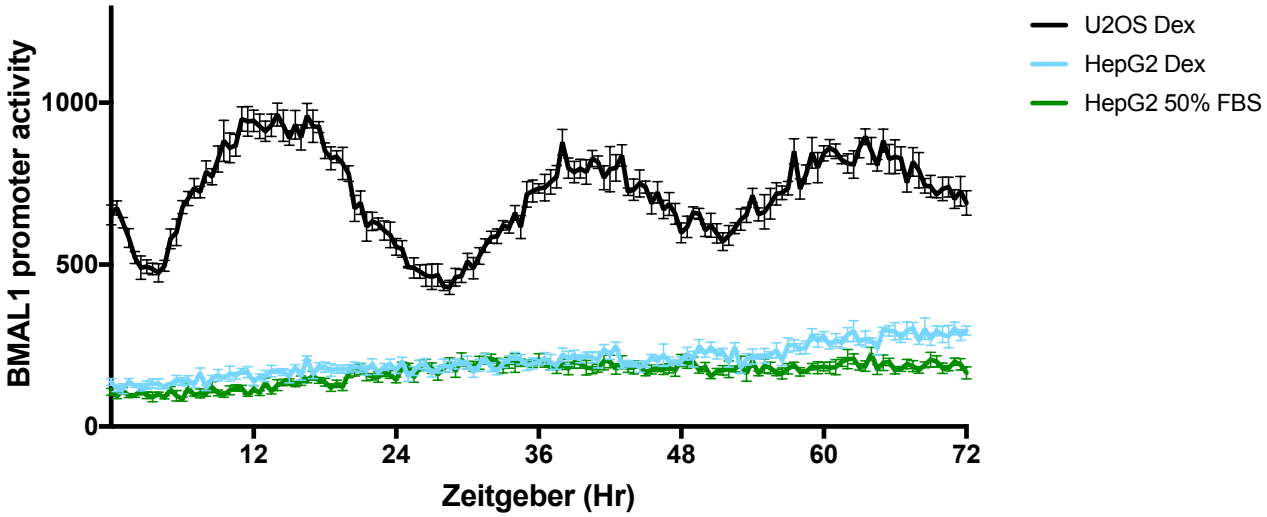
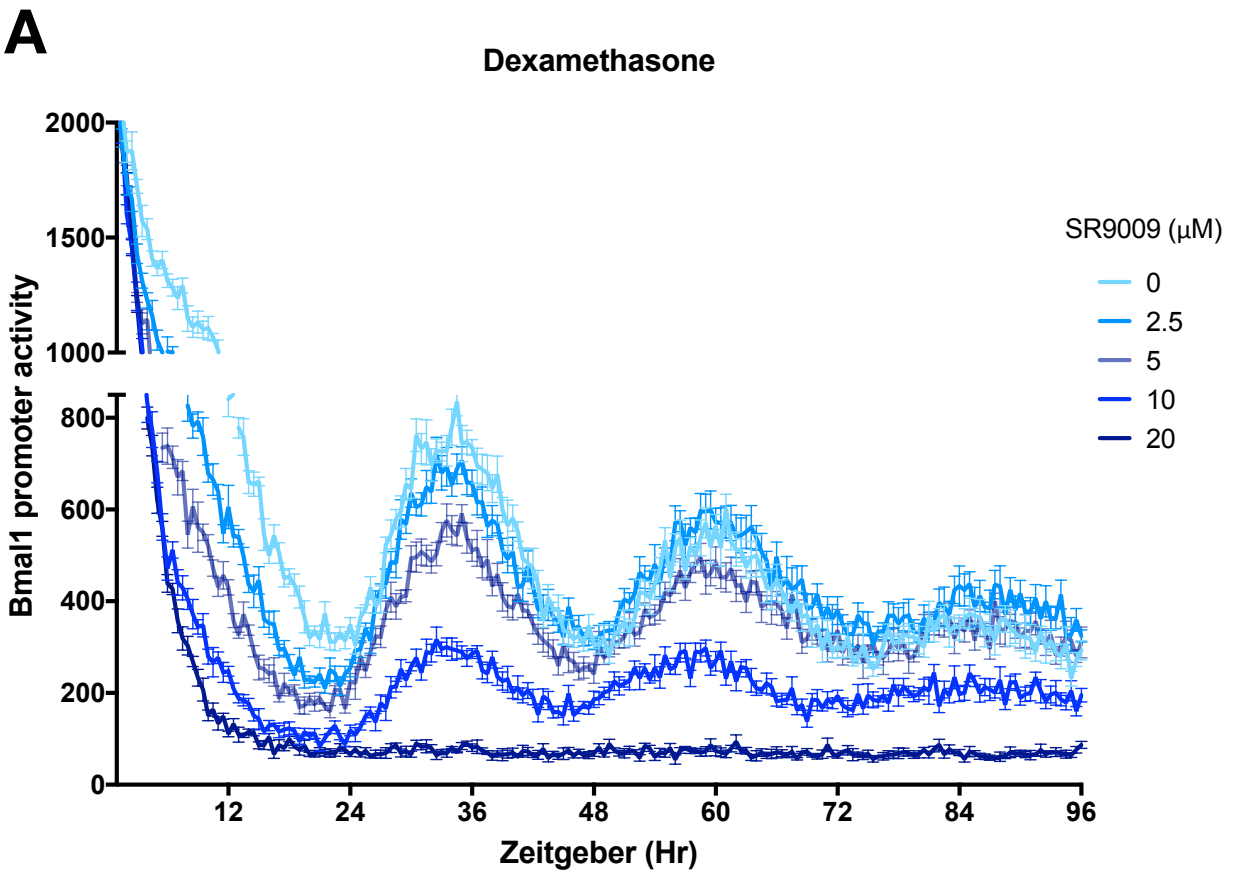


Figure 3.4: The HepG2 cell line does not display a circadian rhythm. HepG2 NTCP cells stably expressing BMAL1 luciferase were shocked using two techniques and the resultant rhythm compared with U2OS BMAL1 luc cells. No discernible circadian rhythm is observed in the HepG2 cells with either shocking technique. The graph is representative of three experiments. Data shown as mean ± SD, n=6 per time point.

3.2.4 HepaRG cells demonstrate a circadian rhythm.

The HepaRG line developed by (Gripon and Rumin, 2002) is a bipotent progenitor cell that can differentiate towards both hepatocyte-like or biliary-like cells. Two notable characteristics align them closer to primary hepatocytes that other hepatoma derived cell lines; comparable enzymatic activity and morphological changes upon differentiation (Guillouzo *et al.*, 2007; Hantz *et al.*, 2009). Only differentiated cells (dHepaRG) support HBV infection.

A HepaRG cell line stably expressing a BMAL1 luciferase promoter was generated by lentivirus transduction as described above. Both dexamethasone and serum shock protocols yielded a clear pattern of BMAL1 promoter activity consistent with a circadian rhythm that persisted for 72 hours (**Figure 3.5**). As expected, REV-ERB agonist SR9009 showed a dose-dependent attenuation in promoter activity. This was a pivotal result as it demonstrated for the first time a hepatic cell line being able to demonstrate a circadian rhythm *in vitro*.


A
Dexamethasone
Bmal1 promoter activity
2000
1500
1000
800
600
400
200
0
12
24
36
48
60
72
84
96
Zeitgeber (Hr)
SR9009 (μM)
0
2.5
5
10
20

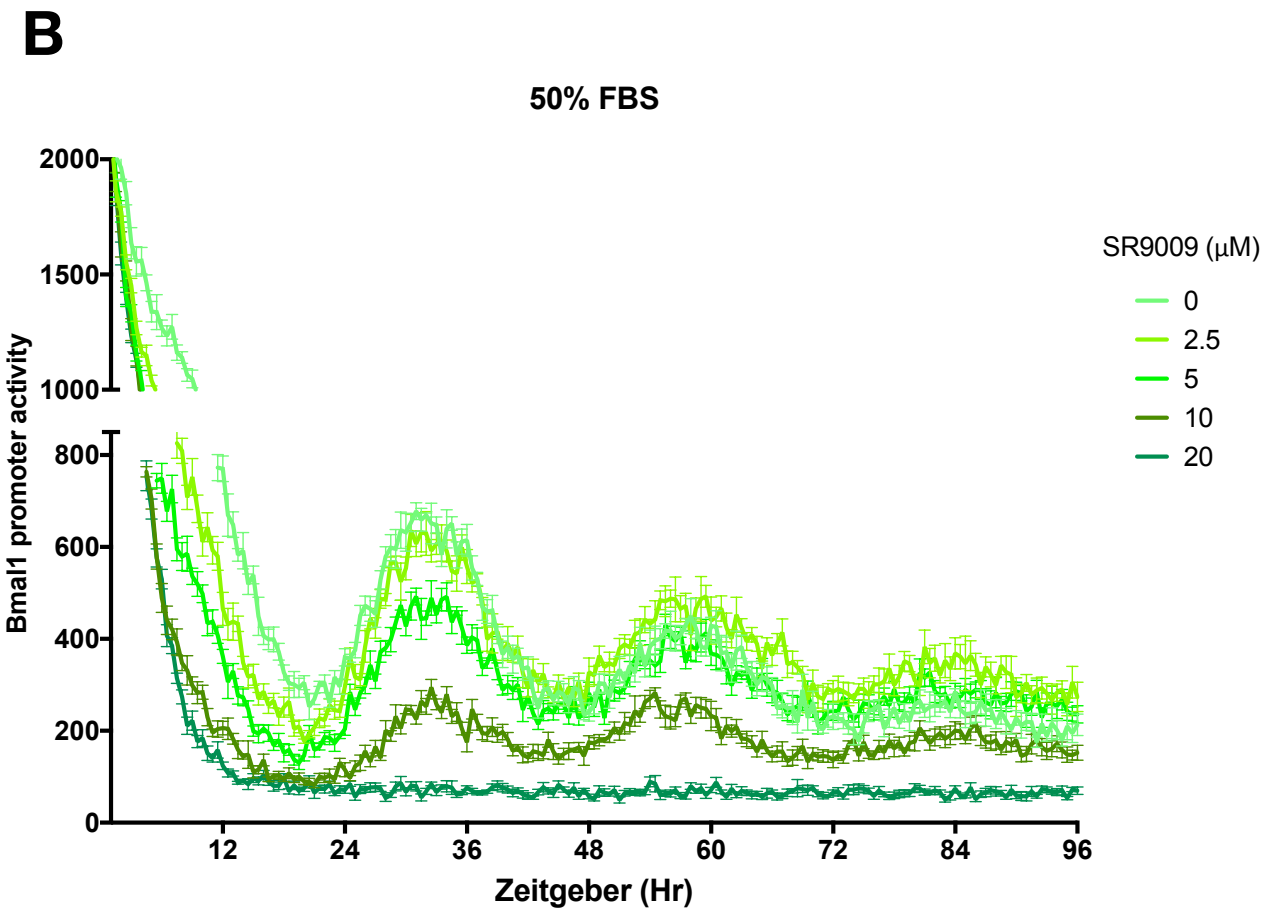

B
50% FBS
Bmal1 promoter activity
2000
1500
1000
800
600
400
200
0
12
24
36
48
60
72
84
96
Zeitgeber (Hr)
SR9009 (μM)
0
2.5
5
10
20

Figure 3.5: The undifferentiated bipotent HepaRG cell line demonstrates a circadian rhythm. Undifferentiated HepaRG cells stably expressing BMAL1 luciferase are a hepatoma cell line that demonstrate a pattern consistent with a circadian rhythm. It shows dose-dependent inhibition to SR9009. A comparable rhythm is seen with either steroid shock (A) or serum shock (B). Data shown as mean ± SD, n=6 per time point.

3.2.5 Evaluating alternate HBV infection models in non-differentiated HepaRG cells.

Having observed a circadian rhythm in undifferentiated HepaRG cells; we wanted to investigate their ability to support HBV replication.

First, an adenoviral-based vector that delivers a 1.3 overlength HBV genome containing a GFP reporter designated AdHBV was used (He *et al.*, 1998; Sprinzl *et al.*, 2001) (**Figure 3.6**). This model efficiently transfers the HBV genome into cells and allows full replication to occur; with detection of HBV proteins, pgRNA and cccDNA (Sprinzl *et al.*, 2001). Accordingly, this model bypasses the conventional NTCP receptor-dependent entry of HBV. It has technical advantages over conventional HBV infection; a two hour inoculation period and detection of most HBV parameters within three days. These are shorter than current HBV infection protocols where HBV parameters cannot be detected until much later post inoculation (Hantz *et al.*, 2009). This would be advantageous for studying a temporary, attenuating, induced circadian rhythm.

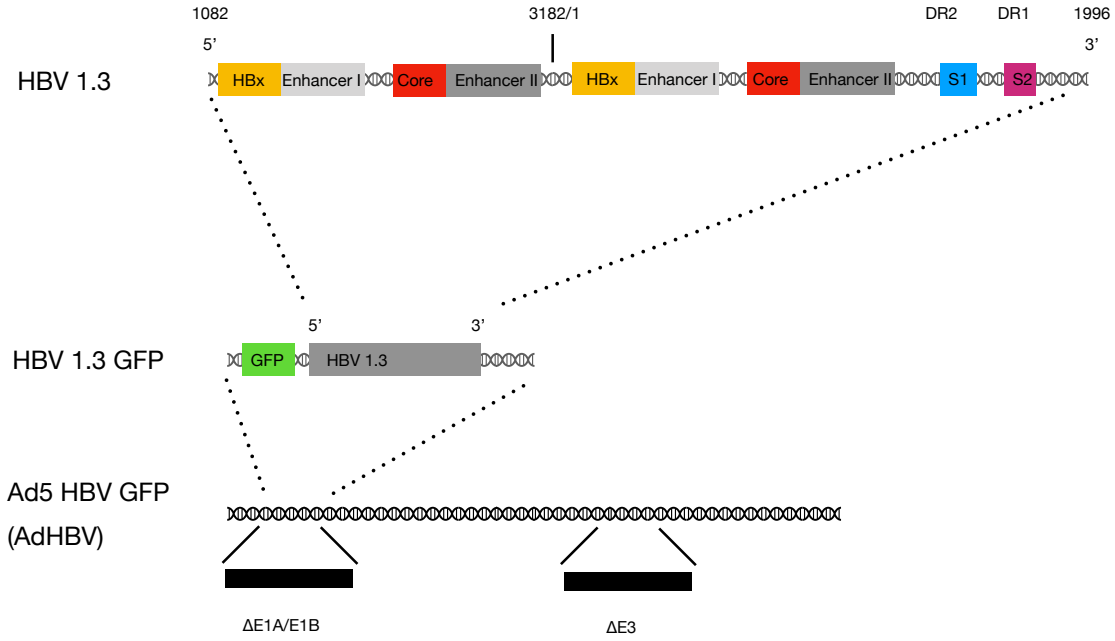
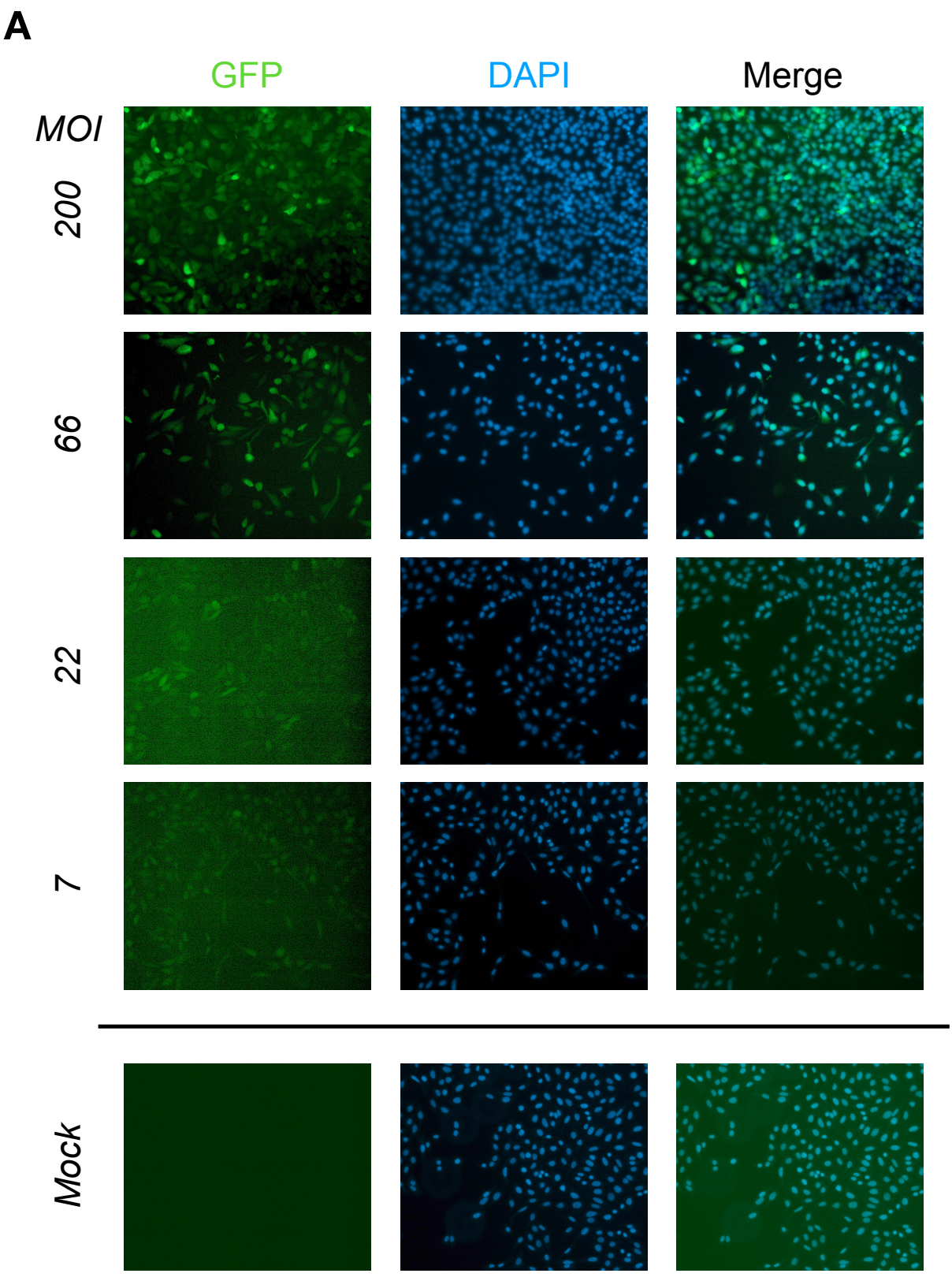


Figure 3.6: Schematic representation of the AdHBV composition. Replication-competent HBV genome was inserted into the E1 region of an adenovirus subtype 5 genome (with deletions of E1A/E1B and E3). A GFP reporter gene was also inserted (He *et al.*, 1998; Sprinzl *et al.*, 2001).

To achieve a 50% infection rate denoted by GFP+ expression in target undifferentiated HepaRG cells, a range of MOIs were investigated. Cells were inoculated with increasing MOI of AdHBV for two hours and the inoculum removed. Fluorescent microscopy was undertaken 24 hours later to evaluate GFP expression, where it was determined that an MOI of 10 would suffice (**Figure 3.7A**). Next, undifferentiated HepaRG cells and control differentiated HepG2 NTCP cells (dHepG2 NTCP)- were compared for their permissivity to support AdHBV replication. Entecavir (ETV) treated cells were used as a negative control as inhibiting reverse transcriptase prevents encapsidated HBV DNA containing virion formation. Comparing both cell lines for encapsidated HBV virions in the supernatant; a sustained infection could not be achieved in the undifferentiated HepaRG cells (**Figure 3.7B).** Both mock and ETV treated cells yield a similar trend. However, in the dHepG2 NTCP cells a sharp increase in HBV virions is seen at 96 hours post infection suggesting a competent HBV replication cycle. Of note, the ETV treated dHepG2 NTCP cells remain negative.


A
GFP
DAPI
Merge
MOI
200
66
22
7
Mock

B

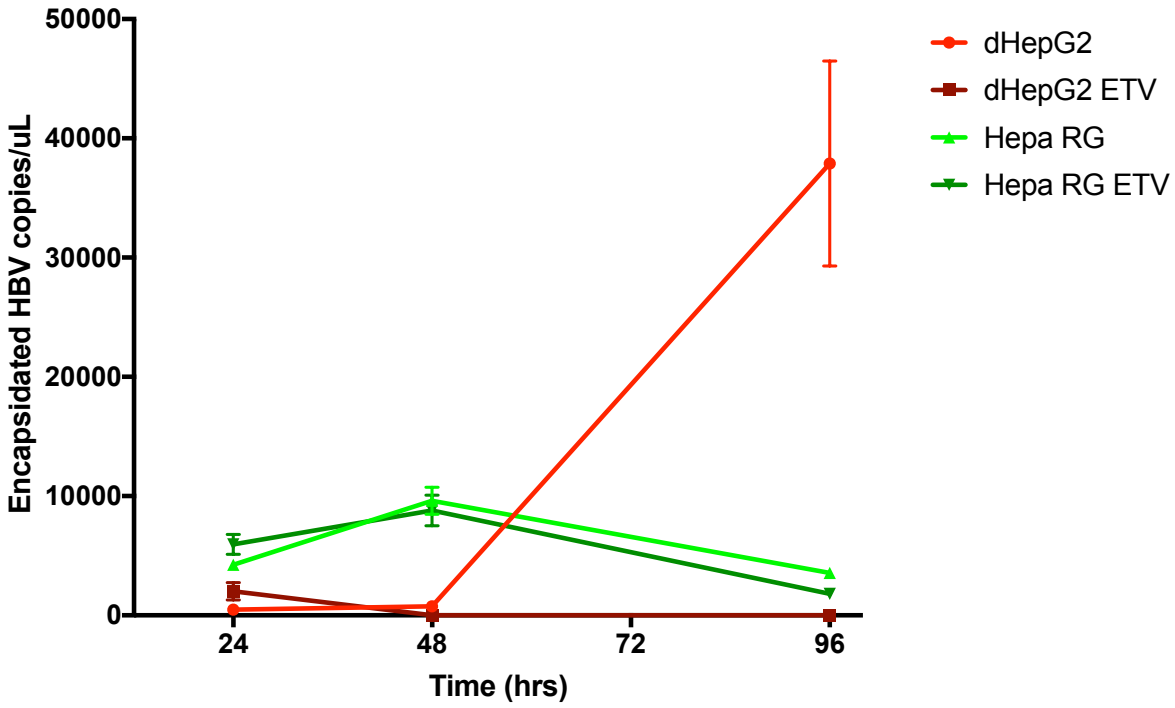
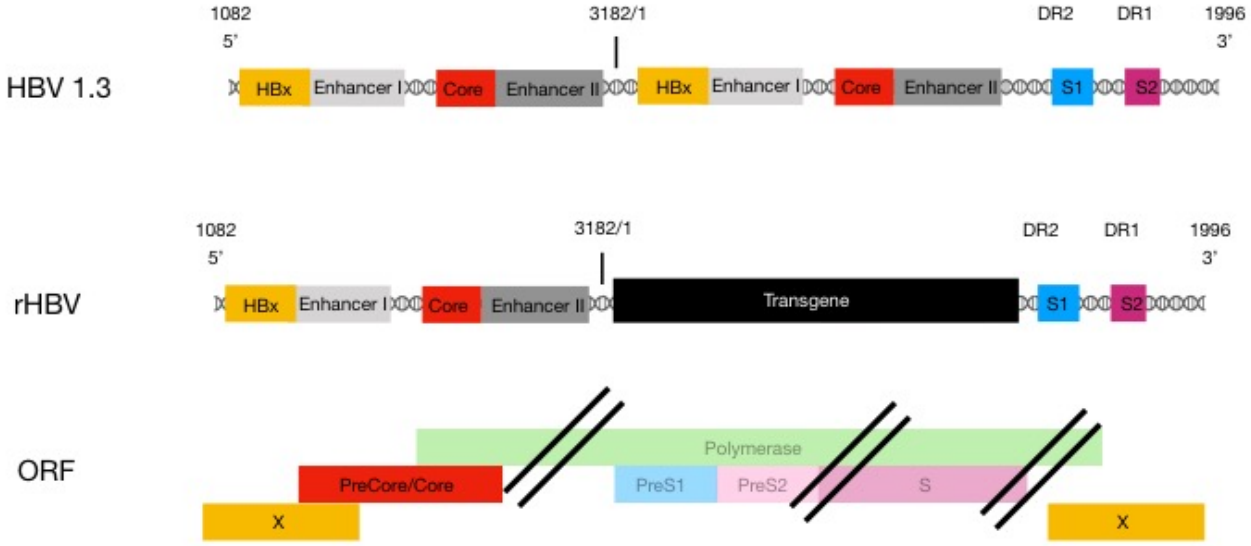


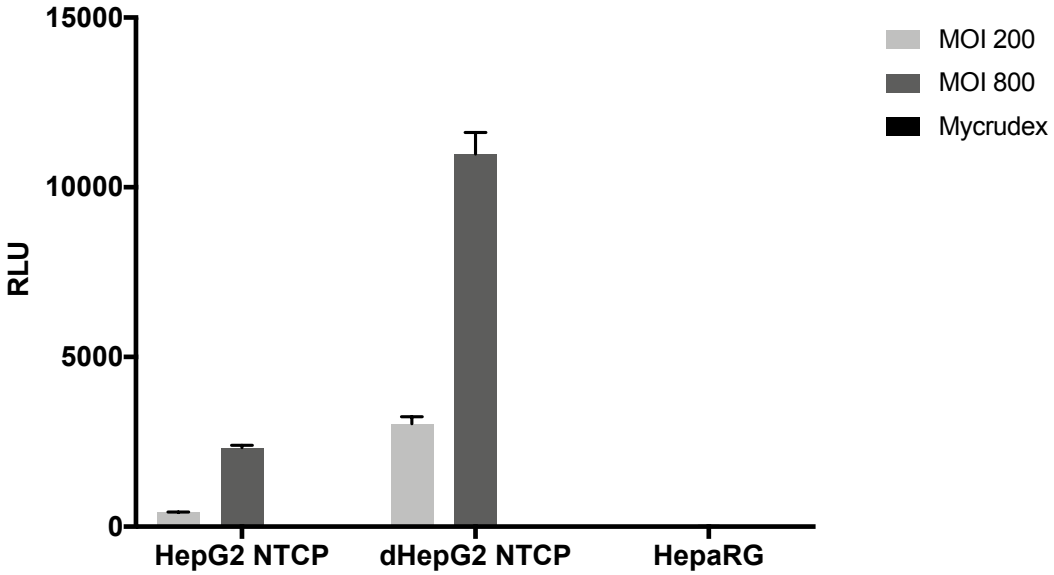
Figure 3.7: Undifferentiated HepaRG cannot sustain the AdHBV infection model.
Undifferentiated HepaRG cells were infected with a range of AdHBV MOI to determine the target infection rate of 50%. Fluorescent microcopy to evaluate GFP expression 24 hours later showed a MOI of 10 to be sufficient (A). dHepG2 NTCP and undifferentiated HepaRG cells were infected with AdHBV and the supernatant analyzed by qPCR for encapsidated HBV DNA containing virions. Undifferentiated HepaRG cells fail to sustain an infection. dHepG2 NTCP cells show an increase in HBV DNA at 96 hours. ETV treated cells acting as remain negative (B). Data shown as mean ± SD, n=10.

Next, a recombinant HBV virus whose genome contains a transgene for a gaussia luciferase cloned in the polymerase region -termed rHBVGLuc- was investigated. Due to disruption of the open reading frames by the transgene insertion; this virus could infect and transcribe but no new particle formation can occur due to disruption in the HBV polymerase ORF (**Figure 3.8A**). This virus was a gift from Dr Jochen Wettengel of Ulrike Protzer group at the Technische Universität München, Munich. This allows for sensitive and real-time measurement of viral transcription. For comparison, both undifferentiated and dHepG2 NTCP cells were infected at two different MOIs. The entry inhibitor mycrludex was used as a control to inhibit NTCP-dependent infection events. All three cell lines were seeded 24 hours before inoculation with rHBVGLuc for six hours. The supernatant was sampled at days 1,3 and 6 post infection and luciferase activity measured. Six days post infection the undifferentiated HepaRG cells showed no evidence of infection (**Figure 3.8B**). As expected, the dHepG2 NTCP cells have higher RLU values than their undifferentiated counterparts, and higher MOI resulted in higher RLU while mycrludex treated cells were negative.

A


1082
5'
3182/1
DR2
DR1
1996
3'
HBV 1.3
HBx
Enhancer I
Core
Enhancer II
HBx
Enhancer I
Core
Enhancer II
S1
S2
1082
5'
3182/1
DR2
DR1
1996
3'
rHBV
HBx
Enhancer I
Core
Enhancer II
Transgene
S1
S2
ORF
Polymerase
PreCore/Core
PreS1
PreS2
S
X
X

B



	MOI 200			MOI 800			Mycrudex		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
HepG2 NTCP	415.250	28.194	4	2326.750	147.380	4	0.500	0.577	4
dHepG2 NTCP	3029.250	424.450	4	10977.750	1271.702	4	0.750	0.957	4
HepaRG	2.000	1.826	4	6.500	3.697	4	0.250	0.500	4

Figure 3.8: Undifferentiated HepaRG cannot sustain the rHBVGLuc model. Schematic representation of the rHBVGLuc virus showing insertion of the gaussian luciferase reporter transgene with resultant disruption of the polymerase and HBsAg ORFs (A). This virus was a gift from Jochen Wettengel of Ulrike Protzer group at the Technische Universität München, Munich. Permissivity to rHBVGluc was evaluated in undifferentiated HepG2 NTCP, dHepG2 NTCP and undifferentiated HepaRG. Supernatant analyzed for gaussian luciferase six days post infection shows no evidence of infection in the undifferentiated HepaRG (B). RLU tabulated as too low to illustrate for HepaRG. Data shown as mean ± SD, n=4.

The results of these two models together convincingly demonstrate that undifferentiated HepaRG cells do not support either of these proposed HBV infection models. Consequently, the investigation of the effect DMSO induced differentiation would have on HepaRG circadian cycling was pursued.

3.2.6 DMSO dampened the BMAL1 promoter-luciferase activity in HepaRG cells.

To explore the effects that DMSO treatment has on circadian rhythm in HepaRG cells, both its introduction and removal on undifferentiated and dHepaRG cells were investigated using real-time luminescence.

First, undifferentiated HepaRG cells were synchronised using either serum shock or dexamethasone and subsequently cultured with or without 1.8% DMSO whilst undergoing real-time luminescence monitoring. The cells where DMSO was introduced showed a decrease in RLU values and reached a lower baseline than mock cells cultured in standard media (**Figure 3.9A**). These mock cells demonstrated a robust circadian pattern of BMAL1 promoter activity comparable with previous experiments (**Figure 3.5**). Both synchronization techniques resulted in a similar pattern of BMAL1 promoter luciferase reporter activity. This suggests the introduction of DMSO either dampened the RLU signal or was detrimental to the induced rhythm.

To differentiate these possibilities, dHepaRG cells were synchronised and maintained in culture with DMSO containing media or mock. In the dHepaRG cells where DMSO was removed, a gradual increase in the RLU is seen from the ZT24 timepoint onwards. This pattern of RLU activity

is consistent with a circadian rhythm (**Figure 3.9B**). This pattern is comparable in its peak and trough activity to the circadian rhythm observed previously in synchronised undifferentiated cells at the same ZT (**Figure 3.9A**). This suggests that removal of DMSO allows for the detection of an induced rhythm by real time luminescence. Together these results indicate dHepaRG cells are capable of sustaining an entrainable circadian rhythm but due to DMSO dampening signal, real-time luminescence is not possible.

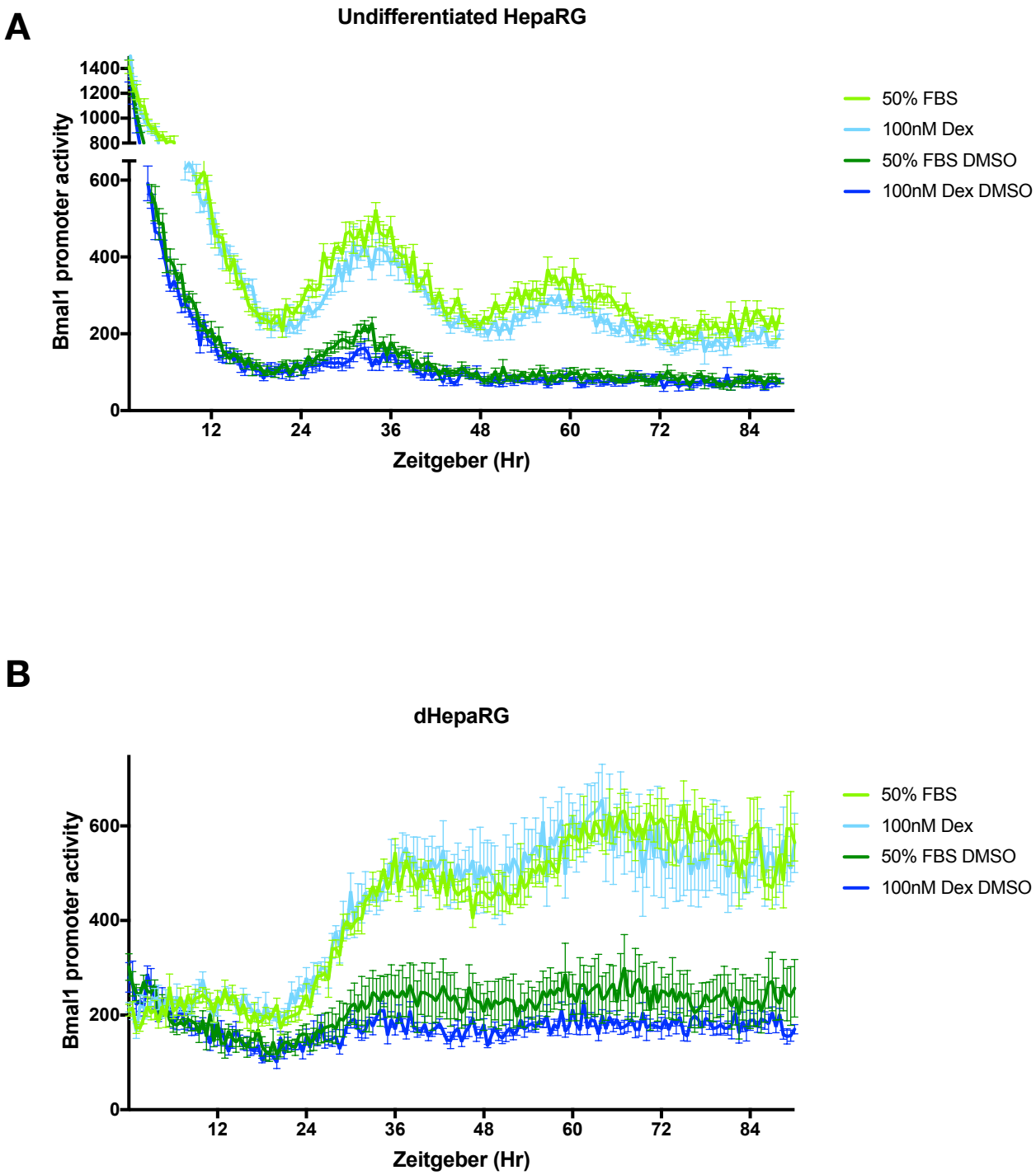


Figure 3.9: DMSO attenuates the BMAL1 reporter luciferase RLU signal in HepaRG cells. Undifferentiated HepaRG cells were synchronised and treated with 1.8% DMSO before being immediately placed for real-time luminescence. Cells treated with DMSO showed a sharp decrease in RLU signal whereas mock cells display a pattern consistent with circadian rhythm (A). dHepaRG cells were synchronised and DMSO removed before being immediately placed for real-time luminescence. Cells where DMSO was removed show a circadian pattern of expression after ZT24 that is not seen when DMSO is maintained (B). Data shown as mean ± SD, n=6 per time point.

This DMSO dampening of a luciferase reporter has been observed in our laboratory previously. HepG2 cells transfected with the HBV core promoter which are subsequently exposed to 2% DMSO showed a dampened response to SR9009 treatment (**Figure 3.10**). This further suggests a technical limitation of firefly luciferase signalling due to DMSO exposure rather than a modulation in the promoter of interest.

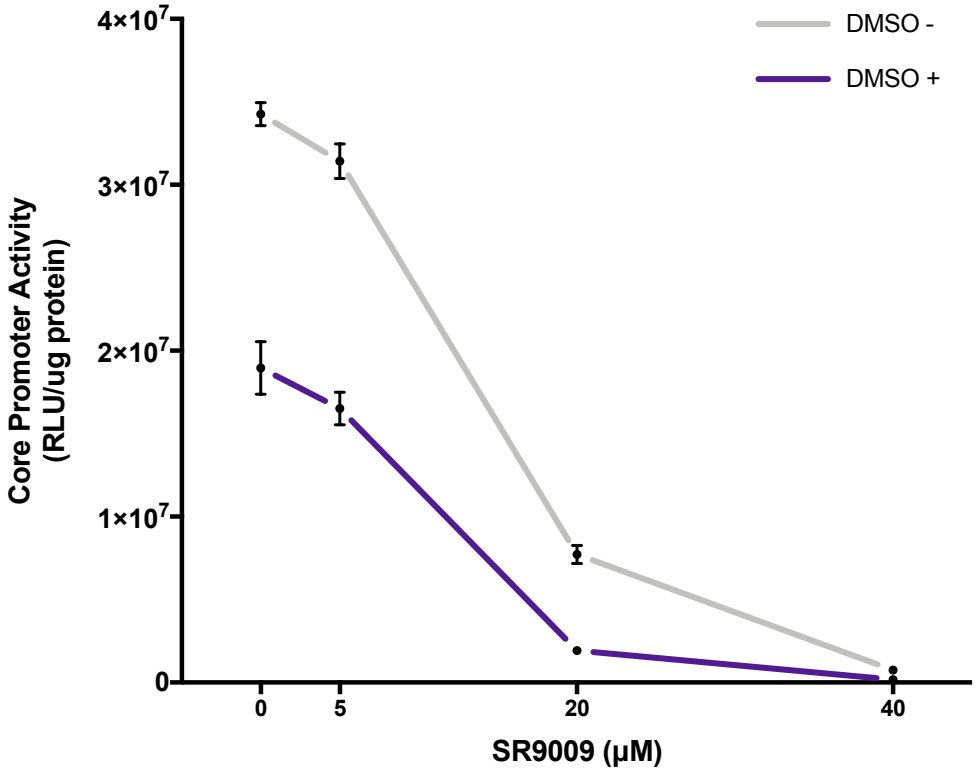
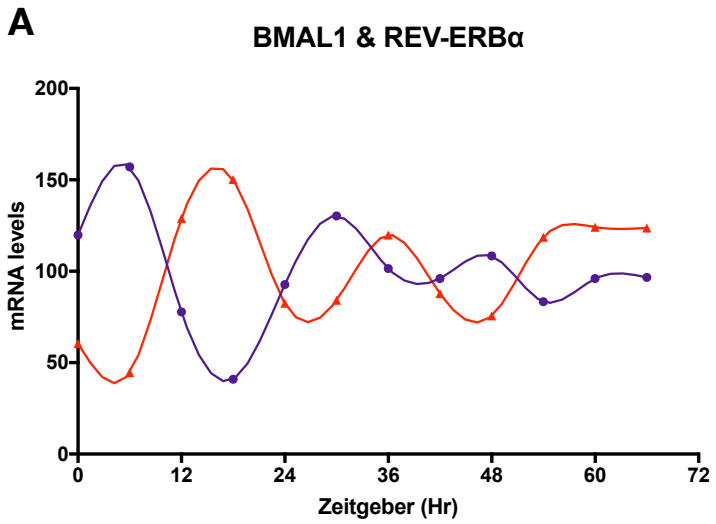


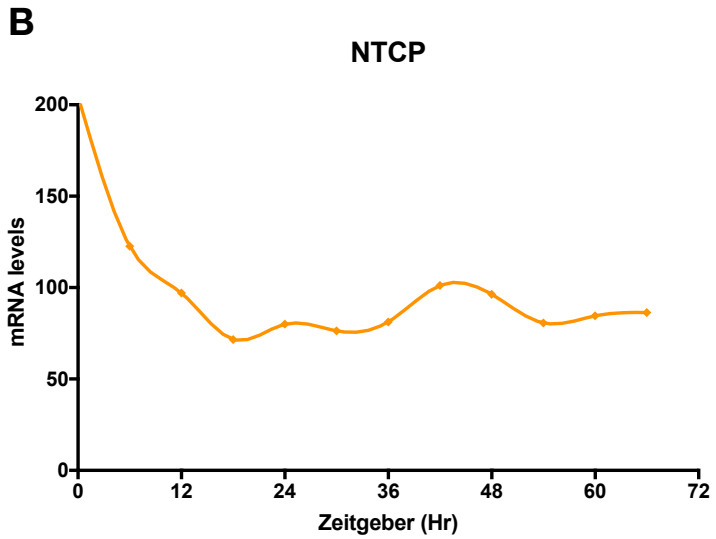
Figure 3.10: DMSO attenuates the RLU signal in the HBV core promoter luciferase reporter. HepG2 cells are transfected with an HBV core promoter luciferase reporter. 2% DMSO and mock treated cells are then exposed to incremental doses of SR9009 for 48 hours before samples taken for luciferase. Data shown as mean ± SEM, n=6.

3.2.7 Differentiated HepaRG cells can show a circadian rhythm and endogenous NTCP protein shows a circadian pattern

To determine whether dHepaRG cells could sustain a circadian rhythm, dHepaRG cells were synchronised with dexamethasone and samples collected every six hours for genomic and protein analysis. When qPCR for BMAL1 and REV-ERBα mRNA was performed, there is a clear circadian anti-phase pattern of their mRNA levels observed which lasts 48h with the amplitude decreasing thereafter (**Figure 3.11A**). The mRNA for NTCP did not show a circadian pattern of expression (**Figure 3.11B**). However, both NTCP and BMAL1 proteins demonstrated a circadian pattern on western blotting; expression is lowest for both proteins at ZT0, ZT24 and ZT48 (**Figure 3.11C**). β-actin protein expression is comparable across the samples.

These results demonstrate that dHepaRG cells do possess an entrainable circadian rhythm, as evidenced by BMAL1 mRNA and protein expression. It illustrates NTCP protein has a circadian pattern of expression that is discordant from its mRNA. Coupled with results from (**Figure 3.9 & 3.10**), it suggests exposure to DMSO dampens the luciferase reporter signal leaving the intrinsic cellular clock intact.


A
BMAL1 & REV-ERBα
mRNA levels
200
150
100
50
0
0
12
24
36
48
60
72
Zeitgeber (Hr)


B
NTCP
mRNA levels
200
150
100
50
0
0
12
24
36
48
60
72
Zeitgeber (Hr)

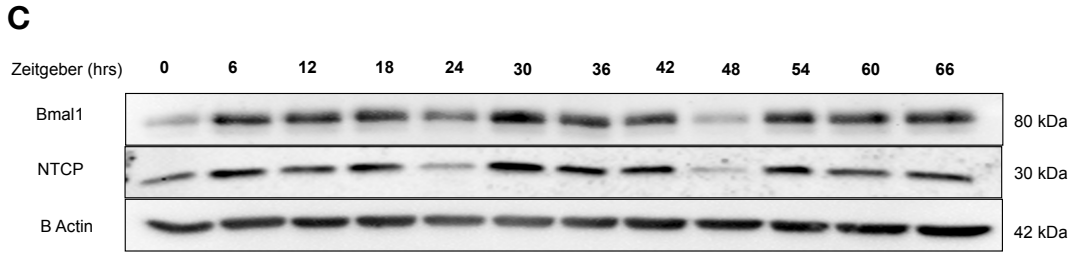
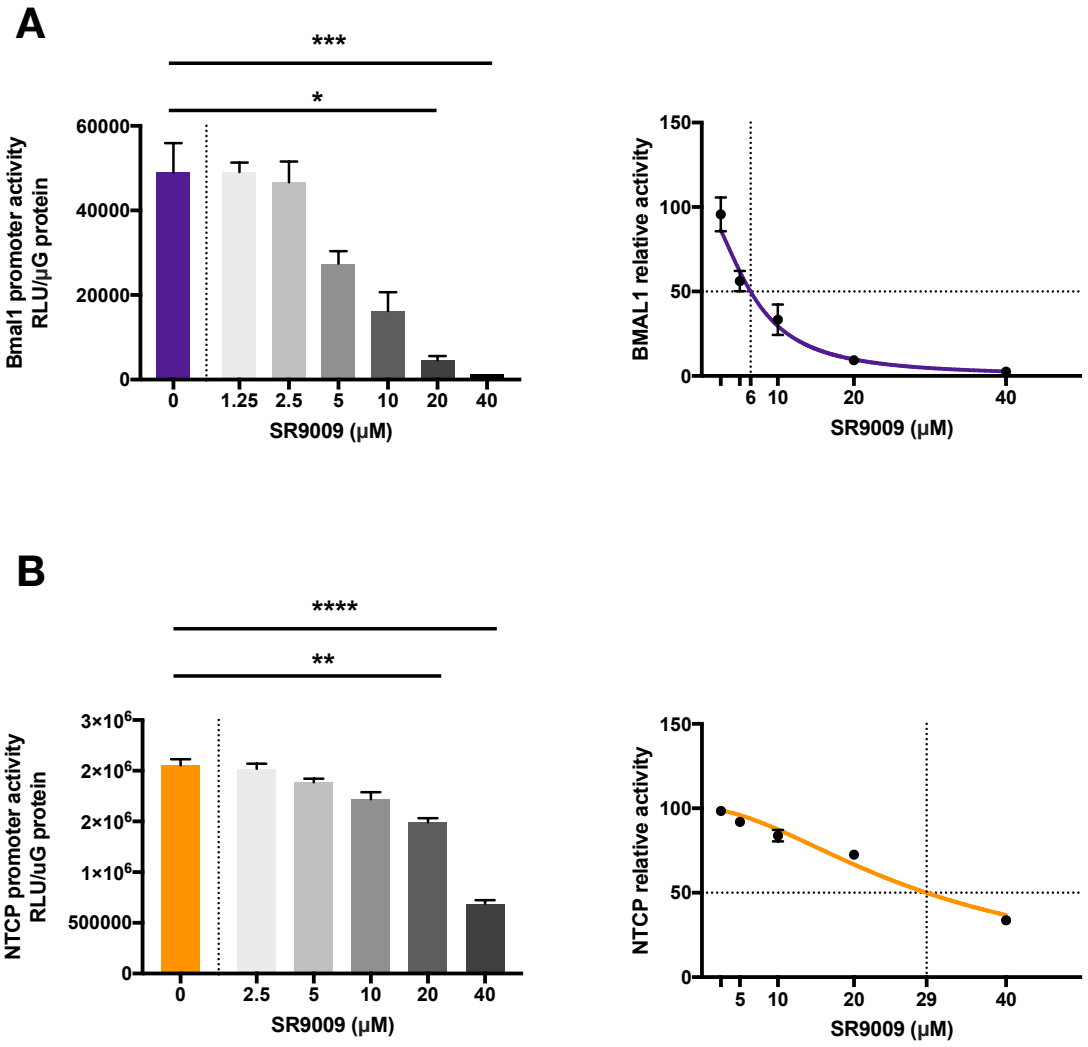

C
Zeitgeber (hrs)
0
6
12
18
24
30
36
42
48
54
60
66
Bmal1
NTCP
B Actin
80 kDa
30 kDa
42 kDa

Figure 3.11: Genomic and protein analysis confirms dHepaRG cells possess an entrainable circadian rhythm; NTCP protein shows a circadian pattern of expression. When dHepaRG cells are shocked and sampled every 6 hours for PCR and protein analysis; BMAL1 and REV-ERBα mRNA show a circadian waveform that is anti-phase to each other (A). Data shown as normalized to mean at ZT0, n=5. NTCP mRNA does not show a pattern consistent with a circadian rhythm (B). Data shown as normalized to mean at ZT0, n=5. Western blot analysis of BMAL1 and NTCP protein demonstrates a circadian rhythm with nadir protein expression seen at ZT0, ZT24 and ZT48 (C).

3.2.8 REV-ERB agonist SR9009 modulates NTCP promoter activity, mRNA and protein expression.

Previous results suggest that NTCP protein may be under circadian control following synchronization to an external ZT (**Figure 3.11C**). It would be desirable to investigate if NTCP promoter and expression could be modulated pharmacologically by circadian modulators such as the well-reported REV-ERB ligand, SR9009.

A HepaRG cell line stably expressing an NTCP promoter driven firefly luciferase reporter was generated using lentivirus transduction with antibiotic selection. When these HepaRG-NTCP-luc cells were treated with increasing doses of SR9009 for 24 hours, a decrease in promoter activity was observed (**Figure 3.12B**) with an IC50 of 29.55 µM. The previously described HepaRG BMAL1 promoter luciferase cells underwent the same treatment as a control (**Figure 3.12A**) with an IC50 of 6.43µM. When dHepaRG cells were treated with SR9009 for 24 hours and samples examined for protein and RNA, a decrease in both was noted (**Figure 3.12C**). Treatment with 40 µM SR9009 significantly decreases NTCP mRNA and effectively abolished NTCP protein expression. These results suggest NTCP is subject to modulation by REV-ERB activation by SR9009 at the promoter, mRNA and protein level.


A
***
*
Bmal1 promoter activity
RLU/µG protein
60000
40000
20000
0
0
1.25
2.5
5
10
20
40
SR9009 (µM)
BMAL1 relative activity
150
100
50
0
6
10
20
40
SR9009 (µM)
B
****
**
NTCP promoter activity
RLU/uG protein
3×10^6
2×10^6
2×10^6
1×10^6
500000
0
0
2.5
5
10
20
40
SR9009 (µM)
NTCP relative activity
150
100
50
0
5
10
20
29
40
SR9009 (µM)

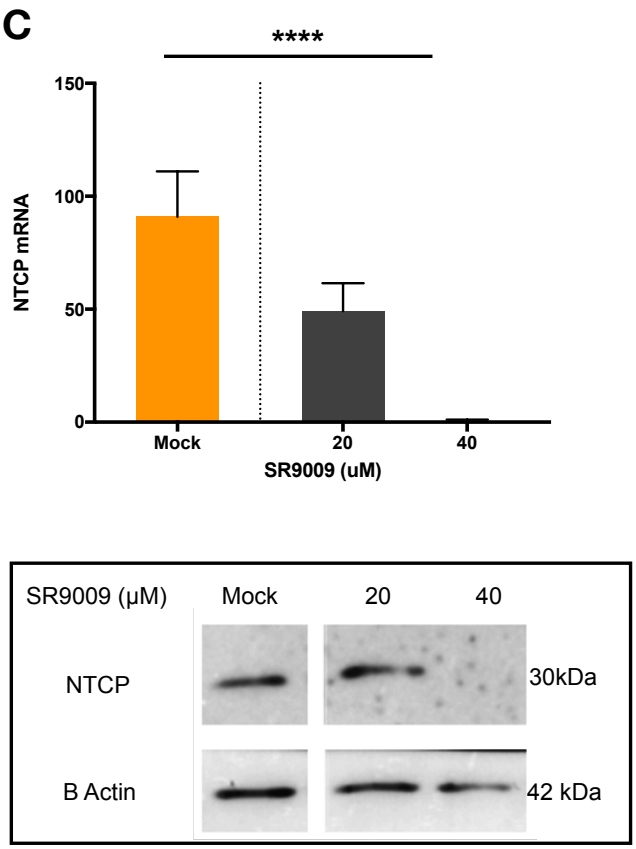


Figure 3.12: The REV-ERB agonist SR9009 modulates NTCP promoter activity, mRNA and protein expression. HepaRG BMAL1 luc cells and HepaRG NTCP luc cells were treated with incremental doses of the REV-ERB agonist SR9009 for 24 hours, before being lysed for analysis. Treatment with SR9009 decreases both BMAL1 and NTCP promoter activity with IC50s of 6.43 and 29.55 µM (A & B). Data shown as mean ± SD, n=6 per time point for both. BMAL1 luc P=0.01 and 0.003 and NTCP luc P= 0.006 and 0.0001 both using Kruskal-Wallis. When dHepaRG cells were treated with SR9009 for 24 hours and samples harvested for protein and RNA, a decrease is noted in both, with the 40 µM exposure abolishing NTCP protein expression (C). Data shown as normalized to mean of control, n=6. P=0.0001 Kruskal-Wallis. Acknowledgement to Dr Zhuang, McKeating group (C).

3.2.9 REV-ERB agonist SR9009 reduces NTCP mediated HBV entry.

In addition to the role of the transporter of bile salts into hepatocytes, NTCP is crucial for HBV entry (Yan *et al.*, 2012; Watashi *et al.*, 2014). Given a clear circadian pattern of NTCP protein expression (**Figure 3.11C**) and being modulated at multiple levels by the REV-ERB ligand SR9009 (**Figure 3.12**), attention was directed towards determining if NTCP receptor function was also subject to the same regulation. A collaboration with Dr Tsukuda of Koichi Watashi group at the RIKEN centre helped to address this.

dHepaRG cells were treated with incremental doses of SR9009 for 24 hours following incubation with radiolabeled taurocholic acid (target bile salt for transport). Compared with the control treatment, there was a dose-dependent decrease of the bile salt uptake in cells treated with SR9009 (**Figure 3.13A**). Na(-) was included as the negative control.

To determine if this decreased receptor function would result in decreased NTCP mediated HBV entry, dHepaRG cells are treated with either SR9009 (60 µM) or HBV derived preS1 peptide for 36 hours. Treated cells were then infected with HBV at 4^{o}C for 3 hours and 37^{o}C for 16 hours and lysed for intracellular HBV DNA quantification. The inoculation at a lower temperature allows viral receptor attachment only as entry will occur upon return to 37^{o}C which allows for an accurate quantification of NTCP mediated HBV entry. A significant decrease in HBV DNA is noted in the samples treated with SR9009, which is comparable in magnitude to the preS1 control (**Figure 3.13B**). These results demonstrate that activation of RER-ERB by SR9009 causes a decrease in bile salt transport and intracellular HBV DNA.

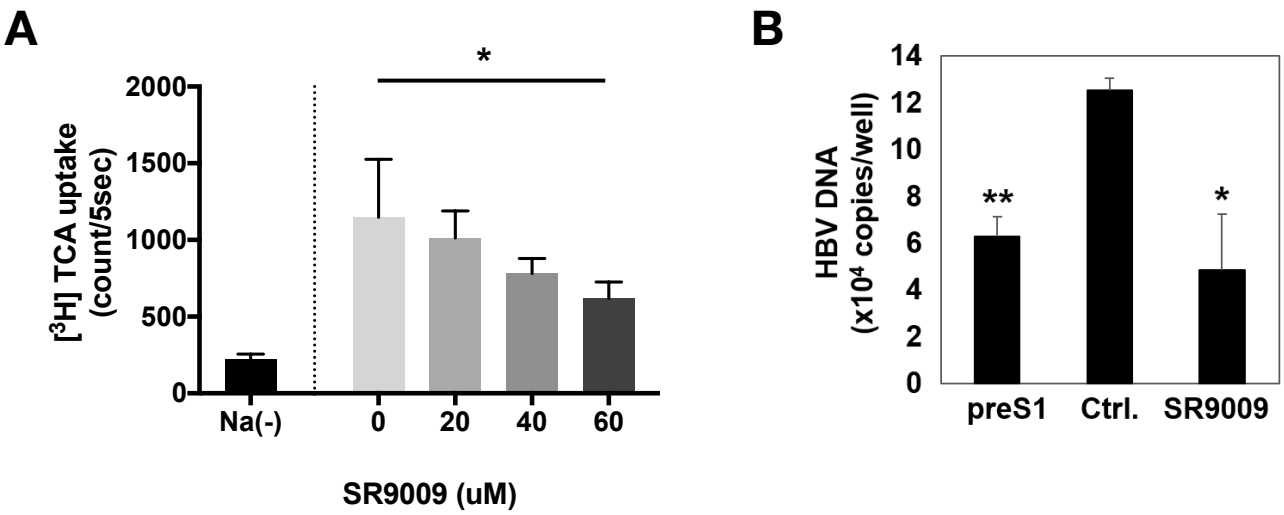


Figure 3.13: REV-ERB activation reduces NTCP mediated HBV entry. dHepaRG cells were treated with SR9009 for 24 hours before exposure to radiolabeled 3H-taurocholic acid. SR9009 reduces uptake of bile salt (A). Data shown as mean ± SD. P=0.041 using Kruskal-Wallis. Next, dHepaRG cells are pre-treated with SR9009 or preS1 before inoculation with HBV. SR9009 decreases intracellular HBV DNA. Data shown as mean ± SD. P=0.041 using one-way Anova. Acknowledgement to Senko Tsukuda of Koichi Watashi group at the RIKEN Centre, Tokyo (A&B).

3.3 Discussion

In this chapter, I have described a series of experiments that support two main observations:

1. The human bipotent progenitor cell line HepaRG can be synchronized and demonstrated a circadian rhythm with endogenous NTCP protein showing a robust circadian pattern.

2. Modulation of REV-ERB activity by its ligand SR9009 inhibited NTCP expression and NTCP mediated bile salt and HBV uptake.

The first step was the optimisation of synchronisation techniques to induce a circadian rhythm and its detection in U2OS cells (**Figure 3.1**). Both serum shock and dexamethasone treatment methods yielded successful rhythm induction of the Bmal1-luc reporter using the real-time luminescence recording machine. Having determined that dexamethasone exposure was the preferred method to synchronise cells, it gave rise to the question of whether dexamethasone per se modulates HBV activity. Clinically, it is well established that CS can reactivate or induce an HBV disease flare and patients with HBV that require CS treatment are often prophylactically administered suppressive antivirals. This reported in numerous case series and accounted for in HBV management guidelines (Perrillo, 2001; Cheng *et al.*, 2003; Chang and Liaw, 2014; European Association For The Study Of The Liver, 2017a; Wong *et al.*, 2019b). This reactivation of HBV disease activity is not limited to the CS alone but common to all immunosuppressant agents. Amongst this group, the B-cell depleting agents are the most likely to induce an HBV flare (Manzano-Alonso and Castellano-Tortajada, 2011). This suggests a modulated host response that is not unique to CS exposure, rather than a direct pro-viral mechanism of CS (Vento, Cainelli and Longhi, 2002). There is a gap in our knowledge when it comes to the molecular basis of CS enhanced HBV replication. There has been little interim progress made since early *in vitro* studies -that identified the presence of a GRE in the HBV genome- into the precise mechanism

driving steroid enhanced HBV replication (Tur-Kaspa *et al.*, 1986, 1988). The observations that this early work reports, have not been replicated with more contemporary molecular techniques and highlights an emergent knowledge gap in the mechanics of GRE functioning. Whilst some *in vitro* models show enhancement of HBV viral parameters in response to CS, the results are not all concordant which in turn makes reaching an overall consensus difficult. Furthermore, these studies differed in the type of CS used, their concentration and duration of exposure in addition to multiple different cell line use. The main differences observed were:

(i) Hep3B cells treated with 100nM dexamethasone for 48hrs showed <5 fold increase in HBsAg and HBeAg (Chou *et al.*, 1992).

(ii) HepG2.2.15 cells treated with methylprednisolone showed no increase in cccDNA formation (Shen *et al.*, 2012).

(iii) Hep3B cells treated with 500nM of dexamethasone for 30 minutes show <5 fold induction in relative HBV core and X promoter activity (Jhuang *et al.*, 2015).

(iv) HepG2.2.15 cells showed a biphasic response in HBsAg production to dexamethasone between 0-200nM over an 8-48 hr period. The maximal observed induction was <4 fold at 36hrs (He *et al.*, 2018).

Of note, the duration of exposure to CS in those studies is all in excess of the two hours for circadian synchronisation used here. Additional preliminary experiments were undertaken which demonstrated dexamethasone had a negligible effect on HBV promoter activity (**Figure 3.2**) or transcription of pgRNA, HBeAg and HBsAg production in the Hepg2.2.15 cell line (**Figure 3.3**). Consequently, the use of CS to synchronise cells was unlikely to modulate any HBV component of proposed circadian work.

Using real-time luminescence as a screening method, two cell lines: HepG2 and HepaRG which are permissive to HBV infection were investigated for their ability to demonstrate an entrainable circadian rhythm. The HepG2-NTCP cell line did not demonstrate a rhythm (**Figure 3.4**). Conversely, the undifferentiated HepaRG cell line illustrated a robust circadian pattern that showed dose-dependent attenuation to SR9009 (**Figure 3.5**). This was a pivotal result as a circadian entrainable hepatic cell line that could serve as a basis for HBV infection studies had been identified.

As the differentiation protocol for the HepaRG cells takes one month and it was unknown what effect the differentiation may have on the circadian rhythm of the cells; two DMSO-free models of infection were pursued. This was correctly predicted to be a challenging yet desirable line of investigation. First, an adenoviral-based delivery vector that would bypass conventional NTCP receptor-mediated entry to otherwise commence a normal HBV replication cycle was undertaken (He *et al.*, 1998; Sprinzl *et al.*, 2001) (**Figure 3.6A**). The main advantages of this system were a short inoculation window of two hours and efficient HBV genome delivery not reliant on the NTCP receptor. Expectedly -due to the adenovirus delivery vector- the AdHBV was able to enter undifferentiated HepaRG successfully as illustrated by GFP visualisation on fluorescence microscopy (**Figure 3.7A**). However, a sustained HBV infection -illustrated

by measurement of encapsidated HBV virions- could not be established in undifferentiated HepaRG cells (Figure 3.7B).

Secondly, infection with a recombinant HBV that had a gaussian luciferase reporter transgene inserted in its polymerase was attempted -rHBVGLuc- (**Figure 3.8A**). This recombinant virus allows for a short inoculation of three hours and a particularly sensitive, real-time analysis of a single step infection. Upon analysis, there was a negligible signal observed in the

undifferentiated HepaRG cells while the dHepG2 NTCP cells demonstrated far higher RLU values consistent with a successful infection (**Figure 3.8B**). Together these results suggest even when caveats to standard *de novo* HBV infection are circumvented; whether its viral entry by using the AdHBV or sensitive detection of HBV transcription by using rHBVGLuc: undifferentiated HepaRG cells are not a permissive cell line. These results made an exploration of the effect DMSO differentiation would have on the circadian rhythm of HepaRG cells, all the more pertinent.

Using real-time luminescence methodology, when DMSO is introduced to synchronised undifferentiated HepaRG; the raw RLU values decreased to a lower baseline than the mock. Closer inspection of the pattern of expression in the DMSO treated cohort suggested it may represent a muted circadian profile (**Figure 3.9A**). Initially, it was unclear whether the cells stopped cycling or DMSO was interfering technically with the real-time luminescence signal. This would be addressed by the second component of the experiment whereby DMSO is removed from dHepaRG cells after synchronisation and there is a reappearance of a circadian waveform from ZT24 onwards (**Figure 3.9B**). Of interest is the remarkable consistency of the circadian pattern between them; the first peak lies just before ZT36 in both. Mindful that hepatocytes *in vivo* are differentiated (Fausto, 1990), these results suggest it is a technical issue with DMSO itself interfering with real-time luminescence measurements. This issue has been observed previously in our laboratory, whereupon the HBV core promoter firefly luciferase reporter once transfected into HepG2 cells and exposed to DMSO shows an attenuated signal (**Figure 3.10**). DMSO interference on other luciferase promoter reporters like Zika has been described by (Zou *et al.*, 2011).

To circumvent this technical limitation of the real-time luminescence; samples for protein and mRNA analysis after synchronisation were investigated. The PCR data for the crucial circadian

transcription factors BMAL1 and REV-ERVα clearly demonstrated a circadian pattern of expression that were anti-phase to each other (**Figure 3.11A**). Despite attempts, a reliable western blotting protocol for endogenous REV-ERBα protein was not possible. Western blotting for BMAL1 and NTCP protein suggests a circadian pattern of expression in both (**Figure 3.11C**). This confirms that dHepaRG cells are capable of sustaining a circadian rhythm. Of interest is the discordance in patterns for NTCP between the mRNA and protein expression. I hypothesise this disparity may be due to a varying location of the NTCP protein within the cell, a long half-life of NTCP RNA or post-translational modification (Ueda *et al.*, 2002; Molina *et al.*, 2008; Watashi *et al.*, 2014). When Nascent-seq technology was used to compare hepatic RNA production to RNA expression in the murine liver, it was found about 70% of the genes that exhibit rhythmic mRNA expression do not have comparable transcriptional rhythms (Menet *et al.*, 2012). This illustrates the significant role post-transcriptional splicing and translation likely plays.

The use of SR9009 as a proxy for REV-ERB activation has both advantages and limitations as an experimental technique. The advantages are a reliable potent and accurate dose dependent induction of REV-ERB activity. The disadvantage is that SR9009 likely has numerous off-target side effects. Overexpression experiments using transfection of BMAL1 and REV-ERB plasmids to overexpress protein would be an alternative option -as used in Chapter 5- but this methodology is not without caveats (transfection efficiency and uncontrolled dynamics of protein induction/decline). Preliminary experimentation whereby BMAL and REV-ERBα were overexpressed following transfection in HepRG cells expressing an NTCP promoter luciferase showed no modulation of NTCP promoter activity (data not shown). In conclusion, pharmacological activation of REV-ERB via SR9009 was chosen as the experimental method.

It is increasingly accepted that HBV is a metabolic virus that has adapted to the enterohepatic circulation of bile salts. Bile salts are secreted by the liver into the duodenum to facilitate absorption of dietary fat and oils by serving as micelle forming surfactants. Bile salts are efficiently reabsorbed (>95%) in the distal part of the small intestine (via the apical bile salt transporter receptor (ASBT)) and reabsorbed into the hepatic portal circulation (via the NTCP receptor) (Hofmann, 2009; Dawson and Karpen, 2015). Together, with two other dedicated bile salt transporters (bile salt export pump (BSEP) and organic solute transporter (OSTα/β)), this circular movement of bile salts is termed the enterohepatic circulation, reviewed by (Meier and Stieger, 2002). The net effect is that each bile salt molecule is used up to 20 times. Secretion is rhythmic, peaking following meals, therefore there is a natural link with metabolism and circadian influence. Additionally, bile salts are recognised ligands for nuclear receptors that exert control of a myriad of processes intrinsically related to metabolism as well as an evolving appreciation of their contribution to the gut microbiome and immunity (Schubert *et al.*, 2017). There is an emerging evidence base that dysfunction of these transporters may interfere with the digestive and signalling functions of bile salts which may contribute to cholestasis, steatohepatitis, hypercholesteremia and even the development of hepatocellular carcinoma (Kullak-Ublick, Stieger and Meier, 2004; Knisely *et al.*, 2006; Alrefai and Gill, 2007). It is possible that pharmacological modulation of NTCP for antiviral intent may carry similar unintentional side effects by disrupting bile salt transport.

NTCP is crucial for effective HBV replication. Patients with the S267F variant of the NTCP receptor were shown to be at lower risk of developing chronic HBV infection and cirrhosis (Lee *et al.*, 2017). This suggests that decreased NTCP mediated HBV entry could contribute to a lower burden of disease with better clinical outcomes. Undoubtedly cell-cell infection through the passing of cccDNA to newly formed hepatocytes is an alternative route of hepatocyte infection but *de novo* entry through NTCP receptor remains integral to chronic infection. The dynamic interplay of NTCP expression due to host metabolism and HBV disease stage remains ill-defined,

although the association of metabolic syndrome with poorer outcomes in patients with HBV infection is well established (Wong *et al.*, 2009; Hsiang *et al.*, 2014; Wang, Tseng and Kao, 2015; Kim *et al.*, 2018). It remains unknown if differing HBV disease activity stages modulate bile salt transport, but it is worth noting that disease flares due to enhanced HBV replication normally constitute a different pattern of liver enzyme derangement (a hepatitis rather than cholestasis pattern) (European Association For The Study Of The Liver, 2017a). To what extent HBV infection modulates NTCP expression predisposing the host to metabolic insult or host dietary habits modulates NTCP expression facilitating an enhanced viral burden, or a combination of both is unknown. Efforts undertaken to attribute proportional causation have found it extremely difficult to adequately control for confounders in the clinical setting to yield a consensus opinion (Hsu *et al.*, 2012; Wong *et al.*, 2012; Jarčuška *et al.*, 2014; Li *et al.*, 2015; Wang, Tseng and Kao, 2015). It is interesting to hypothesise that NTCP may be under circadian regulation with a potential for antiviral activity following its modulation.

Further investigation showed an inhibition of promoter activity, RNA transcripts and protein expression in response to the REV-ERB agonist SR9009 (**Figure 3.12**). In addition, there was a functional modulation where SR9009 decreased bile acid transport and NTCP mediated HBV entry into the cell (**Figure 3.13**). This illustrates that a key facet of the HBV lifecycle, viral entry, may represent a druggable target that could be open to modulation by circadian agents.

3.4 Summary

This chapter describes how the HBV permissive HepaRG cell line is capable of sustaining an entrainable circadian rhythm. It highlights that DMSO is detrimental to measurement of this

rhythm by real time luminescence monitoring. Two DMSO free models of HBV infection are shown not to represent viable options in which to study circadian rhythm. NTCP protein, crucial for HBV replication is modulated by REV-ERB activation by SR9009.

Chapter 4: Circadian regulation of autophagy in the HBV life cycle.

4.1 Introduction.

The large network of cellular processes under circadian regulation makes it largely impossible to infer strictly individual territorial consequences due to its modulation. Suffice to say, to attribute proposed circadian modulation of HBV replication as solely due to clock regulation, fails to acknowledge the myriad of contributing intermediate processes co-regulated by clock. Therefore, it is imperative to investigate such intervening processes, as they represent an opportunity to gain not only a broader understanding of viral replication mechanics, but the chance to narrow molecular targets of interest in the pursuit of novel therapeutics. Autophagy represents one of these intermediate processes regulated by clock that is essential to HBV replication.

HBV has been reported to modulate the early autophagic process and to increase the number of AP but not to alter their rate of degradation or autophagic flux (Sir *et al.*, 2010; Alavian *et al.*, 2011; Li *et al.*, 2011). This perturbation in autophagy has been reported in a range of HBV infection models from cell culture to clinical tissue samples expression (Sir *et al.*, 2010). Murine knockout models of the autophagy pathway showed reduced HBV replication as assessed by HBV DNA and antigen (HBsAg and HBeAg) expression (Tian *et al.*, 2011). It is noteworthy that all of these models -except for *in vitro* cell culture - would have had a synchronised clock.

The autophagic process is recognised to be circadian regulated with a reported role for C/EBP β and REV-ERBα (Sachdeva and Thompson, 2008; Woldt *et al.*, 2013). Pharmacological activation of REV-ERB with SR9009 has been shown to inhibit components of the autophagy at the transcriptional level in breast cancer cell lines (Sulli *et al.*, 2018). AP numbers in peripheral organ tissue and LC3 expression have been reported to exhibit an intrinsic rhythmic pattern,

illustrating a circadian influence (Ma, Panda and Jiandie D. Lin, 2011). In clock deficient murine models, rhythmic expression of C/EBP β was diminished, although the basal autophagy machinery remained intact. However, shRNA silencing of C/EBP β reduced the basal expression of autophagy markers with a consequent breakdown in the autophagic degradative process (Ma, Panda and Jiandie D. Lin, 2011). These studies suggest that although circadian regulation can enhance the autophagic process, it is not a requisite to maintain basal cellular functions. These studies highlight a role for the mammalian clock as a 'conductor' enhancing the performance of various cellular processes rather than unconditional regulation. I hypothesise that a proportion of the overarching circadian modulation of HBV could be attributed to intermediate clock regulation of autophagy.

Autophagy can be quantified by imaging APs using a dual fluorophore LC3 and western blotting for key proteins in the pathway. Enumerating fluorescent AP in an automated manner with a software program called CellProfiler (Jones *et al.*, 2008) was a desirable goal. This provides many advantages over the manual counting of puncta that is subjective and prone to operator error. The manual method is a particularly laborious and time-consuming task when one considers that the puncta from each image require counting in two excitation phases and multiple images to be collected. It is recommended that operators maintain a consistent technique when faced with varying size puncta and clumps of fluorescence. Standard operating procedures for this technique are outlined (Klionsky *et al.*, 2016).

Since the absolute numbers of puncta can vary per cell, we calculate an AP: AL ratio to represent the maturation of AP fusing with lysosomes to generate AL. A high AP: AL ratio suggests a block in autophagy. However, one should note that this ratio does not assess for completion of the pathway termed: autophagic flux which is essential for degradation to occur. To estimate autophagic flux, additional western blotting for LC3-I, LC3-II and p62 are required.

Conjugation of cytosolic LC3-I with phosphatidylethanolamine (PE) to AP membrane bound LC3-II represents the initiation of the autophagic process. Thus, an increase in LC3-II is consistent with an increase in the number of APs which can be due to one of the following scenarios:

(i) Enhanced AP formation (associated with an increase in degradation due completion of the autophagic process)

(ii) Accumulation of APs due to a blockage in AP fusing with lysosomes (with no change in degradation).

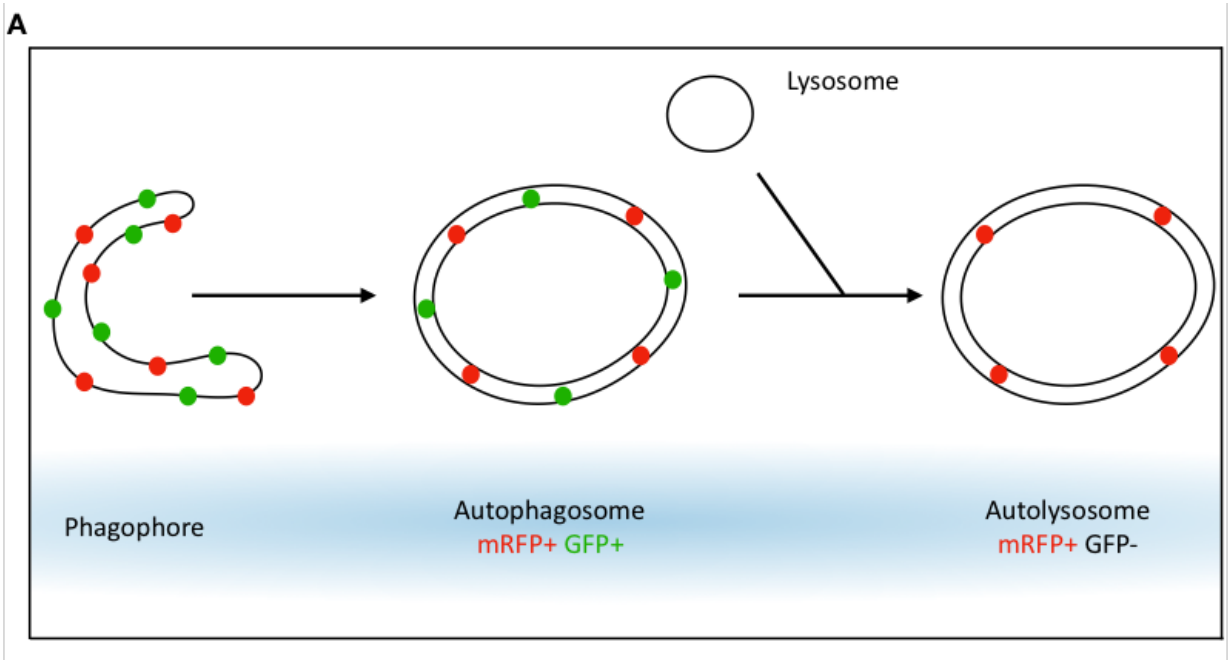
Quantifying p62 provides an indirect marker of AP degradation, whereby its accumulation and reduction represent decreased and increased autophagic flux, respectively. Western blotting for p62 is performed both in the presence and absence of bafilomycin. Bafilomycin inhibits vacuolar ATPase and prevents acidification of lysosomes and their fusion with APs. Broadly speaking, an increase in LC3-II in the presence of bafilomycin relative to its absence suggests an enhanced autophagic pathway. Guidelines for interpretation of LC3 immunoblotting are provided by (Mizushima and Yoshimori, 2007; Klionsky *et al.*, 2016).

In this chapter, the use of a dual fluorescent LC3 containing monomeric red fluorescent protein (mRFP) with an acid labile enhanced green fluorescent protein (EGFP) AP maturation assay is described. It is used to create two LC3 reporter hepatoma cell lines; one of which stably expresses HBV to investigate the role of circadian component REV-ERB to regulate autophagy pathway in human liver derived cell lines.

4.2 Results:

4.2.1 Use of the fluorescent mRFP-EGFP LC3 reporter.

The mRFP-EGFP LC3 fluorescent reporter has been widely used as a measure of AP formation, maturation and fusion to generate AL (Klionsky *et al.*, 2016). It is a dual tagged fluorescent reporter that contains an acid labile EGFP. At the crux of its use is the observation that both fluorophores can be visualised in the AP but after fusing with a lysosome, the EGFP is destroyed leaving only the mRFP expression in the newly formed AL (**Figure 4.1A**). Therefore, caution is required when undertaking visualisation using fluorescence microscopy to estimate AP maturation as both EGFP and RFP excitation phases must be used in tandem. Differentiation between AP and AL is achieved by using the EGFP excitation wavelength: where only AP can be visualised (**Figure 4.1B**). To quantify AP: AL ratio, red and green puncta are independently counted. The number of red puncta (both AP and AL) minus the green puncta (AP) equals the number of AL (**Figure 4.2**). It is impossible to distinguish by mRFP visualisation alone which puncta are AP and which are AL. Subsequent merging of both EGFP and mRFP images, the AP appear yellow whilst AL appear red.



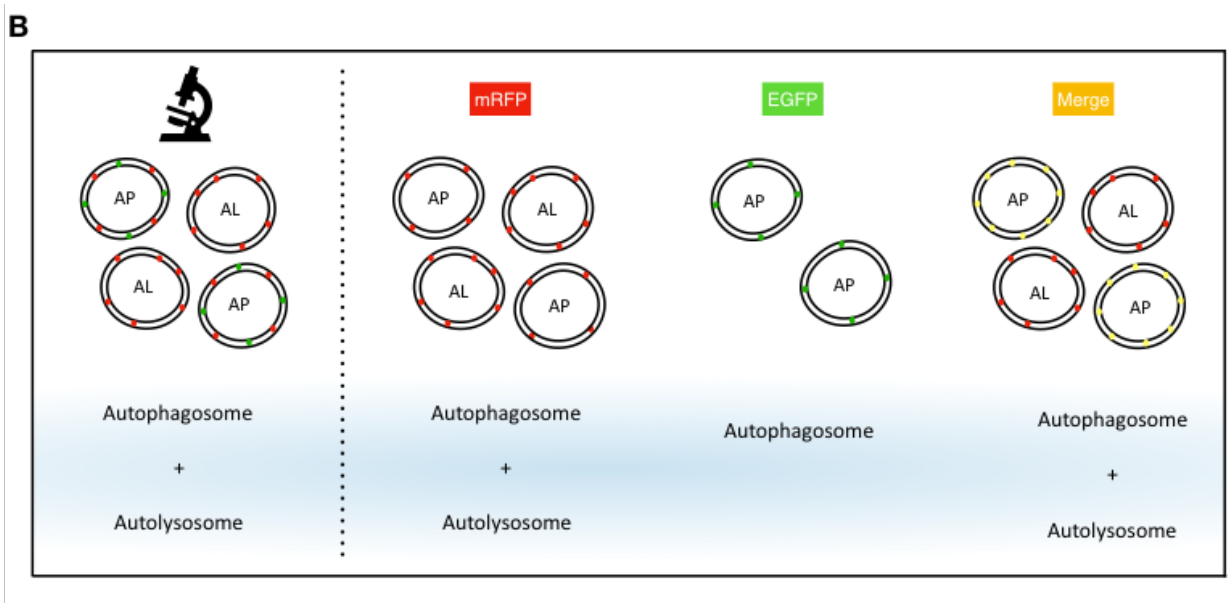


Figure 4.1: The dual fluorophore mRFP-EGFP LC3 reporter can distinguish between autophagosomes and autolysosomes. Autophagosomes express both mRFP and GFP until fusing with a lysosome to form an autolysosome thus quenching the acid labile GFP (A). Upon merging of mRFP and EGFP images, AP appear yellow due to co-localization while AL remain red (B).

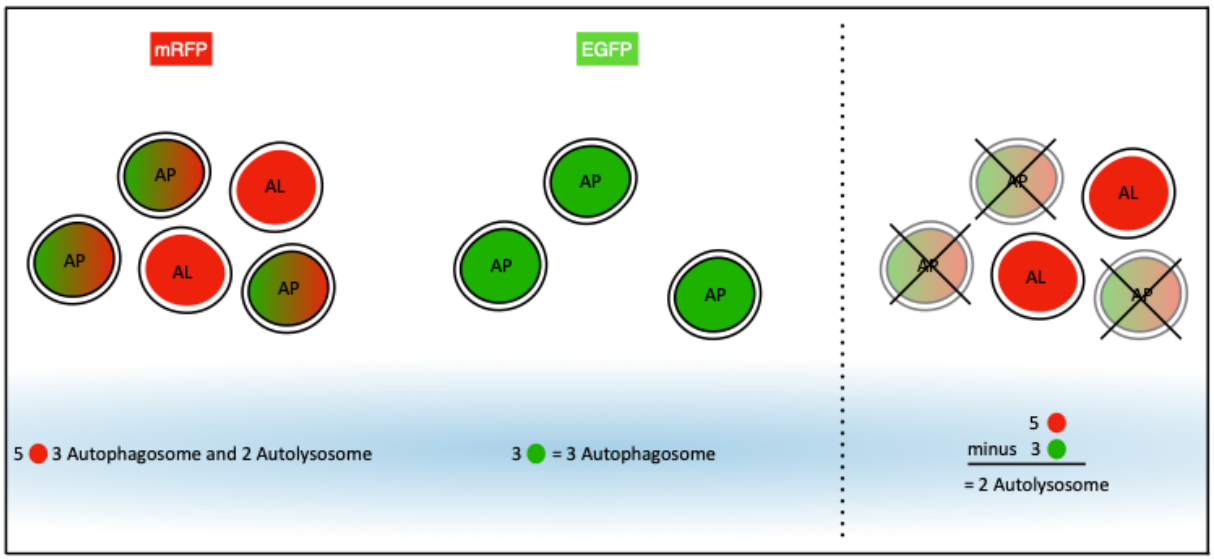


Figure 4.2: Calculation of autophagosome and autolysosome numbers. The number of autophagosomes can be determined by counting the EGFP puncta. The number of autolysosomes can be determined by subtracting the number of EGFP puncta from mRFP puncta.

Imaging parental HepG2 cells along with the newly created HepG2 LC3 reporter line enables one to visualise discrete fluorescent puncta in the reporter line at rest (**Figure 4.3**). The reporter line was created to stably express the fluorescent LC3 by lentivirus transduction that also contained a puromycin resistance gene. These cells were maintained in 10% FBS DMEM supplemented with puromycin, thus the images are representative of basal autophagy. The use of lentiviral transduction with a puromycin resistance gene resulted in a near 100% expression rate of LC3. A high rate of fluorescent LC3 protein expression was essential to enumerate puncta, independent of employing the manual or automated technique. High magnification (x100) images enabled clear visualisation of the cytoplasmic puncta. It can be appreciated that there are more puncta present under mRFP excitation than EGFP indicating the acid labile nature of the EGFP. Merging of the images demonstrates co-localisation of signals in APs, whilst red puncta indicate AL. In addition to a HepG2 LC3 reporter line, I generated a HepAD38 LC3 reporter line using the same methodology. HepAD38 line is derived from HepG2 cells transfected with a single copy of HBV DNA strain *ayw* under a tetracycline-inducible reporter (Ladner *et al.*, 1997). Since its creation, the tetracycline responsiveness has been lost with HBV production occurring constitutively. HepAD38 cells produce infectious virus and are widely used as the source of virus for both *in vitro* and *in vivo* studies.

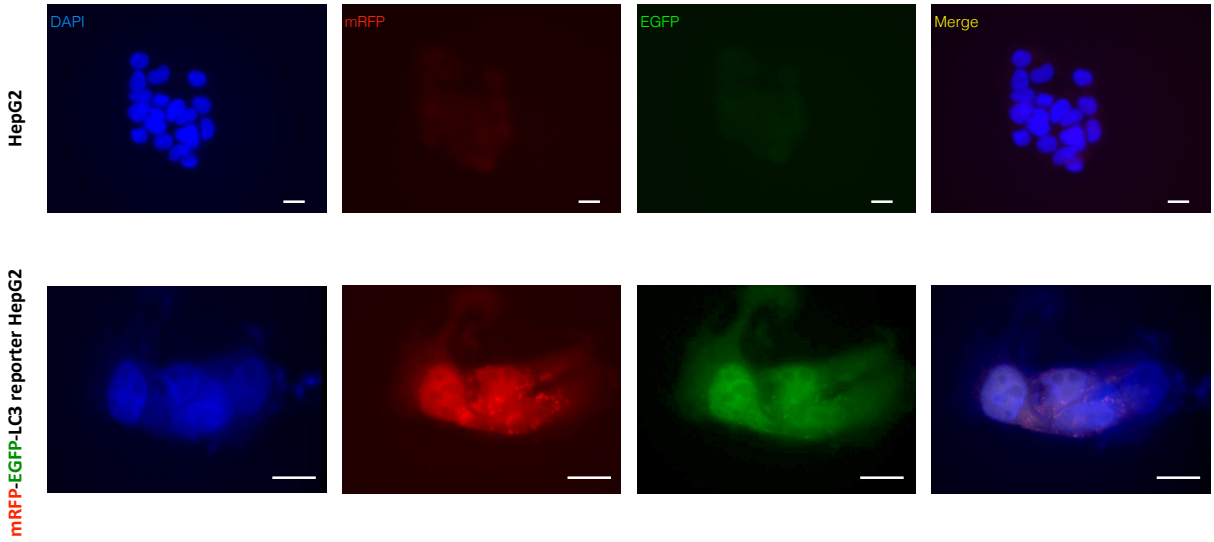


Figure 4.3: Creation of a HepG2 mRFP-EGFP LC3 reporter line. HepG2 cells were transduced to stably express the dual fluorophore LC3 and parental cells are shown for comparison. There are puncta present under mRFP that are not present under EGFP excitation, demonstrating the acid labile quenching of the EGFP fluorophore. Upon merging the images, yellow puncta suggestive of autophagosomes are apparent, whilst those that remain red indicate autolysosomes. Presence of co-localisation was determined manually. Images acquired on a Leica DM6000B fluorescence microscope. Scale bars: 10 µm.

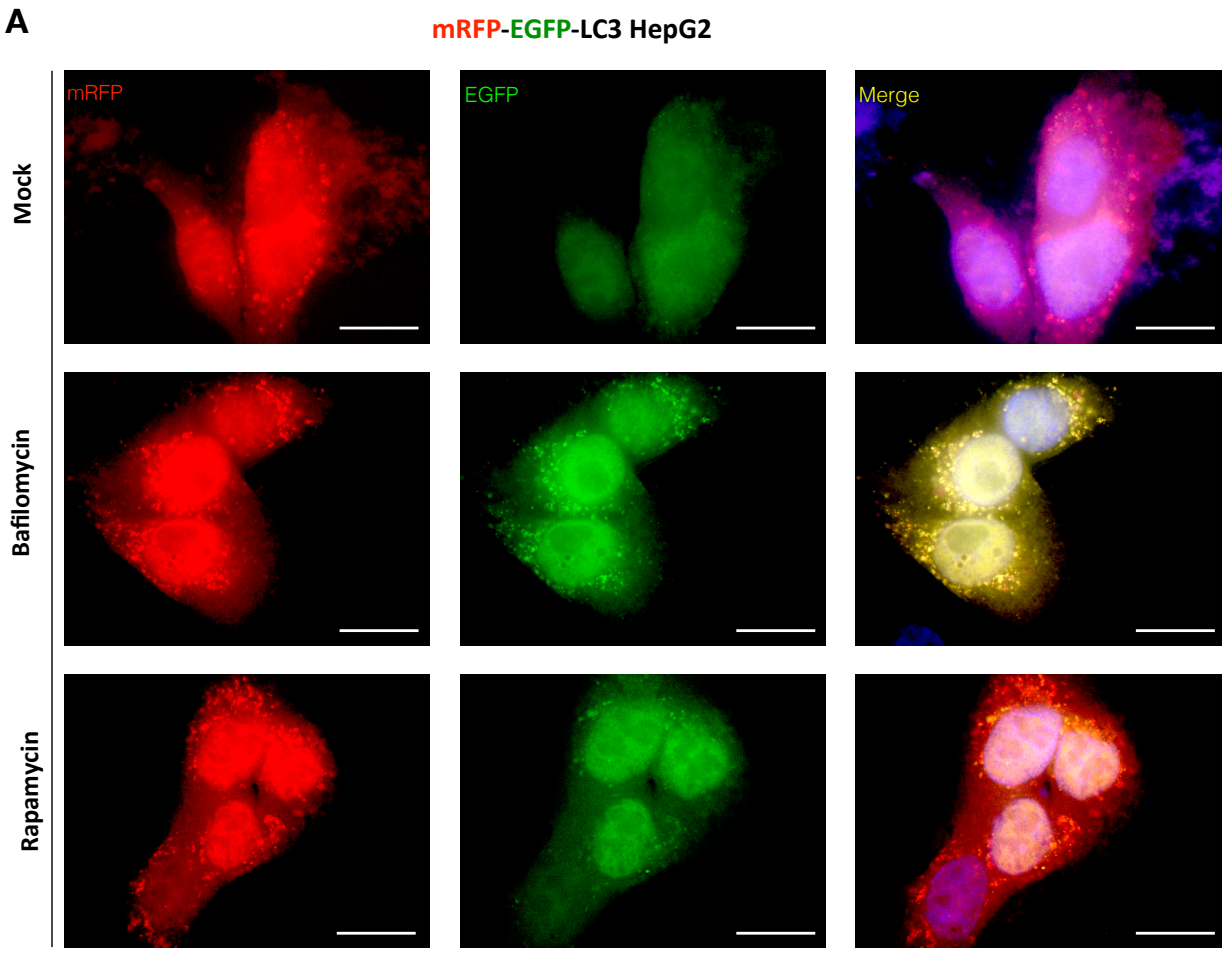
4.2.2 Establishing Cell Profiler to quantify AP: AL ratio

All reported AP: AL ratios are derived from a minimum of 30 cell counts taken over 20 images (**Figure 4.2**). In an attempt to make the counting of puncta objective and faster, an automated software called Cell Profiler was used (Jones *et al.*, 2008). Its calibration and validation took place in tandem to the characterisation of the two newly created LC3 reporter cell lines. Details of the individual calibration software pipelines used are listed in Chapter 2. The resultant AP: AL ratio derived from each method is presented for each reporter cell line.

4.2.3 Characterising the HepG2 mRFP-EGFP LC3 reporter line

A HepG2 line stably expressing the dual mRFP-EGFP LC3 protein was created by lentivirus transduction. Study population lines were used to avoid any inherent specific clonal effects. To characterise the cell line, it was exposed to a known repressor (bafilomycin) and stimulator (rapamycin) of autophagy. Bafilomycin inhibits the vacuolar ATPase and prevents lysosome acidification and rapamycin inhibits the protein kinase in the mTOR complex. Cells were exposed to bafilomycin (400nM) for four hours and rapamycin (400nM) for six hours before fixing onto coverslips using 4% paraformaldehyde. Imaging was undertaken on a Leica DM6000 immunofluorescence microscope. Visual inspection of the images showed that bafilomycin treated cells showed increased yellow puncta that are indicative of increased AP formation; whilst the rapamycin treated cells showed mainly red puncta consistent with increased AL formation (**Figure 4.4A**). In calculating the AP: AL ratio, the results from manual examination are shown (**Figure 4.4B**). As expected bafilomycin inhibits AP lysosome fusion resulting in an increased AP: AL ratio, whilst rapamycin decreased the ratio. An estimate of basal autophagy is illustrated by the mock treated cells. Cell Profiler analysed AP: AL ratios show a comparable

pattern (**Figure 4.4C**), with an acceptable <10% discordance between the methods. Interestingly estimates of basal autophagy showed less than 5% discordance. These data highlight the differing qualitative features of puncta -size and clumping due to aggregates- at the extremes of flux. To demonstrate a competent autophagic process in these cells, western blotting showed an increase in p62 and LC3-II in the bafilomycin treated cells (**Figure 4.4D**). Together these results demonstrate that the HepG2 mRFP EGFP LC3 reporter line responds to known autophagy modulators and CellProfiler provides a viable option to quantify AP maturation. Having established this benchmark, the next step would be how HBV would affect these baseline autophagy characteristics.


A
mRFP-EGFP-LC3 HepG2
mRFP
EGFP
Merge
Mock
Bafilomycin
Rapamycin

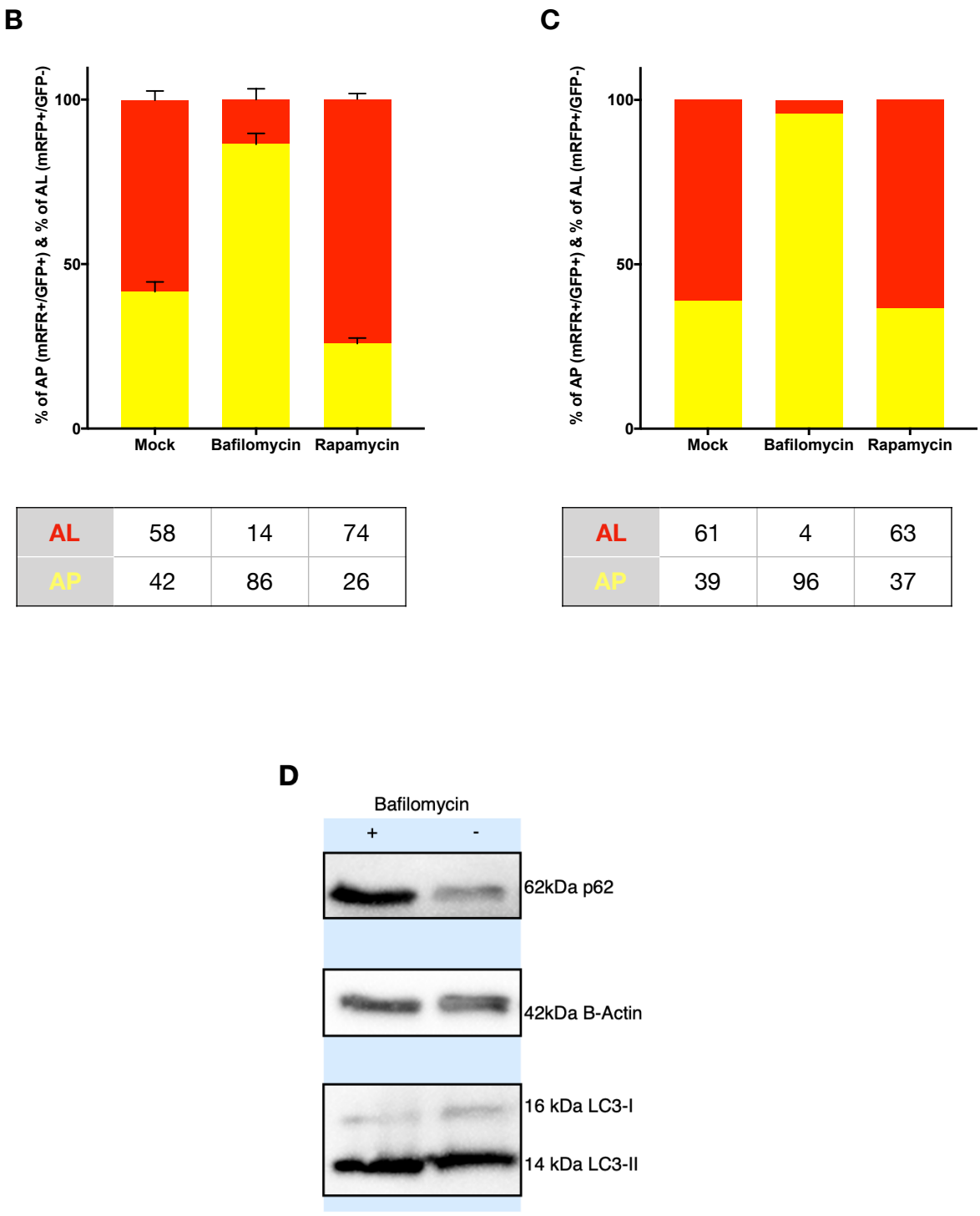
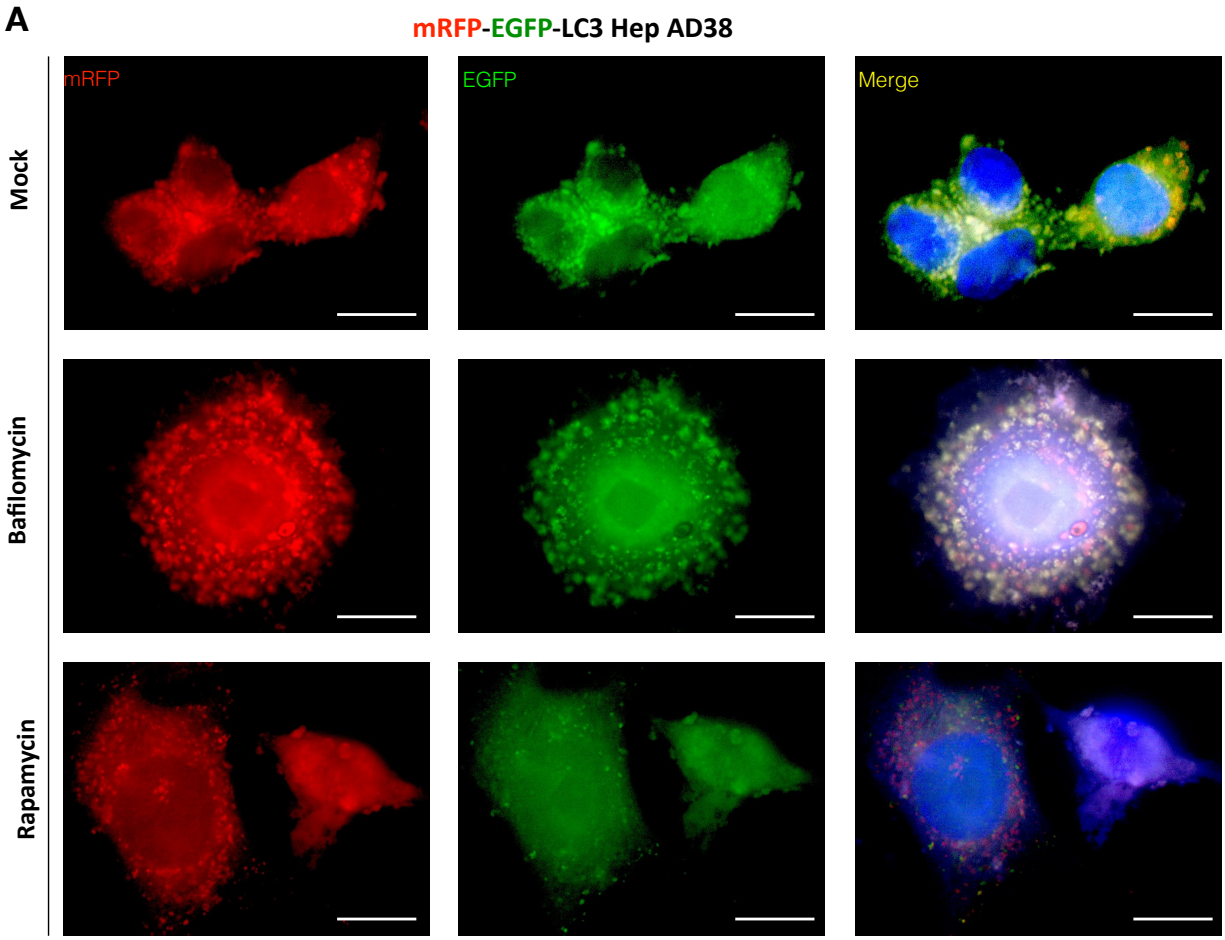
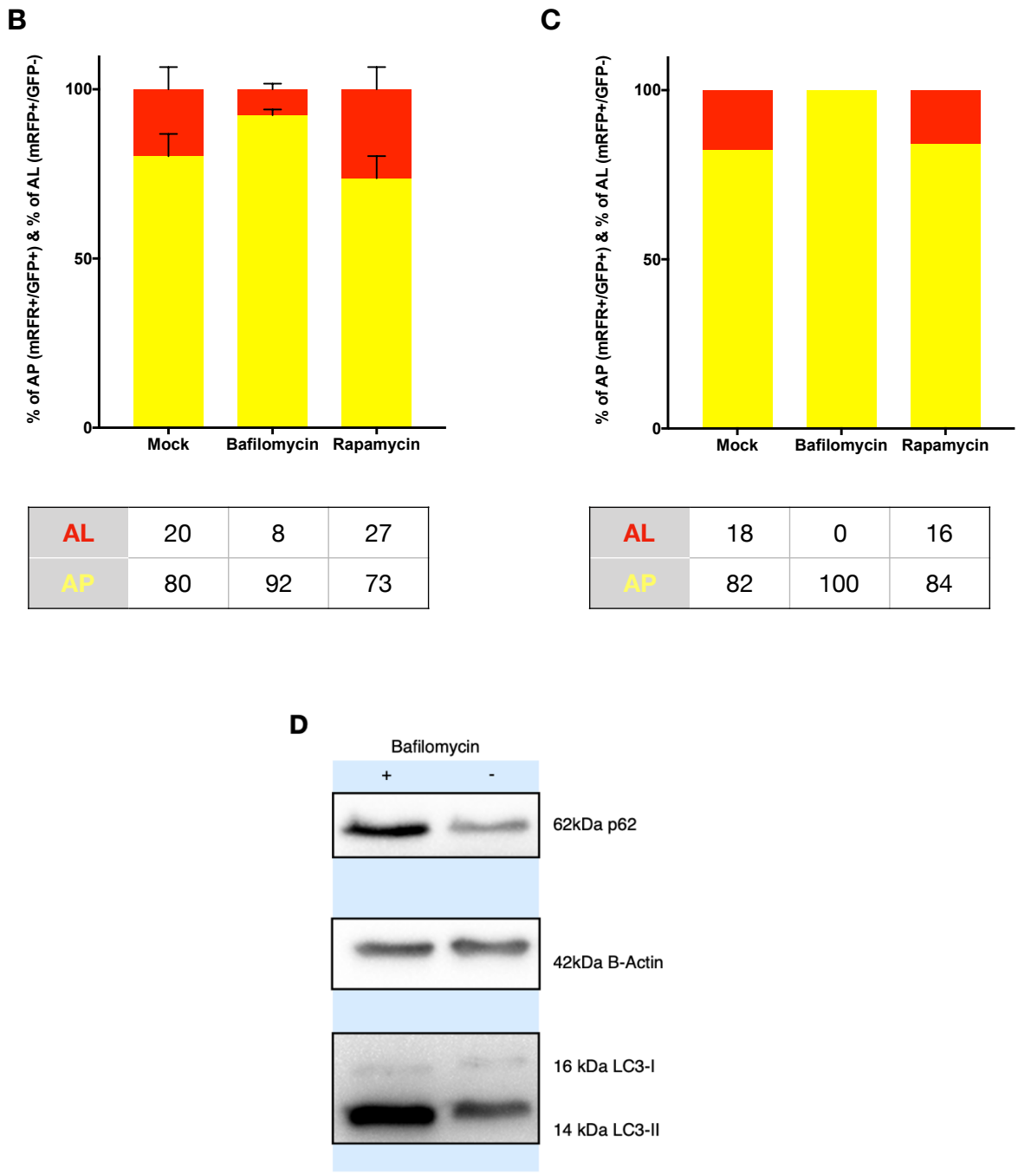


Figure 4.4: Characterizing the HepG2 mRFP-EGFP LC3 reporter line. HepG2 cells stably expressing a dual mRFP-EGFP LC3 fluorescent protein were created by lentiviral transduction. Cells were treated with bafilomycin or rapamycin before fixing in paraformaldehyde and imaging (A). Puncta are counted and expressed as a percentage per image. Manual counting of puncta (B) and automated by Cell Profiler (C) show <10% discordance. Data shown as a single AP:AL ratio expressed as a cumulative percentage ± SEM, n=30. Protein analysis using bafilomycin treated cells show a competent autophagy pathway with expected response of increased p62 and LC3-II. Images acquired on a Leica DM6000B fluorescence microscope. Scale bars: 10 µm. Western blot images representative of 3 experimental replicates.

4.2.4 Characterising the HepAD38 mRFP-EGFP LC3 reporter line

To investigate the effect of HBV on the autophagic process, I generated an HBV producer HepAD38 line that stably expresses the mRFP-EGFP LC3 by lentivirus transduction. Similar to the previous series of characterisation experiments, the cell line was maintained in DMEM media with puromycin and was treated with autophagy modulators before fixing and enumerating the AP: AL ratio. Visual inspection of the merged images shows an accumulation of yellow AP puncta (**Figure 4.5A**). This was seen across all treatments, even in the presence of rapamycin. The AP: AL ratio derived from the manual counting is shown (**Figure 4.5B**). As expected bafilomycin treatment inhibits AP lysosome fusion resulting in increased AP. Strikingly however, in the mock and rapamycin treated cells there remains a significant increase in the AP: AL ratio. Comparable data were obtained using the CellProfiler software (**Figure 4.5C**), showing >90% concordance rate between methods. These data are consistent with an inhibition of autophagy in HepAD38 cells and increase in APs that is resistant to rapamycin. Western blotting shows an increase in p62 and LC3-II in the bafilomycin treated cells (**Figure 4.5D**). Together these results demonstrate the HepAD38 mRFP-EGFP LC3 reporter cell line has increased AP formation that is not associated with a degradation block. I hypothesise this increase in AP is due to the presence of HBV.


A
mRFP-EGFP-LC3 Hep AD38
mRFP
EGFP
Merge
Mock
Bafilomycin
Rapamycin



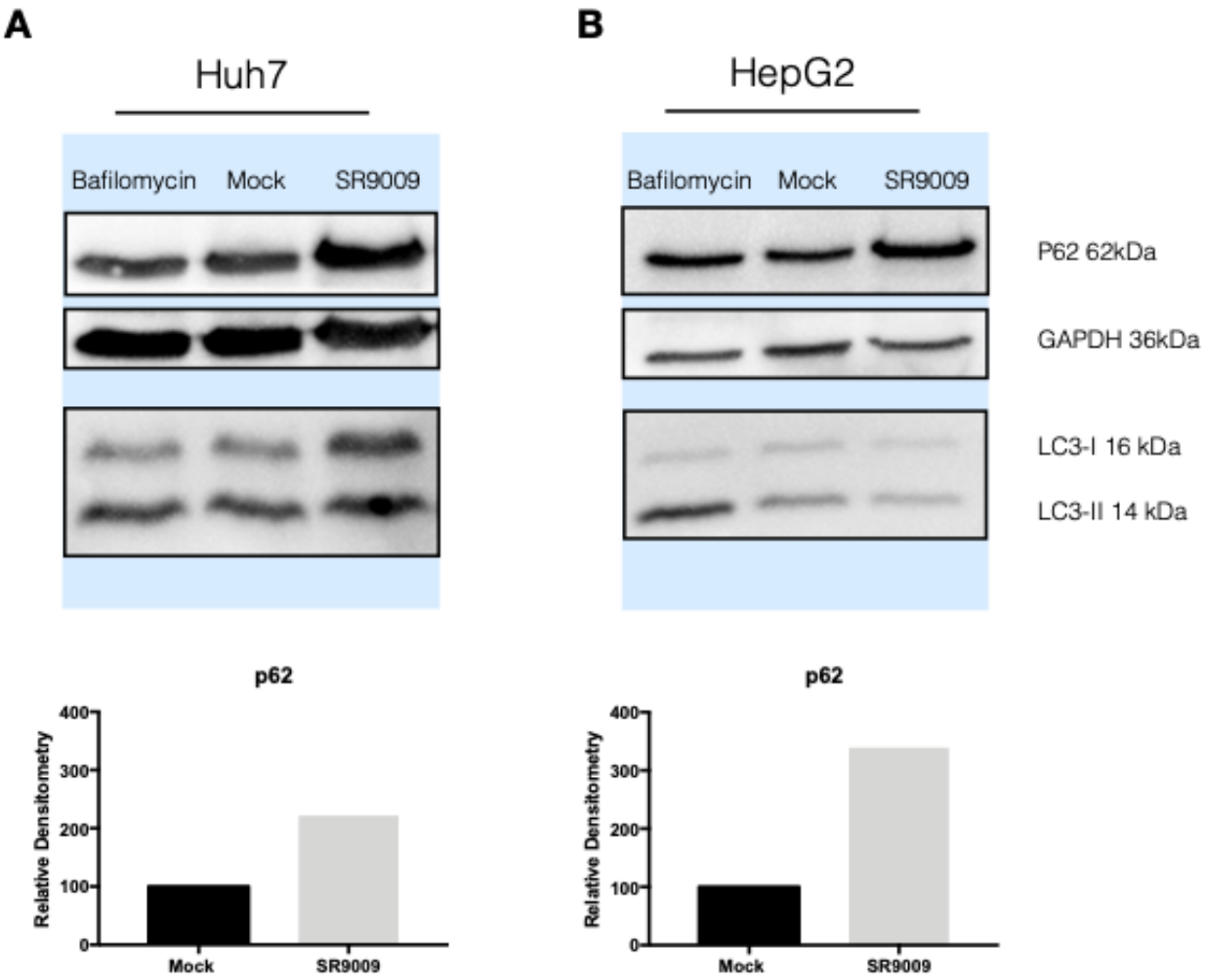
AL	20	8	27
AP	80	92	73

AL	18	0	16
AP	82	100	84

Figure 4.5: Characterizing the HepAD38 mRFP-EGFP LC3 reporter cell line. HepAD38 cells stably expressing a dual mRFP-EGFP LC3 fluorescent protein were created by lentiviral transduction. Cells were treated with bafilomycin or rapamycin before being fixed with paraformaldehyde before imaging (A). Puncta are counted as a percentage per image. Manual counting of puncta (B) and automated by CellProfiler (C) show >90% concordance. Data shown as a single AP:AL ratio expressed as a cumulative percentage ± SEM, n=30. An increase in AP is noted across all treatments suggestive of a basal autophagy inhibition not responsive to stimulation. Protein analysis using bafilomycin treated cells show a competent autophagy pathway with expected response of increased p62 and LC3-II. Images acquired on a Leica DM6000B fluorescence microscope. Scale bars: 10 µm. Western blot images representative of 3 experimental replicates.

4.2.5 REV-ERB activation inhibits autophagy in three human hepatoma cells

The REV-ERB agonist SR9009 was reported to inhibit autophagy at the transcriptional level by decreasing ATG, ULK and BECN mRNA in a breast cancer cell line (Sulli *et al.*, 2018). I was interested to evaluate whether SR9009 would inhibit autophagy in hepatoma cell lines commonly used in hepatitis virus research. Huh-7, HepG2 and HepaRG cells were treated with SR9009 (20μM) for 24 hours before lysing the cells and quantifying key autophagy proteins by western blotting. Cells were treated with bafilomycin (400nM) for four hours as per protocol detailed above to ensure a competent flux pathway. SR9009 treated Huh-7 cells showed a two-fold increase in p62 expression relative to the mock, indicative of autophagic inhibition with a decrease in flux (**Figure 4.6A**). LC3 expression was comparable across the conditions. HepG2 cells exposed to SR9009 showed a three-fold increase in p62 expression relative to mock and reduced LC3-I & II expression (**Figure 4.6B**). Since both proteins are perturbed, this suggests a decrease in transcription of both cytosolic LC3-I and as a consequence membrane bound LC3-II. The HepaRG cells appear to be the most sensitive to SR9009 and show a four-fold increase in p62 (**Figure 4.6C**). SR9009 induced greater expression of p62 than bafilomycin across all three hepatoma lines. These results are consistent with the reported literature and show a role for REV-ERB to negatively regulate autophagy in commonly used hepatoma cell lines. Mindful that autophagy is critical for HBV replication, it was unknown if autophagy modulated via circadian rhythm would be capable of regulating HBV infection parameters.


A
Huh7
Bafilomycin
Mock
SR9009
B
HepG2
Bafilomycin
Mock
SR9009
P62 62kDa
GAPDH 36kDa
LC3-I 16 kDa
LC3-II 14 kDa
p62
Relative Densitometry
400
300
200
100
0
Mock
SR9009
p62
Relative Densitometry
400
300
200
100
0
Mock
SR9009

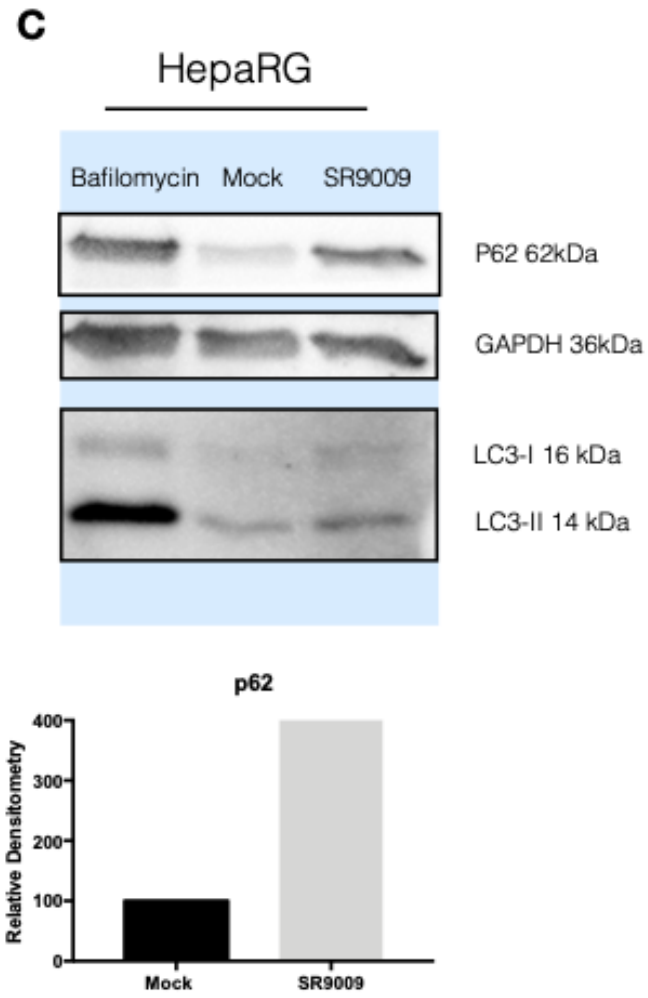


Figure 4.6: REV-ERB activation by SR9009 inhibits autophagy in three hepatoma cell lines. Cells were treated with 20µM SR9009 for 24 hours before protein was harvested for western blot analysis. Exposure to 400nM bafilomycin for four hours prior to protein harvest was performed to illustrate autophagic flux blockade. Densitometry of the p62 band was performed relative to the mock. There is a two, three and four fold increase in p62 across the Huh7, HepG2 and HepaRG cell lines (A-C). LC3 bands in the HepG2 treated cells were weaker than mock suggesting decreased transcription of LC3. Data representative of 3 experimental repeats.

4.2.6 Evaluating the effect of autophagy modulators and REV-ERB agonist on HBV antigen expression.

Chapter 3 illustrates that SR9009 activation of REV-ERB can inhibit circadian cycling of HepaRG cells. Results from this chapter demonstrate that SR9009 can inhibit the autophagy pathway in multiple hepatoma cell lines. I was therefore interested to investigate the effect of autophagy modulators and SR9009 on HBV antigen expression in a model of persistent infection. The HepG2.2.15 cell line carries two integrated dimers of the HBV genome and secretes infectious virions (Sells, Chen and Acs, 1987).

HepG2.2.15 cells were treated with rapamycin (400nM), bafilomycin (100nM) or SR9009 (20μM) for 48 or 72h before the supernatant was collected and analysed for HBsAg and HBeAg expression. Rapamycin exposure showed no significant difference in HBsAg production (**Figure 4.7A**), but HBeAg showed a significant increase after both 48 and 72 hours of exposure (**Figure 4.7B**). Bafilomycin exposure showed a decrease in HBsAg production after 72 hours only (**Figure 4.7C**) and a decrease in HBeAg production was observed after both 48 and 72 hours (**Figure 4.7D**). Finally, SR9009 exposure showed a decrease in both HBsAg and HBeAg at both time points (**Figure 4.7E-F**).

These results suggest that modulation of autophagy whether by the traditional agent bafilomycin or by circadian modulation via SR9009, result in a similar phenotype of decreased HBsAg and HBeAg production. Promoting autophagy via rapamycin had the opposite effect which is consistent with reported literature.

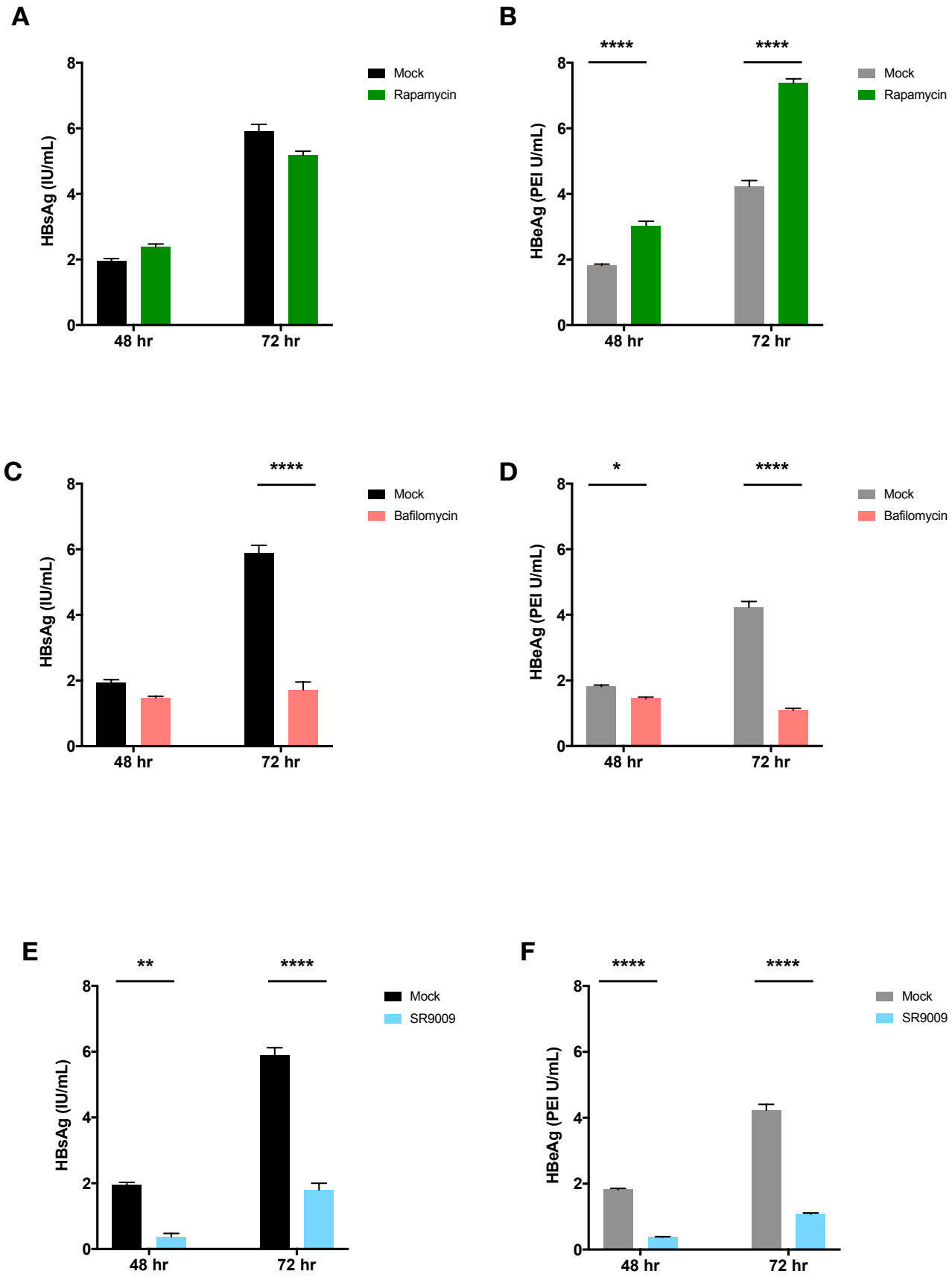

A
HBsAg (IU/mL)
0
2
4
6
8
48 hr
72 hr
Mock
Rapamycin
B
HBeAg (PEI U/mL)
****
****
C
HBsAg (IU/mL)
****
Mock
Bafilomycin
D
HBeAg (PEI U/mL)
*
****
E
HBsAg (IU/mL)
**
****
Mock
SR9009
F
HBeAg (PEI U/mL)
****
****

Figure 4.7: Regulation of HBsAg and HBeAg production in HepG2.2.15 cells by modulators of autophagy and circadian rhythm. HepG2.2.15 cells were treated with 400nM rapamycin, 100nM bafilomycin or 20µM SR9009. The supernatant was collected after 48 and 72 hours for HBsAg and HBeAg quantification. Rapamycin treatment shows no change in HBsAg (A) but a significant increase in HBeAg at both timepoints (B). Bafilomycin treatment shows a decrease in HBsAg at 72 hours (C) and a decrease in HBeAg at both timepoints (D). SR9009 treatment shows a decrease in HBsAg (E) and HBeAg (F) at both timepoints. All are shown as mean ± SD, n=6. P= * <0.05, ** <0.01, ***<0.001, ****<0.0001 using ordinary 2-way ANOVA.

4.3 Discussion

This chapter hypothesises that circadian rhythm exerts some of its regulation of HBV through modulation of the intermediate process of autophagy. A stepwise approach was taken to investigate this. First, HBV mediated perturbation of autophagy was demonstrated using an LC3 reporter AP maturation assay. Then, REV-ERB -a key component in the circadian regulation - was shown to inhibit autophagy in commonly used hepatoma cell lines. Finally, pharmacological activation of REV-ERB and inhibition of autophagy both reduced expression of two key viral proteins: HBsAg and HBeAg.

To illustrate HBV dependent modulation of autophagy; a measurement of AP formation and maturation was needed. This was achieved using a dual fluorophore labelled mRFP-EGFP LC3 where the EGFP component is acid labile (**Figure 4.1**). Analysis of immunofluorescence microscopy images allows an AP: AL ratio to be calculated (**Figure 4.2**). Two hepatoma cell lines were created to stably express this protein by lentiviral transduction. A comparison of the newly created HepG2 LC3 reporter and parental HepG2 cells demonstrated the presence of fluorescent protein (**Figure 4.3**). As the manual counting of fluorescent puncta is prone to significant operator error; a decision to calibrate automated puncta counting software was taken to ensure a standardised method (Peebles, Hochberg and Clarke, 1981; Klionsky *et al.*, 2016). Additionally, this software could be used in the future as part of a wider screening technique for compounds of interest.

The HepG2 LC3 reporter cell line responded appropriately to known modifiers of autophagy as shown by AP: AL ratio and protein expression (**Figure 4.4**). When this characterisation is applied to the HBV producing HepAD38 LC3 reporter line, some striking differences emerge (**Figure 4.5**).

There is a 40% increase in the basal AP: AL ratio suggesting a block in AP maturation. Treatment with bafilomycin augmented this delay in AP maturation whilst treating with rapamycin showed minimal enhancement of autophagy beyond basal levels.

Notably, there is consistency in the generated AP maturation results whether generated by manual (**Figure 5.B**) or automated (**Figure 5.C**) methodologies. This verifies the use of the CellProfiler software to count fluorescent LC3 puncta in two cell lines over six conditions, with >90% concordance (Jones *et al.*, 2008). These results suggest that HBV modulates autophagy and is consistent with previous work that used a single LC3 fluorophore showing that the early autophagic response is stimulated by HBV (Sir *et al.*, 2010).

The next step was to determine whether REV-ERB could modulate autophagy in our model systems. This was investigated by looking at the response following exposure to the REV-ERB agonist SR9009 in three hepatoma cell lines commonly used in our laboratory: Huh-7, HepG2 and HepaRG (**Figure 4.6**). All three showed inhibition of autophagy in response to SR9009 treatment. It is noteworthy that the circadian clock is asynchronous in tissue culture and the HepaRG cell line showed the greatest response with a four-fold increase in p62. These results are in agreement with reported pharmacological activation of REV-ERB inhibiting other tumour cell lines (Sulli *et al.*, 2018).

Consequently, it was proposed that circadian modulated autophagy by SR9009 could in turn regulate HBV replication. This was investigated by treating HepG2.2.15 cells with known autophagy modulators in addition to SR9009.

HBsAg and HBeAg production in Hepg2.2.15 in response to rapamycin and bafilomycin exposure mirrored their expected autophagic responses (**Figure 4.7A-D**). This stimulation of autophagy increasing the production of key HBV proteins verifies the existing -albeit limited- literature (Huang *et al.*, 2014). A striking observation is made when HepG2.2.15 cells are exposed to REV-ERB activation by SR9009; a significant decrease in both HBsAg and HBeAg (**Figure 4.7E-F**). Exposure to SR9009 shows the greatest modulation in parameters out of the three compounds. This is a critical result as it illustrates that SR9009 by dually acting as both a modulator of circadian rhythm as an agonist for REV-ERB (Chapter 3) and an inhibitor of autophagy as illustrated in this Chapter, is capable of regulating the production of two crucial HBV proteins. Inhibition of autophagy whether by bafilomycin or SR9009 produces a similar phenotype of reduced translation of HBV. This initial result hypothesises that HBV replication may be under circadian control.

The *in vitro* modulation of viral antigen expression results presented here must be taken in the context that we are unable to quantify the expression of HBV antigens in hepatocytes in a clinical setting. This represents a substantial knowledge gap in how HBV infection is distributed throughout the human liver. This is further confounded by the likelihood that it is a dynamic process displaying variation by disease stage (European Association For The Study Of The Liver, 2017a). We are dependent on measurement of indirect biomarkers present in the serum, with biopsies of liver tissue now infrequently performed due to the availability of non-invasive modalities. Even when such biopsies are performed, they are subject to significant sample error (**Chapter 1.1.3 &1.1.5**). *In vitro* HBV antigen expression models thus far have been plant based and are largely confined to HBsAg alone (Pasek *et al.*, 1979; Kumar *et al.*, 2005; Yu *et al.*, 2009; Guan *et al.*, 2010). How the HBV mediated modulation of autophagy might affect subsequent viral antigen expression is not a well understood area. Consequently, caution is required before making direct correlations.

At what stage of the HBV replication cycle the key components of the circadian pathway BMAL1, REV-ERBα and the REV-ERB agonist SR9009 can modulate HBV replication will be investigated in the following chapter.

4.4 Summary

This chapter demonstrates that autophagy can be modulated by REV-ERB activation via SR9009 in multiple hepatoma cell lines. It suggests that via circadian modulation of autophagy, HBV replication can be regulated. It validates an automated method for a recognised AP maturation assay.

Chapter 5: The role of circadian components BMAL1 and REV-ERBα in HBV replication.

5.1 Introduction

The cell undergoes seismic changes on a daily basis to respond to its environment and function optimally as part of an organism: a circadian rhythm. The main molecular framework underpinning this is mostly understood and reviewed by (Buhr and Takahashi, 2013). REV-ERB is at the forefront of this modulation and couples circadian rhythm to an increasingly recognised number of cellular homeostatic pathways: energy expenditure, autophagy, regeneration, inflammation and metabolism (Ma, Panda and Jiandie D Lin, 2011; Solt, Kojetin and Burris, 2011; Everett and Lazar, 2014; Poggiogalle, Jamshed and Peterson, 2018). The role REV-ERB plays in regulating immunity has been discussed in Appendix E. Due to increasing recognition of the processes being modulated by circadian rhythm a novel branch of medical science called chronobiology has evolved, this is introduced concisely by (Panda, 2019). As this field widens, an exciting sub-field called chrono-immunology has emerged (Abele *et al.*, 2019). Currently, there is a paucity of research on how HBV is influenced by circadian rhythm, the available research has been discussed in the introduction.

SR9009 is a synthetic REV-ERB agonist that provides a useful tool complementing both *in vitro* and *in vivo* work (Solt *et al*., 2012).

This chapter aims to assess the HBV replication cycle in a stepwise fashion for responsiveness to circadian transcription factors. This would help to understand the how the virus itself has adapted to the dynamic circadian host setting. It has been shown in Chapter 3 that NTCP mediated bile acid transport and HBV entry could both be modulated by REV-ERB activation via SR9009. It has been demonstrated in Chapter 4 that autophagy – which is essential for HBV replication (Sir *et al.*, 2010)- can itself be inhibited by SR9009. Consequently, the key HBV parameters HBeAg and HBsAg show responsiveness to known autophagy regulators as well as

REV-ERB activation via SR9009. This illustrates how HBV is subject to the overall modulation of the cellular environment which is under circadian control.

NTCP protein expression may be under circadian regulation, suggesting resultant HBV entry subject to similar modulation (**Figure 3.11, 3.12 & 3.13**). There are gaps in our knowledge about HBV cccDNA formation which makes studying this aspect particularly challenging (Lucifora and Protzer, 2016). The HBV genome contains four viral promoters: Core, X, Pre-S1 and Pre-S2, which are essential for its transcription and replication. These promoters are conserved amongst replication-competent HBV genotypes (Meier-Stephenson *et al.*, 2018). Using viral promoter constructs linked to a firefly luciferase reporter allows for investigations into their interaction with circadian components. Effects of REV-ERB activation on *de novo* HBV *in vitro* infection models will be tested using dHepaRG cells. Lastly, a collaboration with the Thomas Baumert group of Strasburg University is presented. This made a transition into *in vivo* work possible by facilitating the use of a humanised murine HBV infection model using urokinase plasminogen activator-severe combined immunodeficiency (uPA-SCID) mice. These mice homozygous for the uPA protein have undergone a xenograft with human hepatocytes. These human hepatocytes grow, differentiate and repopulate the murine liver (Meuleman *et al.*, 2005). This partnership represents a valuable opportunity to test the translational therapeutic potential of SR9009 as an HBV anti-viral agent.

5.2 Results

5.2.1 The putative circadian regulatory motifs RORE and E-box in the HBV genome

The transcription factors BMAL1 and REV-ERB bind to specific DNA sequences termed enhancer box (E-box) and RORE, respectively. The existence of these regulatory motifs in the promoter indicates transcriptional regulation through direct binding. A search for these sequences within the HBV genome identified eight E-box motifs with the canonical sequence CANNTG and a single RORE motif with the canonical sequence WAWNTRGGTCA in the 3.2kb HBV awy strain (NCBI Reference Sequence: NC_003977.2) (**Figure 5.1**). Given the small size of the HBV genome and the extent to which they are distributed throughout, it is likely that direct regulation by BMAL1 or REV-ERB exists.

Also illustrated are the compositions of the four HBV promoter luciferase reporter plasmids which allows quick assessment of their ability of being regulated by circadian components such as BMAL1 and REV-ERB. It is noteworthy that the single RORE site does not sit within a promoter region and there is an E-Box in all but one of the viral promoters. Three of the eight E-boxes are located within viral promoters and are designated with an asterisk (**Figure 5.1**). However, it is also worth noting that this illustration represents a linearized HBV genome and most transcription will arise from the circular cccDNA mini-chromosome.

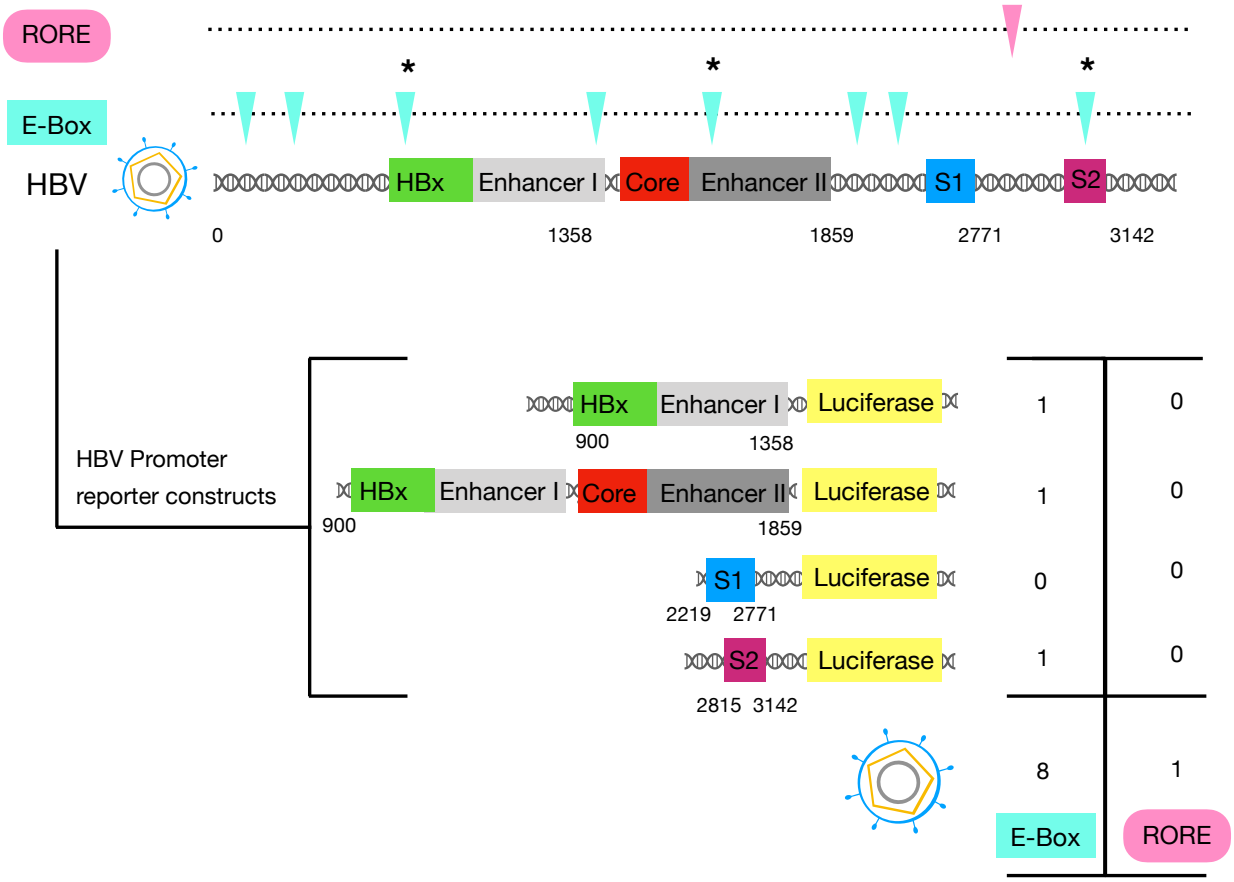
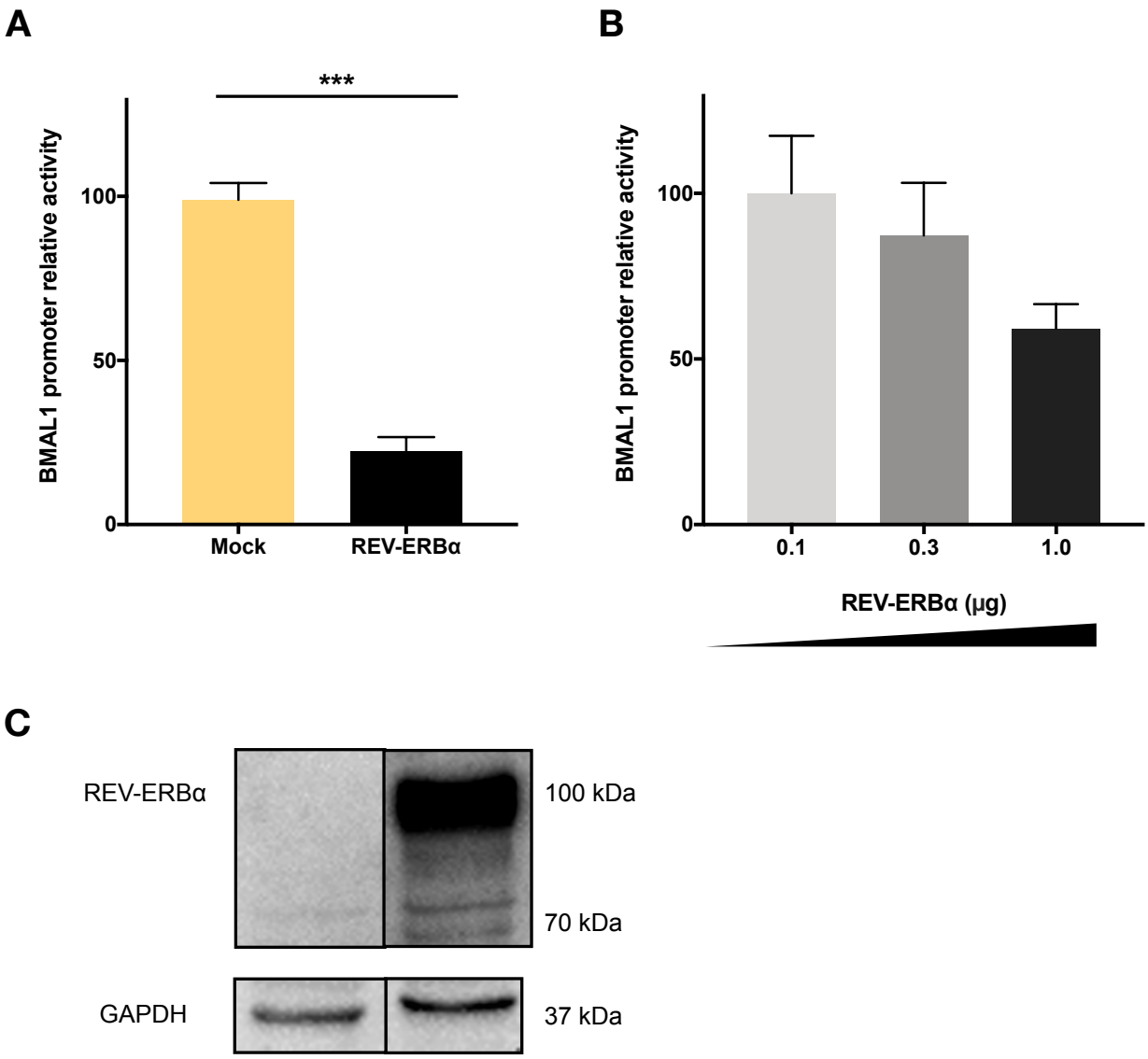


Figure 5.1: Schematic representation of the putative E-box and RORE binding motifs in the HBV genome and four viral promoter reporter constructs. HBV genome ayw strain (NC_003977.2) and promoter reporter constructs were searched for E-Box (CANNTG) and RORE (WAWNTRGGTCA) consensus sequences. Eight E-box and a single RORE matches were found across the whole HBV genome.

5.2.2 REV-ERBα overexpression decreases HBV Core promoter activity.

The HBV core promoter is considered the most crucial element for effective replication. It is unknown if it could be modulated by circadian transcription factors. To address this, a protein overexpression assay to measure the effect of REV-ERBα on the HBV core promoter reporter was established. To ensure that the overexpressed REV-ERBα was functionally active, a BMAL1 promoter reporter was included as a control. A plasmid containing REV-ERBα was co-transfected with either the core or BMAL1 promoter reporter using PEI into HepG2 cells. The total quantity of 2 µg DNA transfected per well was kept constant by the addition of pcDNA3.1 plasmid where necessary. When cell lysates were harvested 48 hours post-transfection and luciferase activity measured, there was an expected decrease in BMAL1 promoter activity (**Figure 5.2A**). Furthermore, a titration in the amount of REV-ERBα DNA transfected suggests a dose-dependent decrease in promoter activity (**Figure 5.2B**). When REV-ERBα was co-transfected with the HBV Core promoter, there was a significant decrease in activity at 48 hours (**Figure 5.2D**). Similarly, it displayed a dose-dependent decrease in activity relative to the quantity of REV-ERBα DNA transfected (**Figure 5.2E**).

Endogenous REV-ERBα is approximately 70 kDa and has been difficult to detect by western blotting. The overexpressed REV-ERBα contains a 34 kDa halo tag which increases the protein size to around 100 kDa. When the lysates were analysed for REV-ERBα protein expression, a faint band of endogenous REV-ERB was apparent at 70 kDa and the halo tagged REV-ERBα runs at 100kDa as expected (**Figure 5.2C&F**).


A
***
BMAL1 promoter relative activity
100
50
0
Mock
REV-ERBα
B
BMAL1 promoter relative activity
100
50
0
0.1
0.3
1.0
REV-ERBα (μg)
C
REV-ERBα
100 kDa
70 kDa
GAPDH
37 kDa

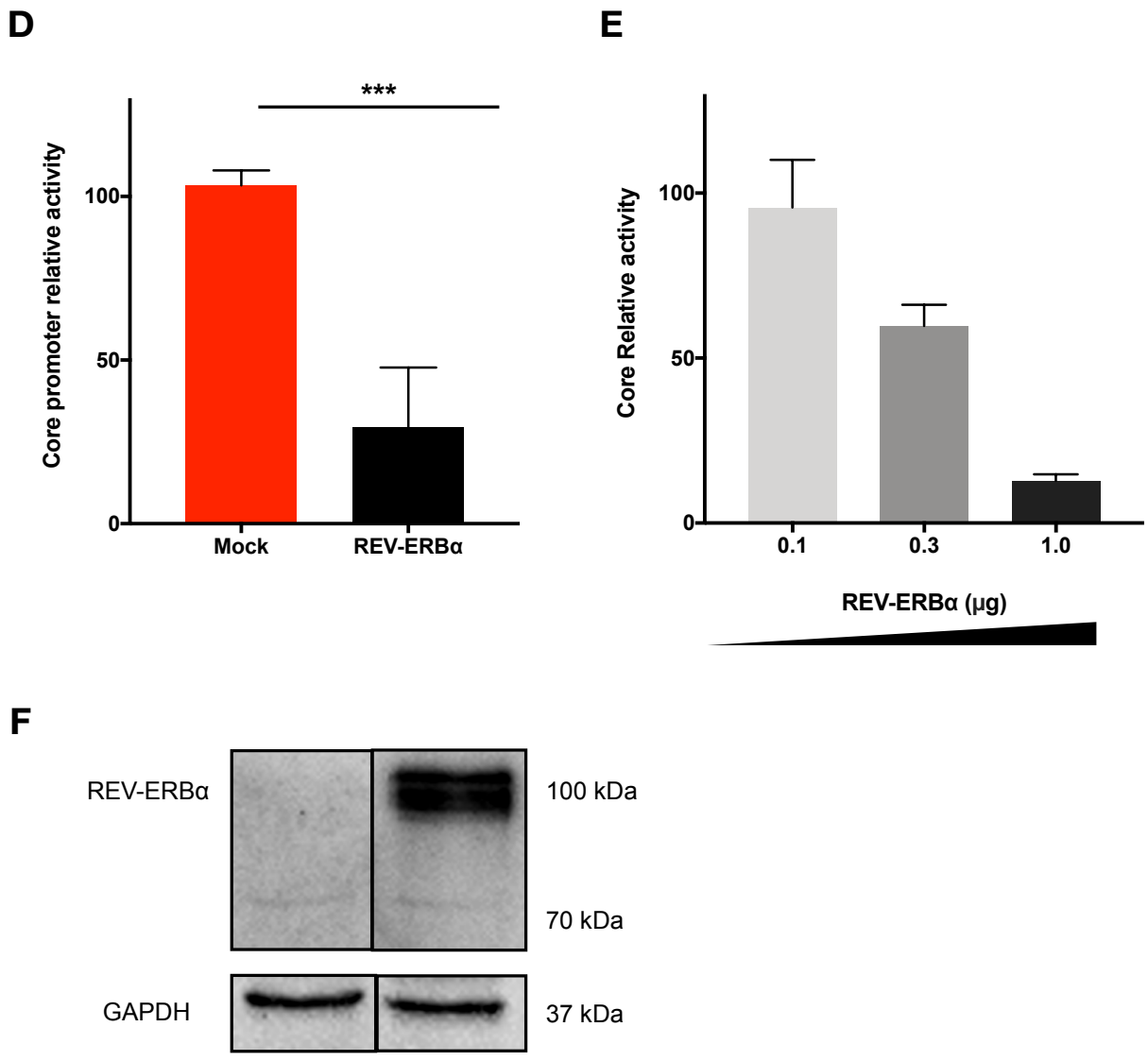


Figure 5.2: REV-ERBα overexpression decreases BMAL1 and HBV core promoter activity in HepG2 cells. HepG2 cells were co-transfected with halo-tagged REV-ERBα and either BMAL1 or HBV core promoter reporter. The total amount of DNA transfected was maintained at a constant 2 µg/well. Cell lysates were collected 48 hours later for luciferase quantification and REV-ERB western blotting. There is a decrease in both BMAL1 and HBV core promoter activity (A&D). This decrease in promoter activity is dose dependent (B&E). Data shown are normalized to mock mean ± SEM, n=18. P=0.001 using Mann-Whitney. Two biological experimental replicates combined. Overexpressed REV-ERBα-halo was confirmed by western blotting (C&F).

5.2.3 BMAL1 and CLOCK overexpression increases HBV Core promoter activity.

BMAL1 forms a heterodimer with CLOCK and together they comprise the activating component of the circadian molecular transcription-translation feedback loop (Buhr and Takahashi, 2013). It was hypothesised that BMAL1 could enhance the activity of HBV Core promoter.

To ensure that the overexpressed BMAL1/CLOCK was functionally active, a CRY1 promoter luciferase reporter was included as a control. BMAL1 and CLOCK plasmids were co-transfected in a 1:1 ratio- with either the Core or CRY1 promoter reporter using PEI into HepG2 cells. The total quantity of 2 µg DNA transfected per well was kept constant by use of pcDNA3.1 plasmid where necessary. When cell lysates were harvested 48 hours post-transfection and luciferase measured, there was an expected increase in CRY1 promoter activity (**Figure 5.3A**). When BMAL1/CLOCK was co-transfected with the HBV Core promoter, there is a significant increase in activity at 48 hours (**Figure 5.3B**).

Endogenous BMAL1 has a size of approximately 70 kDa. The overexpressed BMAL1 plasmid contains x5 Myc (1.2kDa each) and x6 His (1kDa each) tags, this increased the size to 80 kDa. To confirm the correct size band was being identified, a myc western blot was performed (**Figure 5.3C&D**). These results demonstrate that BMAL1/CLOCK overexpression promotes HBV core promoter activity. Coupled with the previous observation that REV-ERBα decreases Core promoter (**Figure 5.2**), they suggest a viral promoter highly susceptible to circadian influence. It was unknown whether these transcription factors were directly acting on the HBV genome.

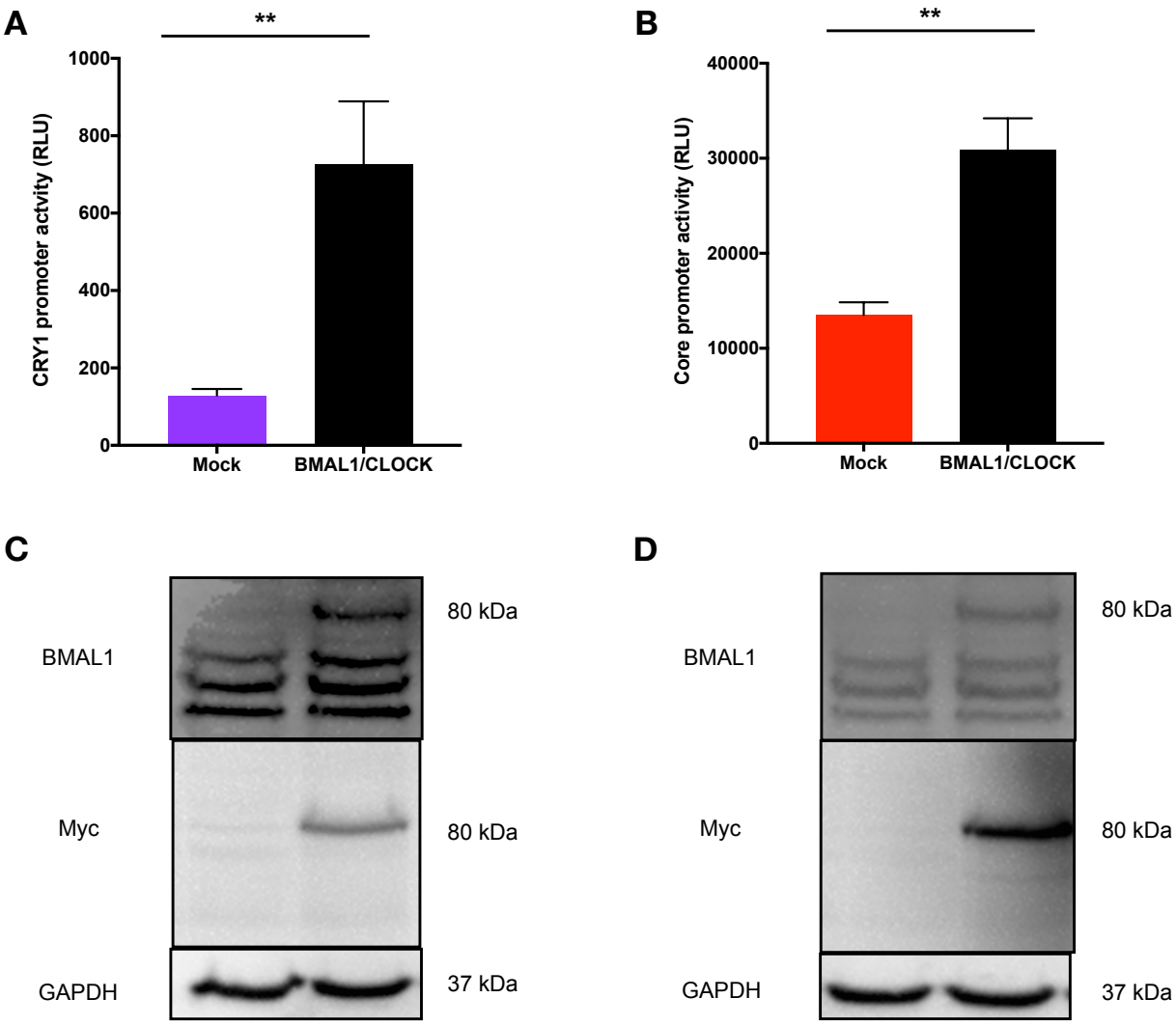


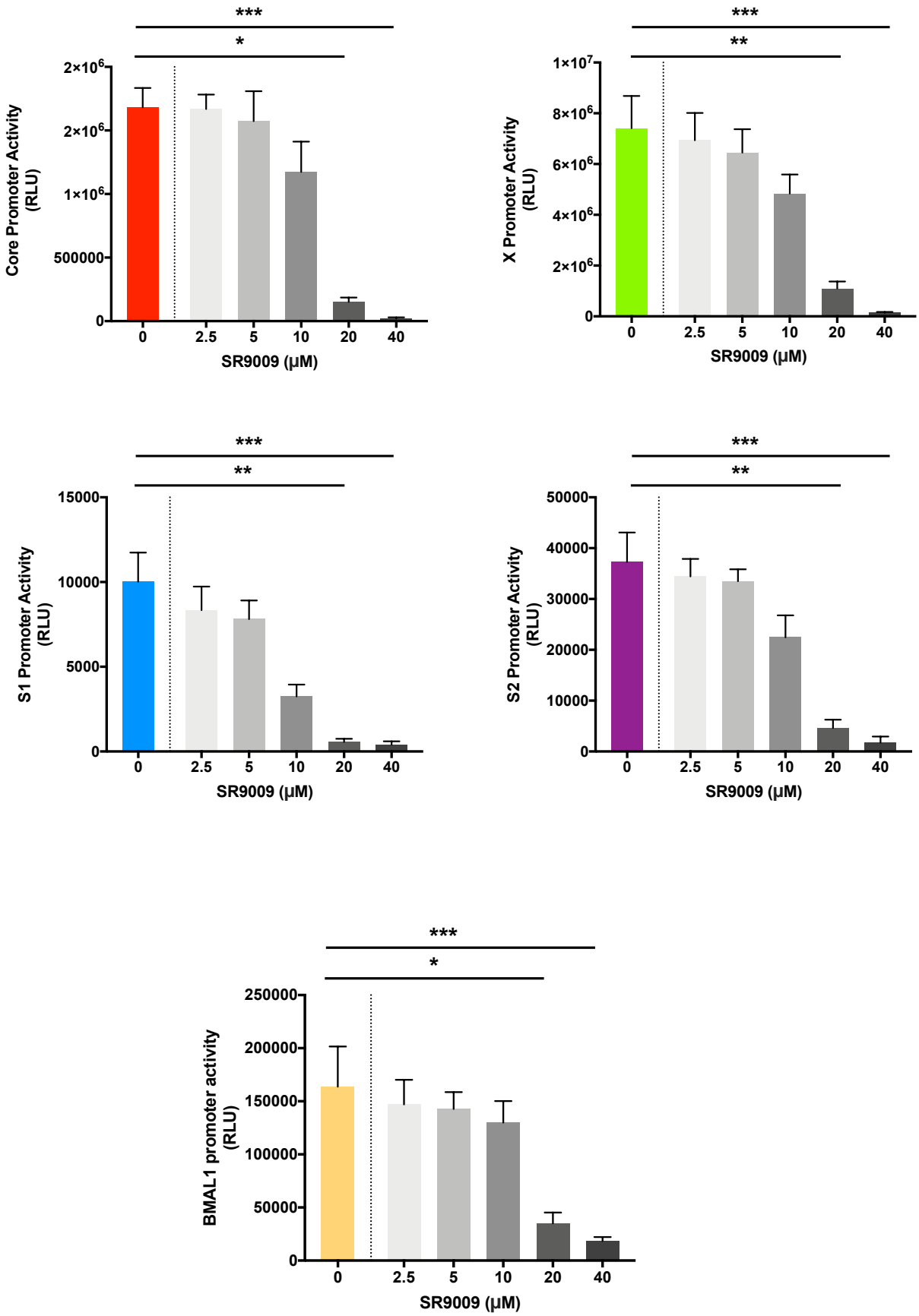
Figure 5.3: BMAL1/CLOCK overexpression increases CRY1 and HBV Core promoter activity. HepG2 cells were co-transfected with BMAL1 (myc-tagged) and CLOCK in a 1:1 ratio with either a CRY1 or HBV core promoter reporter. The total amount of DNA transfected maintained a constant 2 µg/well. Samples for luciferase and protein analysis were collected 48 hours later for luciferase assay and western blotting. There is an increase in both CRY1 and HBV Core promoter activity (A&B). Data shown as mean ± SD, n=6. P=0.002 using Mann-Whitney. Overexpressed BMAL1 was confirmed at 80 kDa by western blotting (C&D).

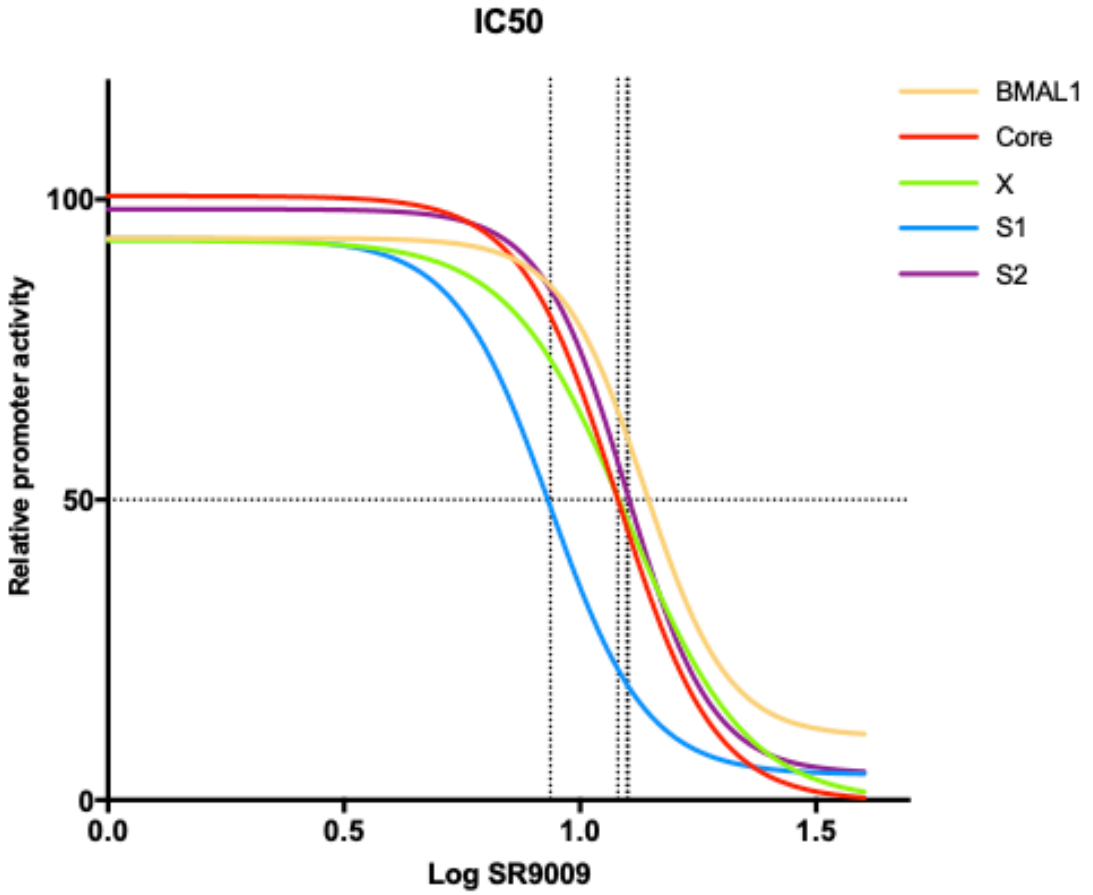
5.2.4 REV-ERB activation by SR9009 decreases HBV promoter activity.

HBV core promoter activity has shown to be decreased by overexpression of REV-ERBα (**Figure 5.2**). To determine if REV-ERB activation would have the same effect, the previously described four HBV promoter luciferase reporters -Core, X, S1 and S2- (**Figure 5.1**) were treated with the REV-ERB ligand SR9009.

HepG2 cells were transiently transfected using each of the four HBV promoter reporters. As a control, the BMAL1 promoter reporter was included. 48h post-transfection, the cells were treated with incremental doses of SR9009 for 48 hours and samples harvested for luciferase assay. There is a significant dose-dependent decrease in activity seen in all four viral promoters in response to SR9009 (**Figure 5.4A)** and the amount of compound required to inhibit 50% promoter activity (ID50) (**Figure 5.4B)**. This demonstrates that all of the HBV viral promoters can be modulated pharmacologically by SR9009 with similar efficacy.

A


***
*
Core Promoter Activity (RLU)
2×10^6
2×10^6
1×10^6
500000
0
0
2.5
5
10
20
40
SR9009 (μM)
***
**
X Promoter Activity (RLU)
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
SR9009 (μM)
***
**
S1 Promoter Activity (RLU)
15000
10000
5000
0
SR9009 (μM)
***
**
S2 Promoter Activity (RLU)
50000
40000
30000
20000
10000
0
SR9009 (μM)
***
*
BMAL1 promoter activity (RLU)
250000
200000
150000
100000
50000
0
SR9009 (μM)



Promoter	Log SR9009	SR9009 (μM)
BMAL1	1.14	13.7
Core	1.08	12
X	1.1	12.6
S1	0.94	8.65
S2	1.1	12

Figure 5.4: REV-ERB activation by SR9009 decreases HBV promoter activity. HepG2 cells were transfected with either a BMAL1 or viral promoter reporter. They were treated with incremental doses of SR9009 for 48 hours before samples taken for luciferase. All viral promoters showed a dose dependent decrease in activity (A). Data shown mean ± SD. P= * <0.05, ** <0.01 and ***<0.001 using Kruskal-Wallis. Composite normalized data to show similar IC50 shown (B).

5.2.5 REV-ERB activation by SR9009 decreases HBV transcription, encapsidated virion production and antigen expression.

After demonstrating that HBV viral promoter activity can be decreased by SR9009 (**Figure 5.4**), its effect on HBV transcription, encapsidated virion production and HBsAg and HBeAg production (key clinical parameters) were investigated next.

HepG2.2.15 cells were treated with 20 µM of SR9009 for 48 hours before cell lysates and the supernatant was collected. HBV pgRNA was determined by qPCR and shows a significant decrease with SR9009 treatment (**Figure 5.5A**). Secreted levels of HBV DNA were quantified by qPCR and there was a significant decrease with SR9009 treatment (**Figure 5.5B**). To ensure only encapsidated HBV rcDNA containing virions were measured, the supernatants were pre-treated with a DNase before performing the qPCR. ELISAs for HBeAg and HBsAg performed on supernatant after 48 and 72 hours treatment with SR9009 showed a significant decrease (**Figure 5.5C&D**). These results demonstrate that the REV-ERB agonist SR9009 inhibits HBV transcription, antigen expression and particle release.

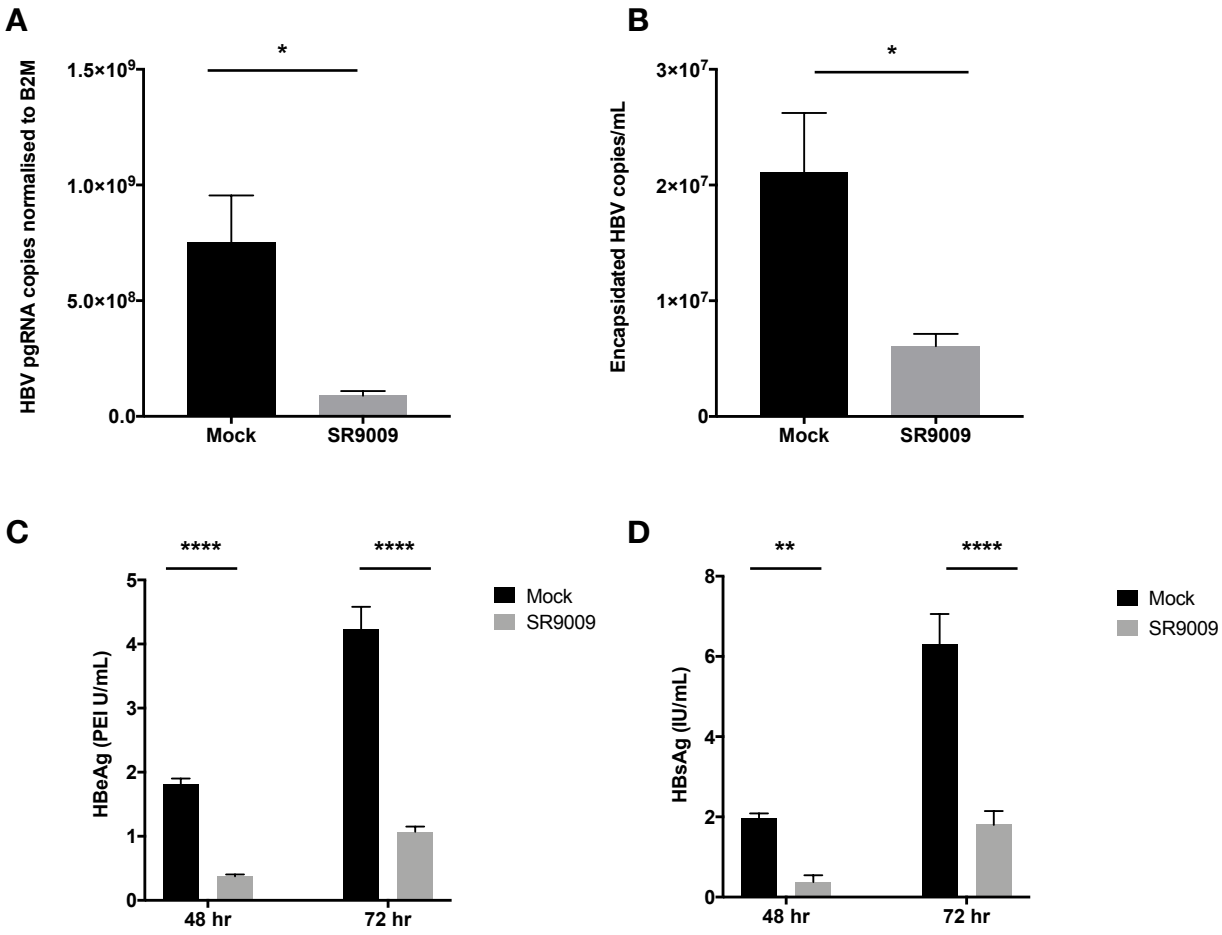


Figure 5.5: REV-ERB activation by SR9009 inhibits HBV transcription, virion production and, translation of E and Surface antigen in HepG2.2.15 cells. HepG2.2.15 cells were treated with 20μM SR9009 for 48 hours before lysates are collected. qPCR for HBV pgRNA shows a significant decrease (A). Data shown as mean ± SEM, n=5. P=0.029 using Mann-Whitney. Supernatant collected at 48 hours was treated with DNase to ensure only encapsidated virions were measured. When qPCR for HBV rcDNA containing virions was performed, this showed a significant decrease (B). Data shown as mean ± SEM, n=6. P=0.03 using Mann-Whitney. ELISAs for HBeAg and HBsAg after 48 and 72 hours treatment with SR9009 both showed a significant decrease at each timepoint (C-D). Both are shown as mean ± SD, n=4. P= * <0.05, ** <0.01 and ***<0.001 using ordinary 2-way ANOVA. Acknowledgement to Dr P Wing, Jane McKeating group, NDM Oxford (A).

5.2.6 REV-ERB activation by SR9009 decreases HBV replication in dHepaRG cells.

To further investigate the impact of SR9009 on the HBV replication cycle, *de novo* infection was undertaken in the dHepaRG cells. This cell line has been shown previously to possess a robust entrainable circadian rhythm and demonstrated modulation of NTCP mediated HBV entry in response to SR9009 (**Figure 3.11 & 3.13**). To account for this modulation of entry, two different infection protocols were investigated where SR9009 treatment occurred pre or post HBV infection.

To evaluate the pre-treatment model, dHepaRG cells were treated with SR9009 for 24 hours before removal and inoculation with HBV for 16 hours. Six days later lysates and supernatant were collected. Two concentrations of SR9009 (10 μM and 20 μM) were used to show a dynamic range. There was a significant decrease in HBV cccDNA and pgRNA in the SR9009 treated cells compared to mock (**Figure 5.6A**). HBsAg and HBeAg in the supernatant showed a significant decrease following treatment with SR9009 (**Figure 5.6A**). The reductions observed in all HBV parameters show a dose-dependent relationship. This protocol does not differentiate entry from post-entry viral replication modulation by SR9009. It is likely that a proportion of the decrease in HBV parameters seen with pre-treatment of SR9009 is due to decreased NTCP expression and so less virus would be able to enter the cell. However, if one considers the investigations of SR9009 modulating NTCP in dHepRG cells (**Figure 3.11, 3.12 & 3.13**) along with the smaller concentrations and duration of SR9009 used here; decreased viral entry is likely to represent a minor contributor rather than the overall predominant mechanism.

Since REV-ERBα has been demonstrated to regulate HBV transcription, a post-infection treatment model was tested. dHepaRG cells were inoculated with HBV for 16 hours and six days later when the infection has been established, the infected cells were treated with SR9009 for 48 hours and lysates and supernatant were collected. It is a limitation of the experiment that the percentage of cells infected was not separately measured. This is due to technical limitations surrounding the measurements of early HBV infection on a discriminatory cellular level (Thomas and Liang, 2016; Revill *et al.*, 2019). Two concentrations of SR9009 (10 µM and 20 µM) were used to show a dynamic range. There was a decrease in HBV pgRNA in the SR9009 treated cells compared to mock (**Figure 5.6B**). Interestingly, the downward trend in cccDNA copies did not reach significance. Extracellular HBsAg and HBeAg in the supernatant showed a significant decrease with SR9009 treatment at 20 µM (**Figure 5.6B**). Taking into account results from (**Figure 5.6A**), the magnitude of decrease in viral parameters is greater when SR9009 is used in a pre-treatment capacity.

These results demonstrate that the REV-ERB agonist SR9009 can modulate key HBV replication parameters in a *de novo* infection model *in vitro*. A greater decrease was observed when SR9009 was used in a pre-infection or prophylactic capacity rather than in an established infection, suggesting that viral entry may be the component of the lifecycle most susceptible to REV-ERB activation.

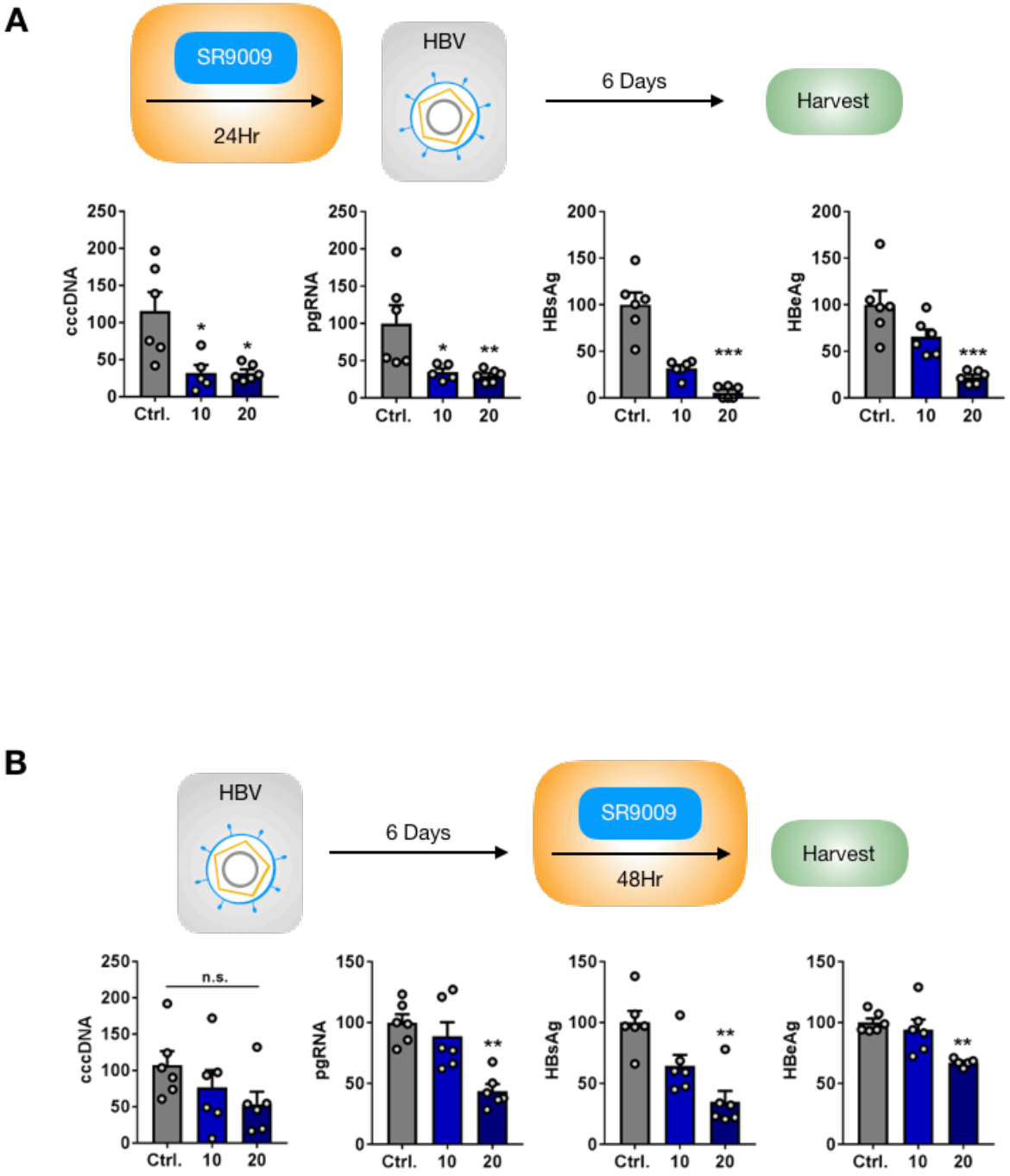


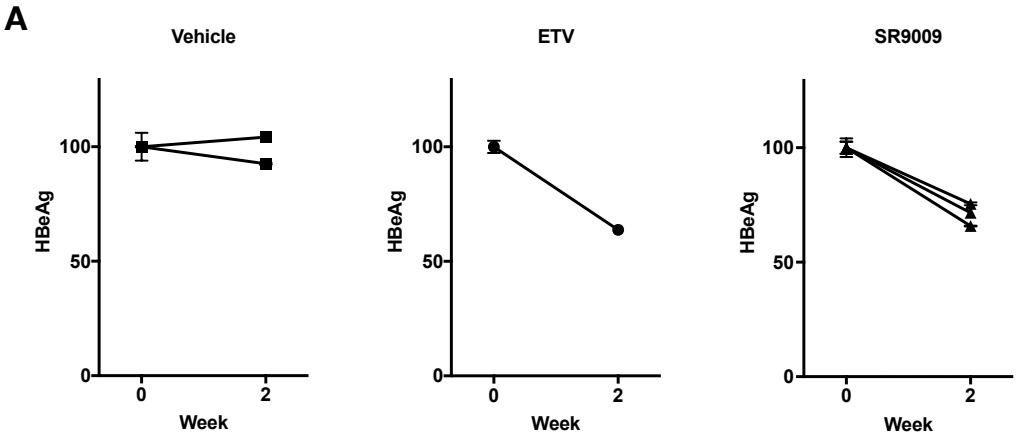
Figure 5.6: REV-ERB activation by SR9009 decreases HBV parameters in *de novo* infection in dHepaRG cells. Differentiated HepaRG cells are treated with SR9009 for 24 hours and then infected with HBV, six days later samples are harvested. There is a decrease observed in HBV cccDNA, pgRNA, HBsAg and HBeAg (A). Data shown as normalized to mock ± SD, n=6. P= * <0.05, ** <0.01 and ***<0.001 using Kruskal-Wallis. Differentiated HepaRG cells are infected with HBV and six days later treated with SR9009 for 48 hours before samples are harvested. There is a decrease observed in HBV pgRNA, HBsAg and HBeAg (B). Data shown as normalized to mock ± SD, n=6. P= * <0.05, ** <0.01 and ***<0.001 using Kruskal-Wallis. Data provided by Dr. V D'Arienzo.

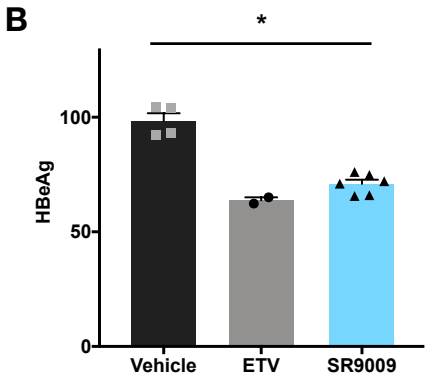
5.2.7 Use of SR9009 in a murine model reduces HBV parameters.

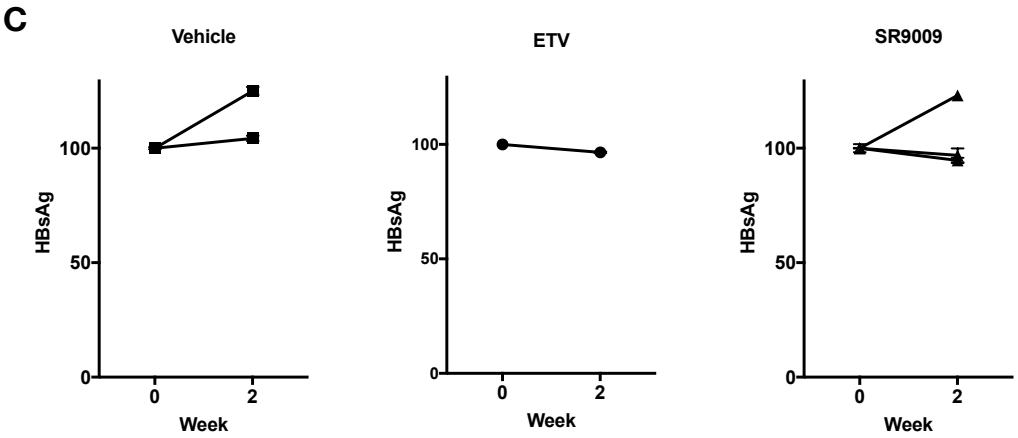
Six uPA-SCID mice with established HBV infection were entered into the experiment. Three underwent treatment with SR9009 (100 mg/kg BD intraperitoneal injection) for two weeks. One was treated with ETV and two with vehicle alone. At the end of the 14-day treatment, the animals were bled before being sacrificed and their livers harvested.

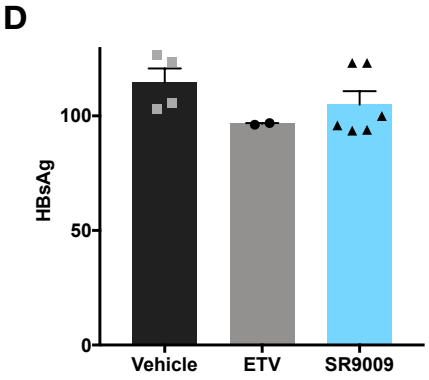
Serum collected at baseline and the final bleed were screened for HBeAg and HBsAg. Individual animal responses are shown in (**Figure 5.7A**). When each response is collated, the mice treated with SR9009 showed a significant decrease of 30% in HBeAg compared to vehicle mice (**Figure 5.7B**). This decrease was comparable in magnitude to the ETV treated mouse. The sera of individual animals analysed for HBsAg are shown in (**Figure 5.7C**). When each response is collated, there is no change in HBsAg levels compared to vehicle mice (**Figure 5.7D**) This is observed across the vehicle, ETV and SR9009 treated animals.

When liver tissue was analysed for HBV pgRNA, a significant decrease was observed in the SR9009 treated animals (**Figure 5.7E**). There was no change in the ETV treated animal. As a control to ensure administered SR9009 was active in this experiment, the mRNA of known host REV-ERB target genes Stearoyl-CoA Desaturase (SCD) and Fatty Acid Synthase (FASN) were measured by qPCR: both showed a significant decrease with SR9009 treatment (**Figure 5.7F**). Together these results demonstrate a potent *in vivo* inhibition of HBV transcription and HBeAg production by SR9009.


A
Vehicle
ETV
SR9009
HBeAg
100
50
0
0
2
Week


B
*
HBeAg
100
50
0
Vehicle
ETV
SR9009


C
Vehicle
ETV
SR9009
HBsAg
100
50
0
0
2
Week


D
HBsAg
100
50
0
Vehicle
ETV
SR9009

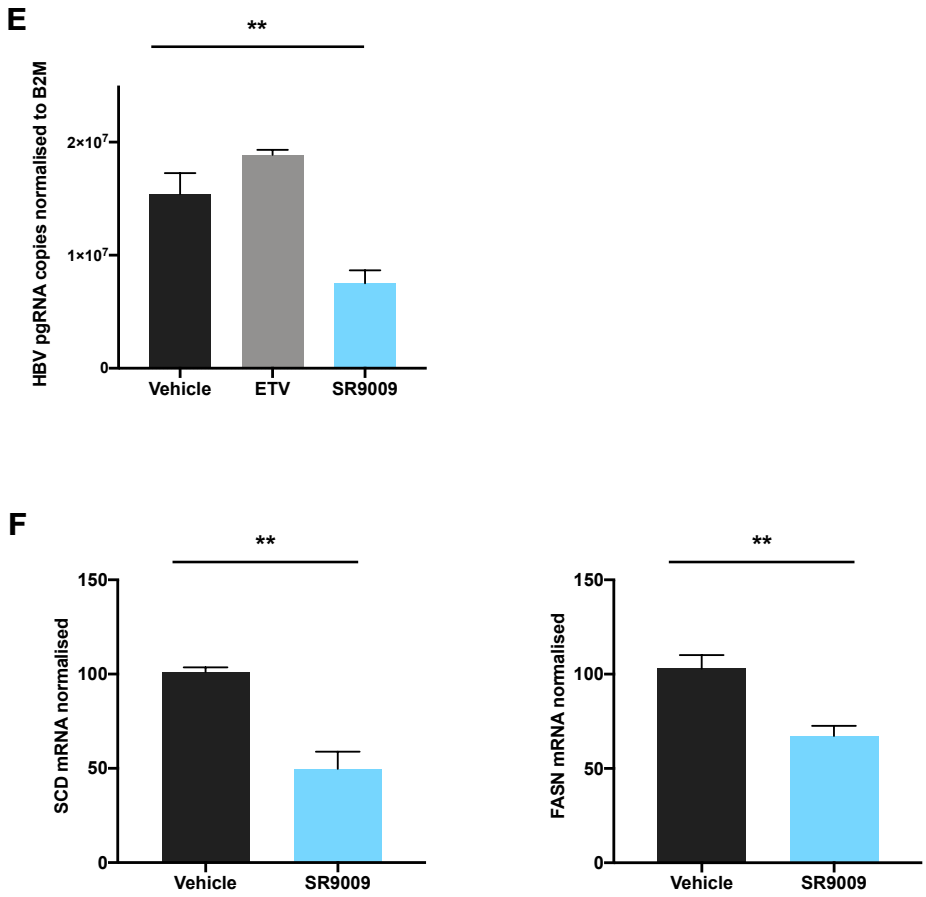


Figure 5.7: HBV infected uPA-SCID mice treated with SR9009 show reduced HBeAg and pgRNA. Six uPA-SCID mice were infected with HBV, three were treated with SR9009 (100mg/kg BD intraperitoneal injection) for two weeks, one with ETV and two left as a control or "vehicle". Final bleeds and organ harvest occurred at the end of this 14 day treatment. Sera was first analyzed for HBeAg and individual animal responses are shown in (A). Data shown as individual normalized to week 0 mean ± SD, n=2. Samples ran in technical duplicate. Collated SR9009 response demonstrated a significant decrease in HBeAg (B) P=0.16, where P= * <0.05, ** <0.01 and ***<0.001 using Mann-Whitney. Sera of individual animal tested for HBsAg are shown in (C). Data shown as individual normalized to week 0 mean ± SD, n=2. Samples ran in technical duplicate. Collated SR9009 response demonstrated no significant change in HBsAg (D). When qPCR for HBV pgRNA is performed on liver tissue, a decrease in response to SR9009 is also observed (E). Data shown as mean ± SD, n=6. P=0.004 where P= * <0.05, ** <0.01 and ***<0.001 using Mann-Whitney. qPCR for known REV-ERB host target genes SCD and FASN mRNA decreased in SR9009 treated animals (F). Data shown as normalized to week 0 mean ± SD, n=6. P=0.03 (SCD) and P=0.01 (FASN) where P= * <0.05, ** <0.01 and ***<0.001 using Mann-Whitney. Acknowledgement to Thomas Baumert Group, Strasburg University and Xiaodong Zhuang, Jane McKeating group, NDM Oxford (E & F).

5.3 Discussion

HBV is a relatively small virus with a genome of 3.2 kb, yet it contains multiple binding motifs for circadian regulators (**Figure 5.1**). Their exact function remains unknown in HBV, however, the presence of these motifs in other viruses has been shown to be important for viral replication. HIV contains four putative E-Box motifs, despite a genome three times larger than that of HBV. The HIV TATA element, which is important for the assembly of the preinitiation complex, is flanked on both sides by an E-Box. When these sites underwent mutagenesis (Ou *et al.*, 1994), there was a decrease in basal and TAT induced gene expression. In patients with HIV on antiretroviral therapy (ART) with a suppressed serum viral load, the levels of cell-associated unspliced RNA were found to have a significant relationship with the time of day the blood was sampled (Chang *et al.*, 2018). This indicates that despite meeting current standard international goals of therapy – a suppressed HIV RNA in serum- (Churchill *et al.*, 2015), viral RNA transcription is occurring and is being modulated by host circadian rhythm. In other viral infections, mutagenesis of the E-Box regions in Bovine Leukemia Virus (Calomme *et al.*, 2002) and Muscovy duck-origin goose parvovirus (Wang *et al.*, 2019) were detrimental to their replication. This illustrates the largely unmet challenge to understand the interaction between circadian rhythm and HBV infection whilst showing the theoretical sites of circadian modulation within its genome.

First, it needed to be established that HBV replication may be under circadian influence. After suggesting in Chapter 3 that NTCP mediated HBV entry is under circadian regulation by REV-ERB activation, an investigation into whether the other components of the HBV replication cycle could be similarly modulated was undertaken. The next aspect of viral replication most amenable to investigation is promoter activity. It is generally accepted that role played by the HBV core promoter is the most critical to replication, as it directs the pre-core and pre-

genomic transcripts that encode HBeAg, HBcAg and pgRNA. HBeAg is a soluble protein that is an important clinical marker as it can serve as an indicator of viral replication and disease staging, whilst pgRNA is the precursor to the DNA contained in the mature HBV virion (European Association For The Study Of The Liver, 2017a). The functionality and regulation of the core promoter are reviewed in more detail by (Kramvis and Kew, 1999; Beck and Nassal, 2007; Quarleri, 2014) and discussed in (**Appendix A**).

To investigate if HBV core promoter was subject to circadian modulation, overexpression of the key regulators REV-ERBα and BMAL1 were first performed. When the transcription factor REV-ERBα is overexpressed, it negatively modulated the activity of the HBV Core promoter (**Figure 5.2 D-E**). An attempt to illustrate the dose-dependent activity of REV-ERBα was made by titrating the quantity of plasmid transfected which can be seen in (**Figure 5.2E**). Conversely, overexpression of BMAL1 and CLOCK enhanced HBV Core promoter activity (**Figure 5.3 A-B**). This demonstrates that crucial regulators in the circadian molecular framework are capable of influencing HBV Core promoter activity. These were a significant set of results: if viral transcription initiation is under circadian regulation then all steps downstream of this may be subject to the same influence. The mechanism behind this modulation was unknown. It was hypothesised that in addition to broader indirect modulation by the cellular environment, there may be direct interaction between circadian transcription factors and the viral genome itself. After identifying the numerous putative binding motifs of BMAL1 and paucity of those for REV-ERBα; it was hypothesised the majority of REV-ERB modulation would occur indirect**ly (Figure 5.1**). However preliminary data using a ChIP-assay in the HepG2.2.15 and HepG2 Epi cell lines suggests REV-ERVα may be directly interacting with the HBV genome. Surprisingly the effect with BMAL1 was modest. This suggests that REV-ERBα alone may exert a portion of its transcriptional effects directly on the virus. However, one must remain mindful that in addition

to any direct viral interactions, there will be indirect modulation due to the endogenous circadian rhythm of the cellular landscape in which the virus occupies.

To investigate if HBV viral promoter activity could be modulated by pharmacological REV-ERB activation, each was treated with SR9009. All four promoters Core, X, S1 and S2 would show decreased activity in response to treatment with SR9009 (**Figure 5.54A**). A composite representation illustrates all four have a similar ID50 (**Figure 5.4B**). This demonstrates that these crucial regulators of subsequent viral transcription and replication are under circadian regulation and can be repressed effectively by pharmacological means. Notably these promoters are lacking a RORE motif suggesting that REV-ERB activation is acting by indirect means.

Up to this point, viral promoter activity was measured in isolation. To investigate viral replication as a whole, HepG2.2.15 cells were treated with SR9009 and HBV pgRNA, encapsidated virion, HBeAg and HBsAg all showed significant decreases in response to SR9009 treatment (**Figure 5.5**). To verify these observations, a *de novo* infection in dHepaRG cells was used. To account for modulation of NTCP by SR9009 suggested previously (**Figure 3.12**), two protocols were investigated whereby SR9009 treatment occurs before infection termed 'pre-infection' or afterwards termed 'post-infection'. In the pre-infection protocol a greater magnitude decrease across all of the measured HBV infection parameters -cccDNA, pgRNA, HBsAg and HBeAg- was observed than in the post-infection protocol (**Figure 5.6 A-B**). In fact, cccDNA formation shows little change in the post-infection protocol with both HBsAg and HBeAg showing less inhibition (**Figure 5.6B**). I hypothesise that SR9009 has modulated NTCP mediated HBV entry which has been bypassed in the post-infection protocol.

A collaboration with the Baumert group in Strasbourg made the valuable transition into *in vivo* work possible. This was the first translational use of SR9009 as an antiviral for HBV. uPA-SCID mice with human hepatocytes and established HBV were administered two weeks of BD intraperitoneal SR9009 treatment. HBeAg showed a significant decrease with SR9009 treatment (**Figure 5.7 A-B**). Decreasing HBeAg is a highly desirable clinical goal of therapy, as it likely precedes seroconversion to anti-HBeAg which is associated with more favourable long term outcomes (European Association For The Study Of The Liver, 2017). Interestingly HBsAg remained mostly unaffected in the SR9009 treated mice (**Figure 5.7 C-D**). It was hypothesised that HBsAg could be derived from integrated double-stranded linear DNA (dslDNA) rather than the episomal cccDNA. This phenomenon was observed by (Wooddell *et al.*, 2017), during a phase 2 clinical trial for a new RNAi therapy for HBV. It was noted that HBsAg was strongly reduced in patients who were HBeAg positive but was reduced significantly less in patients who were HBeAg negative. The authors further demonstrated that in a chimpanzee model, in animals that were HBeAg negative a substantial proportion of HBsAg transcripts arose from dslHBV DNA rather than cccDNA. Murine liver tissue analysed for HBV pgRNA shows a significant decrease in response to SR9009 treatment (**Figure 5.7E**) although serum HBV DNA remained unchanged (data not shown). The half-life of HBV virions in serum is thought to be in the range of a few hours to a day (Nowak *et al.*, 1996; Dandri *et al.*, 2008). However, there are incompletely understood mechanisms that result in complex viral decay patterns that result in a far slower decrease of HBV DNA from serum (Lewin *et al.*, 2001). Therefore, it is hypothesised that the reservoirs of intracellular HBV DNA and a lag in the rate of immune mediated clearance of infected hepatocytes precluded a decrease being detected in a two-week study. This preliminary model has numerous limitations; the precise half-life of SR9009 was not calculated (due to constraints on the permitted number of interventions made to the mice) nor were estimates of NTCP levels undertaken (due to baseline cholestatic liver damage present in the mice due to the immunosuppression required for the human liver xenograft).

5.4 Summary

In this chapter evidence that REV-ERB activation by SR9009 leads to a decrease in multiple HBV infection parameters *in vitro* is presented. Evidence that multiple components of the HBV replication cycle are subject to circadian influence are presented. It is hypothesised that viral entry is most susceptible to circadian regulation. Finally, evidence of SR9009 decreasing HBeAg and pgRNA in a translational murine HBV infection model is illustrated.

Chapter 6: Discussion

6.1 Summary of key findings

The HepaRG cell line is capable of sustaining an entrainable circadian rhythm. Identifying a cell line both permissive to HBV and capable of sustaining an entrainable circadian rhythm was a significant challenge. Although HepaRG cells more closely resemble primary human hepatocytes than HepG2, both are hepatoma derived and possess numerous altered cellular pathways -particularly in growth and metabolism- (Gripon and Rumin, 2002; Hart *et al.*, 2010; Wiśniewski *et al.*, 2016). HepG2 cells would not demonstrate a concordant pattern of circadian expression thereby making them unsuitable for further investigation. In contrast, the bipotent HepaRG cell line maintained a robust entrainable circadian pattern of BMAL1 expression rhythm **(Figure 3.5).**

The almost ubiquitous use of DMSO in *de novo in vitro* HBV infection assays poses a challenge to studying circadian modulation.

DMSO differentiation of hepatoma cell lines remains a mainstay of HBV molecular biology (Thomas and Liang, 2016; Revill *et al.*, 2019). It was demonstrated that DMSO adversely affects real-time luciferase luminescence technology (**Figure 3.9 & 3.10)**. This interference when coupled with the limitations of establishing a sustained HBV infection in the same time frame, led to pursuing a DMSO free HBV infection model. It was correctly predicted to be a challenging avenue of investigation, but one which needed exploration. Two DMSO free HBV infection models failed to sustain sufficient viral replication in which to study the effects of circadian rhythm **(Figure 3.7 & 3.8)**. Subsequently, DMSO dHepaRG cells were shown to still possess an entrainable rhythm as evidenced by protein and RNA analysis, even if real-time luciferase measurements were invalid (**Figure 3.11)**.

NTCP is modulated by REV-ERB activation by SR9009.

The NTCP receptor is crucial for HBV entry and represents a site of investigation for potential HBV therapeutics (Yan *et al.*, 2012; Volz *et al.*, 2013; Ni *et al.*, 2014). NTCP receptor activity and protein expression decreased in a dose dependent manner following exposure to REV-ERB activation by SR9009 **(Figure 3.12).** NTCP protein expression showed a circadian pattern of expression in synchronised dHepaRG cells **(Figure 3.11)**.

The autophagic pathway can be circadian regulated in multiple hepatoma cell lines.

Autophagy is known to be an essential component for HBV replication (Sir *et al.*, 2010; Alavian *et al.*, 2011; Li *et al.*, 2011; Tian *et al.*, 2011). Activation of REV-ERB by SR9009 decreased autophagy indicated by an increase in p62 in three hepatoma cell lines **(Figure 4.6).** This suggests an additional mechanism for modulation of HBV replication parameters by SR9009.

Automated methodology to perform an AP maturation assay is validated.

Measuring fluorescent LC3 puncta is a common method of measuring AP maturation (Mizushima and Yoshimori, 2007; Klionsky *et al.*, 2016). Manually counting puncta is laborious and subjective, therefore development of an automated methodology was a desirable goal. This method was validated and verified to be >90% concordant in its results compared to manual **(Figure 4.4 & 4.5).**

HBV Core promoter activity can be regulated by overexpression of both BMAL1 and REV-ERBα. The HBV core promoter is crucial as it directs transcription of the both precure and core RNA transcripts which will be translated into HBcAg, HBeAg and viral polymerase (Quarleri, 2014)**.** Overexpression of key circadian regulatory proteins can both enhance -BMAL1/CLOCK- and decrease -REV-ERBα- the primary HBV promoter **(Figure 5.2 & 5.3).**

REV-ERB activation by SR9009 downregulates all four HBV promoters' activities. Each of HBV four promoters -Core, X, S1 and S1- show a dose-dependent decrease in activity in response to exposure to SR9009 (**Figure 5.4**). This demonstrates that viral transcription can be modulated by a key component of the circadian molecular framework: REV-ERB.

REV-ERB activation by SR9009 decreases HBV pgRNA transcription, translation of both HBsAg & HBeAg, and mature virion release. The HepG2.2.15 cell line carries two integrated copies of the HBV genome and is capable of producing infectious virions (Sells, Chen and Acs, 1987). Following exposure to SR9009, multiple components of the HBV replication cycle are downregulated **(Figure 5.5).** Exposing dHepaRG cells to SR9009 prior to infecting them with HBV, demonstrated a greater magnitude decrease in multiple HBV parameters **(Figure 5.6)**. This suggests that pre-emptive SR9009 exposure is most efficacious in modulating HBV infection.

A murine model showed reduced HBeAg and HBV pgRNA following treatment with SR9009.

uPA-SCID mice with established HBV infection were treated with injections of SR9009 BD intraperitoneal for 14 days. Blood and liver analysis demonstrated a 30% reduction in HBeAg and pgRNA relative to baseline **(Figure 5.7).** This preliminary result demonstrates a potential role for SR9009 as an agent with HBV antiviral capabilities.

6.2 Limitations and future work

In vitro HBV research is a particularly challenging area with numerous limitations that can be most readily exemplified by the inability to reliably infect a high proportion of target cells (Thomas and Liang, 2016; Revill *et al.*, 2019). One must temper the immediate clinical applicability of our conclusions without underestimating the impact of the result. HBV does appear to be modulated by circadian rhythm and results from an early murine infection model are encouraging. Whether ameliorating morbidity caused by HBV infection by modulation of circadian targets is possible remains unknown. Studying a temporary circadian rhythm *in vitro* is some distance from the self-sustaining rhythms observed *in vivo*. Nevertheless, these early results do represent tentative steps towards a worthy goal.

Research to determine if the identified E-Box and RORE motifs in the ubiquitously used laboratory genotype D HBV are conserved across other genotypes is being investigated by the group. If conserved, it supports their importance for circadian modulation of viral replication.

It is acknowledged that the use of the agent SR9009 -whilst unavoidable- represents a first-generation REV-ERB agonist and will have limitations. SR9009 is an inhibitor of autophagy (Thomes *et al.*, 2016; Sulli *et al.*, 2018) but will also have off-site target effects that have not been adequately controlled for in this thesis. Some of these effects will be impossible to control for within the constraints of first-generation agents. Consequently, results inferred must bear these potential confounding effects in mind, whilst not diminishing their potential therapeutic value.

It is acknowledged that the absence of dedicated cellular toxicity assays for all cell lines exposed to SR9009, bafilomycin and rapamycin is an unfortunate omission and a limitation. It is noteworthy that no toxicity was noted at a macroscopic level for any of these agents. The concentrations used are consistent with those used in other laboratories (Sir *et al.*, 2010; Li *et al.*, 2011; Tian *et al.*, 2011; Solt *et al.*, 2012; Huang *et al.*, 2014; Sulli *et al.*, 2018).

6.3 Concluding remarks

Circadian rhythm is the master metronome to which all life on Earth is subject. Whilst on a macroscopic level this influence is self-evident due to sheer visibility (sleep-wake & activity cycles), the circadian regulation of a broad spectrum of cellular processes including autophagy is only now at the forefront of contemporary global research (**Appendix C**). This has led to an evolving appreciation of the spectrum of pathology that can be caused by a dysfunctional circadian rhythm. The disorders of circadian rhythm -whether extrinsic or intrinsic- affect vast cohorts of the human population and range from metabolism and cardiovascular disorders to

cancer **(Appendix D).** Therefore, continued research into chronobiology is both worthy and imperative (Panda, 2019).

Key components of human health, immunity and infection are increasingly recognised to be subject to circadian influence (**Appendix E**). By increasing our understanding of the mechanisms that underpin their relationship, there is enormous potential for new healthcare initiatives. These initiatives can be broadly divided into two main routes of future investigation:

(i) To augment the efficacy of existing and well established antimicrobial interventions like vaccination (Phillips *et al.*, 2008; Prather *et al.*, 2012; Long *et al.*, 2016) or time of prescribed medication delivery (Hermida *et al.*, 2019). Interventions here could achieve enhanced health outcomes in a relatively short time frame as it involves altering the timing of already established healthcare procedures. Even comparatively minor gains in vaccine or drug response, when applied to such numbers are desirable.

(ii) A novel avenue to identify therapeutic pharmacological targets. As understanding of the circadian molecular framework increases, pharmacologically targeting its components becomes possible. The ROR pathway was targeted initially and CEM drugs developed (He *et al.*, 2016), which have demonstrated potential to diminish the adverse metabolic effects due to SW (Chen *et al.*, 2012; Chen, Yoo and Takahashi, 2013; Nohara, Yoo and Chen, 2015). Both Zika and HCV replication have recently been shown to be modulated by BMAL1 and REV-ERB (Zhuang *et al.*, 2019).

HBV is a global pandemic with no cure. It affects a third of the world's population and is a leading contributor of morbidity and mortality (European Association For The Study Of The Liver, 2017a, 2018; World Health Organization, 2017). Current treatment options are not only limited but unlikely to eliminate HBV (McNaughton, D'Arienzo, *et al.*, 2019). Therefore, new therapeutics are urgently needed (Revill *et al.*, 2019). The core focus of this thesis was to determine if HBV was subject to modulation by components of the mammalian clock. It has illustrated that REV-ERB activation by SR9009 both *in vitro* and vivo models is capable of modulating key HBV parameters. This represents a novel addition to what is currently understood about the circadian regulation of HBV.

Chapter 7: Appendices.

Appendix A: The HBV replication cycle.

The HBV genome contains a complete (-) negative strand with a covalently attached polymerase, and an incomplete (+) positive strand. The positive strand contains two direct repeat (DR1 and DR2) regions which are important in viral DNA synthesis.

HBV replicates almost exclusively in hepatocytes where infectious virions called Dane particles (42nm) are secreted via a non-cytolytic mechanism (Dane, Cameron and Briggs, 1970). Secreted in far greater quantities (over 1,000 fold more) however, are smaller (22nm) genome-free, non-infectious spherical or filamentous sub-viral particles composed of HBsAg alone (Seeger and Mason, 2015). HBsAg is composed of three subunits differing by size, these are discussed in detail later. HBsAg is also known as Australia antigen due to pioneering work carried out by (Blumberg, 1977) that lead to the initial diagnostic serological tests for HBV being developed.

A.1 Entry and nuclear import

The NTCP cell receptor was first described in 2012, and is crucial for both HBV and Hepatitis D virus entry into hepatocytes (Yan *et al.*, 2012; Ni *et al.*, 2014). The authors demonstrated that the receptor binding region of the L or pre-S1 HBsAg glycoprotein interacted with the NTCP receptor by photo-cross-linking and tandem affinity purification. Existing *in vitro* HBV cell culture systems face significant challenges to sustain a complete HBV replication cycle with ease, and identification of this crucial receptor would revolutionise techniques. The authors went on to confer susceptibility to HBV infection in the traditionally non-permissive HepG2 cell line by expressing NTCP.

NTCP is one of many hepatocyte specific cell receptors whose normal physiological function is the transport of bile salts into the basolateral surface of hepatocytes as part of the enterohepatic circulation. It is encoded by the SLC10A1 (solute carrier family 10 member 1) gene (Hagenbuch and Meier, 1994; Stieger, 2011). This discovery would naturally lead on to the pharmacological development of a synthetic lipopeptide derived from the preS1 domain to bind to human NTCP in an attempt to block HBV entry: Myrcludex-B (Petersen *et al.*, 2008). This compound has been shown to efficiently block *de novo* HBV infection both *in vitro* (Gripon, Cannie and Urban, 2005) and *in vivo* (Volz *et al.*, 2013) using human chimeric uPA/SCID mice. This mouse model is discussed further in Chapter 5. A review of NTCP in HBV viral entry is provided by (Watashi *et al.*, 2014).

After attachment, viral entry occurs via internalization through clathrin mediated endocytosis involving Rab5 and Eps15 (Cooper and Shaul, 2006). The envelope is removed in the cytoplasm and HBV capsids are translocated using cellular transport pathways to the nuclear periphery before nuclear import via the nuclear pore complex (NPC) (Schmitz *et al.*, 2010). The subcellular position and transport between cytoplasm and nucleus of the viral capsid has been attributed to a localisation signal pattern contained within the capsid's Arginine-Rich C-terminal Domain (ARD) and its subdomains (ARD I-IV) (Yeh, Liaw and Ou, 1990). These subdomains can initiate transport of the rcDNA containing HBV capsid to the nucleus (H. C. Li *et al.*, 2010). In fact, ARD-I and ARD-III are associated with two co-dependent nuclear localization signals, while ARD-II and ARD-IV behave like two independent nuclear export signals. The authors demonstrated physical interaction between ARD contained in the viral capsid and a host cellular factor called Tip-associated protein/nuclear export factor-1 (TAP/NXF1), which is known to be important for nuclear export of host mRNA and proteins. In fact, when (H. C. Li *et al.*, 2010) went on to perform siRNA knockdown of TAP, it resulted in a

shift of cytoplasmic viral capsids to the nucleus, a 7-fold decrease in viral replication and a 10-fold reduction in HBsAg production.

The NPC is a 'gateway' structure to the nucleus and is composed of approximately 30 different proteins called nucleoporins (Nups). Work by (Rabe *et al.*, 2009; Schmitz *et al.*, 2010; Gallucci and Kann, 2017) demonstrated that only mature rcDNA containing HBV capsids are capable of binding to Nup153, which triggers capsid disintegration with release of the rcDNA into the nucleus.

A.2 HBV cccDNA

In the nucleus, HBV rcDNA can originate from newly imported infectious virions or be re-cycled from recently formed rcDNA as part of the cccDNA amplification pathway (**Figure 1.5**). It is the persistence of this cccDNA that represents the main barrier against HBV cure (Anikhindi *et al.*, 2017). In fact, persistence of cccDNA has been described decades after recovery from acute infection (Rehermann *et al.*, 1996).

There are significant gaps in our understanding in the biosynthesis of HBV cccDNA from rcDNA. It is generally accepted that numerous host DNA repair pathways including polymerases and ligases are employed. This process is illustrated in (**Figure 7.1**). Firstly, upon import into the nucleus, the rcDNA must be separated from the remnants of the prior RT step - the creation of rcDNA from pgRNA - that have occurred in the mature capsid in the cytoplasm. The removal of this leftover viral polymerase and its primer results in a deproteinized rcDNA ready to be repaired to form cccDNA (Guo *et al.*, 2010). HBV utilises components of the host DNA repair pathways called tyrosyl-DNA-phosphodiesterase (TDP) 1 and TDP2 to cleave the tyrosine-DNA

bond releasing polymerase from rcDNA (Königer *et al.*, 2014). This is possibly due to structural similarities between rcDNA and host DNA damage induced by DNA topoisomerase (Ashour, Atteya and El-Khamisy, 2015). These damaged DNA components can be repaired by the MRN complex to preserve genomic integrity and cell homeostasis or risk blockage of RNA transcription and DNA replication (Aparicio *et al.*, 2016). However, notably CRISPR knockout TDP2 cells were still susceptible to HBV infection (Cui *et al.*, 2015). This suggests a pathway that has considerable redundancy to perform its task. This is evidenced by an increasing list of host DNA repair molecular pathways associated in the biosynthesis of cccDNA: flap endonuclease 1 (FEN1) (Kitamura *et al.*, 2018), DNA ligases (Long *et al.*, 2017), MRN complex (Tsutakawa, Lafrance-Vanasse and Tainer, 2014; Aparicio *et al.*, 2016) and DNA polymerase alpha (Tang *et al.*, 2019). To complete the process, the incomplete positive (+) strand and nick in the negative (-) strand must be repaired before, both strands undergo ligation. They then can undergo supercoiling to form cccDNA.

The cccDNA molecule is organised into a chromatin-like structure containing histones that resemble the characteristic 'beads on a string' appearance (**Figure 7.1**). Consistent with the histone code theory, HBV cccDNA transcription is regulated by multiple epigenetic mechanisms such as DNA methylation and histone acetylation (Jenuwein and Allis, 2001; Belloni *et al.*, 2009). This provides numerous options for dynamic epigenetic transcriptional regulation which include both histone-DNA and histone-histone modifications. DNA modifications such as methylation, repressive and activating post-translational modifications (PTMs) of the histones such as acetylation, methylation, phosphorylation and others have been described (Tessarz and Kouzarides, 2014). It was shown by (Pollicino *et al.*, 2006) that HBV replication can be modulated by the acetylation status of cccDNA-bound H3/H4 histones. The authors first demonstrated *in vitro* that class I histone deacetylase inhibitors increased both cccDNA-bound acetylated H4 and HBV replication. Then, using paired serum and liver

tissue samples from patients with HBV they showed that histone hypoacetylation and histone deacetylase 1 recruitment onto cccDNA in liver tissue correlated with low HBV viremia in serum. Additionally, several host TF have been demonstrated to modulate cccDNA transcriptional activity including CREB, ATF, STAT1/2 and histone modifying enzymes (such as CBP, p300, PCAF/GCN5, HDAC1, hSirt1, PRMT1) (reviewed by (Belloni *et al.*, 2009, 2012)).

Amongst these modulating cellular TF, hepatocyte specific TFs like the nuclear hormone receptors hepatocyte nuclear factor 4 (HNF4) and retinoid X receptor α (RXRα), and peroxisome proliferator-activated receptor α (PPARα) have been reported to be essential for HBV replication (Tang and McLachlan, 2001). These hepatocyte specific TFs likely contribute towards hepatotropism. A wider review of known TFs modifying HBV transcription is provided by (Moolla, Kew and Arbuthnot, 2002; Quasdorff and Protzer, 2010).

The non-structural multifunctional HBx protein plays a direct role in the epigenetic regulation of cccDNA transcription. It was shown that HepaRG cells or primary human hepatocytes infected with an HBx deficient virus -despite comparable cccDNA formation and nuclear import with wild type HBV- showed little viral replication (Lucifora *et al.*, 2011). The authors went on to demonstrate that active transcription of virions and viral antigens could be rescued by exogenous HBx. The mechanisms behind HBx modulation of cccDNA are slowly being described. HBx is a perquisite for efficient viral replication to occur by acting as a multifunctional viral regulator through modulating viral transcription and host signalling pathways (Belloni *et al.*, 2009; Lucifora *et al.*, 2011). Recently HBx has been shown to selectively enhance transcription of HBV cccDNA only (and not integrated HBV) by interacting with DNA binding protein 1 containing E3 ubiquitin ligase complex to degrade Smc5/6 (structural maintenance of chromosomes) (van Breugel *et al.*, 2012; Decorsière *et al.*, 2016).

Smc5/6 has recently been identified as a host restriction factor capable of blocking extrachromosomal DNA transcription (Livingston *et al.*, 2017).

Undoubtedly there are more interactions; there are reports linking HBx interacting with components of the basal transcriptional machinery (RPB5, TFIIB, TBP and TFIIH), with transcription factors (ATF/CREB, c/EBP, NF-IL-6 and RXR receptor), as well as with coactivators (CBP/P300 and ASC-2) or a repressor (PRMT1), as described by (Cougot *et al.*, 2007, 2012; Belloni *et al.*, 2009; Benhenda *et al.*, 2009). Once repaired, cccDNA is transcribed from four promoters into five major transcripts by host polymerase II (**Figure 7.1**).

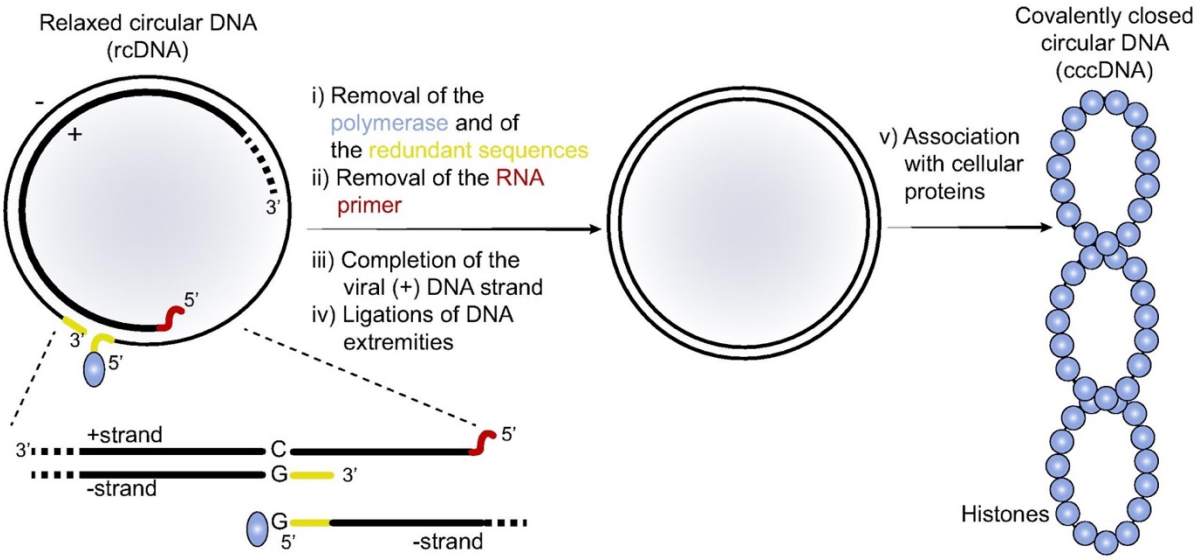


Figure 7.1: Illustration of the conversion of HBV rcDNA to cccDNA. Adapted from (Lucifora and Protzer, 2016).

A.3 Transcription and translation

All viral transcripts arising from cccDNA are unspliced, capped and polyadenylated. The mRNAs are exported to the cytoplasm for translation and nucleocapsid assembly.

The surface ORF generates 2.4kb and 2.1kb transcripts that contain three in-frame translational initiator AUG start codons (Seeger and Mason, 2015; Tong and Revill, 2016; Revill *et al.*, 2019). This results in the production of the three HBsAg proteins, S, M and L, (Prange, 2012). The S1 promoter governs synthesis of the 2.4kb transcript responsible for producing L HBsAg whilst the stronger S2 promoter (under additional regulation by nearby EnhII) govern synthesis of a 2.1kb transcript responsible for producing M and S HBsAg. As a consequence of differential regulation of the S1 and S2 promoters, S represents approximately 84% of all HBsAg with L and M representing the remaining 15% and 1-2% respectively. The differential promoter usage explains the vast production of smaller sub viral particles (SVP) (22nm) composed primarily of S HBsAg that do not compete with the larger infectious Dane particle (42nm) for entry (Seeger and Mason, 2015).

The precise function of this overabundance of SVPs is unknown but is hypothesised to "overwhelm" the immune response during early infection (Coleman, 2006). The L and M proteins are also known as pre-S1 and pre-S2 due to the presence of additional domains. L contains a pre-S1 domain not present on M whilst similarly M contains a pre-S2 domain not present on S (Chai *et al.*, 2007). It is the pre-S1 domain that carries the motif responsible for binding the NTCP receptor.

The Core ORF, under the regulation of the Core promoter, generates two longer than genome 3.5kb transcripts called preCore and Core that contain two in-frame initiator codons (Seeger and Mason, 2015; Tong and Revill, 2016; Revill *et al.*, 2019). The preCore transcript directs

translation of HBeAg. HBeAg is secreted from cells as a soluble protein that is used clinically as an indirect marker of viral replication and therefore features in treatment algorithms (NICE, 2013; Terrault *et al.*, 2016; European Association For The Study Of The Liver, 2017b). It is noteworthy that HBeAg is not necessary for HBV replication. The exact function of HBeAg is unknown although it is postulated to serve an immunoregulatory role (Milich *et al.*, 1990; Milich and Liang, 2003). After clinical reports of patients lacking HBeAg in conjunction with high HBV DNA in serum that were associated with severe liver disease (Fattovich *et al.*, 1988), core promoter mutations were identified. The most common was a G to an A change at position 1986 thereby creating a stop codon, abolishing HBeAg production (Carman *et al.*, 1990; Tong *et al.*, 1993). It has been postulated that this phenomenon represents duration of infection rather than geographic/genotypic influence as suggested in a cohort study (Zarski *et al.*, 1994). This virus - referred to as a precore mutant - often become the dominant quasispecies and represents a challenge in disease management if not recognised (Hadziyannis and Vassilopoulos, 2001). The Core transcript directs translation of HBcAg (which forms the core protein in the nucleocapsid of the virion) and less frequently polymerase. This is due to the polymerase start codon being located several hundred bp downstream from the 5' end, so ribosomes must access this initiator codon by a different process than ribosome scanning. This results in an imbalance to match the stoichiometric structure of the icosahedral nucleocapsid; it is estimated that for every 250 core subunits there is a single polymerase protein translated. The HBV polymerase is composed of four domains: terminal protein (TP), spacer, reverse transcriptase (RT) and ribonuclease H (RNaseH). The TP domain covalently links the polymerase to the complete HBV negative (-) DNA strand. The spacer domain connects the TP to RT. The RT domain is followed by the RNaseH which functions to hydrolyze HBV rcDNA from the pgRNA template after reverse transcription within the nucleocapsid. HBV pgRNA arises from the core transcript, which after translation into its polypeptides packages its own RNA to serve as pgRNA.

HBx is under negative autoregulation -purportedly to avoid excessive activation and expression- through the enhancer EnhI and Core promoter that direct production of the X transcript (Murakami, Cheong and Kaneko, 1994). HBx protein is a particularly interesting component of HBV but the true extent of its function is only partially understood. It is a small (17kDa) non-structural protein with a long list of attributed roles; hepatocarcinogenesis, signal transduction, transcriptional regulator, cell cycle progress, protein degradation pathways and apoptosis (Tang *et al.*, 2006). HBx is known to bind over 100 cellular proteins (Wu *et al.*, 2010). It is crucial for efficient transcription to occur from cccDNA and can interact with an increasing number of recognised transcription factors, host restriction factors and elements of basal transcription machinery, discussed previously (**Appendix A.2**) (Belloni *et al.*, 2009; Lucifora *et al.*, 2011). HBx appears to be able to activate and inhibit the damaged DNA repair process. This can be achieved by HBx binding to damaged DNA binding protein 1 (DDB1) which is an adaptor protein for Cullin 4A RING E3 Ligase and can act as a viral receptor for DDB1 Cullin Accessory Factor (DCAF), which binds substrate proteins for ubiquitination and subsequent degradation (Higa and Zhang, 2007; Lee and Zhou, 2007). This grants HBx modulation over cellular processes of DNA damage repair, cell cycle and innate immunity (T. Li *et al.*, 2010; Hodgson *et al.*, 2012). Conversely HBx has been shown to inhibit both nucleotide and base excision repair in hepatoma and murine primary hepatocytes (Becker *et al.*, 1998; Prost *et al.*, 1998; van de Klundert *et al.*, 2012). It is possible that during a dynamic chronic infection, HBV could -dependent on the host hepatocyte environment- modulate activation or repression of the DNA damage repair pathway through HBx to benefit HBV replication (Slagle and Bouchard, 2018).

The possible role for HBx mediation of hepatocyte metabolism is particular exciting and naturally provides an intersection for circadian regulation (Bar-Yishay, Shaul and Shlomai, 2011). HBx has been shown to be capable of modulating hepatocyte metabolism in hepatoma cell lines, murine models and primary hepatocyte culture systems (Shin *et al.*, 2011; Bagga *et*

al., 2016; Golob-Schwarzl *et al.*, 2017). These studies suggest that HBx can increase the expression of hepatic gluconeogenic factors, such as PPARα and phosphoenolpyruvate carboxykinase, thereby activating major metabolic signalling pathways including the PI3K/AKT, mammalian target of rapamycin complex 1 (mTORC1), and AMP-activated protein kinase (AMPK) pathways. Modulation of metabolic signalling pathways could provide increased substrates (lipids, proteins, and nucleotides) to facilitate HBV replication while also altering normal hepatocyte metabolism (Slagle and Bouchard, 2018). These pathways are well known to be under circadian influence (**Chapter 1.2.1**) and presence of HBV modulating the hepatocyte metabolism could easily perturb the endogenous hepatocyte clock.

Whilst HBV as an aetiological agent of HCC is not disputed, the indirect/direct mechanism(s) by which HBV causes transformation of hepatocytes remains uncertain. Indirectly, HBx is associated with cell cycle checkpoints, cell death, genotoxic cell response and wider signalling interactions including p53. Directly, integration into host DNA is relatively common and may occur in sites important for controlling hepatocyte proliferation (Arbuthnot and Kew, 2001; Murakami, 2001; Tang *et al.*, 2006).

A.4 Replication

HBV replication begins with the encapsulation of pgRNA along with polymerase by the Core transcript. The complex process of reverse transcription of pgRNA into rcDNA is illustrated in (**Figure 7.2)** (Wang and Seeger, 1992; Nassal and Schaller, 1996; Rieger and Nassal, 1996; Nassal, 2015). The polymerase attaches to the 5’ end of pgRNA by a ε (epsilon) stem loop structure. This triggers the protein primed process of RT to commence. HBV polymerase lacks

a proof-reading mechanism and is estimated to carry a mutation rate of 10^{-7} substitutions per replication cycle (Sheldon *et al.*, 2006). This is a high rate of mutation for a DNA virus (McNaughton, D'Arienzo, *et al.*, 2019). The TP domain of viral polymerase contains a tyrosine residue which substitutes for the 3' terminal OH group of the nucleic primer (e.g. a host tRNA) thus, there is a covalent tyrosyl-5'-DNA-phosphodiester linkage that is maintained until the end of rcDNA formation. The first 3-4nt of the growing negative (-) strand are templated by a bulged region contained in ε which is followed by transfer to a matching acceptor motif in the 3' DR1. Extension from there to the 5' end of the pgRNA template yields a unit length negative (-) strand DNA copy of the pgRNA carrying a small terminal redundancy (r). As DNA synthesis progresses, most of the pgRNA is degraded by the viral polymerase RNaseH activity. The non-degraded 5' residues of the pgRNA then act as primer for positive (+) strand DNA synthesis. Occasionally, extension of the RNA primer yields a dslDNA. These structures cannot form new pgRNA and thus represent a replicative dead-end. However, they can be integrated into the host genome. In contrast to their retrovirus counterparts, this is not essential for HBV replication. This integration into host DNA - especially at sites of DNA damage - is thought to occur by a process known as non-homologous end joining, and is hypothesised to contribute to the oncogenic potential of HBV depending on where the insertion occurs (Bill and Summers, 2004; Tu *et al.*, 2017). To complete replication, the RNA primer must be transferred from DR1 to the identical DR2, and extension from there to the 5' end of the (-) strand DNA. As (r) at the other end has the same sequence, exchange of the two ends allows (+)-strand DNA synthesis to proceed, eventually yielding RC-DNA with a slightly overlength (-) strand carrying a covalently linked polymerase protein at its 5' end and an incomplete (+) strand whose 5' end is constituted by the RNA primer. The mature rcDNA containing nucleocapsids are either re-imported back into the nucleus (thus amplifying the cccDNA pool) or undergo envelopment to be secreted as infectious virions (Nassal, 2015).

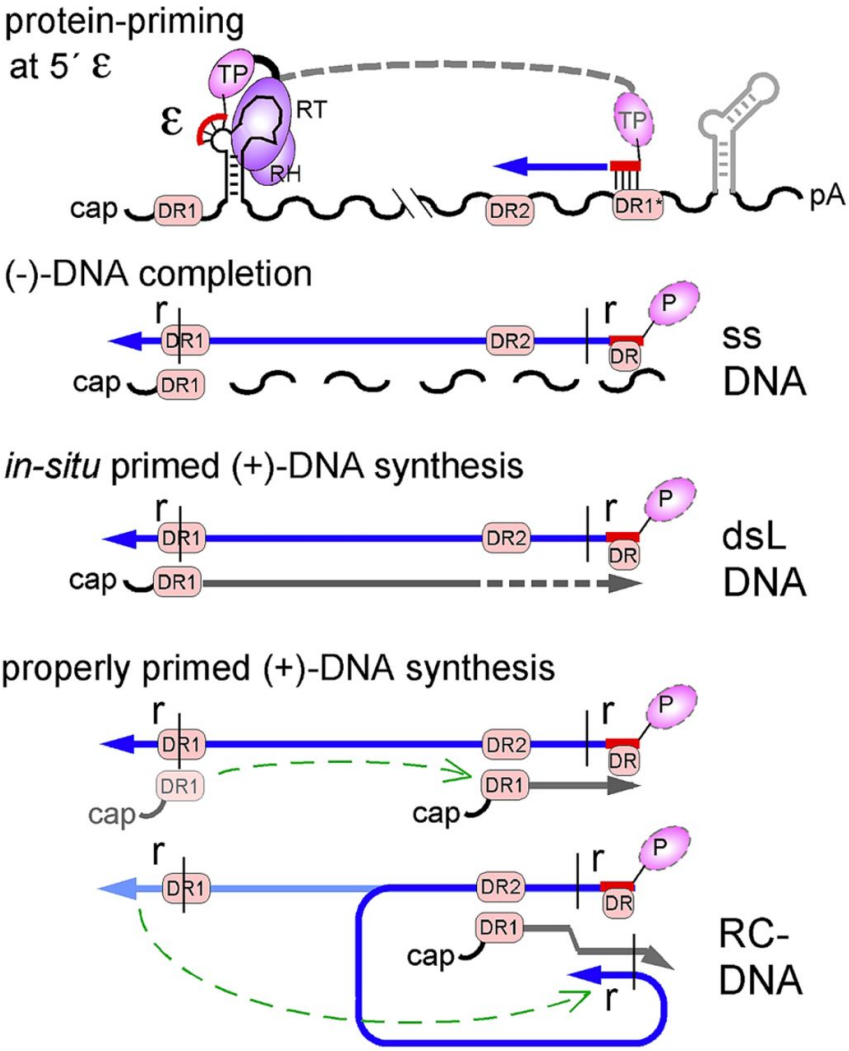


Figure 7.2: Formation of HBV rcDNA via reverse transcription of pgRNA. Adapted from (Nassal, 2015).

A.5 Morphogenesis and assembly

The mature core particle containing rcDNA is subsequently enveloped by HBsAg proteins (generated in the endoplasmic reticulum) and infectious virions bud through multi-vesicular membranes into the extra cellular matrix. There are numerous coordinated virus-host interactions that accompany HBV particle genesis. These include the cellular chaperones for transporting the three viral envelope proteins, intracellular trafficking of the mature nucleocapsids and components of the multivesicular body pathway that enable virion budding at intracellular compartments (Watanabe *et al.*, 2007; Prange, 2012).

Appendix B: Glucocorticoids are a key component of the hepatic clock

The liver is arguably the peripheral organ most subject to circadian influence, putatively contains the highest percentage of CCGs, and is in receipt of numerous zeitgebers to entrain the phase of its clock (**Figure 7.3**). One of the best recognised of these hormonal signals is the glucocorticoid hormone cortisol (Van Cauter, Leproult and Kupfer, 1996). Serum cortisol in humans exhibits a clear diurnal pattern peaking at approximately 8am and reaching its nadir three to four hours after sleep commencement. Rhythmic secretion is due in part to autonomic signals but primarily in response to the hypothalamic-pituitary axis which is composed of a multi-level negative feedback loop. It plays a role in the physiological stress response as well as a central role in glucose metabolism. Exposure to dexamethasone -a potent synthetic analogue- can shift the phase of the clock in peripheral organs in rat models (Balsalobre *et al.*, 2000). Increased hepatic expression of PER1 has been shown despite a refractory SCN phase in response to dexamethasone exposure. Convincing experiments showed that dexamethasone alone was capable of synchronising 60% of the liver circadian transcriptome in surgically SCN-ablated mice (Reddy *et al.*, 2007a). Modulation of the key clock components PER1, PER2, CRY1 and BMAL1 arise in response to dexamethasone, attributed in part to the presence of glucocorticoid response elements in their regulatory regions. Local TFs likely contribute to the regulation of CCGs. Hepatocyte nuclear factor four α (HNF4α) is a TF highly expressed in the liver that is important for ureagenesis and the activity of cytochrome P450 (Inoue *et al.*, 2002). Further work illustrated that two-thirds of the hepatic genes that could be synchronised by dexamethasone also contained putative binding motifs for HNF4α (Reddy *et al.*, 2007b).

Intrinsically linked to glucocorticoids as a hepatic zeitgeber is that of feeding. The central SCN governs activity/rest cycle and indirectly induces a temporal rhythm on eating. This behaviour

can regulate the hepatic clock at a local level by modulating signalling pathways and subsequent metabolic processes. The relatively simple act of feeding acts as a potent zeitgeber as evidenced by a phase shift of 12h in the hepatic clock of mice in response to altered feeding patterns, leaving the central SCN phase unaffected (Damiola *et al.*, 2000). In experiments by (Vollmers *et al.*, 2009), the authors demonstrated that prolonged fasting caused the majority of otherwise rhythmic transcripts to cease oscillating in mice. In double CRY1/2 knock out mice, introduction of a temporally restricted feeding schedule was capable of restoring rhythmic transcription in a cohort of otherwise oscillation deficient genes. There is a vast network of TFs modulated by the process of feeding and the resultant metabolic activities. As well as previously described AMPK (Jordan and Lamia, 2013) and CREB (Zhang *et al.*, 2010), known metabolic signals that can contribute into hepatic clock modulation are PPARα (Canaple *et al.*, 2006; Eckel-Mahan *et al.*, 2013), PCG-1α (Liu *et al.*, 2007; Sonoda *et al.*, 2007), SIRT1 (Asher *et al.*, 2008; Nakahata *et al.*, 2008) and HSF1 which will be discussed separately.

In mammals, ambient temperature fluctuations - whilst a powerful entraining zeitgeber in prokaryotes - do little to alter the phase in the SCN (Refinetti, 2010). The clocks in peripheral organs however, have remained responsive to a temperate zeitgeber *in vitro* (Brown *et al.*, 2002). This regulation is largely controlled by a TF called heat shock factor 1 (HSF1), which has rhythmic transcriptional activity in the liver (Reinke *et al.*, 2008). HSF1 is now understood to be subject to significant post translational modification and is at an intersection for multiple signalling pathways, particularly cellular stress (Åkerfelt, Morimoto and Sistonen, 2010).

The SCN as part of the autonomic nervous system sends signals via the paraventricular nucleus and superior cervical ganglia to the liver to regulate serum glucose. Rats subjected to hepatic sympathectomy show disrupted serum plasma rhythms despite continued rhythmic expression of clock genes (Cailotto *et al.*, 2005). The SCN also directly innervates the adrenal gland responsible for adrenaline release and sensitivity to ACTH hormone (Cailotto *et al.*, 2009).

Together, these inputs and feedback mechanisms reveal the liver as an organ highly subject to circadian regulation, whereby glucocorticoids and metabolism work in tandem with a network of hepatically expressed TFs towards its orchestration. Consequently, perhaps it is not surprising that the main signs of perturbations in circadian rhythm are those of hepatic dysfunction due to metabolic syndrome.

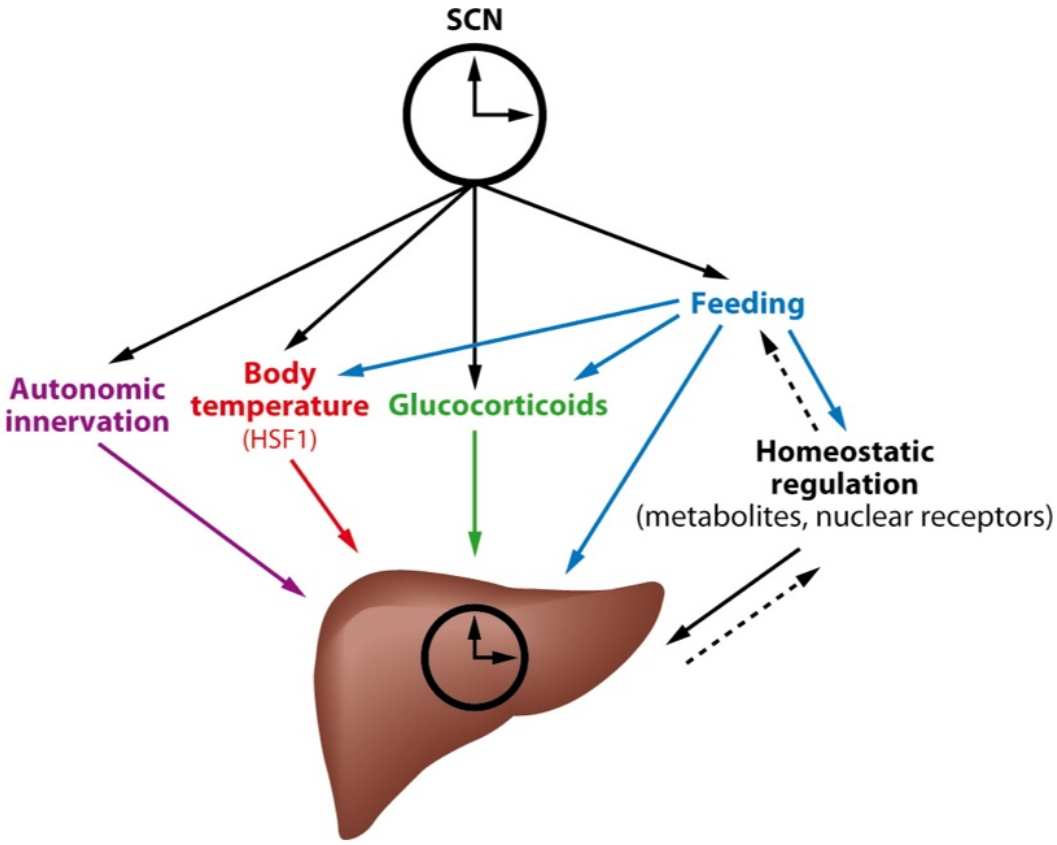


Figure 7.3: The entrainment pathways of the hepatic clock. Taken from (Mohawk, Green and Takahashi, 2012).

Appendix C: Circadian controlled genes.

The molecular TTFL previously described has been observed in almost every mammalian cell as well as the SCN (Stratmann and Schibler, 2006). This allows for each cell to have an autonomous yet synchronised clock as part of the organism as a whole. It is unknown precisely what percentage of genes are under circadian modulation. Any attempt to address this requires a few considerations (Delaunay and Laudet, 2002):

(i) Problems with microarray analysis arise due to noise to signal ratios and reproducibility. This is confounded by the likelihood that few genes are modulated exclusively by the circadian clock; the majority are under the control of multiple pathways. The output of any proposed CCG will be a summation of circadian and metabolic inputs relative to the temporal cellular physiology at that moment.

(ii) Any putative CCGs in an organ are unlikely to be under circadian modulation in a different organ; reflecting the spatial need for different physiological processes in each cell type. Of 650 genes showing rhythmic expression in the murine SCN and liver, there were only 28 in common which were mainly known clock components (Panda *et al.*, 2002).

(iii) There is an increasing awareness of both post transcriptional and/or post translational modifications that act as the component enforcing rhythmic gene expression. Almost half (43%) of protein coding genes in mice showed a circadian pattern of expression in a organ specific manner, based on a combination of RNA-sequencing and DNA arrays in 12 organs (Zhang *et al.*, 2014). The liver has been

identified as the organ with the highest percentage (16%, 3186 genes) of CCGs (Zhang *et al.*, 2014),(Akhtar *et al.*, 2002; Panda *et al.*, 2002; Ueda *et al.*, 2002).

The observation of discrepancies between nascent RNA and mRNA levels prompted the search for post-transcriptional modifications that could account for rhythmic gene expression. Microarray studies suggest that approximately 10% of mRNA in the murine liver transcriptome is rhythmic (Akhtar *et al.*, 2002; Panda *et al.*, 2002; Ueda *et al.*, 2002). When this was compared to Nascent-seq, only about 20% of those identified circadian genes with rhythmic mRNA, have comparable *de novo* transcriptional rhythms (Koike *et al.*, 2012; Menet *et al.*, 2012). This discordance has been further explored using Chip-seq to BMAL1 and Global Run-On sequencing, by exploring complexities in TF and RNA interactions (Rey *et al.*, 2011; Fang *et al.*, 2014). Therefore, some caution is needed when measuring transcripts alone, as this likely underestimates the true extent of the circadian proteome. The likely components involved in mediating these post transcriptional modifications are involved with the regulation of splicing and RNA processing: RNA-binding proteins, regulators of mRNA stability and microRNAs. Work by (Fustin *et al.*, 2013) illustrated that inhibition of methylation by siRNA to Mettl3, results in widespread changes in the transcription of the RNA processing machinery and in elongation of the circadian period. This is reviewed further by (Kojima, Shingle and Green, 2011; Zhao, Roundtree and He, 2016). Although significant gaps persist, our understanding of how these CCGs are regulated by post transcriptional modifications is increasing. This is coupled with a recently evolving appreciation of how post translational modifications contribute to the expression of CCGs.

Post translational modifications have been difficult to study *in vitro* and so knowledge has progressed by studying *in vivo* models, often in the form of spontaneous mutations. An *in vivo* example of phase shift due to disordered PER regulation was provided by through the

identification of a golden hamster with a naturally occurring free running period of 20 hours compared to 24 hours in wild type littermates (Ralph and Menaker, 1988). Subsequent genetic analysis identified a missense mutation causing an arginine to cysteine substitution at position 178 (R178C) in the PER regulator CKε. This mutation, named tau, resulted in a decreased ability to phosphorylate with increased PER expression (Lowrey *et al.*, 2000). This illustrates how altered degradation of a clock regulator can modulate the phenotype.

In human red blood cells - which are anuclear, thus transcription is precluded - a circadian rhythm can still be observed in the peroxiredoxin (PRX) proteins; these highly conserved antioxidant proteins undergo 24-hour redox cycles that persist for many days under constant conditions (O-Neill and Reddy, 2011). These observations demonstrate that post translational modifications can convincingly regulate rhythmic gene expression. It is hypothesised these translational modifications add a necessary time delay to help reach a target 24-hour period; as simple transcription modulation would result in a loop that could be completed in a few hours. Our understanding of how post-translational modifications contribute to the rhythmic expression of CCGs is in its infancy, may be achieved via modulating the localization, activity and degradation of regulators through phosphorylation, sumoylation, histone acetylation and methylation. The net effect is a network of regulators that could impose a rhythm on the TF they modulate, which, in conjunction with described transcriptional modification, contributes towards the resultant rhythmic expression of CCGs (Gallego and Virshup, 2007; Hastings, Maywood and O'Neill, 2008; O-Neill *et al.*, 2011).

In summary, multiple genes could be considered CCGs but there are multiple molecular levels at which those rhythms could be imposed. Estimates can vary due to differing methodologies employed in identification and even the type of analysis used in the resultant datasets (Wu *et*

al., 2014). Consequently, it is possible that current methodologies underestimate how much of our genome is under circadian control; multiple methods of data analysis suggest varying percentages of CCGs (Ptitsyn *et al.*, 2006).

Appendix D: Circadian Disease.

D.1 Circadian Disease I: Circadian rhythm sleep disorders

By studying the various phenotypes of human sleep pathology, an insight into the molecular control of circadian rhythm is obtained. These CRSD fall into two main categories (extrinsic and intrinsic) which are detailed in (**Table 7.1**) (American Academy of Sleep Medicine, 2005).Their broader classification and guidelines for treatment are discussed by (Wagner, 1996; Sack *et al.*, 2007a, 2007b; Sateia, 2014). There is an increasing awareness of the pathology caused by misaligned central and peripheral clocks. This is thought to play a role in the development of multiple chronic systemic diseases. As they are acquired rather than inherited disorders, they are sub-grouped as the extrinsic CRSD.

Disorder Group	Name	Main Characteristic
Intrinsic	Advanced Sleep Phase Syndrome (ASPS)	Evening somnolence & morning insomnia
	Free Running Disorder (FRD)	Evening insomnia & morning somnolence
	Delayed Sleep Phase Syndrome (DSPS)	Associated with blindness
	Irregular Sleep Wake Rhythm (ISWR)	Associated with elderly and advanced dementia
Extrinsic	Shift Work Disorder (SWD)	Emerging independent risk factor for multiple pathologies
	Jet Lag Disorder (JLD)	

Table 7.1: Circadian rhythm sleep disorders. Adapted from (Nassal, 2015).

ASPS is characterised by early evening somnolence and early morning insomnia. Staying up later than 21:00 is difficult. Epidemiology suggests about 1% prevalence in adults with a strong family association. Almost 50% of patients will have a relative with ASPS. The patient's main complaint is difficulty to entrain their sleep phase to match a daily conventional work schedule. Diagnosis is mainly clinical with polysomnography. Treatment consists of rigid entrainment schedules, light therapy and occasionally melatonin (Wagner, 1996; Lowrey and Takahashi, 2004). Whilst ASPS is relatively common -particularly in older adults- the majority is sub-clinical and goes without diagnosis. Patients are simply referred to as morning larks. This is distinct to a more severe form associated with a significant phase shift of four to six hours and strongly associated within families called familial ASPS (FASPS). It is noteworthy that core body temperature and melatonin rhythms were concordant with the proposed phase shift. As the clinical recognition and genetic diagnostic tools available progress, so does our understanding of the underlying genotype and molecular components of the human clock. The first familial cluster of FASPS was described by (Jones *et al.*, 1999). Genetic analysis on this cohort was later performed and identified a missense mutation that resulted in a serine to glycine change at position 662 (S662G) of PER2 (Toh *et al.*, 2001). This results in hypophosphorylation by CKε with a subsequent build-up of PER2 protein. A second missense mutation resulting in a threonine to alanine at position 44 (T44A) in the CKδ gene results in reduced enzymatic activity (Xu *et al.*, 2005). This is analogous to the golden hamster with reduced CKδ function previously described (Ralph and Menaker, 1988; Lowrey *et al.*, 2000). These post translational modifications would lead to a proposed pathogenesis of defective phosphorylation of PER2 by CK, thus causing premature termination of the molecular clock causing the FASPS phenotype (Toh, 2008). This hypothesis was tested by (Xu *et al.*, 2007), by reproducing the S662G PER2 mutation in transgenic mice. This resulted in reduced PER2 transcription rather than decreased degradation as had been proposed. The authors went on to show that CK ε/δ phosphorylates multiples sites, thereby adding an additional layer of complexity. Work by (Vanselow *et al.*, 2006) would illustrate over 20 possible sites for phosphorylation that modulate PER2

transcription, degradation and localisation. In fact, as well as phenocopying FASPS and the tau mutation hamster, differential phosphorylation patterns could both extend and shorten the clock period. Finally, it is particularly noteworthy that a phenotype as broad as FASPS is likely to have an equally broad range of putative genetic mutations in the regulators of the circadian clock. This was evidenced by family cluster of FASPS (Reid *et al.*, 2001) with no discernible mutations in any of the three PER genes (Chang *et al.*, 2002). In addition to a smaller study of two Japanese families which would show no mutations in PER2 (Satoh *et al.*, 2003). Cumulatively, these early steps illustrate the genetic heterogeneity possible to produce a concordant clinical syndrome.

Delayed sleep phase syndrome (DSPS) first described by (Weitzman *et al.*, 1981), has the opposite phenotype to (F)ASPS, whereby patients have early night time insomnia and difficulty in waking at a conventional hour in the morning (Wagner, 1996). It represents the most common of the CRSD and is most frequently reported amongst young adults. Treatment is grossly similar to that of (F)ASPS. There are limited genetic studies on this patient cohort. Five polymorphisms have been described in four haplotypes in PER3, one of which was associated with a DSPS phenotype (Ebisawa *et al.*, 2001). The proposed polymorphism is located close to a CKε phosphorylation site. A study by (Johansson *et al.*, 2003) was able to associated the same polymorphism with diurnal preference in a cohort of 92 patients with seasonal affective disorder. Further investigation focused on this length-polymorphic repeat region composed of four or five units in PER3 described in the earlier paper (Archer *et al.*, 2003). The author demonstrated the length polymorphism correlated highly with diurnal preference; the longer allele with ‘morning lark’ and shorter with ‘evening owl’. Furthermore, the authors showed 75% of DSPS patients were homozygous for the shorter allele. The potential role of PER3 variants is reviewed further by (Archer *et al.*, 2018).

There are significant gaps in our knowledge of the two remaining CSRD. FRD is primarily a condition associated with the totally blind (Sack *et al.*, 1992). It reflects a failure of correct entrainment from an environmental 24 hour period to a longer endogenous period of 24.5 hour (Czeisler *et al.*, 1995). ISWR is characterised by the relative lack of any circadian pattern of a sleep wake cycle (Zee and Vitiello, 2009). It is associated with advancing age and co-morbidity particular degenerative neurological disease e.g. dementia. Total time spent asleep is comparable with normal individuals but is broken up and composed of three-four hour naps. There is a paucity of genetic research in these conditions. Both conditions are reviewed in greater depth by (Wagner, 1996; Sack *et al.*, 2007b). By studying the CSRD, it has illustrated the potential genetic heterogeneity in addition to possible post-translational modifications that contribute to the human circadian clock and its subsequent phenotype.

D.2 Circadian Disease II: Asynchronous rhythm is a novel risk factor for chronic illness

It is increasingly recognised that temporally separated organ functions, when made to perform their intended activity out of synchrony to the master metronome, can result in pathology. While the architecture of the circadian clock remains intact there is asynchrony with the environment (Sack *et al.*, 2007a). Traditionally considered insignificant annoyances SWD and JLD are becoming increasing common as the pace of life increases. Approximately 20-25% of the European working population partake in some form of shift work (Parent-Thirion, 2007).

Shift work is an obvious zeitgeber to induce asynchrony; whereby the activity, feeding and sleep cycles are inversed. It has been already been discussed how altered timing of food intake is capable of inducing a phase shift in the liver. Numerous murine models demonstrate how

disruption of the liver clock can result in increased rates of fatty acid liver disease, cirrhosis and even malignancy (Tahara and Shibata, 2016).

The transition from murine work to the investigation of human shift work patterns as an adverse health effector is a relatively new field of research and faces numerous challenges. There is an increasing body of work linking discordant circadian rhythms with increased rates of cancer, metabolic syndrome and cardiovascular disease (CVD) (Szosland, 2010; Wang *et al.*, 2011). Clearly development of these conditions is multifactorial, with a complex interplay of individual risk factors. Whilst mortality and morbidity due to CVD and metabolic syndrome can represent individual endpoints in the literature, *in vivo* they are interlinked. Metabolic syndrome is a risk factor for both cancer and CVD. Metabolic syndrome is a term for the combination of diabetes, hypertension and obesity. Individually these are significant risk factors for CVD but there occurrence together as part of this syndrome is particularly dangerous. Adjusted hazard ratio for CVD mortality due to metabolic syndrome alone during a follow up period of 18 years was estimated at 1.49 (95% CI 1.35-1.78) (Eberly *et al.*, 2006). Nonetheless, an appreciation is beginning to emerge that not only does shift work augment prevalence of these traditional risk factors but likely represents an independent risk factor itself.

There are numerous observational studies making the association between shift work and increased rates of metabolic syndrome or its components. A Swedish cross-sectional study of 27,485 people demonstrated a raised BMI (>30) and raised triglycerides was more prevalent across all age groups in both women and men after correcting for age and socioeconomic factors (Karlsson, Knutsson and Lindahl, 2001). Work by (Suwazono *et al.*, 2009) showed in a 14 year prospective cohort study on almost 3,000 people that undergoing shift work was an

independent risk factor towards development of impaired glucose tolerance: the clinical precursor stage to diabetes. A cross sectional study illustrated an association between duration of shift work and both blood pressure and cholesterol in male workers over 30 years of age (Ha and Park, 2005). The correlation between shift work and metabolic syndrome or its individual components is further discussed in work by (Morikawa *et al.*, 2005; Knutson *et al.*, 2006; Kroenke *et al.*, 2007).

Shift work can increase the risk of CVD by multiple mechanisms. A meta-analysis of 17 studies evaluating CVD risk estimated a 40% increase due to shift work (Bøggild and Knutsson, 1999). A dose dependent increase in CVD risk factors was associated with shift work in 7,839 participants of the British birth cohort (Thomas and Power, 2010). Under separate shift pattern analysis, this increase was mainly in those working a night/morning rather than evening/weekend pattern. In addition to purely biological risk factors there are additional psychosocial and psychological risks associated with working in a shift pattern. 20% of patients with CVD have depression (Elderon and Whooley, 2013). Work by (Bara and Arber, 2009) identified working a shift work pattern for greater than four years as an independent risk factor for anxiety/depression, suggesting a possible additional contributory CVD risk.

Linking shift work to increased rates of malignancy is difficult. Development of cancer is a complex multifactorial process and for ethical reasons human studies will be largely of an observational quality where controlling for confounding is challenging. Nonetheless, a disturbance of CCGs is common amongst various malignancies including HCC (Filipski, Li and Lévi, 2006; Lahti, Merikanto and Partonen, 2012; Savvidis and Koutsilieris, 2012; Li *et al.*, 2017). It has been postulated that this disruption of CCGs and their downstream targets - grown, cell division- could enhance cancer development. Murine models have demonstrated a

disrupted clock leading to increased carcinogenesis. Amplified rates of liver carcinogenesis have been reported in a murine model of chronic jet lag (Filipski *et al.*, 2009), whilst chronic phase shift disrupted NK cell cytolytic function and enhanced lung tumour growth (Logan *et al.*, 2012). Cumulatively it is plausible that these circadian factors could act synergistically towards HBV mediated oncogenesis.

With no standardised international definitions as to what constitutes shift work, numerous confounding variables, methodological differences between studies and intrinsic bias; a definitive conclusion on the quantitively risk posed to human health by shift work is not yet apparent. Multiple groups have attempted meta-analysis in this area.

(i) A particularly critical review is provided by (Wang *et al.*, 2011), concluding that the evidence is suggestive, but not conclusive, of an association between shift work and breast cancer as well as cardiovascular disease, metabolic syndrome and diabetes.

(ii) A meta-analysis of 14 studies looking at CVD and shift work reported a weak association when using fatal outcomes as an endpoint, this association showed a stronger correlation if non-fatal CVD was included (Frost, Kolstad and Bonde, 2009).

(iii) More recently, a meta-analysis of 13 studies showed a dose response relationship between shift work and metabolic syndrome (Wang *et al.*, 2014). The authors estimated a RR of 1.57 (95% CI 1.24-1.98) in those that had ever undertaken shift work compared with a RR of 1.77 (95% CI 1.32-3.36) in those with continued exposure.

Lastly, numerous chronic diseases of humans are known to exhibit a circadian pattern of symptom exacerbation or presentation. Arguably, most have a basis in immune or inflammatory dysfunction, including rheumatoid arthritis (Cutolo, 2012), allergic rhinitis (Smolensky, Lemmer and Reinberg, 2007), asthma (Durrington *et al.*, 2014), acute coronary syndromes (Suárez-Barrientos *et al.*, 2011), stroke (Gupta and Shetty, 2005) or sickle cell crises (Auvil-Novak, Novak and El Sanadi, 1996). It is increasingly apparent that multiple clock regulated immune factors contribute to the pathogenesis of these conditions.

As our understanding increases of how perturbations in the clock contributes to human morbidity, so too does the potential for therapeutic intervention. In additional to environmental and behavioural modalities, pharmacological avenues are being pursued (Nohara, Yoo and Chen, 2015). Identification of clock amplitude enhancing small molecules (CEMs) allows restoration of attenuated clock amplitude to near normal levels (Chen *et al.*, 2012; Chen, Yoo and Takahashi, 2013). The activating RORs were a target for a CEM called nobiletin (He *et al.*, 2016). The authors went on to show that metabolic syndrome was significantly reduced in diet induced obese mice treated with nobiletin. An additional possibility is enhancing effectiveness of current therapeutics. A recent study showed a decreased hazard ratio of 0.55 (0.50-0.61 95%CI) of their primary CVD endpoint in almost 20,000 patients, by merely switching their anti-hypertensive medication administration from morning to evening (Hermida *et al.*, 2019).

In summary, a perturbed circadian rhythm whether due directly -as occurs in the CSRDs- or indirectly due to environmental cues, is an increasingly recognised contributor to human morbidity and mortality. As our mechanistic understanding increases so does the potential for circadian targeted therapeutics. In particular, questions into the circadian governance of the

immune system have arisen in tandem to questions of how infectious pathogens have evolved to circumvent this.

Appendix E: Regulation of immune response by circadian rhythm

Due to our daily cycle of rest/activity/food intake, exposure to pathogens can follow a circadian pattern. To accommodate this there is an increasing recognition that the immune system has evolved to anticipate this antigenic stimulus by cycling its inflammatory response. Early experiments in mice demonstrated survival after exposure to bacteria (or their toxins) and viruses correlated with the time of inoculation (Halberg *et al.*, 1960; Feigin *et al.*, 1969; Feigin, Middelkamp and Reed, 1972). This showed murine immune systems more reactive at the beginning of the active phase. More recently, it was shown individual components of the immune system are under circadian regulation. In addition to demonstrating rhythmic production of cytokines post stimulation, human mononuclear cells, CD4+ T cells and macrophages have been shown to possess their own clock that persists *ex vivo*, (Boivin *et al.*, 2003; Keller *et al.*, 2009; Bollinger *et al.*, 2011). These cells are subject to the same autonomic cues from the central clock as the other organs. Early work describes how hormonal signals composed of glucocorticoids as well as the catecholamines (adrenaline and noradrenaline) from the sympathetic nervous system are capable of modulating lymphocyte traffic and circulation (Haus *et al.*, 1983; Haus and Smolensky, 1999). They state the production of both pro and anti-inflammatory cytokines occurs in a rhythmic fashion. The levels of the glucocorticoids, catecholamines and the pro inflammatory cytokines - such as IL12 and TNFα- peak during at the beginning of the active phase. The circadian regulation of the immune system via autonomic and glucocorticoid influence is reviewed further by (Elenkov *et al.*, 2000; Scheiermann, Kunisaki and Frenette, 2013; Dumbell, Matveeva and Oster, 2016).

Numbers of haematopoietic stem and progenitor cells (HSPCs) and most mature leucocytes peak in the circulation during the resting phase (Haus *et al.*, 1983; Haus and Smolensky, 1999).

Attention was turned as to the mechanism behind this. At the end of the resting phase they are released from the bone marrow to the circulation in a process shown to be dependent on sympathetic innervation downregulating CXCL12: a chemokine protein that acts as a retention factor for HSPCs in the bone marrow (Méndez-Ferrer *et al.*, 2008). Simultaneously, there is reduced expression of its receptor CXCR4 on HSPCs, CD4+ and CD8+ T cells (Lucas *et al.*, 2008). In healthy human volunteers, cortisol and adrenaline control opposing circadian rhythms in T cell subsets in the circulation (Dimitrov *et al.*, 2009); numbers of naïve CD4+ and CD8+ cells reached a nadir during daytime whilst terminally differentiated effector CD8+ T cells peaked. Naïve T cells were negatively correlated with cortisol levels whilst effector CD8+ positively associated with adrenaline. These relationships were later verified by exogenous administration of each hormone. Cortisol acts to increase CXCR4 thus promoting migration to the bone marrow. It is postulated that this segregation of immune cells serves to facilitate an immediate effector cytotoxic defence during the daytime activity period, via activation of stress hormones. This could be followed by release of naïve T cells from the bone marrow to secondary lymphatic tissues during the rest period, to support the commencement of the adaptive response. Cumulatively, this suggests the immune system is under significant circadian regulation.

An immediate translation of this knowledge could be the timing of vaccination. WHO has estimated the only other health intervention to reduce the global burden on infectious disease more is the provision of clean water (Andre *et al.*, 2008). Vaccination efficacy continues to be hampered by inter-personal variations in response. The adjusted vaccine effectiveness of influenza vaccine for the 2017/18 season in the UK was 15% across all influenza strains (Pebody *et al.*, 2017). There are numerous contributing factors for such a poor response, but time of vaccine administration could represent an immediate way of boosting response.

(i) Morning administration of vaccine has been shown to be an independent factor capable of increasing host response in the Hepatitis A and Influenza vaccines (Phillips *et al.*, 2008). Traditionally, older adults are a population that show a poorer response to vaccines. In a group of 276 adults >65 years of age, an enhanced influenza vaccine response was observed if vaccination took place in a morning session (9-11AM) compared with afternoon session (3-5PM) (Long *et al.*, 2016).

(ii) An observation study by (Prather *et al.*, 2012) illustrated the amount of sleep the recipient had received -measured by actigraphy- was shown to be independently associated with response to the HBV vaccine.

The immune system is both responsive to and governed by the circadian clock. Although our understanding of the mechanisms that underpin this are progressing, there remains numerous gaps. Furthermore, we must be mindful, particularly when dealing with immune responses to infectious disease that there is often the circadian rhythm of the pathogen itself to consider.

Appendix F: Molecular basis of autophagy.

Some of the autophagy regulatory elements are homologues of those first described in yeast called autophagy related genes (ATG) (Ravikumar *et al.*, 2010). Starvation, energy exhaustion or rapamycin are potent stimulators of autophagy via inhibition of mammalian target of rapamycin (mTOR), which is part of a wider macromolecular signalling complex called mTORC1 that negatively regulates autophagy. This signalling complex negatively regulates the mTOR substrate complex: consisting of UNC-51–like kinase 1 (ULK1), ATG13, ATG101, and FIP200 (Kim *et al.*, 2011). Thus, releasing the negative regulation on the MTOR substrate complex stimulates autophagy. Enhanced autophagy can also arise from signals due to energy depletion; this stimulates this complex directly via AMPK and indirectly through AMPK inhibition of the mTOR signalling complex. Conversely, inhibition of autophagy can arise from growth factors or insulin, which are secreted in times of energy abundance. This stimulates the class I-phosphatidylinositol 3 kinase (PI3K) AKT system which activates the negative regulator mTORC1 signalling complex (Kim *et al.*, 2011).

A macromolecular complex that is important in the initiation of autophagy is the beclin-1 class III PI3K complex which is composed of Beclin 1, VPS34 and ATG14L. This complex stimulates phosphatidylinositol 3-phosphate (PI3P) which is crucial for the early part of autophagy by promoting AP membrane nucleation (He and Levine, 2010; Carlsson and Simonsen, 2015; Nascimbeni, Codogno and Morel, 2017). Several factors may participate in this regulation including rubicon and the UVRAG-BIFI complex (Takahashi *et al.*, 2007; Nakamura *et al.*, 2019). The BCL-2 family of apoptotic proteins can interact with this complex to inhibit autophagy at this site (Shimizu *et al.*, 2004). It is only at this point that AP formation can begin.

Appendix G: Role of autophagy in human disease

There is an evolving understanding of the role that disrupted autophagy plays in a number of human diseases (Mizushima *et al.*, 2008; Choi, Ryter and Levine, 2013). Autophagy appears to play a dual role in cancer, by reducing development of malignant cells but enhancing their survival once formed (White, 2012). In non-malignant cells, this process serves a protective function against the development of DNA damage and genetic instability. Mitophagy reduces oxidative stress by efficient turnover of mitochondria whilst autophagy serves to remove oncogenic protein substrates and toxic unfolded proteins in malignant cells. However, autophagy can contribute a survival advantage due to the negative environmental pressures of nutrients, oxygen and potential chemotherapy. Inhibition of autophagy in these cells has been shown to enhance responses to cancer treatments.

(i) Numerous neurodegenerative disorders have components of their pathogenesis attributed to abnormal build-up of protein and deficiencies in autophagic clearance. Huntington's disease is characterised by the cellular build-up of the toxic huntingtin protein. It has been postulated that the huntingtin protein may perturb the autophagic pathway by evading recognition by AP for degradation or sequestering of essential proteins in its aggregates (Shibata *et al.*, 2006; Vicente *et al.*, 2010). Multiple mouse models with deficient autophagic responses have shown increased rates of neurodegenerative disease due to enhanced protein aggregates (Hara *et al.*, 2006; Komatsu *et al.*, 2006; Pickford *et al.*, 2008). Consequent pharmacological activation of autophagy has improved symptoms in these models (Ravikumar *et al.*, 2004; Schaeffer *et al.*, 2012).

(ii) Alzheimer's disease is characterised by accumulation of hyperphosphorylated microtubule associated protein called tau and amyloid β (Jellinger, 2010). As well

as APs being a rich source of amyloid β, dysfunctional lysosome fusion has been attributed to its aggregates (Haung Yu *et al.*, 2005).

(iii) Classic Parkinson's disease is defined by the accumulation of α-synuclein protein aggregates called Lewy bodies; despite being a known substrate for degradation, their accumulation impairs autophagic flux (Winslow *et al.*, 2010).

(iv) Danon's disease is an inherited condition featuring the clinical triad of cardiomyopathy, myopathy and intellectual disability. It is due to mutations in the lysosome associated membrane protein 2 (LAMP-2) which is crucial for AP-lysosome fusion. Histologically it is characterised by increased cytoplasmic vacuoles containing autophagic material with disordered glycogen storage. Cardiomyocytes from patients with this condition also show impaired mitophagy (Nishino *et al.*, 2000). Disordered autophagy is hypothesised in various other cardiac pathologies including ischaemic heart disease and heart failure (Kirshenbaum, 2012).

(v) Murine models with genetic knockout of crucial autophagy genes show both pathological liver enlargement and raised storage of triglyceride in lipid droplets in hepatocytes (Singh *et al.*, 2009). A significant decrease in ATG7 expression has been demonstrated in both dietary and genetic murine models of obesity (Yang *et al.*, 2010), associated with disordered insulin signalling and endoplasmic reticulum stress. Subsequent restoration of ATG7 regulators led to reduced endoplasmic stress, improved insulin sensitivity and glucose metabolic parameters. Exercise-induced autophagy protected mice from glucose intolerance induced by obesity (He *et al.*, 2012).

Studying the wide breath of pathology that can arise due to perturbed components of the autophagic process allows us to gain a better understanding of the many roles autophagy plays

in maintaining cellular homeostasis whilst identifying potential therapeutic targets (Jiang and Mizushima, 2014).

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